reversible formation of a hydroxy-benzo(*a*)pyrene radical upon irradiation (Prof. Laarhoven, personal communication).

Discussion

A number of assays for GSH-T has been described including titrimetric, colorimetric, spectrophotometric and radiochemical methods using a variety of substrates (for review see [8]). However, assays using substrates of the biologically important PAH have only employed radioactive substrates [5, 9–11], most of them using a time consuming TLC separation of the conjugate. In addition, enzyme activity is often measured at suboptimal pH due to the large contribution of non-enzymatic conjugation. The present method combines an extreme sensitivity with the easy separation of glutathione conjugated with a non-radioactive PAH substrate. Optimal conditions (substrate, enzyme and glutathione concentrations) were obtained at 1/5 of those described by Cantfort *et al.* [9] who have used the same extraction principle. pH-optimum, time-dependence and non-enzymatic conjugation were quantitatively comparable between the former assay and the present one. The high pH-optimum is rather unusual for cytosolic enzymes, but in accordance to results of others [5, 9, 11]. Although we have used BPO obtained as a gift from the National Cancer Institute, the compound can be synthesized relatively easily from benzo(*a*)pyrene [12].

Due to the extreme sensitivity the method is especially useful for application in extrahepatic tissues and cultured cells. Since human hair follicles have been introduced as indicator organs for assessment of individual differences in susceptibility to chemical carcinogenesis, possibly reflected by differences in activities of carcinogen metabolizing enzymes among which GSH-T [13–16], we have studied the applicability of the present assay for human hair follicles. It was found that the enzyme could be detected in only one freshly isolated hair follicle. Thus, the present method is particularly useful for screening of high risk populations to identify interindividual differences in carcinogen metabolism.

In summary, a method is described for the quantitative determination of the glutathione-conjugate of 4,5-dihydroepoxybenzo(*a*)pyrene. The sensitivity and practical convenience of the procedure is based on (a) the high specific fluorescence intensity of the product, (b) the very low background obtained by the efficient differential extraction of substrate and product, (c) the use of a non-radioactive substrate from the important class of polycyclic aromatic hydrocarbons and (d) the involvement of a single rapid transfer and extraction step. Due to the sensitivity of the method permitting measurement of 30 pmoles product the procedure is especially useful for assaying transferase activity in minute tissue samples such as human hair follicles or cultured cells.

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Tricyclic antidepressant drug effects on liposomal membranes

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Alterations of membrane lipid structure may be an important mechanism by which hormones and other small molecules may regulate cell function [1–4]. The tricyclic antidepressants inhibit lymphocyte mitogenesis [5–8] and reduce numbers of plaque forming cells [7] *in vitro*, and reduce elevated rheumatoid autoantibody levels [9] and produce leukopenia [10] *in vivo*. Tricyclic antidepressant drug-induced perturbations of membrane lipid structure may be related to tricyclic antidepressant-induced inhibition of lymphocyte mitogenesis [11].

Since biological membranes are enormously complex, phospholipid vesicles or liposomes, representing relatively

simple models of the phospholipid bilayer of the biological membrane, may be effectively used to characterize lipophilic drug:membrane interactions [12, 13].

In this paper, we describe tricyclic antidepressant drug effects on the structure of large multilamellar liposomes prepared from DPPC*, DOPC, and egg PC. 1,6-Diphenyl-1,3,5-hexatriene (DPH), a highly lipophilic fluorescent probe [14], has been used to characterize the concentration and temperature dependence of drug-induced alterations of liposomal membrane structure. The size of the multilamellar liposomes was determined by electron microscopy.

Materials and methods

DPH (Lot No. 2021A) was purchased from Molecular Probes, Inc., Junction City, OR. Imipramine hydrochloride (Lot No. 121F-0086), L-α-phosphatidylcholine, dipalmitoyl (DPPC) and L-α-phosphatidylcholine, dioleoyl (DOPC) were obtained from the Sigma Chemical Co., St. Louis, MO. Egg L-α-lecithin (egg PC) was obtained from Avanti Polar-Lipids, Inc., Birmingham, AL. Desipramine hydrochloride was a gift from Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH. Nortriptyline hydrochloride was a gift from Lilly Research Laboratories, West Point, PA. All other compounds used in this study were of the highest grade commercially available.

Liposome preparation. An aliquot of phospholipid, from a 20 mg/ml stock in chloroform, was placed in a round-bottomed, nitrogen-purged, stoppered test tube. The chloroform was evaporated off with a gentle stream of nitrogen, and the phospholipid was resuspended in phosphate-buffered saline (PBS, pH 7.4). The phospholipid mixture was then heated to at least 10° above the transition temperature for the respective lipid and vortexed for 5 min. To the resulting suspension of large multilamellar liposomes, an equal volume of PBS containing dispersed DPH was added to a final phospholipid to DPH ratio of about 500:1. The final concentration of phospholipid was approximately 0.1 μmole/ml. The liposome mixture was incubated at room temperature for 45 min and was used immediately for spectroscopic measurements at 37° unless otherwise indicated.

