

Regulation of the energy coupling in mitochondria by some steroid and thyroid hormones

Anatoly A. Starkov, Ruben A. Simonyan, Vera I. Dedukhova, Svetlana E. Mansurova,
Larisa A. Palamarchuk, Vladimir P. Skulachev *

Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia

Received 1 August 1996; revised 14 October 1996; accepted 30 October 1996

Abstract

Male sex hormones [dihydrotestosterone (DTS), and testosterone] and progesterone, when added to the isolated rat liver mitochondria before or after some protonophores, lower the respiration rate and increase the $\Delta\Psi$ level, i.e., reverse the protonophore-induced uncoupling. Such a recoupling ability shows specific structural requirements correlating with hormonal activity of steroids studied. For instance, epiandrosterone, a DTS isomer of very low hormonal activity, and deoxycorticosterone, differing from progesterone by additional OH-group and possessing quite different hormonal activity, as well as female sex hormones (estron and estradiol) show no recoupling effect. Like 6-ketocholestanol (kCh), male sex hormones and progesterone recouple mitochondria uncoupled by low concentrations of SF6847, FCCP and CCCP, but not by high concentration of these uncouplers or by any concentration of DNP, palmitate and gramicidin. In contrast to recoupling by kCh, hormonal recoupling requires addition of serum albumin and is inhibited by low concentrations of palmitate. Recoupling can also be shown on the heart and skeletal muscle mitochondria, being absent from the heart muscle submitochondrial particles, the bacterial chromatophores and the cytochrome oxidase proteoliposomes. In mitochondria it does not depend upon the oxidation substrate used (succinate or PMS + ascorbate were tested). Pronounced seasonal effect upon the DTS recoupling degree was revealed. The recoupling is maximal in January, February and from June to November, being minimal in the spring months and in December. In spring, the *in vivo* administration of thyroxine, di- or triiodothyronine improves the recoupling ability of DTS. 2×10^{-6} M Thyroxine, when added *in vitro*, does not affect energy coupling if SF6847 was absent. In the presence of small amounts of SF6847, thyroxine stimulates the uncoupling in a DTS-sensitive fashion, di- and triiodothyronines being less effective. Addition of thyroxine to azide-inhibited mitochondria (oligomycin is present) stimulates respiration and normalizes the $\Delta\Psi$ level. In this system, triiodothyronine is much less effective, whereas diiodothyronine is not effective at all. In the intact cells (thymocytes and the Krebs-II cells were tested), DTS lowers the respiration rate stimulated by low concentrations of SF6846 or FCCP. In this case, serum albumin is

Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; CCCP, carbonylcyanide-3-chlorophenylhydrazine; DNP, 2,4-dinitrophenol; DTS, dihydrotestosterone; EGTA, ethyleneglycol-bis(β -aminoethyl- ether)-N,N,N',N'-tetraacetic acid; FCCP, p-trifluoromethoxycarbonylcyanide phenylhydrazine; kCh, 6-ketocholestanol (5 α -Cholestan- 3 β -ol-6-one); MOPS, morpholinopropane sulphonate; PMS, phenazine methosulphate; SF6847, 3,5-di(tret-butyl)-4-hydroxybenzylidenemalononitrile; TS, testosterone; TTFB, tetrachlorotrifluoromethylbenzimidazole.

* Corresponding author. Fax: +7 095 9390338. E-mail: skulach@head.genebee.msu.su

not required. It is suggested that recoupling effects of male sex hormones and progesterone are involved in their anabolic action just as uncoupling takes part in the catabolic activity of thyroid hormones.

Keywords: Uncoupler; Recoupler; Testosterone; Thyroxine; Noradrenaline; Mitochondrion

1. Introduction

In the preceding paper [1] we reported that 6-keto-cholestanol (kCh) and 3-keto,4-cholesten recouple mitochondria uncoupled by SF6847, FCCP or some other protonophores. It was interesting to elucidate whether this effect is related to action of steroid hormones containing keto group in the same positions, i.e., of some glucocorticoids, male sex hormones and progesterone. Below it will be reported that the most active male sex hormones, namely testosterone (TS) and dihydrotestosterone (DTS), as well as progesterone, when added to the SF6847-uncoupled mitochondria, possess a recoupling activity, whereas female hormones and glucocorticoids do not recouple. Thyroid hormones *in vivo* and *in vitro*, during certain seasons, proved to affect the DTS recoupling. A possible relation of recoupling and uncoupling to the anabolic and catabolic effects of male sex hormones and thyroid hormones, respectively, and their connection to the noradrenaline-induced, fatty acid mediated uncoupling will be discussed. (Some of these data were reported at the Conference 'Thirty years of progress in mitochondrial bioenergetics' [2]).

2. Materials and methods

Isolation of the rat liver and heart mitochondria, as well as measurements of the respiration rate and $\Delta\Psi$ were done as described in the preceding paper [1]. Rat skeletal muscle mitochondria were isolated as described earlier [3].

Hyperthyroidism was induced in white rats (approx. 150 g body weight) by the oral administration of 15 μ g thyroxine, 3,5,3'-tri- or 3,5-di-iodothyronine/100 g of body mass per day for 7 days [4].

Thymocytes were isolated from thymus of white rats (80–100 g body weight) as described [5]. The incubation medium contained 145 mM NaCl, 4.6 mM KCl, 1 mM KH_2PO_4 , 10 mM pyruvate, 8 mM

MOPS (pH 7.4). Isolated thymocytes were suspended in the incubation medium ($2\text{--}3 \times 10^9$ cells/ml) and stored on ice. For the respiration measurements the same incubation medium was used.

Respiration was measured with a Clark-type electrode mounted in 0.5 ml plastic cell. The thymocytes suspension (2×10^8 cells/ml) was continuously stirred by a Teflon-covered magnetic stirring bar. Temperature of the incubation medium was 37°C.

