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TECHNICAL PROGRESS REPORT

The Role of Population-Dependent Factors in
Altering Adrenal Steroidogenesis and Reproduction
in the Cottontail Rabbit in Ohio. *Progress Report*

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PROGRESS REPORT

Introduction

Fifty-one female cottontails were collected during their first pregnancy of the breeding season in 1964. The reproductive performance of these animals has been completely evaluated and the adrenal glands incubated. Chromatographic separation and quantitation have been carried out on the majority of the adrenal incubates.

During 1966, thirteen incubations with freshly collected rabbit adrenals were done within 24 hours of collection. They were stored on ice in the field and frozen until used in the incubations. The laboratory conditions were similar to the conditions used in the previous study, with the exception of the labeled precursor used in these studies. The literature sites examples of loss in the 3B-hydroxysteroids: NAD(P) oxidoreductase with prolonged storage. Accordingly, despite the short storage time, it seemed feasible to utilize 7a-H³-pregn-5-ene-3B-ol, 20-one (Pregnenolone) as the precursor in this study. This would allow an alternate pathway to likewise be studied, i.e., the delta⁵ route through dehydroepiandrosterone and the delta⁴ pathway. It was felt, in as much as the major difference in the quantitative data was a very non-polar compound, that there was the possibility that the unidentified difference (being two or more fold) was a nonpolar androgen or perhaps an estrogen, as well as one of the pregnene derivatives.

Methods

Reproductive performance of each pregnant female collected was evaluated in the following manner:

- 1) All pregnant uteri were dissected open and the viable embryos counted
- 2) The number of implantation sites in each uterus was also noted

- 3) Ovaries from the pregnant females were serially sectioned, strained and the number of corpora lutea counted in each as an indication of ovulation rate

The biosynthetic capacity of adrenal glands from each rabbit were evaluated as follows: The adrenals were allowed to come to 4° C., stripped of all fat and other debris; they were minced to a fine brei (0.2-0.6 cm.) in preweighed 24 dram vials. The vial containing the tissue were again weighed. To the vial was added the incubation media. All incubations were carried out with a Krebs-Ringer bicarbonate buffer at pH 7.4 and the following concentrations of cofactors: adenosine triphosphate, 4.0 mM; nicotinamide-adenine dinucleotide (NAD), 0.4 mM; nicotinamide-adenine dinucleotidephosphate (NADP), 0.1 mM; reduced nicotinamide-adenine dinucleotide phosphate (NADPH), 0.01 mM; glucose-6-phosphate, 0.5mM; sodium fumerate, 70mM; D-glucose-6-phosphate: NADP oxidoreductase 0.5 uc. units, and glucose concentration of 200 mg.%. These values represent final concentrations. The incubations were carried out at 37°C. in an atmosphere at 95% oxygen-5% carbon dioxide. Radiochemical purity of the labeled steroid was established in several chromatographic systems prior to its use in the incubation. Each vial contained 1.0uc. of 4-C¹⁴ -pregnene-3, 20-dione (C¹⁴ -progesterone); the specific activity of the C¹⁴ -steroid was about 70 uc. /mg. The incubations were ended by the addition of 10 ml. of dichloromethane, shaken and the organic solvent removed. Each was re-extracted twice more and centrifuged to break any emulsion that may have formed. All solvents were freshly redistilled to assure purity, and all chemicals were reagent grade. The enzymes and cofactors were purchased from Sigma Chemical Company and were the best grade obtainable.

The combined organic extracts were dried with sodium sulfate (anhydrous) and dried down in centrifuge tubes; to each dried residue was added 1.0 ml. of 25% benzene in hexane. The tube was warmed to 45° C. and an area of activated silica gel "G" equivalent to 50 cm.² was added to the tube and slurried. An additional 5.0 ml. of the solvent was added, mixed and centrifuged. The solvent was evaporated in another tube after transfer. Randomly, aliquots were removed for counting. At no time did the 25% benzene in hexane solution contain greater than 1% of the radioactive counts. The silica gel was eluted three times with dichloromethane. The elutions were combined and dried to a residue. This residue was then chromatographed.

The defatted extraction residue was chromatographed on washed papers in the toluene/propylene glycol (T/PG) system for 72 hours; one milliliter of seven different steroid standards was dried down with the residue to serve as ultraviolet markers. The chromatographic separation of these seven were well established previously. The run-off beakers of the T/PG were rechromatographed in the same system for 24 hours; the run-offs from the 18 hour papers were placed in ligroene/propylene glycol for 24 hours and the run-off in the hexane/propylene glycol system for a single length run. The papers were dried and scanned for radioactivity in the Packard 4 pi radiochromatogram scanner. The radioactive areas were compared with the UV absorbing areas of the added inert carrier compounds. The area where the UV and radioactivity corresponded well were eluted and rechromatographed to resolve any overlapping compounds and to give the best possible resolution.

