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# SITE-SPECIFIC EFFECTS OF STEROIDS ON MITOCHONDRIAL METABOLISM

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SUMMARY

1. Relatively low concentrations of steroids hormones, particularly progesterone and deoxycorticosterone acetate inhibit the oxidation of L-malate–L-glutamate by phosphorylating rat-liver mitochondria in metabolic State 3. The inhibition takes place by direct action upon the NAD-flavoprotein region of the electron-transport chain and is not affected by dinitrophenol, carbonylcyanide–*m*-chlorophenylhydrazone (CCCP) and dicoumarol.

2. Progesterone and deoxycorticosterone acetate inhibit succinate oxidation by ADP-activated mitochondria but relatively higher concentrations of steroid are required to produce the same inhibition as with NAD-linked substrates. The inhibition of succinate oxidation is caused by the interference of steroids with energy transfer at the second phosphorylating site since it can be released by dinitrophenol, CCCP and triamcinolone but not by stilbestrol which uncouples preferentially at the third phosphorylating site.

3. In contrast with oligomycin, progesterone and deoxycorticosterone acetate do not prevent the release of respiratory control by the combined action of $Ca^{2+}$ and $P_i$ neither do they inhibit the dinitrophenol-activated mitochondrial ATPase (ATP-phosphohydrolase, $Mg^{2+}$-activated, EC 3.6.1.4).

4. Progesterone prevents the swelling of mitochondria by stilbestrol monomethyl ether and triamcinolone. At high concentrations, progesterone induces mitochondrial swelling and uncouples oxidative phosphorylation but these effects, as well as the inhibition of energy transfer by progesterone and deoxycorticosterone acetate, are of secondary metabolic importance compared with the inhibition of electron transfer by the same steroids.

5. Progesterone is a more effective inhibitor of mitochondrial respiration than the metabolically related steroids pregnenolone, pregnandione and *allo*-pregnanolone,

Abbreviations: stilbestrol, α,α′-diethylstilbestrol; dinitrophenol, 2,4-dinitrophenol; CCCP, carbonylcyanide–*m*-chlorophenylhydrazone; dicoumarol, 3,3′-methylene-bis (4-hydroxycoumarin); TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

Steroids nomenclature: norprogesterone, 19-norpregn-4-ene-3,20-dione; pregnenolone, 3β-hydroxypregn-5-ene-20-one; pregnandione, 5β-pregnan-3,20-dione; *allo*-pregnanolone, 3α-hydroxy-5α-pregnan-20-one; chlormadinone, 6-chloro-17α-acetoxypregna-4,6-diene-3,20-dione; prednisone, 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione; triamcinolone, 9α-fluor-11β,16α, 17α,21-tetrahydroxypregna-1,4-diene-3,20-dione; other steroids are referred to by their trivial names.

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and also than the artificial gestagens norprogesterone, 4-hydroxy-17α-acetoxyprogesterone, chlormadinone and 1-dehydrochlormadinone. Similarly, deoxycorticosterone acetate is more effective than cortisol, cortisone acetate and prednisone as inhibitor of mitochondrial respiration.

## INTRODUCTION

It is known[1–8] that steroid hormones can inhibit mitochondrial respiration and oxidative phosphorylation but the mechanism of these inhibitions does not seem to be clearly established. Concerning the inhibition of electron transport it has been postulated that there is (a) a direct action of steroids on the respiratory chain, as with non-phosphorylating systems[2,3,6–8], and (b) a steroid-induced alteration of the mitochondrial membranes leading to the loss of intramitochondrial coenzymes which are essential for respiration[4,5]. In addition to their action on electron transfer some steroids, *e.g.* progesterone, are capable of inhibiting energy transfer[6,7] and also of uncoupling oxidative phosphorylation[2]. These different effects are not necessarily linked because triamcinolone, an artificial corticoid, is quite a powerful uncoupling agent but does not inhibit electron or energy transfer[9,10]. These observations[1–10] inspired the present study whose aim was to obtain more information on the mode of action and locus-specificity of steroids on mitochondrial respiration. Most of the experiments were carried out with progesterone but for comparative purposes other steroids were also assayed, particularly deoxycorticosterone acetate and some artificial progestins.

## MATERIAL AND METHODS

### *Mitochondria*

Rat-liver mitochondria were prepared by the method of SCHNEIDER[11]. The liver cells were disrupted with a Potter–Elvehjem homogenizer consisting of a motor-driven (800–1000 rev./min) Teflon pestle (Kontes Glass Co), fitting a 20 cm × 2.2 cm glass tube. The homogenization medium was 0.25 M sucrose–1 mM EDTA–10 mM Tris–HCl (pH 7.4). The homogenates were fractionated according to MYERS AND SLATER[12]. The mitochondria were suspended in the homogenization medium without EDTA and used not later than 3 h after the completion of the preparation. The protein concentration of the mitochondrial suspension was determined with the biuret method[13].

