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EFFECTS OF ATRATONE ON HORMONE-DEPENDENT REACTIONS IN HYPOTHALAMUS, PITUITARY AND PROSTATE GLAND

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SUMMARY

The effect of atratone, a selective s-triazine herbicide, on specific reactions in hypothalamus, pituitary and prostate gland was studied. It was found that atratone influenced the biosynthesis of LRF at the hypothalamic level. Increased concentrations of atratone from 2.5 to 8.0 mmol inhibited the synthesis of LRF from 22% to 94%. The presence of atratone (0.4 mmol) in pituitary inhibited the activity of 5α-reductase in experiments conducted *in vitro* and *in vivo* (s.c. 0.1 mg of atratone/100 g b.wt.) for approximately the same amount (80%). The presence of atratone inhibited the 5α-dihydrotestosterone (5α-DHT) binding to receptor proteins in rat ventral prostate cytosol. It was found by the microcalorimetric technique that 5α-DHT was bound exothermically to cytosol receptors, while in the presence of 0.4 to 2 nmol/s of atratone the energetic level was changed and the binding was endothermic. In sucrose density gradient separation, the presence of 0.4 or 1 mmol of atratone decreased the binding of 5α-DHT to specific receptors in the 8S fraction, which is further proof of the blocking effect of atratone in the hormone-dependent reactions.

INTRODUCTION

The use of pesticides for protecting and improving the field crop yield is steadily rising. Ecological studies of environmental pollution and health hazards induced by various pesticides are in progress, but our knowledge of the interaction of pesticides with the endocrine system of mammals is relatively poor. Most pesticides are lipid soluble, and therefore their presence in the reproductive organs, which contain significant amounts of lipids, is very probable. It was found that pesticides produce changes in hormone-dependent organs such as uterus[1, 2] and prostate gland[3, 4].

This prompted us to study in more detail the effect of atratone*, a selective s-triazine herbicide, on hypothalamus, pituitary and prostate gland. Atratone is used commercially in great quantities for treating fields and therefore there is a strong possibility that it could be present in the organs of mammals.

At the hypothalamic level we followed the influence of atratone on the biosynthesis of LRF. In pituitary we found that atratone exerted a blocking effect on 5α-reductase, inhibiting in this way the reduction of testosterone in 5α-dihydrotestosterone (5α-DHT) [5]. Our experience in the study of hormone–protein[6] and hormone–receptor[7, 8] interactions using the microcalorimetric technique was directly applied for determination of the inhibitory effect of atratone on 5α-DHT binding to androgen receptors in rat prostate cytosol. These findings were supplemented by separation of 5α-DHT–receptor complex in the presence of atratone by sucrose density gradient resolution.

EXPERIMENTAL

Chemicals. Unlabelled 5α-dihydrotestosterone (Steraloids Inc.) was used without further purification. Bovine serum albumin Fraction V (BSA) was purchased from BDH Biochemicals Ltd. and bovine liver catalase was obtained from Boehringer Mannheim GmbH. Atratone was purified by t.l.c. before use.

[1,2,4,5,6,7(*n*)-^{3}H]-5α-Dihydrotestosterone (specific radioactivity 175 Ci/mmol), [4-^{14}C]-testosterone (specific radioactivity 59 mCi/mmol) and L-UL-[^{14}C]-leucine (specific radioactivity 324 mCi/mmol), were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and purified by t.l.c. before use.

Buffer solutions were prepared with demineralized and glass-distilled water. All other chemicals employed were of "Analar" grade.

Animals and tissues. Male rats (Wistar strain), aged 90 to 100 days were used. Standard laboratory food and water were continuously available. The animals were kept in an air-conditioned room at 24°C and relative humidity of about 55%, with 12 h light and 12 h darkness. Under ether anesthesia, the rats were bilaterally orchidectomized by the scrotal route and sacrificed 48 h later by decapitation.

Hypothalamus, pituitary and ventral prostate tissues were removed immediately after decapitation. The hypothalamic tissue was cut out as a block (11–13 mg) limited anteriorly by the optic chiasma, laterally by the hypothalamic fissures and posteriorly by the mammillary body. The depth of the section was approximately 2.5 mm from the basal surface.

