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9. Solutions included sodium L-glutamate (0.2*M*; *p*H 7.0), sodium L-aspartate (0.2*M*; *p*H 7.0), acetylcholine chloride (0.2*M*; *p*H 4.5), and γ-aminobutyric acid (0.2*M*, *p*H 4.0).
10. The TRH (supplied by Abbott, Peninsula, and Beckman laboratories) was dissolved as a 10 m*M* solution in distilled water or as a 50 m*M* solution in 165 m*M* sodium chloride (*p*H 7.0).
11. Possible artifacts associated with the microiontophoresis of TRH were checked by the control application on all cells of an equivalent positive current through the sodium chloride micropipette channel. Sensitive neurons were excluded from the results. Automatic current balancing was also used in studies on certain cells.
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17. Acetylcholinesterase activity was measured by using the technique of G. L. Ellman, K. D. Courtney, V. Andres, R. L. Featherstone, *Biochem. Pharmacol.* **7**, 88 (1961). Protein concentration was estimated by using the procedure of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951), with bovine serum albumin being used as standard. A Cary 14 recording spectrophotometer was used for enzyme assay and optical density measurements.
18. The uptake of ^{14}C-labeled choline was carried out by suspending (for 10 minutes at room temperature) the P_2 fraction in Krebs-Ringer (KR) phosphate buffer with the following compositions: 124 m*M* NaCl, 5 m*M* KCl, 10 m*M* Na_2HPO_4, 1.2 m*M* KH_2PO_4, 1 m*M* $CaCl_2$, 1.3 m*M* $MgCl_2$, and 10 m*M* glucose (*p*H 7.4). The P_2 suspension was then aded to KR phosphate buffer containing the radioactive choline (28 m*M*) in the presence or absence of TRH (10 to 50 μg/ml). The final suspension was centrifuged immediately or following incubation at 37°C for 4 minutes. Pellets were dissolved in 1 percent sodium dodecyl sulfate; radioactivity was counted on a Packard 3320 tricarb liquid scintillation spectrometer, with Cannamix (Canatech Inc., Montreal) being used as scintillator.
19. Release studies involved several steps. First, the P_2 suspension was labeled with radioactive choline as described in (*18*), except that the final choline concentration was 1 μ*M* and the incubation was for 15 minutes at 37°C. Then the [^{3}H]choline-labeled P_2 suspension was separated from the supernatant by centrifugation, washed with buffer, and resuspended in KR phosphate buffer. Equal portions of [^{3}H]choline-labeled P_2 suspension were pipetted into KR buffer and concentrated potassium buffer (final KCl concentration, 40 m*M*). Finally, suspensions in both KR and potassium buffers were incubated at 37°C for 10 minutes and centrifuged, after which the radioactivity in the supernatant was counted.
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Acridine Araphanes: A New Class of Probe Molecules for Biological Systems

Abstract. *The bis-acridine ring system forms the basis for new biophysical probes of novel stereochemistry. Spectral data indicate that certain alkylene bridged bis-9-aminoacridines have a parallel plane conformation of predictable interplane distance. The parallel plane conformation is independent of solvent and thus is different from nucleic acid systems. This stable conformation allows these compounds to be used as sensitive "rulers" for describing binding site geometry in cholinergic enzymes and in the delineation of the mechanism of allosteric control in acetylcholinesterase.*

As a part of a study of cholinergic neural systems, a series of alkylene bridged bis-9-aminoacridines were prepared by the method of Chen *et al.* (*1*), and were found to exhibit major spectral anomalies, which were related to bridge length (*n*) (Fig. 1). They suggested a conformation change from an open (coplanar) conformation ($n > 4$) to a parallel plane conformation when $n = 2, 3$ (or 4).

Parallel plane conformations are known to exist when aromatic molecules form concentration dimers, stacks, or excimers in concentrated solution, or in oriented polymers such as DNA, base pair systems, and in bridged bis-adenines in water solution (*2–4*). In these cases, the parallel plane conformation is either concentration dependent or stable only in the presence of water, and disappears on dilution. DNA base pairs and bridged bis-adenines are denatured or lose spectral effects (hypochromatism), when dissolved in ethanol (*4*).

