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Sex-Steroid Formation in Gonadal Tissue Homogenates During the Testicular Cycle of the Redwinged Blackbird (*Agelaius phoeniceus*)

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Fluctuations in the *in vitro* metabolism of pregnenolone-^{3}H and progesterone-^{14}C were studied in the testes of captive, photoinduced, adult Redwinged Blackbirds (*Agelaius phoeniceus*) during the gonadal cycle. Starting material utilization was high (87–99%) during the photosensitive stage (breeding period), decreased during the regressive stage, and leveled off at about 40% in the refractory stage. The extent of testosterone synthesis varied in a bimodal pattern during the testicular cycle. Peaks in testosterone synthesis occurred during the photosensitive and regressive stages. However, testosterone formation was not detected in the refractory stage.

In addition to testosterone, 20β-dihydropregnenolone (3β,20β-dihydroxypregn-5-ene), 20α-dihydropregnenolone (3β,20α-dihydroxypregn-5-ene), 20β-dihydroprogesterone (20β-hydroxypregn-4-en-3-one), and 20α-dihydroprogesterone (20α-hydroxypregn-4-en-3-one) were isolated. The 20α-pregnenols were detected only during the regressive and refractory stages. Moreover, an inverse relationship was seen between the formation of 20α-pregnenols and testosterone in the photosensitive and refractory stages.

The physiological significance of a bimodal pattern of testosterone synthesis and of a restricted occurrence of 20α-pregnenol formation in blackbirds is discussed in reference to the proposal by Lacy that two major populations of steroidogenic cells are present in the testis.

Initial studies of the *in vitro* production of testicular steroids in birds have revealed that the pathway of androgen synthesis and required cofactors are similar to those reported in mammals (Fevold and Eik-Nes, 1962, 1963; Fevold and Pfeiffer, 1968). In addition, bird testes are capable of *de novo* synthesis of testosterone from acetate (Connell *et al.*, 1966). A major difference, however, is the elevated level of 4-pregnenol formation in birds compared to mammals (Fevold and Eik-Nes, 1963).

In seasonal-breeding birds, testosterone formation has been shown to fluctuate with testis size. Fevold and Eik-Nes (1962) reported that 17α-hydroxylase activity increases with increasing testis size in English Sparrows (*Passer domesticus*). More recently Cardinali *et al.* (1971) have shown that testosterone formation occurs during both the breeding and nonbreeding season in the domestic duck. These findings are consistent with reports that peaks in plasma testosterone concentration (Jallageas and Assenmacher, 1970; Garnier, 1971) and testicular vein plasma testosterone concentration (Garnier and Attal, 1970) occur during both seasons of the gonadal cycle in this species.

It is known that testosterone treatment can modify avian reproductive physiology.

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On the one hand, testosterone treatment can stimulate the completion of spermatogenesis, prevent post-breeding testicular regression, or induce testicular growth and restore spermatogenesis in atrophied gonads in certain species (reviewed by Lofts and Murton, 1973). On the other hand, testosterone treatment has been found to inhibit testicular and pituitary growth, reduce pituitary gonadotropin content, or antagonize the stimulatory effects of light on the cytology of gonadotropic cells in the pituitary (for recent reviews see Kordon and Gogan, 1970; Stetson, 1971; Tixier-Vidal and Follett, 1973).

The Redwinged Blackbird (*Agelaius phoeniceus*) is a seasonal-breeding species (Wright and Wright, 1944; Brenner, 1967; Payne, 1969; Kerlan, 1972). The testicular cycle consists of the photosensitive, regressive, and refractory stages. The photosensitive stage is associated with testicular growth and breeding activity. After the breeding season the gonads regress and in nature testicular regression is associated with photorefractoriness. Photorefractoriness is characterized by the failure of long day lengths to stimulate gonadal growth.

The present studies were undertaken to characterize the relationship between the extent of the *in vitro* production of testosterone, 20β-dihydropregnenolone (3β,20β-dihydroxypregn-5-ene), 20α-dihydropregnenolone (3β,20α-dihydroxypregn-5-ene), 20β-dihydroprogesterone (20β-hydroxypregn-4-en-3-one) and 20α-dihydroprogesterone (20α-hydroxypregn-4-en-3-one) and testis size during each stage of the photo-induced gonadal cycle.