Areas of radioactivity not resolving completely were eluted and rechromatographed in appropriate systems to effect the desired separation. Unknown areas were repeatedly rechromatographed with various standards, run on a side limb, to provide evidence for their identification. Oxidations and reductions, as well as acetylations, were performed as necessary to establish the identity of the compounds, see Table VI.

In the 1966 experiments, the extraction and preliminary clean-up and subsequent chromatography was identical to the procedure reported in the previous report. A total of sixty-five separate chromatograms were run; these involved the separation of the resulting radio-labeled steroids biosynthetically prepared by the adrenal tissue during incubation.

Each chromatogram was fixed tail to head and scanned as a continuous flow. Thus, the resulting scans could be compared with the previous incubations utilizing C^{14} -progesterone. A qualitative similarity was noted with few exceptions to the previous scans. This is expected, as one would encounter some Δ^5 pathway compounds.

Results

Reproductive rates of the cottontails collected in 1964 were significantly different in the two groups collected from different regions (Tables I and II). As was found from previous study, ovulation rates were lower in cottontails collected from the Southeastern region. These differences were comparable to those observed in previous studies.

The reproductive rates of rabbits collected in 1966 were comparable to those collected in 1964 and from previous studies. These data are shown in tables III and IV.

Twenty pairs of adrenal gland incubations were partially resolved in 1965 and the results shown in Table VI. The resolved radiolabeled compounds were eluted, and an aliquot was taken for quantitation in the Packard Tri-Carb Spectrometer. The remaining amount was dried down for further work. The results of these counts, following calculation for conversion to dpm/gm of tissue were further used to express the results as per cent substrate conversion of the C^{14} -labelled steroid.

During the past year, 13 pairs of adrenals were incubated using H^3 Pregnenolone as a steroid precursor. Radioscan patterns of the numerous chromatograms suggests the same steroid profile as glands incubated earlier with C^{14} progesterone as precursor. Rigid identification of all of the steroids resolved has not yet been completed. Some of these compound appear to different from those isolated from adrenals of other species studied (eg. rat, cow, human and dog). The usual chemical reactions (oxidation, reduction and acetylation) are being done on all of these substances. Final identification will be made by recrystillization to a constant specific activity. Although there now appears little, if any, qualitative difference in the steroid profile, a major quantitative difference has been found consistently in a single isolated steroid. Recently, our efforts have centered around the chemical identification of this substance which is produced in approximately twice the quantity in rabbits from the Westcentral region as in the Southeastern region.

As was noted in an earlier report, one chromatographic area contained much more radioactivity in Westcentral rabbits than in Southeastern rabbits. It was noted from extensive chromatography, at that time, that a particular peak in this complex seemed to appear only in the adrenal incubations from the Westcentral region. The conversion of C^{14} progesterone to the 7-(H) complex was 20.69% in Westcentral adrenals as opposed to 10.98% in Southeastern adrenals (Table VI). Thus the conversion data agreed with conclusions from the radioscan, but indicated that the difference was purely quantitative.

The incubation residue had been chromatographed in the following manner: Toluene/propylene glycol (T/PG), 72 hours; run off into T/PG for 24 hours; run off into Ligroine/propylene glycol for 24 hours; and finally into Hexane/propylene glycol, for a single length run. The radioscan demonstrated differences in the area designated as 7-(H). This complex was eluted and radioquantitated and re-chromatographed for twelve hours in Hexane/propylene glycol.

When these papers were radioscaned, they were shown to contain four radioactive areas, the most polar of which was extremely large in the residue of the Westcentral incubation tissues. The Southern zone incubations contained likewise these same areas of radio-compounds, but did not contain the same amount of radiolabeled material. Thus, 7-(H) complex has been resolved into four compounds, the most polar of which is the area of interest as it accounts for the two fold difference in the quantitated complex.

Establishment of the most polar fraction of 7-(H), called 7-(H)mp: It is apparent that definitely sufficient radioactivity, when pooled, is available in the 7-(H) complex, even when it is resolved into the separate compounds, to lend itself to the usual classical analysis for identification.

In fact, 7-(H) mp., from both regions, contained the majority of the label.