Spectral data indicated that the parallel plane conformation in alkylene bridged bis-9-aminoacridines was independent of the nature of the solvent or dilution. Nuclear magnetic resonance (NMR) spectra indicated a dynamic rather than a rigid conformation. The diversity and predictability of dynamic conformations, independent of solvent, suggest that this series of singly bridged bis-acridines are new probes for biological systems. We report data from four spectral methods and uses of a parallel plane, molecular probe in the study of the active site stereochemistry of acetylcholinesterase (E.C. 3.1.1.7).

The bis-acridine series have only a single bridge; they have the potential for a preferred parallel plane conformation. They exist in that conformation in aqueous or organic solvents ($10^{-6}M$) only when the total interplanar forces are sufficient to constrain the movement of the two rings in relation to each other. The parallel plane conformation was identified as follows.

In water and methanol, molecules having a parallel plane conformation manifest three distinct differences in their ultraviolet absorption spectra from the spectra of their parent 9-alkylaminoacridine. (i) The complex three-peak structure at 410 nm (n-π^*) is lost to a broad single peak in the related parallel plane structure. (ii) The strongly absorbant π–π^* transition peak at 268 nm shows a progressively increasing blue shift with decreasing bridge length. (iii) The shift of the π–π^* absorption is accompanied by a progressive decrease in extinction coefficient (Fig. 2). Similar progressive abnormalities are noted in the fluorescence spectra of these compounds. (i) The emission, which consists of a lone, broad peak at 486 nm, is diminished and replaced by a series of two peaks at a shorter wavelength in the bridged bis-acridines, which exist in a parallel plane conformation. A red shift occurs in the emission maximum as solvent polarity decreases. (ii) There is also a decrease in quantum yield as the bridge length shortens between the two rings, stemming from the transannular electronic effects that occur when two aromatic moieties are in close proximity and in parallel planes.

Absorption and fluorescence spectra of parallel plane molecules in highly polar solvents correspond to the spectra of simple 9-alkylaminoacridines in nonpolar solvents. This effect indicates that bis-acridines in which the rings are parallel have a hydrophobic environment between the rings, in aqueous solution (*5*).

The spectral data described above are consistent with a transition from a parallel plane conformation when $n = 2$ or 3 to a coplanar conformation when n is 6 or greater, with a time resolved average parallel conformation observed when $n = 4$.

Proton NMR spectral data (deuterated dimethylsulfoxide) were consistent with conclusions derived from ultraviolet and fluorescence data when the number of bridge carbons is two. When $n = 3$, the increased distance limited NMR-resolvable effects. However, the bis-acridines identified as open (coplanar) conforma-

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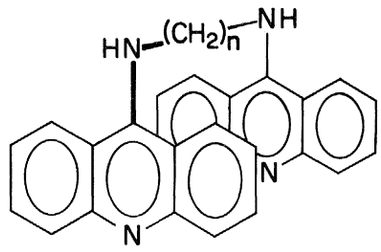

Fig. 1. Structure of alkylene bridged bis-9-aminoacridine.

tion molecules had a proton NMR spectra which corresponded to that of 9-alkylaminoacridines, whereas those identified with a parallel plane conformation exhibited marked changes in the aromatic region when the rings were within an estimated interplanar distance of 3.5 Å. These changes take the form of spin-spin splitting rearrangements that are anomalous to the typical 9-aminoacridine pattern. Such abnormalities are seen as a series of multiplets which replace the familiar doublet (8.4 ppm), doublet (7.9 ppm), triplet (7.5 ppm), triplet (7.3 ppm) sequence (*6*). In the parallel conformation, the induced magnetic lines of force that project perpendicularly from the plane of the aromatic ring interfere with one another. The transannular magnetic field interactions exist when the neighboring acridine rings are parallel and in motion with respect to each other. Carbon-13 NMR spectra were obtained from identical solvent-reference systems as with the proton spectra. Coupled spectra obtained in the gated I mode indicate a tendency for the quaternary 9, 11:12, and 13:14 carbons to broaden, and indicate also a breakdown of splitting patterns of the methine carbons in the acridine ring to occur because of the enhanced transannular magnetic interactions associated with parallel conformation.