DPPC (2/3) + DOPC (1/3) were prepared by adding appropriate amounts of each phospholipid to the same round-bottomed test tube. The remainder of the preparatory procedures for DPPC + DOPC liposomes were as described above.

Drugs were dissolved in either PBS or distilled water and then added to liposome suspensions (sample) with Hamilton microliter syringes. Addition of either PBS or distilled water in equal amounts, and at similar times, to a liposome suspension served as the corresponding control. Unless otherwise indicated, sample and control liposome suspensions were incubated at 37° for 5 min prior to measurement of fluorescence anisotropy or lifetimes.

Following the protocol of Bangham and Horne [15], liposomes were examined with a JEOL-100S electron microscope at instrumental magnifications of 40,000 to 100,000 diameters to determine the liposome size. The large multilamellar liposomes used in this study were sized by electron microscopy and ranged from about 0.1 to 0.8 μm in diameter.

Steady-state fluorescence anisotropy measurements. Instrumentation has been described in detail previously [11, 16]. Briefly, measurements were made with an SLM 8000 interfaced with a Hewlett–Packard 87 computer for on-line acquisition and analysis. The fluorometer was set up to measure fluorescence anisotropy

$$r = A - B/A + 2B$$

where r is anisotropy, A is the ratio of the fluorescence intensities parallel and perpendicular to the plane of polarized excitation light with the excitation polarizer in the vertical position, and B is the ratio of the fluorescence intensities parallel and perpendicular to the plane of polarized excitation light with the excitation polarizer in the horizontal position. The sample was excited with 360 nm light and emission was monitored at 430 nm.

Fluorescence lifetime measurements. Methodology and instrumentation for determination of fluorescence lifetimes have been described elsewhere [11, 17]. Fluorescence lifetimes were calculated from phase and modulation measurements made with an SLM 4800 fluorometer interfaced with a Hewlett–Packard 87 computer for on-line data acquisition and analysis. The phase shift and the modulation of each sample were measured alternately four times, with a time interval between each measurement of about 10 sec. Data were acquired by the computer and averaged, and the lifetimes were calculated from the following basic relationships:

$$T_{\text{phase}} = 1/w \tan O$$

$$T_{\text{modulation}} = (1/w)\,(1/D^2 - 1)^{1/2}$$

where T_{phase} is the phase lifetime, O is the phase shift in degrees caused by a sample of lifetime T, w is the angular frequency of excitation which is $2\pi *$ modulation frequency (30 MHz), $T_{\text{modulation}}$ is the modulation lifetime, and D is the ratio of $M_{\text{fluorophore}}/M_{\text{reference}}$, where M represents the relative modulation of a reference solution.

The sample was excited with 360 nm light, and emission from the sample was monitored through a KV-389 cut on filter. Lifetimes were determined in the presence of a reference fluorophore with a known lifetime (dimethyl-POPOP, 1.4 nsec). A rhodamine quantum counter solution was placed in the reference chamber, and emission was monitored through a RG630 filter.

The contents of the sample cuvettes were stirred with a magnetic stirrer, and the sample chamber temperature was controlled by a circulating water bath.

Results and discussion

As observed in the lymphocyte membrane [11], desipramine, imipramine, nortriptyline, and protriptyline can be placed into two groups based on their effects on the fluorescence anisotropy and lifetime of DPH in the DPPC + DOPC liposome. Nortriptyline and protriptyline effected concentration-dependent decreases in the DPH fluorescence anisotropy of DPH-labeled DPPC + DOPC liposomes (Fig. 1) and had no significant effect on the

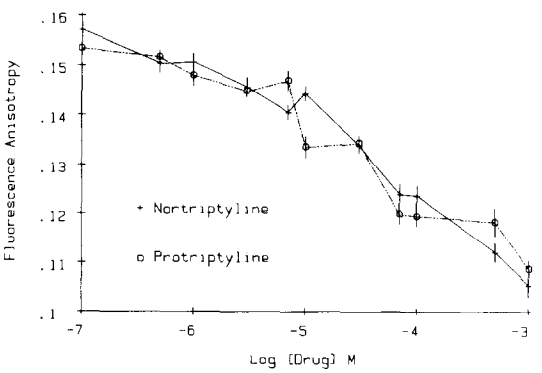

Fig. 1. Effects of nortriptyline and protriptyline on the fluorescence anisotropy of DPH in DPPC (2/3) + DOPC (1/3) multilamellar liposomes. The fluorescence anisotropy value of DPH in a control liposome suspension remained at 0.151 ± 0.004.