This fraction, 7-(H) mp, was chromatographed in several systems of low polarity in an attempt to establish homogeneity of the compound. Presently, it is again being compared with the following compounds as the polarity lies within the limits of these compounds in the same systems:

- 1) Pregn-4-ene-20B-ol, 3-one
- 2) Pregn-4-ene-3a-ol, 20-one
- 3) Pregn-4-ene-3B-ol, 20-one
- 4) Pregnane-3B-ol, 20-one
- 5) Pregn-4-ene-21-ol, 3, 20-dione
- 6) Pregn-4-ene-11B-ol, 3, 20-dione
- 7) Pregn-4-ene-17a-ol, 3, 20-dione
- 8) Pregn-4-ene-3, 6, 20-trione
- 9) Estra-1, 3, 5(10)-triene-3ol, 17-one
- 10) Andrane-3a, 17B-diol

Obviously, it follows, much remains to be done for the identification of this compound. It is interesting to note, the increased presence of this compound, relates directly to the reproductive data. Both in the fresh and long term-frozen adrenals, this generalization holds true when compared as a group. Presently, the pooled collection of S-r-(H) mp and W-7-(H) mp are undergoing intense chromatographic comparison with the above listed compounds. Once a reasonable chromatographic identity has been established, the above micro-organic reactions and finally recrystallization to constant specific activity will be obtained. At this point, biological activity of the compound can be assessed, either from commercially available material or custom synthesized material by our laboratories.

Discussion

Although it has been demonstrated that the frozen storage of adrenal tissue (and the possible loss of 3-B-ol dehydrogenase enzyme) does not effect the steroid profile of cottontail adrenals, this question was important to answer. In addition, although no qualitative difference was found in the 7-(H) region of the chromatograms, there remains the possibility of such differences in as yet unresolved steroid areas. Quantitative evaluations showed only a difference in the 7-(H) region, however, the other regions may have exhibited equal radioactivity from different steroids. Complete resolution of the remaining areas will be done as a continuation of Subproject II.

When final identification of most polar of the four compounds in area 7-(H) is made, this material will be obtained (either purchased or synthesized) and tested for biological activity. Since this compound is present in larger amounts in animals with higher reproductive rates, an inhibition of reproduction by this substance is not expected. If feasible, specific inhibitors of this steroid will be administered to laboratory animals. The reproductive performance of these test animals will also be evaluated.

There is still a possibility of an inhibitory steroid being produced in rabbits from the Southeastern region. Nevertheless, current evidence would suggest that a "hypo-function" of adrenals in this region is more likely than a "hyper-function". Despite many unresolved questions, it can be concluded with certainty that adrenal gland function is different in cottontails collected in the two study regions.

Table 1

Reproductive Performance of Cottontails
Collected in the Westcentral Region (1964)

<u>Rabbit No.</u>	<u>No. of Corpora Lutea</u>	<u>Implantation Sites</u>	<u>Living Embryos</u>
W8	7	7	-
W11	7	-	-
W9	6	5	5
W20	7	7	6
W26	5	5	3
W21	7	5	4
W23	6	5	5
W16	4	4	4
W27	9	8	8
W10	6	4	4
W12	7	5	5
W15	7	6	6
W14	5	5	5
W13	-	6	3
W17	5	5	5
W19	5	4	4
W18	-	5	5
W22	6	6	6
W5	4	4	4
W7	8	-	-
W1	-	-	-
W6	5	5	5
W4	5	5	5
W3	3	-	-

5.90

5.30

4.84

X

Table II

Reproductive Performance of Cottontails
Collected in the Southeastern Region (1964)

Rabbit No.	No. of Corpora Lutea	Implantation Sites	Living Embryos
S 18	4	4	4
S 3	5	5	5
S 5	4	4	4
S 1	7	5	4
S 4	5	5	5
S 2	5	5	5
S 6	3	3	3
S 8	6	4	4
S12	4	4	2
S14	-	5	4
S 7	5	5	5
S 9	4	4	3
S11	4	2	2
S17	5	4	4
S15	4	4	4
S16	5	5	5
S24	4	3	3
S19	6	4	4
S27	4	4	3
S28	4	4	4
S21	5	3	3
S20	4	4	4
S22	5	5	5
S23	4	2	2
S26	5	6	6
S25	4	4	4

X

4.60

4.1

3.88

Table III

Reproductive Performance of Cottontails
collected in the Westcentral Region (1966)

<u>Rabbit No.</u>	<u>Corpora Lutea</u>	<u>Implantation Sites</u>	<u>Living Embryos</u>
W-28	5	5	5
W-29	7	7	6
W-30	6	6	6
W-31	6	6	5
W-32	5	-	-
W-33	4	3	3
W-34	5	5	5

