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Radiopaque Liposomes: Effect of Formulation Conditions on Encapsulation Efficiency

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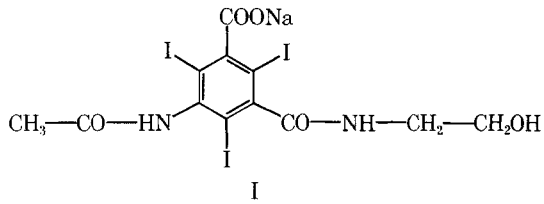
Received May 27, 1983, from the **Pharmacy Department, School of Pharmacy, Hebrew University of Jerusalem, Jerusalem 91120, Israel,* [‡]*Laboratoire de Pharmacie Galenique, Faculte de Pharmacie de Paris-Sud, 92290 Chatenay-Malabry, France,* and [§]*Laboratoire de Biochimie, Faculte de Pharmacie, 63100 Clermont Ferrant, France.* Accepted for publication, September 14, 1983.

Abstract □ Liposomes containing sodium ioxitalamate were prepared by sonication. Suitable amounts of purified soybean phosphatidylcholine and cholesterol were used at various molar ratios. Stearylamine or dicetylphosphate were added to this lipid composition when charged liposomes were required. After sonication and removal of unencapsulated solute, this manufacturing process yielded small multilamellar vesicles as confirmed by electron microscopy. These liposomes did not exhibit a narrow range of size distribution; the mean particle size varied from 135 to 145 nm. With respect to the efficiency of encapsulation, two parameters were distinguishable: the volume of encapsulated aqueous space per unit of lipid weight and the percentage of the contrast agent added that became encapsulated in the liposomes. Investigation of the preparative parameters revealed that increased molar ratios of cholesterol yielded higher aqueous volume and iodine contents in the liposomes, which were attributed to a reduction of the liposome permeability to the contrast agent. However, the inclusion of cholesterol into the bilayer liposomal membrane was limited, probably by solubility restrictions. Negatively and positively charged liposomes had higher rates of encapsulation than did neutral liposomes. This result was expected since efficient encapsulation of polar compounds requires formation of large aqueous spaces within the vesicles per mole of lipids. Increase of the lipid fractions at a constant ratio of phosphatidylcholine–cholesterol, with all the other factors kept constant, reduced the aqueous volume entrapped per millimole of lipid and, consequently, the iodine content in the liposomes. However, an increase in the initial sodium ioxitalamate concentration diminished the aqueous volume entrapped in the liposomes but increased the iodine content.

Keyphrases □ Liposomes—radiopaque, effect of formulation conditions on encapsulation efficiency □ Formulations—radiopaque liposomes, effect on encapsulation efficiency □ Encapsulation—radiopaque liposomes, effect of formulation conditions

The possibility of utilizing liposomes as vehicles for therapeutic agents had been widely considered over the last 16 years. The potential applications have been extensively reported and clearly summarized (1–3). The ultimate goal has been the targeting of drugs and other biologically active ingredients to specific cells within the body. However, this cannot be easily accomplished since it has been shown in numerous reports that intravenously injected liposomes localize predominantly in the liver, spleen, and other organs rich in cells of the reticuloendothelial system (4).

The specific hepatic uptake can be exploited to design a selective delivery system based on liposomes containing sodium ioxitalamate, which is a water-soluble iodinated contrast agent characterized by its high iodine content (57%). The radiopaque agent, sodium ioxitalamate (I), is primarily used for urography because of its pharmacokinetic properties, (5, 6); however, side



