Acacia-Gelatin Microencapsulated Liposomes: Preparation, Stability, and Release of Acetylsalicylic Acid

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Liposomes of dipalmitoylphosphatidylcholine (DPPC) containing acetylsalicylic acid (ASA) have been microencapsulated by acacia-gelatin using the complex coacervation technique as a potential oral drug delivery system. The encapsulation efficiency of ASA was unaltered by the microencapsulation process. The stability of the microencapsulated liposomes in sodium cholate solutions at pH 5.6 was much greater than the corresponding liposomes. The optimum composition and conditions for stability and ASA release were 3.0% acacia-gelatin and a 1- to 2-hr formaldehyde hardening time. Approximately 25% ASA was released in the first 6 hr from microencapsulated liposomes at 23°C and the kinetics followed matrix-controlled release ($Q \propto t^{1/2}$). At 37°C, this increased to 75% released in 30 min followed by a slow constant release, likely due to lowering of the phase transition temperature of DPPC by the acacia-gelatin to near 37°C. At both temperatures, the release from control liposomes was even more rapid. Hardening times of 4 hr and an acacia-gelatin concentration of 5% resulted in a lower stability of liposomes and a faster release of ASA. It is concluded that under appropriate conditions the microencapsulation of liposomes by acacia-gelatin may increase their potential as an oral drug delivery system.

KEY WORDS: acacia-gelatin-microencapsulated liposomes; stabilized liposomes; acetylsalicylic acid release.

INTRODUCTION

The development of liposomes as drug delivery systems is often halted because of poor stability in the biological environment in which they are intended to be administered. The oral route in particular has not been successful because of hydrolytic influences of enzymes and pH and the solubilizing action of the bile salts. Attempts to improve liposome stability have used polymerized liposomes (1), polymer-encased liposomes (2), polymer-coated liposomes (3–6), or microencapsulated liposomes (7–9). In addition, controlled release of protein (10) and sustained release of an ionized drug molecule (6) from these modified liposomes have been demonstrated.

Microencapsulation of liposomes by polymers may have the advantage of stabilizing liposomes against hydrolytic influences in the gastrointestinal tract more efficiently than polymer-coated liposomes because of thicker shells which can be produced. Although synthetic polymers, such as the polyamides (7,8) may be suitable in some circumstances, nontoxic naturally occurring polysaccharides are more appropriate for oral liposomes, with which it is also possible to microencapsulate liposomes without the use of organic solvents.

This paper describes the preparation of microencapsulated liposomes and the effect of this process on the encapsulation of a small ionic solute and its release behavior. In addition, the degree of stability of the microencapsulated liposomes compared to the liposomes themselves in bile salt solutions has been measured.

MATERIALS AND METHODS

Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and gelatin (300 bloom, type A) were obtained from Sigma Chemical Co., St. Louis, MO. Acacia, formaldehyde (Fisher Scientific Co., Fairlawn, NJ), acetylsalicylic acid (ASA), and sodium cholate (Aldrich Chemical Co., Inc., Milwaukee, WI) were used as received. All other chemicals were at least reagent grade and deionized distilled water was used throughout.

Microencapsulated Liposomes by Complex Coacervation

Multilamellar liposomes (MLVs) were prepared by a simple hydration method. Typically, DPPC (34 μmol) and ASA (30 μmol) dissolved in 5 ml of chloroform were dried as a film in a round-bottom flask by rotary evaporation at 50°C, then flushed with N_2 and left overnight in a vacuum oven at 30°C. Subsequently, the film was hydrated with 1 ml of pH 5.6 buffer solution (15 m*M* acetic acid + 157 m*M* sodium acetate) at 50°C and dispersed by vortex-mixing. MLVs (1 ml) were mixed with 0.5 ml of acacia dispersion, then 0.5 ml of gelatin solution, each at the same concentration, over the range 1.5–5% (w/v), then the preparation (2 ml) was adjusted to pH 4.0 using pH 2.5 acetate buffer. The mixture was cooled to 8°C for 10 min to form microencapsulated liposomes, then 37% formaldehyde (1 ml) was added to harden the microcapsule shell. Finally, the excess formaldehyde was removed by centrifugation and washing with pH 5.6 buffer solution at 23°C. At this temperature, the leakage of ASA from the liposomes was insignificant.

