Biochemistry (Moscow), Vol. 67, No. 4, 2002, pp. 468-472. Translated from Biokhimiya, Vol. 67, No. 4, 2002, pp. 561-566.

Thyroxine Reversibly Inhibits the Uncoupling Action of Protonophores on Energy Production in Rat Thymus Lymphocytes

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Received March 14, 2001
Revision received May 3, 2001

Abstract—Earlier we reported that some thyroid and steroid hormones and also 6-ketocholestanol used in micromolar concentrations modulated the effects of protonophoric uncouplers on isolated mitochondria (Starkov et al. (1997) *Biochim. Biophys. Acta*, **1318**, 173-183). In the present study we investigated the effects of a thyroid hormone, thyroxine, on energy coupling of intact rat thymus lymphocytes and mitochondria isolated from these cells. The resting (oligomycin-inhibited) respiration of the isolated intact lymphocytes was stimulated by the addition of protonophoric uncouplers 2,4-DNP, FCCP, or SF6847. Subsequent addition of micromolar concentrations of thyroxin decreased the rate of uncoupler-stimulated respiration and partially reversed uncoupler-induced decrease of membrane potential ($\Delta\Psi$). In experiments with mitochondria isolated from thymus lymphocytes the re-coupling effect of thyroxine was not observed. In this case thyroxine did not influence mitochondrial respiration stimulated with 2,4-DNP, but did potentiate the stimulation of respiration and $\Delta\Psi$ decrease induced with another uncoupler, SF6847. The data are discussed in terms of a hypothesis that aromatic uncouplers are transported into the cell by the thyroxine carrier of the plasma membrane.

Key words: mitochondria, rat thymus lymphocytes, 6-ketocholestanol, thyroxine, uncoupling, uncouplers SF6847, FCCP, and 2,4-DNP

During the last forty years many attempts have been made to demonstrate that the mechanism of the uncoupling effect of amphiphilic anions, protonophores, on mitochondria involves proteins of the inner mitochondrial membrane. Initially a possible involvement of a protein transporter in the effects of DNP and PCP was postulated by Yaguzhinsky et al. [1]. Direct experiments carried out in Skulachev's laboratory revealed that ADP/ATP antiporter is involved in the uncoupling effect of low concentrations of DNA and fatty acids and antiporter inhibitor, carboxyatractyloside, abolishes the uncoupling effect of these substances [2]. Carboxyatractyloside-insensitive uncoupling by SF6847 and FCCP obviously involves other proteins [3]. There is increasing evidence (including our own data [3-6]) for the existence of a so-called "mild uncoupling" mechanism in mitochondria. According to Skulachev's hypothesis [4] thyroid hormones can modulate this process, and this is a mechanism of cell protection against reactive oxygen species [7].

Recently we demonstrated that micromolar concentrations of thyroxine potentiate the effect of low concentrations of uncoupler SF6847 on membrane potential of rat liver mitochondria [6]. In the present study we have continued the investigation of the effect of thyroxine on respiration and energetic coupling of isolated intact lymphocytes and mitochondria isolated from these cells.

Abbreviations: oligo) oligomycin A; DNP) 2,4-dinitrophenol; FCCP) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops) 4-morpholinopropanesulfonic acid; PCP) pentachlorophenol; KCh) 6-ketocholestanol; SF6847) 3,5-di-*tert*-butyl-4-hydroxybenzylidene-malononitrile; T_4) thyroxine; TPP^+) tetraphenylphosphonium; $\Delta\Psi$) transmembrane electric potential of mitochondria.

