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TITLE OF THESIS SOME ASPECTS OF STEROID METABOLISM AND EXCRETION IN CATOSTOMUS COMMERSONNII

UNIVERSITY OF ALBERTA

DEGREE PH.D. YEAR GRANTED 1969

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THE UNIVERSITY OF ALBERTA

SOME ASPECTS OF STEROID METABOLISM AND EXCRETION IN CATOSTOMUS COMMERSONII

by

© RENE ROMAIN ROTH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

Spring, 1969.

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Some aspects of steroid metabolism and excretion in Catostomus commersonnii", submitted by Rene R. Roth in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Supervisor

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External Examiner

Date.....

ABSTRACT

There is general agreement that the lower vertebrates possess endocrine tissues and products similar,if not identical to those in Eutheria. This conception was demonstrated beyond any doubt for Amphibia and Reptilia. However,in fish the evidence is less conclusive. Although hormonal compounds were detected in blood of many species,it is not clear if they all are chemically identical with those found in Eutheria and if they have a similar physiological role. Most of the work done so far,on steroid hormones in particular,was performed on various tissues and blood.

The experimental approach used in the present study had the following goals: (a) to determine if steroid hormones and/or their metabolites were excreted in the urine of normal,untreated suckers; (b) to determine whether and what kind of metabolites could be detected in sucker urine after administration of pharmacological doses of standard steroids; (c) to trace the origin of urinary metabolites by the use of labeled steroids; (d) to determine if and how ACTH affects steroid metabolism and excretion in suckers.

In untreated fish,a relatively great number of urinary steroid compounds could be detected. Seasonal differences and differences between the sexes were recorded. The highest number of urinary steroid metabolites could be detected in females during summer,whereas during

winter there were almost none in the female group. The males presented no extremes but very similar results in winter and in summer.

Only two steroid metabolites could be detected constantly in sucker urine. Both compounds were very polar and gave positive alkaline fluorescence. One of the compounds (SP_1) reacted with blue tetrazolium, indicating the presence of an α-ketol side chain. It was possible to demonstrate that the two compounds (SP_1 and SP_2) were catabolites of cortisone.

Pharmacological doses of steroid standards caused the appearance of new compounds in the urine of treated fish,simultaneously with the suppression of many of the compounds found in untreated animals. While progesterone caused the appearance of material giving a positive reaction with phosphomolybdic acid,and testosterone administration resulted in Zimmermann positive material,estradiol failed to elicit the appearance of phenolic steroids. It was concluded that Catostomus has the ability to alter the phenolic structure of the estrogen molecule.

ACTH caused the appearance of some additional urinary steroids but it seemed that its main role was the effect upon the excretory pathway by shifting the excretion of steroid metabolites from a pararenal to the renal route. On the other hand,ACTH enhanced the excretion of steroid metabolites incorporating ^{14}C from labeled acetate, although the part played by acetate in steroid biosynthesis in the white sucker was found to be of minor importance.

It was possible to show that in Catostomus the main excretory pathway for steroids is the entero-hepatic route.

All the evidence presented indicates that in Catostomus commersonnii the metabolism of steroid hormones is different from that seen in higher vertebrates; that the physiological action, in fish, of different steroids and ACTH cannot be equated with their actions in mammals and that the elucidation of the biochemistry and physiology of endocrine mechanisms in fish needs much more investigational effort.

ACKNOWLEDGEMENTS

I gratefully acknowledge Dr.C.P. Hickman's help in suggesting this research and the interest both he and Dr.D.D.Beatty took in my work. Thanks are also due to the academic and technical staff of the Department of Zoology and to my colleagues whose assistance helped greatly. I am indebted to Dr.W.A.Ayer of the Department of Chemistry for his assistance in processing the mass spectra and to Miss Margaret Harris for the water analyses.

I would like to express my thanks to Dr.J.R.Nursall who took a personal interest in helping me,starting with the invitation for coming to this wonderful country.

My very special and unrelenting gratitude goes to Dr.D.M.Ross who,by his more than generous friendship and helpfulness,made it possible for us to adjust to a new environment and to feel at home in this country.

