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IN VIVO FORMATION OF STEROIDS FROM [1,2,6,7-^{3}H]-PROGESTERONE BY THE SEA LAMPREY, *PETROMYZON MARINUS* L.

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SUMMARY

Intracardiac injection of [1,2,6,7-^{3}H]-progesterone into the blood of the parasitic sea lamprey, *Petromyzon marinus* L. resulted in the formation of 11-deoxycorticosterone (DOC) which was isolated and identified (isopolar and isomorphic with authentic DOC) from blood removed 45 and 120 min post-injection. However, no evidence was obtained for the formation of cortisol, cortisone, corticosterone, 11-deoxycortisol, testosterone, 17α-hydroxyprogesterone or androstenedione. Rapid removal of radioactive progesterone from the blood occurred.

INTRODUCTION

Definitive identification of corticosteroids in the plasma of the sea lamprey, *Petromyzon marinus* have not been reported although low plasma steroid concentrations (e.g., <10 ng/dl) may be present [1]. This possibility has been supported by the presence of 17α-hydroxylase, 21-hydroxylase activities as well as 20-desmolase and possibly 22-desmolase and 3β-hydroxy-steroid dehydrogenase/5-ene isomerase activities in the presumptive adrenocortical tissue (PAT) [11]. Since these enzyme activities appear to be very low, the possibility exists that if steroid precursors of sufficiently high S.A. were injected into the blood, identifiable 3-keto-4-ene steroids may be observed in the plasma. In addition, *in vivo* conversion of a steroid precursor may give information more representative of the *in vivo* capacity to synthesize steroids since qualitative differences between *in vivo* and *in vitro* formation of steroids have been reported [2, 3]. The *in vivo* synthetic capacity of the rat adrenal has been successfully studied by intravenous injections of radioactive steroid precursors [4]. However, the presence of PAT in the pronephric region [5–8], the opisthonephros [9, 10] and the dorsal vessels [10] precludes the measurement of adrenal effluent blood in the sea lamprey. Therefore, intracardiac injections of [1,2,6,7-^{3}H]-progesterone were made and blood sampled from the heart at 45 min and 120 min post-injection.

EXPERIMENTAL PROCEDURES

Parasitic adult sea lamprey, *Petromyzon marinus* were obtained and maintained at 8°C as outlined previously [11]. Unanesthetized lampreys were each given an intracardiac injection of 100 μCi of [1,2,6,7-^{3}H]-progesterone (96 Ci/mmol; New England Nuclear Corp., Boston, MA) dissolved in 0.5 ml of 0.7% NaCl containing 10% ethanol. The radioactive progesterone, which was purified by New England Nuclear Corp., was injected into the lampreys on the same day it arrived at the University of Toronto. Blood was removed from the heart with heparinized syringes 45 min or 120 min post-injection. The parasitic lamprey from which blood was removed 45 min post-injection was a male measuring 40 cm and 84 g. The parasitic lamprey sampled at 120 min post-injection was a female measuring 47 cm and 155 g.

Whole blood was frozen prior to extraction. Radioinert cortisol, cortisone, corticosterone, 11-deoxycortisol, testosterone, 17α-hydroxyprogesterone, androstenedione and progesterone were added to the blood prior to adjustment of the blood to pH 9.0 and extraction with dichloromethane (7–10 vol.). Purification by t.l.c. in solvent systems I–XI have been previously outlined [1]. Solvent systems XIV–XXII have also been previously outlined [11]. An additional solvent system (by vol.) used for t.l.c. was as follows: XXIII, chloroform–methanol (99:1). Recrystallizations followed the procedures of Axelrod *et al.*[12]. Acetylation of androstene dione was attempted with 1 ml of a 10:1 (v/v) mixture of pyridine–acetic anhydride (radio-inert) as outlined by Weisbart and Idler[1]. Oxidation of androstenedione was attempted with 0.5 ml glacial acetic acid and 1.5 ml of 2% chromic acid, at room temperature, in the dark. After 20 h, 5.0 ml H_2O was added and the steroid extracted with 40 ml of dichloromethane. The dichloromethane was washed 4 times with 5 ml of H_2O and subsequently chromatographed.

11.3 μCi of the stock [1,2,6,7-^{3}H]-progesterone was also analysed for the presence of other steroids using the same procedures of purification.

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Table 1. Recoveries of tritium radioactivity from the blood of two parasitic lampreys either 45 min or 120 min following injections of 100 μCi of [1,2,6,7-^{3}H]-progesterone

Time post-injection	Injected μCi/ml blood*	Recovered μCi/ml blood	Percent recovery	Percent of recovered radioactivity isolated as authentic progesterone
45 min	14.0	1.42	10.8	1.54
120 min	7.59	0.44	5.8	0.32

* Assuming an 8.5% blood volume/body weight [16].

Purification of solvents, elution of thin-layer chromatoplates and counting of radioisotopes have been previously outlined [1] with the exception that whole blood samples were counted in Scintisol Complete (Isolab Inc., Akron, OH) with 200 μl ethanol added.