The Erlich ascite Krebs II carcinoma cells were grown in the female white mice. 0.2 ml ascite cells were injected intraperitoneally and harvested 7 days after injection. The cell-containing liquid was added to the cold Erl medium, passed through two layers of gaze, and cells were separated by centrifuging at 800 rpm (centrifuge K-70D) for 10 min. Then cells were resuspended in cold solution of 0.9% NaCl and centrifuged under the same conditions. This step was repeated twice. After this, cells were suspended (10^8 cells per ml) in the isolation medium (see above) and stored on ice. Cells were studied during 5–8 h after isolation. Respiration was measured under the same conditions as for thymocytes except that cells concentration was 5×10^6 per ml.

Testosterone was from 'Sigma', other hormones were from 'Koch-Light'. Zearalenone was generous gift of Professors F. Macri and A. Vianello.

3. Results

Fig. 1 shows effect of DTS and progesterone on respiration of rat liver mitochondria oxidizing succinate in the presence of oligomycin, rotenone and small concentration of SF6847. It is seen that both hormones attenuate stimulation of respiration by SF6847. In contrast to kCh inhibiting this stimulation completely (see Ref. [1]), the hormonal inhibition is incomplete. On the other hand, the inhibition by hormones, like that by kCh, is abolished by DNP.

In Fig. 2A, the effect of DTS on the $\Delta\Psi$ level in mitochondria is shown. It is found that the DTS

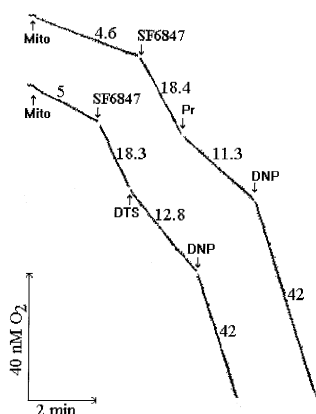


Fig. 1. Recoupling of the SF6847-uncoupled rat liver mitochondria by DTS and progesterone. Effects on the respiration rate. The incubation mixture contained 250 mM sucrose, 5 mM MOPS/Tris (pH 7.4), 5 mM succinate, 2 mM EGTA, 0.2 mg/ml BSA, 2 μ M rotenone, 3 μ g/ml oligomycin, 8 μ M safranin O. Additions: mito, the rat liver mitochondria (1.1 mg protein \times ml $^{-1}$), 40 nM SF6847, 75 mM DTS, 75 mM progesterone, 35 mM DNP. Numbers near curves, the respiration rates (nM O₂ \times min $^{-1}$ \times mg $^{-1}$ protein).

inhibition of the SF6847-stimulated respiration is accompanied by a $\Delta\Psi$ increase. This means that DTS, like kCh, operates as a recoupler. The difference of two recouplers is that (i) the reversal by DTS of the SF6847 effect on $\Delta\Psi$, just as on the respiration rate, is incomplete (ii) somewhat higher concentrations of DTS are required and (iii) serum albumin appears to be necessary for the DTS recoupling (cf. Fig. 2A,B). Other proteins tested, 1 mM dithioerythritol, reduced and oxidized glutathione failed to substitute for serum albumin (not shown). Effect of serum albumin may be due to binding of endogenous fatty acids. As can be seen in Fig. 2C, added fatty acid (palmitate) abolishes the DTS recoupling. The palmitate action occurs at such a low concentration of this fatty acid (6 μ M) which is insufficient per se to cause any measurable uncoupling under conditions used, i.e., in the presence of 3 μ M serum albumin. When added before SF6847, DTS prevented to a large degree a $\Delta\Psi$ drop induced by the subsequent SF6847 addition. The recoupling effect proved to be independent of the oxidation substrate (succinate and PMS and ascorbate were used).

In the next series of experiments, recoupling abilities of various steroid hormones were compared. It was found that TS and progesterone are competent,

like DTS, in an increase in $\Delta\Psi$ which was lowered by SF6847. As for estron, estradiol, epiandrosterone, deoxycorticosterone and cortisone, they were without effect or cause further $\Delta\Psi$ decrease when added after