I

effects, allergic reactions, and intolerance have been reported. The preparation of liposomes containing the radiopaque agent is, therefore, also aimed at reducing or avoiding these adverse reactions since selective liver uptake enables administration of smaller amounts of radiopaque agent for a similar or even improved diagnostic result. The liposomal preparation will probably be able to produce a visible contrast enhancement of the liver, which will help improve the diagnostic accuracy of computerized tomography of this organ. Some attempts, including the preparation of brominated radiopaque liposomes (7) and the encapsulation of diatrizoic acid salts within liposomes (8), have already been performed, yielding preliminary results that are promising. However, this interesting approach is limited by the amount of contrast agent that can be entrapped in the aqueous phase of liposomes and the potential release of the opaque ingredient from the liposomes. The objective of this study, therefore, is to incorporate the contrast agent within liposomes and to investigate some of the physical and chemical factors which may control the efficiency of the encapsulation rate. The results of such an investigation offer the possibility of optimizing liposome formulation, enabling a high encapsulation rate and, consequently, an increase in the opacification of the liposomes caused by their high iodine content.

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EXPERIMENTAL SECTION

Materials—Soybean phosphatidylcholine[1] was purified by the method of Singleton *et al.* (9), and the purity was ascertained by a TLC analytical method (10). Stearylamine and dicetylphosphate were obtained commercially[2]. Cholesterol[3] was purified by two recrystallizations. Sodium ioxitalamate was used as supplied[4].

Methods—*Preparation of Liposomes*—Suitable amounts of purified phosphatidylcholine and cholesterol in various molar ratios were dissolved in 20 mL of chloroform to which stearylamine or dicetylphosphate was added when charged liposomes were required. The lipid mixture was deposited as a thin film on the walls of a round-bottomed flask by rotary evaporation under reduced pressure at 30°C. Twenty-five milliliters of an aqueous solution of sodium ioxitalamate at a given concentration was poured into the flask, shaken by continuous rotation for 30 min at 30°C, and allowed to stand over a 2-h period. The liposomal dispersion obtained was then sonicated[5] for 3 min under a nitrogen atmosphere at 0°C for homogenization. Sonication promoted conversion of the large multilamellar vesicles (MLV) into smaller liposomes which were separated from the excess of free sodium ioxitalamate by gel filtration on a coarse Sephadex G-50 column. The liposomes were mainly collected from fractions three to six after the void volume was eluted; they were identified by the presence of turbidity in the different samples collected. The liposome size was then measured[6].

Drug Encapsulation Efficiency—After vesicle preparation and removal of the unencapsulated solute, the amount of encapsulated compound could be determined. With respect to the efficiency of encapsulation, two parameters can be distinguished: first, the volume of encapsulated aqueous space per millimole of lipid and, second, the percentage of the compound added which becomes encapsulated in the liposomes. The latter, when expressed in percent iodine content of the liposomes, in fact represents a measure of the probable opacification ability of the liposomal preparation. The first parameter, which is defined as the encapsulation capacity (EC), is a function of liposome size and number of lamellae per particle. EC represents the fraction of aqueous volume entrapped per millimole of lipids and is calculated by (11):

$$EC = \frac{C_e}{C_0} \cdot \frac{1}{C_l} \qquad \text{(Eq. 1)}$$

where C_0 is the initial sodium ioxitalamate concentration, C_e is the final concentration found, and C_l is the lipid concentration in the liposomes. In contrast, the molar fraction of drug entrapped per mole of lipid is calculated by the simple ratio of the molar concentration of sodium ioxitalamate to the molar concentration of lipids determined in the liposomes. Obviously, the second parameter, which is defined as the entrapment efficiency or drug content (DC), depends on EC, but in addition, it also depends on the lipid concentration in the vesicles. DC is expressed by (in percent):

