Spontaneous Transfer of Sphingomyelin between Phospholipid Bilayers†

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ABSTRACT: The half-time of spontaneous transfer of pyrene-labeled and ^{3}H-labeled sphingomyelin has been determined as a function of temperature in several matrix lipids. Data obtained by using the two labels are in good agreement. Transfer half-times from liquid-crystalline phase matrix lipids are about 2 orders of magnitude shorter than the half-times obtained in gel-phase systems; however, transfer half-times are not markedly dependent on the molecular species of matrix lipid. The small half-times of transfer seen below 30 °C in 1-palmitoyl-2-oleoylphosphatidylcholine suggest that a gel phase rich in sphingomyelin may exist in this system even at low sphingomyelin concentrations.

In recent years, there has been considerable interest in the spontaneous, noncatalyzed, net interbilayer transfer of membrane lipids (McLean & Philips, 1981; Doody et al., 1980; Roseman & Thompson, 1980; Nichols &Pagano, 1980a,b; Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Papahadjopoulos et al., 1976; Kao et al., 1977; Massey et al., 1980). Previous to the pioneering study by Martin & MacDonald (1976), it had been generally assumed the spontaneous net transfer did not occur at an appreciable rate because of the very low solubility of membrane lipids in aqueous media (Smith & Tanford, 1972). A number of lipid-transfer proteins have been described which accelerate the transfer process, either specifically for certain lipids or nonspecifically [for a review, see Wirtz (1982)].

In this paper, we report the results of studies on the spontaneous interbilayer net transfer of sphingomyelin using fluorescently labeled sphingomyelins. This method depends upon the ability of pyrene-containing molecules to form an excited-state dimer (excimer) between a ground-state and an excited-state monomer (Doody et al., 1978; Charlton et al., 1976; Sengupta et al., 1976; Roseman & Thompson, 1980). The fluorescence emission of the excimer is at a longer wavelength than that of the monomer. Since excimer formation is dependent on the fluorophore concentration, the ratio of excimer fluorescence intensity to monomer intensity is a function of the fluorophore concentration (Birks et al., 1963). When a population of donor vesicles containing a known amount of the pyrene-labeled lipid is incubated with an acceptor vesicle population containing no pyrene label, the dilution of pyrene-labeled lipid resulting from the transfer of lipid from donor to acceptor vesicles can be followed as a function of time by observing the time dependence of the excimer to monomer fluorescence intensity ratio (E/M) (Roseman & Thompson, 1980). In order to assess the degree to which the pyrene-labeled sphingomyelin perturbs the transfer process, we have also carried out studies of spontaneous transfer with [^{3}H]sphingomyelin carrying the radiolabel in the amide-linked fatty acid moiety. A preliminary report of this work has appeared elsewhere (Frank et al., 1982).

Experimental Procedures

Materials. Dimyristoyl-, dipalmitoyl-, 1-palmitoyl-2-oleoyl-, and distearoylphosphatidylcholines (DMPA, DPPC, POPC, and DSPC, respectively)[1] were purchased from Avanti Biochemicals, Inc. These lipids were checked for purity by thin-layer chromatography and stored in sealed ampules under N_2 at −20 °C.

Sphingomyelin (Spm) was prepared from bovine brain following established procedures (Shinitzky & Barenholz, 1974). Semisynthetic sphingomyelins with a single acyl chain in amide linkage to the sphingosine base were prepared by deacylation of porcine brain sphingomyelin followed by reacylation of the resulting sphingosine phosphorylcholine with the desired fatty acid (Correa-Freire et al., 1982; R. Cohen et al., unpublished results). The products were purified by thin-layer chromatography. 12-(1-Pyrenyl)dodecanoic acid was obtained from Molecular Probes. The pyrene-labeled sphingomyelin (PyrSpm) used in these studies was *N*-[12-(1-pyrenyl)dodecanoyl]sphingomyelin. New England Nuclear supplied [^{3}H]oleic acid and [^{3}H]palmitic acid.