MATERIALS AND METHODS

Animals

Adult male blackbirds were initially maintained in an outdoor aviary. Food (Purina Game Bird Chow-Finisher) mixed with mineralized grit and water were provided ad lib. Steroid formation in one bird (Rg-696.3 mg; Table 3) that was continuously maintained in an outdoor aviary was measured. In the remaining studies, birds were transferred from the aviary to photoperiod rooms which provided 18 hr light (0800–0200), 6 hr dark (LD 18:6) and were maintained at room temperature. To monitor changes in testis size, laparotomies were routinely performed on all birds before they were placed in the photoperiod rooms and on representative birds at selected intervals during this treatment. Birds were classified in the photosensitive stage if testicular size increased markedly between two observations. On the other hand, birds that were observed to pass through the photosensitive stage and then displayed a significant reduction in gonadal size were considered in the regressive stage. Since testes of minimal size are not found exclusively in the refractory stage, only birds whose gonads failed to enlarge after 1-mo exposure to a LD 18:6 regimen were considered to be refractory. In one experiment, birds that were continuously maintained on this long photoperiod from 15 July to 5 February remained photorefractory.

Experimental Procedure

Materials. Progesterone-4-^{14}C, specific activity (sp act) 58.5 mCi/mmole and pregnenolone-7-^{3}H, sp act 500 mCi/mmole were obtained from Amersham/Searle (Arlington Heights, IL). Prior to use, radiochemical homogeneity of the starting materials was established by submitting each steriod to two different paper-partition chromatography (PPC) systems and recrystallization of an aliquot with authentic progesterone or pregnenolone.

The following PPC systems were used to purify the products: System 1 heptane; formamide; System 2 heptane:benzene, formamide (1:1); System 3 heptane:methanol:water (5:4:1); System 4 ligroin, propylene glycol; System 5 heptane:benzene:methanol:water (2:1:4:1); System 6 toluene, propylene glycol; System 7 cyclohexane:benzene, formamide (1:1).

PPC was performed on Whatman No. 1 paper. All organic solvents were redistilled. The melting point of all crystalline steroid standards was determined and each steroid was recrystallized prior to use.

Incubation procedures. Birds were decapitated and the testes were removed, measured, weighed, and placed in Krebs–Ringer bicarbonate buffer (pH 7.4) which was kept on ice.

In the majority of experiments, both testes from an individual were used. In two trials during the regressive stage and in all trials during the refractory stage, however, testicular tissue was pooled from several birds to provide adequate material. All gonads were incubated within 45 min of their removal.

The testes were homogenized with a Potter–Elvehjem homogenizer in 1 ml Krebs–Ringer