$$DC = \frac{W_c}{W_c + W_l} 100 \qquad \text{(Eq. 2)}$$

where W_c is the amount of drug found in the liposomes, and W_l is the amount of lipids which form the bilayer membrane found experimentally in the liposomes. The sodium ioxitalamate amount entrapped was determined with a liquid chromatograph[7] consisting of a pump[7], an injector[7], and a UV detector[7] fixed at 254 nm. The stationary phase was C_{18}[8], and the eluant was methanol–water (70:30) containing 1% tetrabutylammonium phosphate, supplied at a flow rate of 1 mL/min. A portion of the liposomal preparation was diluted with water (1:100) before injection. The amount of contrast agent in the liposomal preparation was calculated by using a calibration curve based on standard solutions determined under identical experimental conditions. No interference of the phospholipid fragments by the HPLC method was observed. The phospholipids in the liposomal preparation were assayed by using a phospholipid B test[9] based on a colorimetric reaction (12). No disturbance of the colorimetric reaction was detected as a result of the presence of sodium ioxitalamate, proving that the concentrations of the phospholipid found were correct. For stearylamine-, dicetylphosphate-, and cholesterol-incorporated liposomes which were eluted through the gel column, the original molar ratio to lecithin on mixing was assumed to be maintained (11). All the calculations on drug encapsulation efficiency were carried out on the basis of this assumption. Each batch of liposomes was triplicated. The different values reported are the average of six experimental measurements since the property determinations of each liposome batch were duplicated.

[1] Epikuron 200; Lecithos, Saint Maur, France.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Rhône-Poulenc, Paris, France.
[4] Guerbet Aulnay-sous-Bois, France.
[5] Model B-30 sonifier; Branson.
[6] Nanosizer; Coulter.
[7] 480-HPLC composed of a 6000A pump, a U6K injector, and a 480-UV detector; Waters Associates, Milford, Mass.
[8] μ-Bondapak; Waters Associates.
[9] Wako Chemical, Osaka, Japan.

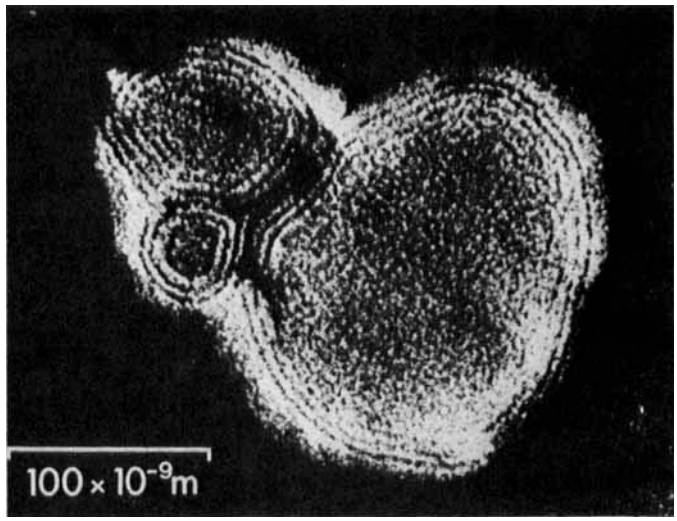

Figure 1—*Electron micrograph of multilamellar liposomes produced by the method described in the text (initial conditions: phosphatidylcholine, 500 mg; cholesterol, 70 mg in 20 mL of chloroform; 25 mL of sodium ioxitalamate aqueous solution equivalent to 100 mg of iodine/mL).*

RESULTS AND DISCUSSION

Water-soluble compounds entrapped within liposomes are found in the internal aqueous compartments of these particles (13) owing to their low affinity and solubility in the lipids which constitute the liposomal membrane. Thus, efficient encapsulation of polar compounds requires formation of large aqueous spaces within the MLV. The manufacturing process used in this study yielded, after sonication and removal of unencapsulated solute, small MLV, as confirmed by electron microscopic examinations (Fig. 1). These liposomes did not exhibit a narrow range of size distribution, and their mean particle size varied from 135 to 145 nm[6]. Polydispersity index values were obtained during routine liposome size measurements and ranged from 3 to 5, which indicated, according to the manufacturer's instructions, a distribution range, by diameter, of 2:1 in the liposome samples.

In preliminary studies, the influence of the phospholipid characteristics on liposome properties was investigated. The chemical purity of the phosphatidylcholine source, which might slightly affect the chemical composition, had little effect on solute retention or particle size of the liposomes formed. The entrapped volume of the total internal aqueous phase remained practically constant, irrespective of the phosphatidylcholine used. In spite of these results, purified soybean phosphatidylcholine, as compared with commercial soybean phosphatidylcholine, was preferred for liposomes production since fewer adverse toxic reactions would be expected after parenteral administration of such liposomes in future animal experiments.

