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Permeation and toxicity of ethylene glycol and methanol in larvae of *Anopheles gambiae*

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Summary

In this study, we applied proton NMR to measure the permeation of two cryoprotective agents (CPAs), ethylene glycol (EG) and methanol, into 1st instar *Anopheles* larvae. Calibration with standard solutions of EG or methanol (0–10 mol l^{-1}) confirmed the reliability of the NMR measurements for determining the concentration of these solutes. To assess permeation, larvae were immersed in 1.5 mol l^{-1} EG or 1.5 mol l^{-1} methanol for different periods of time at 22°C. The concentration of both CPAs in the larvae was then measured as a function of exposure time using ^{1}H-NMR spectroscopy. Results show that after a 6 h exposure to 1.5 mol l^{-1} EG, the larval concentration of EG reaches a maximum value of 1.44 mol l^{-1}, which is 96% of the theoretical maximum. By contrast, after just 1 h exposure to 1.5 mol l^{-1} methanol, the larval methanol concentration reaches its maximum, which, however, is only 75% of the theoretical maximum. Toxicity data show that larval survival remains 91% and 95% after 4 h and 1 h exposure to 1.5 mol l^{-1} EG and 1.5 mol l^{-1} methanol, respectively, at which time the larval concentration of EG and methanol has risen to 1.21 mol l^{-1} and 1.13 mol l^{-1}, respectively. These results suggest that CPAs such as EG and methanol do permeate *Anopheles* larvae to up to 81% and 75% of equilibrium, respectively, before the exposure becomes toxic.

Key words: cryoprotectant, permeation, toxicity, ethylene glycol, methanol, NMR, larvae, mosquito, *Anopheles gambiae*.

Introduction

Mosquitoes are, by far, the deadliest creatures on the planet. They transmit malaria, yellow fever, dengue fever, encephalitis and other diseases to more than 700 million people each year. Among them, malaria, transmitted by *Anopheles* mosquitoes, is the worst and it infects nearly half a billion people, resulting in several million deaths worldwide each year (WHO, 1996). Malaria control strategies have been proposed in which genes for resistance to malaria will be introduced into vector populations in order to convert normal vector populations to mosquitoes that are unable to transmit malaria (Collins et al., 1986; Ito et al., 2002; Zheng et al., 1997). The recently complete genome sequence of both *Anopheles gambiae* (Holt et al., 2002) and the malaria parasite *Plasmodium falciparum* (Gardner et al., 2002) ought to accelerate the development of engineered mosquitoes refractory to *Plasmodium* and of methods for driving this genotype into the wild population. Such genetic studies will require the creation, maintenance and characterization of many genetic lines; however, such maintenance with present breeding techniques, in addition to being costly and time consuming, has problems of cross-contamination among strains, loss of stains through handling errors, and genetic changes caused by laboratory domestication or genetic drift. The ability to preserve *Anopheles* eggs or larvae cryobiologically would eliminate or substantially ameliorate these problems.

Cryopreservation of any biological material requires that cryoprotective agents (CPAs) are present in the cells either to prevent so-called ‘solution-effect’ injury (Mazur, 1970) during classical slow-freezing procedures or to prevent intracellular ice formation during vitrification procedures that involve cooling at high rates. The former approach is used in the cryopreservation of most mammalian cells; the latter, vitrification, was used by Steponkus et al. (1990) and Mazur et al. (1992a) in the successful cryopreservation of *Drosophila* embryos. Both approaches demand that the cells in question be permeable to both water and CPAs. Unfortunately, native *Anopheles* eggs show poor permeability to water and are essentially impermeable to ethylene glycol (EG; Valencia et al., 1996a), the CPA used in the cryopreservation of *Drosophila* (Mazur et al., 1992a; Steponkus et al., 1990) and house flies (Wang et al., 2000). Native eggs of *Drosophila* and house flies are also impermeable to both water and CPA; however, procedures were developed to successfully permeabilize them and thereby permit cryopreservation. The permeability barrier in the eggs of these two species appears to be a wax layer lying on the surface of the endochorion

(=vitelline membrane), the innermost of the two shells that surround the embryo proper. An essential component of the permeabilization procedure is exposure to the alkanes hexane or heptane, compounds that presumably solubilize the wax layer. Unfortunately, these procedures are not applicable to the *Anopheles* eggs (Valencia et al., 1996b) or are applicable to only a limited extent. Early eggs of *Anopheles* also appear to have a wax layer. The problem is that they develop a second permeability barrier between 8 h and 12 h after egg laying that is refractory to removal by alkanes. This second barrier is probably related to tyrosine cross-linking in the endochorion of the egg (Mazur et al., 2001). To date, we have found no method to breach this second barrier or to prevent it from forming without killing the egg.