This study was supported by grants from the National Research Council of Canada to Dr.C.P.Hickman and by the award of a research assistantship and a Dissertation fellowship by the University of Alberta in Edmonton. This assistance is most gratefully acknowledged.

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I. INTRODUCTION

Comparative endocrinology is a rapidly expanding field. This makes it mandatory for students of comparative endocrinology to become more and more specialized in particular aspects of this science. Therefore it can be expected that only relatively few investigators will work on the same problem or the same species.

Fishes exhibit a diversity in their morphology,ecology and physiology which is not found in any other group of vertebrates. Besides,their poikilothermic nature and the aquatic environment are two factors which make the study of fish endocrinology even more difficult.

Therefore it is not astonishing that work in fish endocrinology is proceeding slowly and that many of the results are contradictory and their interpretation sometimes cumbersome.

Investigators of this field of study followed some principal lines of research which might be grouped as:

1. Anatomical and histological studies performed on tissues with assumed endocrine function.

2. Studies on sex reversal,spontaneous or by castration,by administration of various hormones and reimplantation of gonads.

3. Studies on reproductive behaviour in normal and castrated or spayed fish,in fish injected with various hormones and in hypophysectomized animals.

4. Studies on the physiological effects of various hormones in fish.

5. In vitro incubation of secretory tissues,assumed to produce hormones,with or without added precursors.

6. The extraction and purification of hormones from tissues and from blood or plasma of various fish.

To mention just a few works done on these main lines of investigation into the endocrine mechanisms of fish and,in particular,the steroid endocrinology of teleosts,we have to remember the demonstration,by Hildemann (1954),of the development of male sex characters in female guppies,Lebistes reticulatus,after administration of testosterone propionate. On the other hand Tavolga (1955)observed that,after castration,males of the gobiid fish Bathygobius soporator show the same courtship behaviour as intact males and Aronson (1959) reported that in males of the cichlid Aquidens latifrons,all elements of the mating pattern were preserved after castration.

In experiments including a variety of species,estrogenic hormones have been shown to possess the ability to influence sex differentiation and development. Berkowitz (1937) succeeded,by feeding estrogens to young,sexually undifferentiated males of Lebistes reticulatus,to cause the suppression of secondary sex characteristics and of spermatogenesis. By inclusion of estrone and stilbestrol in the diet of genotypic males of Oryzias latipes,Yamamoto (1953) caused complete feminization and sex reversal.

Hypophysectomy has been carried out in a large number of teleostean species and there is quite substantial evidence that the gonads of both

sexes are under the control of the pituitary gland (Dodd,1960).

Because interrenalectomy in teleosts has so far proved impossible because of the arrangement of the tissue in the head-kidney and around blood vessels,the effects of corticoid insufficiency could be studied only indirectly by means of hypophysectomy.

Fontaine and Hatey (1953) found that hypophysectomy in Anguilla was followed by atrophy of the interrenal. They showed that mammalian ACTH restored to normal the weight of the interrenal. This response depended not only on the dose but also on the temperature at which the animals were kept. On the other hand the interrenal of the hypophysectomized male killifish,Fundulus heteroclitus,did not show degenerative changes nor indeed any apparent difference from that of the normal animals (Pickford,1953).

Phillips and Mulrow (1959) demonstrated that the interrenal tissue of Fundulus heteroclitus is capable of producing cortisol and cortisone in vitro,although cortisone was not found in the peripheral blood of this fish. They found that tritiated progesterone was converted in vitro to tritiated cortisol,cortisone and aldosterone by the interrenal tissue of killifish. Butler (1965) succeeded in purifying,by paper chromatography,the extracts from interrenal tissue of Anguilla and Conger,after incubation with ^{14}C labeled progesterone. Cortisol was found in both species but only cortisone in the freshwater Anguilla. Conger eel interrenal tissue converted labeled progesterone to corticosterone.