Electron Microscopic Studies

Negative Staining. The procedure for negative staining of a sample liposome preparation was as follows: a drop of liposome preparation was applied to a Formvar film-coated copper grid and the excess was removed by filter paper. Phosphotungstic acid solution (1%, pH 7.0) was dropped onto the grid, and after 10 sec it was removed with filter paper. The stained samples were examined in a Hitachi H-7000 electron microscope at 75 KV.

Ultrathin Section Technique. Liposomes were fixed with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer solution at pH 7.5 for 2 hr (11). After rinsing three times with the buffer solution, samples were further fixed with 1% osmium tetroxide in the same buffer solution for 2 hr. After rinsing with water to remove excess osmium tetroxide, 1% uranyl acetate was added and the sample left overnight. Sub-

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sequently, after rinsing with distilled water the samples were dehydrated with successive 10-min washings of acetone solutions (50, 70, 95, and 2 × 100%). This was followed by successive 30-min washings with acetone:Spurr's medium (1:1, v/v; 1:3, v/v), then Spurr's medium (12) (twice for 1 hr). The specimens were placed in flat embedding molds containing Spurr's medium and put in an oven at 70°C to polymerize for 8 hr. Thin sections cut by ultramicrotome (60–90 nm) were placed on 400-mesh copper grids, then poststained with 2% uranyl acetate before electron microscope examination.

Particle Sizing and Wall Thickness Determinations

Twenty microliters of liposome preparations (controls, 34 μmol DPPC + 1 ml pH 5.6 acetate buffer solution) was diluted with pH 4.0 acetate buffer solution (3 ml) and sized by dynamic light scattering (Brookhaven Instruments Particle Sizer, Model BI-90) using a protocol of 2500 cycles/run, five runs for each of triplicate samples at a count rate of about 18 kcps. Wall thicknesses of microcapsules were estimated from the differences in mean size between microencapsulated and control liposomes.

Analysis of Encapsulated ASA

Encapsulated ASA was determined by centrifugation (135,000*g* for 10 min, 23°C, Beckman Model L8-55 Ultracentrifuge) of liposomes and subsequent removal of the supernatant. The pellet was then dissolved in chloroform:isopropanol solution (1:4, v/v) and analyzed for ASA by reverse-phase HPLC (Waters, C_{18}, 15 cm, 5-μm Novapak) with spectrophotometric detection at 280 nm. The mobile phase was 20% acetonitrile and 0.1% phosphoric acid at pH 2.7 at a flow rate of 1.5 ml/min. The retention time of ASA was 2.4 min, whereas salicylic acid (SA) had a retention time of 5.2 min under these conditions.

Liposome Stability Studies

A 1-ml aliquot of either control or microencapsulated liposomes (without ASA) of 3.4 μmol/ml of DPPC was mixed with different volumes of 20 m*M* sodium cholate solution buffered to pH 5.6, then diluted to 10 ml with buffer at room temperature. Turbidities were monitored spectrophotometrically at 400 nm (13) (Beckman, Model 25). Decreased turbidities were considered indicative of decreased stability (6,13,14). Some studies were also conducted in pH 7.0 buffer solution (50 m*M* NaH_2PO_4, 35 m*M* NaOH).

Drug Release Studies

Microencapsulated liposomes containing 0.24 μmol ASA at pH 5.6 were incubated (Dubnoff Metabolic Shaker) with gentle agitation at either room temperature (23°C) or 37°C. At 20-min intervals 1-ml aliquots were withdrawn and centrifuged. The amount of ASA in the supernatant was analyzed as before. The fraction of ASA released was calculated from

$$\% \text{ ASA released} = (C - C_o)/(C_t - C_o) \times 100$$

where C_o is the initial ASA concentration in the aqueous medium before incubation, C_t is the total ASA concentration in the liposome suspension, and C is the ASA concentration in the sample supernatant after time, t.