Preparation of Liposomes. In transfer experiments using pyrene-labeled lipid, small unilamellar vesicles (SUV) were used as donor systems. These vesicles, which were composed of a matrix phospholipid containing PyrSpm at a concentration less than 7 mol %, were prepared by sonication of the lipid components in 0.05 M KCl, 0.01 M Tris, and 10^{-4} M EDTA (pH 7.5) under N_2 following the method of Barenholz et al. (1977). Sonication was always carried out above the phase transition temperature of the matrix phospholipid. Small unilamellar vesicles formed from matrix lipid only and prepared by the procedure described above were used as acceptor vesicles. In a few experiments, SUV were prepared by bath sonication for 1 h above the transition temperature of the matrix lipid. The resulting SUV were separated from large vesicles and multilamellar liposomes by differential ultracentrifugation (Barenholz et al., 1977).

In transfer experiments using tritium-labeled lipids, small unilamellar vesicles served as donors and large unilamellar

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Spm, bovine brain sphingomyelin; PyrSpm, *N*-[12-(1-pyrenyl)dodecanoyl]sphingomyelin; PyrPC, 1-palmitoyl-2-[12-(1-pyrenyl)dodecanoyl]phosphatidylcholine; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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vesicles (LUV) as acceptors. Donor vesicles containing the ^{3}H-labeled phospholipid (about 6400 dpm/μmol of matrix lipid) and cholesteryl [^{14}C]oleate (25 500 dpm/μmol of matrix lipid) as a nontransferable marker phospholipid were prepared as described above. Large unilamellar acceptor vesicles were prepared from DPPC by fusion of SUV below the phase transition temperature (Schullery et al., 1980).

Multilamellar liposomes for calorimetric studies were prepared as described by Lentz et al. (1976a,b). Differential scanning calorimetry utilized a heat conduction type apparatus. Studies were carried out as described by Suurkuusk et al. (1976).

Transfer Kinetics from Fluorescence Measurements. Fluorescence measurements utilized a Perkin-Elmer MPF-3 spectrofluorometer. The cuvette temperature was controlled by a Lambda Mk-2 thermoregulated bath and was monitored by a thermistor element located in the cuvette. The quartz cuvettes were sealed with Teflon stoppers at all times. Emission spectra of the pyrene derivatives were recorded by scanning the range from 360 to 600 nm. The excitation wavelength was constant at 345 nm. Uncorrected spectra were used in all calculations.

The experimental procedure for the determination of pyrene-labeled phospholipid transfer was similar to that used by Correa-Freire et al. (1982). Aliquots of donor and acceptor vesicle dispersions were mixed at zero time to achieve the desired concentration ratio (R) of acceptor to donor vesicles. In most experiments, R was 5 or larger, the final sample volume was 2 mL, and the total lipid concentration was between 0.16 and 0.32 mM. The mixed-vesicle system was maintained at the desired temperature in the sealed fluorometer cuvette. Excimer emission intensity (E) at 460 nm and monomer emission intensity (M) at 376 nm were determined from peak heights. Fluorescence intensity measurements were made just prior to mixing, immediately after mixing ($t = 0$), and at subsequent time intervals. Light-scattering corrections proved to be negligible in all cases (Correa-Freire et al., 1982).

The derivation of the equations relating the experimental E/M ratio to the concentration of probe remaining in the donor vesicles during the transfer experiments has been presented by Correa-Freire et al. (1982). The expression relating E/M to fluorophore concentration in the donor vesicles for this system is

$$\frac{E}{M} = \frac{C_D^2(C_{D_0} - C_D) + RC_D^2C_h + (C_{D_0} - C_D)^2(C_D + C_h)}{C_hC_D(C_{D_0} - C_D) + RC_h^2C_D + RC_h(C_{D_0} - C_D)(C_D + C_h)} \times \frac{K_1}{K_2}\frac{N_{E_{max}}}{N_{M_{max}}} \quad (1)$$

