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BERBERINE: A NATURALLY OCCURRING PHOTOTOXIC ALKALOID

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Abstract—The isoquinoline alkaloid berberine, present in nine different plant families was found to be phototoxic to mosquito larvae. In the presence of near UV the LC_{50} for acute 24-hr toxicity was 8.8 ppm compared to 250 ppm for dark controls. Mosquito larvae that were treated with 10 ppm berberine plus near UV for 24 hr and then transferred to berberine-free water showed decreased larval survival and resulted in a smaller cumulative number of pupae and adults as compared to controls, during a subsequent 4-week development period. Berberine was found to be a singlet O_2 generator in experiments with the chemical trap 2,5-dimethyl furan. A slight increase in chromosome aberrations in Chinese hamster cells was also observed with berberine plus near UV treatment. The significance of the phototoxicity of berberine is discussed in relation to plant-insect relations.

Key Words—Berberine, phototoxicity, *Aedes atropalpus*, Diptera, Culicidae, singlet oxygen, UV, alkaloid, secondary plant substance.

INTRODUCTION

In a recent symposium, we reported on the phototoxicity of a range of plant secondary metabolites to insects (Arnason et al. 1983). The photosensitizing properties of polyacetylenes and furanocoumarins are well known. A search for other phototoxic metabolites that might act as protective agents in plants was undertaken. Berberine was one of the substances identified in the survey and forms the basis of the present detailed study.

One of the important isoquinoline alkaloids, berberine (Figure 1) is

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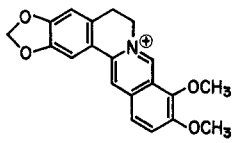
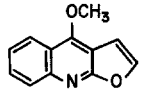


Berberine



Dictamnine

FIG. 1. The molecular structure of berberine and dictamine.

known to occur in at least nine botanical families: Annoncaceae, Berberidaceae, Juglandaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae, and Rutaceae (Jeffs, 1967; Manske, 1968, 1975; Raffauf, 1970; Santavy, 1970). It can be found in any part of the plant and concentrations of up to 10% have been reported (Manske, 1950). To date studies of the compound have been primarily concerned with phytochemical and pharmacological aspects.

Berberine has been shown to possess fungicidal and antibacterial properties (Greathouse and Watkins, 1938; Yoshikaju, 1976; Nakamura, 1977). In a previous study, we found that berberine affected the development rate and survival of insects at levels as low as 0.3% in the diet (Devitt et al., 1980).

The fluorescent nature of berberine was a circumstantial piece of evidence that suggested its possible photodynamic activity, i.e., the production of toxic activated O_2 species such as singlet O_2 ($^1O_2$) in the photoreaction, (Spikes, 1977) and led us to investigate its phototoxic properties. In the current paper we report on its action against the mosquito *Aedes atropalpus* and offer possible explanations as to its mode of action.

METHODS AND MATERIALS

The rock hole breeding mosquito, *Aedes atropalpus* was used to test berberine toxicity. Rearing conditions were previously reported (Philogène and Labaky, 1982).

Berberine (99%, Sigma) was recrystalized twice in ethanol and its purity verified by 2D chromatography on silica gel, using MeOH–$CHCL_3$ (35:36) in the first direction and benzene–MeOH (55:45) in the second direction (Rama Rao and Tandon, 1978).

The following concentrations were tested: 0.1, 1.0, 10, and 100 ppm. Twenty-nine fourth instar larvae were treated in each trial for a period of 24 hr under three different light conditions: (1) complete darkness; (2) two Indorsun fluorescent lamps (Verd-A-Ray Corp.) (5 W/m^2 total and 0.4 W/m^2 UV); and (3) two black light blue lamps (Westinghouse 20 T 12) and two Indorsun lamps (20 W/m^2 total and 3.5 W/m^2 UV). The light intensity was measured with a YSI radiometer and near UV intensity was estimated by using a Kodak Wratten filter No. 2B with a near UV cut off at 400 nm.

Following exposure to the above-mentioned conditions, the larvae were washed several times with dechlorinated water, placed in clean jars, and fed their normal diet of milkbone biscuits. Observations on their development were made to the adult stage.

A special control test consisted of 4th instar *A. atropalpus* larvae treated with a currently used mosquito larvicide, chlorpyrifos (dursban) (diethyl trichloropyridil phosphorothioate). The mosquito and insecticide were subjected to the same light conditions as the berberine-treated larvae.

