Table VIII—Application of the Base Line Technique to Various Nonsteroidal Anti-inflammatory Agents or Their Derivatives

	Optical Purity, %	S, cm	$\left(1 - \frac{\text{optical purity}}{100}\right)$
Ibuprofen[a]	0.0	0.59	1.00
	23.7	0.44	0.76
	47.6	0.29	0.52
	75.0	0.15	0.25
	100.0	0.00	0.00
Naproxen[b]	0.0	1.61	1.00
	24.4	1.39	0.76
	49.0	1.05	0.51
	70.1	0.79	0.30
	100.0	0.40	0.00
Ketoprofen methyl ester[c]	0.0	0.90	1.00
	20.0	0.72	0.80
	45.0	0.48	0.55
	70.0	0.27	0.30
	78.0	0.19	0.22
Fenoprofen methyl ester[d]	0.0	1.80	1.00
	20.0	1.45	0.80
	55.0	0.86	0.45
	80.0	0.46	0.20

[a] Concentration of drug used was 0.23 *M* in carbon tetrachloride, using III–drug molar ratio of 0.366. The analytical peak was $ArCHCH_3$ in the region δ3.14–3.52 ppm. [b] Drug concentration was 0.29 *M* in deuterated chloroform–carbon tetrachloride (3:4), using III–drug molar ratio of 0.355. The analytical peak was at δ1.80–2.60 ppm ($ArCHCH_3$). [c] Drug derivative concentration was 0.30 *M* in carbon tetrachloride, using III–ester molar ratio of 0.406. The analytical peak was $ArCHCH_3$ in the region δ1.90–2.15. [d] Concentration of derivatized drug was 0.215 *M* in carbon tetrachloride using III–ester molar ratio of 0.919. The analytical peak was the $COOCH_3$ resonance in the δ5.20–5.30 region.

Higher molar ratios produced greater downfield shifts. Table IV shows results for two different molar ratios, using I as shift reagent, on successive addition of 0.1 ml volumes of deuterated benzene to a mixture prepared in 0.7 ml of the same solvent.

Data obtained on application of the base line technique to *N*-acetylpropranolol, compound V, ibuprofen, naproxen, ketoprofen methyl ester, and fenoprofen methyl ester are presented in Tables V–VIII and the corresponding calibration curves, when plotted, are linear. Appropriate derivatization was required in those cases of poor solubility of the parent drug in the solvents available, or where the number of points of complexation with the shift reagent was large, with consequent line broadening.

If authentic, optically pure samples of a drug are available, this method offers a useful means of routine optical purity determination up to ~90% optical purity. The susceptibility of a molecule to pseudocontact shifting influences of a lathanide agent can be quickly established using a nonchiral compound, and the appropriate shift reagent–drug molar ratio necessary in a given case found from incremental addition of the shift reagent (Table II).

It has also been observed that, in certain cases, the peak height difference (δh) of overlapping resonances from corresponding groups of optical isomers in the presence of a chiral shift reagent bears a linear relationship to optical purity up to levels of ~50%. However, there was little success in establishing optical purity at levels >50%. The instrumental and other conditions necessary for successful application of the base line technique similarly apply to the peak height difference method.

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Liposome Dialysis for Improved Size Distributions

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Abstract □ A technique is described which allows reproducible preparation of liposomes with improved size–frequency distributions. The recent procedure of extrusion of crude liposome dispersions through controlled-pore polycarbonate membranes is used to control the upper limit of liposome diameter. Subsequent dialysis, using the same type of membrane, can remove the majority of liposomes smaller than a predetermined size. The pattern of dialysis of a liposome preparation is a function of the size–frequency distribution (as well as the membrane pore size) and can be used to approximate the distribution and/or used to monitor the reproducibility of liposome preparations.

Keyphrases □ Liposomes—dialysis for improved size–frequency distribution □ Polycarbonate membranes—dialysis of liposomes, size–frequency distribution □ Dialysis—liposomes, improved size–frequency distribution □ Distribution—size–frequency, liposome dialysis

It is recognized that liposome properties, both as model membranes and drug carrier systems, are dependent on their size (1–4). The differences in the plasma time course and tissue distribution seen between large multilamellar and small unilamellar vesicles are now well established (5), and even different size classes of large multilamellar vesicles can have significantly different pharmacokinetics (6). Unfortunately, the size distribution of the original multilamellar preparation previously described (7) is very heterogeneous and poorly reproducible. The use of this

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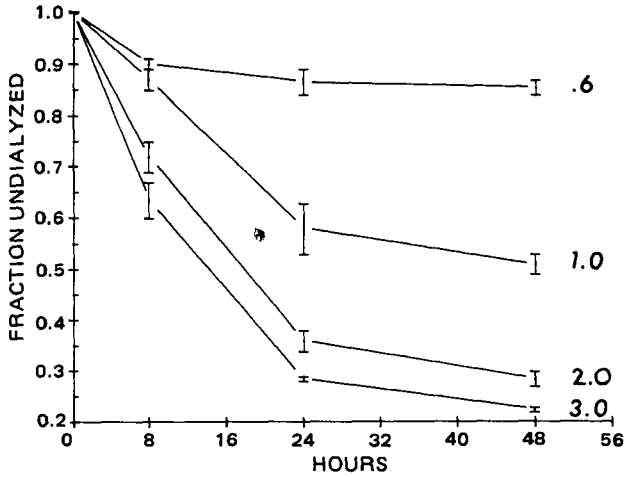

Figure 1—*The fraction of liposomes remaining in the dialysis cells is plotted* versus *time for a suspension [10 μmoles (total lipid)/ml] of mechanically dispersed liposomes dialyzed against either a 0.6-, 1.0-, 2.0-, or 3.0-μm pore-size membrane. Liposomes were quantified by measuring [^{14}C]cholesterol included in the membrane. The vertical bars give the range for duplicate runs using one preparation.*

preparation for *in vivo* tissue distribution studies may mask some differential effects of size. Indeed, complex blood level time courses have been attributed by some investigators to the heterogeneous size distribution of the administered liposomes (4).