RESULTS

Following the injection of 100 μCi of [1,2,6,7-^{3}H]-progesterone into each of two parasitic lampreys, 2 ml of whole blood was removed 45 min post-injection while 5 ml of whole blood was removed from the other lamprey 120 min post-injection. Only 10.8% of the injected radioactivity was recovered 45 min post-injection, whereas 5.8% recovered after 120 min (Table 1). 1.5% and 0.32% of the radioactivity in the blood collected 45 min and 120 min, respectively, after injection was isopolar and isomorphic with authentic progesterone (Table 2).

Although considerable tritium radioactivity was found to be isopolar with the added radioinert steroids following the first thin-layer chromatogram (Table 3), subsequent chromatography and recrystallization failed to establish isopolarity and isomorphicity of the radioactivity with the radioinert cortisol, cortisone, corticosterone, 11-deoxycortisol, testosterone, 17α-hydroxyprogesterone and androstenedione. The ^{3}H/^{14}C ratios of the crystals in each of the purifications resulted in a lack of constant ratios.*

* Data available from M.W.

However, for both the 11-deoxycorticosterone isolated from the blood, 45 min and 120 min post-injection, the ^{3}H/^{14}C ratios of the crystals had a coefficient of variation <5% (Table 4).

Purification of the radioactivity in the androstenedione areas of the first chromatogram resulted in identification of androstenedione in the uninjected [1,2,6,7-^{3}H]-progesterone (Table 5). To further verify this identification, the material remaining after the fourth recrystallization was acetylated, purified in solvent system XX, subsequently oxidized with chromic acid and purified in solvent system XXIII. In both cases, the tritium radioactivity remained isopolar with the radioinert androstenedione. A control acetylation of cortisol and a control oxidation of 17α-hydroxyprogesterone run simultaneously with the same derivative reagents were successful. Therefore, on the basis of chromatography, failure to acetylate or oxidize the compound and the constant ^{3}H/^{14}C ratio of the crystals, the [1,2,6,7-^{3}H]-progesterone contained tritium-labelled androstenedione. The tritium-labelled androstenedione represents approximately 0.4% of the 11.3 μCi of [1,2,6,7-^{3}H]-progesterone which was analysed.

DISCUSSION

The sea lamprey's *in vivo* steroidogenic ability appears to differ from the *in vitro* steroidogenic capacity of its tissues. Considerable 17α-hydroxylase ac-

Table 2. Purification of radioactive progesterone (d.p.m.) by chromatography and recrystallization (R) following extractions of the blood of parasitic sea lampreys injected with 100 μCi of [1,2,6,7-^{3}H]-progesterone. 11.3 μCi of uninjected [1,2,6,7-^{3}H]-progesterone (CTL) was also analysed for progesterone

Purification	Blood obtained: 45 Min post-injection	Blood obtained: 120 Min post-injection	CTL
I, II	224,000*	260,000*	13,000,000*
XX	74,600	45,300	11,000,000
XVIII	63,600	18,400	9,200,000
Before R1	13.4†	2.12	45.3
R1	11.7 (17.9)‡	1.33 (5.19)	46.4§ (50.4)
R2	10.7§ (14.2)	1.05§ (1.90)	46.2§ (48.4)
R3	10.7§ (11.5)	0.960§ (1.32)	45.4§ (52.9)
R4	9.94§ (10.5)	1.03§ (1.07)	45.6§ (50.4)

* Contaminated with radioactivity from adjacent areas of chromatogram. † ^{3}H/^{14}C. ‡ ^{3}H/^{14}C ratio of crystals (mother liquor). § Coefficient of variation of each group <5.0%.

Table 1. Amount of radioactivity (d.p.m.) in the cortisol (F), cortisone (E), corticosterone (B), 11-deoxycortisol (S), testosterone (T), 11-deoxycorticosterone (DOC), 17α-hydroxyprogesterone (17αOHP), androstenedione (AD), and progesterone (P) areas after chromatographic purification* of extracts of blood taken 45 and 120 min post-injection of 100 μCi of [1,2,6,7-^{3}H]-P. 11.3 μCi of uninjected [1,2,6,7-^{3}H]-P were similarly purified (CTL)

	45 Min	120 Min	CTL
P	224,000	260,000	13,000,000
AD			
17αOHP	437,000	443,000	2,770,000
DOC			
T		264,000	
S	312,000	155,000	584,000
B	255,000		
E		1,130,000	435,000
F	110,000	321,000	228,000

* Thin-layer solvent systems (by volume): I, hexane–ethylacetate (4:1); II, chloroform–methanol–water (90:10:1).

