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THE EFFECTS OF *n*-ALCOHOLS ON SARCOPLASMIC RETICULUM VESICLES

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SUMMARY

Short, mild treatments of sarcoplasmic reticulum vesicles with aqueous *n*-alcohols from methanol to *n*-heptanol caused an inhibition of calcium uptake and an enhancement of ATPase activity. The *n*-alcohol treatments increased both calcium-dependent (extra) ATPase activity and calcium-independent (basic) ATPase activity of vesicles. The apparent initial reaction rate of ATPase of *n*-alcohol-treated vesicles was about twice that of control vesicles. With increasing number (n) of carbon atoms of the *n*-alcohols, the maximum increment of ATPase activity increased, and both the alcohol concentration (N_{Ca}) required to inhibit calcium uptake by 50% and the alcohol concentration (N_{ATPase}) required to enhance ATPase activity by 50% of the maximum increment of ATPase activity decreased as follows.

$$N_{Ca} = 23.5 \cdot 10^{-0.593n} \text{ M}$$

$$N_{ATPase} = 35.5 \cdot 10^{-0.593n} \text{ M}$$

The ratio, N_{ATPase} to N_{Ca}, was constant for all n values. The apparent free energy of binding of the methylene groups of *n*-alcohols to sarcoplasmic reticulum vesicles was evaluated (-796 cal/mole) and compared with data from the partition of *n*-alcohols in octanol and water (-670 cal/mole). The effects of *n*-alcohols on membrane vesicles are discussed on the basis of these data.

INTRODUCTION

The calcium transport system of sarcoplasmic reticulum membranes is inhibited by a variety of agents and treatments which affect the lipid moiety of the membranes. Inhibition of both calcium uptake and extra ATPase activity is brought about by detergents[1], acetone extraction[2] and phospholipase C digestion[3–6]. On the other hand, mild diethyl ether treatment[7–9], phospholipase A digestion[10,11], unsaturated fatty acids[10,12] or thymol treatment[9] interferes only with calcium storage while extra ATPase remains unimpaired when these agents are applied in small amounts. This effect may be considered as an uncoupling of energy-yielding ATP consumption from energy-requiring calcium translocation[8]. In contrast, aqueous heptane treatment leads to an increase in both calcium uptake and ATPase activity[13]. But exhaustive

Abbreviation: EGTA, ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

extraction of sarcoplasmic reticulum vesicles with dry diethyl ether or dry heptane does not change these activities[13].

Phospholipids are essential for active transport processes of sarcoplasmic reticulum membranes[5,10]. Addition of ultrasonically prepared dispersions of synthetic lecithin[3–5], lysolecithin[4,5] or phosphatidic acid[4] causes reactivation of both ATPase activity and calcium transport in phospholipase C-treated sarcoplasmic reticulum membranes. Besides, the structure of phospholipid bilayers in the membranes seems to play an important role in the active transport of calcium[9]. Diethyl ether and thymol change the membrane structure to such an extent that, upon negative staining, the membrane-stain interactions may be altered[9].

Low concentrations of diethyl ether[8], phospholipase A[10], unsaturated fatty acids[10] or lysolecithin[10] increase the permeability of sarcoplasmic reticulum vesicles to calcium, and dissociate calcium accumulation and extra ATPase activity. Ca^{2+} cannot be accumulated because they immediately flow out through the leaky membranes[8].

Extra ATPase activity is increased by diethyl ether or thymol treatment, but basic ATPase activity is not affected by these treatments[9]. Extra ATPase activity remains unimpaired after phospholipase A treatment[10]. But when splitting products of phospholipids by phospholipase A, unsaturated fatty acids and lysolecithin are removed and extra ATPase activity is abolished[10].

On the other hand, *n*-alcohols from butanol to octanol increase the cation, especially potassium, permeability of black lipid membranes[14] or liposomes[15,16], and produce changes in the structural arrangement of lipid in lipid bilayers[17].

This paper reports that extra ATPase activity is not proportional to calcium uptake by *n*-alcohol treatment of sarcoplasmic reticulum vesicles, and describes the relationship between the biological activities of *n*-alcohol-treated vesicles and the number of carbon atoms of the *n*-alcohols.

MATERIALS AND METHODS

Materials

ATP was purchased from Sigma Chemical Co., U.S.A. $^{45}CaCl_2$ was purchased from the International Chemical and Nuclear Co., U.S.A.

Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit leg and back skeletal muscle by the method of Weber *et al.*[18] with slight modifications[19].

Total ATPase activity was measured in 1.5 ml of reaction mixture containing 0.1 M KCl, 2 mM $MgCl_2$, 40 mM Tris–maleate (pH 6.5), 50 μM $CaCl_2$ and 0.1 mg/ml sarcoplasmic reticulum protein in the presence of various concentrations of the alcohols which are the same concentrations as those at the pretreatment. The reaction was started by the addition of ATP (final concentration 1.25 mM). After incubation for 5–6 min at room temperature, the reaction was stopped with 1.5 ml of 24% trichloroacetic acid. The amount of P_i liberated was determined by the method of Taussky *et al.*[20]. Basic ATPase activity was measured in the presence of 100 μM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) instead of Ca^{2+}. Extra ATPase activity was defined by total ATPase *minus* basic ATPase.

Calcium uptake experiments were carried out using a reaction mixture

containing 0.1 M KCl, 2 mM $MgCl_2$, 50 μM $CaCl_2$, 0.01 μCi/ml $^{45}CaCl_2$, 40 mM Tris–maleate (pH 6.5) and 0.2 mg/ml sarcoplasmic reticulum protein in the presence of various concentrations of the *n*-alcohols. ATP (final concentration 1 mM) was added to the reaction mixture, and after incubation for 30 s at room temperature, 1.5 ml of the mixture was filtered through a Millipore filter (HAWP 0.45 μm). Remaining free ^{45}Ca was removed by passing 5 ml of a washing solution containing 0.1 M KCl, 2 mM $MgCl_2$, 40 mM Tris–maleate (pH 6.5) and 0.1 mM ATP through the filter. By this treatment, sarcoplasmic reticulum containing calcium was retained on the Millipore filter. The Millipore filters were dried and soaked in 10 ml of toluene liquid scintillation counting medium. The flasks were counted in a Hitachi-Horiba liquid scintillation counter.

Protein was determined by the biuret method; calibration was done by nitrogen determination.

Before addition of ATP, sarcoplasmic reticulum vesicles were pretreated with *n*-alcohols for about 30 min at room temperature in a medium containing 0.1 M KCl, 2 mM $MgCl_2$, 50 μM $CaCl_2$ and 40 mM Tris–maleate (pH 6.5).

RESULTS

The effects of n-alcohols on ATPase and calcium uptake

With increasing alcohol concentration, the calcium uptake of *n*-alcohol-treated sarcoplasmic reticulum vesicles began to decrease and eventually was lost

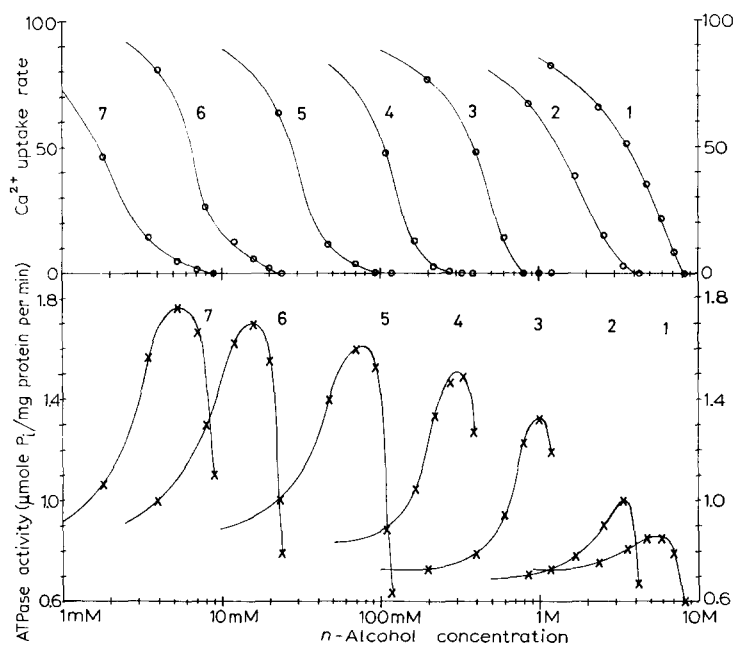

Fig. 1. Effects of *n*-alcohols on calcium uptake ability and ATPase activity of sarcoplasmic reticulum vesicles. ATPase assay was carried out for 5 min by addition of ATP (final concentration 1 mM). ATPase activity and calcium uptake were measured in a medium containing alcohol by the same methods as described in Materials and Methods. ○—○, calcium uptake rate: ×—×, ATPase activity. 100% of calcium uptake rate was that of control vesicles. 1, methanol; 2, ethanol; 3, *n*-propanol; 4, *n*-butanol; 5, *n*-pentanol; 6, hexanol; 7, *n*-heptanol.