An alternative to cryopreserving eggs would be to cryopreserve larvae. Recently, we found (X.-H. Liu, unpublished data) that untreated 1st instar *Anopheles* larvae were uniformly stained by rhodamine B, which has a molecular mass (M_r) of 479, so it is reasonable to predict that the larvae will probably be permeable to CPAs with low molecular masses, such as EG (M_r=62) and methanol (M_r=32). To verify this assumption, we applied nuclear magnetic resonance (NMR) spectroscopy to measure the concentration of CPAs within the larvae. NMR is a phenomenon that occurs when certain nuclei (such as ^{1}H) that possess an intrinsic magnetic property imparted by their spin are immersed in a powerful static magnetic field and are simultaneously exposed to a second oscillating magnetic field (Ault and Dudek, 1976). For example, when a proton with a spin is placed in an external magnetic field, the spin vector of the proton aligns itself with the external field, just as a magnet would. This proton can undergo a transition between the two energy states by the absorption of a photon and can jump to the less stable orientation (higher energy state) when the frequency of the oscillating electromagnetic signal matches the resonance frequency of the proton or the energy difference between the two states. Each proton in a chemical has a characteristic resonance frequency, which is affected by the neighboring electrons since the circulation of the electrons creates small magnetic fields that can oppose (generally) or enhance the externally applied field. The variation in resonance frequency of a nuclear spin due to the chemical environment around the nucleus is referred to as a chemical shift. Removal of the oscillating magnetic field causes the proton to revert to the more stable orientation (lower energy state) and emit electromagnetic radiation, which is the NMR signal. Protons with different chemical environments produce separate NMR signals at different chemical shifts. The intensity of the NMR signal at a given frequency is proportional to the number of protons with that characteristic resonance frequency. In Fourier-transformed spectra, the NMR signals yield NMR peaks. Calculation of the integrated area of the peaks will permit determination of the molar ratio of different protons and thereby measurement of the concentrations of a specific chemical. These principles have been applied to study the permeation of dimethyl sulfoxide (DMSO; Me_2SO) in rat liver (Fuller and Busza, 1994; Fuller et al., 1989), rat carotid artery (Bateson et al., 1994) and rabbit and porcine corneas (Taylor and Busza, 1992; Walcerz et al., 1995; Wusteman et al., 1999), ethylene glycol in the rabbit common carotid artery (Wusteman et al., 1995) and a number of CPAs in human ovarian tissue (Newton et al., 1998). Proton NMR has thus been shown to be a very useful method for measurement of the kinetics of CPA permeation in tissues. The prime objective of the present study was therefore to use proton NMR to determine the kinetics of permeation of EG and methanol into larvae of *Anopheles* mosquitoes. A second objective was to determine the resulting toxicity of the CPAs as a function of permeation. While our underlying goal is cryopreservation, the permeation of solutes in multicellular systems such as larvae is of broader biological relevance. We believe that this study illustrates the applicability of NMR to this broader question.

Materials and methods

Experimental animals

Blood-fed female *Anopheles gambiae* Giles adults were provided weekly by the Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD, USA and the Centers for Disease Control, Atlanta, GA, USA. After arrival at our laboratory, the mosquitoes were placed in an incubator, which was kept at 26°C and 70% relative humidity. A piece of cotton saturated with 15% Karo light corn syrup (Bestfoods, Sarasota, FL, USA) in Millipore water was provided as a food source. Egg collection was carried out 4 days after a blood feed. Approximately 40–60 mosquitoes were aspirated from their container and anesthetized with carbon dioxide gas. Mosquitoes were then placed in an inverted OPTILUX^{TM} Petri dish (100 mm×15 mm; Becton Dickinson, Franklin Lakes, NJ, USA) containing Whatman No.1 filter paper. After the mosquitoes recovered from the carbon dioxide (approximately 15–20 min), 2 ml of Millipore water was added to the filter paper to induce oviposition. After 1 h of egg laying, the mosquitoes were removed from the Petri dish and the eggs were incubated in the 26°C incubator until they hatched (approximately 46 h). Newly hatched 1st instar larvae were collected and maintained in Millipore water for the treatments that followed. No food was given to the larvae before and during the treatments.

Determination of the kinetics of permeation of ethylene glycerol and methanol into 1st instar larvae

The 1st instar larvae were immersed in 1.5 mol l^{-1} ethylene glycol (EG; Sigma Chemical Co., St Louis, MO, USA) or 1.5 mol l^{-1} methanol (GR Anhydrous; EM Science, Gibbstown, NJ, USA), which were prepared in Millipore water, for different period of times (1, 15, 30, 60, 120, 180, 240, 360 and 480 min) in a 15 ml centrifuge tube (Corning Inc., Acton, MA, USA) at room temperature (22–24°C). After the appropriate exposure time, approximately 100–200 larvae were pipetted to a nylon cell strainer (70 μm, Becton Dickinson) to drain the CPA solutions. The strainer was then

blotted with facial tissue and the larvae were briefly washed using 1 ml H_2O to remove the residual surface CPAs.

