Inhibition of testosterone metabolism in the brain and cloacal gland of the quail by specific inhibitors and antihormones

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REVISED MANUSCRIPT RECEIVED 8 July 1986

ABSTRACT

The effects of antioestrogens, antiandrogens and of various inhibitors of testosterone metabolism on testosterone metabolism in the quail hypothalamus and cloacal gland were studied by an in-vitro radioenzymatic assay. It was found that antioestrogens and antiandrogens generally had little or no effect on aromatase and 5α- and 5β-reductases of testosterone, except when used at very high doses. The 5α-reductase inhibitor, 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one, inhibited both 5α- and 5β-dihydrotestosterone production without markedly affecting aromatase activity. Surprisingly, the aromatase inhibitor, 1,4,6-androstatriene-3,17-dione, inhibited not only the production of oestradiol but also that of 5β-dihydrotestosterone and, to a lesser extent, 5α-dihydrotestosterone. These unexpected properties should be taken into account when interpreting the results of in-vivo experiments using these compounds.

J. Endocr. (1987) **112,** 189–195

INTRODUCTION

The involvement of testosterone metabolism in the control of sexual behaviour, gonadotrophin secretion and other androgen-dependent responses such as the growth of secondary sexual characteristics (e.g. prostate in rats, comb in chicken and cloacal gland in quail) has been the subject of much research in the last decade. Several inhibitors of metabolism, or antihormones which block the interaction of androgens and oestrogens with their receptors, have been developed. In order to understand fully the effects of such antihormones *in vivo*, it is necessary to know the full spectrum of their in-vitro properties.

This requirement seems even more important when consideration is given to the paradoxical effects which are sometimes observed in in-vivo tests. For example, androstatrienedione (ATD) is a very good inhibitor of aromatase *in vitro* (Lieberburg, Wallach & McEwen, 1977) and in most experiments inhibits testosterone-induced sexual behaviour (Christensen & Clemens, 1975; Adkins, Boop, Koutnik *et al.* 1980; Bridges & Russel, 1981; Södersten, Eneroth, Mode & Gustafsson, 1985) which supports the notion that aromatization of testosterone is critical for the activation of copulatory behaviour. Landau (1980), however, observed that, at high doses in the rat, ATD did not inhibit testosterone-induced sexual behaviour and, furthermore, activated the behaviour when injected alone. This could result from unidentified properties of this compound. Similarly 17β-N,N-diethyl-carbamoyl-4-methyl-4-aza-5α-androstan-3-one (4MA), a 5α-reductase inhibitor (Brooks, Berman, Hichens *et al.* 1982), does not affect sexual behaviour (Södersten *et al.* 1985) despite the fact that this behaviour seems to depend on both oestrogens and 5α-dihydrotestosterone produced by metabolism of testosterone (e.g. Feder, 1978; Balthazart, Schumacher & Malacarne, 1985). Nevertheless, 4MA blocks prostate growth *in vivo* (Brooks, Baptista, Berman *et al.* 1981; Liang, Rasmusson & Brooks, 1983; Wenderoth, George & Wilson, 1983) but this could be by an antiandrogenic action rather than by inhibition of 5α-reduction (Brooks *et al.* 1981; Liang *et al.* 1983; Wenderoth *et al.* 1983). Its activity on aromatase has never been tested.

Antihormones which block the action of steroids by preventing their normal interaction with intracellular receptors prevent the actions of androgens and oestrogens on secondary sexual characteristics and behaviour *in vivo*, but their mechanism of action is not always clear because some of them also inhibit testosterone metabolism. The antiandrogen cyproterone acetate prevents binding of androgen to receptor (Liao, Howell & Chang, 1974; Tindall, French & Nayfeh, 1981) but also inhibits 5β-reductase (van Doorn & Clark, 1973; Balthazart, Marcelle, Sanna & Schumacher, 1982) and perhaps aromatase (McEwen, Lieberburg, Chaptal *et al.* 1979). Cyproterone acetate

  Printed in Great Britain
0022–0795/87/0112–0189 $02.00/0

also has androgenic, progestagenic and antigonadotrophic properties (Neri, Mohaman, Meyer *et al.* 1967; Neumann & Steinbeck, 1974; Lax & Schiefers, 1981). Cyproterone acetate has been known for a long time and most of its properties have therefore been established, but this is far from being the case for other antihormones whose action on testosterone metabolism have not been studied.