* Abbreviations: DPPC, L-α-phosphatidylcholine, depalmitoyl; DOPC, L-α-phosphatidylcholine, dioleoyl; egg PC, egg L-α-lecithin; DPH, 1,6-diphenyl-1,3,5-hexatrienic; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

fluorescence lifetime of DPH. By contrast, desipramine and imipramine produced a concentration-dependent increase in the fluorescence anisotropy of DPH in DPH-labeled DPPC + DOPC liposomes. However, the increase in fluorescence anisotropy of DPH caused by desipramine and imipramine may be explained in terms of quenching, since both imipramine and desipramine produced a concentration-dependent decrease in the fluorescence lifetime of DPH (Fig. 2). The quenching of DPH fluorescence by desipramine and imipramine is suggested to be due to intermolecular collisions (dynamic quenching) because (1) there is no spectral overlap between DPH and desipramine and imipramine, and (2) the quenching is temperature dependent (greater at higher temperatures), suggesting dynamic diffusion-dependent mechanisms. Another possibility is that a perturbing effect of these agents upon membrane structure could be indirectly producing these lifetime changes. In this eventuality, there would not be the need to invoke direct drug–DPH interactions.

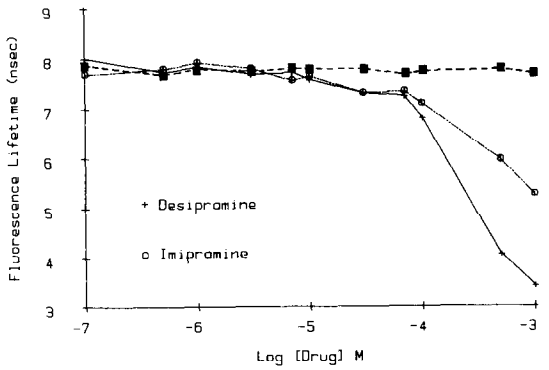

Fig. 2. Effects of desipramine and imipramine on the fluorescence phase lifetime of DPH in DPC (2/3) + DOPC (1/3) multilamellar liposomes. The fluorescence lifetime of DPH in a control suspension of liposomes remained at approximately 7.75 nsec (■---■).

The fluorescence anisotropy of DPH in liposomes is interpreted in terms of changes in the structural order of the phospholipid bilayer [18–20]. Protriptyline- and nortriptyline-induced decreases in DPH fluorescence anisotropy in DPH-labeled DPPC + DOPC liposomes were interpreted as drug-induced perturbations of the structural order of the phospholipid bilayer. Desipramine and imipramine, by contrast, did not penetrate the DPPC + DOPC phospholipid bilayer and perhaps localized in the proximity of the excited DPH molecule, resulting in the possible quenching of DPH fluorescence emission. It is probable that desipramine and imipramine, like protriptyline and nortriptyline, also perturbed the phospholipid bilayer of DPPC + DOPC liposomes. Calorimetric, ESR, and NMR studies have demonstrated that desipramine and imipramine affect the phospholipid bilayer of DPPC liposomes [21–23].

To characterize the effect of the tricyclic antidepressants on the temperature transition profile of DPPC + DOPC liposomes, a 100 μM concentration of either nortriptyline or protriptyline was added to the DPPC + DOPC liposome suspensions (Fig. 3). The typical temperature transition profile of DPH-labeled DPPC + DOPC liposomes in the absence of drug is consistent with the findings of Lentz *et al.* [24]. However, addition of nortriptyline or protriptyline to DPPC + DOPC liposomes shifted the temperature transition profile to the left. These results are consistent with the understanding that molecules which perturb the lipid bilayers of liposomal and biological membranes lower the transition temperature of the lipid bilayer [22].

In our studies we were unable to demonstrate lipid structure perturbations in DPPC liposomes at a 100 μM concentration of any of the tricyclic antidepressants. This fluorometric result should be compared with calorimetric studies in which desipramine produces lipid polar head group structural alterations in DPPC liposomes at 2 moles/100 ml [22]. Cater *et al.* [22] reported that these lipid structural perturbations originated at the polar-head groups of the phospholipid bilayer. In such circumstances, our probe, DPH, which resides in the hydrocarbon region, may not be sensitive to structural changes that mainly occur in the vicinity of the polar-head groups. Also, desipramine and other tricyclic antidepressants may not readily penetrate to the fatty acid regions of DPPC liposomes due to the physical state of the lipid (gel) below the transition temperature (T_m 41°).

