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Rapid Measurement of Toxicity Using Electrochromic Dyes and Frog Embryos

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The membrane potential of a cell is affected by the ionic concentrations inside and outside of the cell and by the carriers and ion pumps located in the cell membrane. If any are disrupted, the membrane potential across the cell will change. One way to measure the membrane potential of many cells is to use a fluorescent dye, usually termed an electrochromic dye, to measure the membrane potential of cells. Di-4-Anepps is a styryl dye that is apparently interspersed in the membrane leaflet. Di-4-Anepps changes fluorescence directly in response to changes in membrane potential (Fluher et al 1985).

Because alteration in a membrane pump or ion channels could explain some toxicity at the cellular level (Blankemeyer and Hefler 1990; Blankemeyer and Bowerman 1992), we examined the effect of 6-aminonicotinamide (6-AN) on the membrane potential of embryos of the South African Clawed Frog, Xenopus laevis. We chose 6-AN because the EC50 for malformation differs greatly from the LC50 (Dawson et al 1989). We hope to discern whether the response of the embryonic membrane potential, measured by fluorescent dye, predicts the EC50 or LC50 as determined by FETAX.

FETAX-Frog Embryo Teratogenicity Assay-Xenopus was developed as an environmental bioindicator for the presence of toxicant in water (Dumont et al 1983). FETAX has been useful in assessing the developmental, environmental, and genetic changes wrought by toxic substances (Bantle et al 1990). FETAX uses 96-hr endpoints of overall length, survival, and malformation to assess the effect of chemical compounds. A teratogenic index is calculated by dividing the LC50 (survival) concentration by the EC50 (malformation) concentration. The teratogenic index

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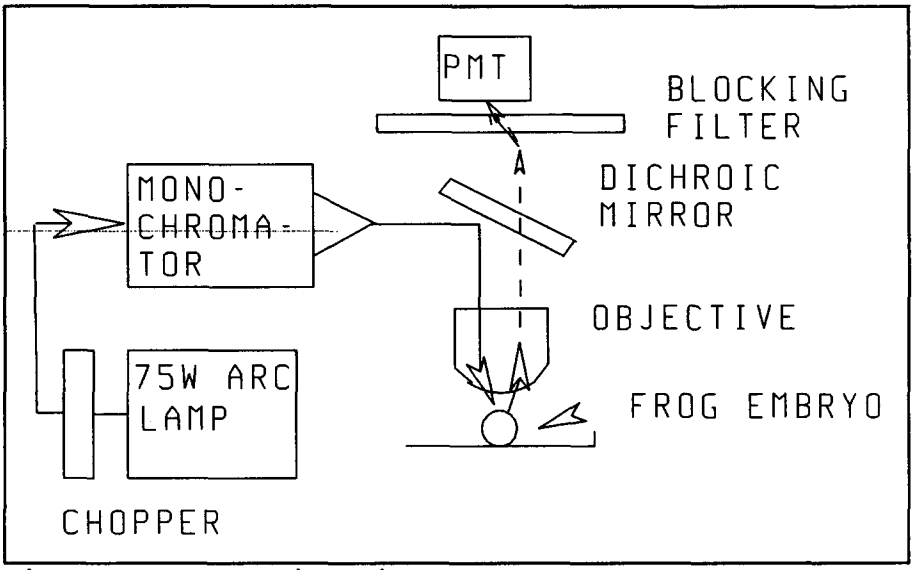

Figure 1. Syntopic diagram of embryo fluorescence instrument including monochromators, microscope, embryo, and photomultiplier tube. Line represents light path.

(TI) gives a measure of the teratogenicity of a chemical relative to its lethal concentration.

