BIOPHYSICS AND BIOCHEMISTRY

Incorporation of Isoniazid into Liposomes with Different Lipid Composition

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Liposomes containing isoniazid were obtained by extrusion of multilamellar phospholipid vesicles containing isoniazid, by ultrasound treatment, and from proliposomes. Optimal conditions for isolation of stable liposomes (200 nm in diameter) and the amount of incorporated isoniazid were determined by varying concentrations of phospholipids and isoniazid and by changing phospholipid composition.

Key Words: *phospholipids; liposomes; isoniazid; gel filtration*

Isoniazid (IN) possessing high bacteriostatic activity in relation to *Mycobacterium tuberculosis* and serves as the main antituberculosis drug [3]. Unfortunately, this preparation is rapidly excreted from the organism. It necessitates treatment with IN in high doses producing toxic effect. Attempts were made to develop new medicinal forms of IN producing prolonged effect, possessing high efficacy, and inducing not toxic reactions. They include multilamellar vesicles containing IN [2], liposomal IN [7], conjugates of IN with natural and synthetic polymers (*e.g.*, dextran [6] and carboxymethyl Sephadex [5]), and nanoparticles with conjugated IN [1]. These medicinal preparations differ from traditional pharmaceutics in the ability to interact with macrophages via endocytosis [9]. It is of particular importance during the therapy of patients with tuberculosis, since mycobacteria can divide in infected cells.

Here we optimized the conditions for isolation of liposomes with IN and evaluated their characteristics (size, stability, and degree of IN incorporation).

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MATERIALS AND METHODS

Experiments were performed with the following preparations: IN (Sigma-Aldrich); soybean phospholipids (Lipoid S-75, Lipoid) containing 90% phospholipids (73% phosphatidylcholine, 10% phosphatidylethanolamine, 3% lysophosphatidylcholine, 3% triglycerides, and 0.5% free fatty acids); cholesterol (Merk); soybean phosphatidylcholine (98% purity, Lipoid); soybean lysophosphatidylcholine (Lipoid); bovine cardiolipin (Biolek); and palmitic acid (Merck).

All solvents were "chemically pure" grade (Khimmed).

Liposomes and IN were obtained by extrusion of vesicles, ultrasound treatment, and by the method of proliposomes.

Weighted samples of phospholipids (10-60 mg/ml) were dissolved in ethanol and stripped to the formation of a lipid film. The film was dispersed in physiological saline (0.89% aqueous solution of NaCl) containing 0.1-200.0 mg/ml IN. The mixture was repeatedly frozen, defrosted, and shaken to obtain homogenous emulsion. The emulsion of multilamellar vesicles was forced through nuclear filters with a pore size of 200 (Tenzor) or 100 nm (Avestin Inc.) using a

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LiposoFast Basic extruder (Avestin Inc.). Liposomes were stored in a refrigerator at 4°C for several months.

The emulsion of multilamellar vesicles was sonicated on an UZDN-21 ultrasound generator at 22.7 kHz and 20 W for 15 min under cooling.

The solution of phospholipids (2 ml) in a 3:1 alcohol-glycerol mixture was added to 18 ml aqueous IN (10-100 mg/ml) under intensive agitation [11]. The final concentration of phospholipids was 10-60 mg/ml.

Optical density of 1% liposome solution was measured on a Beckman DU-6 spectrophotometer at 400-780 nm. The size of liposomes was calculated as described elsewhere [4].

Liposomes (200 μl) were applied to a column (height 150 mm, diameter 10 mm) packed with Sephadex G-25 Fine (Pharmacia) and 3-ml fractions were collected. Opacity of each fraction was determined at 400-780 nm to confirm the presence of liposomes. Fractions were disintegrated by adding 0.5 ml 5% deoxycholate per 3 ml liposomes. The content of IN in fractions was estimated by optical density at 264 nm (IN absorption maximum).

RESULTS

The emulsion of multilamellar vesicles composed of phospholipids and IN was extruded through a nuclear filter. We obtained free IN and unilamellar vesicles (liposomes) containing and not containing IN. The liposomal fraction was separated from free IN by the method of gel filtration. The degree of IN incorporation into liposomes was calculated as the difference between IN concentrations in the fraction of liposomes and initial preparation and expressed in percents.

The lipid composition of liposomes and the amount of IN or phospholipids were varied to optimize the conditions for maximum incorporation of IN into liposomes. Phosphatidylcholine was the major component used to obtain liposomes. Cholesterol or other phospholipids were added. The percent of IN incorporation into liposomes depended on phospholipid composition. The maximum incorporation was observed in liposomes composed of phosphatidylcholine or phosphatidylcholine-cholesterol (10:1) mixture (Fig. 1). The degree of IN incorporation was lower after the addition of zwitterions (phosphatidylethanolamine and lysophosphatidylcholine) or negatively charged lipids (cardiolipin and palmitic acid) to phosphatidylcholine.

