BIPHASIC EFFECT OF EXTRACTS OF LITHOSPERMUM RUDERALE ON THE UPTAKE OF ^{32}P BY TESTES AND THYROIDS OF COCKERELS

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Summary - A polyphenolic fraction of the plant Lithospermum ruderale was injected into White Leghorn cockerels at various time intervals and the effect on testes and thyroids was measured by the uptake of ^{32}P. The ^{32}P uptake 8 hours after lithosperm[2] injection was markedly depressed and 12 hours after administration was notably increased. It is suggested that the hypothalamic regulation of the testes and thyroids is altered by lithosperm interference of the hypothalamic feedback system and/or the binding of receptor sites in the two glands.

The synthesis and release of chick gonadotropins and thyrotropin has been shown to be inhibited by cold water extracts (1) and polyphenolic fractions (2) of the plant Lithospermum ruderale. Several possibilities exist concerning the site of lithosperm[2] action including the inhibition of hypothalamic releasing hormones and/or the altering of the sensitivity of the anterior pituitary cells to releasing hormones. This paper reports experiments in which polyphenolic fractions of Lithospermum ruderale were administered to cockerels at various times before autopsy and the treatments were analyzed for their effects on the release of gonadotropins and thyrotropin from the anterior pituitary as assayed by the uptake of ^{32}P by the testes and thyroids.

Materials and Methods. Single-comb White Leghorn cockerels aged 2, 9 or 11-days were used in the experiments. The chicks were removed from feed 12 hours prior to being killed at which time the glands were quickly removed and weighed to the nearest 0.1 mg. The thyroids and testes were dried and then counted on a Nuclear Chicago gas flow counter. The end-point of the assays was the uptake of ^{32}P in terms of wet-weights of the glands. Counts were expressed as Antilog $L_{10}\left[\sqrt{\frac{CPM}{\text{gland (mg)}}} \times \sqrt{\text{Body weight (gm)}}\right]$ (1,2). Analysis of variance (ANOVA) was employed for the evaluation of data and the

1. Contribution number 1019 from the Department of Zoology, 115 from the Waterman Institute.

2. The term lithosperm is used as a generic designation for the hormone inhibiting principle, or principles, in the genus Lithospermum.

t-test was used when two groups were compared. The anterior pituitary glands were stored in acetone, then air-dried, ground, suspended in distilled water and assayed for their gonadotropin and thyrotropin content.

Injections were made subcutaneously in a volume of 0.2 ml. Carrier free ^{32}P was administered one hour before autopsy. The lithosperm fractions were prepared from water-soluble extracts of the dried roots of Lithospermum ruderale (3). The polyphenolic fraction Ea used in these experiments was prepared from a combination of chromatographic separations on Sephadex and a chemical fractionation (3, Table 1, Footnote a).

Three extraneous variables were analyzed: (a) The weights of the testes and thyroids of different groups were compared and also were corrected for variations in body weights. No significant differences between groups were observed by either analysis. (b) The uptake of ^{32}P by the two glands was expressed in milligrams of wet-weights and of dry-weight and related differences between treatment groups were comparable by either method. (c) Finally, injections began in early morning and continued until early afternoon, therefore, the light stimulation via the pineal gland was considered as a possible source of variation (4). An experiment was performed in which cockerels received only ^{32}P at time intervals which spanned the duration of the lithosperm experiments. No significant group differences were observed in the ^{32}P uptake by either the testes or thyroids.

Results. Fraction Ea (3) was administered in a single injection to groups of eleven-day-old cockerels beginning sixteen hours before autopsy and terminating four hours before autopsy (Table 1A). The ^{32}P uptake responses of the testes were bimodal. There was a notable decrease in ^{32}P uptake in the 6 and 8-hour treatment groups and a very marked increase in ^{32}P uptake when treatment was initiated at 10 or 12 hours before autopsy. The ^{32}P counts overlapped the control ranges at the 4, 14 and 16-hour periods.