NMR measurements require that the larvae are in deuterium oxide (D_2O) rather than water to eliminate proton signals from the solvent. The H_2O on the larval surface was removed by washing them twice with 99.90% D_2O (Aldrich Chemical Co., Milwaukee, WI, USA). The strainer was briefly blotted following each washing. The whole washing procedure took less than 2 min, so the potential washout of permeated CPA was minimal. The larvae were then suspended in 0.2 ml of high-purity D_2O (≥99.96%; Aldrich Chemical Co.) and immediately transferred to a 5 mm symmetrical D_2O matched NMR microtube (Catalog no. BMS-005V; Shigemi, Inc., Allison Park, PA, USA) for measurements. Once the larvae were in the NMR tubes, efflux of CPA from the larvae into the surrounding medium would be of no significance. NMR measurements were carried out using the same NMR parameters as those used for the standard solutions described below. Each time point was repeated three times for both CPAs at each exposure time to the CPA.

For experiments involving determination of the kinetics of CPA efflux (washout), larvae that had previously been exposed to 1.5 mol l^{-1} EG for 4 h or to methanol for 1 h were put in a nylon cell strainer to briefly drain the CPA solution and lightly blotted. The strainer with the larvae was then transferred to a Falcon tissue culture dish (35 mm×10 mm; Becton Dickinson) containing 5 ml Millipore water. After sitting in the water for 15 min, 30 min, 60 min, 120 min (only for EG) or 240 min (only for EG), approximately 100 larvae were removed and placed in another strainer and washed twice with D_2O to remove surface water. The subsequent procedures for the NMR measurements were the same as described above.

NMR measurements on standard solutions of EG and methanol

In order to determine the concentration of EG and methanol in the larvae *versus* exposure time, it was necessary to first determine the NMR proton signals generated by a series of solutions of EG and methanol at a range of concentrations. These standards (0–10 mol l^{-1}) were prepared in Millipore water. Specifically, from the NMR peaks we determined the molar ratio of the NMR peaks attributable to the protons in the CH_2- groups of EG and the CH_3- group of methanol to the area of the peak due to -OH groups. These peaks occur at different chemical shifts. Concentrations can be calculated from the magnitude of the peaks knowing the molecular masses and densities of EG and methanol. Once the standard curves of CH_2/OH and CH_3/OH proton ratios *versus* molar concentration of EG and methanol have been constructed, one can then use these to calculate the concentrations of EG and methanol in the larvae from the proton signals generated by the larvae. Specifically, 20 μl of each standard solution was added to 0.2 ml pure D_2O (≥99.96%) in a 5 mm D_2O matched NMR tube. The NMR tube was preloaded with a sealed glass capillary tube containing chloroform (3.82 mg $CHCl_3$ or 0.032 mg ^1H). Since this internal reference contained a fixed number of protons, it served as a proton quantity comparison for all experiments. A blank control sample that contained only 0.2 ml of the pure D_2O and the reference was measured in order to know the background proton count from residual water in the D_2O, and the data was used for calibration. When calculating the integrated proton peak area, the value of the chloroform reference was set at 100 and then the relative area value of each proton peak could be compared among different NMR spectra so that the proton contributed by the residual H_2O in D_2O could be subtracted from the OH proton peak.

The proton NMR experiments were performed on a Mercury 300 NMR spectrometer (Varian, Fort Collins, CO, USA) with proton resonance frequency at 300 MHz and a vertical 7 T magnet, using a single 20° pulse, 5 s relaxation delay and 32 scans for proton Bloch decay acquisition. The data were recorded at 25°C and processed using MestRe-C software (version 2.1.0; Universidad de Santiago de Compostela, Santiago de Compostela, Spain). The data were multiplied by 1 Hz line broadening faction and were Fourier transformed to yield the final spectra. The integrated proton peak area was determined by the MestRe-C software.

Toxicity of 1.5 mol l^{-1} EG and 1.5 mol l^{-1} methanol

The 1st instar larvae were immersed in 1.5 mol l^{-1} EG or 1.5 mol l^{-1} methanol for 0 (control), 1, 2, 3, 4, 5, 6 or 8 h in a centrifuge tube at room temperature (22–24°C). After the appropriate exposure time in the cryoprotectant solutions, approximately 50 of the larvae were pipetted to a cell strainer to drain the CPA solutions. The strainer was then blotted with facial tissue and transferred into 5 ml water in a tissue culture dish for 15 min to wash out the internal CPAs. The number of larvae was determined under a dissecting microscope and they were then transferred into 50 ml water in a plastic box. Larvae were fed with slurry comprising a mixture of tropical fish food (VitaPro Plus Cichlid Power Flakes; M. Reed Enterprises, Sutter Creek, CA, USA) and active baker's yeast (2% w/v; 2:1 fish food:baker's yeast in water). The larval feeding and culture methods were according to the procedures described by Benedict (1997). After 4 days incubation in a 26°C incubator, larval survival was assessed on the basis of motility and normal morphology. The data are expressed as percentage survival relative to the percentage survival of control untreated larvae.