We are now engaged in a long-term study on the specificity of the hormonal control of sexual behaviour and reproductive physiology in the Japanese quail. By injecting birds with metabolites of testosterone, it has been possible to identify androgen- and oestrogen-dependent aspects of reproduction (Schumacher & Balthazart, 1983; Balthazart *et al.* 1985), but these findings should now be confirmed by the use of inhibitors of steroid metabolism and specific antihormones. Before engaging in these studies, it was felt desirable to analyse the potential effects of all these compounds on testosterone metabolism in the species and target tissues to be studied. This has seldom been carried out in detail and, except in some mammals (usually the rat), it is possible that specificity of some of the compounds could vary between species. Thus the effects of all the inhibitors and antihormones to be used during the in-vivo studies were first determined.

MATERIALS AND METHODS

The effects of inhibitors and antihormones on 5α-reductase, 5β-reductase and aromatase were measured *in vitro* using a radioenzymatic assay described previously and validated for quail tissue (Schumacher, Contenti & Balthazart, 1984) with the modifications described and validated by Hutchison & Schumacher (1986).

In the first experiment, 11 hypothalami and four cloacal glands from sexually mature male quail (*Coturnix coturnix japonica*) were dissected out, pooled in two tubes and homogenized in STMM buffer (0·25 mol sucrose/l, 10 mmol Tris–HCl/l (pH 7·4 at 20 °C), 5 mmol $MgCl_2$/l and 1 mmol 5β-mercaptoethanol/l) at a final concentration of 25 mg/ml. Aliquots (200 µl) of these homogenates were then added to tubes containing [1α,2(n)-^{3}H] testosterone (60 Ci/mmol; Amersham International plc, Amersham, Bucks) at a final concentration of 20 nmol/l. STMM buffer (50 µl) containing 6 mg $NaDPH_2$/ml was then added with another 50 µl buffer containing water-soluble compounds to be tested. Other compounds (such as the radioactive substrate and inhibitors which were not water soluble) had been previously added to the tube in 50 µl toluene and carefully evaporated to dryness. Tubes were incubated for 15 min at 41 °C and androgenic and oestrogenic metabolites separated by phenolic partition, purified by thin-layer chromatography and quantified in a beta counter as described previously (Schumacher *et al.* 1984).

The following compounds were tested at concentrations ranging from 10 nmol/l to 10 µmol/l: the aromatase inhibitor 1,4,6-androstatrien-3,17-dione (ATD; Steraloids Inc, Wilton, NH, U.S.A.), the 5α-reductase inhibitor 4MA (compound L-636028 generously supplied by Merck Sharp and Dohme, Rahway, NJ, U.S.A.), the antioestrogen 1-[2(p-[a(p-methoxyphenyl)-β-nitrosyl]phenoxy)ethyl] pyrrolidine (nitromifene citrate, CI-628; generously supplied by Warner-Lambert Co., Ann Arbor, MI, U.S.A.), the antioestrogen tamoxifen citrate (Sigma, St Louis, MO, U.S.A.), the antiandrogen cyproterone acetate (generously supplied by N.V. Schering S.A., Diegem, Belgium) and the antiandrogen flutamide (generously supplied by Schering Co., Bloomfield, NJ, U.S.A.). Testosterone was also used in the experiment to test for simple competitive inhibition.

In a second experiment, three hypothalami from juvenile male quail (3–4 weeks old) which had been implanted with Silastic capsules (Dow Corning, Midland, MI, U.S.A.; number 602–265) containing testosterone to stimulate aromatase activity (Schumacher & Balthazart, 1986) were dissected out, pooled and homogenized in STMM buffer at a final concentration of 10 mg/ml. Aliquots were incubated with radioactive testosterone under the same conditions as in experiment 1 in the presence of ATD at concentrations between 10 nmol/l and 0·1 mmol/l. All other procedures were the same as in experiment 1.

In both experiments, control tubes without inhibitors ($n=9$ and 4 in experiments 1 and 2 respectively) were analysed in parallel. Duplicate determinations were made for each compound and concentration in experiment 1; quadruplicates were run in experiment 2.

In both experiments, four metabolites of testosterone were quantified: oestradiol-17β, 5α-dihydrotestosterone (5α-DHT), 5β-dihydrotestosterone (5β-DHT) and androstenedione. Proteins were assayed in the homogenates by the Coomassie blue reaction (Bradford, 1976).

