ment given at 14 weeks resulted in marked reductions at 2 and 5 mg/kg (99 and 100%) with a loss of marked activity at 1 and 0.5 mg/kg. Toxic effects were observed only in the 20-mg/kg-group of experiment 1. In this case 1 of the 2 sheep was functionally blind 3 days after treatment, but otherwise clinically normal.

In studies in sheep to assess toxicity it has been found that blindness with an ocular pathology characterized by corneal edema, equitorial lens opacities, papilledema, and changes in the retinal vessels, can follow oral administration of the compound at a level above 20 mg/kg. Blindness in some sheep as a sequel to treatment with a single dose of 20 mg/kg has been confirmed; 15 mg/kg was associated with slight papilledema in 1 of 3 sheep, and no definite abnormalities were noted with a treatment of 10 mg/kg.

Table 2. Efficacy of 2-hydroxy-2′,3,4′,5,5′,6-hexachlorobenzenesulfonanilide against mature *Fasciola hepatica* in the bovine

Dose (mg/kg)	Mean number of live flukes recovered	% Reduction
0	137	0
5	2.5	98
4	39	72
3	72	48
2	132	4

Experimental conditions: 10 mixed-breed calves were each exposed orally to 600 selected metacercariae of *Fasciola hepatica.* 14 weeks after exposure the animals (mean weight 186 kg) in groups of 2 were each given a single oral dose as shown of compound suspended in 1.25% Methocel vehicle (approx. 0.15 ml/kg b.wt). 1 group received vehicle only (placebo). 13 days after treatment the calves were killed and flukes recovered by dissection of the liver.

In a limited efficacy trial in cattle (table 2) the compound, given as a single oral dose of 5 mg/kg, was 98% effective against 14-week-old flukes. Doses of 4, 3, or 2 mg/kg gave apparent reductions of 72, 48, and 4%, respectively. No clinical manifestations of toxicity were noted in any group during the trial.

As illustrated in this report, halogenated benzenesulfonanilides are potent fasciolicides with excellent activity against both immature (< 12 weeks old) and mature flukes. In common with certain known fasciolicides[7,8], the chemotherapeutic index was narrow. Alterations in chemical structure, formulation, and method of administration can, however, significantly influence this parameter.

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Seasonal variation of androgen interconversion in testicular tissue of *Rana temporaria* in vitro

E. Antila and A. Saure

Department of Medical Biology, University of Helsinki, Siltavuorenpenger 20A, SF-00170 Helsinki 17 (Finland), 29 May 1978

Summary. Testicular steroid metabolism of winter and spring frogs, *Rana temporaria,* were studied by in vitro incubation with radioactively labelled dehydroepiandrosterone and androst-4-ene-3,17-dione. Marked seasonal differences were observed which are in line with the findings of others.

The breeding cycle of frogs is known to be due to an independent inherent rhythm[1]. The spawning is timed to occur just after the end of the wintering period and the behavioural change is believed to be caused by increased hormonal activity. A seasonal variation in plasma levels of testosterone, estradiol and estrone has been found to occur in *Rana esculenta*[2]. Δ^5-3β-Hydroxysteroid dehydrogenase activity has earlier been shown histochemically both in anuran and urodelan testes[3]. This reaction was most intense with dehydroepiandrosterone in *Xenopus* testes[4]. Incubation studies have shown the presence of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD), 17α-hydroxylase, C21-C19-desmolase and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities in testes of *Pleurodeles waltlii*[5], *Rana esculenta*[6] and *R. catesbeiana*[7].

We report here seasonal differences in the testicular steroid metabolism of winter (December) and spring (May) frogs, *R. temporaria.* In Southern Finland the spawning starts on the average on 20 April[8].