### *Reagents*

The following were used: stilbestrol was a gift from Glaxo Laboratories or was purchased from Sigma Chemical Co.; stilbestrol monomethyl ether was purchased from Dextran Chemical Inc., New York; ADP, ATP, oligomycin, antimycin A, 17β-estradiol, pregnenolone, 3-methylpregnenolone, 16-dehydropregnenolone, pregnandione, *allo*-pregnanolone and 16,17-epoxy-deoxycorticosterone acetate, were purchased from Sigma Chemical Co; carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was the gift of E. I. DuPont de Nemours and Co., through the courtesy of Dr. P. G. HEYTLER; dicumarol (Marcoumar) was the gift of F. Hoffman-La Roche and Co. Ltd.; and

phenyl-ethyl biguanidine the gift of U.S. Vitamin and Pharmaceutical Corp. Progesterone, testosterone, deoxycorticosterone acetate, cortisone acetate and cortisol, were gifts from Ciba A.G.; pregnenolone acetate from Schering A.G.; norprogesterone, chlormadinone, and 1-dehydrochlormadinone from Syntex; 4-hydroxy-17α-acetoxy-progesterone from Farmitalia; prednisone from LePetit, and triamcinolone from E. R. Squibb and Sons, Argentina. All solutions were made with glass-distilled, deionized water.

*Measurement of mitochondrial respiration*

This was performed polarographically at 30° with a vibrating platinum oxygen electrode (Model K Oxygraph, Gilson Medical Electronics). The oxygen concentration in the reaction media was taken as 0.22 mM at 30°. The following reaction media were used. 'Sucrose–phosphate' medium contained 0.25 M sucrose, 6.0 mM $MgCl_2$, 40 mM KCl, and 10 mM potassium phosphate buffer (pH 7.4–7.5). 'Sucrose-Tris' medium had the same composition except that phosphate was replaced by 10 mM Tris–HCl (pH 7.4–7.5). 'Ascorbate' medium contained 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl, 5 mM $MgCl_2$ and 12.5 mM potassium phosphate buffer (pH 7.5). 'NaCl' medium contained 80 mM NaCl, 10 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.2). The reaction was usually started by adding 0.2 ml of mitochondrial suspension to the medium. Steroids were dissolved in peroxide-free dioxane and added in the smallest possible volume (15–1 μl). Controls with the corresponding volume of dioxane were intercalated during runs and the values given are corrected for the effect of dioxane. Unless stated otherwise, the final volume of the reaction mixture was 1.65 ml.

*Measurement of ATPase activity*

This was based on KIELLEY's method[14]. The composition of the reaction mixture is described in Table V. After 15 min incubation at 30°, with occasional stirring, the reaction was stopped by the addition of 1 ml of 5 % (w/v) perchloric acid. After cooling and centrifugation at 0°, $P_i$ was determined in the supernatant according to FISKE AND SUBBAROW[15]. Zero-time incubation samples were also measured in order to establish the initial $P_i$ concentration.

*Measurement of swelling of mitochondria*

This was done by following the variation of absorbance at 520 mμ (ref. 16). The mitochondrial suspensions were placed in 1-cm optical path cuvettes of a Beckman DU spectrophotometer thermostated at 30°, attached to a Model 200 Gilford absorbance converter and a Leeds and Northrup 10-mV Speedomax recorder. Mitochondria were thoroughly mixed with medium and samples were automatically scanned for recording of initial absorbance at 8-sec intervals, with the aid of the Gilford automatic cuvette positioner. Steroids (or dioxane) were then added and measurements continued as described under RESULTS. From the records obtained, the absorbance *vs.* time curves were plotted. From these plots the diminution of absorbance per min was calculated ($\Delta A \times 10^3$/min). The corresponding values, less the variation in absorbance of the respective control samples, are expressed in Table VI as 'rate of swelling'. Variation in the absorbance of controls was usually negligible even in the presence of dioxane ($\Delta A \times 10^3$/min, 0–10).

*Expression of results*

Unless stated otherwise, the rate of respiration of mitochondrial suspensions is represented as mμatoms O/mg of mitochondrial protein per min. The amount of mitochondria used in each experiment is expressed in mg/ml.

## RESULTS

*Comparative effects of progesterone and other steroids on mitochondrial respiration*

Addition of increasing concentrations of progesterone to mitochondria inhibited the ADP-stimulated respiration in proportion to the steroid concentration. For comparative purposes, inhibition *vs.* concentration curves obtained with progesterone, deoxycorticosterone acetate, 17β-estradiol and testosterone are presented in Fig. 1, with both L-malate–L-glutamate and succinate as substrates. In agreement with previous reports[1,2] the higher effectiveness of progesterone and deoxycorticosterone acetate is noteworthy. Thus, with the NAD-linked substrates, the concentrations giving 50 % inhibition were 27 μM with progesterone, 45 μM with deoxycorticosterone acetate and above 0.1 mM with 17β-estradiol and testosterone. Succinate oxidation was much less inhibited by the steroids and, again, progesterone and deoxycorticosterone acetate were the more effective.

