influx per twitch was equal to 1.1 pmole/cm^2 per twitch on the basis of the increase in total ^{45}Ca uptake and 0.27 pmole/cm^2 per twitch on the basis of the increase in residual ^{45}Ca uptake.

The major difference was observed in the extracted calcium; stimulation caused an additional 0.29 μmole/g of calcium to be extracted in strontium Ringer solution (0.55 μmole/g − 0.26 μmole/g). The additional loss is reflected in the residual calcium, which was lower in concentration in the stimulated muscle (1.35 μmole/g) than in the resting muscle (1.52 μmole/g).

The additional calcium extracted from the stimulated muscle was not matched by a gain of strontium, indicating that there was a net loss of calcium, and it was not replaced by strontium. The specific activity ratio of the calcium extracted from resting muscle was 0.61 and the specific activity of the calcium extracted from stimulated muscle was 0.45. The reduced specific activity was accounted for by increased extraction of calcium from an unlabeled pool. Stimulation apparently led to translocation of a fraction of the unlabeled calcium released from the terminal cisternae to the transverse tubules.

Muscles stimulated at 100 Hz for three 1-second intervals during the last 5 seconds of a 10-minute equilibration period in ^{45}Ca lost more calcium to the extraction medium than the strontium taken up; the excess was 0.24 μmole/g (Table 2). This loss of calcium is in good agreement with the loss of 0.29 μmole when the muscles were stimulated at 1 Hz for 5 minutes.

The calcium mobilized from the terminal cisternae to the transverse tubular element amounted to 0.8 nmole/g per twitch. At the end of 120 twitches, 96 nmole/g would be accumulated in the transverse tubules and by the end of 300 twitches, 240 nmole/g. If we used 0.4 percent of the fiber as an estimate of the transverse tubular volume, 80 percent of which is junctional (*1*), the transverse tubular volume would amount to 3.4 μl/g (wet weight). The calcium concentration in the transverse tubular element at the end of 120 twitches would be 28.2 m*M*, and at the end of 300 twitches, 70.6 m*M*. The marked increase in transverse tubular calcium would raise the threshold for coupling of the action potential to contraction and thus cause uncoupling of the action potential from contraction. The muscle twitch response at 1 Hz is maintained for the first 2 minutes of stimulation and then decreases to 43 percent of the initial twitch tension at the end of 5 minutes (Fig. 1). The translocation of

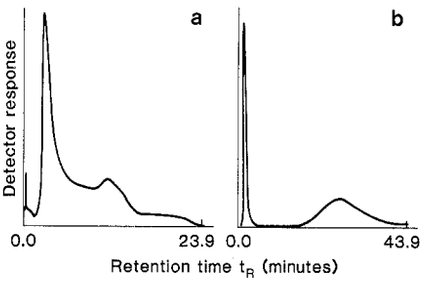

Fig. 1. The decline of twitch response of frog sartorius muscle (in vitro) when stimulated at 1 Hz for 5 minutes. Stimulation was by means of longitudinal current, pulse amplitude was 25 V, and pulse duration was 5 msec.

calcium from the terminal cisternae to the transverse tubular element is more than sufficient to account for the fatigue of muscles during repetitive stimulation at 1 Hz.

The amount of calcium released from the terminal cisternae during a twitch is approximately 91 nmole/g (*8*). The translocation of 0.8 nmole/g of calcium during the twitch to the transverse tubular element represents 0.9 percent of the calcium released from the terminal cisternae. The major portion of the released calcium must still be sequestered by the sarcoplasmic reticulum. The presence of a Ca^{2+}-dependent adenosine triphosphatase in the transverse tubular element (*9*) may provide the means for translocation of the calcium released from the terminal cisternae to the transverse tubular element. Reuptake of calcium from the transverse tubular element to the terminal cisternae could occur during the negative afterpotential, which is prolonged by increasing extracellular calcium from 1.8 to 8.0 m*M*.

The transverse tubular element serves not only as a conduit for the action potential in excitation-contraction coupling but also as a means for feedback through calcium accumulation from the terminal cisternae during the coupling process to decrease the effectiveness of the action potential in excitation-contraction coupling.