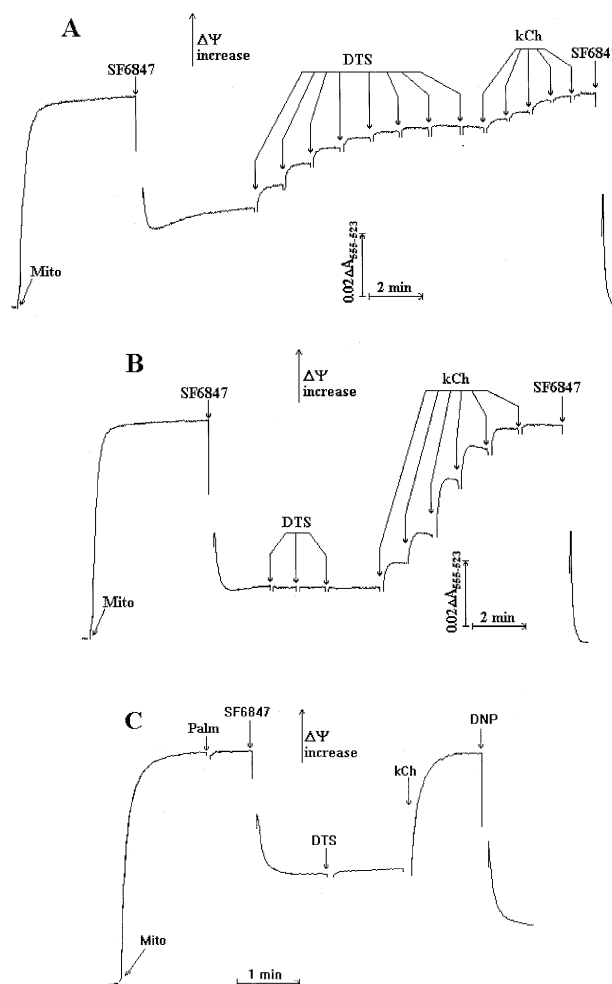


Fig. 2. Recoupling by DTS. Effect on $\Delta\Psi$. The serum albumin requirement and palmitate inhibition of recoupling. The incubation mixture contained 250 mM sucrose, 5 mM MOPS/Tris (pH 7.4), 3 mM succinate, 2 mM EGTA, 2 μ M rotenon, 3 μ g/ml oligomycin, 8 μ M safranin O. In A and C, the mixture was supplemented with serum albumin (0.64 and 0.2 mg \times ml $^{-1}$, respectively). Additions: A, the rat liver mitochondria (0.5 mg protein \times ml $^{-1}$), 80 nM (1st addition) and 240 nM (2nd addition) SF6847, 12 μ M DTS (each addition), and 2.5, 2.5, 5, 5, 10 μ M kCh; B, the rat liver mitochondria, 0.5 mg protein \times ml $^{-1}$, 10 nM (1st addition) and 120 nM (2nd addition) SF6847, 30 μ M DTS (each addition) and 2.5, 2.5, 5, 5, 10, 10 μ M kCh; C, the rat liver mitochondria (0.7 mg protein \times ml $^{-1}$), 6 μ M palmitate, 40 nM SF6847, 75 μ M DTS, 50 μ M kCh, and 40 μ M DNP.

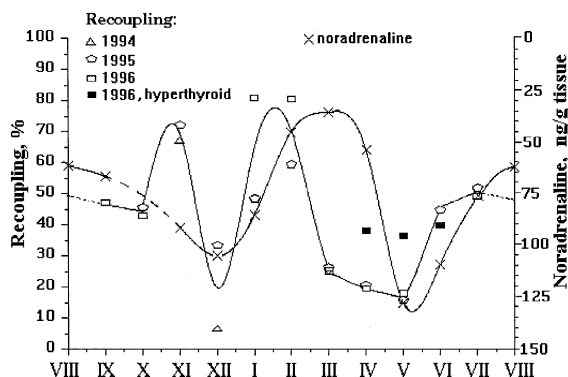


Fig. 3. Seasonal dependence of the DTS recoupling effect in the rat liver mitochondria and of the noradrenaline level in the rat liver tissue. The noradrenaline level data are from Montagu [13]. Note that upward deflection of the noradrenaline trace means decrease in the noradrenaline concentration.

SF6847. Moreover, they decrease the recoupling efficiency of DTS (not shown in the figures).

Just as kCh, male sex hormones and progesterone proved to be effective recouplers at low, but not at high, SF6847 concentrations (see below, Fig. 5).

One more common feature of recoupling effects of the hormones and kCh was revealed when a series of different uncouplers was studied. In both cases, recoupling was observed with SF6847, FCCP and CCCP and was not with fatty acids, DNP and gramicidin.

DTS failed to reverse uncoupling caused by zearalenone, a natural uncoupler from plant, structurally related to steroids (not shown). On the other hand, kCh partially recoupled in samples with zearalenone [1].

Further experiments showed that cyclosporin A can prevent the DTS-induced recoupling. This effect seems to be different from well-known inhibition by cyclosporin A of the permeability transition pore in mitochondria [6] since PSC 888, a cyclosporin A analog, inactive in the pore inhibition, was also inhibitory for the DTS recoupling. Ca^{2+} proved also to be unfavorable for the hormonal recoupling action whereas inorganic phosphate, adenine nucleotides and carboxyatractylate were without significant influence. At the concentrations used, DTS did not affect the State 3 respiration rate (succinate was the substrate), the level of $\Delta\Psi$ in State 4 as well as the $\Delta\Psi$ generation by the mitochondrial H^+ -ATPase. Recou-

pling could be demonstrated not only in the liver but also in the heart and skeletal muscle mitochondria. No DTS effects were observed in beef heart submitochondrial particles, bacterial chromatophores and cytochrome oxidase proteoliposomes (not shown).

The studies carried out in 1994–1996 revealed a pronounced seasonal dependence of the hormonal recoupling. It proved to be maximal in January and February and from June to November. The recoupling was low in the spring months and in December (Fig. 3). From May to February the recoupling activity of DTS on the isolated rat liver mitochondria showed reverse correlation with the level of noradrenaline in rat liver tissue reported by Montagu [13]. In March and April, however, the recoupling was small in spite of a low level of noradrenaline. Just in these months, the recoupling effect increased if animals obtained thyroxine, 3,5,3'-triiodothyronine or 3,5-diiodothyronine (Fig. 3). An example of this kind is also shown in Fig. 4. In this experiment, 'spring rats' were studied. One can see that the DTS recoupling in the euthyroid animal (Fig. 4A) was

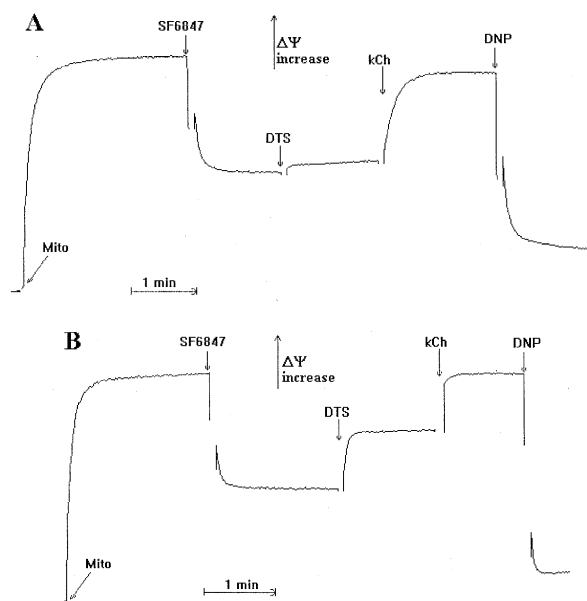


Fig. 4. Improvement of the DTS recoupling effect on mitochondria by the in vivo thyroid hormone treatment. A and B, the eu- and hyperthyroid 'spring' rats, respectively. Additions, the rat liver mitochondria ($0.6 \text{ mg protein} \times \text{ml}^{-1}$), 30 (A) and 40 (B) nM SF6847, 75 μM DTS, 50 μM kCh, 40 μM DNP.