Results for each drug and metabolite studied were analysed by one-way analysis of variance (ANOVA), comparing the different concentrations with the control values. When significant, they were followed by Newman–Keuls tests for multiple comparisons in order to identify the concentrations which produced significant effects by comparison with control.

RESULTS

The production of metabolites in the absence of inhibitors during both experiments was similar to that

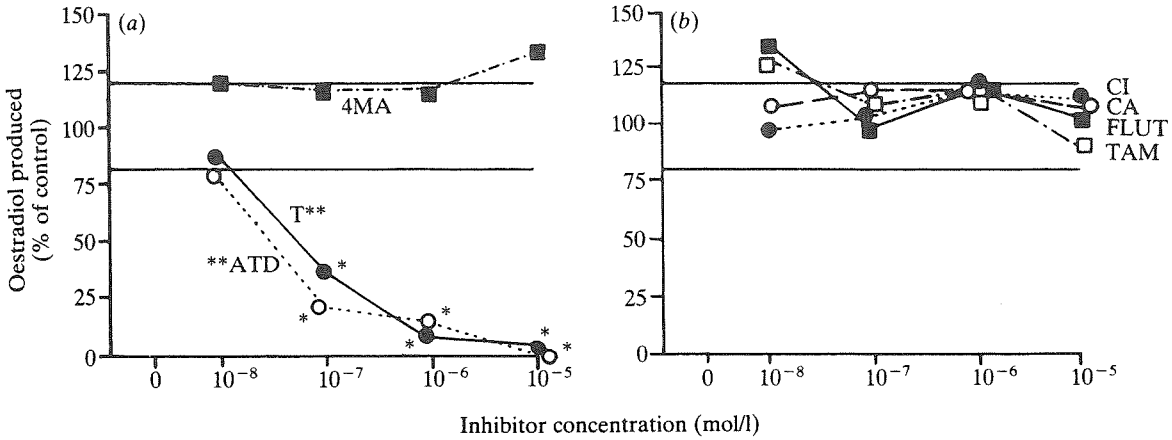

FIGURE 1. Production of radioactive oestradiol from tritiated testosterone by quail hypothalamic homogenates in the presence of (*a*) various inhibitors of steroid metabolism and (*b*) antihormones. The compounds tested were: testosterone (T), 1,4,6-androstatriene-3,17-dione (ATD), 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4MA), nitromifene citrate (CI), tamoxifen citrate (TAM), cyproterone acetate (CA) and flutamide (FLUT). Data are expressed as percentages (means of duplicates) of values observed in controls (99 fmol/mg protein) without inhibitor (100% and S.D. represented by the two horizontal lines). Results of the analysis of variance comparing the different concentrations of inhibitors with the control are represented by the asterisks next to the abbreviation for the inhibitor; asterisks next to the data points represent comparisons with the control group by Newman–Keuls test; $*P<0{\cdot}01$, $**P<0{\cdot}001$.

observed previously in similar experiments (e.g. Massa, Davies & Bottoni, 1980; Balthazart *et al.* 1982; Schumacher & Balthazart, 1984, 1986; Schumacher *et al.* 1984). In the hypothalamus the amounts of oestrogens produced were always smaller than those of androgens. No oestradiol could be detected after incubation of the cloacal gland and, in this tissue, the production of androgenic metabolites was greater than in the hypothalamus. Very large quantities of 5β-DHT were produced by the hypothalamus during experiment 2, which is probably due to the young age of the animals used (Schumacher & Balthazart, 1984).

In both experiments, less than 25% of the radioactive substrate was metabolized, so that production of metabolites was linear with respect to time (see also Schumacher *et al.* 1984). The four metabolites which were quantified represented approximately 50–60% of the metabolites produced. Significant quantities of 5α- and 5β-androstenediols were also detected but these results are not presented here because separation of these compounds was incomplete in the chromatography system used and the kinetics of their production are complex since they are secondary metabolites of testosterone. Minor quantities (less than 10% of metabolized substrate) of unidentified more polar metabolites were also present.

To allow direct comparison of all results, production of metabolites in the presence of various inhibitors was expressed as a percentage of values observed in corresponding controls.

Figure 1 shows the effects of metabolism inhibitors (ATD and 4MA), antiandrogens (cyproterone acetate and flutamide) and antioestrogens (nitromifene citrate and tamoxifen citrate) on aromatase in the hypothalamus.