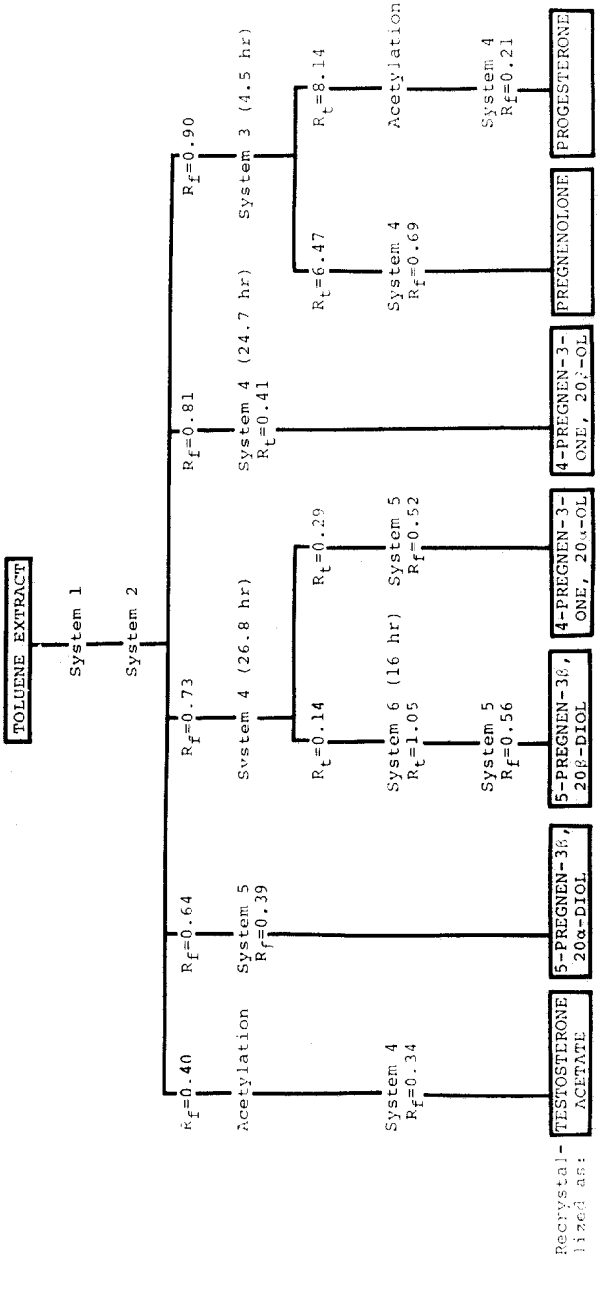

FIG. 1. Flow chart for isolation and identification of metabolites after incubation of isotopic pregnenolone and progesterone with testes of Redwinged Blackbirds. R_t values represent the ratio of distance from the origin to duration of chromatography in hours.

bicarbonate buffer containing 7 μmoles NADP, 7 μmoles NAD, 50 μmoles $MgCl_2$, 150 μmoles glucose-6-phosphate, and 1.6 units glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MO) packed in ice. The homogenizer was rinsed with another milliliter of buffer. Each incubation beaker contained 0.017 μmoles (8.5 μCi) of pregnenolone-7-^{3}H and 0.017 μmoles (1.0 μCi) of progesterone-4-^{14}C in 2 drops of propylene glycol. All incubations were carried out for 3 hr in a Dubnoff metabolic incubator at 41° with 95% oxygen and 5% CO_2 as the gaseous phase. The reactions were terminated by adding diethyl ether–ethyl acetate (4:1, v/v).

Tissueless controls were run simultaneously for each experiment. Four representative controls were processed. Three of four controls contained tritiated and ^{14}C-labeled material with chromatographic mobility similar to 20α-dihydroprogesterone. Mean blank values were subtracted from all conversions reported in this study.

Extraction and chromatographic identification. Progesterone (300 μg), testosterone (300 μg), and in many experiments pregnenolone (100 μg) were added prior to extraction of the reaction mixture. The extraction procedure used was that described by Fevold and Pfeiffer (1968) with minor modifications. Eight milliliters of water were added to the incubation flask and the resultant solution was extracted with 10 ml of diethyl ether–ethyl acetate (4:1, v/v). This extract was mixed thoroughly, centrifuged, and the diethyl ether–ethyl acetate mixture was aspirated. The homogenate was extracted five times with the above mixture, and the fractions soluble in organic solvent ("free" fraction) were combined and evaporated *in vacuo*. The "free" fraction was partitioned between toluene and NaOH in a countercurrent fashion using two separatory funnels with four lower-phase transfers. The toluene fraction ("neutral" steroids) was washed with H_2O, evaporated *in vacuo*, and submitted to PPC in System 1 followed by PPC in System 2. The NaOH fraction ("phenolic") was not processed further. The metabolites in the neutral fraction that were identified in this study were subjected to chromatography as illustrated in Fig. 1.

Testosterone and progesterone were detected on paper chromatography by ultraviolet absorption. Pregnenolone was detected with phosphomolybdic acid (Neher, 1963). Isolation of the radioactive metabolites was completed after the radioactive material behaved similarly to steroid carrier in at least two different chromatography systems. Acetylation was performed at room temperature overnight using acetic anhydride–pyridine (1:2, v/v).

Fifty micrograms each of 20β-dihydropregnenolone, 20α-dihydropregnenolone, 20β-dihydroprogesterone, and 20α-dihydroprogesterone were added after the first chromatogram. The Δ^4-pregnenols were detected by ultraviolet absorption while the remaining two Δ^5-pregnenols were detected with phosphomolybdic acid.

Recoveries of progesterone and testosterone acetate were measured in ethanol at 240 nm, and pregnenolone was quantitated as described by Oertel and Eik-Nes (1959). Corrections were performed as described by Allen (1950).