completely. However, the ATPase activity of *n*-alcohol-treated vesicles first increased, reached a maximum value and then decreased with increasing alcohol concentration (Fig. 1). These effects became more pronounced and became apparent at lower concentrations of *n*-alcohols with increasing number of carbon atoms in the *n*-alcohols (Fig. 1).

Plots of the logarithm of the alcohol concentrations required to decrease calcium uptake by 50% and to increase ATPase activity by 50% of the maximum increment against the number of carbon atoms of the *n*-alcohols gave two parallel straight lines (Fig. 2). From these straight lines, the empirical formulae giving the alcohol concentration (N_{Ca}) required to decrease calcium uptake by 50% and the alcohol concentration (N_{ATPase}) required to increase ATPase activity by 50% of the maximum increment as a function of the number (n) of carbon atoms of the *n*-alcohols were determined:

$$N_{Ca}=23.5\cdot 10^{-0.593n}\,\mathrm{M} \quad (1)$$

$$N_{ATPase}=35.5\cdot 10^{-0.593n}\,\mathrm{M} \quad (2)$$

The ratio, $N_{ATPase}/N_{Ca}=1.51$ was constant for all n values.

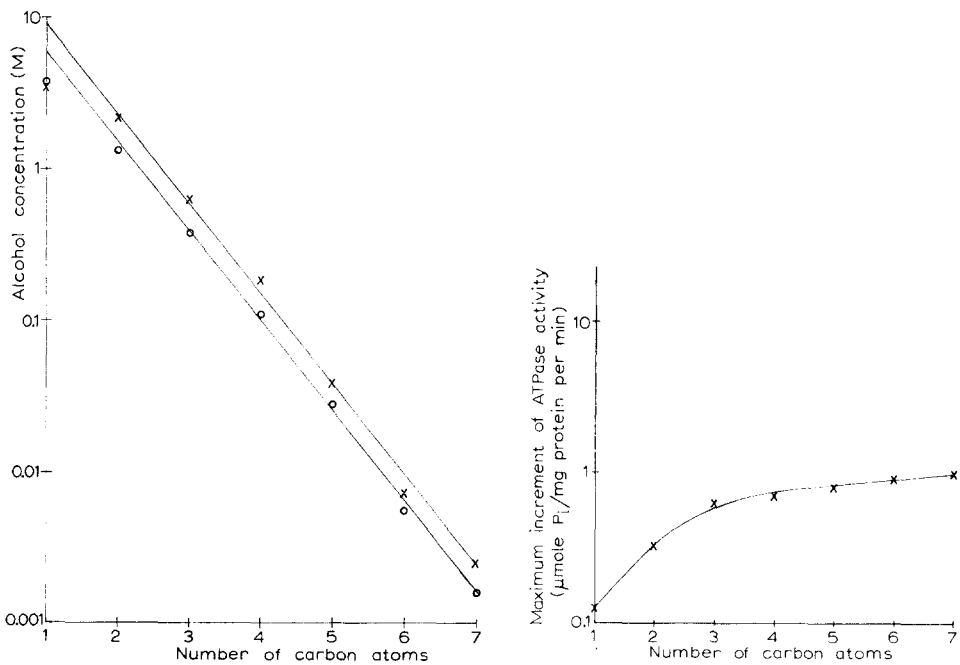

Fig. 2. Alcohol concentrations required to affect ATPase activity and calcium uptake in sarcoplasmic reticulum vesicles against the number of carbon atoms of *n*-alcohols. ×—×, alcohol concentrations required to enhance ATPase activity by 50% of the maximum increment of ATPase activity by *n*-alcohol treatments: ○—○, alcohol concentrations required to inhibit calcium uptake ability by 50%. These values were obtained from Fig. 1.

Fig. 3. Maximum increment of ATPase activity of sarcoplasmic reticulum vesicles by *n*-alcohol treatment against the number of carbon atoms of the *n*-alcohols. The maximum increment of ATPase activity was the difference between control ATPase activity and maximum ATPase activity increased by *n*-alcohol treatments. These values were obtained from Fig. 1.