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Sedimentation Field Flow Fractionation of Liposomes

Abstract. *Quantitative analyses of the particle size distributions of liposomes were performed in 30 to 60 minutes by exponential-field sedimentation field flow fractionation. This gentle new separation method exhibits great potential for the high-resolution fractionation and the size or molecular weight analysis of a wide variety of biological macromolecules and colloidal suspensions.*

The characterization of biological polymers and colloids in the 10- to 1000-nm size range has been hampered by a lack of suitable separation techniques. The problem is particularly important in liposome research, because the increasing use of liposomes in drug delivery applications necessitates a rigorous characterization of the particle size distribution (*1*). Recently, sedimentation field flow fractionation (SFFF), a high-resolution separation technique originated by Giddings and others (*2*), has been introduced to fractionate and analyze colloids and soluble macromolecules within this size interval (*3*). We have applied SFFF to the size analysis of phospholipid vesicle dispersions, both because of inherent interest in this subject and because liposomes are ideal models for demonstrating the general applicability of SFFF to the separation of biological colloids and soluble biomacromolecules. Liposomes represent a model system since they can be prepared with size distributions in the 20- to 1000-nm range. Moreover, the ability of SFFF to handle labile materials can be tested since liposomes are sensitive to shear forces. Furthermore, the currently used electron microscopic analysis method is very time-consuming, which discourages routine particle size analysis by this approach (*4*).

The SFFF equipment and techniques used here have been described in (*3, 5*). Separations are carried out in a channel

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that is formed between two closely spaced parallel surfaces. The channel is rotated in a centrifuge, and the resultant centrifugal force causes dissolved or suspended solutes to migrate toward the wall regions (Fig. 1a). If the components in a sample are of a greater density than the mobile phase, particles migrate toward the outer wall; particles of a lesser density move to the inner wall. The liquid mobile phase is caused to flow continuously through the channel with a characteristic parabolic velocity profile (Fig. 1b), and the movement of particles toward the wall places them in regions of slower flow. The continuously fed mobile phase carries the sample components through the channel for detection at the outlet; smaller particles elute first (Fig. 1c), followed by components of increasing mass (Fig. 1d).

The density of liposomes is so close to unity (*6, 7*) that, when HN buffer (*5*) was used as the mobile phase, insignificant retention was obtained for small, sonicated unilamellar vesicles (SUV). We found, however, that by increasing the density of the mobile phase with a 1.34*M* sucrose-HN buffer (density = 1.1796 g/cm^3), we were able to move the particles to the inner wall of the channel. This procedure increased the SUV particle retention in the channel because of a greater density difference between the solute and the mobile phase, so that a particle size distribution could be calculated. We obtained "raw" fractograms for the SUV particles (Fig. 2a), which indicated a relatively narrow range of particle sizes. The differential particle size distribution in Fig. 3a illustrates a particle weight-average diameter of 26.4 nm and a polydispersity (the weight-average diameter divided by the number-average diameter) of 1.07. For this plot, we transformed the detector response from the "raw" fractogram in Fig. 2a, using the Rayleigh scattering theory with the assumptions that the eluting particles are of sufficiently low refractive indices and do not absorb at the detection wavelength. The speed and sensitivity of this method are demonstrated by the fact that only 15 minutes and about 100 μg of sample were required.

Larger multilamellar vesicles (MLV) could be fractionated directly (Fig. 2b) in HN buffer, because they are larger and therefore more retained than the SUV. The MLV exhibited a relatively wide range of particle sizes, as illustrated by the differential particle size plot in Fig. 3b. The MLV showed a weight-average diameter of 232 nm and a polydispersity of 1.5. The weight-average diameter compared quite favorably to the value obtained in negative-stain electron microscopy (*7*). However, staining artifacts make it difficult to obtain particle size distributions for liposomes by electron microscopy, and it is doubtful that such methods can approach the accuracy of the SFFF technique, particularly for colloids that undergo morphological changes on drying.

To confirm that SFFF did not increase the apparent size of the MLV through aggregation or decrease to smaller particles by shear, the middle third of the MLV distribution was isolated and reconcentrated by centrifugation at about 45,000*g* for 40 minutes. Refractionation of this concentrated isolate by SFFF in the same manner as the original sample produced a narrower peak at the same retention time. This isolate had a weight-average diameter of 251 nm and a polydispersity of 1.14. These results are in keeping with expectations; the weight-average diameter is slightly larger because primarily smaller particles were eliminated by the isolation, and the distribution is narrower since only the central portion of the distribution was isolated. This result indicates that the particle size distribution of nonassociating liposomes can be quantitatively determined by SFFF without fear of changing the particle size. Furthermore, it demonstrates the potential of SFFF to generate liposomes of defined narrow size ranges, an important consideration for their pharmacokinetic behavior.