Paper chromatograms were scanned with a Packard radiochromatogram scanner, Model 7200, and radioactive samples were counted in a Packard Tri-Carb Scintillation Spectrometer, Model 3375 for sufficient time to assure a counting error no greater than 5%. Appropriate quench corrections were performed using an internal-standard technique.

Establishment of radiochemical homogeneity. Varying amounts of authentic crystalline progesterone, pregnenolone, and testosterone acetate were added to aliquots from pools of each of these isolated radioactive steroids. They were successively recrystallized until constant sp act was achieved between crystals and mother liquors. Calculations of reported conversions for these steroids was based upon the amount of starting material added to the incubation beakers with appropriate corrections for recrystallization and recovery data. The identity of each pregnenol was assessed twice by recrystallization of representative samples from various experiments.

RESULTS

Identification of Steroids

A representative series of recrystallizations of pregnenolone, progesterone, and testosterone acetate is presented in Table 1. In addition, radiochemical homogeneity of the pregnenols from selected incubations is illustrated in Table 2. No significant decrease in specific activity of the pregnenols was noted in the initial recrystallization. The conversion to these steroids are minimal estimates, however, since they were not corrected for procedural losses.

Starting-Material Utilization

The extent of starting-material utilization varied during the testicular cycle (Table 3). In the photosensitive stage, the

TABLE 1

REPRESENTATIVE RECRYSTALLIZATION OF ISOLATED COMPOUNDS DURING THE TESTICULAR CYCLE[a]

Metabolite	Fraction	Ps stage (dpm/mg)		Rg stage (dpm/mg)		Rf stage (dpm/mg)	
		^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
Pregnenolone	SM	4301	0	7321	0	7454	0
	C_1	4296	0	7114	0	6984	0
	C_2	4338	0	7233	0	6946	0
	ML[b]	4400	0	7372	0	7211	0
Progesterone	SM	4557	2352	198	2430	1581	2428
	C_1	4409	2311	160	2174	1443	2286
	C_2	4414	2239	160	2156	1466	2238
	C_3	4282	2230	—	—	—	—
	ML[b]	4287	2272	165	2182	1521	2232
Testosterone acetate	SM	5086	547	21909	1453		
	C_1	4946	540	21566	1441		
	C_2	4863	527	20448	1321		
	C_3	4827	525	21043	1349		
	ML[b]	5055	549	21909	1425		

[a] Ps = photosensitive stage; Rg = regressive stage; Rf = refractory stage; SM = starting material; C = crystal.

[b] ML = final mother liquor.

TABLE 2

REPRESENTATIVE RECRYSTALLIZATION OF PREGNENOLS[a]

Metabolite	Fraction	dpm/mg		Solvent system
		^3H	^{14}C	
20β-Dihydropregnenolone	SM	11408	0	
	C_1	11052	0	Dichloromethane
	C_2	10965	0	Ethanol–water
	ML[b]	11586	0	
20α-Dihydropregnenolone	SM	895	0	
	C_1	864	0	Methanol
	C_2	893	0	Ethanol–water
	C_3	895	0	Dichloromethane
	ML[b]	946	0	
20β-Dihydroprogesterone	SM	1349	1505	
	C_1	1233	1533	Hexane–acetone
	C_2	1222	1600	Hexane–acetone
	ML[b]	1259	1562	
20α-Dihydroprogesterone	SM	1700	1926	
	C_1	1417	1845	Hexane–acetone
	C_2	1459	1828	Ethyl acetate
	ML[b]	1438	1864	

[a] See Table 1 for explanation of symbols.

TABLE 3
VARIATIONS IN THE METABOLISM OF STARTING MATERIALS

Stage[a]	Total gonadal weight[b] (mg)	(N)[c]	% Pregnenolone-^{3}H utilized[d]	% Progesterone-^{14}C utilized	Pregnenolone / Progesterone
Ps	176.2	(1)	98.5	97.6	1.01
	254.7	(1)	97.2	87.2	1.12
	256.3	(1)	98.1	94.2	1.04
Rg	696.3[e]	(1)	nm[f]	84.3[g]	nm
	562.4	(1)	nm	74.9	nm
	158.3	(1)	nm	74.8	nm
	73.8	(1)	41.2	71.9	0.57
	22.5	(1)	48.8	63.8	0.77
	18.1	(1)	62.0	85.7	0.73
	17.1	(2)	nm	31.2	nm
	31.4	(4)	16.4	49.3	0.33
Rf	25.8	(5)	30.8	44.1	0.70
	—	(5)	30.3	54.2	0.56
	22.9	(6)	40.4	51.8	0.78
	21.8[h]	(4)	nm	25.1	nm