There has apparently been no prior recognition that two aromatic rings joined by a single bridge linked by four to six atoms can exist in a preferred parallel plane conformation, independent of any stabilization by water, and that there is a progressive, dynamic transition to a preferred coplanar conformation as the bridge length increases. These new bis-aromatic systems are stereochemically related to the parallel plane conformational constraints in bis-aromatic cyclophanes (*7*). Because of the similarity between such bis-aromatic cyclophanes and singly bridged bis-aromatic molecules related to Fig. 1, we suggest the term araphanes for this new class of parallel plane molecules.

The araphane ethylene-bis-9-aminoacridine (Fig. 1, $n = 2$) acts as a stereochemical probe for the topology and subsites of acetylcholine esterase (AChE) and as a probe of the mechanism of allosteric control in this enzyme. That mechanism and the existence of a separate allosteric subsite represents major controversy (*8–10*). Use of the araphane probe provides additional evidence for the existence of a separate allosteric subsite in AChE, and a measure of the steric constraints in the anionic subsite.

Biophysical probe data were obtained in 0.113 ionic strength buffer at *p*H 7, by kinetic and fluorescence measurements as follows. (i) Kinetics obtained from typical Lineweaver-Burk plots with the Ellman (*11*) assay method for AChE activity were constructed for the hydrolysis of ACh by AChE when inhibited by araphane. Pure noncompetitive kinetics of inhibition showed that the araphane did not bind at the anionic subsite and exercised its rate-moderating function from an "exo" subsite (*12*). Typical cationic inhibitors of AChE have a characteristic kinetic mixture of competitive and noncompetitive components (mixed inhibition) (*9*). The competitive component stems from binding of the inhibitor at the anionic subsite. The absence of araphane binding at the anionic subsite suggests that the lateral dimensions of the anionic cleft in AChE are less than the lateral dimensions of the araphane (6 to 7 Å) (*13*). (ii) Double inhibitor studies involving fluorescence measurements (*12*) showed that when the araphane was added to a solution containing *N*-methylacridinium reversibly complexed with AChE, substantial *N*-methylacridinium fluorescence was regained. As evidence in (i) has shown, the araphane does not compete at the anionic subsite, therefore the release of bound *N*-methylacridinium by the araphane stems from competition at the secondary allosteric subsite whose binding with *N*-methylacridinium has been described (*7*). (iii) The fluorescent *O,O*-diethylphosphate of *N*-hydroxynaphthalimide (Maretin) is strongly bound at the anionic subsite of AChE during phosphorylation of the enzyme (*7*). Figure 3 shows the second-order kinetics of that reaction and that addition of an inhibition concentration (I_{50}) of *N*-methylacridinium inhibits the phosphorylation by competition at the anionic subsite. Addition of araphane at its I_{50} concentration has no effect on the Maretin-AChE kinetics (Fig. 3). This is additional evidence that the araphane competes only at the allosteric subsite and exerts its rate-moderating effect from that "exo" (allosteric) subsite. The prevailing viewpoint has been that the rate-moderating step in AChE involves binding of inhibitor cation at the anionic subsite of the acylated enzyme (*9*).