Several procedures have attempted to improve the size distribution or encapsulation efficiency of the liposome preparation: sonication and centrifugation (8, 9), detergent dialysis (10–12), injection methods (13–15), French pressure cell (16, 17), reversed-phase evaporation (18), and others (19–21). All suffer from at least one of three faults: (*a*) the resulting size range is too wide or unpredictable; (*b*) the method produces liposomes in only one size range; or (*c*) there are restrictions on composition or specific solutes. However, a significant improvement in size distribution can be obtained by extrusion of a heterogeneous population through straight bore pore polycarbonate membranes of defined pore size (22, 23); this technique defines the upper size limit for the population but does not

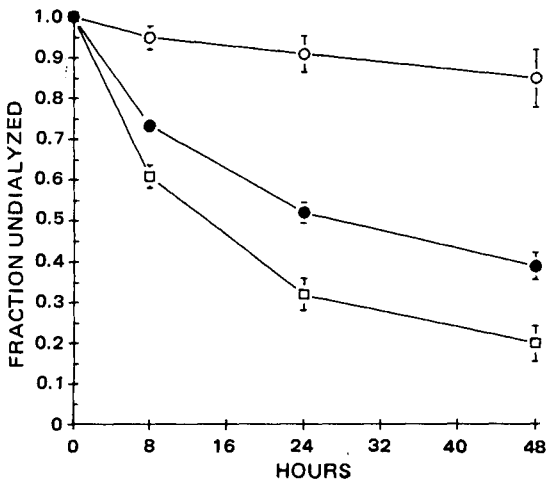

Figure 2—*The fraction of liposomes remaining in the dialysis cells is plotted* versus *time for a suspension [10 μmoles (total lipid)/ml] of small unilamellar French press liposomes dialyzed against either a 0.05 (○), 0.1 (●), or 0.2 (□) μm pore-size membrane. Liposomes were quantified as described in Fig. 1. The values are the means ± 1 SD for four preparations.*

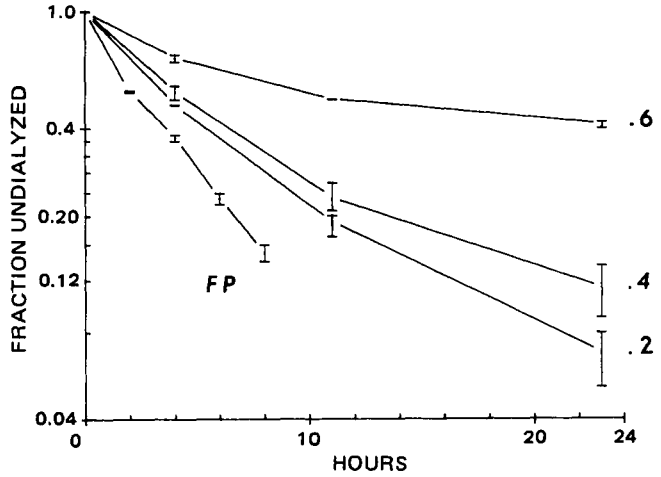

Figure 3—*The fraction of liposomes remaining in the dialysis cells is plotted* versus *time for four different batches of liposomes dialyzed against a 0.8-μm membrane. Liposome concentration and quantitation were as described in Fig. 1. The three upper curves are for liposomes prepared by two extrusions (see text) of mechanically dispersed liposomes through 0.6-, 0.4-, and 0.2-μm membranes. The lower curve is for French press liposomes. The vertical bars give the range for two preparations.*

affect the lower half of the distribution (control of the smaller size may be critical if liposome pharmacokinetics depend in part on the surface area or number of injected liposomes). In this report an improvement on the extrusion technique is described in which the extruded liposomes are subsequently dialyzed with the same type membrane with the object of removing a fraction of the smaller liposomes, thus narrowing the size distribution from its lower end.

EXPERIMENTAL

Chemicals—Purified egg yolk phosphatidylcholine (I), sodium dipalmitoyl phosphatidate (II), cholesterol (III), and α-tocopherol (IV) were chromatographic grade[1], as were sucrose [U-^{14}C] and cholesterol [4-^{14}C][2]. A universal scintillation reagent[3] was used. All other chemicals were

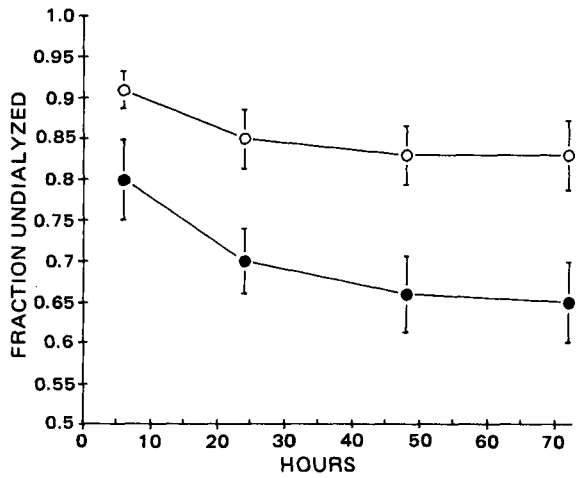

Figure 4—*The undialyzed fraction is plotted* versus *time for 1.0-μm extruded liposomes previously dialyzed against a 0.8-μm membrane. These values are for the previously described liposomes following 20 hr of dialysis against a 0.05-μm membrane (○) (essentially no dialysis of small liposomes), or following 20 hr of dialysis against a 0.8-μm membrane (●). Liposome concentration and quantitation were as described in Fig. 1. The values are the means ± 1 SD for nine preparations.*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] New England Nuclear, Boston, Mass.
[3] PCS, Packard Instrument Co., Inc., Downer's Grove, Ill.