[a] Ps = photosensitive stage; Rg = regressive stage; Rf = refractory stage.
[b] Total weight of tissue/incubation.
[c] (N) = number of birds/incubation.
[d] Initial radioactivity − recovered radioactivity/initial radioactivity × 100.
[e] Three hundred forty-five milligrams of tissue from a bird kept in an outdoor aviary was incubated.
[f] nm = not measured.
[g] Initial amount of progesterone-^{14}C was 0.53 μCi.
[h] Refractory period extended about 27 wk in birds maintained on a LD 18:6 regimen.

ratio of pregnenolone utilization to progesterone utilization was approximately one, but progesterone appeared to be preferentially utilized during the remainder of the cycle. Testes during the photosensitive stage metabolized nearly all of the pregnenolone and 87–98% of the progesterone. During the regressive stage, there was a general trend of decreasing utilization of the starting materials. It is important to note that during the refractory stage about 30–50% of the starting materials were metabolized. The extent of starting-material utilization by testes in the refractory stage was similar to that of minimal-size testes in the regressive stage. The lowest level of progesterone utilization was observed in testes from birds which remained in the refractory stage about 27 wk on a light–dark 18:6 regimen. The level of progesterone utilization in these testes was approximately 50% less than in testes of birds in the refractory stage after only 1-mo exposure to the same photoperiod.

Testosterone Formation

The level of testosterone formation fluctuated dramatically and exhibited a bimodal pattern during the testicular cycle. If the level of testosterone production is expressed as dpm per bird (Table 4), the first peak occurred during the photosensitive stage and was followed by a second peak during the regressive stage. Surprisingly, large testes in the regressive stage produced less testosterone than smaller testes in the regressive or photosensitive stages. In the refractory stage, testosterone was not detected.

If the level of testosterone formation is expressed per unit weight of testicular tissue (Table 5), small testes during the regressive stage produced the most testosterone. Surprisingly, a small pair of testes

TABLE 4

VARIATIONS IN THE *in Vitro* PRODUCTION OF TESTOSTERONE AND PREGNENOLS DURING THE TESTICULAR CYCLE

Stage	Total gonadal weight[a] (mg)	(*N*)	dpm × 10^{-3}/bird							
			T[b]		20β-DΔ⁵P	20α-DΔ⁵P	20β-DP		20α-DP	
			^{3}H	^{14}C	^{3}H	^{3}H	^{3}H	^{14}C	^{3}H	^{14}C
Ps	176.2	(1)	2493.7	271.2	260.1	nf[c]	47.5	12.2	nf	nf
	254.7	(1)	2728.2	308.8	146.9	nf	52.9	9.4	nf	nf
	256.3	(1)	1127.5	130.1	220.6	nf	30.0	4.6	nf	nf
Rg	696.3[d]	(1)	511.5	30.3	291.1	101.2	396.3	36.2	nm[e]	nm
	562.4	(1)	131.0	8.6	769.2	49.4	161.1	36.9	69.7	16.1
	158.3	(1)	111.5	13.5	nf	nf	390.0	71.5	320.7	59.6
	73.8	(1)	373.8	102.0	nm	nm	nm	nm	nm	nm
	22.5	(1)	1568.1	225.6	167.5	nf	44.1	111.8	nm	nm
	18.1	(1)	587.7	42.2	nf	nf	101.0	173.1	nm	nm
	17.1	(2)	59.5	6.7	nm	nm	19.5	24.2	13.8	30.1
	31.4	(4)	10.4	0.8	74.4	49.5	18.2	21.9	10.4	9.7
Rf	25.8	(5)	nf	nf	213.6	119.4	16.1	23.0	23.3	24.0
	—	(5)	nf	nf	268.4	246.4	22.2	29.9	33.3	40.0
	22.9	(6)	nf	nf	173.5	116.1	3.5	9.8	3.6	11.2
	21.8[f]	(4)	nf	nf	68.3	81.8	4.6	5.1	6.1	6.2

[a] Total weight of tissue in each incubation.

