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Transmembrane Potentials and Phospholipid Flip-Flop in Excitable Membrane Vesicles†

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ABSTRACT: Excitable membrane vesicles prepared from the electroplax of *Electrophorus electricus* are permeable to tempocholine, a spin-labeled choline analog. The inside-outside distribution of tempocholine measures the transmembrane electrical potential difference induced in the vesicles by a concentration gradient of sodium sulfate. Dioleoylphosphatidyltempocholine (OPTC), a spin-labeled phosphatidylcholine analog, can be incorporated into excitable membrane vesicles by incubating spin-label vesicles with the membranes at 30°. The rate of inside-outside transitions (flip-flop) of the spin-labeled phospholipid, measured by an ascorbate reduction procedure, is characterized by a half-time of 3.8–7 min at 15°. This rate is an order of magnitude faster than the corresponding rate in pure phospholipid vesicles.

The molecular mechanism of chemical excitation is not known, although the changes in ion permeability and transmembrane potential associated with excitation are well characterized. For cholinergic synapses, the interaction of acetylcholine with the acetylcholine receptor at the postsynaptic membrane is generally proposed to be the primary event in the excitation process and considerable effort has been dedicated to the isolation and purification of the receptor.[1]

The reconstitution of functional membranes from purified membrane components is a problem of current interest in membrane molecular biology (Razin, 1972) and the incorporation of purified receptor into model membrane systems offers a promising approach to understanding the role of the receptor in chemical excitation. Any mechanism proposed for excitation either *in vivo* or in reconstituted membranes must be compatible with the physiochemical properties of the membrane. The molecular motions and interactions of the protein and lipid membrane components are dynamic parameters that clearly affect membrane function.

The dynamic properties of phospholipid bilayer vesicles have been characterized in detail by a variety of physical methods; Kornberg and McConnell (1971a,b) have shown that diffusion in the plane of the bilayer is very fast, whereas diffusion across the bilayer (phospholipid flip-flop) is exceedingly slow (half-time ~6.5 hr at 30°). The permeability of the vesicles to ions is low and Kornberg *et al.* (1972) have developed a spin-label technique for measuring transmembrane potentials induced in the vesicles by antibiotic ion carriers. The phospholipid bilayer vesicles provide a good model system for the phospholipid bilayer region of biological membranes and the development of techniques for incorporating membrane proteins into the vesicles will make them ideal systems for reconstitution studies.

Kasai and Changeux (1971a–d) introduced a membrane vesicle preparation that serves as a good model system for studying the functional properties of excitable membranes. Membrane fragments containing up to 0.7% of their total protein as the acetylcholine receptor can be isolated from the electroplax of the electric eel, *Electrophorus electricus*, and the permeability of the vesicles to ions can be measured. Kasai and Changeux established an impressive correlation between the *in vitro* response of the vesicles to cholinergic agonists and antagonists and the *in vivo* physiological responses of intact electroplax.

We report here the results of two spin-label studies designed to correlate the physical and electrical properties of Changeux's excitable membrane vesicles with corresponding properties of phospholipid bilayer vesicles. In this way, we can estimate the effect that membrane proteins might have on the molecular motions of phospholipids in reconstituted membranes.

In part A of Results, we use the inside-outside distribution of the spin-labeled ion, tempocholine, to measure transmem-

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[1] For a review of the efforts to isolate and characterize the receptor, see Hall (1972). Recent papers include those of Olsen *et al.* (1972), Franklin and Potter (1972), Schmidt and Raftery (1973), Fulpius *et al.* (1972), Eldefrawi and Eldefrawi (1972), DePlazas and DeRobertis (1972), Karlsson *et al.* (1972), and Reiter *et al.* (1972).

brane potentials induced in excitable membrane vesicles under controlled ionic conditions.

In part B we measure the flip-flop rate of a spin-labeled phosphatidylcholine analog incorporated into the excitable membrane vesicles.

Materials and Methods

Dioleoylphosphatidyltempocholine (OPTC).[2] Dioleoylphosphatidyltempocholine was prepared by the 2,4,6-triisopropylbenzenesulfonyl chloride mediated condensation of dioleoylphosphatidic acid with tempocholine. The procedure was similar to that used by Kornberg and McConnell (1971b) for the synthesis of dipalmitoylphosphatidyltempocholine except that the reaction time was shortened to 1.5 hr and the second purification by silicic acid chromatography was eliminated. The product showed one spot on silica thin-layer chromatography with an R_F value identical with that of dipalmitoylphosphatidyltempocholine. Tempocholine was a gift of Dr. R. D. Kornberg.

Dioleoylphosphatidic acid was obtained by phospholipase D hydrolysis of dioleoylphosphatidylcholine according to the procedure of Davidson and Long (1958) and was purified by precipitation as the barium salt.

Dioleoylphosphatidylcholine was synthesized by the method of Cubero Robles and van den Berg (1969) from L-α-glycerylphosphorylcholine, oleic anhydride, and potassium oleate and purified by silicic acid chromatography (Mallinckrodt SilicAR, CC-4, 100–200 mesh, eluted with mixtures of chloroform–methanol).

L-α-Glycerylphosphorylcholine was obtained by alkaline deacylation of egg phosphatidylcholine (isolated from fresh hen egg yolks by the procedure of Singleton *et al.* (1965)) using the procedure of Brockerhoff and Yurkowski (1965); the L-α-glycerylphosphorylcholine was purified by precipitation as the cadmium chloride adduct and regenerated by the method of Baer and Kates (1948).

Oleic anhydride was prepared from oleic acid (Sigma Chemical Co.) by the action of dicyclohexylcarbodiimide in dry carbon tetrachloride according to the procedure of Lapidot *et al.* (1969).