RESULTS AND DISCUSSION

Microcapsules prepared by coacervation usually employ an organic disperse phase of an oil-in-water emulsion to contain the material to be encapsulated. In the present study MLVs constituted the disperse phase on the surfaces of which the coacervate of acacia and gelatin was deposited. Evidence of this is seen in Figs. 1 and 2. In Fig. 1 electron micrographs of a negatively stained DPPC liposome are shown before (A) and after (B) being microencapsulated by a shell of acacia and gelatin coacervate. It is apparent that the microcapsule is nonspherical, with uneven contours on its surface. Figure 2 provides additional evidence from the ultrathin section technique that in the interior of the microcapsules the multilamellar structure of the liposomes has been retained. Particle size data (Table I) also show a larger size of microencapsulated liposomes compared to control liposomes and an apparent increase in size of the microencapsulated liposomes as a function of the acacia-gelatin concentration, suggesting that the polymers had formed an outer polymeric shell surrounding the liposomes. The average shell thickness was estimated to vary from 0.1 to 0.4 μm at 1.5 to 3 or 5% initial acacia-gelatin concentration, which is reasonable with respect to a particle size of 3.0 to 3.5 μm (15). A possible explanation for the lack of a size increase at 5% acacia-gelatin will follow below within the context of liposome stability and ASA release studies. It was also observed that the particle sizes were not influenced by the formaldehyde hardening times of 1 to 4 hr. Since the action of formaldehyde as a hardening agent is relatively slow, cross-linking of the polymers on the liposome surfaces is likely to be incomplete in less than 1 hr (15).

Evaluation of Liposome Stability

As shown previously and confirmed here, turbidity changes occurring in the liposome preparations are proportional to the number of liposomes, and in this case microencapsulated liposomes, after irradiation at 400 nm (13). The sodium cholate reduces the number of liposomes by solubilization without itself interfering with the turbidity measurement. Zero turbidity resulted after complete disruption of liposomes. The relative stabilities of microencapsulated and control liposomes at pH 5.6 and 7.0 are depicted in Fig. 3. The liposomes are shown to be fairly stable in sodium cholate solutions at pH 7.0, hence no difference could be seen between microencapsulated and control liposomes. In contrast, control liposomes rapidly disintegrated upon the addition of cholate solutions at pH 5.6, whereas microencapsulated liposomes were quite resistant, only about 30% reduction in the turbidity occurring with concentrations as high as 8 m*M* sodium cholate. At pH 7.0, cholic acid is mainly in the ionized state, whereas at pH 5.6 more nonionized cholic acid exists (16–18), suggesting that the incidence of mixed micelle formation of phospholipid and cholate over this concentration range is strongly influenced by the fraction of nonionized cholic acid in the system (14). The detergent activity of cholate is influenced by the ionic environment (18) and has been reported to be greater at pH 5.6 due