[b] T = testosterone; 20β-DΔ⁵P = 3β,20β-dihydroxypregn-5-ene; 20α-DΔ⁵P = 3β,20α-dihydroxypregn-5-ene; 20β-DP = 20β-hydroxypregn-4-en-3-one; 20α-DP = 20α-hydroxypregn-4-en-3-one. Corrected for losses.

[c] Not found.

[d] Three hundred forty-five milligrams of tissue from a bird kept in an outdoor aviary was incubated in 8.5 μCi pregnenolone-^{3}H and 0.53 μCi progesterone-^{14}C.

[e] Not measured.

[f] Extended refractory period.

(22.5 mg) in the regressive stage produced the largest relative amount of testosterone observed in this study. A smaller elevation in testosterone production occurred earlier during the photosensitive stage. The occurrence of the major and minor increases in testosterone production during the testicular cycle, therefore, is variable and depends on whether an absolute or relative measurement is used.

Pregnenol Formation

Fluctuations in pregnenol production during the testicular cycle are also presented in Tables 4 and 5. The level of 20β-dihydropregnenolone, expressed as dpm per bird (Table 4), was relatively stable, except for a transient elevation that was associated with large testes in the regressive stage. In contrast, the formation of 20β-dihydroprogesterone was variable. The level of synthesis of this steroid was minimal in the photosensitive stage, but increased to a peak during the regressive stage and then decreased as the testes reached minimal size in the regressive and refractory stages.

The level of 20β-dihydropregnenolone, expressed as dpm per 100 mg tissue (Table 5), was highest in the refractory stage whereas the formation of 20β-dihydroprogesterone was elevated in small testes during both the regressive and refractory stages. It is of interest to note that the production of 20α-dihydropregnenolone and 20α-dihydroprogesterone were sought but

TABLE 5

VARIATIONS IN THE *in Vitro* PRODUCTION OF TESTOSTERONE AND PREGNENOLS DURING THE TESTICULAR CYCLE

Stage[a]	Total gonadal weight[b] (mg)	(N)[c]	dmp × 10^{-3}/100 mg							
			T[d]		20β-DΔ⁵P	20α-DΔ⁵P	20β-DP		20α-DP	
			^{3}H	^{14}C	^{3}H	^{3}H	^{3}H	^{14}C	^{3}H	^{14}C
Ps	176.2	(1)	1415.3	153.9	147.6	nf[e]	27.0	6.9	nf	nf
	254.7	(1)	1071.1	121.2	57.7	nf	20.8	3.7	nf	nf
	256.3	(1)	439.9	50.8	86.1	nf	11.7	1.8	nf	nf
Rg	696.3[f]	(1)	73.5	4.4	41.8	14.5	56.9	5.2	nm[g]	nm
	562.4	(1)	23.3	1.5	136.8	8.8	28.7	6.6	12.4	2.9
	158.3	(1)	70.4	8.5	nf	nf	246.4	45.2	202.6	37.7
	73.8	(1)	508.4	138.7	nm	nm	nm	nm	nm	nm
	22.5	(1)	6962.4	1001.7	743.7	nf	195.8	494.4	nm	nm
	18.1	(1)	3244.1	232.9	nf	nf	557.5	955.5	nm	nm
	17.1	(2)	695.6	78.3	nm	nm	228.2	282.6	160.9	352.2
	31.4	(4)	132.5	9.9	949.3	631.0	232.2	279.8	131.1	123.8
Rf	25.8	(5)	nf	nf	4143.1	2316.4	311.6	446.2	452.4	465.6
	22.9	(6)	nf	nf	4549.2	3045.5	92.2	256.5	95.3	292.4
	21.8[h]	(4)	nf	nf	1253.5	1500.9	84.0	8 93.6	111.5	113.4

[a] Ps = photosensitive stage; Rg = regressive stage; Rf = refractory stage.

[b] Total weight of tissue/incubation.

[c] Number of birds/incubation.

[d] Corrected for losses.

[e] Not found.

[f] Three hundred forty-five milligrams tissue from a bird kept in an outdoor aviary was incubated in 8.5 μCi pregnenolone-^{3}H and 0.53 μCi progesterone-^{14}C.

[g] Not measured.

[h] Extended refractory period.

not found in the photosensitive stage. Moreover, 20β-reductase activity exceeded 20α-reductase activity throughout the photosensitive and regressive stages.