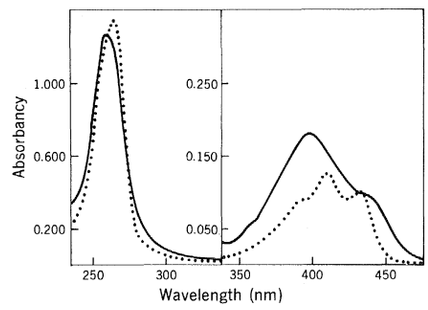

Fig. 2. Ultraviolet visible spectra of the parallel plane 1,2-propylene-bis-9,9′-amino-acridine (solid line) and the coplanar 1,6-*n*-hexylene-bis-9-9′-aminoacridine (dotted line).

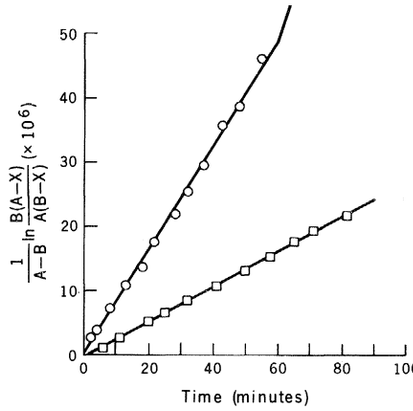

Fig. 3. Second-order reaction equation for the phosphorylation of AChE by Maretin. *A* is the initial Maretin concentration in equivalents ($3.0 \times 10^{-8} M$) and *B* is the initial enzyme concentration ($1.4 \times 10^{-8} N$). *X* indicates the number of equivalents of phosphorylated AChE formed at a given time (from fluorescence assay). ○—○, AChE phosphorylation reaction (no inhibitor added); □—□, AChE phosphorylation in the presence of an I_{50} concentration ($2.5 \times 10^{-7} M$) of *N*-methylacridinium iodide. An I_{50} concentration of the parallel plane alkylene bridged bis-acridines ($2.0 \times 10^{-7} M$) resulted in no significant change from the uninhibited Maretin phosphorylation rate.

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Changes in Carbon Fixation, Tuberization, and Growth Induced by CO_2 Applications to the Root Zone of Potato Plants

Abstract. *The root systems of potato plants* (Solanum tuberosum *L. var. Russet Burbank) treated with CO_2 for 12 hours showed an increase in dry matter as early as 2 days after the treatment. When treated plants were allowed to grow for 3 to 6 weeks there was a substantial increase in tuberization. In addition, there was an increase in stolon length, number of tubers per stolon, and overall dry weight after the enrichment of the root zone with CO_2. Plants treated with CO_2 showed higher concentrations of malic and citric acids and of the cations Mg^{2+} and Ca^{2+}. The effect of CO_2 was more dramatic when CO_2 was applied to the root zone than when it was applied to the shoots.*

Terrestrial plants have been credited with fixing about 25 billion metric tons of CO_2 per year (*1*). Atmospheric CO_2 concentrations appear to be limiting with respect to photosynthesis, because most C_3 plants (*2*) are capable of significantly greater rates of CO_2 fixation in the presence of higher concentrations of CO_2 than are present in ambient air. Carbon dioxide enrichment of the air increases yields of greenhouse-grown vegetable and flower crops (*3*, *4*), but CO_2 enrichment of the air for field crops would not be practical. We have investigated the feasibility of increasing plant productivity by applying CO_2 to underground plant parts.

The response of plants to high concentrations of CO_2 depends on the species. The root growth of *Pisum sativum*, *Vicia faba*, *Phaseolus vulgaris*, and *Helianthus annua* were completely inhibited by high CO_2, whereas *Avena sativa* and *Hordeum vulgare* were unaffected (*5*). Potato plants are particularly well suited to CO_2 enrichment of the root zone because of their ability to tolerate high concentrations of CO_2 without the roots being damaged.