OPTC Vesicles. OPTC (0.2 ml) in ethanol (15 mM) was evaporated to dryness using toluene to remove traces of ethanol. The dry lipid was suspended by Vortex mixing in 0.5 ml of 0.1 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3) at 35°, transferred to a small test tube, and sonicated for 20 min at 35–40° in a Heat Systems-Ultrasonics Model "7" bath sonicator. The effectiveness of the sonication depended critically on the location of the sample tube in the bath and the optimal location was determined empirically for each preparation. The initially cloudy suspension became clear within 10 min and the sonicated dispersion remained clear for several hours at temperatures above 30°. We generally used the vesicles within 10 min of preparation. It was not possible to prepare stable dispersions of pure dipalmitoylphosphatidyltempocholine, a saturated analog of OPTC synthesized by Kornberg and McConnell (1971b).

Preparation of Excitable Membrane Vesicles. The typical procedure outlined below is adapted from the procedures of Kasai and Changeux (1971a).

Live 1.5-ft electric eels (*E. electricus*) were purchased from Paramount Aquarium (Ardsley, N. Y.). A 35-g portion of fresh main electric organ was cut into small pieces and suspended in 90 ml of ice-cold 0.2 M sucrose (Schwarz/Mann Ultra Pure)–0.1 M Na_2SO_4–0.02 M Tris-SO_4 at pH 7.3. The suspension was homogenized at 0° in a Virtis "23" apparatus for 1.5 min at 90% maximum speed and the homogenate was sonicated for 45 sec at 0° with the 0.5-in. tip of a Branson W185D Sonifier cell disruptor at output control setting 6. The suspension was centrifuged at 5000*g* (6500 rpm) for 20 min at 0° in the 9RA rotor of a Lourdes centrifuge. The supernatant was filtered through cheesecloth and a 20-ml portion was layered onto a discontinuous sucrose gradient (4 ml of 1.1 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4 and 4 ml of 0.4 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4, both at pH 7.3) and centrifuged at 2° for 4.5 hr at 64,000*g* (25,000 rpm) in the SW25.1 rotor of a Beckman L2-65B ultracentrifuge. The membrane fraction at the 1.1–0.4 M sucrose boundary is rich in acetylcholinesterase and corresponds to the excitable microsacs of Kasai and Changeux; we designate this fraction as the "main fraction" in subsequent discussion. To obtain more concentrated suspensions of membrane vesicles, the main fractions from several centrifugations were combined, diluted with two volumes of 0.1 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3), and centrifuged at 0° for 1 hr at 28,000 rpm in the no. 30 rotor of a Spinco centrifuge. The supernatant was decanted and the membrane pellet was resuspended in a small volume of 0.3 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4 at pH 7.3. Total phosphate and protein concentrations and acetylcholinesterase activity were routinely determined for each membrane preparation.

Acetylcholinesterase was assayed with acetylthiocholine as the substrate by the method of Ellman *et al.* (1961) using the same conditions reported by Kasai and Changeux (1971a). The activity of our concentrated membrane vesicle suspensions was ~15 mol of acetylthiocholine hydrolyzed per hr per l.

Total protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard and phosphate was determined by the method of McClare (1971). Typical values of protein and phosphate concentrations for our preparations were 15 mg of protein/ml and 12 μmol of phosphate/ml. We have taken the phosphate concentration to be a measure of the phospholipid concentration in the membrane preparations.

For the experiments reported in Results we specify the preparation number of the particular membrane preparation used and the concentration of protein and phosphate.

Millipore Filtration Assay for Membrane Excitability. The efflux of radioactive ions from membrane vesicles was measured by the Millipore filtration technique described by Kasai and Changeux (1971a). Briefly, vesicles are equilibrated overnight at 4° with a radioactive ion (*e.g.*, $^{22}Na^+$). At time zero the vesicles are diluted 50- to 100-fold into a nonradioactive buffer at 22° and at time *t*, 1-ml aliquots are rapidly filtered through 0.45 μ Millipore filters and washed three times with 3 ml of ice-cold dilution buffer. The counts remaining on the filter after appropriate corrections are attributed to radioactive ions inside vesicles trapped on the filter (400–4000 cpm in our experiments depending on the concentration of membrane vesicles).

The effect of carbamylcholine and *d*-tubocurarine on the efflux of $^{22}Na^+$ from membrane vesicles is determined by including one or both of these agents in the dilution buffer. Specific conditions for the assay are provided in the legend to Figure 1.

Radioactive ions were counted in a Nuclear-Chicago Unilex liquid scintillation counter. The dried Millipore filters

[2] Abbreviations used are: OPTC, dioleoylphosphatidyltempocholine; Carb, carbamylcholine; dTC, *d*-tubocurarine; TC^+, tempocholine.

were suspended in 10 ml of scintillation medium consisting of 3 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (Amersham-Searle) in 1 l. of toluene.

$^{22}Na^+$ (0.5 mCi) and $^{35}SO_4^{2-}$ (5 mCi) were purchased from New England Nuclear Co. (Boston, Mass.)

Electron Paramagnetic Resonance (epr) Spectra. Samples in 50-μl glass capillaries were mounted vertically in the variable-temperature accessory of a Varian E-4 spectrometer. For most experiments the peak-to-peak amplitude of the low-field line of the derivative spectrum was used as a measure of relative spin-label concentration. For samples in which spin-exchange broadening or superposition of spectra made this method unreliable, the spectra were doubly integrated to obtain an accurate relative concentration, using a time-shared computer for real-time data acquisition and storage. Actual values of concentration were determined by reference to spectra of known concentration.

Ascorbate Treatment. Ice-cold 0.35 M sodium ascorbate (5 μl) at pH 7 was added at 0° to 50 μl of a spin label containing sample. The mixture was immediately transferred to an epr sample capillary and the spectrum was recorded. The minimum elapsed time between the addition of ascorbate and the recording of the low-field resonance amplitude was ~1 min.

Excess ascorbate rapidly abolishes the paramagnetism of the nitroxide group; Kornberg and McConnell (1971b) and Kornberg *et al.* (1972) have presented detailed methods for relating the amplitudes of epr spectra of samples before and after ascorbate treatment to inside-outside distributions of spin labels in vesicle membranes. In all of our experiments there was at least a tenfold excess of ascorbate over spin label in the treated samples.