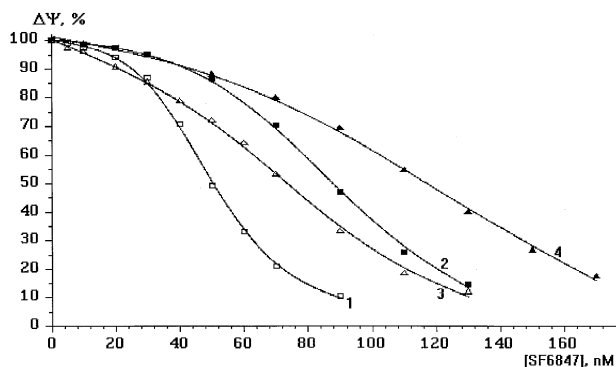


Fig. 5. The $\Delta\Psi$ level in mitochondria as a function of the SF6847 concentration in hyperthyroid rat (curves 1 and 2) and euthyroid rat (curves 3 and 4) 'spring' rats. In all four cases, the $\Delta\Psi$ levels without addition of SF6847 were almost the same. Curves 2 and 4, 75 μM DTS was added before the uncoupler.

very small, whereas in the hyperthyroid one it was quite measurable (Fig. 4B).

In the hyperthyroid rats, respiratory control was found to be decreased (not shown). The data obtained are in line with observations on the uncoupling effect of thyroid hormones (for discussion, see below). In fact, the hyperthyroid mitochondria looked as if they

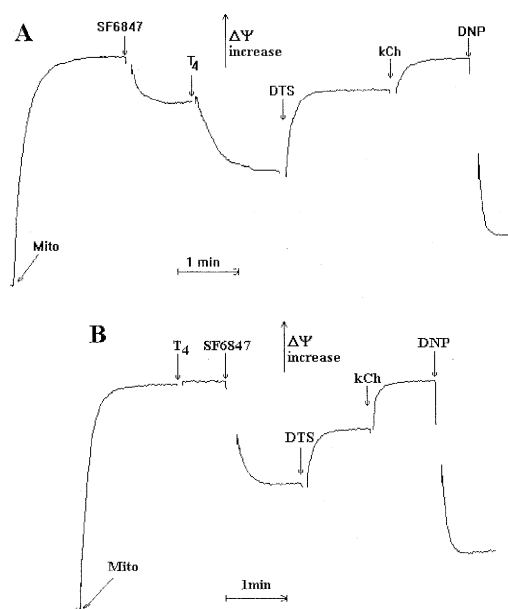


Fig. 6. Effect of the in vitro thyroxine additions on mitochondria partially uncoupled by SF6847. The incubation mixture was supplemented with 2 mM potassium phosphate. Additions, the rat liver mitochondria (0.55 mg protein \times ml $^{-1}$), 30 nM SF6847, 8 μM thyroxine (T_4), 75 μM DTS, 50 μM kCh and 40 μM DNP.

already contain some uncoupler, so the addition of a small amount of SF6847 was without influence. This resulted in the more pronounced sigmoid shape of the SF6847 titration curve in the hyperthyroid rats. The DTS addition shifted these curves to higher SF6846 concentrations both in the eu- and hyperthyroid animals, the shift being stronger in the latter case (Fig. 5).

In the same experiments, a partial recoupling by DTS of the thyroid hormone-induced uncoupling was revealed. To this end, mitochondria were treated with azide to lower activity of the respiration-linked $\Delta\Psi$ generation. In the presence of azide, the $\Delta\Psi$ level decreased since rate of the endogenous H^+ leak became of the same order of magnitude as that of the H^+ pumping by the respiratory chain. Under these conditions, addition of DTS was shown to produce small but reproducible $\Delta\Psi$ increase. Further kCh addition was without effect (not shown). These data, together with those on the seasonal dependence (Fig. 3), suggest that the DTS recoupling like the thyroid hormone uncoupling, is an in vivo-controlled physiological phenomenon rather than an in vitro artifact.

Thyroxine when added to mitochondria in vitro after low SF6847 concentration was shown to stimulate uncoupling in a DTS- and kCh-sensitive fashion. Thyroxine added before SF6847 did not influence $\Delta\Psi$ but increased the $\Delta\Psi$ lowering by subsequent SF6847 addition (Fig. 6). On the other hand, thyroxine did not inhibit respiration. Serum albumin proved to be necessary for both thyroxine-stimulated uncoupling and DTS-induced recoupling. Moreover, it de-

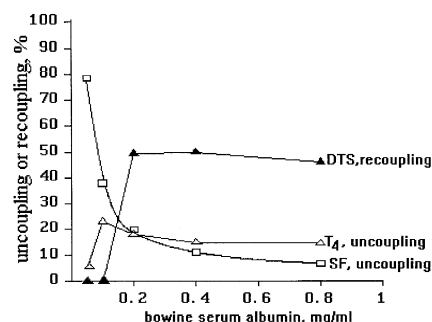


Fig. 7. Effect of serum albumin on the uncoupling action of SF6847 and thyroxine and on the recoupling action of DTS. Uncoupling, a $\Delta\Psi$ decrease by subsequent additions of 30 nM SF 6847 and 8 μM thyroxine; recoupling, a $\Delta\Psi$ increase by addition of 75 μM DTS.

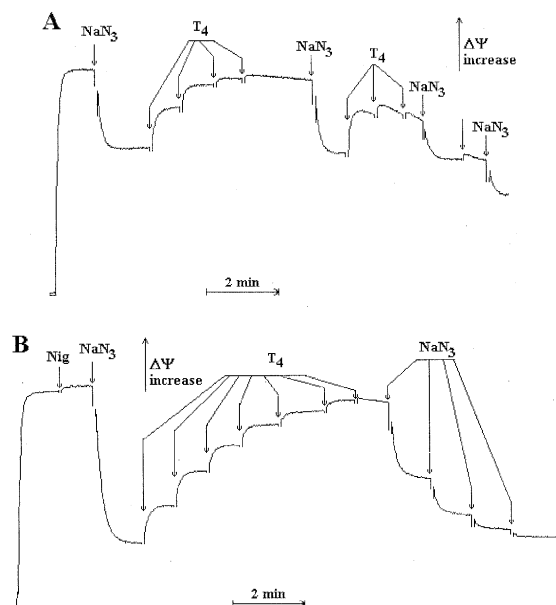


Fig. 8. Reversal by thyroxine of the azide inhibition of $\Delta\Psi$ formation in the rat liver mitochondria. The incubation mixture was as in Fig. 9 but in B the phosphate concentration was increased up to 15 mM. Additions, A, 0.75, 1, 1 and 2 mM NaN_3 (the 1st, the 2nd, the 3rd and the 4th additions, respectively); T_4 , 2 μM (the 1st four additions), 8 μM (the next three additions), 16 μM (the last addition) thyroxine; B, 50 nM nigericin; 6 and 10 mM NaN_3 (the 1st and the other additions, respectively); T_4 , 16 μM (each addition) thyroxine.