Here C_{D_0} is the initial probe concentration in the donor vesicles, C_D is the probe concentration in the vesicles at any time t, and R is the ratio of the concentrations of acceptor to donor vesicles. C_h is the concentration at which the fluorescence of the monomer has been quenched by 50% while that of the excimer has risen to half of its maximum value in concentrated solutions (Parker, 1968), and K_1 and K_2 are proportionality constants. $N_{E_{max}}$ and $N_{M_{max}}$ are the maximum excimer quantum yield as $C \rightarrow \infty$ and monomer quantum yield as $C \rightarrow 0$, respectively (Correa-Freire et al., 1982).

Values of C_D as well as values for the constants C_h and $(K_1/K_2)(N_{E_{max}}/N_{M_{max}})$ were obtained from data sets by using an interative nonlinear curve-fitting procedure (Johnson et al., 1981) on a Control Data Corp. Cyber 730 computer. Experimental determinations of the values of these constants were made in several cases following the procedure of Correa-Freire et al. (1982). The apparent first-order rate constant for transfer (k_{obsd}) was obtained from the following equation:

$$\ln \frac{C_{D_0} - C_{D_{eq}}}{C_D - C_{D_{eq}}} = k_{obsd}t \quad (2)$$

Here C_D is the concentration of the pyrene derivative at time t, C_{D_0} is the initial concentration of the derivative at $t = 0$, and $C_{D_{eq}}$ is the equilibrium value of this variable at $t = \infty$. Values of $C_{D_{eq}}$ can be calculated from the initial concentration parameters for two limiting cases. (i) If the transbilayer migration rate (flip-flop) of the pyrene phospholipid is very fast with respect to interbilayer transfer, then $C_{D_{eq}} = C_{D_0}/(R + 1)$. As described above, R is the concentration ratio of acceptor to donot vesicles. (ii) If the transbilayer migration of the fluorophore is very slow with respect to interbilayer transfer, then $C_{D_{eq}} = C_{D_0}[0.65/(R + 1) + 0.35]$. The second equation is valid only for SUV.

Kinetics of Transfer Using ^{3}H-Labeled Lipids. Small unilamellar donor vesicles containing [^{3}H]sphingomyelin, cholesteryl [^{14}C]oleate as a nonexchangeable marker, and matrix phospholipid were mixed with large unilamellar acceptor vesicles comprised of DPPC and incubated at the desired temperature. Aliquots of the system at various time intervals were placed on a Sepharose 4B-CL molecular sieve column to separate the large unilamellar acceptor vesicles from SUV donors. The recovery from the column was always greater than 80%. The percent transfer of ^{3}H-labeled phospholipid was determined after correction for small vesicle and fusion product contaminants as estimated from the amount of cholesteryl [^{14}C]oleate in the acceptor fraction. Transfer half-times were calculated following the procedure of McLean & Philips (1981). Total phospholipid concentration was estimated in all experiments by determination of phosphorus (Bartlett, 1959).

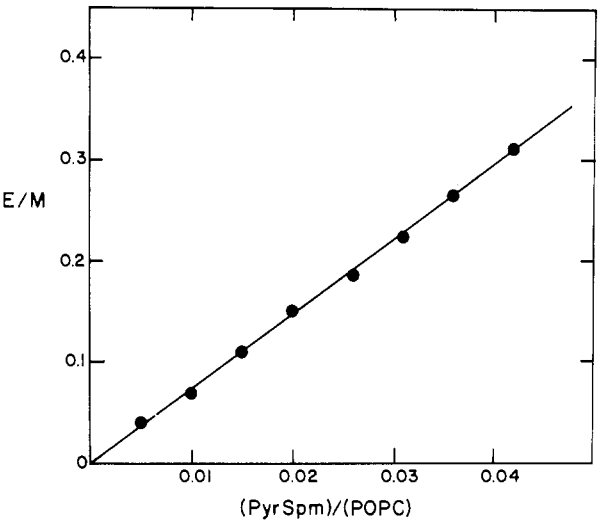

FIGURE 1: Dependence of the excimer to monomer intensity ratio on the PyrSpm concentration in POPC small unilamellar vesicles at 50 °C.