Effect of Cholesterol Concentration—Increased initial molar ratios of cholesterol, with respect to the phosphatidylcholine concentration, yielded higher aqueous volumes and iodine contents in the liposomes (Table I). This was attributed to a reduction in the liposome permeability to the contrast agent

Table I—Influence of Initial Cholesterol Concentration on Liposome Properties [a]

Soybean Phosphatidylcholine–Cholesterol, Molar Ratio	Encapsulation Capacity, mL/mmol	Iodine Content, %[b]	Molar Fraction of Drug Entrapped/mol of Lipid, × 10^2 [c]
8:0	0.78	9.5	20.42
8:2	1.00	11.89	20.79
8:4	1.41	15.97	29.05
8:8	1.52	16.87	29.01

[a] Conditions: 500 mg of phosphatidylcholine, 4.325 g of sodium ioxitalamate, with various amounts of cholesterol. [b] Calculated according to Eq. 2 by using only the molar fraction weight of iodine contained in the sodium ioxitalamate molecule. [c] The ratio was calculated according to the experimental results by using the analytical methods described in the text.

Table II—Influence of a Positive Charge, Stearylamine, or Negative Charge, Dicetylphosphate, on the Liposome Properties[a]

Liposome Composition, Molar Ratio	Mean Particle Size, nm[b]	Encapsulation Capacity, mL/mmol	Iodine Content, %[c]	Molar Fraction of Drug Entrapped/mol of Lipid, $\times 10^2$ [d]
Soybean phosphatidylcholine-cholesterol, 8:2	140	1.00	11.89	20.79
Soybean phosphatidylcholine-cholesterol-stearylamine, 8:2:1	128	1.22	14.03	24.84
Soybean phosphatidylcholine-cholesterol-stearylamine, 8:2:2	145	1.33	15.25	29.87
Soybean phosphatidylcholine-cholesterol-dicetylphosphate, 8:2:1	138	1.19	13.87	25.29
Soybean phosphatidylcholine-cholesterol-dicetylphosphate, 8:2:2	144	1.43	16.25	27.76

[a] Conditions: 500 mg of phosphatidylcholine, 4.325 g of sodium, ioxitalamate, with various amounts of cholesterol. [b] The polydispersity index in all the measurements performed was 4. [c] Calculated according to Eq. 2 by using only the molar fraction weight of iodine contained in the sodium ioxitalamate molecule. [d] The ratio was calculated according to experimental results by using the analytical methods described in the text.

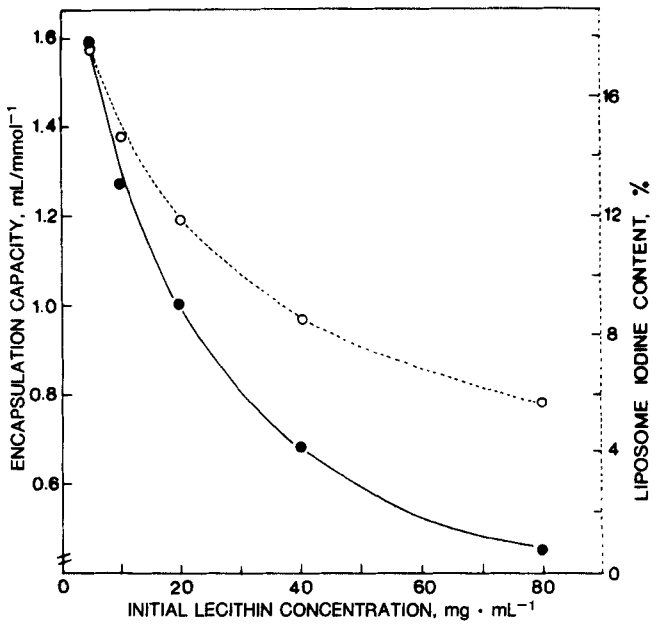

Figure 2—*Effect of initial phosphatidylcholine concentration on encapsulation capacity (●) and iodine content (○) of the liposomes prepared.*