A comparison between the extent of pregnenol production and testosterone formation during the testicular cycle reveals that there was no apparent relationship between the production of 20β-pregnenols and testosterone. However, if the photosensitive stage is compared to the refractory stage, an inverse relationship existed between the synthesis of 20α-pregnenols and testosterone. In the photosensitive stage, 20α-pregnenols were absent, but large amounts of testosterone were produced. This relationship was reversed during the refractory stage.

Table 6 compares the percentage of the starting materials converted to the pregnenols during the photosensitive and refractory stages. The percentage of metabolized starting material converted to each steroid was calculated using the ratio of the radioactivity of each steroid divided by the difference of the radioactivity associated with the starting materials before and after the incubation. In the photosensitive stage, most of the starting materials were metabolized and the most abundant product was testosterone. However, the sum of the percentages of metabolized starting material converted to pregnenols per incubation accounted for only 1.1–1.7% of the pregnenolone-^{3}H and 0.2–0.6% of the progesterone-^{14}C. In contrast,

TABLE 6
COMPARISON OF THE PERCENTAGE OF STARTING MATERIALS CONVERTED TO PREGNENOLS

Stage[a]	Total gonadal weight[b] (mg)	(N)[c]	Percentage of metabolized starting materials					
			20β-DΔ⁵P	20α-DΔ⁵P	20β-DP		20α-DP	
			³H	³H	³H	¹⁴C	³H	¹⁴C
Ps	176.2	(1)	1.4	nf[d]	0.3	0.6	nf	nf
	254.7	(1)	0.8	nf	0.3	0.2	nf	nf
	256.3	(1)	1.2	nf	0.2	0.2	nf	nf
Rf	25.8	(5)	18.5	10.4	1.4	11.9	2.0	10.5
	—	(5)	23.6	21.7	1.9	12.5	2.9	15.3
	22.9	(6)	13.8	9.3	0.3	5.2	0.3	4.4

[a] Ps = photosensitive stage; Rf = refractory stage.
[b] Total weight of tissue/incubation.
[c] Number of birds/incubation.
[d] Not found.

the decrease in the extent of starting material metabolism during the refractory stage was correlated with a marked increase in the production of these pregnenols. The sum of the percentages of metabolized starting material converted to these steroids per incubation accounted for 23.7–50.1% of the pregnenolone-³H and 9.6–27.8% of the progesterone-¹⁴C.

DISCUSSION

Utilization of pregnenolone and progesterone in steroid biosynthesis occurs during each stage of the gonadal cycle. There is a general trend of decreased starting material utilization during the regressive and refractory stages compared to the photosensitive stage. These findings imply that qualitative as well as quantitative changes in steroidogenesis are probably associated with variations in testicular size and histology during the annual gonadal cycle.

Testosterone formation in Redwinged Blackbird testes agrees with earlier studies of other avian species (Fevold and Eik-Nes, 1962, 1963; Connell *et al.*, 1966; Fevold and Pfeiffer, 1968; Nugara and Edwards, 1970; Cardinali and Rosner, 1971; Cardinali *et al.*, 1971; Nakamura and Tanabe, 1972; Galli *et al.*, 1973). Moreover, testosterone has been isolated from extracts of Redwinged Blackbird testes (Höhn and Cheng, 1967). The synthesis of testosterone from pregnenolone and progesterone in blackbird testes appears to fluctuate in a bimodal pattern during the testicular cycle. One rise is associated with intermediate size testes in the photosensitive stage. It is surprising to find a second rise associated with small testes in the regressive stage. Testosterone formation was not found in the refractory stage.

A second rise in testosterone formation during the nonbreeding period has also been reported in the domestic duck (Cardinali *et al.*, 1971). In this species, testosterone formation in the nonbreeding period is about double that in the breeding period. In both ducks, and Redwinged Blackbirds, the level of testosterone formation, expressed on the basis of testis weight, is higher during the nonbreeding period. However, in both species the extent of testosterone synthesis, expressed per bird, is higher during the breeding period.