* The following trivial names and abbreviations are used: Atratone = 2-methoxy-4-ethylamino-6-isopropylamino-s-triazine; 5α-Dihydrotestosterone (5α-DHT) = 17β-hydroxy-5α-androstan-3-one; LRF = luteinizing hormone-releasing factor; 5α-Reductase = 3-oxo-5α-steroid 4-ene-dehydrogenase; Testosterone = 17β-hydroxy-4-androsten-3-one.

Table 1. Incorporation of [^{14}C]-leucine into LRF by hypothalamic tissue of male rats in presence of various concentrations of atratone

Treatment	Atratone (mmol)		LRF† (d.p.m./mg)
Intact	none	(12)	689 ± 43*
Intact	8.5	(2)	67 ± 7
Castrated 48 h	none	(17)	837 ± 47‡
Castrated 48 h	2.5	(4)	660 ± 88
Castrated 48 h	5.0	(6)	399 ± 36
Castrated 48 h	7.5	(4)	220 ± 99
Castrated 48 h	8.0	(3)	48 ± 18

* Mean ± S.E.; † Results are expressed as radioactivity incorporated by tissue during 3 h; ( ) = number of determinations. Student's t test was used to calculate the significance of differences among mean values. ‡ $P < 0.02$ vs intact animals.

Ventral prostate tissue was cut into small pieces in a buffer (pH 7.4) containing 50 mmol Tris–HCl, 1 mmol EDTA and 0.5 mmol dithiothreitol. After washing the tissue was homogenized (while holding the whole system in an ice bath) in the same buffer, in a glass–Teflon tissue homogenizer, Kontes Glass Co., NJ. The homogenate was centrifuged at 105,000 *g* for 60 min in a Beckman ultracentrifuge Model L-3-50, rotor SW 41.

In vitro *biosynthesis of LRF*. Hypothalamic tissue obtained from one animal was incubated in 1 ml Krebs–Ringer bicarbonate solution, pH 7.4, containing 0.005% penicillin, 0.005% streptomycin[9], 0.7 μCi[^{14}C]-leucine and 2.5 to 8.0 mmol of atratone. Incubation was carried out for 3 h in an atmosphere containing 95% oxygen and 5% carbon dioxide, at 37°C under continous shaking. The tissue was then transferred into cold Krebs–Ringer solution. Extraction and detection of LRF were performed as outlined previously[10].

Pituitary 5α-reductase. Pituitary used in *in vitro* experiments, was cut into small pieces of about 2 mg, and immersed into 2 ml of glucose Krebs–Ringer solution, pH 7.4, containing 34 nCi (about 0.56 nmol)[^{14}C]-testosterone and 0.38 mmol of atratone.

In *in vivo* experiments, injections of atratone in parafine oil (1 mg/ml) were given subcutaneously to rats immediately after castration. Dose of atratone per animal was constant (0.1 mg/100 g b.wt.). The animals were sacrificed 48 h later. Extraction of pituitary, incubation and determination of 5α-reductase activity were performed as described in detail elsewhere[5].

Sucrose density gradient separation. The supernatant fraction (105,000 *g*) of ventral prostate tissue (1 ml cytosol) was incubated for 15 min at 25°C with 0.38 mmol or 0.95 mmol of aratone, with gentle shaking. From the incubated supernatant fraction, 0.8 ml was transferred into a scintillation flask and incubated for 2 h at 4°C and periodically shaken with 2.5×10^{-12} mol of purified [^{3}H]-DHT. Separation in sucrose density gradient (5–20%) was performed by ultracentrifugation for 20 h at 180,000 *g*. The fractions were collected by means of Beckman Recovery System and measured for radioactivity in a Packard Tri-Carb Liquid scintillation counter, using Permablend TM III scintillant. Correction for quenching in all samples was made by the internal standard method. Apparent sedimentation coefficients (S) were determined using BSA (4.6 S) and bovine liver catalase (11.3 S) as reference standards, according to the method of Martin and Ames[11].

