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Induced Adrenal and Ovarian Enlargement in Nursing Mothers by a Single μg Injection to their Pups of a 3β-Hydroxy-Δ⁵-Steroid Oxidoreductase Inhibitor[1] (38922)

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The administration of certain inhibitors of 3β-hydroxy-Δ⁵-steroid oxidoreductase (3β-HSOR), cyanoketone (2α-cyano-4,4,17α-trimethylandrost-5-en-17-βol-3-one) and isoxazole (17β-hydroxy-4,4,17α-trimethyl-androst-5-en-[2,3d]-isoxazole) to pregnant rats produces hypospadias in males and clitoral hypertrophy in female fetuses by inhibiting fetal testicular and adrenal hormone secretion (1). Furthermore, an additional postnatal injection of cyanoketone delays the onset of puberty and adult feminine development in rats (2). In our attempt to study the effects of neonatal blockade of 3β-HSOR we often found that pups injected with large doses (30 mg/kg) of cyanoketone had subnormal body weight gain (unpublished observation). However, when the pups were placed with foster mothers 2 or 3 days after the injection their body weight gain was normal. These findings suggested that administration of the inhibitor to the pups interfered with milk production in the mothers.

The present investigation examines the effects of an injection of radioactive isoxazole to nursing pups on 3β-HSOR containing tissues in the mothers.

Materials and Methods. Preparation of labeled isoxazole. 17β-Hydroxy-4,4,17α-trimethylandrost-5-en-[2,3d]-^{14}-C-2-isoxazole was prepared by New England Nuclear Company (Boston, Mass.) according to the method of Manson *et al.* (3) by the formation of the ^{14}C-formate, and a subsequent production of steroidal isoxazole from the reaction with hydroxylamine. The final product had a UV max of 230 nm and had mobility identical with that of the standard in thin-layer chromatography on silica gel G in chloroform:ethyl acetate (55:45 v/v). The specific activity of the isoxazole was 63 dpm/ng.

Animals. Virgin Sprague–Dawley female rats were mated by the supplier, Charles River Breeding Laboratories (Wilmington, MA) and received by us on day 16 of pregnancy. Following parturition, litters were reduced to seven pups. All the pups in each litter were injected, subcutaneously, with ^{14}C-labeled isoxazole at a dose of 2 mg/kg in dimethylsulfoxide (DMSO; 6 μg/μl) or DMSO alone on either day 1,5, 11 or 22. In order to reduce the possibility of leaking at the injection site and subsequent ingestion by the mother, the inhibitor was administered in μl doses in a microsyringe with a long 27 gauge needle cleaned with alcohol wipes after filling. The contents of the syringe were deposited at least half an inch from the injection site which was pinched closed for 30 sec during and after withdrawal of the needle. Following an additional 30 sec the pups were examined for delayed leaking after which the injection site was cleaned with alcohol. The pups were separated from their mothers at 24 days of age and the mothers were killed 27 days after parturition. A few mothers were killed during the nursing period. All rats were decapitated and trunk blood was collected in centrifuge tubes. After clotting overnight at 4°, the blood was centrifuged and the serum stored at −20°. Maternal tissues were quickly removed, cleaned and weighed to the nearest 0.1 mg. Tissue samples were analyzed for ^{14}C content.

Measurement of ^{14}C. Samples of wet tissue weighing between 10 and 100 mg were solubilized in counting vials with Soluene-100 (Packard Instrument Co.), 1 ml/100 mg wet tissue. Into each vial was added 10 ml

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of Omnifluor (New England Nuclear), 4 g/1 toluene solution. Radioactivity was determined in a Packard Tri-Carb 33/35 liquid scintillation spectrometer and the instrument gave an absolute counting efficiency with the solubilized tissue of 70–80%. Serum, 2.5–3.5 ml, were mixed with 11.5 ml of Aquasol liquid scintillator (New England Nuclear) and water to make a final volume of 15 ml. The absolute counting efficiency was 40–50%. Sufficient counts were accumulated to achieve a 3.5% standard deviation. Uptake, retention and excretion of ^{14}C-isoxazole in the rat does not result in metabolism of the inhibitor (4, unpublished observation), and for this reason radioactivity was expressed as ng of steroidal isoxazole-^{14}C.

Statistics. Significance was calculated by Student's *t* test.

Results. A single injection of ^{14}C-isoxazole to nursing pups at 1 or 5 days of age resulted in significant increases in maternal adrenal, ovarian, preputial and pituitary weights 27 days after parturition (Table I). Administration of the inhibitor to older pups (11 or 22 days of age) had no effect on maternal preputial or pituitary weights, but there was a significant increase in ovarian weights. Mothers with 11-day-old treated pups, also had enlarged adrenals.

At the time of sacrifice ovaries and adrenals from mothers nursing ^{14}C-isoxazole treated pups all contained radioactive label (Table II). In fact, the concentration of isoxazole in the maternal adrenals and gonads were similar, irrespective of the age of the pups when injected and the differences in total dosage administered to the various size pups.