It is also feasible with SFFF to demonstrate the effects of environment on the conformation of bioparticles. For example, the MLV are osmotically active (*8*) and should be expected to lose water and shrink in a sugar solution. The original MLV sample was fractionated in a sucrose-HN buffer with a density of 1.1796 g/cm^3. The resulting retention data

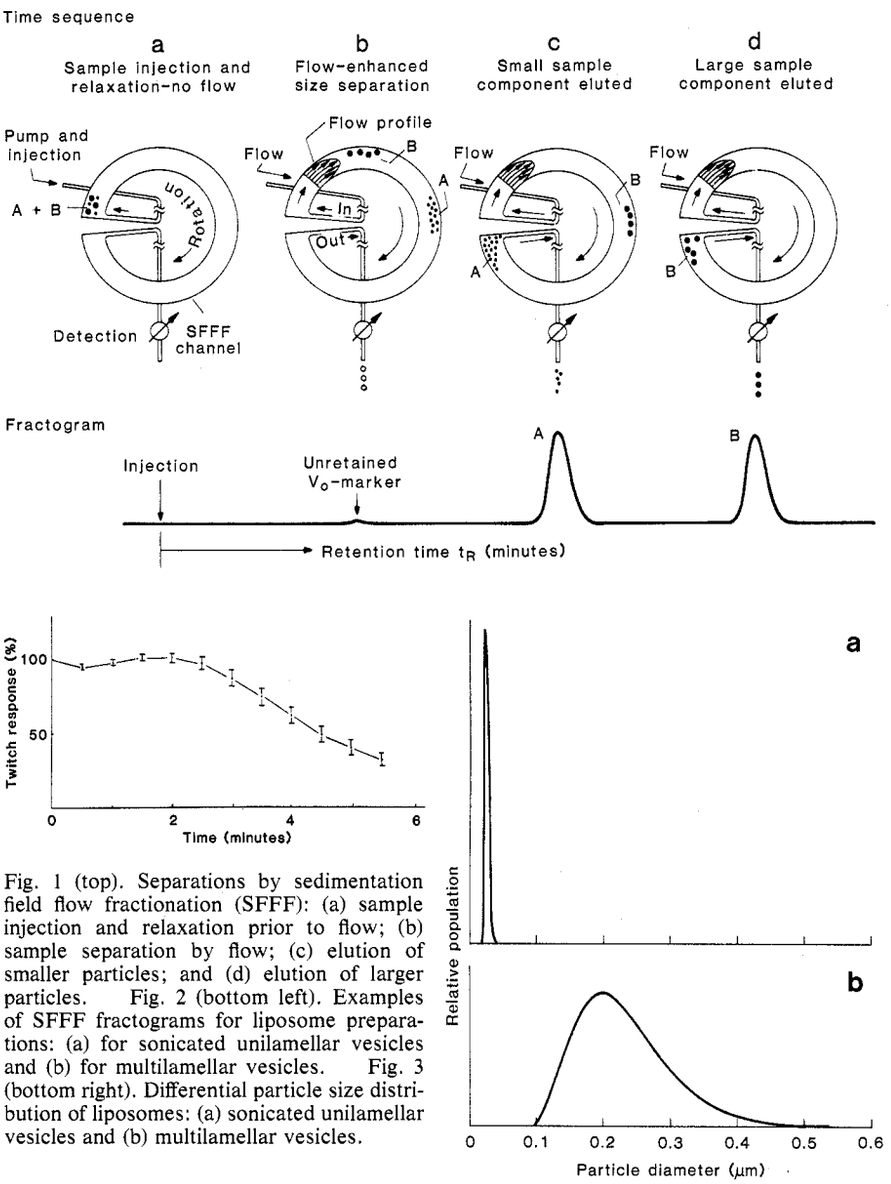

Fig. 1 (top). Separations by sedimentation field flow fractionation (SFFF): (a) sample injection and relaxation prior to flow; (b) sample separation by flow; (c) elution of smaller particles; and (d) elution of larger particles. Fig. 2 (bottom left). Examples of SFFF fractograms for liposome preparations: (a) for sonicated unilamellar vesicles and (b) for multilamellar vesicles. Fig. 3 (bottom right). Differential particle size distribution of liposomes: (a) sonicated unilamellar vesicles and (b) multilamellar vesicles.

showed MLV particles with a weight-average diameter of 152 nm as compared to the 232 nm found in HN buffer. This represents a water loss of 67 percent in the MLV and an increase in particle density to 1.0472 g/cm^3, as compared to the 1.0154 g/cm^3 assumed for the original particles.

The particle size distribution data for the SUV particles are believed to be reasonably accurate even though no correction for the osmotic effect of the sucrose-buffer mixture was used in the measurement. The osmotic activity for the much smaller SUV particles is expected to be considerably less than that for MLV as a result of molecular constraints caused by the minimal radius of the SUV particles.