Microcalorimetric method. Microcalorimetric measurements were carried out with a LKB Flow Microcalorimeter Model 10700-1 using an 18-carat gold reaction channel, designed by Monk and Wadsö[12]. Approximately the same volumes of hormone or hormone–atratone solution and ventral prostate cytosol solution were mixed, and the flow rate was about 0.0033 ml/s. The steady-state heat flux ranged from 0.5 to 9 μcal/s. The heat of dilution for hormone solution, hormone-atratone solution and protein solution used were determined separately and subtracted from the measured heat of mixing to obtain the heat of reaction. The stock solutions of unlabelled 5α-DHT were prepared in absolute ethanol and then diluted in 50 mM Tris–HCl buffer, pH 7.4; the final concentration of ethanol was 10 μl/ml. 5α-DHT solutions in 50 mM Tris–HCl buffer, pH 7.4, were prepared daily in the concentration range: 32.4 to 97.3 μmol. Hormone–atratone solutions, in the same buffer, contained atratone in the range of 147.6 to 590.0 μmol. The ventral prostate cytosol solution was adjusted with 50 mM Tris–HCl buffer, pH 7.4, to a protein concentration of 1 mg/ml. The molar

Table 2. Conversion of [^{14}C]-testosterone into [^{14}C]-dihydrotestosterone in pituitary of male rats castrated 48 h earlier

		Tissue wt. (mg)	d.p.m.	d.p.m./mg
Control	(6)	6.9 ± 0.3	3258 ± 126	473 ± 18*
In vitro addition of atratone (0.38 mmol)	(5)	7.3 ± 0.2	570 ± 87	79 ± 11†
In vivo s.c. administration of atratone (0.1 mg/100 g b.w.)	(3)	7.2 ± 0.2	619 ± 98	86 ± 17†

* Mean ± S.E.; ( ) = number of samples. † $P < 0.001$ vs control.

enthalpy change was determined as described in our previous papers[6–8].

RESULTS

LRF biosynthesis in vitro

It was found that biosynthesis of LRF is enhanced significantly 48 h after castration (Table 1). The presence of 8.5 mmol of atratone in the incubation medium decreases the incorporation of [^{14}C]-leucine into the hypothalamic tissue of intact rats by 90%.

Significant inhibitory effect of atratone was noted in experiments with castrated animals. As the dose of atratone added to the medium increased, the incorporation of [^{14}C]-leucine gradually decreased, reaching the level of 94% inhibition in the presence of 8.0 mmol of atratone in the incubation medium.

Pituitary 5α-reductase activity

Castration of male rats is followed by an increase of pituitary 5α-reductase activity[5]. Therefore, in the present study, experiments were performed with castrated animals (Table 2). Addition of 0.38 mmol of atratone to the reaction mixture, in *in vitro* experiments, strongly inhibited the 5α-reductase activity and only 16.7% of the activity, in comparison to the control animals, could be detected. Similar results were obtained in *in vivo* experiments, where atratone was subcutaneously injected immediately after castration. In this case only 18.8% of 5α-reductase activity could be observed (Table 2).

Inhibitory effect of atratone on hormone–receptor complex formation

Sucrose density gradient pattern of the ventral prostate cytosol from rats, castrated 48 h earlier, following *in vitro* administration of [^{3}H]-5α-DHT, is shown in Figure 1. Binding of 5α-DHT to prostate cytosol receptor, with a sedimentation coefficient of 8 *S*, is only observed if the binding procedure is per-