The day after the administration of ^{14}C-isoxazole to 1 day old pups (6–7 g body weight) the greatest concentration of label was found in the maternal adrenals with lesser concentrations, in decreasing order, in the ovaries, uterus, liver, preputials, kidneys, mammary glands and serum (Fig. 1). Five days following treatment the concentration of the label was reduced in all the tissues except the ovaries, which now contained the greatest concentration. Within this 5-day period ^{14}C-isoxazole concentration in the uterus was reduced to 1/66 the initial value. On day 11 postpartum the ovaries still contained the greatest concentration of label while isoxazole content in the adrenals and preputials continued to decline. Isoxazole concentration in the uterus, mammary glands, liver, kidneys and serum were similar to values found on day 6 postpartum. Twenty-six days after the injection only the adrenals and ovaries contained the label. At no time was radioactive label detected in the pituitary.

TABLE I. EFFECTS OF A SINGLE INJECTION OF ^{14}C-ISOXAZOLE (2mg/kg) TO PUPS ON MATERNAL ORGAN WEIGHTS 27 DAYS AFTER PARTURITION.

Treatment	Age of Pups when injected (days)	Mothers no.	Body weight(g)	Adrenal	Ovary	Preputial	Pituitary
				mg/100 g body weight			
DMSO	1, 5, 11 and 22[a]	8	298 ± 28[b]	20.4 ± 1.5	24.1 ± 2.7	24.6 ± 4.6	3.8 ± 0.3
Isoxazole	1	4	271 ± 11	23.2 ± 1.0[d]	38.2 ± 1.0[f]	33.6 ± 8.1[c]	4.6 ± 0.5[d]
Isoxazole	5	4	267 ± 9	25.2 ± 2.0[e]	36.1 ± 4.4[f]	41.4 ± 8.9[e]	5.2 ± 0.4[f]
Isoxazole	11	4	257 ± 19	23.5 ± 0.9[d]	31.0 ± 3.2[e]	26.7 ± 5.6	3.5 ± 0.3
Isoxazole	22	4	287 ± 34	19.7 ± 2.6	28.8 ± 3.6[c]	25.6 ± 9.5	3.6 ± 0.2

[a] Data for the control group was pooled because there were no significant differences between the various treatment schedules.

[b] Mean ±1 standard deviation.

[c] $P < 0.05$.

[d] $P < 0.01$.

[e] $P < 0.005$.

[f] $P < 0.001$.

TABLE II. CONCENTRATION OF ^{14}C-ISOXAZOLE 27 DAYS POSTPARTUM IN VARIOUS MATERNAL TISSUES AFTER A SINGLE DOSE (2mg/kg) OF ^{14}C-ISOXAZOLE TO THE PUPS.

Age of Pups when injected (days)	Mothers no.	Adrenal	Ovary	Preputial	Pituitary
			ng isoxazole/100 mg wet tissue		
1	4	2.62 ± 0.35[a]	2.03 ± 0.05	0	0
5	4	2.11 ± 0.79	2.52 ± 1.06	0	0
11	4	2.83 ± 1.49	2.29 ± 0.49	0.13 ± 0.11	0
22	4	2.35 ± 1.98	2.68 ± 1.76	0.17 ± 0.11	0

[a] Mean ±1 standard deviation.

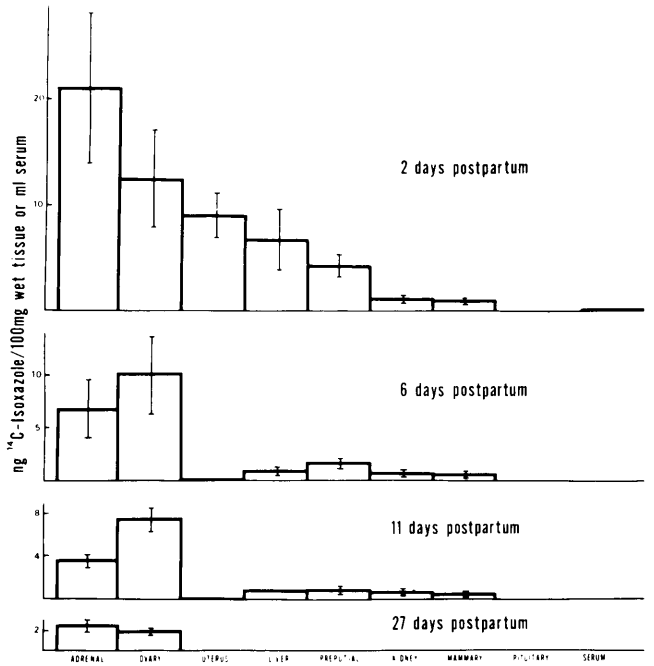

FIG. 1. Distribution and retention of ^{14}C-isoxazole in various maternal tissues after a single dose (2 mg/kg) of ^{14}C-isoxazole to their one day old pups. The mean values represented by the open-blocks are determined from four mothers in the "27 days post-partum" group, and three, each, in the other groups. The vertical lines are ±1 standard deviation.