Results and Discussion

Concentration Dependence of PyrSpm E/M Intensity Ratio. A representative plot of E/M at 50 °C vs. the molar concentration ratio of PyrSpm to POPC, the matrix lipid, is shown in Figure 1. A linear concentration dependence, as predicted theoretically, was observed in all systems examined in this

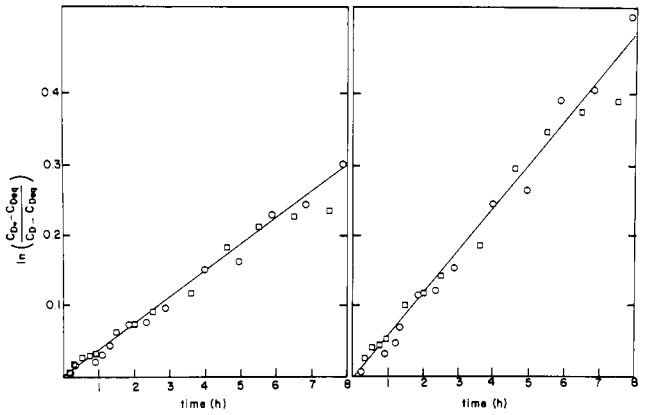

FIGURE 2: Kinetics of PyrSpm transfer between small unilamellar POPC vesicles at 50 °C. Ratios of acceptor to donor vesicles are 1 (○) and 5 (□). Values of $C_{D_{eq}}$ are calculated for rapid transbilayer migration (left panel) and no transbilayer migration of PyrSpm (right panel). The concentration of PyrSpm in POPC is 4 mol %.

study below 5 mol % of the pyrene derivative (Birks et al., 1963; Roseman & Thompson, 1980). In certain systems at low temperatures, the slope of the plot decreased markedly.

PyrSpm Transfer Half-Times. Figure 2 shows representative plots of ln (normalized concentration function of PyrSpm in POPC) vs. time at 50 °C. In each panel, data for the acceptor to donor vesicle concentration ratio (R) equal to 1 and 5 are shown. The left panel presents the data analyzed for case i in which the transbilayer migration of PyrSpm is very fast with respect to interbilayer transfer. The right panel shows the data analyzed for case ii in which no transbilayer migration occurs. It is clear from these data that the rate of transfer does not depend on vesicle concentration. This results, which was obtained in all systems examined, is similar to that obtained with other types of lipids (Roseman & Thompson, 1980; Doody et al., 1980; Nichols & Pagano, 1980a,b; McLean & Philips, 1981; Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Papahadjopoulos et al., 1976; Kao et al., 1977; Masey et al., 1980). A transfer rate which is independent of vesicle concentration has generally been taken to indicate that transfer of lipid is through the aqueous phase, rather than by a process involving vesicle collision, and that transfer is limited by the rate at which molecules leave the donor bilayer (Roseman & Thompson, 1980; McLean & Philips, 1981; Nichols & Pagano, 1982).

It is impossible to distinguish between the two limiting cases (i and ii) discussed above on the basis of the data in Figure 2. However, except for the special case in which the rate of interbilayer transfer equals the rate of flip-flop, the linearity of the plots rules out the possibility that interbilayer transfer and transbilayer migration are in the same time regime. No information is available about the flip-flop rate of PyrSpm. However, based on studies of other phospholipid systems (Thompson, 1978), it seems probable that case i, no flip-flop, describes the correct situation.