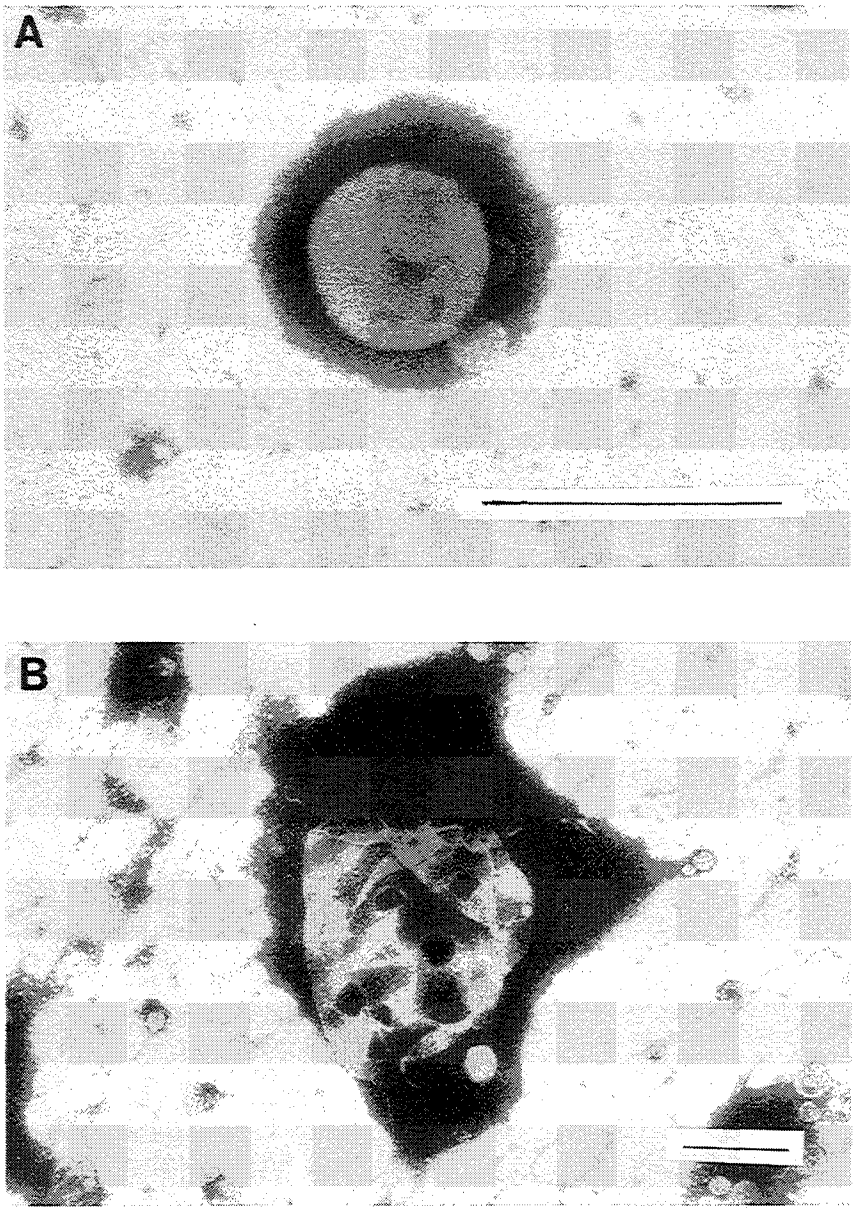

Fig. 1. Negative stain electron micrographs showing the microstructures of (A) control liposomes and (B) 3% acacia-gelatin-microencapsulated DPPC liposomes. Bar = 1 μm.

to protonation, which can be near the pH in the duodenum where bile salts are excreted into the gastrointestinal (GI) tract (19), than at pH 7.0 (14,20). Hence, microencapsulated liposomes could have a distinct advantage in this situation.

When the concentration of acacia-gelatin is increased, the coating thickness and, hence, the stability in cholate solution might be expected to increase accordingly. Figure 4 shows these results at pH 5.6 and 23°C. Not unexpectedly, there exists a fairly narrow range of concentration over which microencapsulation is able to occur and the wall thickness reaches a maximum. Thus, at 1.5% acacia-gelatin no increase in stability of the liposomes was found, indicating insufficient polymer coacervate to form a uniform layer around the liposomes. At the other extreme, 5% acacia-gelatin at a hardening time of 1 hr yielded microcapsule wall properties that were more susceptible to cholate than at 3%, which yielded the greatest stability. Since the particle size also did not increase at 5%, it appears that the excess polymer penetrated the liposomal bilayers, causing a reduction in the overall integrity of the liposomes leading to a lower energy requirement of the cholate to mix with the phospholipid molecules and cause partial solubilization, i.e., a reduction in turbidity, compared to liposomes microencapsulated with 3% acacia-gelatin. A similar observation has been made previously with other polysaccharide-coated liposomes (6). Another factor affecting the formation and coherent structure of the microcapsule wall is the formaldehyde hardening time. Indeed, it was found that 1 hr of hardening time was sufficient prior to harvesting the microcapsules, and a hardening time of 2 hr made no significant difference (results not shown). On the other hand, there appeared to be a deterioration in the microcapsule wall if it was left to harden for 4 hr