Potato plants were propagated by taking 1-cm plugs from the "eye" area of the tuber (*Solanum tuberosum* L. var. Russet Burbank). The plugs were arranged in holes bored into Styrofoam sheets and floated on water with continuous aeration in the dark for 2 weeks. When sprouts were 6 to 8 cm in length, they were detached from the plug and surface-sterilized in 10 percent sodium hypochlorite solution. The sterilized plants were transferred to 3-liter containers with full-strength Hoagland nutrient solution (*6*) and kept in a growth chamber under 16 hours of light (21.1°C) and 8 hours of darkness (15.6°C). Containers were covered with black polyethylene (5 mil in thickness) to keep light (which would inhibit tuberization) from reaching the bottom portion of the plant. Plants were grown for 3 weeks with continuous aeration. Nutrient solutions were changed weekly. After the 3-week growing period, uniform plants were selected for each treatment. The root zones of potato plants were aerated for varying time intervals with a gas stream that was 45 percent CO_2 with 21 percent O_2 and 34 percent N_2. Plant roots were immersed in aerated nutrient solution while stolons and tubers were suspended above the solution. Carbon dioxide enrichment was restricted to the root zones by isolating the roots in an airtight chamber with an outlet attached to a 25 percent sodium hydroxide trap for escaping CO_2, and the nutrient solution was maintained at a constant *p*H (5.5). All CO_2 experiments were conducted when the shoots were exposed to the light unless otherwise stated. We found that the 12-hour treatment period showed the best results with respect to dry matter increase and also the tuberization response.

Plants that had CO_2 applied to the roots showed a significant increase in shoot dry weight as early as 2 to 6 days after the treatment (Table 1). When plants were allowed to grow for 3 to 6 weeks after the CO_2 treatment, there was also a significant increase in the underground biomass (Fig. 1 and Tables 2 and 3). Three weeks after the 12-hour CO_2 treatment, stolons of the treated plants were not only longer than untreated controls, but also had multiple tubers as opposed to the single tubers per stolon typical of controls (Fig. 1 and Table 2).

Table 3 shows that root zone enrichment with CO_2 enhanced shoot weight. When potato shoots were treated with

Table 1. Effects of CO_2 enrichment of the root zone on dry matter content, organic acid, total chlorophyll, and mineral levels in nontuberizing potato plants. The CO_2 (45 percent CO_2, 21 percent O_2, and 34 percent N_2) was applied for 12 hours, at 2, 4, or 6 days before the plants were harvested. Control plants received ambient air. All plants were the same age at the time they were harvested. All values are expressed on a dry weight basis (± standard error).

Days after CO_2 treatment	Plant dry weight (g)	Malic acid (mg/g)		Citric acid (mg/g)		Minerals* (total)					Total chlorophyll (mg/g)
		Leaf	Root	Leaf	Root	Ca^{2+} (mg/g)	Mg^{2+} (mg/g)	Mn^{2+} (μg/g)	K^+ (mg/g)	Cl^- (μg/g)	
Control	10.6 ± 2.3	9.2 ± 1.6	0.14 ± 0.005	4.3 ± 1.7	0.05 ± 0.003	43.7 ± 4.1	15.4 ± 0.25	2.5 ± 0.02	64.2 ± 1.6	928.4 ± 30.0	9.0 ± 0.46
2	14.9 ± 2.1	11.9 ± 0.63	1.4 ± 0.03	7.9 ± 2.3	0.29 ± 0.11	63.3 ± 4.9	12.5 ± 0.22	2.0 ± 0.01	61.0 ± 1.5	711.8 ± 26.5	9.7 ± 0.73
4	19.8 ± 4.5	15.2 ± 0.54	2.9 ± 0.86	10.9 ± 0.65	0.62 ± 0.28	76.8 ± 5.6	19.5 ± 0.28	2.1 ± 0.01	63.9 ± 1.5	681.3 ± 25.6	9.8 ± 0.95
6	25.8 ± 1.8	14.4 ± 0.29	3.9 ± 0.75	11.2 ± 0.38	1.1 ± 0.54	91.5 ± 6.2	18.4 ± 0.24	1.6 ± 0.01	69.7 ± 1.6	691.4 ± 25.8	12.7 ± 0.78

*Calcium, magnesium, and potassium ions were measured in milligrams, and manganese and chloride ions were measured in micrograms per gram of dry weight.

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