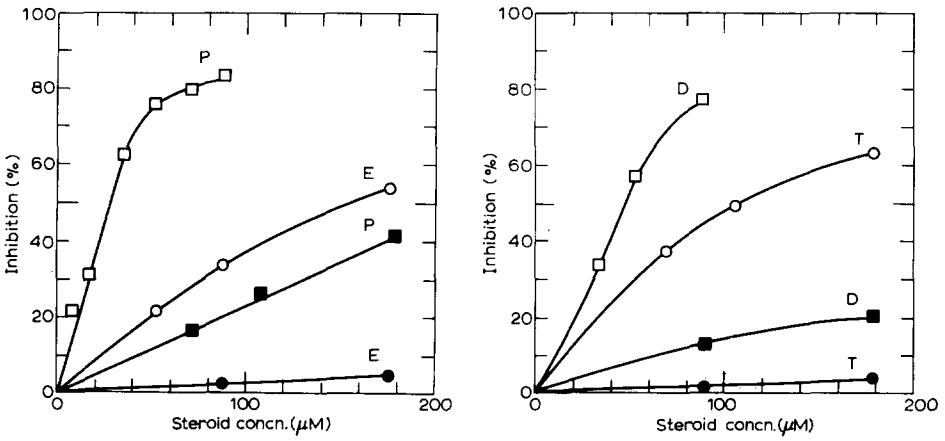


Fig. 1. Effect of steroids on L-malate–L-glutamate and succinate oxidation by mitochondria in State 3. All samples contained mitochondria (1.5 mg/ml), 'sucrose–Tris' medium, 1.3 mM ADP, and 6.0 mM $P_i$. Additions: 6.0 mM L-malate, 6.0 mM L-glutamate and 3.0 mM malonate (□, ○ experiments) or 6.0 mM succinate (■, ● experiments). Steroids were added last in the concentration stated in the abscissa. P, progesterone; E, 17β-estradiol; D, deoxycorticosterone acetate, and T, testosterone. The points represent the average values of duplicate experiments.

Besides affecting electron transfer, progesterone inhibited energy transfer. In fact, with succinate as substrate, dinitrophenol completely relieved the inhibition of respiration by progesterone (Fig. 2, left) which is evidence for the inhibition of energy transfer[7]. The same occurred with deoxycorticosterone acetate (Fig. 2, right). The site-specificity of these inhibitions can be better recognized when compared with other actions of progesterone (and deoxycorticosterone acetate) on mitochondrial respiration. These actions are presented in Table I and can be summarized as follows. (a)

No inhibition of energy transfer could be detected in the NAD-flavoprotein area of the electron-transport chain since three uncouplers (dinitrophenol, CCCP and dicoumarol) were incapable of stimulating the oxidation of NAD-linked substrates by mitochondria pretreated with progesterone or deoxycorticosterone acetate (Table I, Expts. A and B). Similar results were obtained with testosterone (90 $\mu$M) or 17$\beta$-estradiol (180 $\mu$M) as inhibitors. (b) With succinate as substrate, the inhibition of energy transfer was not accompanied by release of respiratory control because progesterone (180 $\mu$M) did not stimulate mitochondrial respiration in metabolic State 4 (Table I, Expt. C). Identical results were obtained with deoxycorticosterone acetate. (c) Progesterone did not inhibit electron transfer at the cytochrome $c \longrightarrow O_2$ link of the electron-transport chain as shown with ascorbate–TMPD as substrates (Table I, Expt. D). The same occurred with deoxycorticosterone acetate. (d) With ascorbate–TMPD, the inhibition of respiration by oligomycin was relieved to a limited extent by 360 $\mu$M progesterone which suggests a weak uncoupling effect at the third energy-conserving site. This assumption agrees with the experiment illustrated in Fig. 3 where 270 $\mu$M progesterone partially reactived the respiration of mitochondria preincubated with succinate and a lower concentration of progesterone.

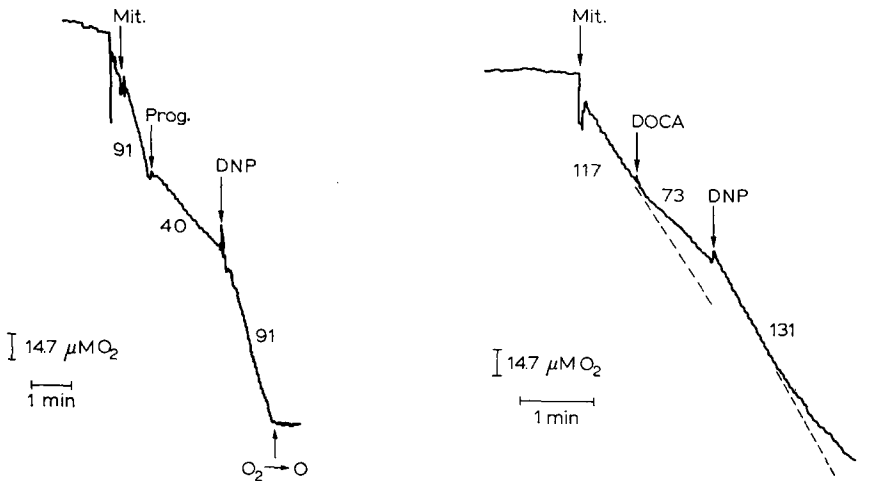


Fig. 2. Reversal by dinitrophenol of progesterone and deoxycorticosterone acetate inhibitions of respiration (mitochondria in State 3). Experimental conditions as in Fig. 1. Mitochondria (Mit.) (mg/ml), 1.7 (left) and 1.2 (right). Succinate as substrate. At the points indicated 180 $\mu$M progesterone (Prog.), 90 $\mu$M deoxycorticosterone acetate (DOCA) and 61 $\mu$M dinitrophenol (DNP) were added. The figures near the traces represent rates of respiration (m$\mu$atoms O/mg per min).