The maximum increment of ATPase activity produced by *n*-alcohol treatment increased with the number of carbon atoms in the *n*-alcohols, until it reached a constant level (Fig. 3).

Properties of ATPase of n-alcohol-treated vesicles

The time course of ATPase activity of *n*-alcohol-treated sarcoplasmic reticulum vesicles after addition of ATP was similar, irrespective of the kind of *n*-alcohol used. The initial reaction rate of vesicles treated with *n*-alcohols was about twice that of control vesicles (Figs 4 and 5).

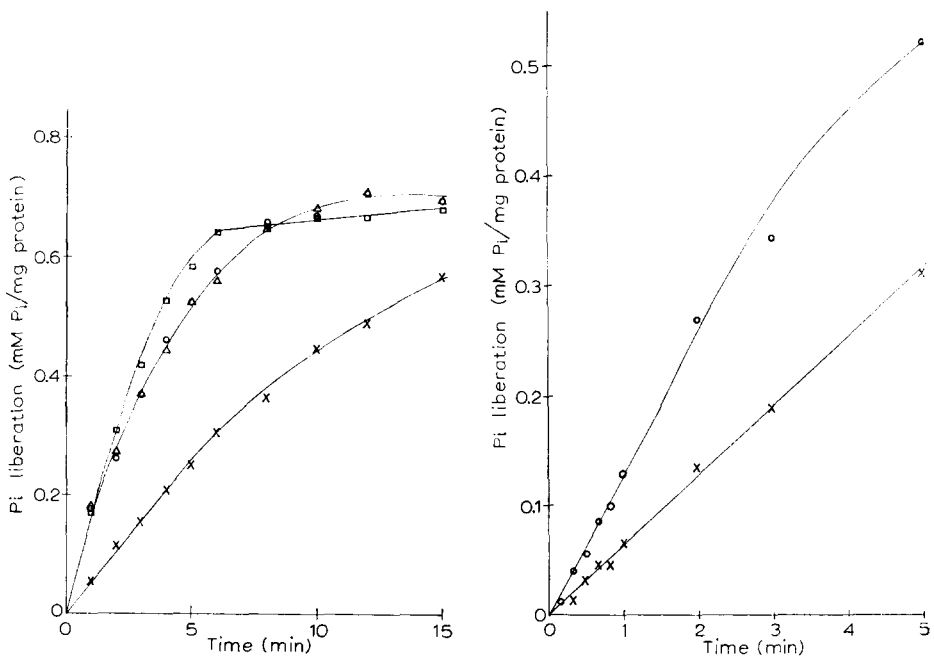

Fig. 4. Time course of ATPase activity of sarcoplasmic reticulum vesicles after *n*-alcohol treatment. Vesicles were pretreated with 0.78 M *n*-propanol (○—○); 0.13 M *n*-butanol (△—△); or 37 mM *n*-pentanol (□—□). ATPase assay was carried out by adding ATP (final concentration 1 mM) to the same, medium as described under Materials and Methods in the same concentration of *n*-alcohols of the pretreatment. ×—×, control vesicles (no alcohol).

Fig. 5. Time course of ATPase activity of sarcoplasmic reticulum vesicles after *n*-pentanol treatment. ATPase assay was carried out by adding ATP (final concentration 1.25 mM) to the same medium as described under Materials and Methods in the presence of *n*-pentanol. ○—○, 37 mM *n*-pentanol pretreated vesicles; ×—×, control vesicles (no alcohol).

Sarcoplasmic reticulum vesicles treated with lower concentration of *n*-alcohols retained both basic and extra ATPase activities (Figs 6 and 7). Both ATPase activities were increased by *n*-alcohol treatment, but the extra ATPase activity was increased to a greater extent than the basic activity (Figs 6 and 7).

The magnesium dependence of the basic ATPase activity was not changed by *n*-alcohol treatment, but that of extra ATPase activity was (Fig. 6).