Singlet Oxygen Measurement. The method of $^1O_2$ measurement was adapted from Ito (1978). Berberine (4.44×10^{-4} M) and 0.1 μl/ml 2,5-dimethyl furan (DMF) were incubated in 1 cm quartz cuvettes under two black light blue tubes (as above). Singlet O_2 generation was observed as the decrease in absorbance of DMF at 220 nm which is consumed in the reaction with $^1O_2$. An alpha-terthienyl (α-T) standard at the same concentration was used. Controls included direct irradiation of DMF without the sensitizer.

Chromosome Aberration Test. Chinese hamster ovary cells (DMO) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), antibiotics (streptomycin sulfate 29.6 μg/ml, penicillin "O" N.F. sodium 125μg/ml, kanamycin 100 μg/ml, fungizone 2.5 μg/ml), and 7.5% sodium bicarbonate (1 μg/ml).

The cells of stock cultures were grown in 240-ml plastic culture flasks (Falcon) at 37°C in water and resuspended in the fresh medium. For seeding, the suspension was diluted to an approximate density of 70,000 cells/ml. An aliquot (2 ml) of this dilution was seeded on each 22-m^2 coverslip in plastic dishes (Falcon 35 × 10 mm) and kept in MEM with 10% FCS at 37°C for 2 days (60–80% confluency).

Berberine was dissolved in 95% ethanol and diluted in MEM with 2.5% FCS. The ethanol concentration in the first dilution did not exceed 1%. Subsequent twofold dilutions were made and 1 ml of each was added to the Petri dishes after removing tissue culture medium. Tests were carried out in duplicate, one series being irradiated and the second being maintained in the dark.

Cultures were incubated in the dark for 30 min at 37°C and the series to be irradiated was placed under a bank of four black light blue UV lamps (max. 350 nm, 15 W/m^2) for 30 min.

After irradiation, test solutions were removed, the coverslips washed two

times with MEM, and fresh medium with 10% FCS was added to the Petri dishes. Samples were incubated for 16 hr. Four hours prior to the harvesting, the cells were pretreated with 0.2 ml of colchicine (0.01%) in 2.5% (MEM). Sodium citrate (1%) was used for 20 min during harvesting. Air-dried coverslips were stained with 2% acid orecin, mounted, and 100 metaphase plates were analyzed for chromosome breaks and exchanges.

RESULTS AND DISCUSSIONS

Insecticidal Activity. Larval, pupal, and adult survival of *A. atropalpus* was significantly affected following treatment with berberine (Figures 2–4). The toxicity of the alkaloid not only increased with concentration but was enhanced by the presence of near UV light (Figure 2) which was not the case in tests with chlorpyrifos, a nonphototoxic insecticide (data not shown). The LC_{50} for acute 24-hr toxicity of berberine with 3.5 W/m^2 near UV, was 8.8 ppm as opposed to 250 for dark exposure.

Larvae treated with 10 ppm berberine and 0.4 W/m^2 near UV for 24 hr and then transferred to a berberine-free medium exhibited chronic toxicity effects which resulted in significant cumulative mortality in the presence of near UV (Figure 3). At the pupal state there were further effects, the UV and berberine-exposed individuals metamorphosing into a lower cumulative number of pupae (Figure 4). The same situation could be observed at the adult stage (Figure 5). Because of the long depuration time of larvae in berberine-

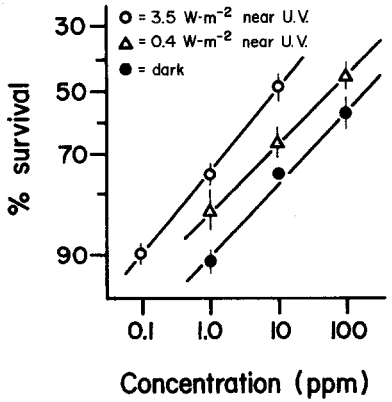


FIG. 2. Probit plots for the effect on 4th instar larvae of *Aedes atropalpus* of 24-hr berberine exposure in the dark (closed circles), exposure to berberine plus irradiation from 2 indoor sun lamps (triangles), or exposure to berberine plus irradiation from 2 indoor sun and 2 black light blue lamps (open circles). Bars represent standard errors.