Materials and methods. 20 adult frogs caught in May in Southern Finland were used immediately (spring frogs). 40 frogs caught in September were kept for a few months in captivity at 4 °C and in the dark. This latter group was used in December (winter frogs). Body and testis weights were determined before incubation. The reproductive condition of the spring frogs was seen by the occurrence of spermatozoa in the vesicula seminalis. To 10 of the winter frogs, 500 IU of HCG (Pregnyl®, Organon) was given per animal a day before the experiment. Pooled testes were homogenized in frog-Ringer buffered by Trizma® (Sigma) solution pH 7.5 at 15 °C to obtain a 10% homogenate which was incubated as 20 5-ml portions at 15 °C for 30 min under a continuous flow of carbogen. 10-min incubation time was also tested and found insufficient for the measurable conversion of the substrate. Substrates were 4-^{14}C-DHA (0.58×10^{-3} mmole/l, 0.15 µCi) and 4-^{14}C-androst-4-ene-3,17-dione(Δ^4A, 0.58×10^{-3} mmole/l, 0.15 µCi). When NAD or NADH was used concentration was 1.51 mmole/l. 6 control incubations were carried out using acetone denatured homogenates and 4-^{14}C-DHA as substrate. Isolation by diethyl ether and characterization of the radioactive metabolites were done as previously described[9]. The meta-

Table 1. Distribution of radioactivity in TLC-fractions (% of the recovery)

	Incubation season	Cofactor	n	TLC-fractions 9	8	7	6	5	4	3	2	1
A	S	–	1	–	–	13.2	2.2	8.1	9.6	13.9	41.1	4.8
	W	–	2	–	5.1±0.4	10.1±1.1	–	54.3±4	–	14.4±3	12.1±0.2	2.8±0.7
	W+HCG	–	1	–	3.7	10.7	–	31.3	–	16.3	35.5	2.5
	S	NAD	1	1.0	5.4	19.0	2.9	7.1	7.9	29.2	19.5	3.5
	W	NAD	2	3.7±0.5	14.0±0.4	15.3±3.7	–	30.2±5.4	–	28.3±1.8	6.3±0.1	2.1±0
B												
	S	–	2	–	2.9±0	19.3±1.4	24.2±2.4	–	–	7.1±0.4	43.1±0.8	3.1±0
	W	–	1	–	6.3	27.4	21.4	–	–	18.0	22.7	3.9
	W+HCG	–	1	–	21.2	17.7	28.0	–	–	25.9	3.8	3.1
	S	NADH	2	2.3±0.3	9.1±0.2	13.4±0.2	47.7±0.5	–	–	9.7±0.3	11.8±0	3.8±0
	W	NADH	1	–	3.6	20.1	31.7	–	–	24.5	15.8	3.6

W: winter, S: spring, A: DHA incubations, B: Δ^4A incubations.

bolites of DHA and Δ^4A were distributed in 8–6 fractions in TLC (numbered in decreasing order of polarity including substrate, 1–9). Radioactive steroids in these fractions were studied in repeated bidimensional TLCs. A tentative identification was based on the identical mobilities of a radioactive steroid and an unlabelled authentic carrier steroid. The conclusive identification was based on the determinations of CSA[9] and constant isotope ratio[10]. Authentic reference steroids were mainly obtained from the Steroid Reference Collection or purchased from commercial dealers.

The solvent systems used in TLC were as follows: 1. chloroform:ether (3:1); 2. chloroform:acetone (8:2); 3. chloroform:ethanol (19:1); 4. chloroform:ethanol (9:1); 5. dichlormethane:acetone (5:1); 6. dichlormethane:acetone (5:2); 7. benzene:ethanol (4:1); 8. benzene:ethylacetate (1:1).

