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LATENT CREATINE PHOSPHOKINASE ACTIVITY IN THE LIPOSOMES

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To study the conditions for preparing liposomes with a high internal protein content the uptake of creatine phosphokinase by the liposomes was studied quantitatively under different experimental conditions. Addition of 20% of a positively charged lipid (stearylamine) to the lipid mixture followed by ultrasonic treatment of the liposomes for 1 min gave the highest levels of latent activity of the enzyme. Maximal uptake (6.1%) was obtained by the use of a 300 mM glucose solution, pH 7.0, containing 0.5 mg protein/ml, as the aqueous phase. Washing the preparations of liposomes with physiological saline at 205,000g gave minimal external enzyme activity. When the liposomes were kept for 7 days under argon protection at 4°C there was no decrease in the internal protein content and no increase in external activity.
KEY WORDS: liposomes; creatine phosphokinase.

One way of correcting inborn errors of metabolism is by replacement enzyme therapy. It has been shown that cells can take up proteins in vitro in cultures of mutant fibroblasts [2]. After parenteral administration of purified enzymes to patients a sharp increase in their activity has been found in the liver and cerebrospinal fluid [4, 8]. The absence of a clinical effect following treatment of this sort is attributed to the slow rate of penetration of proteins through the plasma membrane of the cells and to the development of hyperthermic crises and immunological reactions during repeated administration of the enzymes [3].

Recently published investigations have shown that these disadvantages can be overcome by protecting the injected proteins with artificial lipid membranes [10]. These structures, which have been called liposomes [13], are vesicles, surrounded by multilayered membranes, formed by the swelling of phospholipids in an aqueous medium. If the procedure is carried out in a solution of proteins, these can be incorporated into the interlamellar spaces of the liposomes [10].

This paper gives the results of an investigation into the conditions for preparing liposomes with a high internal protein content and minimal external activity, their purification from unbound protein, verification of the integrity of the liposomes, and their keeping properties.

EXPERIMENTAL METHOD

Creatine phosphokinase (CPK; Olaine factory, USSR) was used as the test system. This protein has high stability when kept in solution. Phosphatidylcholine (PCh) was obtained from phospholipids of egg yolks [9].

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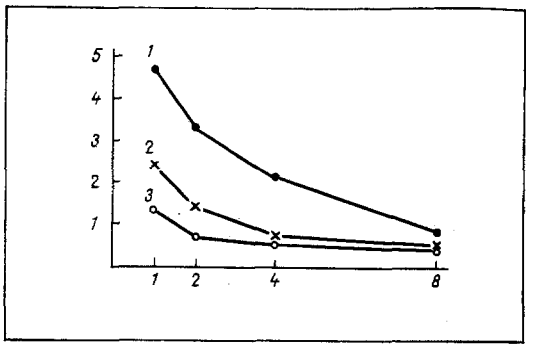

Fig. 1. Latent CPK activity in liposomes as a function ultrasonic treatment. Abscissa, duration of sonication of liposomes (in min); ordinate, percentage uptake of protein by liposomes (latent activity) after washing twice by centrifugation at 140,000g for 1 h. 1) Liposomes made up in 300 mM glucose; 2) liposomes made up in 12 mM glucose; 3) liposomes made up in 3.3 mM phosphate buffer. All solutions contained 0.5 mg CPK/ml, pH 7.0.

TABLE 1. Dependence of Creatine Phosphokinase Uptake into Liposomes on Their Stearylamine Content

Stearylamine content, %	Quantity of CPK used for preparation of liposomes, mg	Latent activity after second washing		Free activity			
				first washing		second washing	
		%	μg	%	μg	%	μg
0	1,75	0,016	0,28	0	0	—	—
5	1,75	2,94	51,45	0,6	10	—	—
10	1,75	4,57	80,00	0,8	14	0	0
15	1,75	5,71	99,92	0,8	14	0	0
20	1,75	6,10	106,70	0,6	10	0,2	3,5

Legend. Aqueous phase contained 0.5 mg CPK to 1 ml of 300 mM glucose, pH 7.0. Preparations sonicated for 1 min. Percentages of latent and free activity calculated relative to quantity of protein used for preparing liposomes.