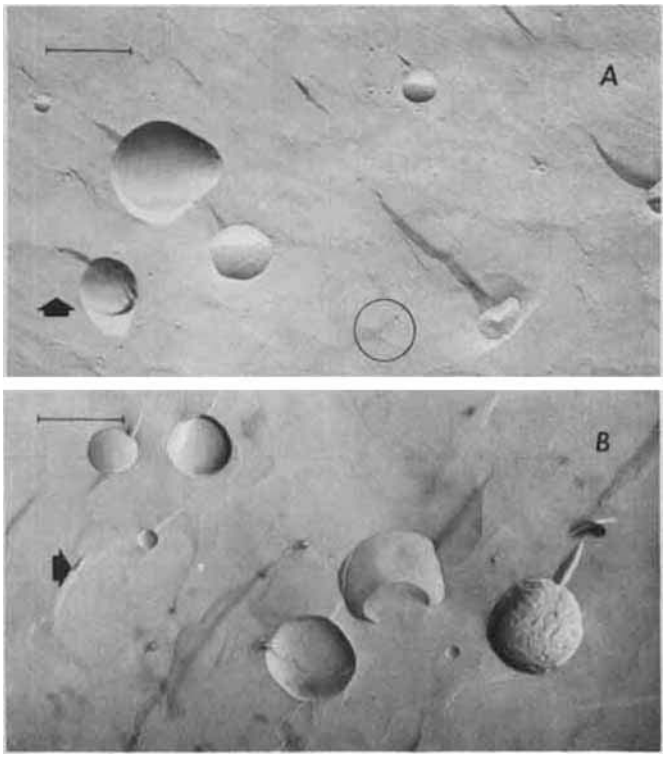

Figure 5—*Freeze-fracture electron micrographs of a mixture of liposomes before (A) and after (B) dialysis. The lipid in the original mixture (A) was from two sources: 70% consisted of a preparation of 1.0-μm extruded liposomes, the larger liposomes (Fig. 6), and 30% consisted of French press liposomes, the smaller liposomes (Fig. 2). Preparation is described in the text, and the mixture is described in Table I; in this mixture liposomes ≤80 nm in diameter account for ~99% of the total number (Fig. 8), ~58% of the total surface area (Fig. 9) and only 7% of the total liposome volume. Frame B shows the same mixture after 21 hr of dialysis against a 1.0-μm membrane; most of the small liposomes were removed. The bar in the upper left of each frame shows 1.0 μm. Within the circle in frame A are five of the small French press liposomes ranging in diameter from 16 to 57 nm.*

analytical reagent grade or better. The buffer, phosphate-buffered saline, contained 92 m*M* NaCl, 23 m*M* dibasic sodium phosphate, and 11 m*M* monobasic sodium phosphate. Prior to use, phosphatidic acid was obtained by chloroform extraction of an aqueous 0.4 *N* HCl solution of sodium phosphatidate containing 20% methanol.

Preparation of Liposomes—Multilamellar liposomes were prepared essentially as previously described (6, 22) and were composed of I–II–III–IV in the molar ratios 4:1:4.5:0.05 or 4:1:1:0.05. The α-tocopherol was added to retard autoxidation and improve stability (24). The lipids were dissolved in chloroform, mixed in a round-bottom flask, and subsequently evaporated to dryness under vacuum. An aliquot of buffer was added to the dried lipid; the mixture was then agitated by hand (at 20° after nitrogen purging) until all lipid was suspended, giving a final concentration of 10 μmoles of lipid/ml. In most cases a small amount of [^{14}C]cholesterol, as a liposomal membrane marker, was included with the other lipids. In other cases, 5 m*M* [^{14}C]sucrose, as an aqueous space marker, was included in the buffer used to disperse the lipids. This hand dispersal system results in the heterogeneous multilamellar population designated as being mechanically dispersed. The 1.0-μm extruded liposomes were prepared by placing the crude mechanically dispersed preparation in a 25-mm stirred ultrafiltration cell[4] fitted with a 1.0-μm pore-size polycarbonate membrane[5] and extruding it with positive nitrogen pressure at 10 ml/min. These liposomes could then be sequentially extruded through 0.8-, 0.6-, 0.4-, and 0.2-μm pore-size membranes to further reduce the size.

In selected cases the suspension was extruded twice through each membrane. The liposome size type was then designated by the smallest membrane size through which the suspension was extruded. The lipid recovery at each extrusion step was 100%, except at or below the 0.2-μm level; the extrusion was always done twice through the final membrane.

French press liposomes were prepared as previously described (16). A multilamellar liposome preparation was placed in a French pressure cell[6] and extruded three times at room temperature (flow rate ~15 ml/min) at 20,000 psi. The French press liposomes were then centrifuged at low speed to remove rubber particles sheared from the cells' O-rings during the extrusion.

Liposomes were dialyzed against the selected polycarbonate membrane as follows: 1 ml (or larger) dialysis cells (actual capacity 1.3 ml/side) were fitted with 25-mm membranes, and 1.0 ml of liposome suspension was placed in the sample chamber and 1.0 ml of buffer was placed in the second compartment. The cells were shaken at 5° on a horizontal shaker at 1.5–2 cycles/sec with a 5-cm amplitude. The dialysate side was replaced frequently with fresh buffer so as to maintain sink conditions. The total counts per minute of carbon on the sample side was monitored with time using scintillation-counting techniques. In the case where [^{14}C]sucrose was used as the liposomal marker, the nonentrapped sucrose was removed prior to the polycarbonate membrane dialysis by overnight dialysis *versus* excess buffer at 5° in a dialysis bag with a molecular weight cutoff of 10,000.