The effects of lithosperm on the testes and thyroids are known to be dose related (2) and in this experiment were shown to be modified by the duration of treatment, therefore, both variables were examined simultaneously (Table 1B). The total treatment effects on ^{32}P uptake by the testes reflected the influence of both the duration of treatment and of the dosage of lithosperm. The combined one-hour injection periods slightly elevated the ^{32}P uptake of the testes: $F = 7.52$, $P < 0.007$ in spite of the fact that the range of the means of the 0.4 mg and 0.8 mg dosages overlapped the control range. A lowered uptake of ^{32}P occurred in the three groups which received treatment of 8 hours duration; $F = 5.10$, $P < 0.025$. Notable increases in ^{32}P uptake occurred following exposure to lithosperm for 12 hours and the combined responses of these three groups had the maximum deviation from the control value; $F = 106.75$, $P < 8.0^{-10}$. The

TABLE 1

A. Testes Uptake of ^{32}P in Eleven-Day-Old Cockerels Which Received 0.4 mg of Fraction Ea. 2.0 μ Ci ^{32}P Was Injected One Hour Before Autopsy To All Animals. f = 14.

Duration of Lithosperm Treatment

Testes:	Control	4 Hours	6 Hours	8 Hours
$\bar{x}$	180.57	188.36	138.77*	128.30***
Range	203.98-159.86	210.23-168.77	154.91-124.30	145.00-112.75
	10 Hours	12 Hours	14 Hours	16 Hours
$\bar{x}$	378.07***	263.11***	196.92	218.99
Range	412.74-346.32	290.78-238.09	216.92-178.77	243.26-181.60

ANOVA		MSQ	F	P
	Treatments 7	0.3745_2	47.38	$< 2.0^{-29}$
	Error 104	$S^2 = 0.0066$		

B. Testes and Thyroid Gland Uptake of ^{32}P in Nine-Day-Old Cockerels. f = 14.

Amount and Duration of Lithosperm Treatment

1 Hour

Testes:	Control	0.2 mg	0.4 mg	0.8 mg
$\bar{x}$	176.84	227.14**	210.00	198.09
Range	198.18-157.79	255.91-201.60	235.57-187.20	221.83-176.87

8 Hours

$\bar{x}$	171.85	154.07	134.36**
Range	191.97-153.83	172.40-137.69	150.88-119.64

12 Hours

$\bar{x}$	432.16***	330.37***	291.60***
Range	523.92-356.49	365.95-298.26	319.11-266.43

ANOVA (Excluding Controls)		MSQ	F	P
	Treatments 8	0.3774_2	44.93	$< 4.2^{-32}$
	Error 117	$S^2 = 0.0084$		

1 Hour

Thyroids:	Control	0.2 mg	0.4 mg	0.8 mg
$\bar{x}$	203.10	266.25***	297.90***	241.70
Range	223.01-184.98	305.70-231.89	341.53-259.82	276.59-211.20

8 Hours

$\bar{x}$	181.40	157.29**	149.84**
Range	203.59-161.64	173.68-142.27	164.56-136.44

12 Hours

$\bar{x}$	348.68***	305.59***	236.98
Range	425.49-285.71	350.21-266.65	265.35-211.65

ANOVA (Excluding Controls)		MSQ	F	P
	Treatments 8	0.2402_2	24.06	$< 2.1^{-21}$
	Error 117	$S^2 = 0.010$		

Range = 95% Confidence Limits. *P = < 0.05, **P = < 0.01, ***P =< 0.001 by t - test.

responses of testes to lithosperm in this experiment, therefore, paralleled those described for similar treatments in Table 1A.

The data for the ^{32}P uptake by the thyroid glands were more variable than those for the testes, however, the ANOVA F-value for treatment effects suggested that very significant differences were present. Comparisons of each of the three time periods with the control thyroid values showed similarities with respect to the relative time-dose effects observed for the testes. There was a pronounced stimulation by lithosperm in the three groups which were treated one-hour before autopsy; $F = 15.08$, $P < 1.5^{-04}$. Treatments which began 8 hours before autopsy had a substantial inhibitory effect; $F = 9.96$, $P < 0.003$ whereas treatments which were of 12 hours duration markedly augmented ^{32}P uptake by the thyroid glands; $F = 26.78$, $P < 6.4^{-07}$. The 0.8 mg dosage in all three time-groups was the most inhibitory and was comparable with the responses at this dosage for ^{32}P uptake by the testes.