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MATERIALS AND METHODS

Rat thymus lymphocytes were isolated as described by Dedukhova and Mokhova [8] with modifications. Thymuses were placed into ice-cold RPMI 1640 medium with glutamine. Each gland was treated separately

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on a cooled Teflon bar; after the removal of non-lymphoid tissue, glands were cut into 4-6 pieces and thymus lymphocytes were gently squeezed from the gland tissue with a Teflon rod. Cells were placed into centrifuge tubes containing the same medium (1-2 ml per thymus). Cell suspension was filtered through two layers of cheesecloth. The cells were pelleted by centrifugation for 5 min at 800*g*, and the pellet was gently resuspended with the Teflon rod. After the addition of 4 ml H_2O, cells were accurately mixed for 5-10 sec and after the addition of 0.5 ml of Hanks' 10X medium they were filtered through two layers of cheesecloth. Lymphocytes were pelleted again by centrifugation (800*g* for 5 min), resuspended in medium containing 145 mM NaCl, 4.6 mM KCl, 8 mM Mops, pH 7.4, 1 mM KH_2PO_4, and 10 mM pyruvate and washed under the same conditions. The resulting cell suspensions containing about $(3\text{-}5)\cdot10^9$ cells per ml were kept in ice. The intactness of cells was evaluated by lack of respiration on succinate in the presence of rotenone. The medium was used as the incubation medium.

Thymus lymphocyte mitochondria were isolated as described by Konoshenko et al. [9]. Briefly, lymphocytes isolated from four thymuses were pooled in 5 ml of medium containing 140 mM sucrose, 70 mM NaCl, 5.6 mM KCl, 10 mM pyruvate, and 8 mM Mops, pH 7.4. The suspension was diluted with 5 ml of medium containing 5.6 mM KCl, 1 mM EGTA, 1% BSA, 10 mM pyruvate, 8 mM Mops, pH 7.4. After incubation for 2 min the cell suspension was homogenized in 40 ml of mitochondria isolation medium containing 350 mM sucrose, 1 mM EGTA, 1% BSA, 5 mM Mops, pH 7.4, using a Teflon homogenizer and Teflon pestle. The homogenate was centrifuged for 10 min at 1000*g* to remove nuclei and cell membranes, and the supernatant was centrifuged for 10 min at 12,000*g* to sediment mitochondria, which were then washed under the same conditions. All procedures were carried out at 0-4°C. Mitochondria with respiratory control of at least 4.0 were used in the experiments. The incubation medium contained: 250 mM sucrose, 5 mM Mops, pH 7.4, 1 mM EGTA, 5 mM succinate, and 1 mM KH_2PO_4.

Respiration of cell and mitochondrial suspensions was registered polarographically using a Clark type oxygen electrode and PA-2 polarograph (Czechia) at 25°C (mitochondria) or 37°C (cells) under constant stirring. Concentrations of lymphocytes and mitochondria in the polarographic cell were $2\cdot10^8$ cells per ml and 1-2 mg of protein per ml, respectively. The volume of the polarographic cell was 1.0 and 0.5 ml for mitochondria and lymphocytes, respectively.

Mitochondrial electric potential difference ($\Delta\Psi$) was determined using safranin O as a penetrating cation. Changes of $\Delta\Psi$ value were evaluated by changes of safranin O absorbance at 555-523 nm [10] using an Aminco DW-2000 double-beam spectrophotometer. The incubation medium (final volume 2 ml) contained 250 mM sucrose, 5 mM Mops, pH 7.4, 2 mM EGTA, 5 mM succinate, 2 μM rotenone, 3 mg/ml oligomycin A, 2 mM KH_2PO_4, 0.2 mg BSA, 8 μM safranin O, and mitochondria (1-2 mg/ml).

Changes of $\Delta\Psi$ on the mitochondrial membrane inside lymphocytes were evaluated by redistribution of penetrating cation TPP^+ using a selective TPP^+-electrode [11]. The concentration of TPP^+ was 1.6 μM. The incubation medium contained 145 mM NaCl, 4.6 mM KCl, 8 mM Mops, pH 7.4, 1 mM KH_2PO_4, 10 mM pyruvate, and 3 mg/ml oligomycin A. Total volume of the cell was 1 ml, and the number of cells was $2\cdot10^8$.

The following chemicals were used: thyroxine, 6-ketocholestanol, FCCP, SF6847, rotenone, oligomycin A, and pyruvate (Sigma, USA); TPP^+ (Fluka, Switzerland); 2,4-DNP and safranin O (Serva, Germany). RPMI 1640 and Hanks' 10X media were produced by the Institute of Poliomyelitis and Viral Encephalitis, Russian Academy of Medical Sciences (Moscow).