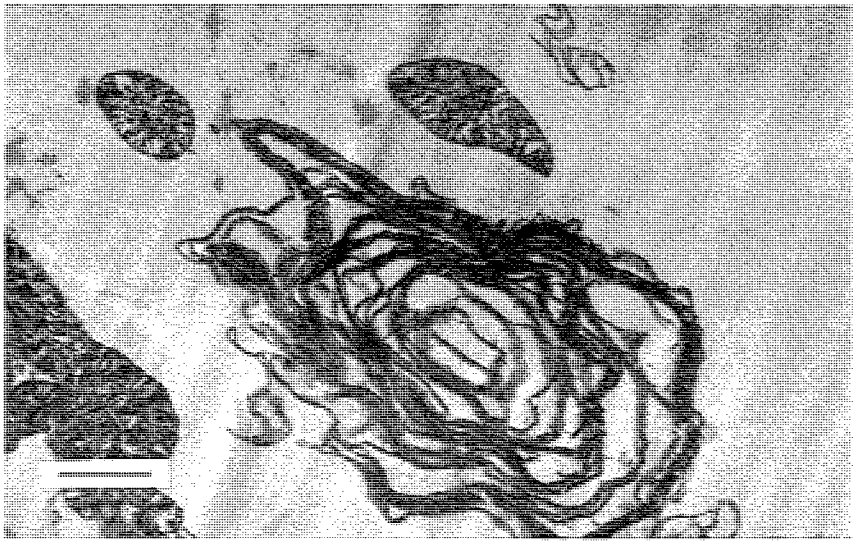

Fig. 2. Electron micrograph of acacia-gelatin-microencapsulated DPPC liposomes by fixation and thin section technique. Bar = 1 μm.

since the turbidity decreased significantly at 3 m*M* cholate compared to 1 or 2 hr of hardening time. Although the reason for this is unclear, the prolonged presence of the formaldehyde may have influenced the integrity of the microcapsule wall through denaturation and cracking (15).

Effect of Microencapsulation on the Release of ASA from Liposomes

Although the encapsulation efficiency of ASA in liposomes was relatively low at 8.3 ± 1.3% (n = 3) using simple hydration techniques, the levels were found to be unchanged after the microencapsulation process. The influence of acacia-gelatin concentration and temperature on the release of ASA from microencapsulated liposomes is shown in Figs. 5 and 6. In each case, sink conditions prevailed. There was a dramatic difference in the amount of ASA released from control liposomes within the first 20 min at 23 compared to 37°C, less than 5 and approximately 90%, respectively. Nevertheless, concentrations of acacia-gelatin in microencapsulated liposomes reduced the extent of ASA released. Even at 1.5% acacia-gelatin, some reduction in the release of ASA was observed, although at this concentration it had been shown that no increase in particle size or stability of the liposomes occurred. However, at 3.0 or 5.0% acacia-gelatin the release of ASA during the initial phase was diminished the most, about a 40% improvement after 400 min, and was sustained with kinetics which were approximately equal for all formulations, including the control liposomes. These results at 23°C (Fig. 7) fit the equation (21),

$$F = kt^{1/2}$$

representing the fraction (F) of ASA released as a linear function of $t^{1/2}$ with a slope k = 1.36 and a correlation coefficient r of 0.987 after a lag time of about 5 min. This release pattern kinetically resembles that found for progesterone from a monolithic hydrogel device (21). At 37°C, 70% of ASA was released after about 40 min from 3% acacia-gelatin-microencapsulated liposomes (Fig. 7), about a 25% improvement over the control liposomes. Thereafter, the remainder was slowly released. Compared to control liposomes at 23°C, which also obeyed $t^{1/2}$ kinetics, the slower release of ASA from microencapsulated liposomes appears to be due to the necessity of diffusion through two barriers: the liposomal bilayers, then the microcapsule wall. This mechanism has even been suggested as a means of delivering a drug by