Lower dosages of Fraction Ea (3) were tested next (Table 2). Total treatment effects were marked. The ^{32}P uptake of the testes for the three one-hour dosage groups, however, did not differ from the control either as individual groups or when all groups were combined. The 0.18 mg dosage did approach significance being similar to the 0.2 mg dosage in Table 1B. The ^{32}P uptake at the 0.02 mg dosage in the 8-hour group did not differ from the control average but the 0.06 mg and the 0.18 mg dosage groups had substantially lower uptakes than that of control animals. The animals which received lithosperm 12 hours before autopsy had ^{32}P counts higher than those of the controls at all three dosage levels. Once again the ^{32}P uptakes in the 8-hour and 12-hour series declined as the dosage of lithosperm was increased.

The data for ^{32}P uptake by the thyroids in general paralleled those of the testes but were less affected by the lithosperm. The combined effects of the three one-hour dosages differed from the control at an F value of 4.46, $P < 0.04$. This significance is questionable, however, because ANOVA indicated an interaction of dose x time. After 8 hours of treatment the mean for ^{32}P

TABLE 2

Testes and Thyroid Gland Uptake of ^{32}P in Nine-Day-Old Cockerels Which Received Fraction Ea. 2.0 μ Ci ^{32}P Was Injected One Hour Before Autopsy To All Animals. f = 13.

	Amount and Duration of Lithosperm Treatment			
	1 Hour			
Testes:	Control	0.02 mg	0.06 mg	0.18 mg
$\bar{x}$	187.23	185.09	190.34	206.10
Range	205.50-170.60	213.58-160.39	212.89-170.19	218.94-194.30
	8 Hours			
$\bar{x}$		175.51	139.27***	129.40***
Range		192.17-160.29	154.85-125.24	145.75-114.
	12 Hours			
$\bar{x}$		291.02***	242.10***	225.14**
Range		327.96-258.24	278.04-210.81	243.46-208.19

ANOVA (Excluding Controls)		MSQ	F	P
	Treatments 8	0.1610_2	25.56	$< 1.0^{-21}$
	Error 108	$S^2 = 0.0063$		

	1 Hour			
Thyroids:	Control	0.02 mg	0.06 mg	0.18 mg
$\bar{x}$	174.40	156.73	150.05	153.87
Range	191.14-159.12	169.77-144.70	168.75-133.42	168.05-140.88
	8 Hours			
$\bar{x}$		145.70	106.30***	105.84***
Range		161.35-131.57	117.60-96.08	118.53-94.50
	12 Hours			
$\bar{x}$		225.10***	182.45	161.72
Range		253.14-200.14	201.91-164.87	192.97-135.54

ANOVA (Excluding Controls)		MSQ	F	P
	Treatments 8	0.1286_2	19.78	$< 4.4^{-10}$
	Error 108	$S^2 = 0.0065$		

Range = 95% Confidence Limits. *P = < 0.05, **P = < 0.01, ***P = < 0.001 by t - test.

uptake at 0.02 mg dosage was slightly lower than that of the control but the depression of ^{32}P uptake at the 0.06 mg and 0.18 mg dosages was more marked. The 12-hour series in contrast with the testicular data, did not show significantly elevated ^{32}P uptake except at the 0.02 mg dosage. There was interaction of time x dosage, however, when the interaction mean was employed as an estimate of variance, the effect of time was apparent; F = 17.57, P < 0.01.

In order to determine the effect of the lithosperm treatments on anterior pituitary gland hormone content, 11-day-old cockerels were injected with 0.4 mg

of Ea (3) at 1, 2, 8 and 12 hours before autopsy. The effects of the lithosperm on the thyroids and testes of the "donor" birds were essentially the same as those previously described, therefore, these data are not tabulated. Each assay animal received a single dose of 1.0 mg equivalent of donor fresh anterior pituitary gland. The responses of the testes in the assay chicks (Table 3A) indicate clearly that the ^{32}P uptake in the groups which received glands from the one-hour, 8-hour and 12-hour donor groups differed from the control groups. It is noteworthy that the one-hour and 8-hour recipient groups had a lower titer of gonadotropin whereas the 12-hour recipient group had a much higher level of gonadotropin. Differences in the ^{32}P uptake by the thyroids of the assay animals were less striking than were those of the testes and there were two varia-

TABLE 3

Testes and Thyroid Uptake of ^{32}P in Two-Day-Old Cockerels Given Anterior Pituitary from Donor Birds Which Received 0.4 mg Lithosperm Fraction Ea for One, Two, Four, Eight or Twelve Hours. 1.0 μ Ci ^{32}P Was Administered One Hour Before Autopsy. 1.0 mg Equivalent of Fresh Anterior Pituitary Gland Was Injected Five Hours Before Autopsy. f = 15.