Discussion. The results demonstrate that the potent inhibitor of 3βHSOR, ^{14}C-isoxazole, injected in μg quantities into nursing pups can be transferred to their mothers where it remains for at least a month in the enzyme containing tissues. Although the maternal ovaries and adrenals retain only ng quantities of the inhibitor it is apparently sufficient to block steroidogenesis, resulting in compensatory enlargement of these endocrines.

Previous reports have shown that administration of cyanoketone to pregnant rats causes ovarian and adrenal enlargement 1 mo later (5). The inhibitor induced blockade of ovarian and adrenal steroidogenesis probably stimulates pituitary hormone secretion and subsequent pituitary enlargement (6) which in turn results in adrenal, and gonadal enlargement. The preputials, modified sebaceous glands, are androgen responsive (7) and the inhibition of adrenal

3βHSOR causes an increase in the secretion of such precursors as dehydroepiandrosterone (1) which metabolized peripherally to Δ^4-3 keto-androgens could stimulate preputial gland growth. The temporal distribution patterns of ^{14}C-isoxazole in the mothers are similar to past findings when the inhibitor was administered directly into mature female rats (4, 8). The ovaries and adrenals contain the greatest concentration of 3βHSOR (1, 4) and thus would be expected to retain the highest concentration of the stoichiometric inhibitor for the longest period of time. The relatively high concentration of label in the liver and kidneys is probably due to the fact that these organs are concerned with steroid excretion and isoxazole being a steroid is excreted in the urine and feces (4). The initially high levels of ^{14}C-isoxazole in the uterus followed by a rapid and sharp decline in its concentration may suggest transitory accumulation from initially elevated serum levels in this highly vascularized tissue rather than binding to 3βHSOR enzymes. The rodent preputial gland contains 3βHSOR activity (9) and thus, the retention of the label indicates a binding of the isoxazole with the enzyme. The retention of ^{14}C-isoxazole in the mammary glands may also suggest the presence of 3βHSOR in this tissue (10). However, it is possible to speculate that inhibitor from the blood, accumulated in the milk, is retransferred back to the pup. Radioactive thyroid hormone has been shown to pass from the pup to the mother and back to the pup again (11). Thus, when working with synthetic or nonmetabolizable drugs or even viruses in newborns, it might be helpful to consider the mother-neonate as a unit, in some respects similar to the fetoplacental unit.

Due to the special precautions taken during the administration of the ^{14}C-isoxazole (see Materials and Methods) and the rapid solubility of DMSO in biological systems (12), it seems unlikely that the mothers obtained the label at the injection sites on the pups. Nursing rats instinctively lick the anus and external genitalia of their newborn. This behavior is essential for the pups to elicit, reflexly, urination and defecation (13). Thus, the results suggest that the mothers obtain the label by ingesting the urine and feces of the pups and that the inhibitor remains biologically potent when taken orally. Although the 22-day-old treated pups would be expected to be self-weaned, the presence of ^{14}C-isoxazole in the maternal tissues indicate that their mothers were still, at this late date, licking the external excretory organs of the pups.

Summary. Isoxazole-^{14}C, a potent inhibitor of 3β-hydroxy-Δ^5-steroid oxidoreductase, injected into nursing pups is naturally transferred to their mother where it is retained, for at least a month, in ng quantities in the adrenals and ovaries and results in adrenal, ovarian, preputial and pituitary enlargement.

1. Goldman, A. S., *in* "XXI Mosboach Koll., Mammalian Reproduction" (H. Gibian and E. J. Plotz, eds.), p. 389. Springer-Verlag, Heidelberg (1970).
2. Shapiro, B. H., Goldman, A. S., and Root, A. W., Proc. Soc. Exp. Biol. Med. **145,** 334 (1974).
3. Manson, A. J,, Stonner, F. W., Neumann, H. C., Christiansen, R. G., Clarke, R. L., Ackerman, J. H., Page, D. F., Dean, J. W., Phillips, D. K., Potts, G. O., Arnold, A., Beyler, A. L., and Clinton, R. O., J. Med. Chem. **6,** 1 (1963).
4. Goldman, A. S., Endocrinology **86,** 678 (1970).
5. Goldman, A. S., Yakovac, W. C., and Bongiovanni, A. M., Endocrinology **77,** 1105 (1965).
6. Goldman, A. S., Root, A. W., Russ, R. D., and Rudd, B. T., Proc. Soc. Exp. Biol. Med. **136,** 672, (1971).
7. Freinkel, R. K., Adv. Biol. Skin **4,** 125 (1963).
8. Ryo, U. Y., and Bierwalters, W. H., J. Nucl. Med. **14,** 321 (1973).
9. Goldman, A. S. and Klingele, D. A., Proc. Soc. Exp. Biol. Med. **146,** 539 (1974).
10. Deshpande, N., and Carson, P., J. Endocrinol. **61,** liii (1974).
11. Beltz, A. D., and Reineke, E. P., Gen. Comp. Endocrinol. **10,** 103, (1968).
12. Rosenkrantz, H., Hadidian, Z., Seay, H., and Mason, M. M., Cancer Chemother. Rep. **31,** 7 (1963).
13. Reyniers, J. A., Lancet II, 933 (1953).

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