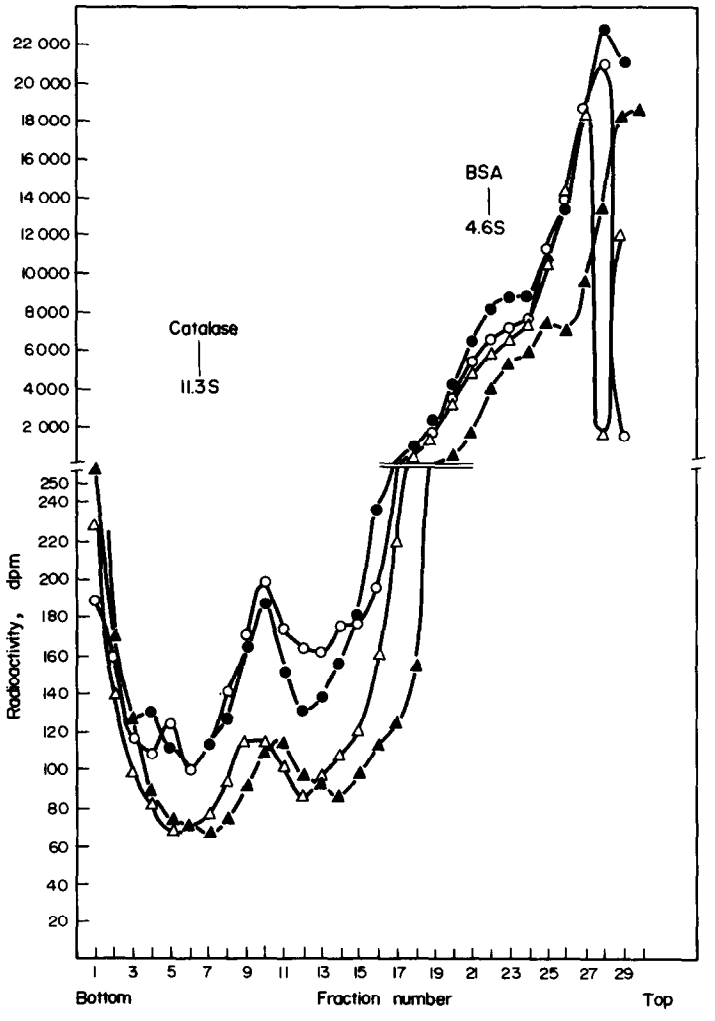

Fig. 1. Sucrose density gradient pattern of ventral prostate cytosol. The ventral prostate cytosol from male rats aged 90 days, castrated 48 h earlier (containing 12.2 mg of protein/ml) was incubated with [^{3}H]-5α-DHT (2.5×10^{-12} mol) for 2 h at 4°C (○——○), or the same quantity of [^{3}H]-5α-DHT with 20 μl of absolute ethanol (●——●), 0.38 mmol of atratone (△——△) or 0.95 mmol of atratone (▲——▲).

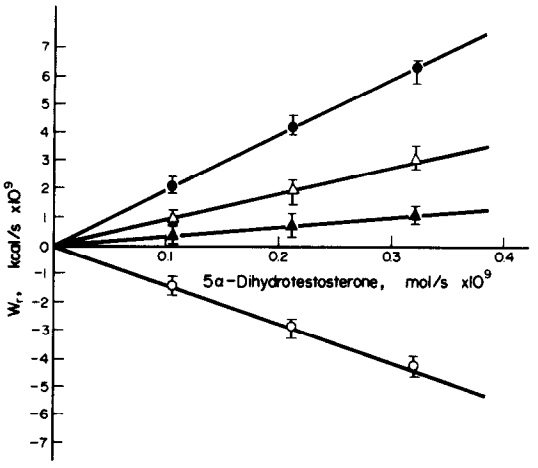

Fig. 2. Concentration dependence of the heat of binding of 5α-DHT to cytosol (1 mg protein/ml) from rats castrated 48 h earlier; in the absence of atratone (○——○) and in the presence of atratone (nmol/s): 1.947 (●——●), 0.974 (△——△) and 0.487 (▲——▲). Heat measurements were performed in a flow microcalorimeter at 37°C, in 50 mM Tris–HCl buffer, pH 7.4.

formed at 4°C. At 37°C, under the same experimental conditions, the radioactivity peak in this region was not detectable. To establish the specificity of 5α-DHT binding to prostate cytosol receptors at 8 *S*, the experiments were performed with [^{3}H]-5α-DHT in the presence of unlabelled 5α-DHT (1000 fold higher conc.); the binding of [^{3}H]-5α-DHT was completely abolished. As illustrated in Fig. 1 addition of atratone decreases the amount of binding in 8 *S* fraction by approximately 40%.

Energy requirements for hormone–receptor complex formation

Figure 2 shows the concentration dependence of the heat of binding of 5α-DHT in 50 mM Tris–HCl buffer, pH 7.4, at 37°C. Results are presented for binding to rat ventral prostate cytosol in the presence and absence of atratone. The amount of hormone was sufficient to completely saturate the cytosol solution in the concentration range used, as indicated by the straight-line fits of the data shown in Fig. 2. The straight lines displayed are the least-square fits through the origin. As is described in our previous papers[6–8], the values of the molar enthalpy changes are directly computed from the slopes of these lines. These values are presented in Table 3. In the absence of atratone the molar enthalpy change of −13.4 kcal/mol of 5α-DHT was observed. Addition of atratone to the reaction medium caused an endothermic reaction. The molar enthalpy change gradually rose from +3.2 to +19.6 kcal/mol of 5α-DHT in the presence of 0.4 to 2 nmol/s of atratone.