*Relief by triamcinolone of progesterone and deoxycorticosterone acetate inhibitions*

Table II (Expts. A and B) shows that the inhibition of succinate oxidation by progesterone and deoxycorticosterone acetate was relieved by a relatively small concentration of triamcinolone, an uncoupler of oxidative phosphorylation[9,10]. In order to elucidate the triamcinolone *vs.* progesterone (or deoxycorticosterone acetate) antagonism, further information on the site of action of triamcinolone was sought. Triamcinolone released the inhibition of energy transfer by phenyl-ethyl biguanidine which

TABLE I

EFFECT OF PROGESTERONE AND DEOXYCORTICOSTERONE ACETATE ON MITOCHONDRIAL RESPIRATION

Mitochondria (mg/ml): 1.5 (Expt. A), 1.6 (B), 1.2 (C), 0.74 (D) and 0.82 (E). Medium: 'sucrose–phosphate' (A, B), 'sucrose–Tris' (C) and 'ascorbate' (D, E). Additions: 3.0 mM L-malate, 3.0 mM L-glutamate and 1.5 mM malonate (Mal–Glu experiments); 6 mM succinate (Suc experiment); 15 mM ascorbate, 0.3 mM TMPD and 1.2 μg/ml antimycin A (Asc–TMPD experiments). ADP and oligomycin (1.2 μg/ml) were added where indicated. The mitochondrial suspension was added to the medium containing additions as stated above. Inhibitors (steroids, uncouplers and oligomycin) were added successively, in the order given, at about 1-min intervals.

| *Expt.* | *Additions* | | *Metabolic state* | *Inhibitors (μM)* | *Rate of respiration (mμatoms O per mg per min)* | *Inhibition of respiration (%)* |
|---|---|---|---|---|---|---|
| | *Substrates* | *ADP (mM)* | | | | |
| A | Mal–Glu | 1.3 | 3 | None | 90 | — |
| | Mal–Glu | 1.3 | 3 | Progesterone (180) | 7 | 92 |
| | Mal–Glu | 0 | 3u | Dicoumarol (48) | 76 | — |
| | Mal–Glu | 0 | 3u | Dicoumarol (48) + progesterone (180) | 5 | 93* |
| | Mal–Glu | 1.3 | 3 | Progesterone (18) | 58 | 36 |
| | Mal–Glu | 0 | 3u | CCCP (3) | 77 | — |
| | Mal–Glu | 0 | 3u | CCCP (3) + progesterone (18) | 52 | 33* |
| B | Mal–Glu | 1.3 | 3 | None | 58 | — |
| | Mal–Glu | 1.3 | 3 | Deoxycorticosterone acetate (80) | 13 | 78 |
| | Mal–Glu | 1.3 | 3 | Deoxycorticosterone acetate (80) + dinitrophenol (61) | 15 | 74 |
| | Mal–Glu | 1.3 | 3 | Deoxycorticosterone acetate (80) + CCCP (3) | 12 | 79 |
| C | Suc | 0 | 4 | None | 27 | — |
| | Suc | 0 | 4 | Progesterone (180) | 28 | −4 |
| D | Asc–TMPD | 1.3 | 3 | None | 171 | — |
| | Asc–TMPD | 1.3 | 3 | Progesterone (360) | 171 | 0 |
| E | Asc–TMPD | 1.3 | 3 | None | 158 | — |
| | Asc–TMPD | 1.3 | 3 | Oligomycin | 100 | 37 |
| | Asc–TMPD | 1.3 | 3 | Oligomycin + progesterone (360) | 115 | 27 |

* Calculated with respect to the dicoumarol (or CCCP)-treated control.

acts preferentially at the second site of energy conservation[17] (Table II, Expt. C). On the other hand, triamcinolone did not affect the inhibition of ascorbate–TMPD oxidation by oligomycin (Table II, Expt. D). Since energy transfer at the third site is inhibited by oligomycin[18], comparison of Expts. C and D allows one to conclude that triamcinolone acted preferentially at the second site. In view of this, the relief of progesterone and deoxycorticosterone acetate inhibitions by triamcinolone may be explained on the basis that the two former steroids inhibit energy transfer preferentially at the second site.

*Effect of progesterone in the presence of stilbestrol*

Stilbestrol uncouples oxidative phosphorylation at the third site of energy conservation[19]. According to the preceding paragraph progesterone acts at the second

site and therefore stilbestrol would not relieve the inhibition of mitochondrial respiration by progesterone. The validity of this reasoning is confirmed by the results presented in Table III. With succinate as substrate, addition of stilbestrol to pro-

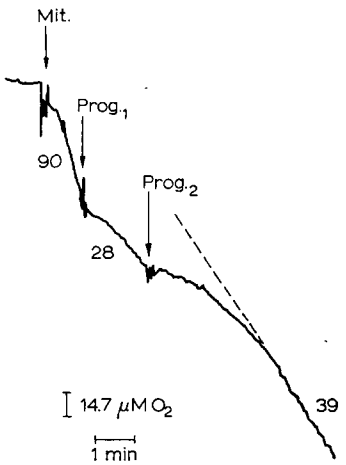


Fig. 3. Effect of a high concentration of progesterone on ADP-activated succinate oxidation. Mitochondria (Mit.), 1.75 mg/ml. 'Sucrose–Tris' medium; 6 mM succinate, 1.3 mM ADP and 6.0 mM $P_i$. Progesterone ($\mu$M): 180 (Prog.$_1$) or 270 (Prog.$_2$). The figures near the traces are as in Fig. 2.