RESULTS AND DISCUSSION

Figure 1 shows that thyroxine exhibits different effects on uncoupled respiration of thymus lymphocytes and mitochondria isolated from these cells. Mitochondria of intact lymphocytes are in the active phosphorylation state and addition of ATP synthetase inhibitor, oligomycin, inhibited cell respiration. Subsequent addition of low concentrations of uncoupler SF6847 (Fig. 1a, curve *1*) or 2,4-DNP (Fig. 1b, curve *1*) restored cell respiration to the initial level. Addition of thyroxine (20 μM) significantly reduced the rate of uncoupled respiration. This effect was not due to possible inhibition of mitochondrial respiratory chain because subsequent addition of the uncoupler stimulated respiration again (Fig. 1, a and b, curves *1*). This re-coupling effect of thyroxine was observed in experiments with different uncouplers: SF6847, DNP, and FCCP (data not shown). These uncouplers differ by effective concentrations (low nanomolar, high nanomolar, and high micromolar range of concentrations of SF6847, FCCP, and DNP, respectively) and mechanisms of proton transport across lipid membranes [12]. Nevertheless, uncoupling effect of these compounds was inhibited by similar concentrations of thyroxine (10-20 μM).

Addition of thyroxine to isolated lymphocyte mitochondria caused a completely different effect. Thyroxine did not inhibit respiration; it induced further increase of mitochondrial respiration stimulated by SF6847. Subsequent addition of 6-ketocholestanol reduced the rate of mitochondrial respiration. The mechanism of the re-coupling effect of 6-ketocholestanol on mitochondria was studied in detail earlier [3]. This compound is sug-