The phospholipids (5.0 g) were applied to an alumina column (2.5 × 30 cm) at the rate of 1 g to 25 g of sorbent. Elution was carried out with a mixture of chloroform and methanol (9:1) at the rate of 10 ml/min [11]. Subsequent purification of the PCh to remove traces of sphingomyelin was carried out on a column (1.5 × 30 cm) using silica gel (granule size 100-250 μ, Chemapol, Czechoslovakia), previously activated at 110°C for 16 h [6, 7], as the sorbent. The fractions obtained on elution from both columns were analyzed by unidimensional thin-layer chromatography (TLC) on mark KSK silica gel (granule size 15-30 μ). The resulting PCh gave one spot on two-dimensional TLC using systems of chloroform–methanol–water (65 : 25 : 4) and chloroform–methanol–7 N NaOH (70 : 35 : 5). As far as possible all procedures were carried out under argon protection and the PCh also were kept under argon protection at −20°C. Lipid phosphorus was determined by ignition with perchloric acid [12]. Cholesterol was recrystallized, after which no impurities were found on TLC in a system of benzene–diethyl ether–ethanol–acetic acid (50 : 40 : 2 : 0.2). Stearylamine (Eastman, USA) was used as the positively charged lipid and its purity was verified from its melting point.

To prepare liposomes, the PCh, stearylamine, and cholesterol in chloroform in molar proportions of 7 : 2 : 1 [10] were poured into a round-bottomed flask and the solvent evaporated to dryness on a rotary vaporizer in order to obtain a thin lipid film. The flask was kept for 1 h under a pressure of 0.5 mm Hg to remove traces of chloroform. The lipid film was emulsified with solutions of CPK of different concentrations containing glucose or phosphate buffer. The aqueous phase was transferred to a flask which was quickly shaken at 37°C. Emulsification was then continued on a magnetic mixer for 15-30 min and the completeness of emulsification of the lipid was verified visually. The suspension was kept for 2 h at 22°C and sonicated at 22 kHz on

an ultrasonic disintegrator with UZDN-1 (USSR) titanium rod under argon protection at 4°C. After incubation for 2 h at 22°C the liposomes were centrifuged for 10 min at 10,000g to remove titanium particles [1]. The liposomes were then washed to remove unbound protein with physiological saline during centrifugation at 140,000 and 205,000g on the BAK-601 (East Germany) centrifuge, and the residue was resuspended in 1 ml of 0.14 M NaCl. The liposomes were kept under argon protection at 4°C. Activity of CPK in the washed liposomes was investigated by means of test systems (Boehringer, West Germany). Latent activity was determined 3 min after the addition of 0.2 ml of 1% Triton X-100 in 0.14 M NaCl to the liposomes and free activity was determined with 0.2 ml 0.14 M NaCl. The protein content of the liposomes was calculated from the specific activity of a standard solution of CPK. The increase in optical density at 340 nm after the addition of 1 μg CPK in 0.1 ml 0.14 M NaCl to the incubation medium was 0.05 optical density unit after 1 min. Treatment of the CPK solution with Triton X-100 in the same concentration did not change the enzyme activity. The lipid content in the preparation of liposomes was estimated from the absorption at 410 nm in a cuvette with a working layer 3 mm thick, in 0.14 M NaCl [10]. The absorption curve at 410 nm was calibrated for content of lipid phosphorus [10, 12].

EXPERIMENTAL RESULTS

Preliminary experiments showed that the optimal proportions of lipids and aqueous phase for producing a good emulsion (without clumping) was 10 mg/ml. The maximal protein concentration in the solution to satisfy these conditions was 0.5 mg/ml when tested in concentrations of between 0.1 and 5 mg/ml of aqueous phase. The completeness of emulsification of the lipids in these experiments was estimated from their yield in the final preparation.