X	5.43	5.33	5.00

Table IV

Reproductive Performance of Cottontails
in the Southeastern Region (1966)

<u>Rabbit No.</u>	<u>Corpora Lutea</u>	<u>Implantation Sites</u>	<u>Living Embryus</u>
S-28	4	-	-
S-29	3	3	2
S-30	4	4	4
S-31	5	4	4
S-32	5	5	5
S-33	4	4	3
<u>X</u>	4.17	4.00	3.60

Table V

<u>Mobility and Reactions Used to Identify the Various Areas</u> <u>Of The Radiochromatogram Scans</u>			
<u>Area (a)</u>	<u>Mobility Of</u>	<u>Reaction Or Manipulation</u>	<u>Mobility Of</u>
1-(A)	Unknowns	Extensive Chromatography	1. Unknowns 2. Tetrahydrocortisol (?)
2-(B)	Hydrocortisone	1. NaBiO ₃ oxidation 2. CrO ₃ oxidation 3. Acetylation	1. II-OH-Androstene 2. II-oxo-Androstene 3. Hydrocortisone Acetate
3-(C)	Cortisone	1. CrO ₃ oxidation 2. NaBiO ₃ oxidation 3. Acetylation	1. II-oxo-Androstene 2. II-oxo-Androstene 3. Cortisone Acetate
4-(D)	Unknown	Extensive Chromatography	Unknown
5-(I)	Unresolved as Yet	Extensive Chromatography	1. 17-OH-Progesterone 2. Corticosterone 3. Unknown
6-(E)	Desoxycorticosterone	Acetylation	Desoxycorticosterone Acetate
7-(H)	Unresolved As Yet	Extensive Chromatography	1. Progesterone 2. Unknown
8-(G)	Allopregnandione	1. CrO ₃ oxidation 2. Acetylation 3. NaBH ₄ reduction	1. Allopregnandione 2. Allopregnandione 3. Allopregnandiol (G. C.) (b)

(a) The numbers in this column refer to the compound or groups of compounds seen in Table IV.

(b) Refers to chromatography on gas chromatograph.

Table VI

Per cent Substrate Conversion of C¹⁴-Progesterone
to Various Compounds Isolated from the Incubations (a)

Incubation	Compound or groups of compounds (b)							
	1	2	3	4	5	6	7	8
W-4	0.83	0.46	0.31	0.79	12.83	1.60	50.69	3.94
W-5	0.81	0.22	0.18	0.69	9.71	1.55	4.15	3.05
W-6	0.64	0.32	0.26	0.59	10.41	0.82	----	3.06
W-8	1.35	0.38	0.31	0.24	12.48	1.43	8.03	3.84
W-11	0.80	0.38	0.37	0.58	9.73	1.95	29.00	2.67
W-23	0.63	0.25	0.30	0.60	8.07	1.65	4.70	2.72
W-1	0.59	0.19	0.13	1.28	9.05	1.29	6.94	3.96
W-7	0.77	0.34	0.27	0.85	14.85	2.60	22.38	5.07
W-2	0.48	0.06	0.07	1.45	7.72	1.47	47.65	3.74
W-18	1.50	0.51	0.39	1.08	13.64	2.70	12.65	3.66
Average	0.84	0.31	0.26	0.82	10.85	1.71	20.69	3.57
S-20	0.77	0.34	0.18	0.84	8.02	0.75	4.86	1.67
S-19	0.81	0.27	0.13	2.32	8.04	1.70	4.15	1.83
S-4	0.66	0.23	0.19	0.69	6.76	1.10	6.59	1.68
S-16	1.05	0.28	0.32	1.69	9.03	1.81	8.02	2.71
S-2	0.56	0.22	0.13	1.16	7.28	1.71	11.45	2.11
S-6	1.06	0.33	0.39	0.96	9.76	1.83	11.50	2.07
S-5	1.54	0.40	0.58	1.48	10.58	1.65	28.37	3.25
S-21	1.82	0.74	0.34	2.09	14.32	3.08	8.98	5.53
S-22	0.73	0.22	0.25	1.19	5.94	0.67	14.36	1.63
S-25	0.40	0.16	0.15	2.09	4.87	0.96	11.52	1.15
Average	0.94	0.32	0.27	1.45	8.46	1.53	10.98	2.36

a) Calculated from conversion expressed as dpm./gm/ tissue.

b) See Table I for identification of compounds.