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NET ATP SYNTHESIS IN H^+-ATPASE MACROLIPOSOMES BY AN EXTERNAL ELECTRIC FIELD

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SUMMARY: Application of electric pulses (1000 V/cm, 20 m sec duration) to macroliposomes containing pure stable H^+-ATPase ($F_o \cdot F_1$) resulted in synthesis of ATP. Microliposomes containing $F_o \cdot F_1$ showed very little ATP synthesis under the same conditions. The amount of ATP synthesized was increased by increasing the number of electric pulses applied and decreased by addition of either an uncoupler or an energy transfer inhibitor.

In studies on the mechanism of ATP synthesis by $F_o \cdot F_1$, rapid energization of $F_o \cdot F_1$ is necessary. Acid-base treatment of $F_o \cdot F_1$ liposomes results in synthesis of ATP (1, 2), but analysis of the reaction by this method has the disadvantages that time resolution is poor and the energy components are complex, i.e. both electric and osmotic energy. An alternate method is ATP synthesis driven by an external electric field. This method has been used for chloroplast particles (3, 4), and gives excellent time resolution of pure electrical energy. It can be applied to pure reconstituted $F_o \cdot F_1$ liposomes if sufficient membrane potential of about 200 mV is attained (5). For this purpose, the external electric field strength or the diameter of the reconstituted

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The abbreviations are: DCCD; *N,N'*-dicyclohexylcarbodiimide, FCCP; carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, Tricine; *N*-tris (hydroxymethyl) methylglycine. $F_o \cdot F_1$; proton translocating adenosine triphosphatase (H^+-ATPase).

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liposomes must be increased. Owing to the production of Joule heat by the current, even at low buffer concentrations, the electric field strength cannot be raised above 1000 V/cm. Therefore, the diameter of the liposomes must be increased to the order of micrometers. When these conditions were satisfied, net ATP synthesis sensitive to an uncoupler or energy transfer inhibitor was observed.

MATERIALS AND METHODS

Preparation of $F_o \cdot F_1$ macroliposomes Stable $F_o \cdot F_1$ was prepared from thermophilic bacterium PS3 as described previously (6) except that the DEAE-cellulose step was repeated twice and gel filtration was omitted. $F_o \cdot F_1$ macroliposomes were reconstituted by the dialysis method in the presence of Sephasorb (Pharmacia, Sweden, particle size 10-23 μm, HP superfine). A mixture of 75 μl of $F_o \cdot F_1$(1.5 mg protein), 1.2 ml of phospholipid solution (6) and 400 μl of washed Sephasorb suspension (50 mg/ml) was dialyzed against 500 ml of 10 mM Tricine-NaOH, pH 8.0 containing 0.2 mM EDTA, 2.5 mM $MgSO_4$ and 0.25 mM dithiothreitol at 30°C for 18 hours. The dialyzed mixture (1.6 ml) was centrifuged at 10,000 × g for 10 min. The resulting pellet was suspended in 400 μl of the dialyzing solution and was used as the macroliposome fraction, while the supernatant fraction was used as a microliposome fraction. Electron microscopy showed macroliposomes of 0.5 to 1.5 μm diameter in the macroliposome fraction and microliposomes (average diameter 0.1 to 0.2 μm) in the microliposome fraction. The macroliposome fraction contained 0.94 mg/ml of protein and 0.665 units/ml of ATPase activity (7) while the microliposome fraction contained 1.63 mg/ml of protein and 0.84 units/ml of ATPase activity. Details of these two fractions will be reported elsewhere.

Composition of the assay mixture The assay mixture in a final volume of 0.78 ml in an electrical cell consisted of 400 μl of macro- or micro- liposome