A. Testes

Donors:	Control AP	1 Hour LSPM	2 Hours LSPM	4 Hours LSPM
$\bar{x}$	179.78	149.21***	166.95	173.97
Range	191.21-169.02	160.55-138.68	184.59-151.00	195.84-154.55

Donors:	8 Hours LSPM	12 Hours LSPM
$\bar{x}$	124.39***	240.99***
Range	131.32-117.84	276.16-210.28

		MSQ	F	P
ANOVA	Treatments 5	0.3832_2	58.95	$< 3.0^{-25}$
	Error 84	$S^2 = 0.0065$		

B. Thyroids

Donors:	Control AP	1 Hour LSPM	2 Hours LSPM	4 Hours LSPM
$\bar{x}$	100.20	73.10***	93.01	87.28*
Range	110.51-90.86	82.91-64.45	103.62-83.49	94.46-80.66

Donors:	8 Hours LSPM	12 Hours LSPM
$\bar{x}$	67.91***	100.00
Range	76.06-60.64	110.61-90.41

		MSQ	F	P
ANOVA	Treatments 5	0.0792_2	11.65	$< 1.8^{-08}$
	Error 84	$S^2 = 0.0068$		

Range = 95% Confidence Limits. *P = < 0.05, **P = < 0.01, ***P = < 0.001 by t - test.

tions in the responses (Table 3B). The ^{32}P uptake in the chicks which received pituitaries from the 4-hour donors was slightly lower than the control and the uptake in the recipients of the 12-hour donor glands did not differ from control. The birds which were given the glands from the 8-hour donors had a ^{32}P uptake which was markedly lower than the control.

Discussion. Testicular responses demonstrate that animals which received lithosperm 8 hours before autopsy had markedly depressed uptakes of ^{32}P, whereas the testes of birds administered lithosperm 12 hours before autopsy had strikingly elevated ^{32}P uptakes. Other time intervals were somewhat more variable. The thyroids glands responded to the lithosperm injection to a lesser degree than did the testes but the pattern was similar. The thyroidal ^{32}P was increased after the one-hour treatment in one experiment. The analysis of lithosperm effects, therefore, must be evaluated with respect to the duration of treatment as well as in terms of the dosages employed.

The mechanism of action of lithosperm is not known but the lithosperm data are similar in some respects to results which have been reported in the female rat (5,6,7,8) in which a biphasic effect on the anterior pituitary gland was observed following the administration of estradiol. One hour following injection the rat pituitary showed decreased sensitivity to luteinizing hormone releasing factor (LH-RF) but, eight hours after estradiol administration an enhancement of the response to LH-RF occurred. It was suggested that the cells of the rat anterior pituitary are cyclic in their sensitivity to LH-RF, that the cells may be "primed" by endogenous LH-RF following prior estrogen secretion, and that pituitary responsiveness is caused by variations in a hypothalamic feedback axis. The timing of these responses are the reverse of the lithosperm effects on cockerels but assay of the anterior pituitary glands of lithosperm-treated chicks suggest parallels. The glands from the 8-hour treated group had low titers of both gonadotropins and thyrotropin and the testes and thyroids of these animals had marked depression of ^{32}P uptake. It is evident that both synthesis and release of the anterior pituitary hormones was inhibited. The

pituitary glands of the assay birds which received pituitary material from the 12-hour treated donors, on the other hand, had high titers of both gonadotropins and thyrotropin and the testes and thyroids of the 12-hour donors had enhanced ^{32}P uptakes. Both synthesis and release of the pituitary hormones were greatly augmented at this time period. This suggests that the action of hypothalamic releasing factors is blocked in some fashion by the 8 hour treatment. Secretion and/or release from the hypothalamus may not occur or the receptors for the releasing factors in the anterior pituitary cells may complex with the lithosperm and decrease the ability of the cells to respond. The marked increase in secretion and release after 12 hours of treatment could be explained if lithosperm has only a short-term action. A sudden release of hypothalamic factors would be especially effective if either a receptor-lithosperm complex in pituitary cells had broken or if additional receptors had been synthesized during the interval of inhibition. The occurrence of either or both of these events would increase the sensitivity of the pituitary cells to stimulation; essentially a "priming" effect would occur.

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