Corticosteroid Production *in vitro* by the Interrenal Tissue of Killifish, *Fundulus heteroclitus* (Linn).* (24904)

J. G. PHILLIPS† AND P. J. MULROW‡ (Introduced by P. K. Bondy)
Bingham Oceanographic Laboratory, Yale University, and Dept. of Internal Medicine, West Haven Vet. Admin. Hospital, Conn.

Earlier work(1) has shown that an adrenocorticosteroid, cortisol, is present in blood obtained from tails of killifish (*Fundulus heteroclitus*). In teleostean fishes the adrenocortical (interrenal) tissue is usually situated in the walls of cardinal veins as they pass through the head kidney. *Fundulus heteroclitus* lacks a left posterior cardinal vein, and the major part of interrenal tissue is found in relation to the right head kidney(2). Evidence that these cells are teleostean adrenocortical tissue is indirect but decisive(3,4), depending on their response to mammalian ACTH and the effects of hypophysectomy. The method of tissue incubation of adrenal tissue has proved a useful tool in the study of corticoid biosynthesis in mammalian preparations(5) and the present investigation resulted from an attempt to apply similar methods to fishes.

Methods. In preliminary study (series A), 45 healthy fish of both sexes were used. Right head kidneys were removed and incubated in 3 batches in manner identical with series B. In series B 163 fish of both sexes were used. Fish for both series were caught at low tide near the mouth of West River, New Haven, June, 1958 (series A) and Sept. 1958 (series B). In series B, right and left head kidneys were removed and pooled separately. Nine batches of each pool, comprising 15-20 head kidneys were incubated for 3 hours at 37°C in 10 ml Krebs Ringer bicarbonate buffer with 200 mg % glucose (pH 7.4) in an atmosphere of 95% O_2 and 5% CO_2 in Dubnoff metabolic incubator. Prior to incubation, 273,493 cpm of progesterone-16-H^3 (representing 0.56 μg) in 0.1 ml propylene glycol were added to each flask. Incubation media of right and left head kidneys were combined separately and each extracted twice with 1.5 volumes of redistilled methylene chloride. The methylene chloride extract was washed once with 1/10th vol. 0.05 N NaOH, twice with redistilled water, evaporated *in vacuo* at 37°C and chromatographed (Fig. 1). Acetylation of the aldosterone area was carried out at room temperature with 25 lambda of 15% acetic anhydride in anhydrous benzene and 20 lambda of absolute pyridine. The reaction was terminated after 48 hrs by adding 0.5 ml absolute ethanol (personal communication). A methylene chloride extract of the mixture was washed with distilled water, dried in air, and applied to paper chromatogram. The acetylated aldosterone area was chromatographed in the cyclohexane-benzene-methanol-water (100:50:100:25) system with adrenosterone as standard reference substance; eluted and rechromatographed in the system cyclohexane-dioxane - methanol - water (100:100:50:250) with Δ^4-androstene-11β-ol-3, 17-dione as a standard. Adrenosterone and Δ^4-androstene-11β-ol-3, 17-dione have the same mobility as aldosterone diacetate in these systems (confirmed in this laboratory using aldosterone - diacetate - C^{14}). Acetylation of

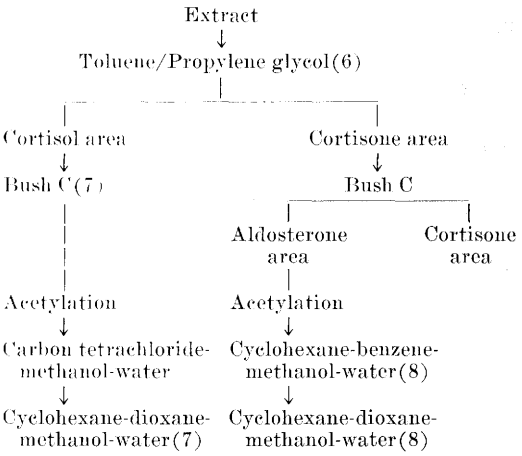

FIG. 1. Chromatographic flow sheet.

* Supported in part by U.S.P.H.S. grant.

† Fellow of Commonwealth Fund of N. Y. Present address: Dept. of Zoology, University of Sheffield, Sheffield, England.

‡ Clinical Investigator, Vet. Admin.

TABLE I. Measurements of Radioactivity at Indicated Points during Chromatographic Analysis. "Control" refers to left head kidneys.

Exp.	Cortisol areas		Cortisone areas		Aldosterone areas	
	Right head kidney cpm	"Control" cpm	Right head kidney cpm	"Control" cpm	Right head kidney cpm	"Control" cpm
Series A, 45 fish						
Counts added			3 × 273,493 cpm			
Counts after Bush C	1456		1359		157	
Series B, 163 fish						
Counts added			9 × 273,493			
Counts after Bush C	11,884	5248	4779	2458	642	1272
No. counts aliquoted	8913	3936				
Counts after E2B	3004	359				
% recovery	34	9				
No. counts acetylated	2250	270			483	954
Counts after acetylation and 2 systems (Fig. 1)	1518	88			462	630
% recovery	67	33			96	66

the cortisol area was carried out in similar manner except that 100% acetic anhydride was used and 20 μg cortisol was added as carrier; then chromatographed in the system carbon tetrachloride - methanol - water (100:100:25); eluted and rechromatographed in the system cyclohexane-dioxane-methanol-water (100:100:50:250). Authentic cortisol monoacetate was run in parallel in both systems.