Electron Microscopy of Liposomes—For electron microscopy, ali-

Table I—Measured Liposome Diameter (μm) for Four Batches of Liposomes [a]

Liposome Type	Number Counted	Corrected mean Diameter	Liposome Diameter, Mean Surface Area[f]	Liposome Diameter, Mean Volume[g]
1.0 μm Undialyzed[b]	979	0.156	0.23	0.31
1.0 μm Dialyzed[c]	195	0.30	0.40	0.50
1.0 + FP Undialyzed[d]	9923	0.033	0.040	0.076
1.0 + FP Dialyzed[e]	1066	0.047	0.105	0.213

[a] Diameters were measured on freeze-fracture electron micrographs as described in the text; the frequencies were then corrected (25) to eliminate the freeze-fracture artifacts. [b] Mechanically dispersed liposomes extruded through 1.0-μm membrane (Fig. 6). [c] 1.0-μm extruded liposomes dialyzed against a 0.8-μm membrane (Fig. 6). [d] The mixture of 1.0-μm extruded and French press (FP) liposomes described in Fig. 8. [e] The dialyzed mixture of 1.0-μm extruded and French press liposomes described in Fig. 8. [f] The corrected diameter of each liposome was used to calculate its surface area; the average surface area of the sample was then calculated and these values are the diameters of the liposome having this average area. [g] The volume of each liposome was calculated from its corrected diameter; the average volume of the sample was then calculated and these values are the diameters of the liposome having this average volume.

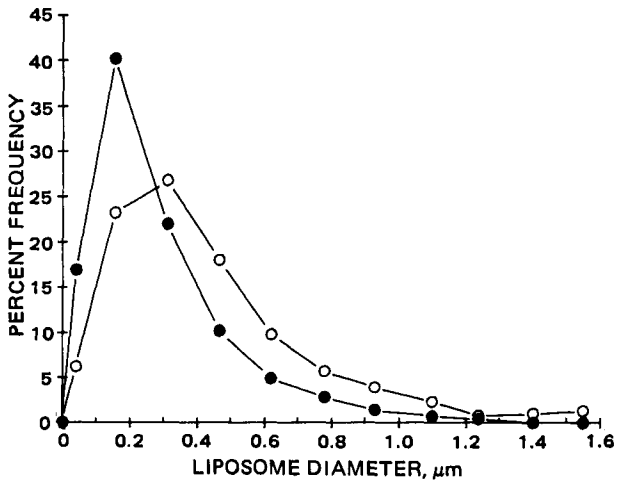

Figure 6—*The corrected percent of liposomes within equal diameter ranges is plotted* versus *the midpoint of the size range for 1.0-μm extruded liposomes before (●) and after (○) dialysis for 20 hr against a 0.8-μm membrane. The observed frequencies in each size range were corrected using the Wicksell method (25); the original data before applying the correction procedure is shown in Fig. 7.*

[4] Millipore Corp., Bedford, Mass.
[5] Nucleapore, Pleasanton, Calif.
[6] Aminco, Silver Spring, Md.

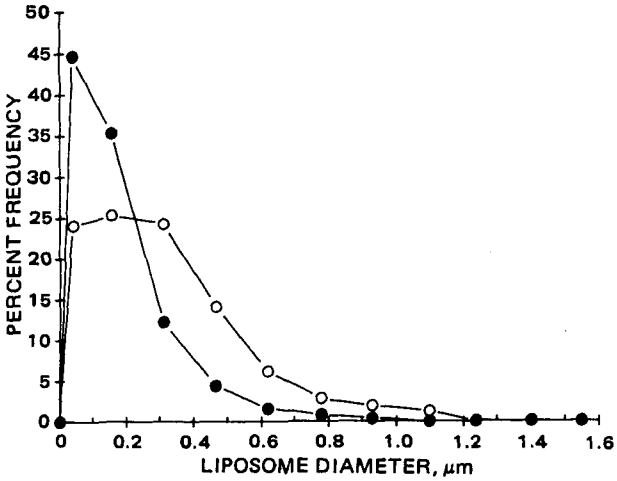

Figure 7—*The observed percent of liposomes within equal diameter ranges, as measured on the electron micrographs, is plotted* versus *the midpoint of the size range for 1.0-μm extruded liposomes before (●) and after (○) dialysis for 20 hr against a 0.8-μm membrane. The corrected data are shown in Fig. 6.*

quots of the liposome suspensions were diluted twofold with a 25% (v/v) solution of glycerin in buffer and kept at room temperature for 2 hr. The samples were then quickly frozen in liquid freon at ~−155° and stored at −190° in liquid nitrogen. Stored samples were transferred to the specimen stage of a freeze-fracture device[7], fractured at −115°, and then platinum-carbon coated. The replicas then were cleaned in dilute (1%) hypochlorite solution overnight, and rinsed for 10 min in several successive distilled water baths. A final 30-sec acetone rinse removed any remaining lipid. Replicas were then transferred to flamed 200-mesh grids and viewed at 80 kV. Magnification was determined using a carbon calibration grid.

RESULTS

Dialysis of Liposomes—Figure 1 shows results when mechanically dispersed liposomes were dialyzed against several different pore-size membranes. The initial rate and extent of dialysis increases with increasing pore size. The results suggest that the majority of liposomes are between 0.6 and 3.0 μm in diameter. When the French press liposomes, averaging 28 nm in diameter, were dialyzed against 0.05-, 0.1-, and 0.2-μm pore-size membranes (Fig. 2), the half-life of dialysis increased, as the ratio of the pore size to mean liposome diameter increased.

Figure 3 shows the results of a study in which 0.6, 0.4, and 0.2 μm extruded and French press liposomes were dialyzed against 0.8-μm membranes. There is a clear trend of more rapid and extensive dialysis as the liposome size decreases relative to membrane pore size. A first-order dialysis was seen only for the French press liposomes, where the membrane pore size is significantly larger than the largest liposomes in the suspension. Figure 4 shows the combined results of three separate studies in which 1.0-μm extruded liposomes were made and treated as follows: half of the batch was dialyzed for 20 hr against 0.8-μm membranes, the other half against 0.05-μm membranes. Both subbatches were subsequently dialyzed a second time against 0.8-μm membranes for 72 hr. There is a significant difference in dialysis patterns because a fraction of the smaller liposomes dialyzed across the 0.8-μm membrane, whereas no significant dialysis across the 0.05-μm membrane was detected. Aliquots of both subbatches, taken after the first dialysis step, were used to construct size frequency distributions.