Half-times for the spontaneous transfer of PyrSpm in several matrix lipids at various temperatures are summarized in Table I. The values listed for systems at 20 and 16 °C are lower limits calculated from the precision of the E/M measurements, since no transfer could be detected. A value of the transfer half-time of PyrPC in DMPC at 36 °C, taken from the results of Roseman & Thompson (1980), is included for comparison. The data in Table I show that the transfer half-time for PyrSpm in the gel phase at 20 °C is longer than that in the liquid-crystalline phase at 50 °C (Barenholz & Thompson, 1980; Silvius, 1982) by a factor of at least 270. A similar

Table I: Transfer Half-Times Using Pyrene-Labeled Sphingomyelin

system	temp (°C)	$t_{1/2}$ (h)[a] fast flip-flop	$t_{1/2}$ (h)[a] no flip-flop
PyrSpm[b] in POPC	20	$>2.4 \times 10^3$	$>1.6 \times 10^3$
	30	4.1×10^2	2.6×10^2
	40	3.0×10	2.0×10
	50	2.0×10	1.2×10
PyrSpm in Spm	20	$>2.4 \times 10^3$	$>1.6 \times 10^3$
	50	1.0×10	5.9
PyrSpm in DPPC	50	1.1×10	6.4
PyrSpm in DMPC	16	$>2.4 \times 10^3$	$>1.6 \times 10^3$
	36	3.0×10	1.9×10
	50	3.6	2.4
PyrPC in DMPC[c]	36	4.3×10	2.7×10

[a] Error ± 15%. [b] PyrSpm T_m = 36 °C. [c] Roseman & Thompson (1980).

Table II: Transfer Half-Times Using Tritiated Sphingomyelin

system	temp (°C)	$t_{1/2}$ (h)[a] fast flip-flop	$t_{1/2}$ (h)[a] no flip-flop
[^{3}H]palmitoyl-Spm[b] in POPC	22	2.3×10^3	1.7×10^3
	50	5.3×10	3.3×10
[^{3}H]oleoyl-Spm[c] in POPC	22	5.3×10^3	3.3×10^3
	30	1.7×10^2	4.3×10^2
	40	9.0×10	5.7×10
	50	5.7×10	3.4×10

[a] Error ± 25%. [b] T_M = 41 °C for N-palmitoylsphingomyelin (Barenholz et al., 1976). [c] T_M lies between 22 and 30 °C for oleoylsphingomyelin.

situation obtains for PyrSpm in DMPC. In light of these results, it is surprising the PyrSpm in POPC also shows a large increase in $t_{1/2}$ in going from 50 to 20 °C although POPC, with a phase transition temperature at −5 °C (Silvius, 1982), is liquid crystalline over this temperature range.

It is possible that the low rates of interbilayer transfer of PyrSpm in POPC at 30 °C and below are a special property of the pyrene derivative. To examine this possibility, transfer experiments were carried out with sphingomyelins containing a tritiated fatty acid moiety. The results of studies using N-[^{3}H]palmitoylsphingomyelin or N-[^{3}H]oleoylsphingomyelin in POPC at various temperatures are presented in Table II. These results compare well with the corresponding data in Table I. It seems clear that the strong temperature dependence of interbilayer transfer of PyrSpm is also a property of N-palmitoyl- and N-oleoylsphingomyelins.

The temperature dependence of the rate of interbilayer transfer from POPC of both [^{3}H]oleoylsphingomyelin and PyrSpm can be calculated from an Arrhenius plot (not shown). At high temperatures, the apparent activation energy is about 10 kcal mol^{-1}, but at low temperature, this increases to at least 65 kcal mol^{-1}.

The marked dependence of the transfer rate of sphingomyelin in POPC on temperature suggests the presence in the low-temperature range of a gel-like phase containing the sphingomyelin which is in equilibrium with a liquid-crystalline phase comprised of POPC. This conclusion is supported by an extensive study carried out by Untracht & Shipley (1977) on systems comprised of very similar lipids. In this study, X-ray diffraction, differential scanning calorimetry, and polarizing microscopy were used to determine the ternary phase diagram for egg phosphatidylcholine–bovine brain sphingomyelin–water over the temperature range 10–44 °C. These authors present evidence for the existence in excess water at