MATERIALS AND METHODS

Albino Xenopus laevis frogs were purchased from Xenopus-I(Ann Arbor, Michigan). Breeding pairs were conditioned for 1 mo to 6 wk prior to usage. Twelve hours prior to amplexus both male and female frogs were injected with Human Chorionic Gonadotropin (Sigma, St. Louis). Following successful amplexus, eggs were collected, dejellied with 2% (w/v) cysteine, adjusted to pH 8.1, and separated into viable and non-viable groups using a dissecting microscope. Twenty mid-to-late blastula stage embryos were selected; viable embryos were collected in covered plastic petri dishes. Embryos were maintained in FETAX solution which contained 10.8 Mm NaCl, 1.2 Mm $NaHCO_3$, 0.58 Mm $MgSO_4$, 0.44 Mm $CaSO_4$, 0.4mM KCl, and 0.14 Mm $CaCl_2$ and was at Ph 8. Valinomycin, gramicidin, and 6-aminonicotinamide were purchased from Sigma (St. Louis, Missouri) and dissolved into FETAX solution without a carrier solvent. Di-4-Anepps was purchased from Molecular Probes (Eugene, Oregon).

Each concentration of 6-AN was tested with at least three groups of embryos. Each set of experimental embryos was compared against a control of 20 embryos co-cultured with the experimental embryos. 6-AN was added to the petri dish containing the embryos at the

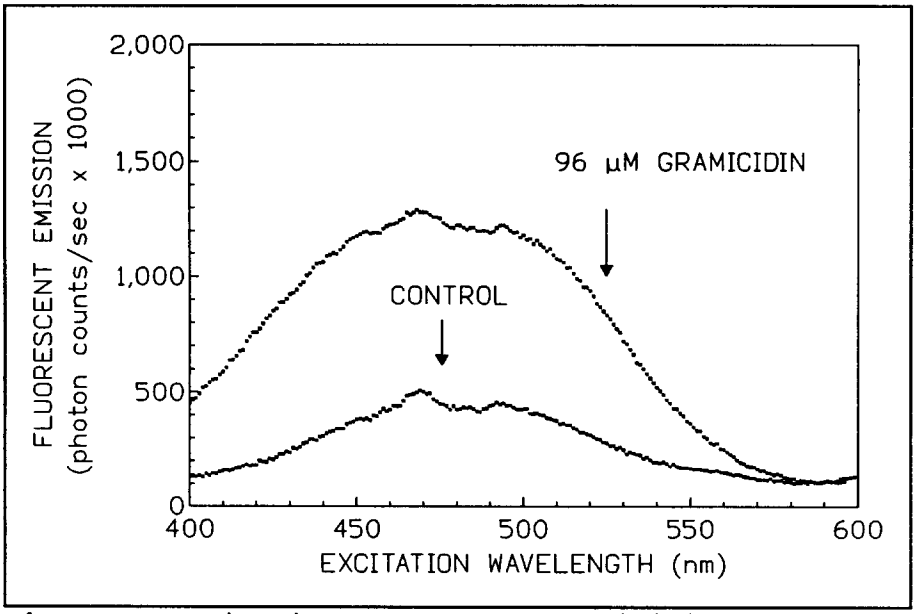

Figure 2. Excitation spectrum of Gramicidin and control embryos

same time as the styryl dye Di-4-Anepps. The final concentration of Di-4-Anepps was nominally 10^{-6} M and was identically concentrated in control and experimental petri dishes. After 30 min of exposure to Di-4-Anepps, and to 6-AN for the experimental, the embryos were placed on a microscope well-slide and the fluorescence data collected. The embryos were excited with 480 nm light from a PTI DeltaScan (Photon Technology Inc., Princeton, New Jersey) monochromator-based excitation source. The excitation light passed through a dichroic mirror and microscope objective (see Figure 1) . The reflected excitation light and the fluorescence from the embryos returned through the objective. The excitation light was removed by a high-pass optical filter (580 nm). The fluorescence was measured by a photomultiplier tube operating in photon-counting mode for increased sensitivity. The experimental fluorescence counts were divided by the control fluorescence counts to normalize the data for the age and condition of the embryos.

Statistical analysis of the data was performed with GraphPad (San Diego, California). EC50's were determined using the 50 % point of the maximum effect of the chemical. Concentration-response curves were fitted by non-linear regression techniques using iteration to find the best fit employing a sigmoidal model.