The effects of dialysis on a heterogeneous population, consisting of 1.0-μm extruded liposomes and French press liposomes (70 and 30%, respectively, of the total lipid), pictured in Fig. 5, were dramatic. This mixture was selected to represent a worse-case example (it is similar in size composition to those resulting from sonication of mechanically dispersed liposomes for short periods of time). A sample of this heterogeneous batch was saved and the rest was dialyzed for 21 hr using 1.0-μm membranes. Electron microscopy was then done on the dialyzed and undialyzed samples

Control Studies—The effect of the dialysis system on liposome stability was assessed as follows: aliquots of 0.8-μm extruded liposomes of either the I–II–III–IV = 4:1:1:0.05 or 4:1:4.5:0.01 composition, containing [^{14}C]sucrose, were dialyzed against 0.05-μm membranes following the same procedures used in other studies; these two compositions (differing in cholesterol content) were chosen as medium- and low-permeability liposomes (1). It was previously established that these same liposome types, when labeled with [^{14}C]cholesterol, would not dialyze across 0.05-μm membranes, so the appearance of [^{14}C]sucrose on the dialysate side would be evidence of liposome instability. After 24 hr a maximum of 3% had leaked from the more permeable liposomes, whereas no leakage was detected from the less permeable liposomes. For the case where the label did dialyze, it was important to know if the liposomes dialyzed across intact. To answer this question, 0.4-μm extruded liposomes of the I–II–III–IV = 4:1:4.5:0.05 composition containing [^{14}C]sucrose were dialyzed to equilibrium against 3.0-μm membranes (the size distribution of these liposomes is such that all are small enough to dialyze). Samples of the dialysate side were chromatographed on a sepharose gel column[8], and all of the radioactivity appeared in the void volume, indicating that the liposomes had dialyzed across intact.

The rate and extent of liposome dialysis was found to be a function of several system variables. The dialyses were concentration-dependent; that is, with all other variables held constant (*e.g.*, liposome size, mem-

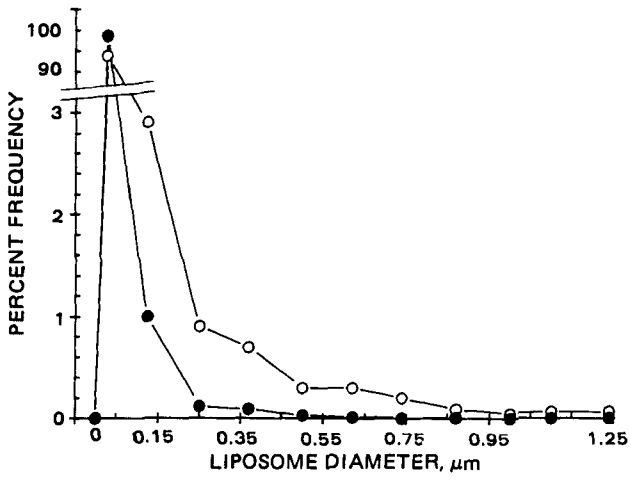

Figure 8—*The corrected percent of liposomes within equal diameter ranges is plotted* versus *the midpoint of the range for the liposome preparation consisting of a 1.0-μm-extruded (70% of total lipid) and French press liposomes (30% of total lipid) before (●) and after (○) dialysis for 20 hr against a 1.0-μm membrane. The original frequencies (not shown) were corrected to the observed frequencies using the Wicksell method (25).*

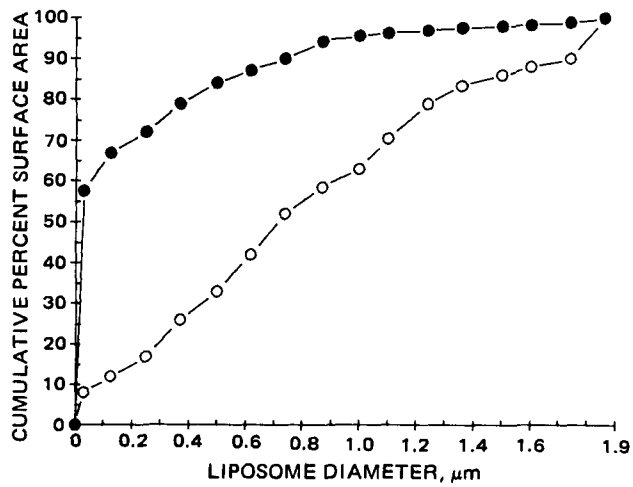

Figure 9—*The cumulative percent of total surface area (calculated from the corrected frequency data) for the two liposome populations shown in Fig. 8 is plotted* versus *the midpoint of the diameter range before (●) and after (○) dialysis.*

[7] Balzers, Berkhansted, U.K.

[8] G-50-80 Pharmacia Fine Chemical, Sweden.

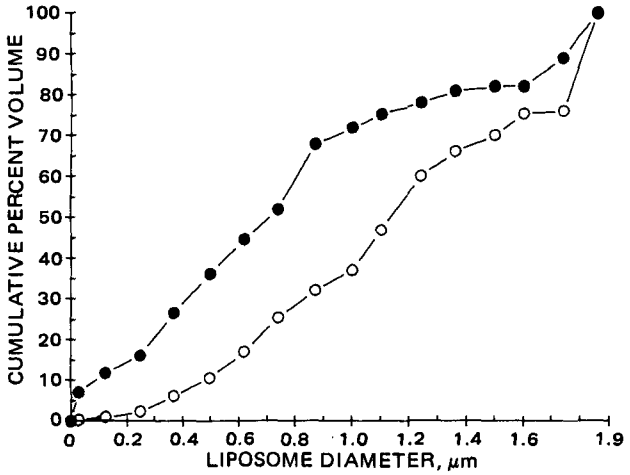

Figure 10—*The cumulative percent of the total volume (calculated from the corrected frequency data) for the two liposome populations shown in Fig. 8 is plotted* versus *the midpoint of the diameter range before (●) and after (○) dialysis.*

brane size), samples with higher lipid concentration dialyzed more slowly than more dilute samples. The agitation rate and the cell fill-volume were important. Three different modes of agitation rates (*i.e.*, motionless, gentle test tube inverter, and the horizontal shaker described previously) gave dialysis rates that covered a sixfold range. It was necessary to leave a small head space of air (~300 μl) when filling the cells, so that the dialyzing solution had the opportunity to agitate sufficiently. When these variables were held constant, the dialyses were reproducible.