20 °C and below of an intermediate gel phase or compound in liquid-crystalline egg phosphatidylcholine in the concentration range 0–33 mol % Spm. Since POPC is the principal component of egg yolk phosphatidylcholine (Porter et al., 1978) and since most sphingomyelins have similar phase transition temperatures (Barenholz & Gatt, 1982), it seems possible to us that our transfer measurements reflect the existence of a similar sphingomyelin-rich phase or compound in our system. Less detailed calorimetric studies on mixtures of POPC and synthetic *N*-stearoylsphingomyelin support this interpretation (Barenholz et al., 1976). The existence of a gel-like phase dispersed in a liquid-crystalline bilayer has also been suggested for the structure of systems comprised of small amounts of glucosylceramide in DPPC or DMPC above the phase transition temperatures of these phospholipids (Correa-Freire et al., 1982).

Very recently, Pownall and co-workers (Pownall et al., 1982) have reported data for the spontaneous transfer of *N*-[9-(1-pyrenyl)nonanoyl]sphingomyelin between single-bilayer vesicles composed of DMPC, beef brain sphingomyelin, or L-α-1-palmitoyl-2-palmitoleoylphosphatidylcholine. These workers conclude as we do that the transfer process is first order and independent of the composition of the acceptor vesicles. They also note that the transfer rate in bovine brain sphingomyelin is about an order of magnitude smaller below the phase transition of this lipid than above it. Where comparable, however, their transfer half-times are about an order of magnitude smaller than ours. The origin of this difference almost certainly rests in the length of the pyrenyl fatty acid linked to the nitrogen of the sphingosine phosphorylcholine moiety of the sphinogmyelin. The data in Table I were obtained by using the 12-(1-pyrenyl)dodecanoyl derivative. Preliminary studies carried out with *N*-[16-(1-pyrenyl)hexadecanoyl]sphingomyelin give substantially longer transfer half-times than those shown in Table I (data not shown). It thus appears that the transfer half-time increases with increasing chain length of thee amide-linked fatty acid in sphingomyelins. A similar acyl chain length dependence has been noted for a variety of phospholipids labeled with the fluorophore 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) (Nichols & Pagano, 1982). Pownall and co-workers have also described this phenomenonon for a number of pyrene-labeled phospholipids and other amphiphiles (Massey et al., 1982; Pownall et al., 1983).

From the biological standpoint, an interesting question is whether or not spontaneous transfer of sphingomyelin can be a contributory process in the distribution of this molecule between plasma membranes of neighboring cells and between the internal membranes of a single cell. The answer to this question depends upon the half-time of transfer. Since our PyrSpm data are in agreement with the ^{3}H-labeled sphingomyelin data, it appears that the *N*-[12-(1-pyrenyl)dodecanoyl]sphingomyelin reasonably mimics the behavior of *N*-palmitoylsphingomyelin, a major sphingomyelin component of mammalian membranes (Barenholz & Thompson, 1980). This view is strengthened by the fact that the gel to liquid-crystalline phase transition of *N*-[12-(1-pyrenyl)dodecanoyl]sphingomyelin is about 35 °C as we determined by using differential scanning calorimetry. This is very close to the transition temperature for natural sphingomyelin preparations (Barenholz et al., 1976). It thus may be that the spontaneous transfer process is too slow to be of importance in comparison to the in vivo process catalyzed by one or more exchange proteins (Pownall et al., 1982; Wirtz, 1982).

Registry No. DMPC, 13699-48-4; DPPC, 2644-64-6; POPC, 6753-55-5; DSPC, 4539-70-2.

