Folic Acid Protects against Potato Glycoalkaloid α-Chaconine-Induced Disruption of Frog Embryo Cell Membranes and Developmental Toxicity

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To demonstrate whether folic acid can protect *Xenopus* embryos against reported adverse effects of the potato glycoalkaloid α-chaconine, the frog embryos were exposed simultaneously to the glycoalkaloid, folic acid (pteroylglutamic acid), and an electrochromic fluorescent dye, Di-4-ANEPPS, in a specially designed instrument that measures embryonic membrane potential. Folic acid decreased the chaconine-induced fluorescence, with a maximum decrease occurring at about 10 mg/L of both folic acid and the glycoalkaloid dissolved in solution. The protective effect was also operative in the frog embryo teratogenesis assay—*Xenopus* (FETAX), in which survival and teratogenicity of the whole embryos were the endpoints. Possible mechanisms of the protective effect and the possible significance of the results to food safety and health are discussed.

Keywords: *α-Chaconine; folic acid; food safety; frog embryos; glycoalkaloids; membrane potential; potatoes; teratogenicity*

INTRODUCTION

The membrane potential of a cell is affected by ionic concentrations inside and outside the cell and by the permeabilities of the carrier and ion pumps located in or near the cell membranes. One way to measure the membrane potential is to use a fluorescent electrochromic dye. Di-4-ANEPPS is a dialkylaminostyryl pyridiniumsulfonate dye with positive and negative charges (Figure 1a). It is interspersed in the membrane leaflet and changes fluorescence directly in response to changes in membrane potential (Fluher et al., 1985). Glycoalkaloids are nitrogen-containing steroidal glycosides synthesized in numerous plants species (Friedman and McDonald, 1997). Previous studies have described (a) changes in membrane potential in embryos of the South African clawed frog, *Xenopus laevis*, induced by glycoalkaloids such as α-chaconine (Figure 1b); (b) α-chaconine-induced reduction in transepithelial active transport of sodium, as measured by changes in interstitial short-circuit current (ISC) of frog skin; and (c) protective effects of glucose 6-phosphate and nicotine adenine dinucleotide phosphate (NADP) against α-chaconine-induced malformations and mortality in the frog embryo teratogenesis assay—*Xenopus* (FETAX) (Blankemeyer et al., 1992, 1995; Rayburn et al., 1995).

Since data showed that the membrane potential, as measured by Di-4-ANEPPS, changed rapidly in the presence of α-chaconine, with a concentration dependence similar to that of the full-scale FETAX assay in which this compound induced gut and brain malformations (Friedman et al., 1991), it was concluded that the cellular mechanisms involving membrane disruption are causal to the effects observed in the assay. These considerations and the reported protective effect of folic acid (Figure 1c) against neural tube defects in human fetuses (Milunski, 1996; Oakley et al, 1996; Rieder, 1994) prompted us to investigate whether folic acid would protect frog embryo membranes and organs against α-chaconine-induced changes in membrane potential and embryo malformations. The results extend earlier work on protective effects of certain compounds against chaconine-induced damage to *Xenopus* embryos.

MATERIALS AND METHODS

α-Chaconine (99%+ purity) was isolated from NDA-1725 potato sprouts (Friedman et al., 1993). Di-4-ANEPPS was obtained from Molecular Probes, Eugene, OR, and folic acid from Sigma, St. Louis, MO.

Albino *X. laevis* frogs were purchased from Xenopus-I (Ann Arbor, MI). Breeding pairs were conditioned for 1 month to 6 weeks prior to usage. Four hours prior to mating, the frogs were injected with human chorionic gonadotropin (Sigma). Following successful amplexus, eggs were collected, dejellied with 2% (w/v) cysteine adjusted to pH 8.1, and separated into viable and nonviable groups. Mid-to-late blastula to neurula stage embryos were selected under a dissecting microscope. Viable embryos were collected in covered plastic dishes. Embryos were maintained in pH 8 FETAX solution, which contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.44 mM $CaSO_4$, 0.4 mM KCl, and 0.14 mM $CaCl_2$. Addition of the maximum amount of folic acid used in this study to the FETAX solution did not affect the pH at the highest concentration of folic acid used.

The setup for measuring fluorescence emanating from embryos loaded with Di-4-ANEPPS was adapted from that of Blankemeyer et al. (1992). Each concentration of α-chaconine was tested with at least three groups of 50 embryos. Each set of experimental embryos was compared against three control groups of 50 embryos. The test compounds were dissolved in FETAX solution, which was then added to the Petri dish containing the embryos at the same time as the dye (10^{-6} M). After 30 min, the embryos were placed in a cuvette and the fluorescence intensity was measured.