Table 4. Purification of radioactive 11-deoxycorticosterone (d.p.m.) by chromatography and recrystallization (R) following extraction from the blood of parasitic adult sea lampreys injected with 100 μCi of [1,2,6,7-^{3}H]-progesterone for 45 min or 120 min. 11.3 μCi of uninjected [1,2,6,7-^{3}H]-progesterone were similarly purified (CTL)

	Blood obtained		
Purification	45 Min post-injection	120 Min post-injection	CTL
I, II	437,000*	443,000*	2,770,000*
XVIII	117,000*	243,000	795,000*
XXI	66,800	154,000	274,000
IX	24,600	131,000	67,000
Before R1	7.22†	20.5	10.5
R1	6.89 (7.82)‡	21.0 (20.0)	3.89 (18.3)
R2	7.00§ (7.23)	20.9§ (21.3)	3.45 (6.87)
R3	7.00§ (7.10)	20.5§ (20.3)	3.04 (4.05)
R4	7.09§ (7.17)	20.2§ (21.2)	2.94 (3.39)

* Contaminated with radioactivity from adjacent areas of chromatogram. † ^{3}H/^{14}C. ‡ ^{3}H/^{14}C ratio of crystals (mother liquor). § Coefficient of variation <5.0%.

Table 5. Purification of radioactive androstenedione (d.p.m.) by chromatography and recrystallization (R) following extractions from the blood of parasitic adult sea lampreys injected with 100 μCi of [1,2,6,7-^{3}H]-progesterone for 45 min or 120 min. 11.3 μCi of uninjected [1,2,6,7-^{3}H]-progesterone was also analysed (CTL) for androstenedione

	Blood obtained		
Purification	45 Min post-injection	120 Min post-injection	CTL
I, II	224,000*	260,000*	13,000,000*
XX	23,800	105,000	563,000
XVIII	27,600	56,500	189,000
VIII	10,200	14,200	113,000
Before R1	2,87†	4.14	30.7
R1	1.29 (7.50)	0.957 (18.3)	18.4 (88.4)
R2	0.568 (2.83)	0.463 (7.56)	15.7§ (29.7)
R3	0.279 (1.15)	0.173 (1.82)	15.0§ (19.6)
R4	0.249 (0.562)	0.125 (0.465)	14.9§ (16.3)

* Contaminated with radioactivity from adjacent areas of chromatogram. ‡ ^{3}H/^{14}C. ‡ ^{3}H/^{14}C ratio of crystals (mother liquor). § Coefficient of variation <5.0%.

tivities have been reported in *in vitro* incubations of presumptive steroidogenic tissue [11]. However, no 17α-hydroxylase activity was found in the present study. The only compound formed from injected [1,2,6,7-^{3}H]-progesterone was 11-deoxycorticosterone indicating the presence of 21-hydroxylase activity. This finding is in keeping with the *in vitro* capacity of testicular and presumptive adrenocortical tissues to synthesize 11-deoxycorticosterone from progesterone [11]. It is of interest to note that the amount of 11-deoxycorticosterone in the female sea lamprey sampled 120 min post-injection is approximately 5× that in the male sea lamprey sampled 45 min post-injection (Table 4). Whether this difference is related to the sex of the fish or to other factors remains to be determined.

The *in vivo* and *in vitro* results are also in agreement with regard to 11β-hydroxylase activity. In both types of studies, this enzyme activity has not been demonstrated. The failure to observe *in vivo* radiolabelled 17α-hydroxyprogesterone, cortisol, cortisone and any of the other compounds studied may be due to a rapid metabolic clearance rate of these compounds. The data for [1,2,6,7-^{3}H]-progesterone suggests that it is rapidly cleared from the blood (Table 1). These results are supported by the report of Boffa *et al.*[13] who observed that plasma protein binding of progesterone was low in sea lampreys. The rapid clearance of progesterone from the blood under the conditions of low plasma protein binding is in keeping with the general concept of high metabolic clearance rate being correlated with low plasma protein binding [14, 15]. Part of the rapid clearance of progesterone is due to the conversion to other unidentified compounds (Table 3). Of the radioactivity remaining 45 min and 120 min post-injection, only 1.54% and 0.32%, respectively, was progesterone (Table 1). However, part of the apparent high clearance rate of [1,2,6,7-^{3}H]-progesterone from the blood is due to the instability of the compound (Table 3). This radioactive steroid (CTL) was kept frozen under the same conditions as the blood prior to extraction. Of the original 24,900,000 d.p.m. of tritium radioactivity, only about 40% of this radioactivity was found to be isopolar with progesterone (Table 2).

Only 27.1% of the tritium radioactivity in the blood sampled at 45 min post-injection was extracted from the blood into the dichloromethane. This low efficiency compares to the much higher efficiency of 96.8% in the 120 min post-injection sample. This substantial difference in the efficiency of dichloromethane extraction may be the result of differences in the chemical composition of the steroids in male and female lampreys since the free (unconjugated), radioinert steroids appeared to have been extracted with about equal efficiency in these two extractions.

The identification of androstenedione as a 0.4% contaminant of the 11.3 μCi of [1,2,6,7-^{3}H]-progesterone was confirmed by establishment of isopolarity and isomorphicity (Table 5) followed by attempts at derivative formation. Whether the androstenedione was a contaminant of the progesterone as obtained from New England Nuclear Corporation, or the result of decomposition of the progesterone is not known. If the androstenedione was a contaminant in the radioactive progesterone injected into the blood of our lampreys, then the absence of androstenedione in the blood of these two lampreys (Table 5) may reflect the rapid removal of androstenedione from the blood.

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