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Characterization of Blue and Yellow Pigments in Eggs of the Mexican Leaf Frog†

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ABSTRACT: Blue and yellow pigments were isolated from eggs of the Mexican leaf frog, *Agalychnis dacnicolor*. The major yellow pigment has chemical and spectral properties similar to the xanthophyll lutein. It is readily extracted from yolk platelets with acetone or methanol and has major absorption peaks in hexane at 441 and 470 nm. The dimethyl ester of the blue pigment has absorption maxima in methanol at 374 and 640–680 nm. The blue pigment is protein bound and requires acetone–HCl for extraction. The dimethyl ester of the major blue pigment has chemical, chromatographic, ultraviolet–visible, infrared, and proton magnetic resonance spectra identical with those of the dimethyl ester of biliverdin IXα. Although biliverdin IXα is the major component of the blue pigment, two other minor blue pigments occur which are believed to be biliverdin isomers. Analysis of eggs varying in color from brilliant blue to pale yellow-green show that the color is dependent on the ratio of the two pigments present. These pigments may provide protective coloration to the eggs which are laid on leaves of trees.

During our study on the lipid composition of the eggs and embryos of the Mexican leaf frog, *Agalychnis dacnicolor* (Marinetti et al., 1981), we observed that their green coloration was due to the presence of two pigments, one blue and one yellow. In the present study we have identified the major blue pigment as biliverdin IXα and the major yellow pigment as lutein. The presence of the latter pigment is not surprising since lutein is widely distributed among plants and animals (Fox, 1979). On the other hand, biliverdin is more restrictive as a pigment, although it has been found in the dog placenta, in the shells of bird eggs, bones and skin of some fishes and amphibians (Rudiger, 1970), the eggs and larvae of the tobacco hornworm (Cherbas, 1973), and the serum and eggs of *Xenopus* (Redshaw et al., 1971). It seems likely that the utilization of these two diverse pigments by *A. dacnicolor* evolved as a mechanism for producing green eggs. The green coloration of eggs of leaf frogs which are laid on green vegetation may afford camouflage in protecting eggs and embryos from predation. The biochemical, physiological, ecological, and evolutionary significance of these pigments seem far reaching and have provided the impetus for our elucidating their chemical structures.

Materials and Methods

Eggs of *A. dacnicolor* (900 eggs) were obtained from greenhouse reared frogs. The eggs varied in color from yellow-green to brilliant blue. Silica gel 60 TLC glass plates 20 × 20 cm (0.25 mm) were obtained from VWR. Chloroform, ethyl acetate, hexane, methanol, acetone, and glacial acetic acid (ACS grade) were obtained from Fisher Scientific. Chloroform, methanol, and acetone were redistilled before use. Unisil silicic acid (100–200 mesh) was purchased from Clarkson Chemical Co.

^{1}H NMR spectra and infrared spectra were run in $CDCl_3$ at ambient temperature on a Brüker WHFT 400 mHz spectrometer and Perkin-Elmer Model 220 spectrometer, respectively. Electron microscopy was done on a Zeiss 10-Å electron microscope. Ultraviolet–visible spectra were performed on a Cary Z19 spectrophotometer. Lutein and zeaxanthin were supplied by Hoffmann-La Roche, Switzerland. α- and β-carotene and biliverdin were obtained from Sigma Chemical Co. Commercial biliverdin is primarily biliverdin IXα but does contain other biliverdin isomers (Lehner et al., 1978; McDonagh & Palma, 1980).

The blue pigment and Sigma biliverdin were converted to their dimethyl esters by heating in methanol–3 N HCl (Supelco) for 1 h at 80 °C in a sealed tube under nitrogen. The dimethyl esters were purified by column chromatography on Unisil silicic acid and then by TLC in chloroform–acetone, 95:5.

Results

Isolation and Microscopic Analysis of the Yolk Platelets. Fifteen eggs were homogenized in 3 mL of Krebs–Ringer's bicarbonate (KRB) buffer, pH 7.4, by using 50 strokes of a Dounce homogenizer. The sample was filtered through two layers of 86 mesh nylon (Southern Sign Supply Co., Glen Burne, MD). The filtrate was treated with 1 mg of collagenase (ICN Pharmaceuticals, 183 U/mg) for 18 h at 21 °C. The sample was centrifuged at 2400*g* for 5 min, and the pellet (yolk platelets) was washed twice with 3 mL of KRB buffer. The

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