Figure 2 shows the setup for measuring of fluorescence emanating from embryos loaded with Di-4-ANEPPS. A 75-W xenon arc lamp (USHIO) provided the light source. The collimated beam from the xenon lamp passed from a collimating and focusing mirror to a monochromator set at 490 nm. The 490-nm excitation passed through a dichroic mirror and

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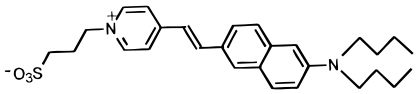
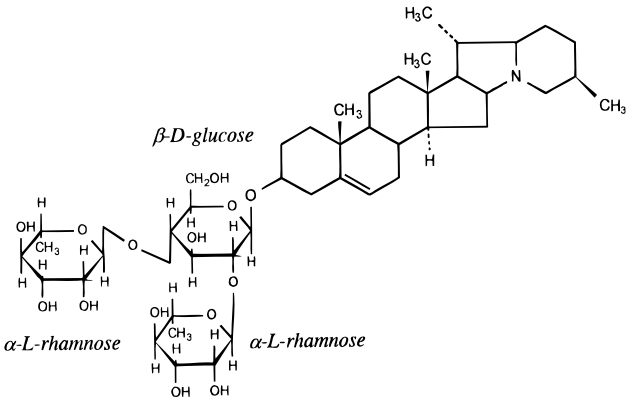
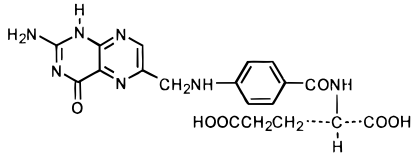


(a) Di-4-ANEPPS



(b) α-CHACONINE



(c) FOLIC ACID

Figure 1. Structures of (a) fluorescent dye Di-4-ANEPPS, (b) α-chaconine, and (c) folic acid.

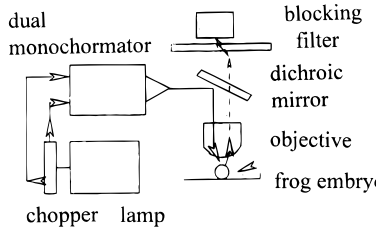


Figure 2. Schematic of apparatus used to assay frog embryo cell membranes.

was reflected through a microscope objective unto the embryo. Reflected light and fluorescent emission from the embryo returned through the objective. The reflected 490-nm light was filtered out by a dichroic mirror and a blocking filter (590-nm high pass). The intensity of fluorescent emission was measured by a photomultiplier tube operating at 900 V in a photon-counting mode. The photon count was divided by 10 and collected via a module attached to a computer (Photon Technology Inc., Princeton, NJ). The emission data (in counts per second; Figure 3) were collected to a disk and fitted by linear regression over the sample period (20 s). The calculated value for the midpoint in the time-based record was used as the emission intensity. The fluorescence was corrected by dividing the experimental counts by the control embryo counts and expressed as a percentage.

Embryo survival and teratogenicity in the presence of α-chaconine and α-chaconine plus folic acid were estimated by the FETAX assay (ASTM, 1991; Bantle et al., 1991; Friedman et al., 1991). Statistical methods used are described in SAS (1987).

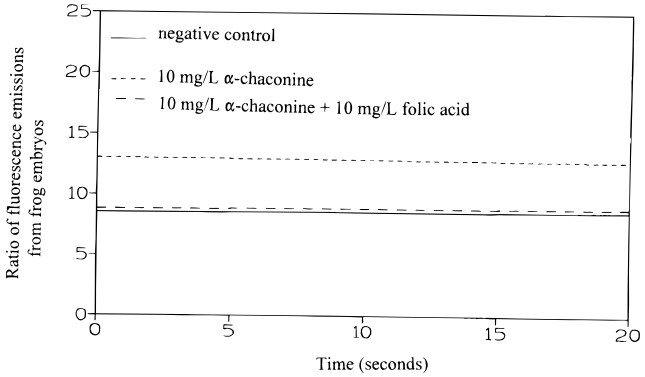


Figure 3. Plot of a time course experiment of fluorescence emission comparing the effect of α-chaconine to that of α-chaconine plus folic acid.