Results and discussion. The recoveries in ether of DHA and Δ^4A were 80.8±6.8% and 87.5±2.8%. The minor amounts of radioactivity remaining in aqueous and NaOH phases were not studied. The radioactive steroids found to be contained in TLC fractions were characterized as follows:

Fraction 1: 19α-hydroxyandrost-4-ene-3, 17-dione was identified (table 2).
Fraction 2/DHA: androst-5-ene-3β, 17β-diol (Δ^5 A) was tentatively identified (TLC systems 6,4,3).
Fraction 2/Δ^4 A: androst-4-ene-3β, 17β-diol was tentatively identified (TLC systems 3,7).
Fraction 3: testosterone (T) was identified (table 3).
Fraction 4: remained unidentified.
Fraction 5: unchanged substrate (DHA) was found (TLC syst. 3, repeated 2×).
Fraction 6: 17β-hydroxy-5α-androstan-3-one (DHT) was tentatively identified (TLC syst. 3, repeated 2×).
Fraction 7: androst-4-ene-3,17-dione (Δ^4 A) was identified, determination of constant isotope ratio (table 4).
Fraction 8: 5α/β-androstane-3,17-dione was tentatively identified in TLC (syst. 2,3).
Fraction 9: remained unidentified.

Table 2. Identification of 19-hydroxy-4-androstene-3,17-dione by determination of constant specific activity (cpm/µg)

Chromatography Numbers		Sample 1
Before chromatography		123
1	(3)	97
2	(7)	92
3	(10)	95

Sample: 1,4-^{14}C-DHA incubations: fraction 1 pool.

Table 3. Identification of testosterone by determination of constant specific activity (cpm/µg)

Chromatography Number	System	Samples 1	2
Before chromatography		669	147
1	(3)	577	109
2	(10)	560	108
3	(7)	540	102

Samples: 1,4-^{14}C-Δ^4A incubations: fraction 3 pool;
2,4-^{14}C-DHA incubations: fraction 3 pool.

Table 4. Identification of 4-androstene-3,17-dione by determination of constant isotope ratio

Chromatography Number	System	Samples 1	2	3
Before chromatography		330	197	335
1	(3)	327	297	335
2	(10)	337	306	335
3	(7)	323	316	344

Samples: 1,4-^{14}C-Δ^4A control incubations: fraction 7 pool;
2,4-^{14}C-DHA incubations: fraction 7 pool;
3,4-^{14}C-Δ^4A incubations: fraction 7 pool.

As shown in table 1, both substrates were actively metabolized. When DHA was used as substrate, its conversion occurred at the lowest rate in W-frogs testes. About equal amounts of Δ^4A, Δ^5A and T were formed amounting ca. 37% of the dose. When HCG was added the conversion of DHA to Δ^5A was increased about 3-fold. The formation rates of Δ^4A and T remained unchanged. This suggests the stimulation of 17β-HSD activity by HCG.

The addition of NAD enhanced the formation of T about 2-fold and also of 5α/βA about 3-fold, possibly via Δ^4A because the formation of Δ^5A remained low. This suggests the presence of more $\Delta^5$3β-HSD activity than demonstrated by control incubations. The formation of DHT could not be detected in any of these experiments.

DHA was very effectively metabolized in spring. Especially Δ^5A was increased, amounting to nearly half of the radioactivity; DHT was also found but in tiny amounts. The addition of NAD clearly increased the formation of T and also 5α/βA but not of DHT. The formation of Δ^5A was reduced about 2-fold.

DHT was the main metabolite found in all incubations with Δ^4A as the substrate. The addition of HCG mainly enhanced the formation of 5α/βA, but not significantly that of T and DHT. The addition of NADH resulted in the increased formation of DHT.

In spring Δ^4A was strongly converted to Δ^4A-diol (nearly half the radioactivity). The amount of T was significantly reduced (as compared to winter) but the ratio of DHT/T was higher. The addition of NADH increased both the amount DHT (2-fold) and DHT/T ratio. Δ^4A-diol was significantly reduced. Hibernating frogs are known to live in temperatures below 4 °C[11]. We did not test these temperatures. However, DHA metabolism in testicular homogenate of hibernating hedgehogs has been shown to be reduced but had not changed its pattern when temperature is lowered to 4 °C[9].