Results suggest that cortisol, cortisone, and aldosterone were produced (Table I). Series A gave some indication that these 3 corticoids were synthesized from the added precursor. Series B confirmed these findings and added extra evidence for authenticity of the steroids produced. It should be pointed out that a different number of fish were used in the 2 series and since the volume of interrenal tissue is unknown, the data are not directly comparable. Further, the results must take into account 3 facets of experimental technic. Optimal temperature for this species is 15-20°C and therefore incubation of tissues at elevated temperatures may introduce a complicating factor. Also, objections can be raised against the use of a mammalian incubation medium for fish tissue. Finally, it was assumed that progesterone is an intermediary product of adrenocorticosteroidogenesis in fish as it is in mammals. Nevertheless, under these conditions a small percentage of added radioactivity was recovered in steroid areas studied. The head kidneys consist chiefly of lymphoid tissue with only a small amount of adrenocortical cells. It is conceivable, therefore, that only a small portion of the added progesterone-16-H^3 was accessible to the interrenal cells, the excess being metabolized by lymphoid tissue or remaining unaltered.

In incubates of "control" tissue (left head kidneys) there was a striking decrease in amount of cortisol-like steroid after successive chromatographic systems. This emphasizes the necessity for rigorous purification before the authenticity of a steroid can be established. The number of counts/minute and percentage recovery for cortisol from right head kidneys is consistently higher than that of "control" tissue, which suggests that a large portion of radioactivity in "control" incubates can be accounted for by non-specific contamination. The analysis of cortisone was not carried beyond the second chromatography. It is interesting that again the greatest amount of radioactivity was found in the right head kidney incubates. Cortisone has not been found in peripheral blood of Fundulus (1), but it has been identified in a large sample of salmon plasma (unpublished), in adrenal effluents of mammals, the snake and the capon (9,10,11), but not in peripheral blood of humans.

In contrast to findings with cortisol and

cortisone, the amount of radioactivity in aldosterone areas from "control" media was greater than that of right head kidney media. This radioactivity persisted despite 2 chromatographies as the free alcohol, and 2 as the diacetate. However, the magnitude of the differences between right head kidney and "control" results for aldosterone diminished after acetylation. These findings would indicate that either further purification is necessary to establish the identity of the aldosterone believed present or that the "control" tissue can synthesize this mineralocorticoid despite little or no ability to synthesize glucocorticoids. If radioactivity is indicative of aldosterone it raises the question as to whether aldosterone plays a role in electrolyte homeostasis in the fish.

Conclusions. With the above reservations in mind, the following tentative conclusions can be drawn. 1. It seems probable that cortisol, in plasma obtained from *Fundulus heteroclitus,* is synthesized by interrenal tissue of head kidney. 2. Production of cortisol and probably cortisone by both left and right head kidneys suggests that interrenal tissue is present in both, although in lesser amounts on the left. If this is so, no explanation can be given at present for slightly larger amounts of aldosterone produced by left head kidneys. 3. Interrenal tissue of Fundulus possesses enzyme systems, capable of converting progesterone-16-H^3 to tritiated cortisol, cortisone, and aldosterone through biosynthetic pathways not known.

The authors are grateful to Dr. P. K. Bondy and Dr. G. E. Pickford for help and encouragement, and to Albert Kuljian for technical assistance.

1. Chester Jones, I., Philipps, J. G., Holmes, W. N. *Symposium on Comparative Endocrinology,* John Wiley and Sons, 1959, in press.

2. Pickford, G. E., *Bull. Bingham Coll.,* 1953, vXIV, art. 2.

3. Pickford, G. E., Atz, J., *Physiology of Pituitary Gland of Fishes.* N. Y. Zool. Soc., 1957.

4. Chester Jones, I. *The Adrenal Cortex. C.U.P.* 1957.

5. Giroud, C. J. P., Stachenko, J., Piletta, P. *An International Symposium on Aldosterone.* Little Brown and Co., 1958.

6. Burton, R. B., Zaffaroni, A., Keutman, E. H., *J. Biol. Chem.,* 1951, v188, 763.

7. Bush, I. E., *Biochem. J.,* 1952, v50, 370.

8. Chen, P. S., Shedl, H. P., Rosenfeld, G., Bartter, F. C., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 683.

9. Bush, I. E., Ferguson, K. A., *J. Endocrin.,* 1953, v10, 1.

10. Hechter, O., Pincus, G., *Physiol. Rev.,* 1954, v34, 459.

11. Phillips, J. G., Chester Jones, I., *J. Endocrin.,* 1957, v16, iii.

Received April 3, 1959. P.S.E.B.M., 1959, v101.

Bovine Ocular Squamous Cell Carcinoma. III. Tissue Culture Studies of Carcinoma.* (24905)

J. A. SYKES, L. DMOCHOWSKI AND W. O. RUSSELL†‡

Section of Virology and Electron Microscopy, and Dept. of Pathology, University of Texas M. D. Anderson Hospital and Tumor Inst.; and Department of Microbiology, Baylor University College of Medicine, Houston, Texas

Genetic studies of bovine ocular squamous cell carcinoma have demonstrated relationship of incidence of this disease to age of animal and degree of ocular and circumocular pigmentation(1). Studies on pathological anatomy revealed presence of eosinophilic, cytoplasmic inclusion-like bodies in cells of both benign precursor and malignant lesions(2). Tissue culture studies of cells from benign precursor lesions, plaque and papilloma, have shown cytoplasmic changes and inclusions suggestive of presence of a virus(3,4,5,6). Dis-

* This work was supported by Research Grants from Nat. Cancer Inst., N.I.H., U.S.P.H.S.

† Invaluable cooperation of ranch owners in this study and of Dr. E. S. Wynne in histological diagnosis of specimens is gratefully acknowledged.

‡ Publication No. 9 of Cancer Eye Study Section.