creased the uncoupling efficiency of SF6847 (Fig. 7). Triiodo- and diiodothyronines proved to be less efficient than thyroxine (not shown).

One more *in vitro* effect of thyroxine was found when mitochondrial respiration was partially inhibited by azide. It was shown that thyroxine reverses the inhibition of mitochondrial respiration and the $\Delta\Psi$ decrease caused by low azide concentration. It was shown that 1 mM azide caused a two-fold decrease in the respiration rate of the oligomycin-treated mitochondria. Thyroxine stimulated the inhibited respiration by about 40% (not shown in the figure). This was accompanied by complete reversal of the azide-induced $\Delta\Psi$ decrease (Fig. 8A). Such an effect could hardly be explained by azide release from mitochondria due to dissipation of ΔpH . The azide inhibitory efficiency was really decreased by such ΔpH -abolishing agents as nigericin and 15 mM phosphate but effect of thyroxine was still present if higher hormone concentrations were used (Fig. 8B). In the

other experiment, addition of 6 nM SF6847 to the oligomycin-treated mitochondria caused 5-fold stimulation of respiration. This stimulation was abolished by 2 mM azide. Subsequent addition of thyroxine increased the respiration rate by factor 1.5. Serum albumin proved to be unnecessary for these thyroxine effects. Triiodothyronine was almost ineffective and diiodothyronine was completely ineffective in release of the azide inhibition. In the cytochrome oxidase proteoliposomes, thyroid hormones failed to decrease the azide inhibition. Inhibition of the respiration rate and $\Delta\Psi$ formation by cyanide, formate, malonate or antimycin A were not affected by thyroxine (not shown).

In the last series of experiments, the recoupling activity of DTS was tested in the intact animal cells. It was shown that DTS added after oligomycin and small concentrations of SF6847 or FCCP gave rise to significant inhibition of the respiration rate of isolated thymocytes or Krebs-II cells. Subsequent addition of high concentration of uncouplers stimulated respiration. In fact, these relationships are similar to those shown in isolated mitochondria. However, there was also some difference. It consists in that serum albumin which was required to be added to mitochondria to show the DTS recoupling, proved to be unnecessary in the case of the intact cells.

4. Discussion

4.1. Effects of steroid hormones

The above-described recoupling effect of male sex hormones and progesterone resembles, to some degree, that of kCh. In both cases, recoupling took place with low concentrations of SF6847, FCCP and CCCP. Recoupling did not occur at high concentrations of the uncouplers listed and at any concentrations of DNP, pentachlorophenol, TTFB, fatty acids and gramicidin. The effect of both kCh and hormones could not be shown in planar bimolecular phospholipid membrane [1]. At the same time, some differences in, e.g., DTS and kCh recoupling were revealed. (i) kCh induced complete reversal of the SF6847 uncoupling effect, whereas the DTS recoupling was always incomplete. (ii) The kCh recoupling

could be shown in animal mitochondria [1], plant mitochondria [7], bacterial chromatophores [1] and cytochrome oxidase proteoliposomes [1]. Hormones proved to be active in animal mitochondria only. (iii) Serum albumin proved to be required for recoupling of mitochondria by hormones, not by kCh. (iv) Added palmitate abolished effect of hormones, not of kCh. (v) The recoupling by hormones, not by kCh, depended on season and thyroid status of the animal (on seasonal changes in the thyroid and male sex hormone levels, see [8–12]). It should be stressed that the DTS recoupling activity showed, in fact, reverse correlation with the seasonal variation of the noradrenaline level in rat liver, reported by Montegu [13] (Fig. 3). On the other hand, no correlation with the tissue adrenaline concentration was revealed. These relationships may be due to the well known ability of noradrenaline to increase the plasma and tissue levels of non-esterified fatty acids, small concentrations of which are, according to our data, inhibitors of the

DTS recoupling, whereas serum albumin, which binds fatty acids, stimulates the recoupling (see Figs. 2 and 7).

Generally, the hormone effect looks more specific and delicate, i.e., more ‘biological’ than that of kCh. One may speculate that more hydrophobic kCh possessing a hydrocarbon tail at 17th position does not require specific receptor which is apparently involved in action of less hydrophobic hormones lacking such a tail.

Specificity of the hormonal effect is clearly demonstrated when a series of structurally related compounds was tested (Fig. 9). For instance, progesterone and deoxycorticosterone differ only by a single OH-group (at 17th position, $\text{CH}_3\text{CO-}$ in progesterone, $\text{HOCH}_2\text{CO-}$ in deoxycorticosterone), and this difference results in that the former is a recoupler, the second is not. Remember that these two compounds show quite different hormonal effects *in vivo*. Similarly, epiandrosterone, the DTS isomer of very low