Results

Calibration curve

Fig. 1 shows the ^1H-NMR spectrum of 1 mol l^{-1} EG. In addition to the small reference peak at the left for $CHCl_3$, there are two proton peaks to the right. The far-right peak at a chemical shift of approximately 3.5 p.p.m. represents methyl (CH_2) protons from EG. The large peak at a chemical shift of approximately 4.7 p.p.m. arises from the hydroxyl (OH) protons from water in EG solution, -OH groups of EG and residual water in D_2O (the contribution of which is known from the blank control and subtracted).

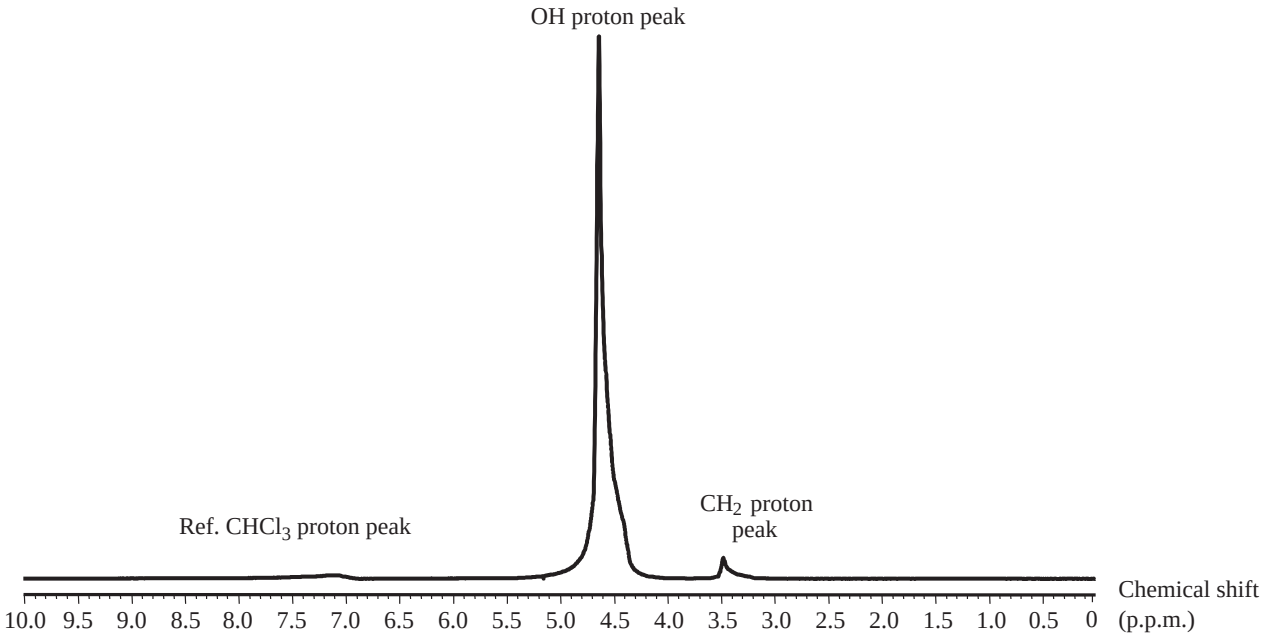

Fig. 1. Proton NMR spectrum of 1 mol l^{-1} ethylene glycol (EG) solution showing three distinguishable peaks. The peak at a chemical shift of approximately 3.5 p.p.m. represents CH_2 protons from EG, and the peak at a chemical shift of approximately 4.7 p.p.m. arises from the OH protons from both water and EG. The left-hand peak is the reference $CHCl_3$ proton peak. The data were acquired using a Varian Mercury 300 NMR spectrometer with proton resonance frequency at 300 MHz and subjected to Fourier transformation.

The signal intensity of each peak (integrated peak area) is proportional to the amount of each proton in the sample. The relative intensities of the CH_2 proton peak and the OH proton peak represent the relative molar concentrations of CH_2 protons and OH protons, respectively. Therefore, the molar ratio of CH_2 protons to OH protons in each standard solution of EG can be measured from its ^{1}H-NMR spectrum. The proton molar ratio (CH_2/OH) of each EG solution can be calculated on the basis of the relative molecular mass (62.07) and density (1.11 g ml^{-1}) of pure EG (CH_2OHCH_2OH). For example, 1.5 mol l^{-1} EG=93.11 g (1.5×62.07 g), i.e. 83.88 ml (93.11/1.11) EG + 916.12 ml (1000–83.88 ml), i.e. 50.90 moles (916.12×1/18) water. Each EG molecule has a ratio of four CH_2 protons to two OH protons, and each water (HOH) molecule contributes two OH protons. Thus, a 1.5 mol l^{-1} EG solution contains 6 mol l^{-1} (4×1.5) CH_2 protons and 104.80 mol l^{-1} [(2×1.5)+(2×50.90)] OH protons. Therefore, proton molar ratio (CH_2 protons/OH protons)=6/104.80=5.73%.