TABLE II

RELIEF OF PROGESTERONE AND DEOXYCORTICOSTERONE ACETATE INHIBITIONS BY TRIAMCINOLONE. UNCOUPLING EFFECT OF TRIAMCINOLONE

Mitochondria (mg/ml): 0.83 (Expt. A), 0.46 (B, C), and 0.61 (D). Medium: 'sucrose–Tris' except in Expt. D where 'ascorbate' medium was used. All samples contained 6 mM $P_i$ and 1.3 mM ADP. Other additions were 6.0 mM succinate (Suc experiments), 15 mM ascorbate, 0.3 mM TMPD and 1.2 $\mu$g/ml antimycin A (Asc–TMPD experiments) and 1.2 $\mu$g/ml oligomycin where indicated. Inhibitors were added as in Table I.

| *Expt.* | *Substrate* | *Inhibitors (mM)* | *Rate of respiration (mμatoms O per mg per min)* | *Inhibition of respiration (%)* |
|---|---|---|---|---|
| A | Suc | None | 78 | — |
| | Suc | Progesterone (0.18) | 49 | 37 |
| | Suc | Progesterone (0.18) + triamcinolone (0.09) | 73 | 6 |
| B | Suc | None | 89 | — |
| | Suc | Deoxycorticosterone acetate (0.09) | 53 | 41 |
| | Suc | Deoxycorticosterone acetate (0.09) + triamcinolone (0.09) | 93 | −4 |
| C | Suc | None | 126 | — |
| | Suc | Phenyl-ethyl biguanidine (3.0) | 69 | 45 |
| | Suc | Phenyl-ethyl biguanidine (3.0) + triamcinolone (0.09) | 137 | −9 |
| D | Asc–TMPD | None | 128 | — |
| | Asc–TMPD | Oligomycin | 90 | 30 |
| | Asc–TMPD | Oligomycin + triamcinolone (0.18) | 83 | 35 |

TABLE III

PROGESTERONE INHIBITION OF SUCCINATE OXIDATION IN THE PRESENCE OF STILBESTROL (MITOCHONDRIA IN STATE 3)

Mitochondria (mg/ml): 1.12 (Expt. A) and 1.74 (B). Medium: 'sucrose–Tris' (A) and 'sucrose-phosphate' (B). Additions: 6 mM succinate, 1.3 mM ADP, 6 mM $P_i$ and inhibitors as indicated. Other experimental details as in Table I.

| *Expt.* | *Inhibitors* ($\mu M$) | *Rate of respiration* (*mμatoms O per mg per min*) | *Inhibition of respiration* (%) |
|---|---|---|---|
| A | None | 142 | — |
| | Progesterone (180) | 93 | 34 |
| | Stilbestrol (45) | 110 | 22 |
| | Progesterone (180) + stilbestrol (45) | 48 | 66 |
| | Progesterone (180) + stilbestrol (45) + CCCP (2) | 96 | 32 |
| | Stilbestrol (45) + progesterone (180) | 47 | 67 |
| | Stilbestrol (45) + progesterone (180) + CCCP (2) | 100 | 29 |
| B | None | 120 | — |
| | Progesterone (180) | 79 | 34 |
| | Progesterone (180) + stilbestrol (144) | 36 | 70 |

gesterone-pretreated mitochondria, instead of releasing respiration, augmented the inhibition to an extent compatible with the action of stilbestrol itself. The same occurred when progesterone was added after stilbestrol. On the other hand, addition of CCCP (a general uncoupler of oxidative phosphorylation[20]) partially reactivated the respiration of the stilbestrol *plus* progesterone-inhibited mitochondria. Since inhibition of respiration by stilbestrol is not affected by uncouplers[19], the effect of CCCP on mitochondria previously subjected to the combined action of stilbestrol and progesterone must be attributed to the release of progesterone inhibition. The effect of CCCP confirms that stilbestrol was unable to uncouple the progesterone-sensitive mechanism.

*Effect of progesterone and deoxycorticosterone acetate on $Ca^{2+}$ + $P_i$-activated mitochondrial respiration*

In the absence of $P_i$, addition of $Ca^{2+}$ ions to rat-liver mitochondria elicits a stoichiometric stimulation of respiration, but in the presence of $P_i$ the oxygen uptake fails to return to that of the initial resting state. The effect of $P_i$ is counteracted by oligomycin[21] and inhibitors of energy transfer. As stated above, progesterone and deoxycorticosterone acetate would also inhibit energy transfer and therefore it seemed appropriate to investigate whether the steroids had an oligomycin-like effect on the $Ca^{2+}$ + $P_i$-activated respiration. The results obtained with succinate as substrate are presented in Fig. 4. Control Traces A–C are consistent with the previous report by ROSSI AND LEHNINGER[21] with β-hydroxybutyrate as substrate. Traces D and E show the effect of deoxycorticosterone acetate and progesterone after addition of $Ca^{2+}$ and $P_i$. In contrast with oligomycin, the steroids did not counteract the $P_i$ effect since the final rates of oxygen uptake in Traces B, D and E were nearly the same (0.96–0.87 $\mu M$ $O_2$/sec), whereas with oligomycin (Trace C) the final rate of

respiration (0.40 $\mu$M $O_2$/sec) approached that of the control sample after the $Ca^{2+}$ ion effect was exhausted (Trace A: 0.34 $\mu$M $O_2$/sec).