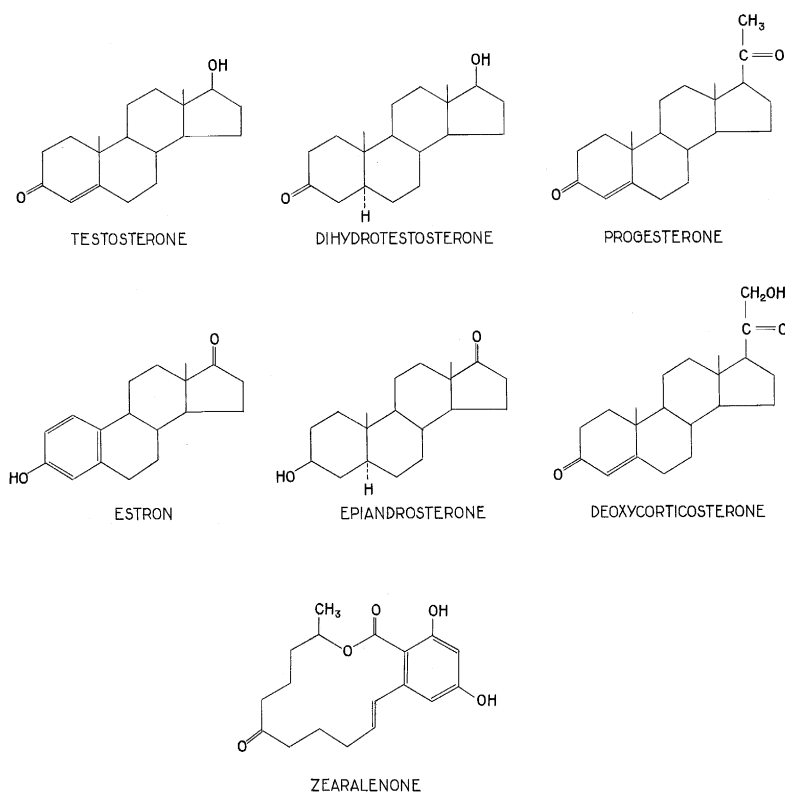


Fig. 9. Steroid hormones active and inactive as recouplers (the upper and the middle rows, respectively), and zearalenone (the lower formula), a natural uncoupler from some plants and fungi.

hormonal activity (3-ol, 17-one instead of 3-one, 17-ol), does not show any recoupling effect. These facts point to correlation of recoupling effects of DTS, TS and progesterone *in vitro* and their hormonal action *in vivo*. It is known that male sex hormones, besides their sex-regulating activity, possess some *in vivo* anabolic effects generalized to many tissues [8]. These effects, requiring much higher hormone concentrations than the sex-regulating ones, might be related to their recoupling action on mitochondria, described in this paper. It seems possible that the *in vivo* recoupling, if it exists, is produced by concentrations of hormones lower than used in our *in vitro* experiments (tens of $\mu\text{mol per l}$; cf. $1\ \mu\text{M}$, maximal progesterone concentration in blood [8]). The *in vivo* effects may consist of slow saturation of a mitochondrial hormonal receptor. It should be mentioned that the above-described *in vitro* recoupling by, e.g., progesterone includes a very slow phase of the $\Delta\Psi$ increase.

The simplest explanation of the DTS recoupling consists in that the mitochondrial DTS receptor is identical to the inner membrane protein responsible for translocation of the main portion of the SF6847 anions. When combined with this protein on the outer surface of the inner mitochondrial membranes, DTS may cause dipole potential asymmetry just as it takes place with kCh [1]. However, DTS, in contrast with more hydrophobic kCh, cannot traverse the hydrophobic membrane barrier. This is why the DTS effect does not disappear in time and cannot be shown in the inside-out submitochondrial particles. The latter fact indicates that the DTS recoupling cannot be accounted for by such trivial explanations as binding of SF6847 by DTS, a DTS interaction with the membrane lipids, etc. No indications of a SF6847-DTS or T_4 -DTS complex formation in solution were obtained by the spectral measurements.

Anabolic effect of male sex hormones might be explained in terms of an increase in efficiency of respiratory energy transduction due to recoupling provided that mitochondria *in vivo* are, to some degree, uncoupled by a natural analog of SF6847. In this group, it was proposed that the analog in question is a thyroid hormone namely diiodothyronine [14,15]. In any case, antagonism of thyroid hormones as catabolics and steroid hormones as anabolics is a well known fact. It is also known that hypo- and

hyperthyroidism are accompanied with a decrease and increase in the H^+ conductance of mitochondrial membrane, respectively [14–19].

There is a natural uncoupler operating in a kCh-sensitive, DTS-insensitive fashion. We mean zearalenone, a compound found in plants and fungi. Zearalenone was shown to be responsible for development of the estrogenic syndrome in animals fed by *Fisarium*-infected grains [20]. As was recently shown, there is a correlation between the endogenous zearalenone level and induction of floral buds in tobacco [21]. Macri, Vianello and co-workers discovered uncoupling effect of zearalenone on plant mitochondria [22,23] and its sensitivity to kCh [24]. Zearalenone structurally resembles steroid hormones (Fig. 9).

In connection with the problem of a possible physiological role of the steroid hormone-linked recoupling, it seems noteworthy that the DTS inhibition of respiration stimulated by low SF6847 concentrations was demonstrated in intact animal cells, i.e., in thymocytes and Krebs-II. Interestingly, in this case serum albumin addition proved unnecessary. This might indicate that inside the cells, DTS was assisted by an intracellular protein playing the role performed by serum albumin in the experiments on isolated mitochondria. The role of this hypothetical protein as well as of serum albumin, might consist of DTS binding, assuming that recoupling is carried out by a DTS-protein complex rather than by free DTS. Specific stoichiometric binding of male sex hormones and progesterone with serum albumin was described [25–29].

An alternative possibility is that serum albumin extracts from mitochondria an endogenous compound preventing the DTS recoupling. This might be small amounts of fatty acids which were shown to abolish the DTS recoupling when added to mitochondria. The saturating character of the dependence of DTS recoupling upon the albumin concentration (Fig. 7) supports such an explanation of the mechanism of albumin effect.

Discussing the regulation of the mitochondrial functions by male sex hormones, we can mention the observation by Konths and co-authors [30] that testosterone increases the mitochondrial protein synthesis when administered to castrated animals. As for *in vitro* effects, steroid hormones added to mitochondria were found to inhibit the NADH-CoQ span of the

respiratory chain [31–33], the State 3 respiration with succinate as the substrate [34] and uniport of monovalent cations through the mitochondrial membrane at high pH [35]. These unfavorable effects could be accounted for by damage to some mitochondrial membrane systems by rather high concentrations of such hydrophobic compounds as steroids. Recoupling described in this and related [1,2,36] papers may be regarded as precedent of a favorable effect of steroid hormones improving the mitochondrial energy transduction damaged by some uncouplers. Investigation in potential antidote activity of DTS against some xenobiotics possessing uncoupling effect [37] seems to be promising.