(14). In addition to its barrier-controlling property, the incorporation of cholesterol was found to provide more physical stability against the ultrasonic vibration force as compared with that of the cholesterol-free liposomes (11). This effect might reduce precocious sodium ioxitalamate release during liposome size homogenization by sonication. However, the concentration of radiopaque agent entrapped in the liposome augmented and reached a maximum (*i.e.*, a plateau) with increasing cholesterol molar ratio (Table I). This phenomenon could be expected since the inclusion of cholesterol into the bilayer liposomal membrane is limited, probably as a result of solubility restrictions which were corroborated by the existence in the highest initial cholesterol concentration of a crystallization process, which is attributed to an excess of cholesterol after vesicle formation.

Effect of Electric Charge on the Surface of the Bilayer Membrane—Various concentrations of stearylamine (cationic substance) or dicetylphosphate (anionic substance) were included in the liposomal bilayer membranes, rendering the surface electrically charged. This resulted in a repulsion and, hence, an increase in the distance between the different bilayers, which appear to be very close to one another in neutral liposomes (Fig. 1); consequently, more hydrophilic solute can be trapped in the aqueous compartments than in neutral liposomes. Negatively and positively charged liposomes gave larger amounts of radiopaque agent and higher rates of aqueous volume encapsulation than did neutral liposomes, confirming the previous hypothesis (Table II). It should be noted that an increase in the charge membrane concentration (*i.e.*, the stearylamine or dicetylphosphate concentration) while all the other factors were kept constant enhanced the sequestration volume capacity and iodine content of the liposomes, indicating that the interlamellar spacing within the vesicles depended on the extent of bilayer ionization. No significant difference with respect to the various properties (Table II) could be detected between negatively or positively charged liposomes at an ionic compound concentration of ~10 mol%. However, a distinct increase in the encapsulation capacity was observed in negatively charged liposomes, as compared with positively charged liposomes (Table II), at an ionic compound concentration of ~20 mol%. This effect could be attributed to a minimization of the leakage during liposome production due to electrostatic repulsion between the similar negative charges of the lipid membranes and the entrapped ioxitalamate ion (15). This electrostatic repulsion reduced the diffusion of the contrast agent through the bilayer membrane. It is interesting to note that in contrast with previous reports (16), the addition of charged lipid in excess of 10 mol% of the total lipid mixture increased volume entrapment (Table II). Moreover, no marked particle size alteration (Table II) was observed among the various species of liposomes, and enlargement of the space between the lamellae did not contribute to an increase in the final liposome size.

Effect of Initial Phosphatidylcholine Concentration on Liposome Properties—It is a well-accepted fact that MLV possess a low encapsulation capacity as compared with LUV (17). The preparation method previously described yielded mainly liposomes of the MLV type, which were characterized by a large core lamella and adjacent bilayer membranes separated by small aqueous spaces. It could be considered that these resulting MLV have a similar encapsulation ability to LUV since the main part of the sequestered solution volume should be stored inside of the core space. Therefore, any variable experimental factor which could modify the physical characteristics of these MLV liposomes alters their encapsulation capacity. An increase in the phospholipid concentration at a constant ratio of cholesterol-phosphatidylcholine reduced the aqueous volume entrapped per millimole of lipid and, subsequently, the iodine content in the liposomes (Fig. 2). These results indicate that although all the other experimental factors were kept constant, larger phosphatidylcholine concentrations produced liposomes of the MLV type consisting of a larger number of lamellae per single vesicle, even if the sonication time was maintained constant during the preparation process in all the different liposome batches. It appeared that fewer liposomes were