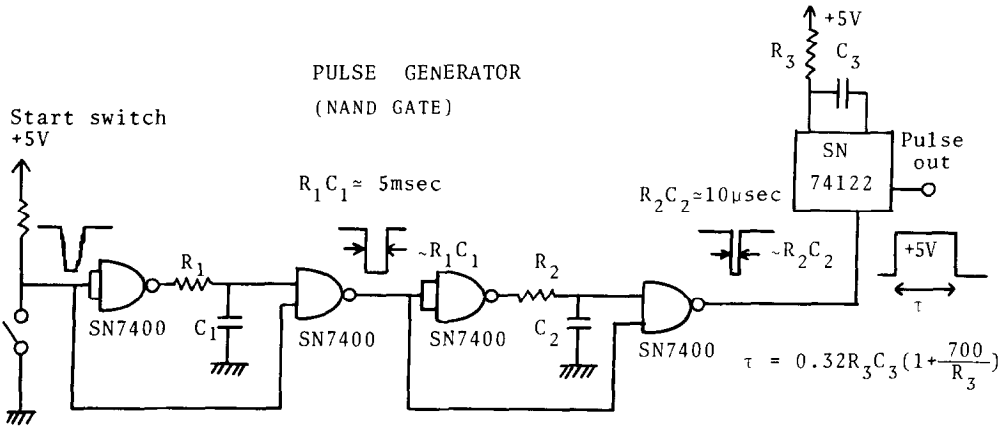

Fig. 1. Electronic circuit of the pulse generator. The shapes of the pulse at four points are indicated.

fraction, 1 mM Pi (^{32}Pi, 4 μCi/ml), 20 units of hexokinase (Sigma, U.S.A., yeast, lyophilized), 25 mM glucose and 0.5 mM ADP. The liposome fraction was added just before application of electrical pulses. After the reaction, 2 ml of a mixture (1:1) of perchloric acid (4N) and ammonium molybdate (8%) was added to the reaction mixture. Then addition of triethylamine (0.1 ml) resulted in precipitation of phosphomolybdate and liposomes which were removed by centrifugation at 600 × g, 5 min (2). The supernatant was extracted with 2 ml of isobutanol-benzene (1:1, water saturated), and an aliquot of the water phase was mixed with 50 % ethanol without any scintillator and its radioactivity was counted in a liquid scintillation counter, as described previously (2).

Electric field cell and circuit Two circular platinum electrode (5 cm^2, 0.1 mm thick) were placed 2 mm apart in a polycarbonate cell. The sample solution was introduced and removed through a capillary tube inserted into a hole between the electrodes. The switching circuit was connected to a pulse generator (Fig. 1) and a high voltage source to raise the voltage (5 V) of the pulse to 200 V. Rectangular pulses of 200 V, 20 msec (unless specified) duration with a rise time of 1 μsec were applied to the electrodes; hereby the polarity of the voltage was

Table I Temperature rise by a pulse

Temperature rise ($\Delta\theta$) = 2.4°K (calculated).

$\Delta\theta = \frac{E}{m\,c} = \frac{V^2\,\tau}{m\,c\,R}$ $E = \frac{V^2\,\tau}{R}$ = current heat.

V = 200 volts : voltage of the pulse.

$\tau = 2 \times 10^{-2}$ second : duration of the pulse.

m = 0.78 gram : mass of the sample solution.

R = 100 Ω : resistance of the sample solution.

c = 4.2 joule/gram·°K : specific heat of the sample solution.

$\Delta\theta$ (measured with a thermistor) = 2.5 - 2.8°K

The $t_{1/2}$ of temperature decrease after the pulse was 15 seconds at about 10 - 15°C during experiments.

changed after each pulse. The pulses were recorded. To avoid heat production by the current, ion concentrations were reduced and the cell was cooled in an ice bath during the 30 second interval between pulses. Heat production in the sample in the cell during a pulse was calculated and measured directly with a thermistor as shown in Table I. Experiments were carried out at 10 to 15°C.

RESULTS AND DISCUSSION

Preliminary experiments showed that the glucose hexokinase system was essential for demonstrating ATP synthesis, because unlike the chloroplast system (3, 4), which does not manifest ATPase activity, $F_o \cdot F_1$ showed ATPase activity and thus an ATP-trapping system was required.

ATP synthesis in this system increased on increasing the number of pulses applied to the $F_o \cdot F_1$ macroliposomes (Fig. 2), but was not proportional to the number of pulses, as in the case of chloroplasts (3, 4), perhaps because the macroliposomes were unstable. $F_o \cdot F_1$ microliposomes did not show ATP synthesis under the same conditions (Fig. 2, Table II), because the membrane potential induced was not sufficient for ATP synthesis.