Results

Throughout this work, we have used membrane vesicles derived from the innervated faces of the electroplax of the electric eel, *E. electricus*, by the procedure of Kasai and Changeux (1971a). These vesicles (~1000 Å in diameter) are rich in acetylcholinesterase, contain the acetylcholine receptor, and retain *in vitro* excitability and binding properties characteristic of the intact electroplax. Kasai and Changeux (1971a) introduced a Millipore filtration assay for assessing the excitability of the membrane vesicles and in Figure 1 we present evidence for the excitability of our membrane preparations. The rate of $^{22}Na^+$ efflux from the vesicles is tripled in the presence of 4×10^{-4} M carbamylcholine, an acetylcholine receptor agonist and this effect is blocked by 5×10^{-5} M *d*-tubocurarine, a receptor antagonist. Adopting the notation of Kasai and Changeux we express the excitability as $(\tau_0/\tau) - 1$, where τ and τ_0 represent the half-times for $^{22}Na^+$ efflux in the presence and absence of carbamylcholine; for the membrane preparation in Figure 1 the excitability was 2.1, a value in agreement with results reported by Kasai and Changeux (1971a).[3] We used the sodium efflux assay as an indicator of the structural and functional integrity of membrane vesicles under the conditions of our experiments. In general, we found it convenient to concentrate the vesicles by a second centrifugation after we purified them by the Kasai and Changeux procedure (see Methods). This treatment usually decreased the apparent excitability of the membranes by decreasing τ_0. We present data in a later section (section B and Table II) that illustrates this point.

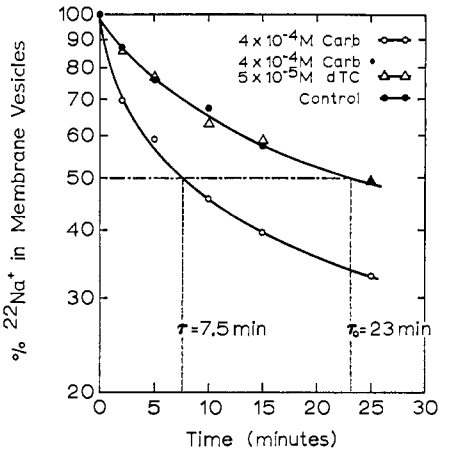


FIGURE 1: The Kasai and Changeux (1971a) sodium efflux assay for membrane excitability. Carbamylcholine (Carb), an acetylcholine receptor gonist, increases the $^{22}Na^+$ efflux rate in membrane vesicles from *Electrophorus electricus*. The effect is blocked by *d*-tubocurarine (dTC), a receptor antagonist. $^{22}Na^+$ (0.1 ml; 0.2 mCi/ml) was mixed with 1.0 ml of membrane vesicles [prep 2 (main fraction), 8 mg of protein/ml] in 0.5 M sucrose–0.05 M Na_2SO_4–0.01 M Tris-Cl (pH 7.3) and incubated overnight at 4°. At time zero, 0.1 ml was diluted with 6.0 ml of 0.17 M KCl–0.01 M Tris-Cl (pH 7.3) at 22°. The rate of $^{22}Na^+$ efflux was measured by the Millipore filtration technique (Methods) and the relative per cent $^{22}Na^+$ inside the vesicles *vs.* time is shown above (●); the effect of Carb was determined by including 4×10^{-4} M Carb in the dilution medium (○) and the blocking effect was determined by including 4×10^{-4} M Carb and 5×10^{-5} M *d*-tubocurarine in the dilution buffer (△). τ and τ_0 represent the half-times for efflux in the presence and absence of Carb.

Results for spin-label measurements of transmembrane potentials and phospholipid flip-flop are presented below in separate sections.

A. Transmembrane Potentials

Excitable Membrane Vesicles Are Permeable to Tempocholine. The inside-outside distribution of a spin-labeled ion $X^{\pm}$ that traverses a vesicle membrane in a charged form is related to the transmembrane electrical potential difference (V) by

$$\frac{[X^{\pm}]_i}{[X^{\pm}]_0} = \exp(\mp eV/kT) \qquad (1)$$

where e is the fundamental unit of charge and k is Boltzmann's constant.

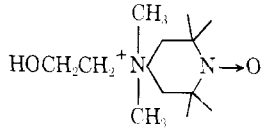
We determined the permeability of excitable membrane vesicles to tempocholine, a positively charged spin-labeled ion, by the spin-label–ascorbate procedure used by Kornberg *et al.* (1972) to determine the permeability of egg phosphatidylcholine vesicles to tempotartrate. We added tempocholine to a concentrated suspension of membrane vesicles at a given temperature and at time t we treated an aliquot with sodium ascorbate at 0° (see Methods). The amplitude of the epr spectrum remaining after this treatment is associated with spin label inside the vesicles protected from ascorbate reduction. We measured half-times for tempocholine influx of 28 min at

[3] We did not usually include Ca^{2+} in our dilution buffers since all of our membrane preparations were prepared in sodium sulfate containing buffers. Kasai and Changeux (1971b) reported that Ca^{2+} increased the apparent excitability of membrane vesicles by increasing τ_0 and they routinely supplemented the dilution buffer with 2×10^{-3} M $CaCl_2$.