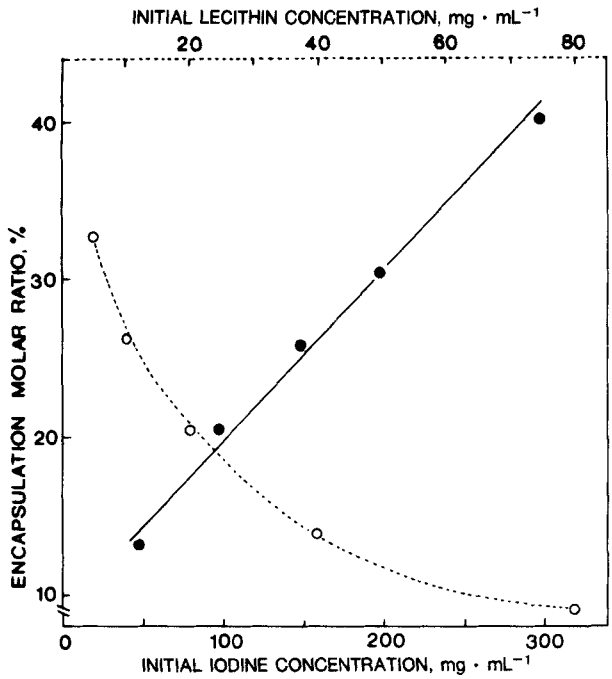

Figure 3—*Effect of initial iodine concentration (●) and initial phosphatidylcholine concentration (○) on the sodium ioxitalamate encapsulation molar ratio.*

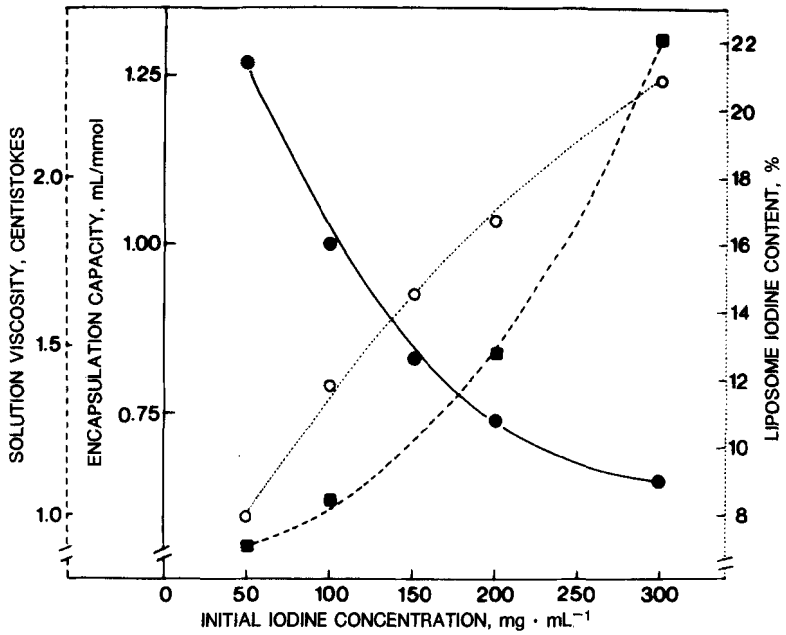

Figure 4—*Effect of initial iodine concentration on encapsulation capacity (●), iodine content of liposomes (○), and viscosity of the aqueous solution (■).*

formed in proportion to the initial amount used, but more membranes were produced per single particle by increasing the initial phosphatidylcholine concentration. Thus, the effective volume entrapped within these liposomes diminished, and since the radiopaque agent concentration remained unchanged, it resulted in a reduction in the iodine content, as seen in Fig. 2. It should be noted that an identical parallel decrease between the encapsulation volume capacity (Fig. 2) and the encapsulation molar ratio (Fig. 3) was observed. The plot obtained yielded a curved line that fell sharply at the beginning and moderately on approaching the highest phosphatidylcholine concentrations (Fig. 3), indicating probable formation of smaller interlamellar spaces within the liposomes and supporting the fact that the polar compound sodium ioxitalamate is found totally in the aqueous compartments of the MLV. No part of it was bound to the lipid membrane, as was reported for some other water-soluble drugs (13).