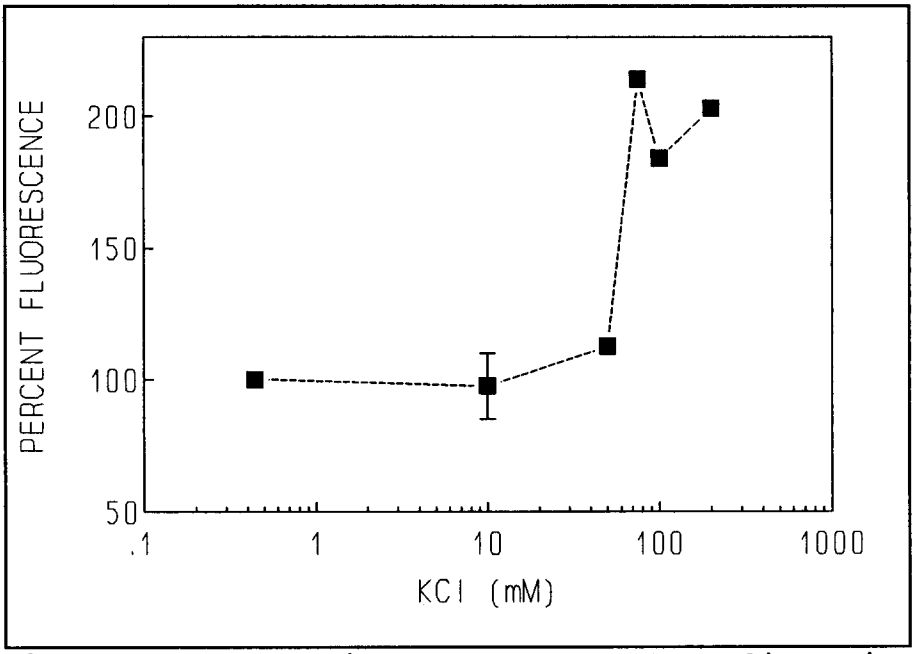

Figure 3. Concentration response plot for valinomycin-treated embryos. Concentration of valinomycin was 20 μM.

RESULTS AND DISCUSSION

Although the relationship of Di-4-Anepps fluorescence to membrane potential has been developed using liposomes, we extended the voltage calibration to frog embryos. Figure 2 illustrates the results of an experiment to calibrate the response of the embryos to gramicidin, an ionophore. Gramicidin forms large cation-selective channels that make the cell membrane permeable to any cation in the bath solution. The membrane potential will be near zero volts when gramicidin is applied. In Figure 2, the fluorescence of a gramicidin-treated embryo, loaded with Di-4-Anepps, is plotted against excitation wavelength. Also plotted in Figure 2 is the fluorescence of a control embryo, loaded only with Di-4-Anepps. Figure 2 shows that the fluorescence of Di-4-Anepps, loaded into the embryos, increases when gramicidin is added. Since the normal membrane potential of the embryo cells is negative, the increase in fluorescence represents a depolarization of the embryonic cells. When gramicidin was mixed with Di-4-Anepps(without embryos), the fluorescence did not increase above the background fluorescence of the Di-4-Anepps.

Valinomycin is a potassium-selective ionophore that makes the membrane permeable to potassium. When 20 μM