4.2. Effects of thyroid hormones

Three kinds of thyroid hormone effects were described in this paper.

(1) The in vitro addition of thyroxine to mitochondria partially uncoupled by SF6847 causes further uncoupling which is reversed by DTS and kCh. Tri- and diiodothyronines are less effective than thyroxine.

(2) The in vitro addition of thyroxine to the azide-inhibited mitochondria in State 4 reverses the effect of azide on respiration and $\Delta\Psi$ if the azide concentration is not too high. Respiration inhibition by azide in State 3 is partially reversed by thyroxine. Triiodothyronine is much less effective than thyroxine, diiodothyronine being without any effect.

(3) The in vivo administration of thyroxine, tri- and diiodothyronines stimulates the in vitro recoupling of the SF6847-uncoupled mitochondria by DTS in the spring when this recoupling is small.

Effect (1) was partially reversed by DTS and completely by kCh, it required serum albumin to be present, and took place in the case of uncoupling by SF6847 or FCCP but not by DNP or palmitate. All these features are inherent in uncoupling induced by SF6847 without thyroxine. As for effect (2), it proved to be DTS- and kCh-resistant and did not require albumin.

Micromolar concentrations of thyroxine causing effects (1) and (2) are higher than those used to saturate the nuclear thyroid hormone receptor but lower than concentrations causing the in vitro uncoupling [16].

It is not clear yet whether these in vitro effects are related to the in vivo hormone action. Dependence of the above activity upon number of iodine atoms in the hormone molecule, resembling that for some biological actions of thyroid hormones, might point to some physiological meaning to the effects in question.

The in vivo effect (3) is remarkable in that 3,5-diiodothyronine proved to be effective. This is in contrast to effects (1) and (2) and resembles observation by Horst et al. [17] that the oxygen consumption by perfused liver of the hypothyroid rat (which is lower than that of the euthyroid animal) is specifically increased by diiodothyronine, whereas similar effects of thyroxine and triiodothyronine are abolished by a deiodinase inhibitor. Our experiments showed that diiodothyronine does not induce the glycerophosphate dehydrogenase in the liver mitochondria, whereas thyroxine does, indicating that the diiodothyronine effect is mediated by a mitochondrial (rather than by a nuclear) receptor (see also Ref. [16]). Just this effect might be related to the well-known antagonism of thyroid and steroid hormones as catabolics and anabolics, respectively [8,15].

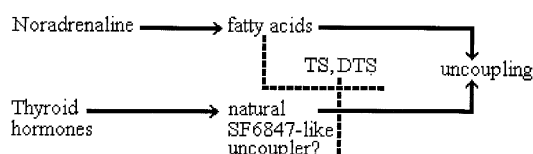
4.3. Possible relationships of thyroid and steroid hormones and noradrenaline

In this group, it was postulated [14,15,38] that thyroid hormone-mediated uncoupling is involved in the anti-oxygen defense system preventing 'parasitic' reactions of the one electron O_2 reduction. Such an uncoupling decreases the thermodynamic efficiency of respiration since respiratory substrates and O_2 are consumed with no ATP formed. However, it can be favorable for maintenance of the concentrations of both O_2 and one electron O_2 -reductants at low levels irrespective of the ADP availability. It was also suggested that male sex hormones and progesterone attenuate this energy-dissipative mechanism [15].

On the other hand, noradrenaline-mediated uncoupling is shown to participate in thermoregulatory responses of the warm-blooded animals [37]. The latter effect includes an increase in the mitochondrial level of non-esterified fatty acids which operate as protonophores [37]. Apparently noradrenaline uncoupling develops in addition to the thyroid hormones uncoupling when more heat should be produced.

Within the framework of this reasoning, it will not be surprising if noradrenaline abolishes recoupling by male sex hormones. Such a cross-talk of thyroid and noradrenaline regulations favorable for the heat production could be realized by abolishing the male sex hormone recoupling with fatty acids released due to the lipolysis activation by noradrenaline. In this way, one may explain reverse correlation of the noradrenaline concentration in rat liver tissue and magnitude of the DTS recoupling in rat liver mitochondria. Such a correlation was revealed from May to February (see Fig. 3). In March and April, the DTS recoupling was small although the noradrenaline level was low. Just in these months strong lowering of the thyroid hormones production was reported [11]. This can account for the fact that the *in vivo* administration of thyroid hormones during this season increases ability of the male sex hormones to recouple (see Figs. 3 and 4).

Thus, the following relationships of the thyroid, steroid and noradrenaline control of the mitochondrial energy coupling may be assumed:



According to this scheme, noradrenaline increases the fatty acid level, which results in uncoupling. Thyroid hormones are also competent in uncoupling but it is mediated by a natural SF6847-like uncoupler rather than by fatty acids. This uncoupling is attenuated by TS and DTS. The recoupling effect of the male sex hormones is arrested by fatty acids. As a result, fatty acids not only uncouple *per se* but also stimulate the thyroid hormone-induced uncoupling by preventing the TS and DTS recoupling. This effect requires much smaller fatty acid concentrations than those causing uncoupling. If the level endogenous fatty acids in isolated mitochondria is low, serum albumin can remove them to allow DTS and TS to recouple. If it is high, serum albumin fails to do this.

Existence of a natural SF6846-like uncoupler in the above scheme is certainly quite speculative at present. However, all the logic of this study suggests that such an uncoupler is more than a product of our

imagination. As was mentioned in Section 3, in hyperthyroid rats, small but reproducible recoupling action of DTS was revealed in samples without any added uncoupler. In other words, DTS is competent, to some degree, in reversal of endogenous uncoupling taking place in the hyperthyroid mitochondria.