TABLE I: Tempocholine (TC^+) Measures an Induced Na^+ Diffusion Potential in Excitable Membrane Vesicles.[a]

Initial	Equilibrium (Observations)		Expected Potential (Eq 2)
$[Na^+]_i/[Na^+]_0$	$[TC^+]_i/[TC^+]_0$	Potential (mV)	
1.0	1.00	0	0
2.0	1.21	−5.0	−18.0
3.0	1.33	−7.4	−28.7
4.0	1.52	−11.0	−32.2
20.0	3.27	−30.9	−78.5
0.4	0.67	+10.3	+23.9

[a] Excitable membrane vesicles (prep 16, 12.9 mg of protein/ml) in 0.33 M sucrose–0.10 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3) were equilibrated with 3 mM tempocholine for 4 hr at 30° (so that $[TC^+]_i = [TC^+]_0$ initially). The 2.0, 3.0, and 4.0 Na^+ distributions were established by diluting the vesicles with one, two, and three parts of 0.5 M sucrose–0.02 M Tris-SO_4 (pH 7.3)–3 mM TC^+; the 0.4 value by diluting with three parts 0.3 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3)–3 mM TC^+ and the 1.0 value by no dilution and by dilution with three parts of 0.33 M sucrose–0.10 M Na_2SO_4–0.02 M Tris-SO_4–3 mM TC^+. The 20 value was established by passing 0.6 ml of vesicles through a G-25 (coarse) Sephadex column (11 mm × 7 cm, 4-ml void volume) equilibrated with 0.48 M sucrose–0.005 M Na_2SO_4–0.02 M Tris-SO_4. TC^+ was added to the eluate to give a concentration of 3 mM. The samples were incubated for 3.5 hr at 30° and the inside-outside TC^+ distribution was determined at 0° by the ascorbate procedure (Methods); observed and expected transmembrane potentials were calculated from eq 1 and 2, respectively.



tempocholine

30°, 250 min at 15°, and >500 min at 0°. For three different membrane preparations, we found that the inside volume determined from the distribution of tempocholine at equilibrium was comparable to the inside volume calculated from the distribution of $^{22}Na^+$ ions by the Millipore filtration method. The epr spectrum of tempocholine inside the vesicles was indistinguishable from tempocholine in water minimizing the possibility that specific binding accounts for the apparent uptake of sodium ions and tempocholine.

In general the inside volume amounted to ~1–1.5% of the total volume for our concentrated membrane preparations, corresponding to a specific volume of 0.6–0.8 μl/mg of total protein depending on the particular preparation.

Tempocholine Measures an Induced Na^+ Diffusion Potential in Excitable Membrane Vesicles. The permeability of excitable membrane vesicles to a large number of radioactive cations, anions, and neutral permeants was determined by Kasai and Changeux (1971b) using the Millipore filtration technique. They found and we have also observed that vesicles are permeable to $^{22}Na^+$ (see Figure 1) but impermeable to [^{35}S]sulfate. A concentration gradient of sodium sulfate across the membrane vesicles is therefore expected to give rise to a sodium ion diffusion potential that can be simply related to the inside-outside sodium ion activities by the Nernst relation

$$V = -\frac{kT}{e} \ln \frac{[Na^+]_i}{[Na^+]_0} \quad (2)$$

We tested this prediction as follows. (i) We induced, by dilution or gel filtration, concentration gradients of sodium sulfate in excitable membrane vesicles that had been prepared in the presence of a known concentration of sodium sulfate. (ii) We allowed tempocholine to equilibrate between the inside and outside of the vesicle at 30°. (iii) We determined the inside-outside distribution of tempocholine by the ascorbate procedure at 0°. (iv) We calculated the transmembrane potential from eq 1.

The validity of the spin-label method for measuring transmembrane potentials has been demonstrated quantitatively by Kornberg *et al.* (1972) for the special case of valinomycin-induced K^+ diffusion potentials in phospholipid vesicles permeable to tempotartrate. Table I summarizes the results for the spin-label measurements of Na^+ diffusion potentials in excitable membrane vesicles.

There exists a good qualitative correlation between the initial inside-outside sodium ion distribution and the measured inside-outside equilibrium distribution of tempocholine. In all cases, however, the potentials are much smaller than would be predicted from the Nernst relationship (eq 2) if we assume that the initial sodium ion distribution provides a reasonable estimate of the equilibrium sodium ion distribution. (We expect, based on calculations by Kornberg *et al.* (1972), that a detailed consideration of activity coefficients and buffer effects would account for about a 10% decrease in the expected potentials in our experiments.)

The low values for the potential may be due to a transient instability of the vesicles over the time scale (~3 hr) of the experiment. Breaking and resealing of some of the vesicles would discharge the ion gradient in those vesicles and the observed potential would be a weighted average for vesicles with no potential and those with a full potential. From the data presented in Table I we estimate that at least 30% of the vesicles remain intact.

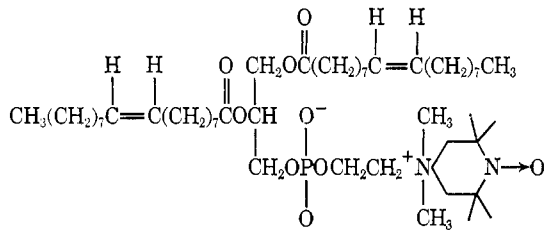
At another extreme, all the vesicles may remain intact and the low values may be attributable to a finite sulfate permeability which would result in a lower expected potential.

The equilibrium potentials measured here are induced under controlled ionic conditions and their existence rests on specific permeability properties of the excitable membrane vesicles. The long time scale for the spin-label procedure and the overall high ion permeability of the vesicles limits the usefulness of the technique as a dynamic probe of potential changes during receptor activation. However, in phospholipid bilayer vesicles containing the acetylcholine receptor we might expect to demonstrate the effect of receptor agonists on membrane potential; the permeability of the phospholipid vesicles to sodium ions is very low and only during receptor activation might a sodium ion diffusion potential be established. Kasai and Changeux (1971c) have estimated that the ionophore properties associated with the activated receptor are equilavent to ten gramicidin molecules, and Kornberg *et al.* (1972) have previously demonstrated that one ionophore (*e.g.*, valinomycin) per five vesicles is sufficient to induce transmembrane potentials in phospholipid bilayer vesicles.

B. Phospholipid Flip-Flop

Incorporation of Phospholipid Spin Label (OPTC) into

Excitable Membrane Vesicles. Dioleoylphosphatidyltempocholine (OPTC), a spin-labeled analog of phosphatidylcholine with the paramagnetic nitroxide group in the polar head group region, forms stable dispersions in aqueous buffer when sonicated in a bath sonicator at 35–40°.