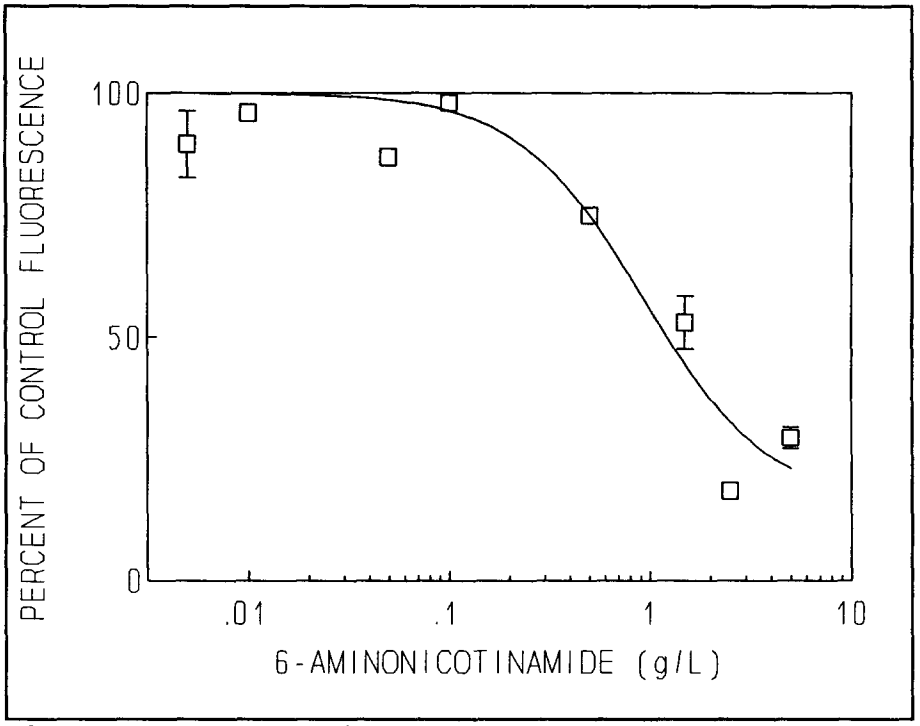

Figure 4. Concentration response of fluorescence versus 6-AN concentration

valinomycin was mixed with 1 μM Di-4-Anepps and excited by 480 nm illumination, the fluorescence did not increase above the background fluorescence of the Di-4-Anepps. The cell membrane potential is a function of the ion concentrations inside and outside the cell membrane and any electrogenic ion transport. The membrane potential of a cell treated with ionophore permeable to only one ion has a membrane potential predicted by the Nernst equation. When various concentrations of potassium are in the bath solution with valinomycin, the cell membrane potential can be calculated by using the Nernst equation for potassium:

$$V_{i-o} = \frac{R*T}{F} * \ln \frac{K_o}{K_i}$$

where:

V_{i-o}	is the membrane potential referenced to the outside of the embryo (Volts).
R	is the gas constant (8.314 Joules $^{\circ}K^{-1}$ mol^{-1}).

T	is the temperature in degrees Kelvin (°K).
F	is Faraday's constant (96,486 Coulombs mol^{-1}).
K_o	is potassium activity outside the embryo (molar).
K_i	is the intracellular K activity (molar).

In the experiment reported in Figure 3, various concentrations of potassium with 20 μM valinomycin were used to calibrate the fluorescent response of the frog embryos. Note that the fluorescence decreased as the bath potassium decreased showing that the decrease in fluorescence represents a hyperpolarization of the membrane potential. The data in Figures 2 and 3 are a calibration curve for the embryos and present a framework to estimate the effect of toxicants on the embryonic membrane potentials.

Figure 4 represents the results of several trials with the teratogen 6-aminonicotinamide. Note that as the concentration of 6-aminonicotinamide increased, the fluorescence from the dye Di-4-Anepps decreased. We interpret this result, based on the calibration in Figures 2 and 3, to mean that 6-AN causes a hyperpolarization of the embryo's membrane potential. Higher concentrations of 6-AN produced greater hyperpolarization.

The data in Figures 2 and 3 demonstrate that the response of the embryos to standard ionophores is predictable and consistent with literature reports on Di-4-Anepps. Although there is still a possibility of direct dye interaction with the ionophores wherein the interaction is mediated by the embryo, our control experiments with Di-4-Anepps and both gramicidin and valinomycin suggest that the dye is detecting the membrane potential of embryonic cells. The data in Figure 4 show that the teratogen 6-aminonicotinamide effected a response in membrane potential with an EC50 at 0.9 g/L. Since the reported 96 hr LC50 for 6-AN is 3 g/L in FETAX whereas the EC50 for malformation is 5.5 mg/L (Dawson et al 1989), we conclude that the membrane potential EC50 is predictive of the lethal effect of 6-AN rather than the teratogenic effect of 6-AN. Thus EC50 from the membrane potential assay using blastula-stage embryos and requiring a thirty minute assay is near the LC50 of 6-AN on 96 hr frog embryos. We can speculate that membrane potential is a

bioindicator signaling the effect of toxicant on frog embryos.

The membrane potential assay is part of a bioassay named CHAWQ- Cell Health Assay of Water Quality. CHAWQ uses cellular bioindicators with quantitative endpoints to rapidly assess water quality through optical transduction of bioindicators. The assay can be used in water quality testing, assessment in bioremediation and toxicity reduction, and in determination of mode of action of toxicants. Validation of the membrane potential bioassay and the other bioindicators of CHAWQ is in progress.

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