In the SFFF analytical mode, it is relatively easy to collect submilligram quantities of isolate with very high resolution. If the resolution is compromised somewhat, milligram to gram quantities can be isolated by having the instrument function as a selective filter (*3*), retaining large liposomes while allowing smaller ones to be eluted in the mobile phase. In conjunction with extrusion techniques (*7, 9*), SFFF should make possible the rapid preparation of appreciable amounts of liposomes with a polydispersity close to unity.

It thus appears that SFFF is a rapid, precise, and gentle method for the analysis of the particle size distributions of noninteracting biological colloids, as we have illustrated with liposomes. We believe that SFFF will be an equally important technique in the fractionation and size analysis of other biopolymers such as nucleic acids and cellular organelles such as ribosomes, mitochondria, and nuclei.

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6. Lipids were obtained, stored, and assayed as described by F. C. Szoka and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4194 (1978). Liposomes were composed of phosphatidylserine, phosphatidylcholine, and cholesterol in a molar ratio of 1:4:5. Small unilamellar vesicles were prepared in HN buffer at a lipid concentration of 10 μmole/ml in a bath type Sonicator at 20°C and centrifuged at 115,000*g* for 90 minutes to remove large vesicles. The supernatant containing SUV was stored under nitrogen at 4°C at a lipid concentration of 5 μmole/ml. The MLV were prepared in HN buffer at a lipid concentration of 10 μmole/ml and sequentially extruded through a 0.2-μm polycarbonate membrane to obtain a defined particle size distribution (*7*) and stored under nitrogen at 4°C at a lipid concentration of 5 μmole/ml. Negatively charged liposomes were used to minimize the possibility of aggregation during the transfer of vesicles. C. Huang, *Biochemistry* **8**, 344 (1969); ——— and J. P. Charlton, *J. Biol. Chem.* **246**, 2555 (1971); S. M. Johnson, *Biochim. Biophys. Acta* **397**, 27 (1973).
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Human T Cell Antigen Expression by Primate T Cells

Abstract. *Phylogenetic study in primates of the reactivity of 11 monoclonal antibodies to human T cells and rosette formation in the reaction of primate T cells with sheep erythrocytes demonstrate that the sheep erythrocyte receptor and the determinants of T cell subset antigens are highly conserved during primate evolution. Other T cell antigen determinants are less well conserved. Human, gorilla, and chimpanzee T cells showed identical reactivity with the monoclonal antibodies to human T cells. The expression of human T cell antigen determinants by primate T cells suggests a constant rate of evolution for this group of molecules and provides additional evidence that man and African apes shared a relatively recent common ancestor.*

Comparisons of proteins from humans and other primates for variations in amino acid sequences, DNA restriction endonuclease cleavage patterns, and immunological cross-reactivity have provided biochemical data in support of a relatively recent common ancestor (5 million years ago) of man and African apes (*1–3*). Data have come from studies of primate albumin, transferrin, and α and β globin proteins (*1*). Recent fossil evidence, although controversial, also supports this notion (*4*). Studies have been done on the reactivity of determinants of human histocompatibility antigens A and B with lymphocytes of nonhuman primates (*5*) and on the use of monoclonal antibodies to T cells as therapeutic reagents in primates (*6*), but few phylogenetic studies have been performed to evaluate the evolution of human lymphocyte–related cell surface proteins. Hybrid cell methodology has provided the reagents needed to define the repertoire of lymphocyte surface antigens. In particular a large number of monoclonal antibodies to human antigens specific to T cells have been produced (*7–13*); the specificity and classification of these reagents have been reviewed (*14*).

Highly specific monoclonal antibodies react with only one antigenic determinant on a given molecule (*15*), and we have used a large panel of these reagents to study the expression of various human T cell antigen determinants on the T cells of nonhuman primates. Our goal was to seek evolutionary patterns of proteins specific to the immune system. Blood was drawn from one to five animals of each of the following primates: gorillas (*Gorilla gorilla*), chimpanzees (*Pan troglodytes*), gibbons (*Hylobates lar*), Old World monkeys (rhesus, *Macaca mulatta*; pig-tailed, *Macaca nemistrina*; and stump-tailed, *Macaca speciosa*), and New World monkeys (Cebus, *Cebus atella*, and spider, *Ateles fusciceps*). Mononuclear cell preparations were made on Hypaque-Ficoll density gradients. We used two methods to determine the reactivity of the monoclonal reagents with primate T cells; indirect immunoflu-

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