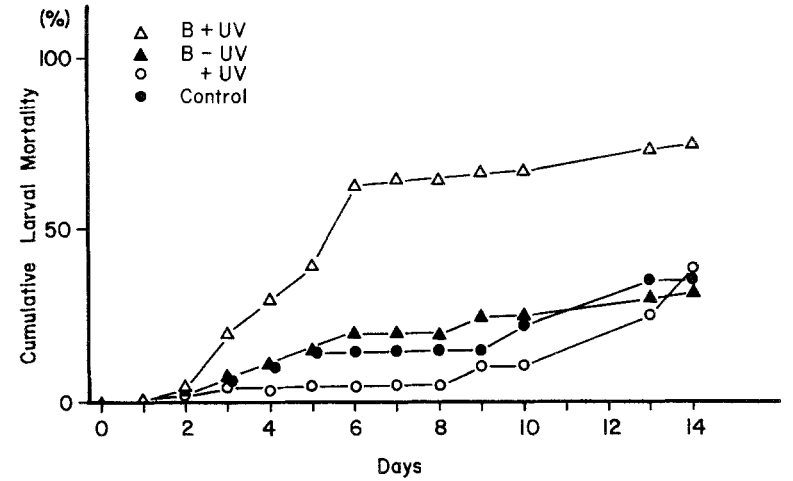


FIG. 3. Variation in cumulative larval mortality of *Aedes atropalpus* 4th instar larvae following 24-hr treatment with 10 ppm berberine plus exposure to near UV (B + UV) and subsequent transfer to berberine-free solution. Other treatments include 24 hr treatment with berberine but without UV exposure (B − UV), UV exposure without berberine treatment (UV), and a control with neither berberine nor UV exposure.

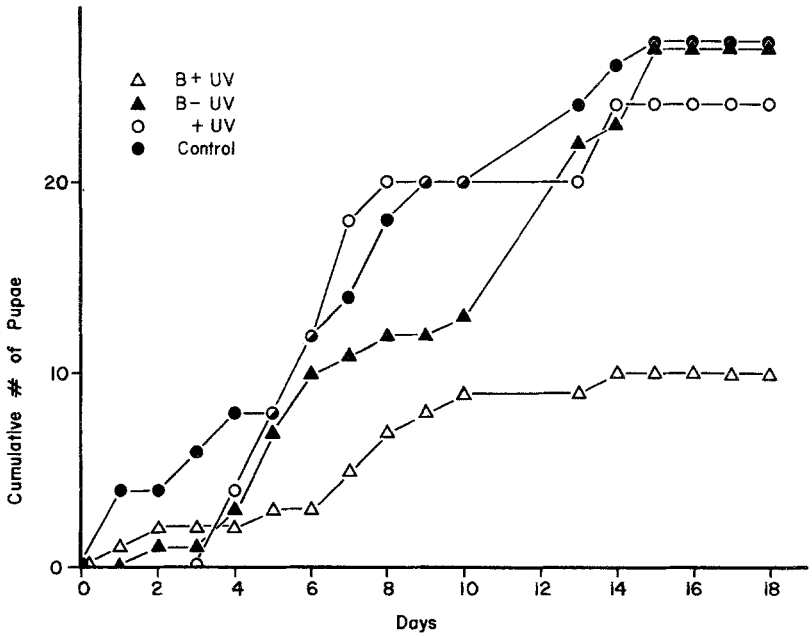


FIG. 4. Variation in the cumulative number of pupae produced from treatments described in Figure 3.

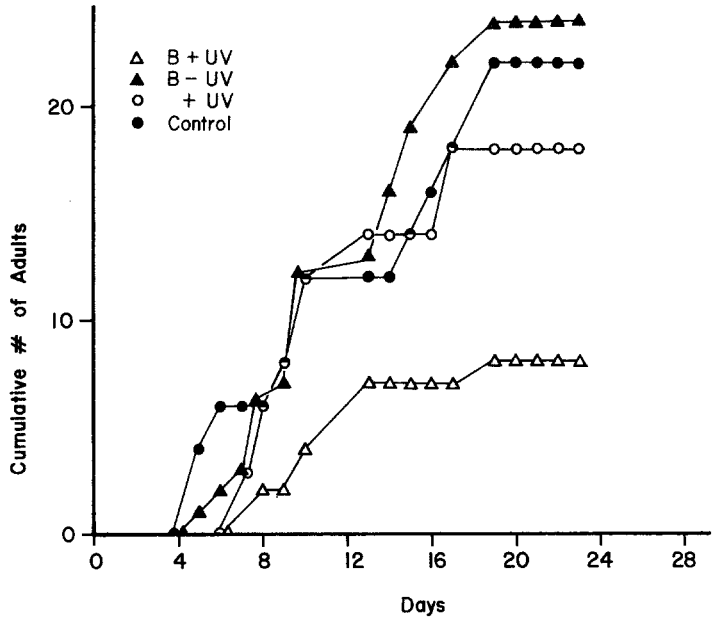


FIG. 5. Variation in the cumulative number of adults produced from treatments described in Figure 3.

free water, we believe there was very little toxic compound in contact with the mosquitoes beyond the larval stage. These results are suggestive of long-term carry-over effects following a brief initial exposure to berberine.