OPTC

The unpaired electrons in vesicles of pure OPTC interact strongly giving rise to a single broad epr resonance line as shown in Figure 2a (the sharp three-line spectrum superimposed on the broad line is due to a small amount (<1%) of tempocholine released during the sonication procedure). After mixing excitable membrane vesicles with OPTC vesicles at 30° (~10 μmol of membrane phospholipid/μmol of OPTC), a three-line resonance pattern distinct from the tempocholine spectrum appears superimposed on the broad resonance spectrum (Figure 2b). A similar phenomenon was first observed by Scandella *et al.* (1972) for the interaction of spin-labeled phospholipid vesicles (spin labeled in the fatty acid chain) with rabbit sarcoplasmic reticulum and the three-line pattern was attributed to spin labels that had been incorporated into the membrane and that were undergoing rapid diffusion in the plane of the membrane. Our spectra are consistent with this interpretation. The epr spectrum of OPTC in excitable membrane vesicles is similar to the spectrum in egg phosphatidylcholine vesicles and suggests that the overall orientation and degree of motion is comparable in the two systems.[4]

We used the amplitude of the low-field resonance line to measure the extent of spin-label incorporation and we monitored the total spin-label concentration by double integration of the spectra in order to compensate for the slow reduction of OPTC (~5%/hr) in the membrane vesicles at 30°. After 2-hr incubation at 30° the rate of spin-label incorporation decreased and membrane vesicles containing OPTC were separated from unincorporated spin label by high-speed centrifugation through a sucrose step gradient at 30°. Spectra of the membrane fraction and the OPTC vesicle fraction are shown in Figure 2c,d, respectively, and indicate that a reasonably good separation was achieved.

We estimate that 25% of the total spin label has been incorporated into the excitable membrane vesicles during 2 hr at 30° corresponding to a 40:1 ratio of total phospholipid to OPTC in the membrane vesicles. Thin-layer chromatography (tlc) analysis of the membrane lipids, extracted from the vesicles with chloroform–methanol (2:1, v/v), indicates that >90% of the spin label migrates as OPTC and that at most 50% of the total phospholipid is phosphatidylcholine (phosphatidylethanolamine is the other major component). In our preparations, therefore, 5% of the phosphatidylcholine is OPTC.

[4] In egg phosphatidylcholine vesicles, spin labels inside and outside have slightly different line shapes. The vesicles are very small (d ~ 250 Å) and spin labels in the inner monolayer are apparently more immobilized than those in the outer monolayer. We did not observe any difference between inside and outside labels in the excitable membrane vesicles (d ~ 1000Å).

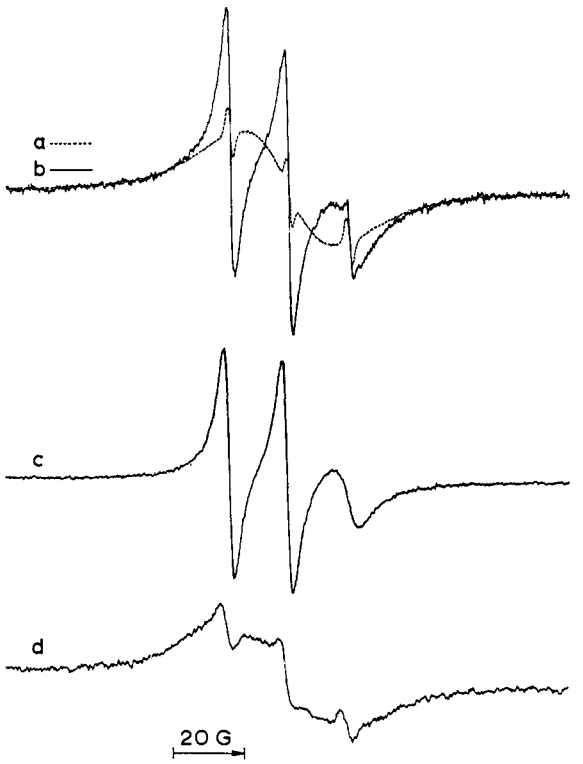


FIGURE 2. The epr spectrum of OPTC changes when OPTC is incorporated into excitable membrane vesicles. Pure OPTC vesicles (0.4 ml; 2.4 μmol of spin label, spectrum a) and 2.0 ml of membrane vesicles (prep 4, 18 mg/ml of protein, 13.5 μmol/ml of phosphate) in 0.2 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3) were mixed at 30°. After 2-hr incubation (spectrum b), the mixture was resolved by centrifugation through a sucrose step gradient (0.5-ml vesicles applied to 4 ml of 0.5 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4 and centrifuged at 30° for 1 hr at 50K rpm in the SW56 rotor of a Beckman L2-65B centrifuge). The membrane pellet was resuspended in 0.3 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3) (spectrum c); the unincorporated OPTC vesicles remain at the top of the sucrose gradient (spectrum d). Spectra a and b are normalized to the same spin-label concentration and all spectra were recorded at 30°. The sharp component in spectrum a is due to a small amount of tempocholine released during sonication.

Membrane Vesicles Containing OPTC Are Excitable. We have determined the excitability of membrane vesicles containing OPTC by the sodium efflux assay described earlier. The half-times for $^{22}Na^+$ efflux in the presence and absence of carbamylcholine and the apparent excitabilities are presented in Table II for membrane vesicles before and after incubation with OPTC vesicles. We have also included data for the main fraction of the same vesicle preparation to illustrate the effect of the concentration procedure on membrane excitability. *The OPTC has no apparent effect on the sodium permeability or excitability of concentrated membrane vesicles.*

Flip-Flop of OPTC in Excitable Membrane Vesicles Is Fast. The procedures used by Kornberg and McConnell (1971b) to measure the flip-flop rate of a spin-labeled phospholipid in phospholipid bilayer vesicles can be used to obtain an estimate for the flip-flop rate of OPTC in excitable membrane vesicles.

Treatment of excitable membrane vesicles containing OPTC with a 50-fold excess of sodium ascorbate at 0 or 15° results in the reduction of ~80% of the spin label within 1 min; the remaining 20% of the spin label is reduced at a slower rate characterized by a half-time for reduction of 3.8 min at 15° (as indicated in Figure 3) and 8 min at 0°. If the vesicles are disrupted by treatment with 1% Triton X-100 before addition of ascorbate, all of the spin label is reduced within 1 min.