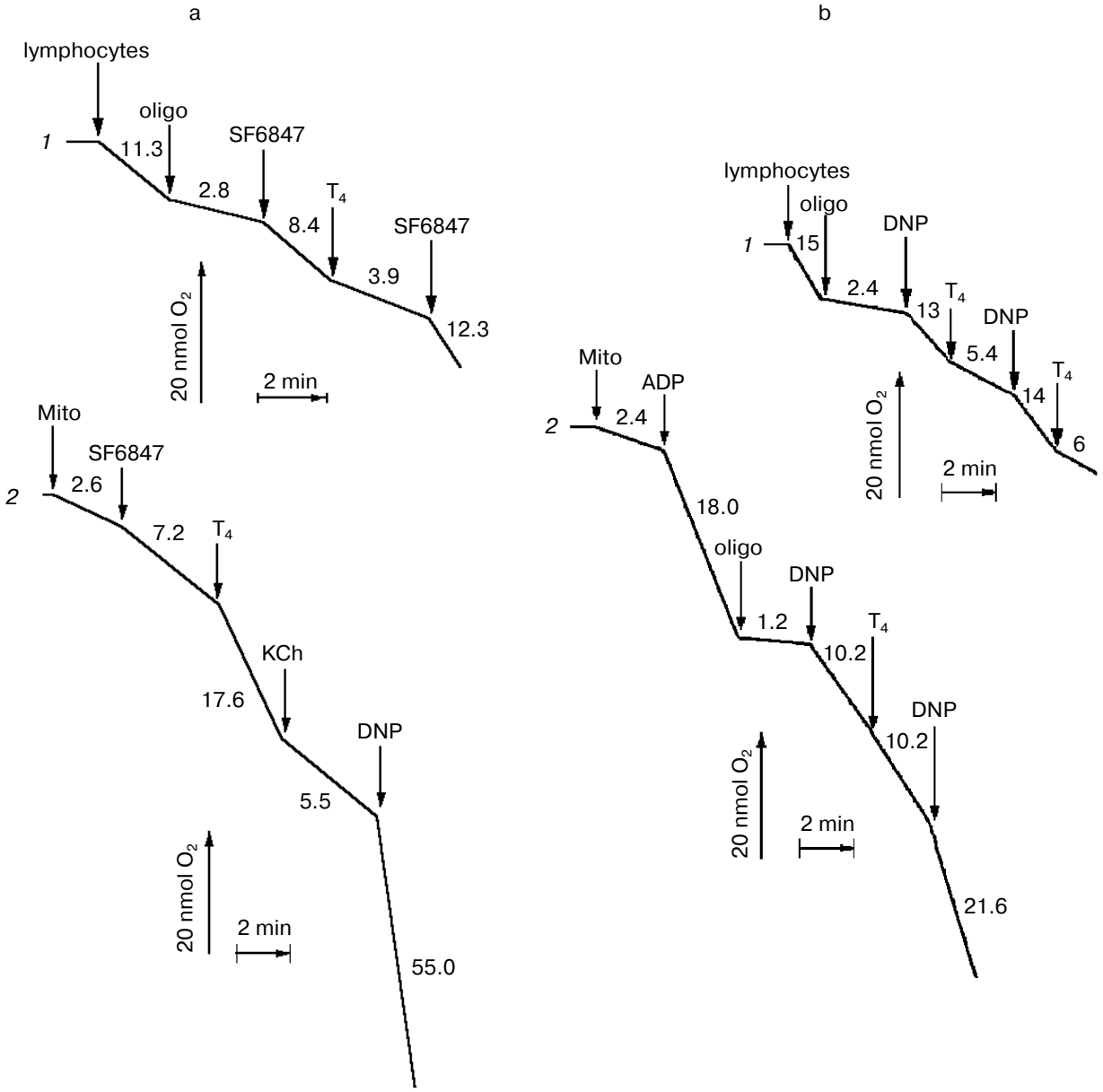

Fig. 1. Effect of thyroxine (T_4) on respiration of rat thymus lymphocytes (a, b, curves *1*) and isolated thymus lymphocyte mitochondria (a, b, curves *2*) weakly uncoupled with SF6847 (a) and DNP (b): *1*) intact cells; *2*) isolated mitochondria. Numbers above the curves designate the rate of oxygen consumption. Respiration medium contained $2 \cdot 10^8$ cells/ml lymphocytes or 1 mg/ml lymphocyte mitochondria. The following additions were made in the case of cells: 4 μg oligomycin A, 40 and 200 nM SF6847, 20 μM T_4, 20 and 40 μM DNP. In experiments with isolated mitochondria there were the following additions: 50 μM ADP, 2 μg oligomycin A, 10 and 20 μM DNP, 10 μM T_4, 75 μM 6-ketocholestanol (KCh), 40 nM SF6847. Total volume of the polarographic cell was 0.5 and 1.0 ml for lymphocytes and isolated mitochondria, respectively.

gested to increase asymmetrically the negative dipole potential of the lipid bilayer of the inner mitochondrial membrane [13, 14]; this attenuates translocation of the anion form of the uncoupler [3]. Stimulation of mitochondrial respiration after the addition of DNP suggests lack of inhibition of the respiratory chain by thyroxine and 6-ketocholestanol. Similar results were also obtained in experiments with rat liver mitochondria (Fig. 2).

Previously we demonstrated that addition of thyroxine to mitochondria partially uncoupled by SF6847 and FCCP potentiated fall of membrane potential [6]. However, such effect was not observed when thyroxine was added to mitochondria uncoupled by DNP. Thyroxine did not influence mitochondrial respiration stimulated by DNP (Fig. 1b, curve *2*; Fig. 2b). The effect of thyroxine on SF6847-induced uncoupling and its inef-