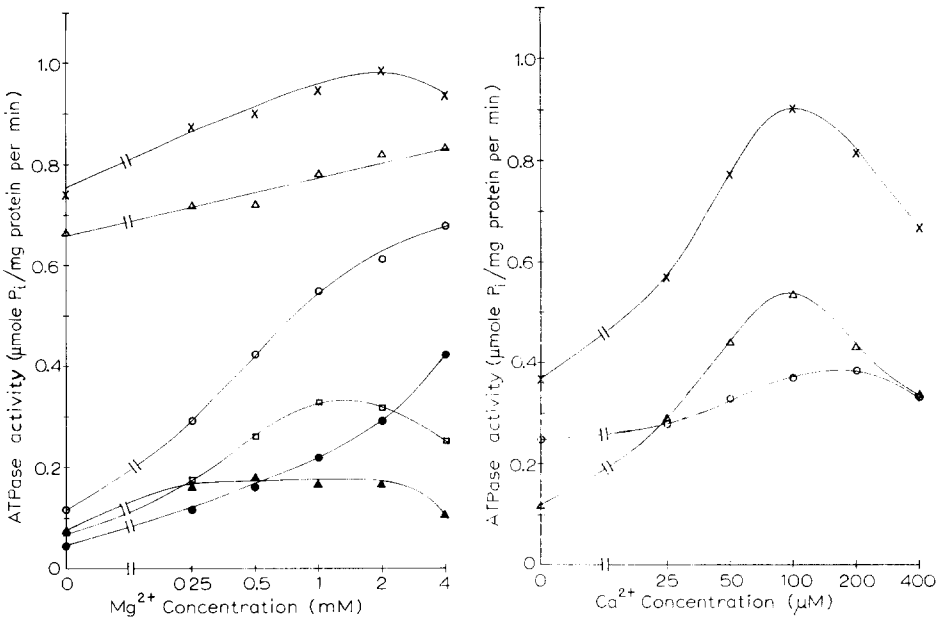

Fig. 6. Magnesium dependence of ATPase activity of sarcoplasmic reticulum vesicles after *n*-pentanol treatment. ATPase activity was measured for 6 min at room temperature by adding ATP (final concentration 1.25 mM) to the medium containing 0.1 M KCl, 100 μM EGTA, 0 or 150 μM $CaCl_2$, 0–4 mM $MgCl_2$, 40 mM Tris–maleate (pH 6.5) and 0.1 mg/ml vesicle protein. △—△, basic ATPase, control vesicles without calcium; □—□, extra ATPase, control vesicles with 150 μM calcium; ×—×, basic ATPase, 56 mM *n*-pentanol-treated vesicles without calcium; ○—○, extra ATPase, 56 mM *n*-pentanol-treated vesicles with 150 μM calcium; ●—●, increment of extra ATPase activity by 56 mM *n*-pentanol treatment; ▲—▲, increment of basic ATPase activity by 56 mM *n*-pentanol treatment.

Fig. 7. Calcium dependence of ATPase activity of sarcoplasmic reticulum vesicles after *n*-pentanol treatment. ATPase activity was measured for 6 min at room temperature by adding ATP (final concentration 1 mM) to the medium containing 0.1 M KCl, 2 mM $MgCl_2$, 100 μM EGTA, 0–400 μM $CaCl_2$, 40 mM Tris–maleate (pH 6.5) and 0.1 mg/ml vesicle protein. ○—○, ATPase activity of control vesicles; ×—×, ATPase activity of 56 mM *n*-pentanol-treated vesicles; △—△, the difference between ATPase activity of control vesicles and that of 56 mM *n*-pentanol-treated vesicles.

Time dependence and reversibility of alcohol effect

To elucidate the mechanism of the alcohol effect on sarcoplasmic reticulum membranes, the time course of the effect was measured, using methanol as an example. Just after addition of 10% methanol, calcium uptake was decreased to the value shown in Fig. 1 and did not change for a few hours. It is concluded that the onset of the effect of alcohol is instantaneous (within 1 min). From this observation, the alcohol effect may be expected to be reversible. A sample with high concentration of sarcoplasmic reticulum vesicles (5 mg protein/ml) containing 20% (4.7 M) methanol was diluted 25 times by the solvent containing various concentrations of methanol and 0.1 M KCl, 2 mM $MgCl_2$, 50 μM $CaCl_2$, 0.01 μCi/ml $^{45}CaCl_2$, and 40 mM Tris–maleate (pH 6.5) as in the case of the effect of methanol in Fig. 1 after about 30 min incubation. Calcium uptake was measured by addition of ATP. Calcium

uptake ability varied with methanol concentration in the same fashion as in the experiment shown in Fig. 1. This shows that the effect of alcohol at low concentration was reversible. However, sarcoplasmic reticulum vesicles exposed to 35% (8.2 M) methanol completely lost the ability to take up calcium even after dilution of the alcohol. This shows that alcohol treatment at higher concentrations caused an irreversible denaturation of sarcoplasmic reticulum vesicles.