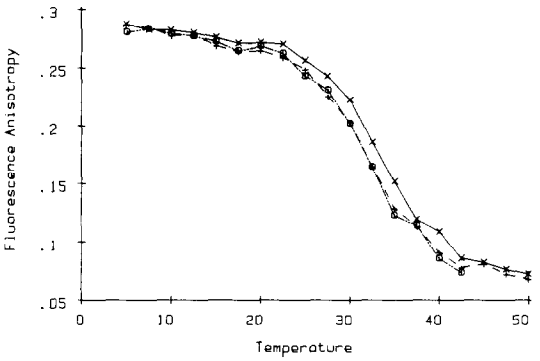

Fig. 3. Temperature dependence of the effects of nortriptyline and protriptyline on the fluorescence anisotropy of DPH in DPPC (2/3) + DOPC (1/3) multolamellar liposomes. Nortriptyline (100 μM) (+), protriptyline (100 μM) (○), or an equal amount of PBS (control, ×) was added to DPH-labeled multilamellar suspensions and the fluorescence anisotropy was recorded at 2.5° intervals.

Lower concentrations (<100 μM) of desipramine and imipramine produced an increase in the fluorescence anisotropy and a corresponding decrease in the fluorescence lifetime of DPH in egg PC liposomes. Nortriptyline and protriptyline at concentrations of 10^{-7} to 10^{-3} M, by comparison, had no effect on the fluorescence lifetime or anisotropy of DPH in egg PC or DOPC liposomes. Since the quenching of DPH requires that desipramine and imipramine localize in close proximity to the excited DPH molecule, these results suggest that desipramine and imipramine must diffuse into the fatty acid domains of egg PC liposomes. The fatty acid chains of DOPC (T_m − 20°) and egg PC liposomes are less tightly packed due to the presence of double bonds. Consequently, the lecithin bilayers of the DOPC and egg PC liposomes are much less ordered than DPPC bilayers. In this study, high concentrations (>100 μM) of nortriptyline and protriptyline apparently were not capable of perturbing the bilayers of the less ordered DOPC and egg PC liposome bilayers to a greater degree than they already are.

DPPC, DOPC and DPPC + DOPC liposomes have been well characterized with DPH fluorescence [24–26]. Concentrations (<100 μM) of tricyclic antidepressants that perturb DPH-labeled bilayers of DPPC + DOPC liposomes did not perturb DPH-labeled bilayers of either DPPC or DOPC liposomes. Since DPPC and DOPC mix less than ideally in both small vesicles and large multilamellar liposomes [22], the tricyclic antidepressants may perturb DOPC + DPPC bilayers at phase boundaries. Alternatively, the tricyclic antidepressants may access DPH-labeled DPPC + DOPC bilayers through less ordered DOPC domains where permeation of the drug is less restricted. Concerning this latter point, there is a correlation between liposome permeability and the presence of unsaturated hydrocarbon chains [27].

The DPPC + DOPC liposome is probably not an adequate model of the more complex biological bilayer. However, the formation of separate lipid domains, as in the DPPC + DOPC liposomes [28], may represent lipid–lipid relationships characteristic of some biomembranes [24, 29–31]. The fact that tricyclic antidepressants interact differently with DPPC + DOPC liposomes than with either DPPC or DOPC liposomes suggests the possible importance of phase boundary regions as possible sites for drug effects.

The results of our study indicate that the tricyclic antidepressants, nortriptyline and protriptyline, perturb the DPH-labeled bilayers of DPPC + DOPC liposomes but do not perturb the DPH-labeled bilayers of DPPC, DPC, or egg PC liposomes. Nortriptyline- and protriptyline-induced perturbations of the bilayers of DPPC + DOPC liposomes occurred at concentrations that are similar to the concentrations of nortriptyline and protriptyline that perturb lymphocyte membranes [11]. In addition, nortriptyline and protriptyline decreased the transition temperature of the lipids in DPPC + DOPC liposomes.

In contrast, desipramine and imipramine quenched the fluorescence emission of DPH in DPPC + DOPC and egg PC liposomes at concentrations similar to those concentrations of desipramine and imipramine that quenched DPH fluorescence emission in murine lymphocytes. The concentrations of desipramine and imipramine that quenched DPH fluorescence were also similar to the concentrations of nortriptyline and protriptyline that perturbed the phospholipid bilayers of liposomes and lymphocytes. Other methods will be required to determine the presence of desipramine and imipramine effects on the lipids of DPPC + DOPC liposome and lymphocyte membranes. Finally, desipramine and imipramine did not alter the fluorescence anisotropy or lifetime of DPH in DPPC liposomes.

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Effect of uncharged anesthetics on ion binding to liposome surfaces

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Calcium binds more strongly to negatively charged lipid surfaces (phosphatidylserine) than to electrostatically neutral phospholipids (phosphatidylcholine) [1]. Trivalent praseodymium, however, strongly binds to both types of lipids. Binding probably occurs via electrostatic interactions between the positively charged ion and the negatively charged phosphate oxygen of the phospholipid or the carboxylic group of phosphatidylserine. As reported recently,