The dependence of IN incorporation on its concentration was studied in liposomes containing phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine (7:1:0.3, 40 mg/ml). IN incorporation into liposomes increased with increasing its concentration in the medium and reached a plateau at a concentration of 100 mg/ml (Fig. 2). Liposomes

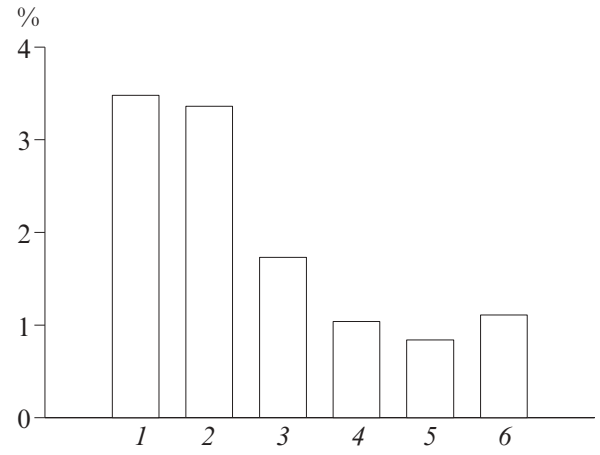

Fig. 1. Incorporation of isoniazid (IN, 60 mg/ml) into liposomes of different phospholipid composition (40 mg/ml). Phosphatidylcholine and cholesterol (7:3, *1*); phosphatidylcholine (*2*); phosphatidylcholine and cardiolipin (10:1, *3*); phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine (7:1:0.3, *4*); phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and cardiolipin (7:1:0.3:0.83, *5*); phosphatidylcholine and palmitic acid (8:1, *6*).

were stable under these conditions. At higher concentrations IN was precipitated.

For evaluation of the dependence of IN incorporation into liposomes on phospholipid concentration was studied in the mixture of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine in the 7:1:0.3 ratio. The amount of IN incorporated into liposomes progressively increased with increasing the amount of phospholipids (Fig. 3).

In further experiments we studied IN incorporation into liposomes obtained by ultrasound treatment of the emulsion of multilamellar vesicles and technique of proliposomes [1]. Analysis of liposome frac-

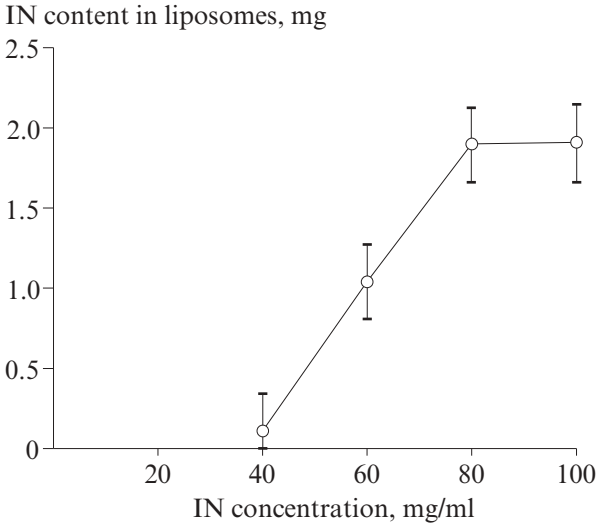

Fig. 2. IN incorporation into liposomes of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine (7:1:0.3, 40 mg/ml) as a function of its concentration.

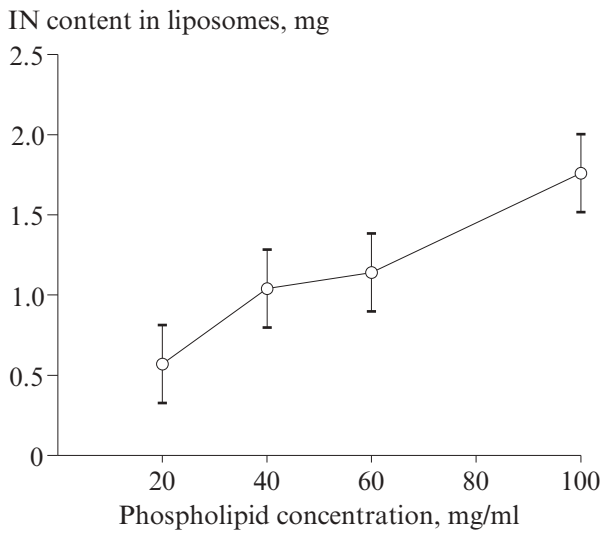

Fig. 3. IN incorporation into liposomes of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine (ratio 7:1:0.3, 40 mg/ml) as a function of phospholipid concentration.

tions containing IN (purified from free IN by gel filtration) showed that IN incorporation did not exceed 2%. Low incorporation of IN was probably related to high membrane permeability for IN and its rapid release from liposomes during gel filtration.

The mean diameter of liposomes was estimated by the method of turbidimetry. The diameter of liposomes obtained by extrusion did not depend on phospholipid composition and was equal to 180-220 nm. Liposomes obtained by the method of proliposomes were larger (1 μ). Our results are consistent with the calculated value [10]. Study by the method of dynamic light scattering showed that optical density of liposome solution depends on their size.

The size of liposomes did not change during storage for several months. The only exception was liposomes composed of phosphatidylcholine and lysophosphatidylcholine (10:1 ratio). During storage these liposomes formed 2.5-μ particles.

In our work the degree of IN incorporation into liposomes was much lower compared to that observed in experiments with other colloid forms (nanoparticles) [1]. We optimized the conditions for isolation of stable liposomes with IN. Bacteriostatic activity of liposomes in relation to *Mycobacterium tuberculosis* should be evaluated in further studies.

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