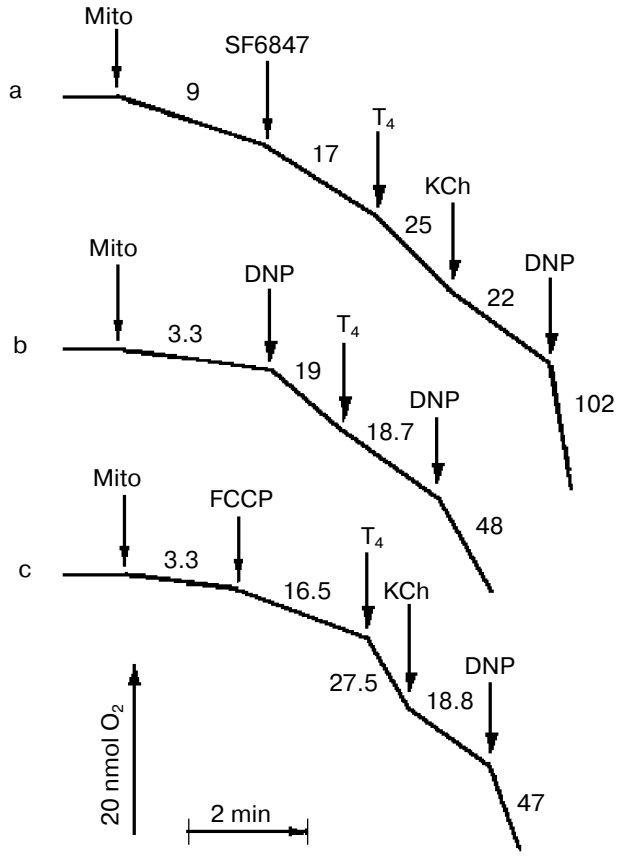

Fig. 2. Effect of thyroxine on respiration of rat liver mitochondria uncoupled by SF6847 (a), DNP (b), and FCCP (c). The respiration medium contained mitochondrial protein (1 mg/ml) and the following additions: 20 nM SF6847, 10 μM T_4, 75 μM 6-ketocholestanol (KCh), 20 μM DNP, and 60 μM FCCP.

fectiveness in the case of DNP-induced uncoupling were quite predictable; these data support the hypothesis on the mechanism of "mild uncoupling" [7]. According to this hypothesis there is a special protein-mediated mechanism of "mild uncoupling" responsible for uncoupling of mitochondria by some (unknown) natural compound(s) similar to SF6847; this mechanism is sensitive to activation by thyroid hormones. This mechanism does not involve uncouplers like 2,4-DNP and therefore thyroid hormones must not influence DNP-induced uncoupling (if the hypothesis is correct).

Measurement of mitochondrial membrane potential (ΔΨ) provides additional information on the mechanism of the effect of thyroxine on lymphocytes and isolated mitochondria. Figure 3 shows that thyroxine caused further decrease of ΔΨ caused by addition of SF6847 to mitochondria (Fig. 3a); however, similar treatment of intact cells resulted in ΔΨ increase of uncoupled mitochondria (Fig. 3b).

Figure 3b shows that thyroxine exerts a re-coupling effect on the uncoupled cells, because the hormone not only decreases the rate of uncoupled respiration of lymphocytes (Fig. 1), but also increases uncoupler-induced mitochondrial membrane potential inside cells.

The mechanism of thyroid hormone transport into cells is not completely understood [15-17]. It is suggested that transport of thyroid hormones through plasma membranes involved at least two proteins, and this process is inhibited by a wide range of aromatic compounds [16-19] including 2,4-DNP [15]. An inhibitory effect of thyroxine binding to adenohypophysis proteins suggests a possibility for recognition of thyroid hormone binding sites by uncouplers [20].