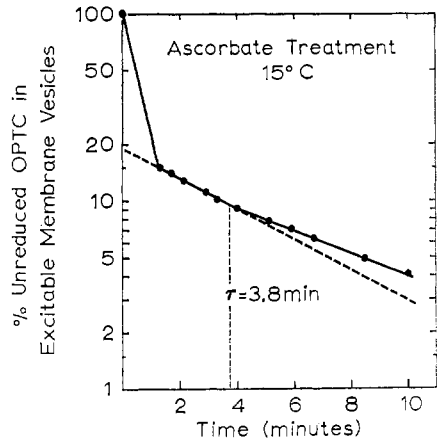


FIGURE 3: The kinetics of OPTC reduction by ascorbate are biphasic in intact excitable membrane vesicles. OPTC containing vesicles (50 μl) (prep 15, 20 mg/ml of protein, 15 μmol/ml of phospholipid, and 0.6 μmol/ml of OPTC) were mixed at 0° with 5 μl of 0.35 M sodium ascorbate and the relative amplitude of the low-field epr line was monitored *vs.* time at 15°. The semilog plot of per cent unreduced OPTC *vs.* time at 15° is used to estimate the percent of OPTC not rapidly reduced by ascorbate (by extrapolation to $t = 0$) and to obtain a half-time (τ) for the slow reduction.

TABLE II: Membrane Vesicles Containing Spin-Labeled Phospholipid (OPTC) Are Excitable.[a]

Membrane Vesicle Sample	Half-time (min) of $^{22}Na^+$ Efflux		Excitability
	τ_0	τ	$(\tau_0/\tau) - 1$
a. Main fraction	10	3.5	1.9
b. Concentrated	6	3.5	0.7
c. Concentrated + OPTC	6	3.5	0.7

[a] The half-times for $^{22}Na^+$ efflux with Carb (τ) and without Carb (τ_0) were measured by the procedures outlined in Figure 1 and Methods. $^{22}Na^+$-loaded vesicles (50 μl) (prep 12) in 0.2 M sucrose–0.05 M Na_2SO_4–0.01 M Tris-Cl (pH 7.4) were diluted with 6 ml of 0.1 M Na_2SO_4–0.01 M Tris-Cl at 22° with and without 5×10^{-4} M carbamylcholine (Carb). Sample c contained 0.02 μmol of OPTC/μmol of total phospholipid and had been incubated for 2 hr at 30° before addition of $^{22}Na^+$. The protein (mg/ml) and phospholipid (μmole/ml) concentrations of the samples were: (a) 7.5, 6.3; (b) 17.1, 13.4, and (c) 11.8, 10.7.

We assume that for intact vesicles, only the OPTC in the outer monolayer of the membrane bilayer is rapidly reduced by ascorbate. For spherical vesicles with a diameter of 1000 Å and a membrane thickness of 100 Å (the average values reported by Kasai and Changeux (1971d) on the basis of electron microscopic studies), we estimate that a random distribution of OPTC should result in a 60–40% distribution of the OPTC between the outside and inside surfaces of the membrane. Our results suggest that close to 80% of the OPTC is effectively on the outside of the vesicles. This could reflect an asymmetric equilibrium distribution of phosphatidylcholine on the two sides of the membrane[5] but more likely reflects the existence of some defective vesicles for which all of the spin label is accessible to ascorbate.

The slow component of the observed reduction is a measure of the rate at which OPTC molecules not initially reduced become accessible to the action of ascorbate and at least two processes must be considered:[6] (1) the penetration of ascorbate into the vesicles and (2) the flip-flop of OPTC from the inner monolayer to the outer monolayer of the bilayer. The rate of slow reduction is the sum of the rates for these two processes and, for single bilayer vesicles, the observed rate provides an upper limit for the rate of OPTC flip-flop.[7] We estimated the contribution of ascorbate penetration to the observed reduction rate by measuring the rate at which ascorbate reduces tempocholine inside the vesicles. (At 0 and 15° the vesicles are relatively impermeable to tempocholine.) The half-time for ascorbate reduction due to penetration is 11 min at 15° and 21 min at 0°, indicating that ascorbate penetration may be too slow to account for all of the reduction of OPTC and that phospholipid flip-flop may make a significant contribution to the reduction rate.

We obtained a lower limit for the rate of phospholipid flip-flop by a two-stage ascorbate treatment; 50 μl of 0.35 M sodium ascorbate was added to 0.5 ml of excitable membrane vesicles and the mixture was applied to a G-25 (coarse) Sephadex column (11-mm diameter and 5-ml void volume) equilibrated with 0.3 M sucrose–0.1 M Na_2SO_4–0.02 M Tris-SO_4 (pH 7.3) at 15°. The vesicle fraction eluted at the void volume contains no ascorbate and has a stable epr spectrum with a low-field amplitude ~20% that of the untreated vesicles after correction for vesicle dilution. Treatment of an aliquot of this eluate with ascorbate 7 min after the original ascorbate treatment results in the reduction of 80% of the remaining spin label within 1 min. Subsequent treatments with ascorbate show no further change in the amount of reduction indicating that the OPTC not initially reduced by ascorbate has completely equilibrated between the inside and the outside of the membrane vesicle within 7 min at 15°. This equilibration is not attributable entirely to disruption of the vesicles by Sephadex chromatography; tempocholine-loaded vesicles can be eluted from a Sephadex column without extensive equilibration of inside-outside contents.

The results are consistent with our assumption that the membrane vesicles consist of a single bilayer (see footnote 7) since we would not expect such a rapid equilibration process to occur for OPTC distributed among the bilayers of different vesicles trapped within one another. We suggest, therefore, that the OPTC not rapidly reduced by ascorbate is in the inner monolayer of a vesicle bilayer and that this OPTC equilibrates (flip-flops) across the membrane with a half-time of less than 7 min at 15°. Combining this result with the upper limit we obtained by the one-stage ascorbate treatment, we

[5] Gordesky and Marinetti (1973) and Bretscher (1972) have presented evidence that there is an asymmetric distribution of amino phospholipids in human erythrocyte membranes. Their results suggest that the outside of the membrane bilayer may be enriched in phosphatidylcholine.