Electron Microscopy and Liposome Sizing—Size–frequency distributions are obtained directly from diameter measurements on freeze-fracture electron micrographs. Figure 5 shows the mixture of 1-μm extruded and French press liposomes. A large number of small liposomes (Fig. 5A) were seen before dialysis, but were rare after dialysis (Fig. 5B).

Size distributions were constructed by assigning each liposome (on the photomicrograph) to size categories of 1-mm width (*e.g.*, 1–2 mm, 2–3 mm, *etc.*). Diameters of liposomes with oblong shape were calculated by averaging the values for their long and short axes. Diameter measurements were made for the above mixture from 8070× micrographs, except for the smallest liposomes, which were measured on 26,090× blow-ups of regions on the same 8070× micrographs. The ratio of smaller to larger liposomes found on each blowup was assumed to hold constant on the small scale micrograph. On the 26,090× micrographs all liposomes with diameters 0–3.3 mm were counted and measured; on the 8070× micrographs all liposomes >1.0 mm were counted and measured. After all liposomes had been counted and measured, the appropriate scaling correction was made to yield actual diameters. The total number of liposome profiles measured and counted in each experiment is shown in Table I.

There are certain inherent biases in the previously described method of sizing. A given fracture plane may not make an equatorial cut through a specific liposome, and it is more probable that a large liposome will be cut than a smaller one; this has the effect of making the apparent average diameter larger than the actual average diameter. The magnitude of the overall bias depends on factors such as the population range and the shape of the actual distribution. These biases are correctable using the mathematical approach developed by Wicksell (25), and the cumulative surface area or cumulative total liposome volume was plotted *versus* diameter and their parameters tabulated in Table I.

The corrected and uncorrected frequency plots for dialyzed (*i.e.*, those remaining after dialysis) and undialyzed 1.0-μm extruded liposomes are shown in Figs. 6 and 7. It can be seen that the correction procedure shifts the size distribution to smaller sizes. Nevertheless, there is a clear upward shift in average size for the dialyzed liposomes, with a doubling in average corrected diameter (from 0.156 to 0.3 μm, Table I). The corrected cumulative surface area and volume curves, not shown, also exhibit dramatic shifts toward larger diameters, which is reflected in the changes in the diameters of the liposomes with the average surface area and volume (Table I).

The data in Figs. 8–10 for the mixture of 1-μm extruded and French press liposomes illustrates an extreme case but serves as an example for a number of points. The corrected frequency *versus* size curve before

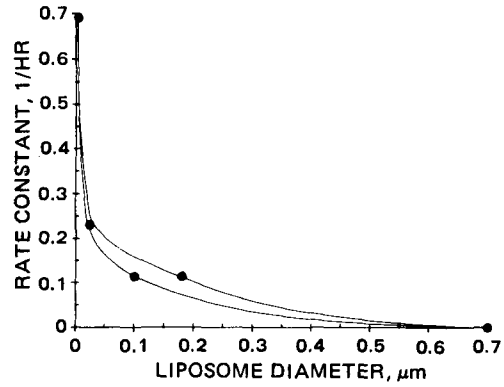

Figure 11—*The apparent first-order rate constant for various size liposomes against a 0.8-μm membrane is plotted* versus *their diameter. From left to right: (a) the rate constant for [^{14}C]inulin, assumed to be the upper limit for rate constants under the conditions used; (b) French press liposomes; (c) 0.2-μm extruded liposomes having an average diameter of 0.1 μm; (d) 0.2-μm extruded liposomes having an average diameter of 0.18 μm; (e) 0.7-μm extruded liposomes assuming negligible dialysis. The curves are arbitrary and drawn to approximate the actual underlying relationship.*

dialysis (Fig. 8) shows that ~99% of the liposomes are in the smallest size class, although French press liposomes comprised only 30% of the liposomal lipid; this class accounted for 58% of the total surface area, but only 7% of total liposome volume. Following dialysis the number of liposomes in the smallest size class decreased dramatically, but still accounted for ~95% of the total number (liposome dialysis was not limited to this size class; liposomes in the next few larger classes also dialyzed but to a lesser extent). Although dialysis of this mixture did not greatly increase the average diameter of the liposomes (Table I), it did produce a large increase in the diameter of the lipsomes with the average surface area and the average volume (Table I).

All differences noted between dialyzed and undialyzed preparations were significant; in the case where the fewest number of liposomes was measured (n = 195, Table I), the difference between mean diameters for the uncorrected dialyzed and undialyzed distributions was significant at the p <0.01 level (1-tailed t test using frequency *versus* log diameter distributions). For the corrected distributions (Fig. 5A) the level of significance between mean diameters before and after dialysis was greater.