The formation of C-19 steroids from DHA and androstenedione in testis tissue of *R. temporaria* showed a marked seasonal variation in the present incubations. Distria et al.[3] have shown that the plasma levels of T in males of *R. esculenta* were maximal from December to March and minimal from August to September. T and DHT plasma levels of *R. pipiens* have been shown to be low during summer months and higher already in October[12]. In our experiments, T was produced at a considerable rate by the testes of winter and spring frogs. DHT was formed from Δ^4A in a substantial rate already in winter frogs. This is in line with the findings of Wada et al.[12] with leopard frog and with those of Müller[13,14] with bullfrog, demonstrating DHT as the major testicular product. Its formation was specifically stimulated by bullfrog LH[13,14]. Furthermore, the formation of DHT from T in incubations of testicular tissue obtained from 2 anuran species (*R. temporaria* and *Discoglossus pictus*) has been previously shown[15,16].

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Cytoplasmic contaminants in Triton X-100 washed rat liver nuclei – a possible way of further purification[1]

C. Viticchi and F. Szeszák

Center of Biochemistry, Gerontological Research Department of the INRCA, Ancona (Italy), 24 April 1978

Summary. The presence of cytoplasmic contaminants is demonstrated in preparations of rat liver nuclei, even after washing with Triton X-100. They can be removed by incubation at 37 °C in a medium of low-salt concentration.

In previous experiments[2], when Triton-treated nuclei were incubated with [γ-^{32}P]ATP in low-salt medium[3] in order to phosphorylate their proteins in vitro, components amounting to 10% of the total protein content of the nuclear preparations were solubilized. The extract, called fraction 2, was found to contain extensive cytoplasmic contamination. The application of incubation in low-salt medium is suggested as an additional step in the purification of rat liver nuclei.

Material and methods. Wistar rats of both sexes were given 4 mCi/200 g ^{32}P orthophosphate i.p. 1 h before killing. Nuclei were purified by the method of Blobel and Potter[4], washed in 0.25 M sucrose-3.3 mM $CaCl_2$ (SC), and treated with 1% v/v Triton X-100 dissolved in SC[5]. The low-salt medium (used originally for labelling in vitro[3]) contained 30 mM Tris-HCl, pH 7.5; 0.25 M sucrose; 25 mM NaCl; 10 mM Na_2SO_3; 5 mM Mg acetate and 0.5 mM ATP. The incubation time was 10 min at 37 °C. Saline extract from the incubated nuclei was also separated[5] with 0.14 M $NaCl^-$ 20 mM Tris-HCl, pH 7.5. The different fractions obtained were analyzed as detailed in the figures and the table.

Results and discussion. Figure 1 shows that the electrophoretic profile of fraction 2 differs considerably from that of the 0.14 M NaCl extract. Isopycnic density gradient centrifugation on metrizamide (figure 2) revealed light components in fraction 2. The nucleic acid contents of the pooled fractions, however, apparently cannot be responsible for their low buoyant density. In fact, quantitative analysis of fraction 2 shows a 30% lipid content. This explains the presence of the light components and is also an

Glucose-6-phosphatase activity of different cellular protein fractions obtained during the purification of rat liver nuclei

Fraction	µg P liberated/mg protein
Cytoplasmic components	
12,000 rpm pellet	98.46
40,000 rpm pellet	181.40
40,000 rpm supernatant	17.10
Nuclear components	
0.25 M sucrose, 3.3 mM $CaCl_2$ wash	27.33
0.25 M sucrose, 3.3 mM $CaCl_2$, 1% w/v Triton X-100 wash	22.30
Whole nuclei	5.51
Fraction 2 (supernatant of nuclei after incubation in the medium given in figure 1, B)	32.40
Nuclei after the extraction of F2	2.60
Nuclei, after the extraction of F2, extracted in saline-Tris (figure 1, C)	2.24
Whole nuclei extracted in saline-Tris	2.13

Liver homogenate was first subjected to low-speed centrifugation and cytoplasmic fractions were obtained from the supernatant. G-6-P-ase activity was measured by determining the liberated orthophosphate[11].