Effect of Initial Sodium Ioxitalamate (or Iodine Equivalent) Concentration—Dependence of the different liposome properties on the initial radiopaque agent concentration was examined. A plot of the volume encapsulation capacity against the initial iodine concentration yielded a curved line that fell almost linearly initially and curved off at the highest iodine concentration (Fig. 4). Obviously, the iodine content of these liposomes was enhanced, but the iodine encapsulation efficiency decreased with increasing initial contrast agent concentration (Fig. 4); no linear relationship was observed. Since fixed amounts of lipids were used, the decrease in EC was probably caused by other experimental factors which were affected by the initial sodium ioxitalamate concentration. Some of these factors, *e.g.*, the ionic strength or the viscosity of the dispersion medium, might alter the physical characteristics of the vesicles formed (*i.e.*, the size and the number of bilayers per particle). However, in a previous report, it has been shown that no variation in trapped volume within neutral liposomes was observed over a full range of salt concentrations corresponding to an increase in the ionic strength of the aqueous phase (15). Therefore, the explanation should be sought elsewhere. Apparently, the viscosity of the dispersion medium affected the encapsulation ability of the vesicles. Viscosity measurements of the different sodium ioxitalamate solutions were carried out with a viscometer[10] according to the British Pharmacopoeia (18). The relationship of the sodium ioxitalamate concentration effects on encapsulation capacity, iodine content, and viscosity of the aqueous phase are more clearly evident when seen graphically (Fig. 4). The encapsulation capacity function follows an inverse pattern to that of viscosity; it is clearly indicated that the sequestration ability falls off, whereas the viscosity increases drastically with increasing contrast agent concentration. It seems that the liposome entrapment process reflects fundamental changes arising from the viscosity variation of the dispersion medium. The reasons for this are not clear, but it might be due to any or all of the following factors. By raising the viscosity the shape of the vesicles formed is probably altered, thus increasing the proportion of spheroids (oblate or prolate) to spheres. If it is assumed that the surface area of these vesicles remains almost constant, then the volume of these spheroids diminishes as compared with that of the spheres. Consequently, a reduction in volume encapsulation ability should be expected, as was observed experimentally (Fig. 4). This solution is in reasonable agreement with the electron microscopic observations. Other factors which should be considered that account for an encapsulation capacity decrease are the formation of a vesicle structure that is not tightly closed at higher viscosities, enhancement of permeation (*i.e.*, loss) of the trapped ionic compound through the membrane, and a diminution in the hydration extent of the head groups which reduce the electrostatic interactions with increasing medium viscosity, leading to smaller distances between the lamellae within the liposomes. It is interesting to note that the sodium ioxitalamate encapsulation molar ratio increased linearly with the initial equivalent iodine concentration, whereas it declined nonlinearly with the initial phosphatidylcholine concentration (Fig. 3). These results indicate that the radiopaque agent incorporated within the liposomes was not influenced by the moderate lack of volume encapsulation efficiency on approaching the maximal contrast agent concentrations.

Finally, it can be concluded that any variable factor which can affect the vesicle volume encapsulation ability determines the properties of the radiopaque liposomes formed. However, by choice of suitable experimental parameters, full efficiency can be achieved without altering the fundamental characteristics of the liposomes.