Dialysis Simulations—The dialysis data for the French press liposomes (Fig. 2) indicates that when the preparation is homogeneous, dialysis is first order. Thus, the dialysis of a heterogeneous population may be simulated by a sum of first-order processes (or exponentials), one for each narrow size class as given by:

$$N_T = \sum_{i=1}^{n} N_{0,i}e^{-k_i t} + M \qquad \text{(Eq. 1)}$$

where N_T is the total number of liposomes remaining in the dialysis cell at time t, $N_{0,i}$ is the number in the ith narrow size class (*e.g.*, Fig. 5), k_i is the apparent first-order rate constant for dialysis of the ith class across a specific membrane, and M is the number of liposomes which cannot dialyze across that pore-size membrane. When a dialysis cleanup is complete, or after some time t, when dialysis is terminated, the fraction of liposomes in each size class will be:

$$N_{t,i}/N_T, N_{t,2}/N_T \ldots N/N_T \qquad \text{(Eq. 2)}$$

where

$$N_{t,i} = N_{0,i}e^{-k_i t} \qquad \text{(Eq. 3)}$$

Thus, if estimates of k_i can be obtained and the predialysis size–frequency distribution is known or can be approximated, the postdialysis size–frequency distribution can be determined. (If size–frequency distributions are not available, demonstrating the reproducibility of the dialysis pattern should ensure reproducibility of the final size–frequency distribution.) For example, the relationship between dialysis rate constant and diameter for the 0.8-μm membrane system described previously can be approximated (see Fig. 11). The maximum value of k_i is set equal to that obtained for a solution of [^{14}C]inulin. A rate constant of zero is assigned to liposomes >0.7-μm diameter, which were shown not to dialyze to any significant extent in this system over a 24-hr period. Additional rate constants are shown for French press liposomes of known mean diameter

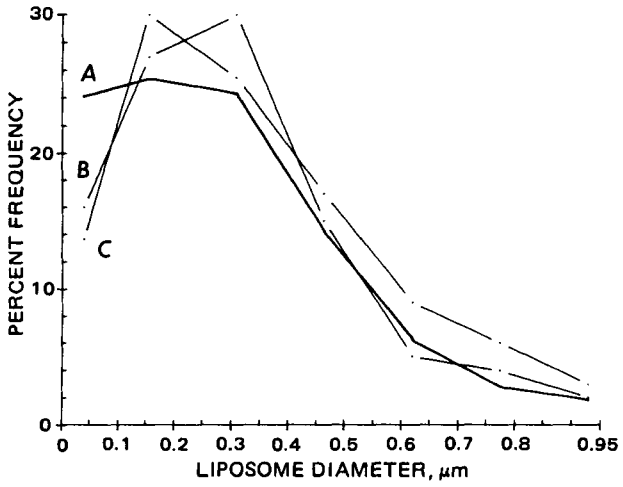

Figure 12—*Frequency, as percent of total, is plotted* versus *diameter. Curve* A *is replotted from Fig. 6 and is for 1.0-μm extruded liposomes following dialysis. Curves* B *and* C *are estimates of curve* A *calculated using Eq. 3, the undialyzed data in Fig. 6, and apparent dialysis rate constants interpolated from Fig. 10 as described in the text.*

and 0.2-μm extruded liposomes with mean diameters of 0.1 and 0.18 μm.

No attempt was made to fit the data in Fig. 11 to any specific function, rather the points were connected by two arbitrary curves to approximate the actual underlying pattern. Two sets of approximate rate constants were interpolated from this curve for the medium size of the seven smallest size categories used for construction of Fig. 6. Values of $N_{0,i}$ and M, obtained from the predialysis data in Fig. 6, along with seven estimates of k_i taken from each of the two curves in Fig. 11, were substituted into Eq. 1 to calculate the postdialysis in size–frequency distribution. The solid curve in Fig. 12 is the actual postdialysis size–frequency distribution. The broken curves are the two estimated size–frequency distributions and are reasonable given the limitations of both the method of calculation and the dialysis system: (*a*) the pores are not uniform in diameter; the manufacturer states that maximum pore size is 0—20% of the rated pore size; (*b*) pore size itself has a size–frequency distribution; (*c*) the technique for estimating the dialysis rate constants is crude; (*d*) the potential exists for large liposomes to block pores, thus reducing the effective dialysis rate constant for each size class.

DISCUSSION

A prerequisite for understanding the *in vivo* properties of liposomes is the availability of techniques to reproducibly prepare liposomes with defined size properties. With the exception of small unilamellar liposomes produced by prolonged sonication or carefully prepared French press liposomes, it is difficult to compare results between or within laboratories because the number, average size, size–frequency pattern, or surface area of the liposome dose rarely is known.

Because each liposome preparation has a characteristic dialysis profile for a given membrane and set of dialysis conditions (Figs. 1–4), dialysis data can be used as a means to compare the similarity of preparations and as a measure of their reproducibility. From the data presented (Figs. 1–4), the relative reproducibility of these preparations can be compared. Although the described technique is unsophisticated and simple, it does allow one to narrow the size–frequency distribution of any liposome batch while improving the reproducibility of preparation. Membrane extrusion under controlled conditions allows one to define and control the upper limit of liposome size anywhere between ~0.1 and 3.0 μm in diameter. This dialysis procedure allows control of the lower end of the size–frequency distributions.

Other techniques can be used to minimize the fraction of smaller liposomes in a preparation, but each has limitations. Separation of larger from smaller liposomes by centrifugation poorly discriminates between adjacent size classes and is further limited because liposomes of the same diameter may have different densities (*i.e.*, number of lamellae) and *vice versa*. Separation by filtration is possible in some cases when there is minimal interaction between liposomes and membranes (fiber types), but even at low pressure, some extrusion (22) occurs for all fluid-phase liposomes with the membranes used here. Filtration is more reliable when the process is carried out at a temperature below the phase transition temperature of the liposomal lipids (26, 27). Gel exclusion chromatography can be successfully used to obtain relatively narrow size distributions of small liposomes between ~20 and 80 nm in diameter and can also be used to control the lower end of the size–frequency distribution of larger liposomes when the adsorption of liposomal lipid onto the column can be minimized (this depends on liposome composition and column). Sedimentation field flow fractionation (28) is specifically designed for separation and analysis of particles in the liposome size range but is not designed for large-scale preparation; as the technique becomes more widely available it will, however, provide a technique to characterize the size–frequency distribution of liposome suspensions.

Control of liposome size may be critical when liposomes are designed for use as *in vivo* drug carriers. When *in vivo* fate is being followed using an aqueous space or liposomal membrane marker, the pharmacokinetic pattern seen will be a function primarily of the larger liposomes (Fig. 10), *i.e.*, the volume adjusted average size (29). Yet, the actual mechanisms governing liposome disposition are expected to be a function of the number of liposomes used and/or their total surface area, as well as diameter, when composition is held constant.