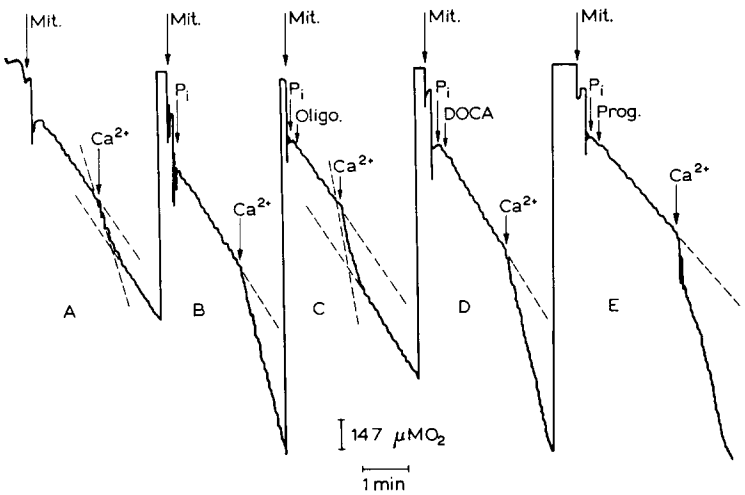


Fig. 4. Different effects of oligomycin (Oligo.), deoxycorticosterone acetate (DOCA) and progesterone (Prog.) on $Ca^{2+}$-activated mitochondrial respiration. Mitochondrial suspension, 0.1 ml. 'NaCl' medium; 6 mM succinate. At the points indicated, 0.19 mM $CaCl_2$, 2.4 mM $P_I$, 1.2 $\mu$g/ml oligomycin, 0.18 mM deoxycorticosterone acetate or 0.22 mM progesterone were added.

*Effects of other steroids on mitochondrial respiration*

Table IV shows the action of the following steroids: pregnenolone (a precursor of progesterone); 3-methylpregnenolone, pregnenolone acetate and 16-dehydropregnenolone; pregnandione and *allo*-pregnanolone (2 metabolic products of progesterone); norprogesterone, 4-hydroxy-17$\alpha$-acetoxyprogesterone, chlormadinone and 1-dehydrochlormadinone (artificial gestagens); 16,17-epoxy-deoxycorticosterone acetate; cortisone acetate, cortisol and prednisone (glucocorticoids). Steroids were tested at 2 concentrations, namely 45 $\mu$M with L-malate–L-glutamate and 150 $\mu$M with succinate as substrates. The results obtained are presented as percentages of progesterone or deoxycorticosterone acetate inhibition and allow the following considerations. (a) The NAD-flavoprotein area of the respiratory chain was preferentially inhibited by all the steroids assayed and, except with 1-dehydrochlormadinone, the inhibition at that site was on electron transfer, as shown by the lack of response towards uncouplers. (b) With the NAD-linked substrates, progesterone was more effective than the other gestagens listed in Table IV, except norprogesterone. (c) Similarly, deoxycorticosterone acetate inhibited to a greater extent than the other corticoids the oxidation of NAD-linked substrates. (d) With succinate as substrate, progesterone and deoxycorticosterone acetate were as a rule the most active inhibitors in the respective groups. Nevertheless, 1-dehydrochlormadinone was somewhat more inhibitory than progesterone. (e) A comparison of the effectiveness of progesterone, chlormadinone and 1-dehydrochlormadinone, with L-malate–L-glutamate and succinate as substrates, suggests that the artificial gestagens were relatively more effective on the succinate chain. (f) Lack of response towards dinitrophenol shows that the inhibitions of succinate oxidation by chlormadinone and 1-dehydrochlormadinone

TABLE IV

COMPARATIVE EFFECT OF STEROIDS ON MITOCHONDRIAL RESPIRATION

Mitochondria, 1.1–0.9 mg/ml; 'sucrose–Tris' medium; 3 mM L-malate, 3 mM L-glutamate and 1.5 mM malonate (Mal–Glu experiments) or 6 mM succinate (Suc experiments). All samples contained 1.3 mM ADP and 6 mM $P_i$. Final vol., 2.0 ml. The figures represent the average of duplicate assays. R, inhibition relieved by 60 $\mu$M dinitrophenol; NR, inhibition not affected by dinitrophenol. Inhibition of respiration by progesterone (%): 60 (Mal–Glu) and 24 (Suc); same by deoxycorticosterone acetate: 50 (Mal–Glu) and 28 (Suc).

| *Steroid* | *Relative effect of steroids with respect to progesterone (or deoxycorticosterone acetate) inhibitions* (%) | |
|---|---|---|
| | *Substrate: Mal–Glu* *Steroid: 45 $\mu$M* | *Substrate: Suc* *Steroid: 150 $\mu$M* |
| Gestagens and related compounds | | |
| Progesterone | 100 (NR) | 100 (R) |
| Norprogesterone | 127 (NR) | 46 (R) |
| Pregnandione | 87 (NR) | 23 |
| 3-Methylpregnenolone | 59 (NR) | 24 |
| Pregnenolone | 53 (NR) | 0 |
| 4-Hydroxy-17$\alpha$-acetoxyprogesterone | 30 (NR) | 33 |
| 1-Dehydrochlormadinone | 30 (R) | 117 (NR) |
| Pregnenolone acetate | 23 | 0 |
| 16-Dehydropregnenolone | 25 | 0 |
| Chlormadinone | 12 | 64 (NR) |
| *Allo*-pregnanolone | 0 | 0 |
| Corticoids | | |
| Deoxycorticosterone acetate | 100 (NR) | 100 (R) |
| 16,17-Epoxy-deoxycorticosterone acetate | 88 (NR) | 0 |
| Cortisone acetate | 34 (NR) | 0 |
| Cortisol | 18 | 0 |
| Prednisone | 22 | 0 |

were on electron transfer. This is in contrast to the inhibitory effects of progesterone and norprogesterone, which were on energy transfer. (g) Similar experiments were performed with triamcinolone. Instead of producing inhibition this steroid stimulated respiration, in agreement with its uncoupling action already described (refs. 9 and 10, and Table II).

