Differential binding of vitamin E to sarcoplasmic reticulum from fast and slow muscles of the rabbit[1]

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Summary. The distribution of [^{3}H]-tocopherol between the intracellular membranes of fast white and slow red muscles was studied in vivo. In vitro experiments confirmed a higher incorporation of the vitamin into the reconstructed sarcoplasmic reticulum membranes from slow muscle.

Muscular dystrophy can be induced in the rabbit by a vitamin E-deficient diet. Although the differential sensitivity to vitamin E-deficiency of fast (white) and slow (red) rabbit muscles is well known[3], very few data are available on the content and distribution of tocopherol in the intracellular membranes of the muscles. A study was therefore undertaken to compare the rate of incorporation and the distribution of [^{3}H]-tocopherol into representative fast-white muscles (adductor magnus) and slow-red muscles (soleus and semitendinosus) of normal rabbits.

Material and methods. The fast adductor muscle and the slow soleus and semitendinosus muscles of adult New Zealand male rabbits fed ad libitum with a stock laboratory diet were used. 10% muscle homogenates in 0.3 M sucrose were centrifuged at 650 × g 10 min to sediment nuclei and myofibrils. Mitochondria were isolated by centrifuging at 6500 × g 20 min and were washed once with 0.3 M sucrose. Sarcoplasmic reticulum (SR) was sedimented by centrifuging the mitochondria-free supernatant (15000 × g 20 min) at 150000 × g 60 min, and was further purified on discontinuous sucrose gradient[4]. Partial extraction of the SR fragments at low concentrations of cholate was carried out according to Meissner et al.[5]. Phospholipids were extracted from the SR membranes by the method of Rouser and Fleischer[6]. Protein was determined by the method of Lowry et al.[7], using bovine serum albumin as standard. Radioactivity was measured in a liquid scintillation spectrometer using a toluene-Triton X 100 (2:1) scintillation mixture. D-α [5-methyl-^{3}H]-tocopherol (sp.act. 5.6 Ci/mmole) was purchased from The Radiochemical Centre, Amersham, U.K.

Results and discussion. In table 1 a typical experiment on the fractionation of subcellular membranes from fast and slow muscles of the rabbit is reported. 1 h after administration, the level of the labelled vitamin in the homogenate and in the subcellular fractions is higher in slow muscle than in fast muscle. SR membranes have the highest specific activity in both muscles; a difference from rat liver, where the highest concentration of [^{3}H]-tocopherol was found to be in the mitochondria[8,9]. While the ratio of labelling of mitochondria and soluble fraction from fast and slow muscles was similar to the ratio for the homogenates, a 4 times higher specific activity was detected in the isolated SR from slow muscle, as compared to the corresponding fraction from fast muscle.

To investigate better the binding of [^{3}H]-tocopherol to SR membranes of the 2 types of muscle, purified SR preparations were treated with a low concentration of cholate and 0.5 M KCl[5]. By this procedure loosely-bound proteins and about 50% of the phospholipids are extracted[5]. This treatment results in a 60% decrease of the bound radioactivity (cpm/mg protein) in fast muscle SR and a 170% increase in slow muscle with respect to untreated preparations. Thus, this result suggests that a higher fraction of [^{3}H]-tocopherol is extracted along with loosely-bound protein and/or phospholipids in fast muscle.

A further experiment was performed to investigate the pattern of incorporation in vitro of [^{3}H]-tocopherol into reconstructed SR vesicles. For this purpose, partially delipidated SR membranes from fast and slow muscle were first solubilized with cholate in the presence of [^{3}H]-tocopherol and of natural phospholipids from the homologous type of membrane. The SR vesicles were reconstructed by centrifugation on a discontinuous sucrose gradient[10]. Under these conditions, the reconstructed vesicles from slow muscle were resolved into 2 subfractions, banding at the 8–30% and the 30–50% sucrose interface respectively, whereas a single heavy subfraction was obtained in the case of fast muscle. As shown in table 2 the radioactivity recovered in these fractions, as well as in the pellet, was much higher for the reconstructed SR membranes from slow muscle.

Therefore, both in vivo and in vitro, incorporation studies clearly show that skeletal muscle SR is effective in binding [^{3}H]-tocopherol and that the greater binding ability of SR membranes from slow muscle is dependent on intrinsic membrane characteristics, such as the higher content of phospholipid arachidonic acid and the presence in these membranes of a cythochrome b_5-linked fatty acid desaturase system[11]. These findings, which are in agreement with the suggested correlation between the content of vitamin E and of arachidonic acid in biological membranes[12], might

Table 1. Distribution of α-[^{3}H]-tocopherol into subcellular fractions of rabbit fast and slow muscles

	α-[^{3}H]-tocopherol (dpm/mg protein)	
	Fast muscle	Slow muscle
Homogenate	1530	2859
Mitochondria	3375	4208
SR membranes	9760	43440
Final supernatant	3000	5429

Rabbit fed a stock diet was injected i.v. with 1 mCi/kg of [^{3}H]-tocopherol emulsified in 1.0 ml saline-1% Tween 20, and killed after 1 h. Subcellular fractions from 10% homogenate in 0.3 M sucrose were separated by differential centrifugation[4]. Radioactivity was measured by liquid scintillation spectrometry.