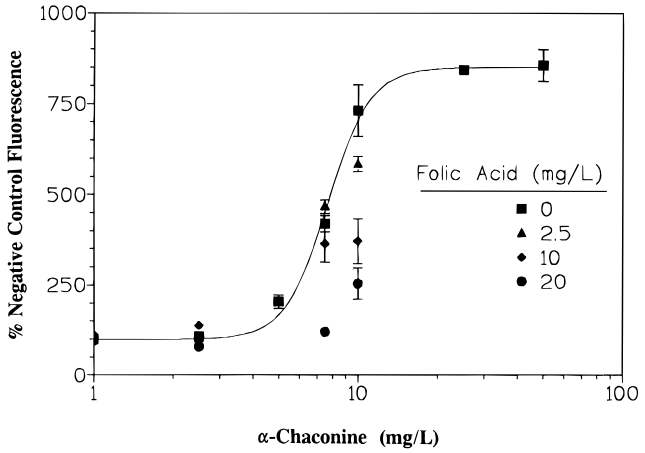


Figure 4. Plot of combined effect of folic acid (0, 2.5, 10, and 20 mg/L) and α-chaconine (0, 2.5, 5.0, 10, and 50 mg/L) on Di-4-ANEPPS fluorescence. All experimental values were corrected for background and normalized by dividing by the fluorescence of the control. Each point (a mean of three) is accompanied with standard error bars.

RESULTS AND DISCUSSION

Figure 4 shows that membrane-associated fluorescence intensity markedly decreased in the presence of various concentrations of folic acid. The fluorescence of the electrochromic dye is directly related to the electrical potential of the cell membrane. These findings suggest that folic acid protected the embryo cell membranes against disruption by the glycoalkaloid, α-chaconine. The protection increased with folic acid concentration up to about 10 mg/L and then leveled off.

Folic acid also protected frog embryos exposed to α-chaconine against mortality and malformations, as illustrated in Figures 5 and 6. A two-phase linear regression model was fitted to the 24-h survival data for embryos exposed to α-chaconine (5 mg/L) and folic acid (0−50 mg/L). This allowed estimation of a threshold of 12.5 mg/L folic acid, with 95% confidence intervals of 6.73 and 22.2. These and additional findings, based on a large number of individual observations, produced somewhat variable results, depending on the concentration of α-chaconine and folic acid, the time of exposure, and the viability of the embryos. However, the general trend in all cases was an observable protective effect against embryo mortality.

The relatively high concentrations of folic acid needed to achieve a protective effect in both assays suggested that protection required a "pharmacological" rather than a much lower "nutritional" concentration of folic acid.

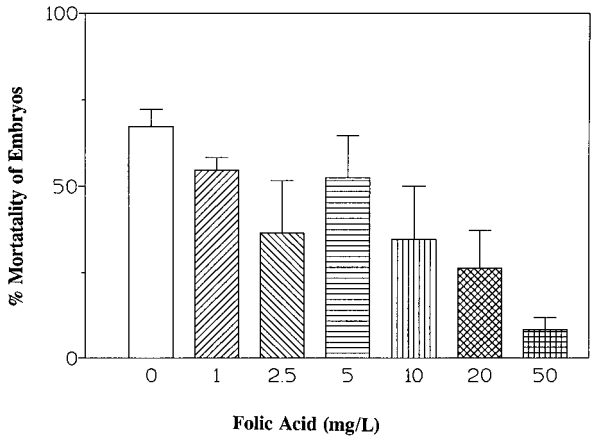


Figure 5. Protective effect of folic acid against α-chaconine-induced mortality of frog embryos. Each point (mean of three) is accompanied by standard error bars. Conditions: embryos were exposed for 24 h to α-chaconine (2.5 mg/L) in the absence and presence of folic acid.

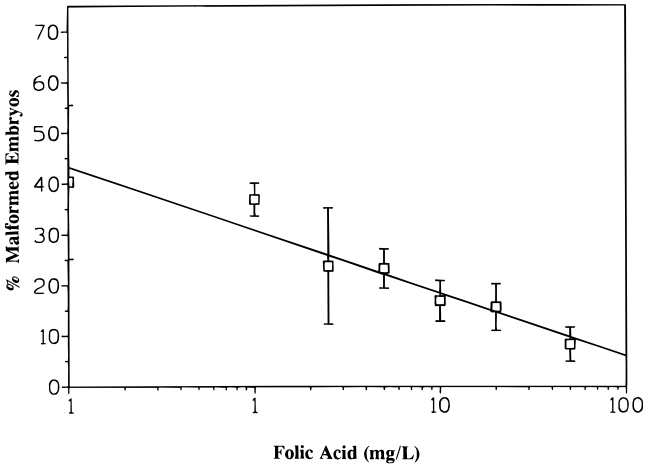


Figure 6. Protective effect of folic acid against α-chaconine-induced malformations in frog embryos. Malformation data were collected for three trials. The plot along with standard error bars shows the relationship of log folic acid concentration versus percent malformation in animals exposed for 24 h to α-chaconine (2.5 mg/L). Increasing folic acid concentration resulted in fewer malformations among the surviving embryos.