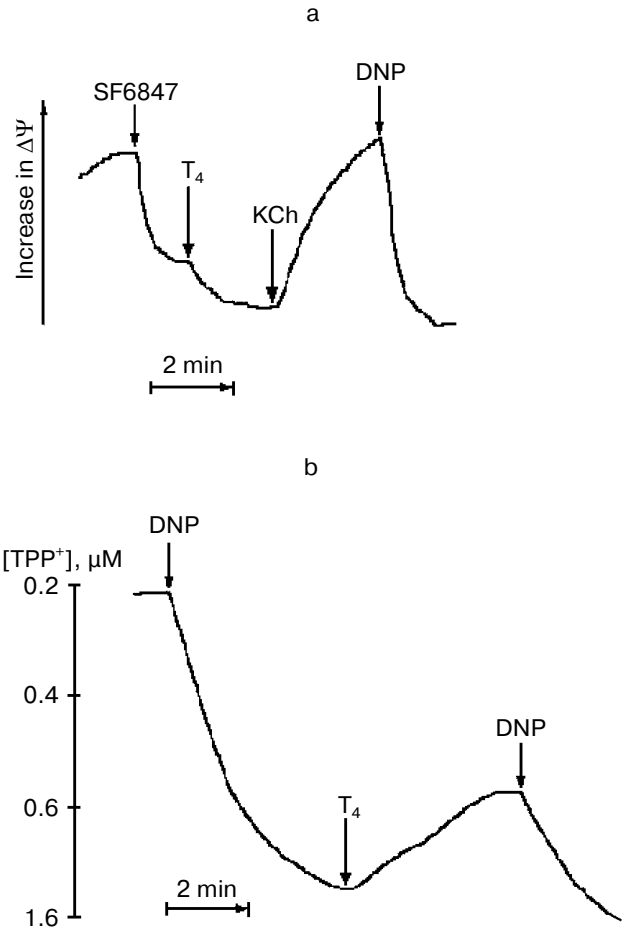

Fig. 3. Effects of 6-ketocholestanol (KCh) and thyroxine on ΔΨ of isolated thymus lymphocyte mitochondria (a) and of native thymus lymphocytes (across the inner mitochondrial membrane) (b). The amounts of mitochondrial protein and number of cells were 0.5-0.8 mg/ml and $2 \cdot 10^8$ cells/ml, respectively. In the experiments with mitochondria the following additions were made: 50 nM SF6847, 8 μM T_4, 75 μM 6-ketocholestanol (KCh), 30 μM DNP. In the case of experiments with intact cells 20 and 20 μM DNP, 20 μM T_4.

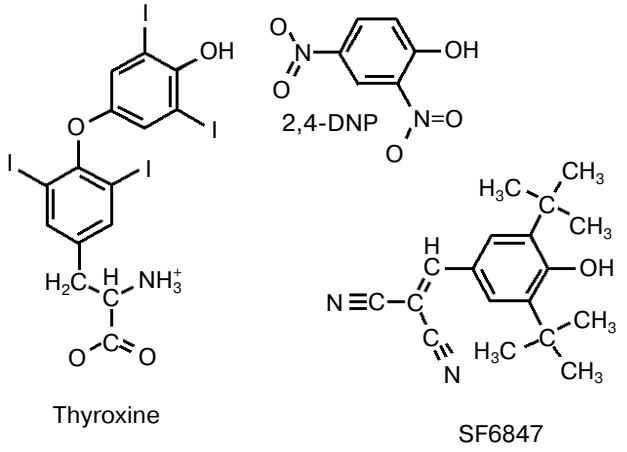

Fig. 4. Structural formulas of thyroxine (T_4) and the uncouplers SF6847 and DNP.

Certain similarity exists between thyroxine and uncouplers used in this study [21]. The thyroxin molecule as well as SF6847 and 2,4-DNP (Fig. 4) contain aromatic rings carrying a hydroxyl group and two symmetric electron acceptor groups (-I, -C≡N, and -NO_2 in thyroxine, SF6847, and DNP, respectively). However, the thyroid hormone molecule also contains a hydrophilic alanine residue and therefore it is membrane impermeable, whereas the presence of hydrophobic groups in SF6847 provides high lipophilicity of this compound. However, high affinity for lipid membranes does not imply that the substance will be readily transported through the cell plasma membrane (or any other biological lipid membrane). It is possible that protein mediated transport will be more effective than that of simple diffusion, especially at low concentrations of the transported compound.

Thus, it is possible that the re-coupling effect of thyroxine observed in experiments with intact cells depends on processes occurring at the level of lymphocyte cell membrane rather than mitochondria. It is also possible that there are competitive interrelationships between the uncoupler and thyroid hormone during their transport into cells. Perhaps, by analogy with ATP-dependent glycoprotein which is responsible for efflux of hydrophobic venoms and drugs from cells, thyroxine may trigger a mechanism of DNP efflux from the cell [22-24]. Within this hypothesis the re-coupling effect of thyroxine may be attributed to the possible hormone binding to a protein transporter in competitive manner with respect to the uncoupler.

This work was supported by the Russian Foundation for Basic Research (grant No. 96-04-00022C).

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