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Gastric and Intestinal Absorption of Captopril in Acutely and Chronically Treated Rats: Comparison with Salicylic Acid

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Abstract □ The gastric and intestinal absorption of captopril, an orally active angiotensin-converting enzyme inhibitor was determined using rat *in situ* gastric pouch and intestinal loop techniques and compared with the absorption of another acidic drug, salicylic acid, whose absorption has been well established from both gastric and intestinal sites. Captopril absorption was determined at two initial intraluminal concentrations in acute (untreated) rats and in rats that had been chronically treated with captopril. Salicylic acid absorption was determined at one concentration in acute rats. During the 40-min experimental period, captopril absorption at the 4.6 mM dose from the gastric pouch was 17.0 ± 1.8% and 17.9 ± 5.4% in acute and chronically treated rats, respectively, and 33.6 ± 9.2% and 23.7 ± 7.6%, respectively, from the intestinal loop. At the 11.5 mM dose the captopril absorption in 40 min was 13.7 ± 2.7% and 17.3 ± 4.2% from the gastric pouch of acutely and chronically treated rats, respectively, and 17.8 ± 4.2% and 22.9 ± 3.3%, respectively, from the intestinal loop. As similar fractions of the different administered doses were absorbed from the respective gastric and intestinal sites in both acutely and chronically treated rats, the absorption process of captopril appears to be principally by passive diffusion and unaffected by chronic administration of captopril. In comparison, salicylic acid was absorbed more rapidly and to a greater extent from both the gastric and intestinal preparations. The percent of salicylic acid absorbed into the plasma at the 11.5 mM dose was 44.8 ± 4.4% and 65.3 ± 5.3% from the gastric and intestinal preparations, respectively. It is concluded that gastric absorption of captopril does occur, but its relative importance to intestinal absorption is dependent on the transit time from the gastric to intestinal site.

Keyphrases □ Captopril—*in situ* absorption, comparison with salicylic acid, rats □ Salicylic acid—*in situ* absorption, comparison with captopril, rats

Captopril, 1-[(2*S*)-3-mercapto-2-methylpropionyl]-pyrrolidine-2-carboxylic acid is the first orally effective, antihypertensive drug in a new class of angiotensin-converting enzyme inhibitors. This drug contains a reactive thiol group postulated to be necessary for its binding to the Zn^{2+} of the angiotensin-converting enzyme (1). However, this functional group also reacts to form low molecular weight disulfide conjugates of captopril in tissues such as liver and kidney (2, 3). Captopril also forms covalent links with plasma proteins *via* disulfide linkages with thiol-containing residues (4) and this may explain the extensive tissue binding of captopril (3). Possible significant alterations in the pharmacokinetics of captopril, particularly following chronic treatment, could result from tissue uptake saturation and binding processes.

Several studies on the disposition of captopril in humans and laboratory animals (3, 5–8) have found an early appearance of captopril in the plasma following oral administration with peak drug levels usually occurring within 1 h. As captopril is a structural derivative of the amino acid proline, there exists the possibility that it could be a substrate for an amino acid transport process that rapidly transports captopril across the gut wall. Additionally, the acidic nature of the drug would, according to the pH partition hypothesis (9), allow passive absorption from the highly acidic gastric site prior to entry into the intestinal absorptive sites. Recently, a clinical study with captopril observed much higher blood levels of the drug following chronic treatment than acute captopril administration, suggesting an increase in the rate and extent of absorption and bioavailability (5). However, no studies to date have directly examined whether changes in the absorption rate occur during chronic therapy.

The definition of the sites and processes involved in the absorption of captopril into the systemic circulation has an important place in the description of the disposition of captopril. In this study, the recently described *in situ* gastric pouch and *in situ* intestinal loop preparations (10, 11) have been utilized to investigate captopril absorption at these sites using acute (untreated) rats and rats chronically treated with captopril over 40 d. These techniques offer the advantage of allowing direct determination of drug traversing the gut wall and also enable the quantitation of any metabolites that may be produced that contribute to a "first pass" effect from either the gastric or intestinal sites. For comparison, we have included in the study the acidic drug salicylic acid, which is known to be readily absorbed from both sites (12).

EXPERIMENTAL SECTION

Materials—Captopril[1], captopril disulfide dimer[1], and salicylic acid[2] were used in the experiments. For the measurement of captopril using a gas chromatographic-mass spectrometric (GC–MS) assay, 3-(3-mercapto-2-methyl-propionyl)-4-thiazolidine carboxylic acid[3] (YS-980) was used as an

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