The functional significance of elevated testosterone formation during the photosensitive stage may involve a stimulatory effect of testosterone on spermatogenesis whereas testosterone produced during the regressive stage may act through a negative feedback mechanism to inhibit the hypothalamo-pituitary axis (Lofts and Murton, 1973). However, Farner (1967) and Stetson and Erickson (1971) have suggested that

the onset of testicular regression may be regulated entirely by negative pituitary gonadotropin feedback. In contrast, testicular growth may be regulated by negative steroid feedback from the testes (Farner *et al.*, 1968).

In addition to testosterone, four pregnenols (20β-dihydropregnenolone, 20α-dihydropregnenolone, 20β-dihydroprogesterone, 20α-dihydroprogesterone) were formed by Redwinged Blackbird testes. The synthesis of 20β- and 20α-dihydropregnenolone is the first demonstration of their formation by avian testes. On the other hand, formation of 20β-dihydroprogesterone has been identified in the testes of English Sparrows (Fevold and Eik-Nes, 1962, 1963) roosters (Galli *et al.*, 1973), and chick embryos (Galli and Wassermann, 1972, 1973) and production of the 20α-isomer was reported in the above species and domestic duck (Cardinali and Rosner, 1971; Cardinali *et al.*, 1971). Moreover, 20β-dihydroprogesterone was found in an extract of cockrel testes (Delrio *et al.*, 1967). It is noteworthy that in Redwinged Blackbirds 20β-reductase activity is equal to or greater than 20α-reductase activity. A similar relationship exists in English Sparrow (Fevold and Eik-Nes, 1962, 1963) and chick embryo testes (Galli and Wassermann, 1972, 1973). In mammals, however, 20α-reductase activity is higher (Tamaoki *et al.*, 1969).

The formation of 20α-pregnenols by Redwinged Blackbird testes only during the regressive and refractory stage of the gonadal cycle raises the possibility that their synthesis is independent of gonadotropin control. This assumption is consistent with the observation that only hypophysectomized pigeons with atrophied and lipoidal seminiferous tubules contain a progestin-like steroid in the testes and the blood (Lofts and Marshall, 1959).

In the Redwinged Blackbirds an inverse relationship occurs between the extent of 20α-pregnenol and testosterone formation. In the photosensitive stage, 20α-pregnenols are not found but testosterone is produced in large amounts. This relationship is reversed during the refractory stage. An inverse relationship between the extent of 20α-pregnenol and testosterone formation also occurs in the domestic duck, but both steroids are found throughout the testicular cycle (Cardinali *et al.*, 1971).

The inverse relationship between the level of 20α-pregnenol and testosterone formation in blackbird testes may be produced by 20α-pregnenol inhibition of testosterone production. Recent studies in mammals have shown that 20α-dihydroprogesterone is a potent inhibitor of C_{17}-C_{20}lyase, a side-chain-cleaving enzyme in the pathway of testosterone synthesis (Neher and Kahnt, 1965; Shikita *et al.*, 1967; Tamaoki *et al.*, 1969). Although similar studies have not been performed in birds, it is noteworthy that the addition of 17α,20α-dihydroxy-4-pregnen-3-one reduces conversion of progesterone to testosterone about 50% in English Sparrow testes (Fevold and Eik-Nes, 1963).

Discovery of a bimodal pattern of testosterone formation and the restricted occurrence of pregnenol synthesis in blackbirds is consistent with the concept proposed by Lacy (1967, 1969) of two populations of steroidogenic cells in the testis. These populations, located in the interstitium and seminiferous tubules can probably be distinguished by differences in steroid biosynthesis. In rats, the extent of 20α-dihydroprogesterone formation is greater in the seminiferous tubules than in the interstitium (Lacy *et al.*, 1969). Moreover, heat treatment of rat testes only increased 20α-dihydroprogesterone production in the seminiferous tubules. This elevation was associated with a dramatic increase in the amount of lipid and cholesterol visualized by histochemical techniques (Lacy and Pettitt, 1970). It is of interest that seasonal changes in testicular histochemistry of Redwinged Blackbirds (Payne, 1969) and many other avian species (reviewed by Lofts and Murton, 1973) involve a shift in the location of sudanophilic material from the interstitium during the photosensitive stage to the seminiferous tubules in the regressive and refractory stages.

This shift in sudanophilic material in bird testes may reflect a change in the site

of steroid formation. Moreover, it remains to be established whether this change is associated with a transition from androgen synthesis to pregnenol formation during the testicular cycle of Redwinged Blackbirds.

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