Mechanism of Action. The experiments with the singlet oxygen acceptor DMF demonstrated that berberine is a singlet oxygen generator. Initial rates of $^1O_2$ generation are linear with respect to DMF and a first-order rate constant was calculated (Figure 6). The value for berberine was $k = 2.1 \times 10^{-4}/S^1$, which is somewhat less than $k = 2.0 \times 10^{-2}/S^1$ determined for the potent phytodynamic sensitizer α-T (Arnason et al., 1981) at the same concentration. As the absorption band shapes and extinction coefficients are very similar, these values also reflect relative quantum yields for $^1O_2$ generation.

Slight cytogenetic damage was observed in the experiments in which berberine was used to photosensitize Chinese hamster cells. In the chromosome aberration test, near UV treatment slightly increased the number (from 0 to 0.05) of exchanges and breaks per metaphase plate and percent metaphase plates with chromosome aberrations (from 0 to 3.1) of a 50-ppm berberine treatment over the dark control. At higher concentrations berberine was toxic when irradiated, while at lower concentrations, no effect was observed (Table 1). This damage was considerably less than that created by dictamnine (Figure 1), a compound thought to form monofunctional adducts to DNA

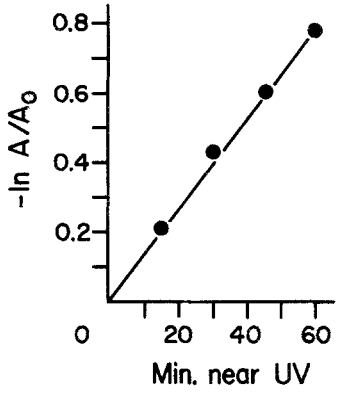


FIG. 6. Singlet O_2 generation by berberine irradiated with near UV as measured by the chemical trap DMF. The graph represents the first-order plot ($\ln A/A_0$) of the disappearance of DMF. Berberine concentration was 4.44×10^{-4} M and irradiation was provided by 2 black light blue tubes with a near UV intensity of 3.5 W/m^2.

(Pyffer et al., 1982) but greater than that observed with α-T (McCrae et al., 1981).

Other workers (Faddejeva et al., 1980; Maidi and Chauduri, 1981; Rungstitiyakorn et al., 1981) have recently shown that berberine intercalates DNA. This suggests that a possible mode of action for cytogenetic damage is the production of $^1O_2$ by berberine molecules bound to DNA, as is thought to be the case with the mutagen acridine orange (Ito, 1978). Further studies

TABLE 1. CHROMOSOME ABBERATION TEST WITH CHINESE HAMSTER CELLS TREATED WITH BERBERINE AND EXPOSED TO DARKNESS AND NEAR UV

		Exchange and breaks per metaphase plate		Percent metaphase plates with chromosome aberrations	
Compound	Conc. (ppm)	Near UV	Dark	Near UV	Dark
Berberine	100	T[a]	0.04	T[a]	2.30
	50	0.053	0	3.1	0
	25	0	0	0	0
Dictamnine	5	MI[b]	0	MI[b]	0
	2.5	2.7	0	82	0
	1.25	1.8	0	60	0

[a]toxic
[b]mitotic inhibition

are necessary, however, to ascertain the photodynamic and mutagenic properties of berberine.

Convergent Evolution. Secondary plant substances (SPS) have so far been studied primarily from the point of view of their phagostimulant or phagodeterrent effects on insects. Ecologists have been interested in the coevolution aspects (Gilbert, 1977; Zwölfer, 1978; Pesson, 1980) while physiologists have focused on the ability of insects to detect the compounds (Dethier, 1980). The current discovery of the phototoxicity of berberine, a widely distributed SPS, constitutes yet another example in the convergent evolution of the phototoxic protective mechanism in plants.

The biosynthetic routes leading to the synthesis of phototoxic substances are very diverse and include derivatization of lipids (polyacetylenes), phenolic biosynthesis from phenylalanine (hypericins, furanocoumarins), and transformation of several amino acids (isoquinoline, β-carboline, and furoquinoline alkaloids). In addition, these phototoxins are found in a very diverse group of plant families. The common phototoxicity of these metabolites would suggest a selective evolutionary advantage to this particular property. We submit that the absorption of light leads to a chemically excited state that is significantly more toxic (and hence protective) than interaction in the dark of secondary metabolites with targets in test species. Excited-state chemistry allows a variety of new processes to occur: covalent bond formation and electron and energy transfer processes. The ability of phytophagous insects to deal with the presence of such compounds will depend on the duration of their exposure to light, the opacity of their integument, and their ability to shield themselves from various wavelengths, particularly near UV.

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