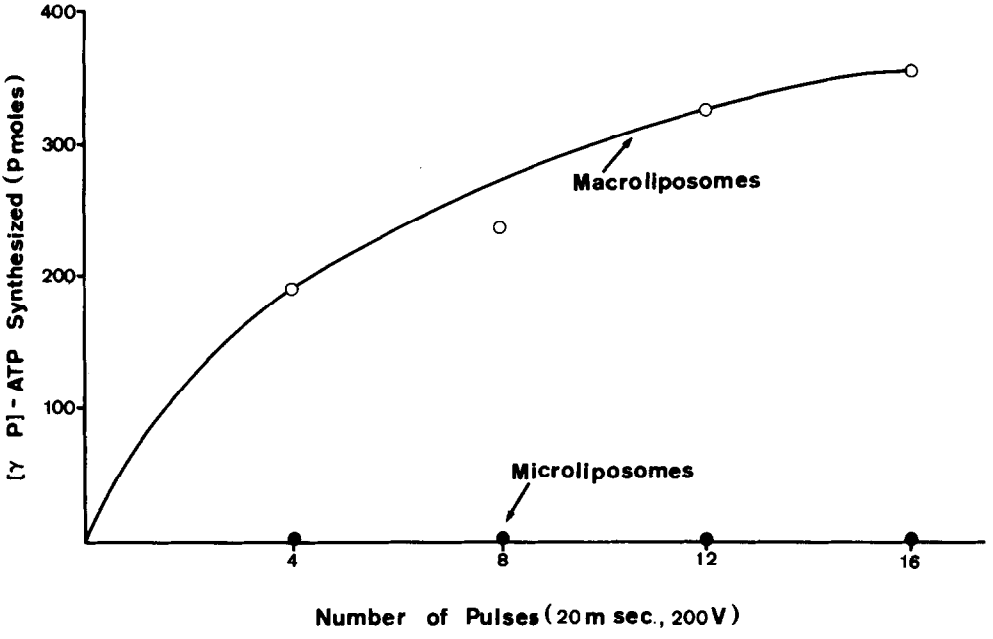

Fig. 2. Effect of the number of pulses on the amount of ATP synthesized.
The conditions are described under the Materials and Methods. ○; macroliposomes. ●; microliposomes.

Table II. Effects of vesicle size, pulse and inhibitors on net ATP synthesis in the reconstituted $F_o \cdot F_1$ liposomes.

vesicle size, inhibitor	number of pulses (20 m sec each)	ATP synthesized (p moles, total)
Experiment I		
macroliposomes + microliposomes*1	8	78
macroliposomes + microliposomes*1	12	105
macroliposomes + microliposomes*1	16	117
macroliposomes	12	139
macroliposomes + DCCD (0.6 μmole)	12	38
macroliposomes + FCCP (20 μg)	12	33
Experiment II		
macroliposomes	4	142
macroliposomes	8	396
macroliposomes	8 (=2.5 msec × 64)	178
microliposomes	4	0
microliposomes	8	0
microliposomes	12	0
microliposomes	16	0

*1 Dialysate before the separation of macro- and micro-liposomes.

When ATP was added to $F_o \cdot F_1$ microliposomes their membrane potential monitored by fluorescence method (6) increased several times faster than that of $F_o \cdot F_1$ macroliposomes, and addition of hexokinase-glucose mixture to these energized liposomes showed that the smaller the diameter, the faster was the decrease of the membrane potential.

The synthesis of ATP was inhibited by the additions of DCCD and FCCP (Table II). When the pulse duration was decreased to 2.5 msec, the yield of ATP was lowered (Table II). This may reflect the turnover number of $F_o \cdot F_1$, since in chloroplasts this was reported to be 5 msec (3, 4). The yield of ATP per pulse was about 25 % of that of chloroplast particles with a chlorophyll concentration of 4×10^{-4} M (M. Rögner unpublished result)

Thus, $F_o \cdot F_1$ liposomes were shown to translocate H^+(8), and synthesize ATP by an electrochemical gradient of H^+(1, 2) and by an external electric field.

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