[6] A third process involves the possibility of exchange of spin labels between protected and unprotected sites in the outer monolayer. The epr signal we observe, however, is due to rapidly diffusing spin labels so we assume that all of the spin label in the outer monolayer is rapidly reduced by ascorbate.

[7] For multicompartment vesicles, the rate of flip-flop in any one bilayer could be much faster than the overall reduction rate and the penetration of ascorbate would be the rate limiting step for reduction of spin label in the internal bilayers. However, electron microscopic evidence presented by Kasai and Changeux (1971c) indicates that most of the vesicles consist of a single layer ~100 Å in thickness. (The vesicles, however, are not homogeneous and we have not attempted a detailed kinetic analysis of the observed rates.)

conclude that the *half-time for flip-flop of OPTC in excitable membrane vesicles is in the range of 3.8–7 min at 15°*.

Discussion

The flip-flop rate of dioleoylphosphatidyltempocholine (OPTC) in excitable membrane vesicles is an order of magnitude faster than the rate measured by Kornberg and McConnell (1971b) for the flip-flop of dipalmitoylphosphatidyltempocholine in egg phosphatidylcholine vesicles. The difference is not due to a difference in the properties of the two spin labels; the flip-flop rate of OPTC in egg phosphatidylcholine vesicles (half-time ~4 hr) is comparable to the rate measured for dipalmitoylphosphatidyltempocholine (M. G. McNamee and H. M. McConnell, unpublished data).

In egg phosphatidylcholine vesicles, Kornberg and McConnell (1971b) observed that progressive lipid oxidation over a period of several days increased the apparent rate of flip-flop by a factor of 2–3 and they speculated that oxidized lipids might be involved in the translocation process. We do not know to what extent oxidized lipids affect the flip-flop rate we measure in excitable membrane vesicles but we have never observed a time-dependent increase in the rate of ascorbate reduction.

There are other reasonable mechanisms, aside from oxidized lipid catalysis, that would explain the fast flip-flop rate in our membrane preparations. Discontinuities in the membrane's phospholipid bilayer, particularly at interfaces with membrane proteins, may present sites at which phospholipids can exchange readily from one side of the membrane to the other. Since lateral diffusion is fast in biological membranes (Scandella *et al.*, 1972), only a few such sites would be required to affect the apparent flip-flop rate of a large population of phospholipids. It is also possible that conformational changes in the protein could affect the degree of disorder in the membrane and indirectly affect the rate of flip-flop. The excitable membrane vesicles are too complex to permit a direct test of such a mechanism. We did add carbamylcholine to some vesicle preparations to see if the increase in $^{22}Na^{+}$ permeability was associated with a change in the flip-flop rate but we observed no effect. The acetylcholine receptor accounts for at most 1% of the protein in the excitable membrane vesicles and any effect associated with the receptor is probably obscured by nonspecific effects of other proteins or lipids.

There is some evidence that there may be significant variations in the flip-flop rate of phosphatidylcholine in different biological membranes. In preliminary experiments, W. Huestis and H. M. McConnell (work in progress) have measured a half-time of 20–30 min for flip-flop in red blood cells at 37° using techniques similar to those described here,[8] whereas Grant and McConnell (1973) have speculated that flip-flop in *Acholeplasma laidlawii* may have a half-time of less than 1 min at 0°.

Rapid flip-flop in biological membranes implies that the distribution of phospholipids in the membrane will always reflect an equilibrium distribution and will not be a consequence of how the lipids were incorporated into the membranes. Rapid flip-flop would allow charged phospholipids to respond to potential differences across the membrane and asymmetric distributions of lipids might provide information about the electric fields at the membrane surface. Measuring the inside-outside distribution of charged phospholipid spin labels (or other amphiphilic spin labels that bind to membranes) might overcome some of the limitations discussed previously (part A, Results) in the tempocholine–ascorbate technique for measuring transmembrane potentials in biological membranes. At the present time, B. McFarland and H. M. McConnell (work in progress) are investigating the use of long-chain ionic spin labels as probes of potentials in erythrocytes and in model membranes, and the techniques developed should be applicable to further studies on the excitable membrane vesicles.

[8] Specifically, the flip-flop rate of dipalmitoylphosphatidyltempocholine incorporated into erythrocytes was measured. The labelling experiments of Gordesky and Marinetti (1973) and Bretscher (1972) suggest that the flip-flop rates of phosphatidylethanolamine and phosphatidylserine may be much slower although they do not explicitly consider flip-flop in their experiments.

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References

Baer, E., and Kates, M. (1948), *J. Amer. Chem. Soc. 70*, 1394.
Bretscher, M. S. (1972), *J. Mol. Biol. 71*, 523–528.
Brockerhoff, H. and Yurkowski, M. (1965), *Can. J. Biochem. 43*, 1777.
Cubero Robles, E., and van den Berg, D. (1969), *Biochim. Biophys. Acta 187*, 520–526.
Davidson, F. M., and Long, C. (1958), *Biochem. J. 69*, 458–466.
DePlazas, S. F., and DeRobertis, E. (1972), *Biochim. Biophys. Acta 274*, 258–265.
Eldefrawi, M. E., and Eldefrawi, A. T. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1776–1780.
Ellman, G. L., Courtney, K. D., Andress, V., and Featherstone, R. (1961), *Biochem. Pharmacol. 7*, 88–95.
Franklin, G. I., and Potter, L. T. (1972), *FEBS Lett. 28*, 101–106.
Fulpius, B., Cha, S., Klett, R., and Reich, E. (1972), *FEBS Lett. 24*, 323–326.
Gordesky, S. E., and Marinetti, G. V. (1973), *Biochem. Biophys. Res. Commun. 50*, 1027–1031.
Grant, C. W. M. and McConnell, H. M. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 1238.
Hall, Z. W. (1972), *Annu. Rev. Biochem. 41*, 925–952.
Karlsson, E., Heilbronn, E. and Widlund, L. (1972), *FEBS Lett. 28*, 107–111.
Kasai, M., and Changeux, J.-P. (1971a), *J. Membrane Biol. 6*, 1–23.
Kasai, M., and Changeux, J.-P. (1971b), *J. Membrane Biol. 6*, 24–57.
Kasai, M., and Changeux, J.-P. (1971c), *J. Membrane Biol. 6*, 58–80.
Kasai, M., Changeux, J.-P. (1971d), *J. Membrane Biol. 6*, 81–88.
Kornberg, R. D., and McConnell, H. M. (1971a), *Proc. Nat. Acad. Sci. U. S. 68*, 2564–2568.
Kornberg, R. D., and McConnell, H. M. (1971b), *Biochemistry 10*, 1111–1120.
Kornberg, R. D., McNamee, M. G., and McConnell, H. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1508–1513.
Lapidot, Y., Barzilay, I., and Hajdu, J. (1969), *Chem. Phys. Lipids 3*, 125–134.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall,