*Effect of progesterone and deoxycorticosterone acetate on mitochondrial ATPase*

Inhibitors of energy transfer, like oligomycin, also inhibit mitochondrial ATPase[22] whereas uncouplers of oxidative phosphorylation elicit latent ATPase activity[23]. In view of this it seemed necessary to examine the response of latent and activated mitochondrial ATPase towards progesterone and deoxycorticosterone acetate. In the absence of $Mg^{2+}$ ions (latent state), progesterone produced a limited increase of ATPase activity (Table V), but the stimulation was less than that elicited by dinitrophenol. This is consistent with previous reports by other workers[24–26]. On the other hand, the dinitrophenol-stimulated ATPase was not inhibited by progesterone

TABLE V

EFFECT OF PROGESTERONE AND DEOXYCORTICOSTERONE ACETATE ON MITOCHONDRIAL ATPase

Mitochondria, 1.0 mg; 5 mM ATP; 40 mM KCl; 0.1 mM EDTA and sucrose 0.15 M (Expt. A) and 0.25 M (B); pH 7.4. Final vol. of reaction mixture, 1.0 ml. Incubation for 15 min at 30°. Controls were added 5 $\mu$l of dioxane, a volume equal to that of the steroid solution. Other experimental details as described under METHODS.

| *Expt.* | *Additions (mM)* | *$P_i$ liberation ($\mu$moles $P_i$ per min per mg protein)* | *Stimulation of ATPase activity (%)* |
|---|---|---|---|
| A | None | 0.72 | — |
| | Progesterone (0.15) | 1.54 | 114 |
| | Dinitrophenol (0.10) | 13.35 | 1750 |
| | Progesterone (0.15) + dinitrophenol (0.10) | 13.75 | 1810 |
| B | None | 0.85 | — |
| | Deoxycorticosterone acetate (0.15) | 1.10 | 29 |
| | Dinitrophenol (0.10) | 11.40 | 1200 |
| | Deoxycorticosterone acetate (0.15) + dinitrophenol (0.10) | 11.50 | 1310 |

TABLE VI

EFFECT OF STEROIDS ON MITOCHONDRIAL SWELLING

Mitochondria (0.87 mg) were added to the reaction medium containing 0.125 M KCl and 10 mM Tris–HCl (Expt. A) or 0.25 M sucrose and 10 mM Tris–HCl (B and C); pH 7.4. Final vol., 3.0 ml. Absorbance ($A$) was measured as described under METHODS. To control samples ($A$, 1.000) was added a volume of dioxane equal to that of the corresponding steroid solutions (5–10 $\mu$l). The values given are corrected for the variation in control absorbance. $\Delta A$, diminution in absorbance.

| *Expt.* | *Additions ($\mu M$)* | *Rate of swelling ($\Delta A \times 10^3 \cdot min^{-1}$)* | *Prevention of triamcinolone and stilbestrol monomethyl ether swelling effect (%)* |
|---|---|---|---|
| A | Progesterone (50) | 1 | — |
| | Progesterone (200) | 0 | — |
| | Progesterone (400) | 42 | — |
| | Triamcinolone (50) | 44 | — |
| | Progesterone (50) + triamcinolone (50) | 34 | 23 |
| | Progesterone (200) + triamcinolone (50) | 13 | 71 |
| | Stilbestrol monomethyl ether (30) | 169 | — |
| | Progesterone (200) + stilbestrol monomethyl ether (30) | 141 | 16 |
| B | Progesterone (200) | −7 | — |
| | Triamcinolone (50) | 20 | — |
| | Progesterone (200) + triamcinolone (50) | −9 | 110 |
| | Stilbestrol monomethyl ether (30) | 60 | — |
| | Progesterone (200) + stilbestrol monomethyl ether (30) | 0 | 100 |
| C | Deoxycorticosterone acetate (100) | 0 | — |
| | Stilbestrol monomethyl ether (30) | 18 | — |
| | Deoxycorticosterone acetate (100) + stilbestrol monomethyl ether (30) | 18 | 0 |

(Table V) and similar results (not shown in the table) were obtained in the presence of $Mg^{2+}$ ions. Deoxycorticosterone acetate (Table V), testosterone and 17$\beta$-estradiol (results not shown in Table V) did not affect mitochondrial ATPase either as stimulants of latent activity or as inhibitors of the activated enzyme.

*Effect of progesterone and deoxycorticosterone acetate on mitochondrial swelling*

The importance of the physical alteration of mitochondria for the response of respiration towards progesterone and deoxycorticosterone acetate was established as described in Table VI. The steroids were assayed with respect to the spontaneous swelling and also to the stilbestrol monomethyl ether- and triamcinolone-stimulated swellings. For the latter, triamcinolone and stilbestrol monomethyl ether were added to mitochondria preincubated for about 1 min with progesterone or deoxycorticosterone acetate. The second addition was taken as starting point for measuring the rate of swelling.

With the KCl–Tris medium, addition of 400 $\mu$M progesterone stimulated mitochondrial swelling to a limited extent (Table VI, Expt. A) but with the sucrose–Tris medium the reverse occurred. Furthermore, preincubation with progesterone prevented the swelling effect of triamcinolone and stilbestrol monomethyl ether, especially with the sucrose–Tris medium (Table VI, Expt. B). In contrast to these results, deoxycorticosterone acetate did not affect either the spontaneous or the stilbestrol monomethyl ether-elicited swelling (Table VI, Expt. C).