Concentration dependence of atratone vs the heat of binding during 5α-DHT–receptor complex formation is presented in Fig. 3. The arrow on Fig. 3 indicates the concentration of approximately 0.25 nmol of atratone/s, at which a change of the energetic balance takes place.

DISCUSSION

Taking the present evidence overall, the influence of atratone, a selective s-triazine herbicide, on hormone-dependent reactions in hypothalamus, pituitary

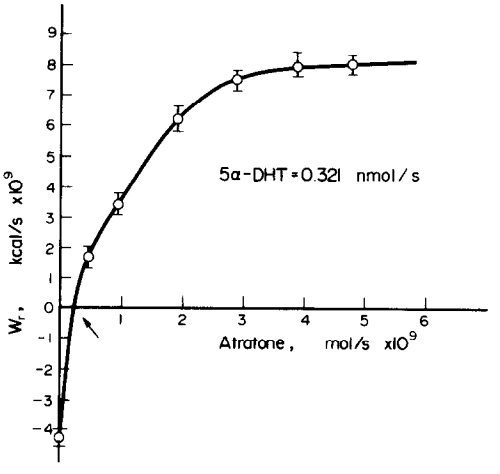

Fig. 3. Concentration dependence of atratone vs heat of binding during 5α-DHT–receptor complex formation. at constant 5α-DHT (0.321 nmol/s) and protein concentration in rat prostate cytosol (1 mg/ml). Heat measurements were performed in a flow microcalorimeter at 37°C, in 50 mM Tris–HCl buffer, pH 7.4.

Table 3. Heat of binding of 5α-dihydrotestosterone to rat prostate cytosol in absence and in presence of atratone, in 50 mM Tris–HCl buffer, pH 7.4 and at 37°C

Atratone* concentration (mol/s × 10^9)	Atratone:5α-dihydrotestosterone*†	Molar enthalpy change‡ (kcal/mol of 5α-DHT) ΔH ± S.D.§
none	0	−13.40 ± 0.352
0.487	4.5, 2.3 and 1.5:1	+3.23 ± 0.541
0.974	9.1, 4.6 and 3.1:1	+11.53 ± 0.237
1.947	18.2, 9.1 and 6.1:1	+19.65 ± 0.569

* Three to 5 measurements were made for each concentration. † Molar ratios were calculated on the basis of total pesticide and total hormone present in the calorimetric system. ‡ Molar enthalpy change observed at protein concentration of 1 mg/ml in rat prostate cytosol. § Deviation from observed experimental values.

and prostate gland in rats, is of a great interest for understanding the possible mechanism of action of such pollutants on human beings in the above mentioned tissues.

The results of this study indicate that the most obvious effect of atratone on hormone-dependent cells is an inhibition of the main metabolic reactions. The biosynthesis of LRF in the hypothalamus is decreased by raising the atratone concentration in intact as in castrated rats. In pituitary the 5α-reductase activity was strongly inhibited by *in vitro* addition of atratone. By *in vivo* administration of atratone nearly the same inhibitory effect was observed.

As shown by Wakeling and Visek[4], the inhibition of 5α-DHT binding to specific receptors in rat ventral prostate in the presence of chlorinated hydrocarbon insecticides may be representative of a biochemical mechanism by which insecticides modify hormonal action in mammalian reproductive organs. Our results from the sucrose gradient pattern of rat prostate cytosol, show the decrease in 5α-DHT binding to specific receptors in the presence of atratone.

In our previous papers[7, 8] it was demonstrated that the formation of hormone–receptor protein complexes either from prostate or hypothalamic tissue was always followed by liberation of energy. In the presence of atratone the total energetic equilibria are changed during the hormone–receptor complex formation. The reactions are endothermic and therefore from the energetic point of view the chance for 5α-DHT–receptor complex formation is very small. As illustrated in Fig. 3 the concentration of 0.25 nmol of atratone/s indicated by the arrow, is the critical amount where the energetic equilibrium is changed and a chance for spontaneous hormone–receptor complex formation is gradually decreased. To confirm that in prostate cytosol used in micro-calorimetric determinations the specific receptor for 5α-DHT is present, sucrose density gradient separations were performed.

The results presented in this study are the best support for the caution which has to be taken in the field of herbicide application.

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