Table I. Particle Size Range and Wall Thickness of Acacia-Gelatin-Microencapsulated Liposomes as a Function of Acacia-Gelatin Concentration with a Hardening Time of 1-hr

Acacia-gelatin concentration (%)	Average diameter, μm (SD)	Diameter range, μm (P = 0.1)	Wall thickness μm (SD)
0	2.76 (0.16)	1.25–4.69	
1.5	2.96 (0.23)	0.92–5.74	0.10 (0.12)
3.0	3.53 (0.13)	1.08–6.86	0.39 (0.07)
5.0	3.50 (0.21)	0.97–6.98	0.37 (0.11)

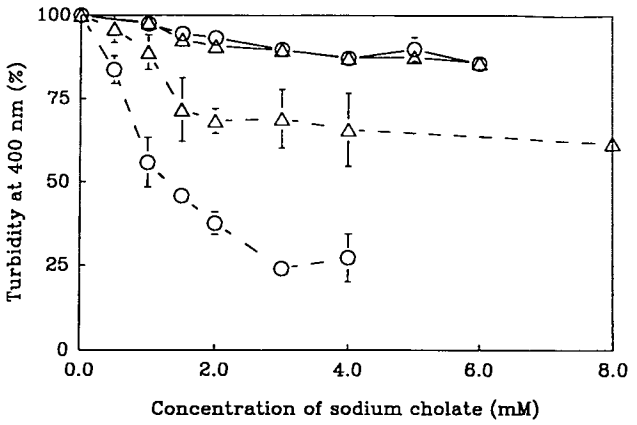

Fig. 3. Physical stability of liposomes at 23°C as a function of pH. (○) Control liposomes; (△) 3.0% acacia-gelatin-microencapsulated DPPC liposomes at pH 7.0 (——) and pH 5.6 (---).

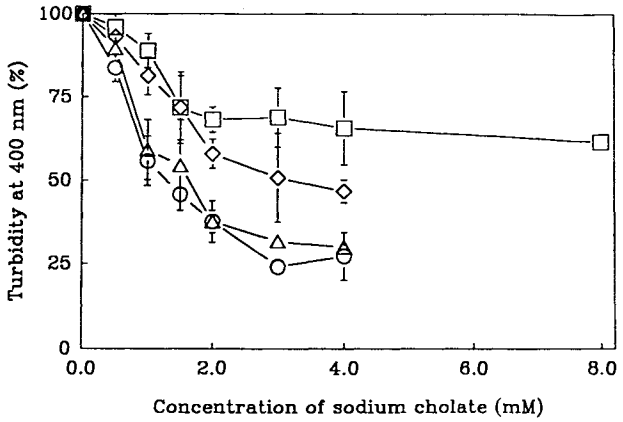

Fig. 4. The effect of acacia-gelatin concentration on the physical stability of microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. (○) Control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of (△) 1.5%, (□) 3.0% and (◇) 5.0%.

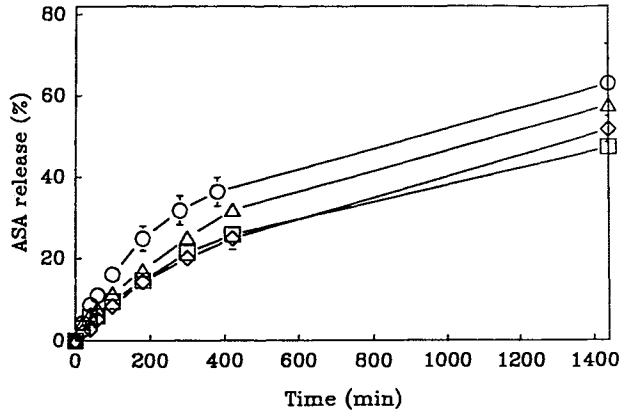

Fig. 5. The effect of acacia-gelatin concentration on the release of ASA from microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. (○) Control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of (△) 1.5%, (□) 3.0%, and (◇) 5.0%.