According to the above calculations, there exists the following theoretical relationship between the proton molar ration (percentage), R, and the molar concentration of EG (C_{EG}):

$$R = \frac{4C_{EG}}{2C_{EG} + 2 \times [(1000 - 62.07C_{EG}/1.11)/18]} \times 100 . \quad (1)$$

Similarly, each methanol (CH_3OH) molecule provides three CH_3 protons to one OH proton, and, based on the relative molecular mass (32.03) and density (0.79 g ml^{-1}) of pure methanol, the calculated relationship between the proton molar ratio (percentage of CH_3 protons/OH protons), R, and the molar concentration of methanol (C_{met}) is given by:

$$R = \frac{3C_{met}}{C_{met} + 2 \times [(1000 - 32.03C_{met}/0.79)/18]} \times 100 . \quad (2)$$

Fig. 2 compares these calculated proton molar ratios for a series of concentrations of EG and methanol with those obtained experimentally from NMR measurements. The agreement is very close, confirming that the ^{1}H-NMR measurement method is reliable.

On the basis of this agreement, one can use measured proton molar ratios to calculate the concentration of EG and methanol in an unknown sample. This is achieved by rearranging equations 1 and 2 to derive the following equations:

$$C_{EG} = \frac{1000R}{37.92R + 3600} \quad (3)$$

and

$$C_{met} = \frac{1000R}{37.54R + 2700} . \quad (4)$$

In the present study, the unknowns are the concentrations of EG and methanol in the larvae as a function of the time of exposure to these two solutes; i.e. as a function of the permeation kinetics. These measurements are possible because the washing procedure described in the Materials and methods removes nearly all the external EG and methanol, and the contribution of the external water hydroxyls is essentially eliminated by the suspension of the larvae in high-purity D_2O.

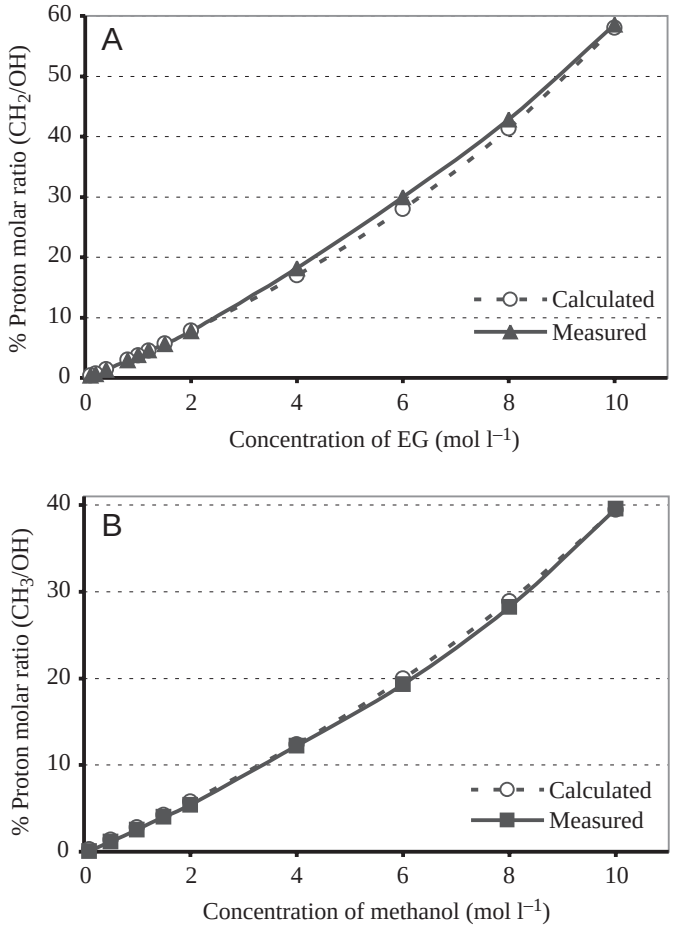

Fig. 2. Comparison of calculated curves and the NMR-measured curves of the proton molar ratio (CH_2/OH or CH_3/OH) *versus* the molar concentration of (A) ethylene glycol (EG) and (B) methanol. The NMR-measured values are means ± S.E.M.; *N*=2 or 3.