R. J. (1951), *J. Biol. Chem. 193*, 265–275.
McClare, C. W. F. (1971), *Anal. Biochem. 39*, 527–530.
Olsen, R. W., Meunier, J. C., and Changeux, J.-P. (1972), *FEBS Lett. 28*, 96–100.
Razin, S. (1972), *Biochim. Biophys. Acta 265*, 241–296.
Reiter, M. J., Cowburn, D. A., Prives, J. M., and Karlin, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1168–1172.
Scandella, C. J., Devaux, P., and McConnell, H. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2056–2060.
Schmidt, J., and Raftery, M. (1973), *Biochemistry 12*, 852–856.
Singleton, W. S., Gray, M. S., Brown, M. L. and White, J. L. (1965), *J. Amer. Oil Chem. Soc. 42*, 53–56.

Hybrid Formation between Collagen and Synthetic Polypeptides†

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ABSTRACT: The α1 chain of calf-skin collagen can form hybrids in the presence of synthetic polypeptides, *e.g.*, $(Pro\text{-}Ala\text{-}Gly)_n$, $(Pro\text{-}Gly\text{-}Pro)_n$, and, to a very much lesser extent, $(Pro\text{-}Ser\text{-}Gly)_n$. The extent of the hybridization reaction increases with an excess of the synthetic polypeptide and also with its molecular weight. The hybrid may be isolated by molecular sieve chromatography and identified by amino acid analysis. There is no evidence for hybrid formation between these polypeptides and the two-chain α-helical tropomyosin. Interaction between the α chain and the synthetic polypeptides is also indicated from optical rotation data. The hybridization experiments show that a single type of synthetic polytripeptide containing the collagen fold can be recognized and accepted to form a common triple helix structure by the many different types of tripeptides which make up the α1 chain of collagen. This hybrid-forming capacity apparently depends both on the nature of the tripeptide in the polymer and the sequence in the collagen chains. Since a collagen chain cannot bind $(Pro\text{-}Ser\text{-}Gly)_n$ to give a collagen-like structure it appears that the collagen chain cannot induce a structural fold in the chain $(Pro\text{-}Ser\text{-}Gly)_n$.

The polypeptide chains of collagen represent a primary structure composed of the following four types of tripeptides (Traub and Piez, 1971)

Gly- (Hyp) Pro-(Hyp) Pro
I
Gly- (Hyp) Pro-X
II
Gly-X(Hyp) Pro
III
Gly-X-X
IV

where X is any amino acid other than Gly, Hyp, or Pro.

The sequence determination of the CNBr peptide, α1-CB6,[1] from calf-skin collagen has shown that the distribution of the tripeptides I–IV, respectively, in this peptide is in the ratio 10:20:20:50 (Mark *et al.*, 1970). The distribution is approximately the same in some of the relatively small peptides of the collagen chains, as for example, in α1-CB4 and α1-CB5 from rat skin (Butler, 1970). The proportion of types I–III is, however, higher in the peptides α1-CB2 and α2-CB2 from chick skin and other sources of collagen (Highberger *et al.*, 1971).

The formation of the collagen triple helix has been investigated for many examples of synthetic polypeptides with known sequences, representing the four types of tripeptides defined above: type I (Yonath and Traub, 1969); type II (Traub and Yonath, 1967); type III (Andreeva *et al.*, 1967); and type IV (Doyle *et al.*, 1970). Triple helix formation in water occurs very readily with type I polytripeptides (Engel *et al.*, 1966), moderately well with type II, and somewhat less readily with type III (Heidemann and Bernhardt, 1967a,b). This difference between II and III was confirmed by Doyle *et al.* (1971). However, it does not seem possible to induce triple helix formation in type IV polytripeptides, *e.g.* $(Gly\text{-}Ala\text{-}Ala)_n$ (Doyle *et al.*, 1970). The tendency for triple helix formation depends mainly on the nature of the amino acids in the type IV unit. It is also possible, however, that the insertion of types I–III units into appropriate places in the polypeptide chain in the vicinity of the type IV tripeptides can promote the formation of triple helix in the type IV unit, *i.e.*, the presence of neighboring triple helix stimulators may give rise to this structure in type IV units. This has been demonstrated by Segal (1969) for polyhexapeptides composed of two different types of tripeptides. The combination of types IV and I in $(Gly\text{-}Ala\text{-}Ala\text{-}Gly\text{-}Pro\text{-}Pro)_n$ gives a triple helix for the whole chain. It may be for these reasons that small CNBr peptides such as α1-CB2 can form triple helices *in vitro* (Piez and Sherman, 1970) even though this peptide has two type IV tripeptide units out of a total of 12. The formation of triple helices from collagen chains or from their CNBr peptides seldom proceeds 100% *in vitro* to give discrete compounds be-

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[1] The nomenclature used for the CNBr peptides is reviewed by Traub and Piez (1971).