A combination of extrusion followed by dialysis, both using controlled-pored membranes, allows reproducible preparation of liposomes having a variety of definable size–frequency distributions. Subsequent use of dialysis rates can be used to obtain information about the size–frequency distribution and may be useful as a quality control technique.

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Disposition of Quinidine in the Rabbit

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Abstract □ Quinidine shows two-compartment characteristics in rabbits with a terminal half-life of 67 min for total drug and 58 min for unbound drug. Statistically, the values are not significantly different from each other ($p > 0.05$). The clearances for total and unbound drug are 52 and 464 ml/min/kg, respectively, and the total and unbound apparent volumes of distribution at steady state are 4.2 and 27.3 liters/kg, respectively. The unbound clearance and unbound apparent volume of distribution were inversely related to the unbound fraction of quinidine in plasma. The total clearance and apparent volume of distribution showed no relationship to the binding. Approximately 0.5% of the dose was excreted as unchanged quinidine. Six identifiable metabolites were found in the urine, accounting for ~14% of the dose. Two unknown metabolites were also observed in the urine. With the exception of 2′-quinidinone, these metabolites were formed in the rate-limiting step in the metabolite kinetics. The quinidine unbound fraction ranged from 0.06 to 0.23 in the eight rabbits studied. The binding of the metabolites was less pronounced, and only 3-hydroxyquinidine showed a significant correlation with quinidine binding.

Keyphrases □ Quinidine—disposition in rabbits, metabolites, binding □ Metabolites—binding, quinidine disposition, rabbits □ Binding—quinidine disposition, metabolites, rabbits □ Pharmacokinetics—disposition of quinidine, rabbits

Very little information exists regarding quinidine disposition in the rabbit, especially when unbound quinidine and metabolite concentrations are considered. The rabbit is, however, an attractive animal model for quinidine studies because it allows for sampling of sufficiently large blood volumes to determine both total and unbound concentrations; blood and urine sampling is simple; and if drug responses are desired, the response, measured as EKG changes, can be readily obtained (1).

As part of a long-term study of the disposition and response interrelationship between binding of quinidine to plasma proteins the pharmacokinetic picture of quinidine was evaluated in the rabbit.

EXPERIMENTAL

Quinidine Administration—Eight male New Zealand white rabbits (2.0–3.3 kg) were injected with 5.2 mg/kg of quinidine base as the gluconate salt[1] dissolved in 1 ml of saline into an ear vein over 2 min. Blood samples of 3-ml volume were obtained from the marginal vein in the other ear before the injections and again at 4, 8, 20, 40, 60, 90, 120, 150, 195, and 240 min after the injections. The blood was heparinized[2] to a final concentration of 5 U/ml. The blood was centrifuged and the plasma was stored at −20° until assayed. In addition, a 300-μl whole blood aliquot from the 4- and 150-min sampling time was also stored frozen until assayed. The red blood cells from the samples obtained at 20 min and at later time points were suspended in an equal volume of 6% dextran 75 in isotonic saline and reinfused within 10 min of sampling.

The urethra of each animal was cannulated with a catheter[3]. To ensure complete urine collection over the 4-hr study period, the bladder was rinsed twice with normal saline at the end of the study. The total urine collected was stored frozen until assayed.

Hepatic Blood Flow Determination—The hepatic blood flow was estimated in the individual animals 20–40 min after the end of the quinidine experiment by determining the indocyanine green blood clearance. Indocyanine green[4] (1 mg/kg) was infused over 30 sec into a marginal ear vein. Blood was collected from the marginal ear vein in the other ear by continuous withdrawal at a speed of 0.36 ml/min over a 12-min period, starting at the time of indocyanine green infusion.

The indocyanine green concentration in plasma of the withdrawn blood (C_{ICG}) was determined spectrophotometrically at 800 nm. Because the half-life of indocyanine green is 1 min and it does not enter the red blood

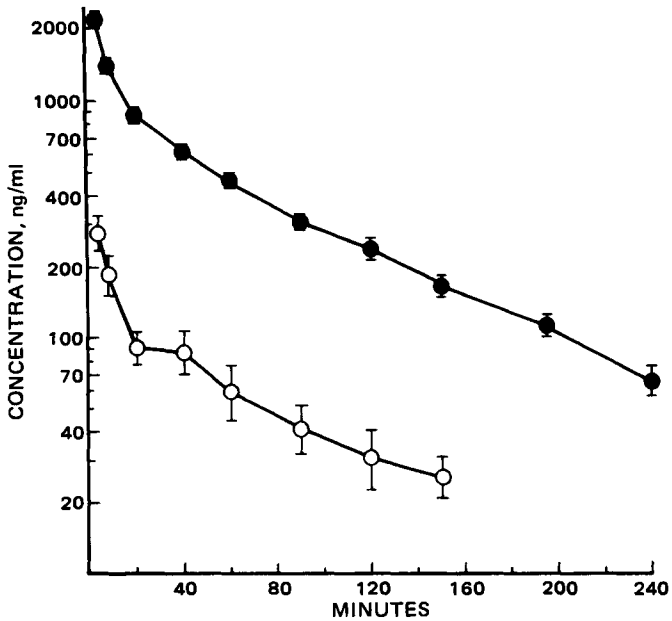

Figure 1—*Log-average plasma concentrations of total (●) and unbound (○) quinidine in eight rabbits after a 5.2-mg/kg intravenous injection.*

[1] Quinidine gluconate injection, USP, 80 mg/ml; Lilly, Indianapolis, Ind.
[2] Sodium heparin injection, USP, 1000 U/ml, Lilly, Indianapolis, Ind.
[3] French Foley Catheter #8, D. R. Bard, Ind., Murray Hill, N.J.
[4] Hynson, Westcott, & Dunning, Inc., Baltimore, Md.

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