## DISCUSSION

In accordance with earlier claims by WADE AND JONES[2] and other workers[3,6–8,27], relatively low concentrations of progesterone and deoxycorticosterone acetate inhibit mitochondrial respiration by a direct action on the NAD-flavoprotein area of the respiratory chain (Fig. 1). This seems to be a general mechanism of inhibition since other steroids including androgens, estrogens, corticoids and gestagens act in the same manner on mitochondrial respiration (Fig. 1 and Table IV). The hypothesis[4,5] that loss of soluble respiratory cofactors is the cause of inhibition may be partially valid at steroid concentrations above 0.5 mM but not in the range of 0.01–0.10 mM, because at these levels progesterone and deoxycorticosterone acetate are capable of inhibiting respiration (Fig. 1) without inducing mitochondrial swelling or facilitating the swelling action of stilbestrol monomethyl ether and triamcinolone (Table VI). In this connection it must be recalled that swelling is usually taken as a sign of the alteration of mitochondrial structure, the cause of leakage of respiratory cofactors. The present observations are also in contrast to the inhibition by steroids of energy transfer at the first phosphorylation site, as occurs with pigeon-heart mitochondria[6,7]. In fact, with rat-liver mitochondria and NAD-linked substrates, the inhibition of respiration by steroids is not released by uncouplers (Table I) in contrast to the positive response of pigeon-heart mitochondria under similar experimental conditions. However, with liver mitochondria there is a much more effective inhibition of electron transfer than with heart mitochondria and that inhibition may mask a possible action of steroids on energy transfer.

In contrast to the inhibition of oxidation of NAD-linked substrates, progesterone and deoxycorticosterone acetate inhibit succinate oxidation chiefly by acting

upon energy transfer. This is shown by the effect of dinitrophenol in Fig. 2 and by the effect of triamcinolone in Table II. Release of progesterone and deoxycorticosterone acetate inhibitions by triamcinolone but not by stilbestrol leads one to postulate a selective inhibition of energy transfer at the second phosphorylation site. This is consistent with the lack of action of progesterone on ascorbate–TMPD oxidation (Table I, Expt. D), which in coupled mitochondria involves energy transfer only at the third site[18]. Comparison of the actions of progesterone, deoxycorticosterone acetate and oligomycin on energy transfer reveals some significant differences. Thus, (a) oligomycin inhibits at the 3 phosphorylation sites whereas progesterone and deoxycorticosterone acetate act chiefly at the second one; (b) oligomycin prevents the effect of $P_i$ on the $Ca^{2+}$ activation of mitochondrial respiration whereas progesterone and deoxycorticosterone acetate do not (Fig. 4), and (c) oligomycin inhibits activated ATPase[22], which is insensitive towards progesterone and deoxycorticosterone acetate (Table V). It may be suggested that points (b) and (c) are just a consequence of the different site-specificity of steroids and oligomycin. In support of this view it must be recalled that the mitochondrial ATPase reaction corresponds predominantly to first phosphorylation site[28].

Besides the inhibition of electron and energy transfer, progesterone displays a weak uncoupling action on oxidative phosphorylation. The release of respiratory control requires a relatively high concentration of steroid as shown by the stimulation of succinate oxidation in Fig. 3 (270 $\mu$M progesterone) and by the partial release of oligomycin inhibition with ascorbate–TMPD as substrates. Lower concentrations of progesterone (180 $\mu$M) did not affect respiratory control as shown in Table I, Expt. C. The uncoupling effect agrees with (a) the small though significant stimulation of ATPase activity presented in Table V; (b) the diminution of P:O quotients obtained by WADE AND JONES[2]; and (c) the release of respiratory control described by PACKER AND BACILA[4]. The activation of latent ATPase activity by progesterone and other steroids has already been described[24–26], but in those studies steroids have been used at concentrations relatively higher than in the present one, for example 0.6 mM (ref. 26). At this concentration steroids may swell mitochondria and therefore it is impossible to ignore an unspecific activation of ATPase analogous to that caused by detergents[29]. In summary, progesterone and deoxycorticosterone acetate belong to that class of inhibitors[7] from which several effects on phosphorylating mitochondria may be expected, namely (a) inhibition of non-phosphorylating electron transfer; (b) inhibition of energy transfer; (c) uncoupling effects, and (d) activation of latent ATPase activity.

Concerning the relationship between the structure of the steroids and their effect on mitochondrial respiration, the results obtained allow the following comments. Omission of the C-19 angular methyl group of progesterone slightly increases the inhibition of the NAD-flavoprotein chain but diminishes that of the succinate chain (Table IV). Moreover, the introduction of 1 chlorine atom at C-6, 1 hydroxyl at C-4 and 1 acetoxyl group at C-17 significantly diminishes the action of progesterone on mitochondrial respiration, as shown with 4-hydroxy-17$\alpha$-acetoxyprogesterone, chlormadinone and 1-dehydrochlormadinone (Table IV). These effects are in contrast to the relatively high progestational activity of norprogesterone, chlormadinone and 1-dehydrochlormadinone which *in vivo* are much more effective than progesterone[30]. On the other hand, pregnenolone, pregnenolone derivatives and two metabolic pro-

ducts of progesterone (pregnandione and *allo*-pregnanolone) are less effective than progesterone on both mitochondrial respiration (Table IV) and in the 'stromal nuclear hypertrophy' test[31], a measure of progestational activity. Similarly, 4-hydroxy-17α-acetoxyprogesterone is less active than progesterone *in vitro* (Table IV) and *in vivo* when administered subcutaneously[32]. With corticoids, the presence of an oxygen atom at C-11 seems to hinder all actions on mitochondrial metabolism which contrasts with the scarcely significant variation of activity after the introduction of an oxygen atom at C-16, 17 (Table IV). Whether these facts reflect the operation of molecular mechanism related to the physiological action of steroids hormones is a subject for further investigation.

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