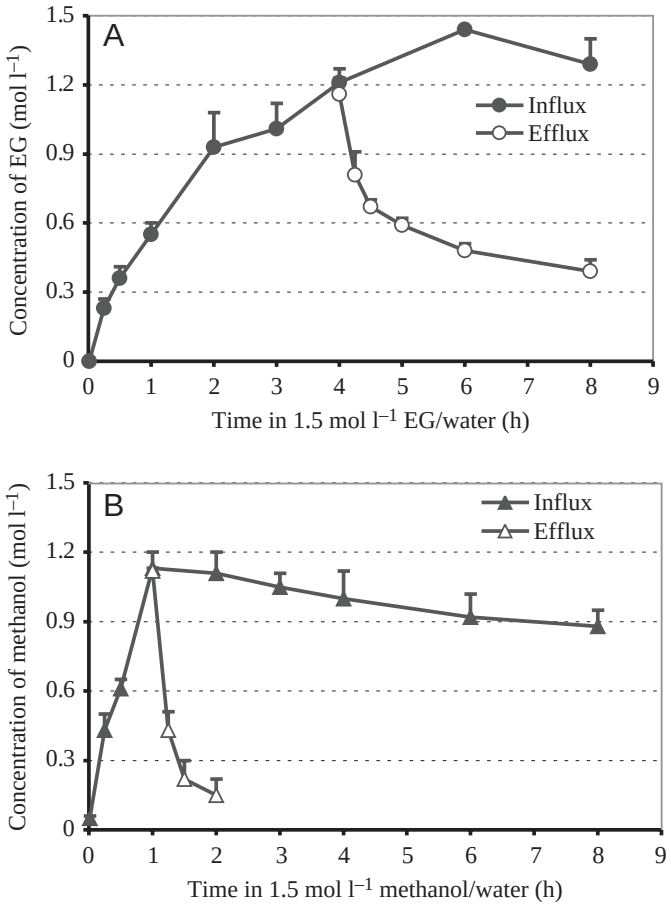

Fig. 3. Influx and efflux kinetics of cryoprotective agents in and out of 1st instar *Anopheles* larvae at room temperature. (A) Influx and efflux of 1.5 mol l^{-1} ethylene glycol (EG) in *Anopheles* larvae as a function of time. (B) Influx and efflux of 1.5 mol l^{-1} methanol in *Anopheles* larvae as a function of time. Values are means ± S.E.M.; *N*=3.

Kinetics of influx and efflux of EG and methanol

The filled circles in Fig. 3A show the measured influx curve for the permeation of EG into the larvae suspended in 1.5 mol l^{-1} EG at room temperature. It can be seen that the larval concentration of EG increased with exposure time during the first 6 h. At that point, the concentration of EG in the larvae was 1.44 mol l^{-1}, which is 96% of the theoretical maximum if full equilibration were achieved.

In separate experiments, larvae were exposed to 1.5 mol l^{-1} EG for 4 h, at which time the larval concentration of EG had risen to 1.21 mol l^{-1}, and then transferred to EG-free water for various times before initiating the NMR measurements. The open circles in Fig. 3A show the resulting curve for the efflux of EG. The efflux occurred at a somewhat similar rate as influx, but approximately 0.4 mol l^{-1} EG remained in larval tissue after 4 h washing.

Fig. 3B shows the results of analogous experiments with methanol. Methanol shows much faster rates of both influx and efflux compared with those of EG. After just a 1 h exposure to 1.5 mol l^{-1} methanol, the concentration of methanol in the larvae reached a maximum of 1.13 mol l^{-1}, which is only 75% of the theoretical maximum. The internal larval concentration of methanol also appears to drop slowly with permeation times longer than 1 h, but the drop is not statistically significant (P>0.05, N=3). When, after 1 h, the larvae are transferred from methanol to water; the rate of efflux of the methanol (open triangles) is almost as high as the rate of influx.

Toxicity of 1.5 mol l^{-1} EG and 1.5 mol l^{-1} methanol

The results of Fig. 3 clearly show that EG and methanol permeate the larvae. The next question is how high a concentration and exposure time they can tolerate. The toxicity studies (Fig. 4) show that larvae can tolerate 1.5 mol l^{-1} EG and 1.5 mol l^{-1} methanol at room temperature up to 4 h and 1 h, respectively, with a normalized survival of 90.6±5.81% and 95.1±2.6%, respectively. When the exposure time was extended to 5 h in EG and 2 h in methanol, larval survival dropped significantly (P<0.01 for EG and P<0.05 for methanol; N=4) when compared with that of controls. However, larval survival remained rather high (77.9±3.8%)

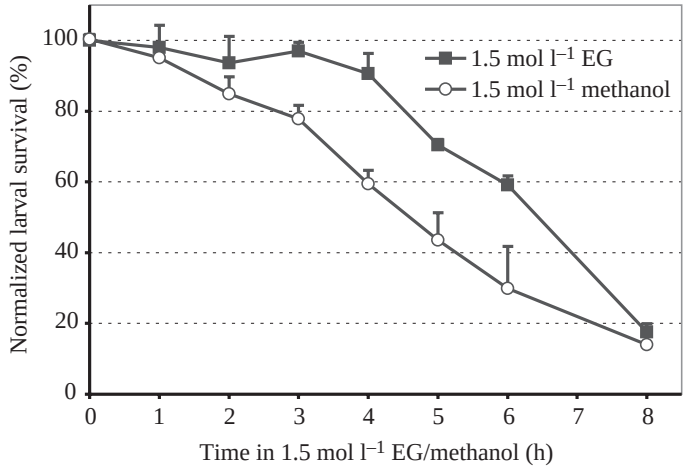

Fig. 4. Toxicity of 1.5 mol l^{-1} ethylene glycol (EG) and 1.5 mol l^{-1} methanol to 1st instar *Anopheles* larvae at room temperature. Larval survival was normalized with respect to that of untreated controls, which was 87.9±2.5%. Values are means ± S.E.M.; *N*=4.

after 3 h exposure in 1.5 mol l^{-1} methanol. About 59% of the larvae survived a 6 h exposure in 1.5 mol l^{-1} EG, by which time the concentration of EG within the larvae reached the maximum of 1.44 mol l^{-1}.