Dilution of substrate by non-radioactive testosterone or addition of ATD inhibited the formation of radioactive oestradiol to the same extent (ANOVA comparing control values with those of inhibitors; $P<0{\cdot}001$ in both cases; the effect was significant for concentrations above 0·1 μmol/l). The other compounds had no significant effect at this level. Both inhibitors of metabolism, however, had large effects on the production of androgenic metabolites of testosterone by the cloacal gland (Fig. 2) and hypothalamus.

In the cloacal gland, 4MA inhibited 5α- and 5β-reduction of the radioactive substrate to a similar extent to that caused by dilution of the substrate with unlabelled testosterone, but had little effect on androstenedione production (moderate increase at intermediate concentrations). Unlabelled testosterone also inhibited the production of the latter compound (Fig. 2). Unexpectedly, ATD inhibited the production of 5β-DHT and increased the production of androstenedione. Antioestrogens and antiandrogens had no clear effect on the production of the androgenic metabolites by the cloacal gland (results not shown). However, at the highest concentrations (10 μmol/l) 5β-reductase was slightly inhibited by cyproterone

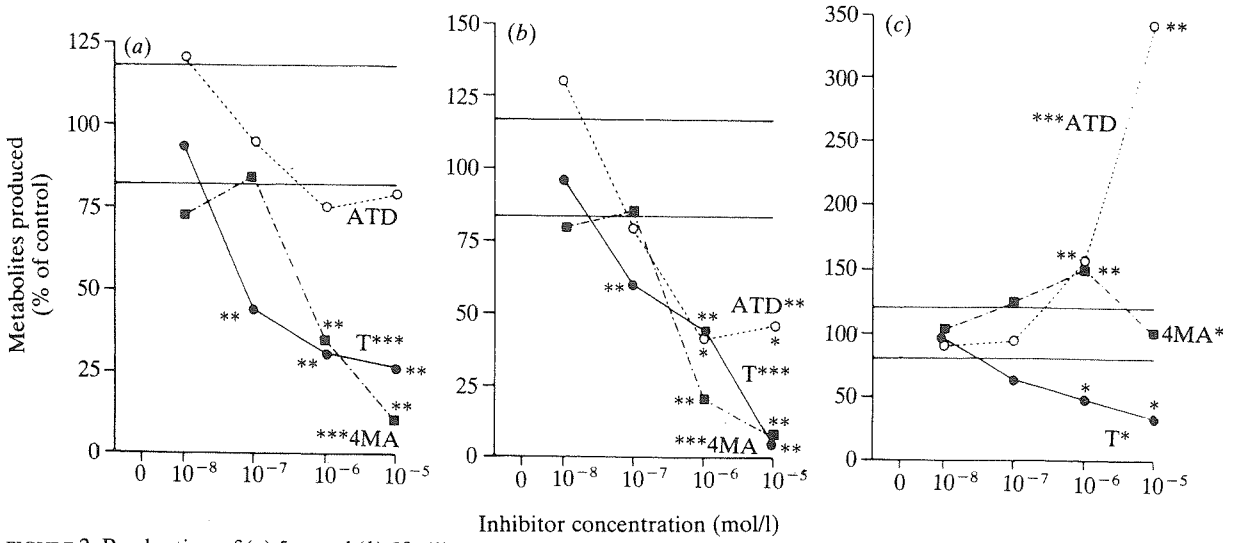

FIGURE 2. Production of (*a*) 5α- and (*b*) 5β-dihydrotestosterone (5α-DHT and 5β-DHT) and (*c*) androstenedione by homogenates of quail cloacal gland in the presence of unlabelled testosterone (T), the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) or the 5α-reductase inhibitor 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4MA). Data are expressed as percentages (means of duplicates) of values observed in controls without inhibitors (534, 548 and 517 fmol/mg protein for 5α-DHT, 5β-DHT and androstenedione respectively; 100% and S.D. represented by the two horizontal lines). Results of the analysis of variance comparing the different concentrations of inhibitors with the control are represented by the asterisks next to the abbreviation for the inhibitor; asterisks next to the data points represent comparisons with the control group by Newman–Keuls test; $*P<0{\cdot}05$, $**P<0{\cdot}01$, $***P<0{\cdot}001$.

acetate ($P<0{\cdot}05$ compared with control; Newman–Keuls test).