Acknowledgements

We are very grateful to Professor F. Macri and Professor A. Vianello for the gift of a sample of zearalenone and to Dr. T. Dmitrieva for providing us with mice injected with ascite carcinoma cells. This study was made possible by Grant 95-04-12799a from The Russian Foundation for Basic Research.

References

- [1] Starkov, A.A., Bloch, D.A., Chernyak, B.V., Dedukhova, V.I., Mansurova, S.A., Severina, I.I., Simonyan, R.A., Vygodina, T.V. and Skulachev, V.P. (1997) *Biochim. Biophys. Acta* 1318, 159–172.
- [2] Starkov, A.A., Dedukhova, V.I., Bloch, D.A., Severina, I.I. and Skulachev, V.P. (1995) in *Thirty Years of Progress in Mitochondrial Bioenergetics and Molecular Biology* (F. Palmieri et al., eds.), Elsevier, Amsterdam, pp. 51–55.
- [3] Andreyev, A.Yu., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.I. and Vygodina, T.V. (1989) *Eur. J. Biochem.* 182, 585–592.
- [4] Carr, F.E., Bingham, C., Oppenheimer, J.H., Kistner, C. and Mariash, C.N. (1984) *Proc. Natl. Acad. Sci. USA* 81, 974–978.
- [5] Dedukhova, V.I. and Mokhova, E.N. (1987) *Biokhimiya* 52, 1324–1334 (in Russian).
- [6] Zoratti, M. and Szabo, I. (1995) *Biochim. Biophys. Acta* 1241, 139–176.
- [7] Vianello, A., Macri, F., Braidot, E. and Mokhova, E.N. (1995) *FEBS Lett.* 365, 7–9.
- [8] Heftmann, E. (1970) *Steroid Biochemistry*, Academic Press, New York.
- [9] Nilssen, K.J., Bye, K., Sundsfjord, J.A. and Blix, A.S. (1985) *Gen. Comp. Endocrinol.* 59, 210–213.
- [10] Laplaud, P.M., Saboureau, M., Beaubatie, L. and el-Omari, B. (1989) *Biochim. Biophys. Acta* 1005, 143–156.
- [11] Webster, J.R., Moenter, S.M., Woodfill, C.J. and Karsch, F.J. (1991) *Endocrinology* 129, 176–183.
- [12] Shi, Z.D. and Barrell, G.K. (1994) *Reproduction, Fertility and Development* 6(2), 187–192.
- [13] Montagu, K.A. (1956) *Nature* 178, 417–418.

- [14] Skulachev, V.P. (1995) *Mol. Biologiya* 29, 709–715 (in Russian).
- [15] Skulachev, V.P. (1996) *Quart. Rev. Biophys.* 29, 169–202.
- [16] Soboll, S. (1993) *Biochim. Biophys. Acta* 1144, 1–16.
- [17] Horst, C., Rokos, H. and Seitz, H.J. (1989) *Biochem. J.* 261, 945–950.
- [18] Brand, M.D., Steverding, D., Kadenbach, B., Stevenson, P.M. and Hafner, R.P. (1992) *Eur. J. Biochem.* 296, 775–781.
- [19] Horrum, M.A., Tobin, R.B. and Ecklund, R.E. (1996) *Biochem. Mol. Biol. Intern.* 38, 61–72.
- [20] Mirocha, C.J., Christensen, C.M. and Nelson, G.H. (1971) *Microbial Toxins* (Kadis, S., Ciegler, A. and Ajl, S.J., eds.), Vol. VII, Academic Press, New York, pp. 107–138.
- [21] Fu, Y., Li, H. and Meng, F. (1995) *J. Plant Physiol.* 147, 197–202.
- [22] Vianello, A. and Macri, F. (1981) *Planta* 153, 443–446.
- [23] Macri, F. and Vianello, A. (1990) *J. Plant Physiol.* 136, 443–446.
- [24] Macri, F., Vianello, A., Braidot, E., Petrusa, E. and Mokhova, E.N. (1996) *Biochem. Mol. Biol. Intern.*, in press.
- [25] Attallah, N.A. and Lata, G.F. (1968) *Biochim. Biophys. Acta* 168, 321–333.
- [26] Sergeev, P.V., Uliankina, T.I., Sejfulla, R.D., Grebenshchikov, Yu.B. and Lichtenstein, G.I. (1974) *Mol. Biologiya* 8, 206–217 (in Russian).
- [27] Romeu, A.M., Martino, E.E. and Stoppani, A.O.M. (1975) *Biochim. Biophys. Acta* 409, 376–386.
- [28] Ogurtsov, S.I., Wesela, I.W., Kamernitsky, A.N., Moshkovsky, Yu.Sh., Terekhina, A.I., Kharakhonicheva, N.V. and Kuznetsov, A.N. (1978) *Biofisika* 23, 432–435 (in Russian).
- [29] Ogurtsov, S.I. and Kuznetsov, A.N. (1978) *Biofisika* 23, 538–539 (in Russian).
- [30] Koths, K.E., Godchaux, W., Doeg, L.H. and Doeg, K.A. (1972) *Endocrinology* 91, 125–134.
- [31] Yielding, K.L., Tomkins, G.M., Munday, J.S. and Cowley, I.J. (1960) *J. Biol. Chem.* 235, 3413–3416.
- [32] Vallejos, R.H. and Stoppani, A.O.M. (1967) *Biochim. Biophys. Acta* 131, 295–309.
- [33] Varricchio, F. and Sanadi, D.R. (1967) *Arch. Biochim. Biophys.* 121, 187–193.
- [34] Aleksandrowicz, Z., Swierczynski, J. and Zelewski, L. (1972) *Eur. J. Biochem.* 31, 300–307.
- [35] Jung, D.W. and Brierly, G.P. (1981) *Experientia* 37, 237–238.
- [36] Starkov, A.A., Dedukhova, V.I. and Skulachev, V.P. (1994) *FEBS Lett.* 355, 305–308.
- [37] Skulachev, V.P. (1988) *Membrane Bioenergetics*, Springer, Berlin.
- [38] Skulachev, V.P. (1994) *Biochemistry (Moscow)* 59, 1910–1912.