Discussion

To achieve successful cryopreservation, CPAs must be able to permeate into cells and tissues. The design of cryopreservation procedures needs the information about the kinetics of CPA permeation into biological samples and also the toxicity of the CPAs. There exists a number of methods to assess CPA permeability. Measurements of volume change by light microscopy have been most used to study cell and embryo membrane permeability to CPAs (Lin et al., 1989; McCaa et al., 1991; Valencia et al., 1996b), but this method would be difficult to apply to an organism that has an irregular shape and contains several compartments of gut, intracellular and extracellular spaces. Other methods such as radioisotopes (Jackowski et al., 1980; Mazur and Rajotte, 1981), depression of freezing or melting points using differential thermal analysis or differential scanning calorimetry (Jacobsen, 1978; Liu et al., 2001), measurements of mass changes (Elford, 1970) and staining with rhodamine (Mazur et al., 1992b) have been used to assess the permeation of CPAs into cells, embryos and other tissues. Proton NMR was first applied to study the permeation of DMSO into rat liver by Fuller et al. (1989) and it provides not only a rapid and noninvasive method but also a direct measurement of CPA concentration in tissues. In the present study, the extremely close agreement between the NMR-measured proton molar ratios of standard solutions of both CPAs and the theoretically calculated values further confirm the reliability of proton NMR spectroscopy.

Ethylene glycol and methanol were chosen here because EG is the main permeating CPA used for the cryopreservation of insect eggs (Mazur et al., 1992a; Nunamaker and Lockwood, 2001; Steponkus et al., 1990; Wang et al., 2000) and because methanol has been reported to be an effective cryoprotectant in both slow-cooling (Ali and Shelton, 1993; Czlonkowska et al., 1991; Rall et al., 1983, 1984; Rapatz, 1973) and vitrification (James, 1980; Liu et al., 1998) approaches to cryopreservation.

Our data indicate that the permeation of EG into larvae is rather slow (only 37% equilibration in 1 h and nearly full equilibration in 6 h), whereas the permeation of methanol is much faster; the maximum internal concentration of methanol (75% of equilibrium) being reached in just 1 h. The rates of efflux of the two CPAs were similar to those for influx. It is not surprising that methanol permeates rapidly. It has been found to be the only CPA that is able to penetrate the embryo of zebrafish (*Danio rerio*; Hagedorn et al., 1996; Liu et al., 2001; Zhang and Rawson, 1996b) and also to penetrate mammalian cells and embryos very rapidly (Naccache and Sha'afi, 1973; Rall et al., 1984). Although it permeates rapidly, it seems to be a paradox that the maximum larval tissue concentration of methanol falls shorter of the theoretical maximum (1.5 mol l^{-1}) than does that of EG. Other investigators also found that the proton NMR-measured concentration of CPA in tissues such as cornea, liver or ovaries does not reach the full theoretical maximum, usually with an equilibration of 50–82% of the theoretical achievable maximum (Fuller and Busza, 1994; Taylor and Busza, 1992; Thomas et al., 1997). Taylor and Busza (1992) suggested that this could be partly due to a fraction of tissue water that is inaccessible for exchange with CPAs, namely 'non-solvent' water, and this might reflect mitochondrial compartments as proposed by Garlid (1979). Thomas et al. (1997), however, suggested another explanation in their work with porcine ovaries; that the tissue organization could produce an effective diffusion barrier for CPAs. Both of the two explanations are inapplicable here, however, since EG does permeate into the larvae to a level of nearly full equilibration. Rather, we think the explanation here is that significant quantities of the internal methanol in larval tissues are lost by efflux during the several washes during sample preparation just before the NMR measurements are initiated. The purpose of the washing procedure was to remove the surface CPAs with minimal possible loss of internal CPAs, and it appears to effectively serve this aim for larvae treated with EG, but less so for the methanol-treated larvae. We also tried using cold (0°C) H_2O and D_2O to wash the larvae, considering that low temperature might slow down the methanol efflux; however, the results were similar (data not shown).