Similar results were obtained for the production of androgens by the hypothalamus. The 5α-reductase inhibitor, 4MA, significantly decreased the production of 5α- and 5β-DHT ($F=6{\cdot}68$, $P<0{\cdot}003$ and $F=3{\cdot}53$, $P<0{\cdot}04$ respectively). The aromatase inhibitor, ATD, decreased the production of these two metabolites by more than 50% at the highest concentrations tested, although the effects did not reach significance possibly because of a large intra-assay error in these samples as a consequence of a technical problem with the thin-layer chromatography. A significant increase in androstenedione production was also observed in the group treated with ATD ($F=10{\cdot}13$, $P<0{\cdot}001$). Antioestrogens had no effect on the production of androgenic metabolites. Antiandrogens had no clear effect although at the highest concentration, cyproterone acetate again inhibited 5β-reduction ($P<0{\cdot}05$; Newman–Keuls test). In addition, flutamide increased 5β-reductase in the hypothalamus ($F=9{\cdot}84$, $P<0{\cdot}001$) but the effect was not directly related to the dose of antiandrogen and was significant only at intermediate concentrations (10 nmol/l and 1 μmol/l) which makes this finding difficult to interpret.

It was especially surprising that ATD inhibited 5β-reductase although this effect did not reach significance for the hypothalamic samples. Considering the importance of this finding for the interpretation of the in-vivo effects of this frequently used compound, it was decided to test the effects of ATD on hypothalamic testosterone metabolism in a second experiment. Strong inhibition of aromatase and 5β-reductase was observed ($F=15{\cdot}56$, $P<0{\cdot}001$ and $F=6{\cdot}38$, $P<0{\cdot}009$ respectively). This inhibition was significant (Newman–Keuls test) for ATD concentrations as low as 0·1 μmol/l. A clear effect on 5α-DHT production was also noted at higher concentrations ($F=45{\cdot}49$, $P<0{\cdot}001$; Fig. 3). However, the increase in androstenedione production did not reach significance.

DISCUSSION

During these experiments, the production of metabolites of testosterone measured in the absence of inhibitors was within the same range as in previous studies and the relative activity of aromatase and 5α- and 5β-reductases was similar to that observed previously (Schumacher *et al.* 1984; Schumacher & Balthazart, 1984, 1986). The very high production of androgenic metabolites in the cloacal gland and the high 5β-reductase activity in the brain of immature animals was clearly confirmed.

Interestingly, no oestradiol was formed in the cloacal gland, while significant aromatase activity was always found in hypothalamic samples which had been processed in parallel. This accords with previous

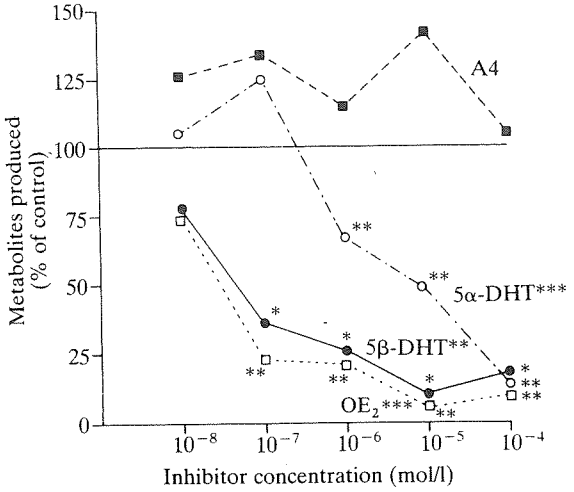

FIGURE 3. Effects of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) on the metabolism of testosterone by the quail hypothalamus. Values are percentages (means of quadruplicates) of relative control values (73, 200, 2424 and 293 fmol/mg protein for the production of oestradiol (OE_2), 5α-dihydrotestosterone (5α-DHT), 5β-dihydrotestosterone (5β-DHT) and androstenedione (A4) respectively) shown by the horizontal line. Results of the analysis of variance comparing the different concentrations of inhibitor with the control are represented by the asterisks next to the abbreviation for the metabolite; asterisks next to the data points represent comparisons with the control group by Newman–Keuls test; $*P<0{\cdot}05$, $**P<0{\cdot}01$, $***P<0{\cdot}001$.

physiological studies showing that growth of the cloacal gland is strictly under androgenic control (Massa *et al.* 1980; Schumacher & Balthazart, 1983) while sexual behaviour, which is regulated by the hypothalamus, depends for its activation on both androgens and oestrogens (Schumacher & Balthazart, 1983; Balthazart *et al.* 1985). There is thus an anatomical correlation between the metabolites of testosterone which are produced by one structure and the hormonal specificity of the physiological response which is controlled at that level.