Since the molecular weight of α-chaconine (852) is about twice that of folic acid (441), the results may also suggest that an approximately stoichiometric amount of folic acid is needed for the observed protection against the action of α-chaconine on the cell membranes. If this is indeed the case, then the protective effect may be due to a competition between α-chaconine and folic acid for receptor sites or channels of the membranes. The possible induction of protective enzymes and/or metabolites by the high levels of folic acid cannot be ruled out, however.

To place these findings in proper perspective, it is instructive to briefly examine some of the postulated molecular events that may be involved in the protection of fetuses by folic acid against neural tube defects.

The main biochemical function of folic acid appears to be in transmethylation reactions (Gumbmann and Friedman, 1987; Hoffman, 1986; Smolin and Benevenga, 1991; Wagner, 1995). There is an apparent negative correlation between folate consumption during pregnancy and the occurrence of neural tube defects in newborns. The defect can be partially prevented by consumption of folate (Milunski, 1996; Oakley et al., 1996; Rieder, 1994).

According to Coelho and Klein (1990) and Coelho et al. (1989), the neural folds in cultured rat embryos failed to close in the absence of methionine. Methionine's function appears to be post-translational methylation of arginine, histidine, and lysine residues of microfilament proteins to form methylated derivatives, which appear to play a key role in neural tube closure. This interpretation is reinforced by the observation that this amino acid also protected rat fetuses against valparoic-acid-induced spina bifida (Ehlers et al., 1996). Valparoic acid may exert its teratogenic effect by interfering with folic acid metabolism in the transfer of methyl groups (Wagner and Na, 1992).

Mills et al. (1996) cited compelling evidence in support of the hypothesis that neural tube defects in children are the result of a metabolic defect in the enzyme methionine synthase, which along with vitamin B_{12} is involved in the methylation of homocysteine to methionine. The vitamin is presumably an independent risk factor. Young (1993) reported that the lowering of plasma levels of *S*-adenosylmethionine due to folate deficiency also seems to induce neurological and psychiatric symptoms such as depression, presumably due to lowering of serotonin levels. Related studies suggest that DNA repair activity is less effective under folate-deficient conditions, presumably due to hypomethylation of DNA (Cravo et al., 1995; James et al., 1994; Kim et al., 1997). Dietary folate also affects the response of rats to nickel ion deprivation, presumably due to its influence on one-carbon metabolism (Uthus and Poellot, 1996).

All of the cited studies were done with animals other than frogs. We do not know whether the embryo of the frog has similar metabolic pathways of folate reported for laboratory animals and humans (Wagner, 1995). It should be noted, however, that the results of in vitro teratogenicity assay with frog embryos show about a 90% correlation with in vivo teratogenic responses of laboratory animals exposed to >60 teratogens (Bantle et al., 1990, 1991).

These considerations and our findings on the protective effects of folic acid, glucose 6-phosphate, and NADP against the action of α-chaconine on frog embryo cells suggest that certain nutrients and other food ingredients may protect against adverse effects of glycoalkaloids in the diet (Friedman et al., 1996; Rayburn et al., 1995). This raises the question whether, in addition to the cited effects on cell membrane potentials and active transport across cell membranes, glycoalkaloids may also affect sulfur amino acid metabolism. It may therefore be worthwhile to ascertain whether cysteine, methionine, and corresponding peptides can ameliorate adverse effects of glycoalkaloids (Friedman, 1973, 1994; Friedman and Gumbmann, 1986, 1988). Possible protective effects of reduced folates such as 5-formyltetrahydrofolate and related folypolyglutamates (Shane, 1989) also merit study. Finally, the simple and inexpensive in vitro endpoints used in this study may validate and possibly replace animal studies, thus minimizing the need to use animals in safety evaluations of other membrane-disruptive and teratogenic compounds (Armstrong et al., 1994; Louise and Obrig, 1995).

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