D_2O was used as the medium to finally wash away the residual water from the larval surface and to suspend the larvae for NMR measurements, since pure D_2O would not contribute any proton signal under proton NMR. However, commercially pure D_2O (≥99.96%) still contains a very small amount of H_2O and it can be contaminated easily during the experiments. The residual and contaminated water in D_2O could make a

significant difference since a relatively large amount of D_2O was used to suspend the relatively small amount of larvae. Therefore, a CH_3Cl reference was used for all samples in order to calibrate the data by subtracting the amount of protons from residual H_2O in D_2O, the value of which is obtained from a blank sample of D_2O.

Another concern is that larval tissues contain hydroxyl protons from sources other than water, which also contribute to the OH peak. A pilot experiment with both fully dehydrated and fully hydrated larvae found that the OH protons from larval tissues only consist of 0.52% of the total OH protons of fully hydrated normal larvae. It is a reasonable figure considering that approximately 82% of the total mass of larvae is water (Liu and Mazur, in press). Therefore, the amount of OH protons contributed by larval tissue is negligible. A final point is that proton exchange occurs between OH (of H_2O, EG or methanol) and OD of D_2O to give HOD and partially deuterated EG or methanol. However, there exists no proton exchange between CH_2 (of EG) or CH_3 (of methanol) and OD. Moreover, once the larvae are in the NMR tube, any proton exchange between OH and OD will not affect the results of NMR measurements since HOD is visible to NMR and the resonance frequency of HOD is the same as that of HOH.

The larva is, at a minimum, a three-compartment system consisting of a gut, extracellular tissue space and intracellular space. One important question regarding the CPA concentration in larvae is whether CPA permeation is primarily limited to the gut or whether permeation occurs throughout the larval tissues. While the present study provides no direct answer, it seems unlikely that EG permeation is just confined to the gut since after 6 h exposure the larval EG concentration reaches about 96% of the theoretical maximum, indicating that full equilibration has almost been reached. NMR measured the concentration of CPA in the larvae and not the absolute amounts. Consequently, one possibility is that the larval tissues could have been dehydrated by the EG solution in the gut, thus raising the larval EG concentration in those tissues closer to equilibrium. But we did not see obvious larval shrinkage following up to 12 h exposure in 1.5 mol l^{-1} EG at room temperature based on microscope observation. Our recent differential scanning calorimetry data on the amount of freezable water (X.-H. Liu and P. Mazur, unpublished data) also indicate that after 4–6 h exposure in 1.5 mol l^{-1} EG, the larvae contain a similar amount of freezable water to that of untreated control larvae, suggesting no significant larval dehydration. Therefore, it seems to be the case that EG does fully permeate into the *Anopheles* larvae following approximately 6 h exposure. Finally, we need to point out that, from the viewpoint of cryopreservation, the concentration of CPA is more important than the absolute amount.

What is the mechanism of CPA permeation? The outer surface of mosquito larvae is covered by a chitin–protein cuticle, which is presumably not permeable to CPAs. However, *Anopheles* larvae are freshwater species and they possess osmoregulatory mechanisms (Bradley, 1987). In hyperosmotic medium, the larvae tend to lose water due to dehydration by the outward diffusion of water down an osmotic gradient, but they maintain normal volume by constant drinking. Presumably, when the hyperosmotic medium contains CPAs, these will enter the gut in the course of this drinking. The gut is separated from the intestinal epithelium and other tissues by an acellular chitin-containing sheath, the peritrophic matrix membrane (PM). Recently, the PM of *Anopheles* mosquitoes has been found to be permeable to particles of ≤148 kDa (Edwards and Jacobs-Lorena, 2000). Therefore, the PM is presumably permeable to EG and methanol, both of which are much smaller than 148 kDa. It is also reasonable to assume that these low-molecular-mass CPAs are able to permeate through the larval intestinal epithelium, which is a cellular layer. As far as the cells themselves, a wide variety of types have been found to be permeable to both EG and methanol. Although this study sheds no direct light on the exact mechanism of CPA permeation, it probably involves both passive transport and a physiological mechanism such as that involved in osmoregulation.

EG appears to be less toxic to *Anopheles* larvae than is methanol for the same time exposure. However, the permeation of EG is considerably slower than that of methanol, and, if we consider larval survival after the tissues are exposed to the two CPAs for the times required to attain equivalent concentrations, these are similar. For example, the larval survival is 95% when, after 1 h exposure, the tissue concentration of methanol reaches 1.13 mol l^{-1}, and the survival is about 93% after exposure to 1.5 mol l^{-1} EG for 3.5 h, at which time the EG concentration in the larvae rises to the same concentration of 1.1 mol l^{-1}. EG and methanol have been reported to be less toxic to a variety of cells and tissues than other commonly used CPAs such as DMSO and glycerol (Ali and Shelton, 1993; Pollock et al., 1991; Zhang and Rawson, 1996a). Our study shows that high percentages (>90%) of *Anopheles* larvae survive the exposure in EG and methanol for a sufficient time period that allows them to permeate up to reasonably high concentrations without excessive injury. These promising results encourage us to conduct further investigation on the cryopreservation of these larvae.

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