The compounds tested here generally had similar actions on testosterone metabolism in both the hypothalamus and cloacal gland. A few exceptions to this were noted, but usually only the magnitude of the effect changed, thus making it significant in one case and not in the other; the same trends were always present. No major conclusion should be drawn from these apparent discrepancies which could be related to the difference in the physiological state of the birds or in the tissue analysed.

Antiandrogens seemed to have little effect on testosterone metabolism. When slight inhibition was observed, it was always at high concentrations of the antihormone (10 μmol/l compared with 20 nmol/l for the substrate) which are never likely to be encountered during in-vivo experiments. Furthermore, these effects were very seldom statistically significant. High concentrations of cyproterone acetate, however, clearly inhibited 5β-reductase activity both in the cloacal gland and in the brain, which confirms earlier observations obtained by different techniques (Van Doorn & Clark, 1973; Balthazart *et al.* 1982). No inhibition by this compound of the production of 5α-DHT nor of oestradiol could be detected despite the fact that cyproterone acetate inhibited placental aromatase in mammals (Schwartzel, Kruggel & Brodie, 1973) and decreased nuclear oestrogen receptor occupation following injection of testosterone in rats (McEwen *et al.* 1979). It is impossible to evaluate whether this discrepancy reveals species or procedural differences. Although cyproterone acetate appears to have a limited action on testosterone metabolism in quail, data collected during in-vivo tests using the compound must be interpreted cautiously because of its many properties in addition to antiandrogenicity (see Introduction).

Flutamide, which is a non-steroidal compound, is usually considered a 'pure' antiandrogen and, indeed, it did not affect testosterone metabolism in the present experiments, with the exception that it seemed to increase 5β-reductase in the brain although not in a dose-dependent manner. Its in-vivo efficacy is, however, limited (Liao *et al.* 1974; Gladue & Clemens, 1980) and it only partly blocks androgen-dependent responses, possibly because it inhibits the binding of androgens to receptors less effectively than cyproterone (Liao *et al.* 1974).

No significant effect of the antioestrogens in the cloacal gland and hypothalamus could be detected for the three metabolites of testosterone which were analysed. At the highest concentrations, marginal effects on metabolism were sometimes observed but they never reached significance. This supports the notion that the biological effects of these compounds *in vivo* reflect their interaction with oestrogen receptors.

The 5α-reductase inhibitor, 4MA, strongly inhibited 5α-reduction in quail but also strongly depressed 5β-reductase activity as has been shown previously for the cloacal gland (Balthazart *et al.* 1982). The inhibition of 5α- and 5β-reductase was observed in the present study in both the cloacal gland and hypothalamus. 5β-Reduction is usually considered an inactivation pathway for testosterone in birds (Steimer & Hutchison, 1981) and is extremely active especially at high substrate concentrations (Schumacher *et al.* 1984). Its blockage by 4MA could have important physiological consequences by increasing the amount of substrate remaining in a tissue (this could for example explain the small increase in oestradiol production observed in the hypothalamus during

experiment 1 at the concentration of 10 μmol 4MA/l) and this effect should probably be considered in the interpretation of in-vivo experiments where birds are treated with high doses of this compound.

The aromatase inhibitor, ATD, revealed the most unexpected properties during these experiments. Besides suppressing oestradiol formation it drastically decreased 5β-reduction and substantially decreased 5α-reduction when used at concentrations higher than 1 μmol/l. Marked stimulation of androstenedione production was also observed in two out of three experiments. These effects on the production of androgenic metabolites were not significant in each experiment but the same trends were always present. This aromatase inhibitor is thus acting on several independent enzyme systems and is not as specific for aromatase as is usually thought. The mechanism of these actions remains unclear at the present time and could only be determined by additional kinetic experiments. It is, however, intriguing to note that the three enzymes which were inhibited by ATD use $NaDPH_2$ as a coenzyme and thus involve reductive reactions, while the only oxidation which was measured (transformation of testosterone into androstenedione) was not affected. Whether this represents a coincidence or reveals a feature of the mechanism of action of ATD could only be evaluated by additional experiments.

ACKNOWLEDGEMENTS

We are indebted to Professor E. Schoffeniels for his continued interest in our work. This research was supported by a grant from the Belgian FNRS to J. B. (Crédit aux Chercheurs) and a grant from the FRFC (number 2.4518.80) to Professor Schoffeniels. We thank Merck Sharp and Dohme, Warner-Lambert Co., Schering S.A. and Schering Co. for gifts of anti-hormones and metabolism inhibitors.

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