DISCUSSION

The effects of *n*-alcohol treatments of sarcoplasmic reticulum vesicles may be explained as follows by analogy with the facts such as the effects of *n*-alcohols on cation permeability of lipid membranes and on the structural arrangement of lipid in lipid bilayers, and the importance of lipid in sarcoplasmic reticulum vesicles mentioned in the Introduction. Alcohol molecules penetrate into lipid bilayers of vesicle membranes, produce changes in the structural arrangement of lipid, and consequently increase calcium permeability. Therefore, Ca^{2+} immediately escape through the leaky membranes, extra ATPase activity is not parallel to net calcium uptake, and Ca^{2+} cannot be accumulated. Recently Makinose *et al.* showed the direct coupling of ATP synthesis and efflux of calcium[21,22]. The net outward movement of 2 moles of Ca^{2+} is stoichiometrically related to the net formation of 1 mole of ATP under conditions where ADP- and P_i-dependent Ca^{2+} release takes place from Ca^{2+}-loaded sarcoplasmic reticulum vesicles. On the other hand, it is suggested that the increase of inner calcium concentration inhibits the ATPase coupled with calcium translocation[23]. It is considered that in the steady state, extra ATPase is balanced to the leak efflux of calcium which is not coupled with ATP synthesis. By the treatment with *n*-alcohol, the leak efflux of calcium is increased with increasing alcohol concentration. ATPase coupled with calcium uptake must be continued at higher levels because the concentration of inner calcium cannot reach a fully loaded level. If only this effect was operative in *n*-alcohol-treated sarcoplasmic reticulum vesicles, a linear relationship between the increase of ATPase and the decrease of calcium uptake would be expected. As shown in Fig. 1, the increase of ATPase activity was accompanied with the decrease of net calcium uptake, but they are not linear. This effect, therefore, seems to be a major part of the alcohol effects. However the direct effect that the alcohols dissociate the coupling between the extra ATPase and the calcium translocation may be operative.

Because the effect of *n*-alcohols is reversible at lower concentrations, an adsorption equilibrium can be expected. The standard free energy of adsorption per methylene group, ΔF is (ref. 24)

$$\Delta F = 2.303\mathrm{RT}\log\frac{a_{i+1}}{a_i} = 2.303\mathrm{RT}\log\frac{C_{i+1}}{C_1}$$

where a_i and a_{i+1} refer to the activity of the ith and $i+1$th homologues, respectively. We assume that a definite number of alcohol molecules bind to sarcoplasmic reticulum vesicles, and make them inactive for ATPase or calcium uptake activity. In practice ΔF is evaluated, assuming the activity of *n*-alcohol equals its mole fraction (C_i, C_{i+1}), from the slope of the linear portion of plots of the logarithm of the mole fraction of *n*-alcohol required to decrease calcium uptake by 50% and to increase ATPase

activity by 50% of the maximum increment against the number of carbon atoms of *n*-alcohols. The values for *n*-alcohols obtained under the assumption that the extent of enhancement of ATPase activity or of inhibition of calcium uptake is directly proportional to the number of molecules absorbed at the sites responsible for these phenomena were both – 796 cal/mole at 20 °C. The free energy of transfer per mole of methylene group from water to octanol was – 670 cal/mole, obtained from the partition coefficient between *n*-octanol and water, using the slope of the linear relationship between the logarithm of the partition coefficient and the number of carbon atoms of *n*-alcohols. The ΔF value for sarcoplasmic reticulum vesicles is close to the value obtained from the partition coefficient of *n*-alcohols between octanol and water. This indicates that non-polar residues of *n*-alcohols interact with the hydrophobic sites of sarcoplasmic reticulum membrane vesicles. The hydrophobicity of the sites for *n*-alcohols may be comparable or more than for octanol.

Now, the coefficient in front of the exponent of the empirical formulae 1 and 2 has a dimension of molar concentration and seems to proportional to the number of hydrophobic binding sites for alcohols. On the other hand, the exponent of the empirical formulae 1 and 2 has no dimension and seems to indicate a reciprocal of strength of hydrophobic interaction between alcohol molecules and membrane molecules. From the same values obtained for ATPase activity and calcium uptake, it seems that these two activities are coupled directly at the molecular level.

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