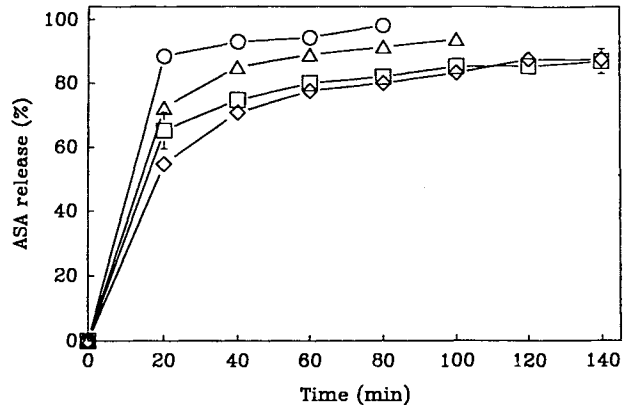

Fig. 6. The effect of acacia-gelatin concentration on the release of ASA from microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 37°C. (○) Control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of (△) 1.5%, (□) 3.0%, and (◇) 5.0%.

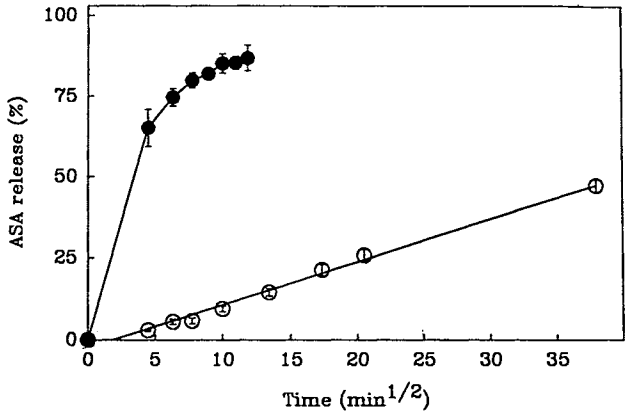

Fig. 7. Release of ASA as a function of $t^{1/2}$ from 3% acacia-gelatin-microencapsulated DPPC liposomes at 23°C (○) and 37°C (●) at pH 5.6.

pulsed release (9). However, the results at 37°C suggest that the marked increase in permeability to ASA may be due to a lowering of the phase transition temperature (T_c) of DPPC from 41 to near 37°C. Liposomes normally exhibit maximum permeabilities to solutes at the T_c (22). Thus, the size data, stability results in cholate solutions, and ASA release behavior of microencapsulated liposomes indicate that the acacia-gelatin increases the integrity and decreases the permeability of gel phase liposomes by means of a partial penetration and interaction with the bilayers. This apparently is limited to polymer concentrations not much greater than 3% (Figs. 4 and 5) and a formaldehyde hardening time of not much longer than 2 hr. The presence of formaldehyde had no effect on ASA stability, as revealed by analysis. Otherwise, at acacia-gelatin concentrations greater than 3% and formaldehyde hardening times greater than 2 hr, the degree of interaction with the bilayers causes instability and increased permeability of the liposomes but without a significant consequence to the size of the particles.

CONCLUSIONS

The microencapsulation of DPPC liposomes using acacia-gelatin and a coacervation technique yielded irregularly shaped particles ranging in size from 1 to 7 μm in diameter. The stability of the microcapsules in sodium cholate solutions was much greater than the corresponding liposomes. The encapsulation efficiency of ASA was unaltered by the microencapsulation process. It is concluded that the formation of an acacia-gelatin microcapsule wall around liposomes produced effects similar to those found previously with polymer-coated liposomes (6). Thus, microencapsulated liposomes represent another formulation approach which may be considered as a possible means to deliver drugs by the oral route.

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