The results of experiments to determine the latent activity of CPK in the liposomes depending on the duration of sonication and the composition of the aqueous phase are shown in Fig. 1. The emulsifying solution contained 0.5 mg protein to 1 ml of the following solutions: 300 mM glucose solution, 12 mM glucose solution, or 3.3 mM phosphate buffer, and the pH of all mixtures was 7.0. The greatest latent activity of CPK in the liposomes was found when a mixture in 300 mM glucose solution was used. Previously [5] a 3.3 mM phosphate buffer, pH 7.0, was used to prepare liposomes containing protein. The smallest values of latent activity were obtained in such a solution. The highest uptake of protein by liposomes in all the solutions tested was observed after sonication of the suspension for 1 min. With an increase in the duration of ultrasound treatment the internal protein content fell sharply. Evidently the difference in latent activity of protein in liposomes prepared with solutions of different ionic strengths was due to changes in the volume of the aqueous phase of the interlamellar spaces of the liposomes, and shortening the sonication treatment leads to an increase in the size and the number of layers of the vesicles. In the series of experiments described above washing twice with physiological saline at 140,000g in a fixed-angle rotor (8 × 35) of the BAK-601 centrifuge for 1 h was used. Under these circumstances liposome material could still be detected in the supernatant. Accordingly, the washing was subsequently carried out twice at 205,000g in a fixed-angle rotor (8 × 11) of the same centrifuge for 1.5 h each time. This led to a sharp decrease in the loss of liposomes.

In the next series of experiments (Table 1) the quantitative uptake of protein by the liposomes was studied in relation to their content of positively charged lipid. In the absence of stearylamine the uptake of protein was found to be small. The increase in latent activity was proportional to the increase in the stearylamine content in the lipid mixture up to 15 mole %, and a further increase in content caused only slight activation of protein uptake. The effect of a sharp increase in the protein content in the liposomes on the addition of stearylamine is connected with an increase in the distance between lamellae of the vesicles [9, 10, 13].

Investigation of the external CPK activity in the preparations, i.e., activity detectable without the addition of Triton X-100, showed that after the first washing it was readily detectable (Table 1). However, it was much lower than that found by Sessa and Weissman [10], who separated liposomes from unbound protein with lysozyme by column chromatography on Sephadex G-75. After the second washing, external activity in the liposomes was sharply reduced.

Phase-contrast microscopy of the liposome population showed that even after sonification for 1 min the main mass of the vesicles was of submicroscopic size and only a small part of the population measured above 1 μ.

Investigation of CPK activity in the liposomes for 7 days during keeping at 4°C under argon protection revealed neither a decrease in the internal protein content nor an increase in free activity. Since CPK is stable in aqueous solutions for a long period, this is evidence that the integrity of the liposome membranes was good.

It should be noted in conclusion that the establishment of conditions for the preparation of liposomes with sufficiently high latent activity but close to zero free activity is the first step toward the development of transport measures capable of supplying enzymes to the tissues of patients with hereditary diseases.

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ACTIVATION OF RESPIRATION OF LIVER MITOCHONDRIA BY CATECHOLAMINES

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Catecholamines activate respiration (V_{O_2}) of rat liver mitochondria in vivo and in vitro on high concentrations of pyruvate, 2-oxoglutarate, and succinate. The effect is characteristic equally of free oxidation (state 4) and phosphorylating (state 3) or even dinitrophenol-uncoupled V_{O_2}. The addition of EDTA and bovine serum albumin did not abolish the effect. Activation of oxidoreductases by catecholamines is postulated.

KEY WORDS: catecholamines; tissue respiration; mitochondria.

Catecholamines (CA) increase the oxygen consumption (V_{O_2}) of adipose and muscular tissue [8-10]. The data for the liver are more contradictory [4, 8-10], which accords ill with the generally accepted universality of the metabolic effects of CA [10]. A major role in the mechanism of the calorigenic effect (CE) of CA is ascribed to the simple accumulation of oxidation substrates as a result of activation of glycogenolysis and lipolysis [7, 9] or to the uncoupling action of the accumulated fatty acids [7, 8, 10] or of calcium [7]. Moreover, these mechanisms are regarded as universal and unique, for the direct action of CA on terminal oxidation processes is denied [7]. This view contradicts the general principle that an important process must be controlled not only by unspecialized regulators such as substrates or allosteric effectors, but also by specialized regulators, more progressive from the evolutionary standpoint, such as hormones and cyclic nucleotides [2].

The object of this investigation was to reveal any possible effects of CA on V_{O_2} of the liver mitochondria and to investigate the mechanism of this effect. Preliminary reports were presented to the 10th and 11th All-Union Symposia on Biochemistry of the Mitochondria [3, 6].

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