Table 2. Incorporation of α-[^{3}H]-tocopherol into sarcoplasmic reticulum membranes reconstructed after solubilization with cholate

Fraction	% of radioactivity	
	Fast SR	Slow SR
8–30% sucrose interface	6.6	23.5
30–50% sucrose interface	–	10.0
Pellet	2.4	13.5

4 mg of SR membrane protein[4] in 0.05 M K-phosphate, pH 7.6, 5 mM Mg-ATP, 1 M KCl were solubilized with 8 mg of cholate in the presence of 4 mg of SR phospholipids and 10 μCi of [^{3}H]-tocopherol. After incubation at 37 °C for 30 min, the SR membranes were reconstructed by centrifuging at 300,000 × g for 240 min on a discontinuous sucrose gradient[9]. The membrane fractions were washed twice with 0.3 M sucrose 1 mM HEPES, pH 7.5, and radioactivity was counted by liquid scintillation spectrometry.

also suggest a causal relationship between the lower vitamin E content of the SR membranes from fast muscle and the greater susceptibility of these muscles to becoming dystrophic in vitamin E-deficiency.

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Quantification of digoxin by enzyme immunoassay: synthesis of a maleimide derivative of digoxigenin succinate for enzyme coupling

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Summary. We synthesized the m-maleimidobenzoyl derivative of digoxigenin-3-0-succinate (through a p-phenylenediamine bridge) as a hapten derivative directed towards coupling to sulfhydryl groups of β-galactosidase. Prepared enzyme conjugate had about 97% of the enzyme labeled with the hapten derivative while retaining full enzyme activity. The enzyme immunoassay for digoxin we prepared showed a maximum sensitivity of 30 pg per assay (c.v. = 3%) with minimal cross-reaction with digotoxin (3.8%). Our method for hapten conjugation to β-galactosidase is highly efficient and is simple and easily replicated.

Digoxin is used in the treatment of chronic heart diseases. Due to the relatively narrow limits between therapeutic and toxic effects, the accurate determination of plasma or serum digoxin levels is of high importance. The incidence of intoxication is greatly reduced by digoxin blood level monitoring during therapy. The determination of digoxin can be carried out by radioimmunoassay[2-6] and enzyme immunoassay[7]. Employing a highly efficient coupling of a m-maleimidobenzoyl derivative of digoxigenin succinate to the enzyme, we were able to develop an enzyme immunoassay (EIA) for the measurement of digoxin by using β-galactosidase as an enzyme label. We describe here the synthesis of our novel maleimidobendzoyl derivative of digoxigenin succinate and the preparation of an enzyme conjugate for the development of a digoxin EIA.

Materials and methods. Chemicals. Digoxigenin, digoxin and digotoxin were obtained from Sigma Chemical Co., St. Louis, MI 63178. Maleic anhydride, m-aminobenzoic acid and p-phenylenediamine were obtained from Aldrich Chemical Co., St. Louis, MI 63178.

Enzyme. Beta-galactosidase from *E. coli* was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana 46250.

Preparation of digoxigenin-3-0-succinate. Digoxigenin-3-0-succinate was synthesized following the procedure of Oliver et al.[8]. Both 0.7 g of succinic anhydride and 2.0 g of digoxigenin were dissolved in 12 ml of pyridine. The solution was protected from light and allowed to react at room temperature for 3 months. The solution was then poured into 75 ml of cold 2 N H_2SO_4. The solid product was isolated by filtration and washed with cold water. It was then redissolved in 150 ml of chloroform-methanol (2:1). The chloroform-methanol solution was washed once with 25 ml of 1 N H_2SO_4, and 3 times with water. 25 ml of methanol was added after each washing. The organic phase was dried over anhydrous sodium sulfate and taken to dryness on a rotary evaporator. The residue was dissolved in 15 ml of hot ethanol, and hot water was added to turbidity. The solution was allowed to cool to room temperature and then left at 4 °C for 48 h. The resultant crystals were isolated by filtration and washed 3 times with cold ethanol-water (3:2). The final product was a white powder with melting point of 190–197 °C. Esterification at position 3 was confirmed by its immunoreactivity to digoxin antiserum as reported in the results and discussion section.

Production of anti-digoxin antibody. Digoxin-bovine serum albumin (BSA) conjugate was prepared according to the procedure of Smith et al.[11]. 1 mg of the immunogen was dissolved in 0.5 ml of saline and emulsified in an equal volume of complete Freund's adjuvant. Goats were then immunized by i.m. injections 3 times at 2-week intervals and boosted bimonthly thereafter. For booster injection, incomplete adjuvant was used in the preparation of emulsion. The animals were bled 10 days after the 3rd primary injection and after each booster injection.

Preparation of enzyme-hapten conjugate. Conjugation of DSA-MB to β-galactosidase was done by the procedure similar to that described previously[9]. A solution of DSA-MB in THF (0.2 mg/ml, 10 nmoles) was added to 1.5 ml of 0.05 M phosphate buffer (pH 7.0) containing β-galactosidase (0.5 mg, 0.93 nmoles). The mixture was incubated for 2 h at room temperature. Following overnight dialysis in the same phosphate buffer, the mixture was chromatographed on a Sephadex G-25 column (1.5 × 40 cm). The fractions of eluate containing the peak of enzyme activity were used for the eluate containing the peak of enzyme activity were used for the digoxin assay. Beta-galactosidase activity was assayed by the method of Dray et al.[12] using O-nitrophenyl β-D-galactopyranoside as substrate.