ACTH was used by Nandi and Bern (1965) for incubation of interrenal tissue of some teleosts. They could detect the formation of cortisol,cortisone and corticosterone in the interrenal of Salmo gairdnerii,cortisol,cortisone,17 α-hydroxy-11-deoxycorticosterone

and 11-dehydrocorticosterone from Tilapia mossambica and cortisol, corticosterone and 17 α-hydroxy-11-deoxycorticosterone in Bodianus bilunulatus. It has to be mentioned here that the same authors (Nandi and Bern,1960) were unable to detect any adrenocorticosteroids in Bodianus interrenal tissue when extracted without previous ACTH incubation.

While there is no evidence for progesterone to have a hormonal action in fish,androgens are universal hormones among vertebrates. Chieffi (1962) reported the isolation of testosterone and androstenedione from the testes of Scyliorhinus stellarius. Idler and his co-workers succeeded in isolating 17 α-hydroxy-progesterone (Idler et al.,1959) and adrenosterone (Idler et al.,1961 a) from the testes and blood of sockeye salmon,Oncorhynchus nerka,and the Atlantic salmon,Salmo salar. Idler et al.(1960 b) isolated and identified 11-ketotestosterone from salmon plasma,showing that in fish there are biosynthetic pathways for steroid biogenesis which differ from those usually found in higher vertebrates. Simpson et al.(1963 a) found 11-deoxycorticosterone, progesterone,pregnenolone,androstenedione and dehydroepiandrosterone in the semen of dogfish.

A number of investigators succeeded in extracting adrenocorticosteroids from blood and plasma of various species of fish. Bondy et al.(1957) demonstrated the presence of cortisol in carp blood. Phillips and Chester Jones (1957) reported the detection of cortisol and corticosterone in blood of ray,dogfish,cod and the lungfish Protopterus annectens. Phillips et al.(1959) claimed to have extracted cortisol,corticosterone and aldosterone from sockeye salmon plasma. However Idler et al.(1960 b) showed that the assumed corticosterone was mainly 11-ketotestosterone and,later,Idler and Ronald (1962) demonstrated that the material which

appeared to be aldosterone was not identical with this steroid. On the other hand,using highly sensitive methods and very precise techniques, Schmidt and Idler (1962) detected in salmon plasma cortisol,cortisone, 11-ketotestosterone,20 β-dihydro-17 α-hydroxyprogesterone,17 α-progesterone and testosterone. In 1966 Idler and Truscott succeeded in isolating for the first time testosterone from the blood of an elasmobranch,finding much higher amounts than those reported in mammals.

Although relatively many investigators worked on blood steroids in various species of fish,there are practically no publications on urinary metabolites of steroid compounds in this group of vertebrates. The only work done to date in this field,as far as I know,was that of Brull and Cuypers (1954). They pooled urine from 25 male and female angler fish, Lophius piscatorius,and found "folliculin",11-oxosteroids and 17-ketosteroids. It is difficult to judge the validity of these findings due to the inadequacy of the methods used.

Although incubation experiments yielded some information about the possible pathways of steroid metabolism in fish,these results do not imply that the in vitro findings reflect implicitly the metabolic processes which take place in vivo. Therefore it seemed of some importance to investigate the steroid compounds presumably present in fish urine,the more so because there was a chance to learn something about the fate of the blood steroids and the factors influencing them.

II. MATERIALS AND METHODS

1. Fish handling and care.

The fish used in the present study is the common white sucker, Catostomus commersonnii commersonnii, Lacépède,known also as black sucker,mullet etc.

The suckers were caught in Lac St.Anne,a eutrophic lake situated about 50 miles north-west of Edmonton,in central Alberta. It is a dimictic lake,presenting a spring overturn in April and a fall turnover in October. The water temperature is highest in July when it may reach on rare occasions even 25°C at the surface. The bottom layers however,never exceed 20°C. The lake is usually ice covered from mid-November to mid-May (Fig.1).

The spawning season of the white sucker starts in April,when the water temperature rises above 5°C. During this period the fishes may ascend tributaries of Lac St.Anne where they can be caught without difficulty. The peak of the spawning season lasts 2-3 weeks. After spawning,most of the fish return to the lake.

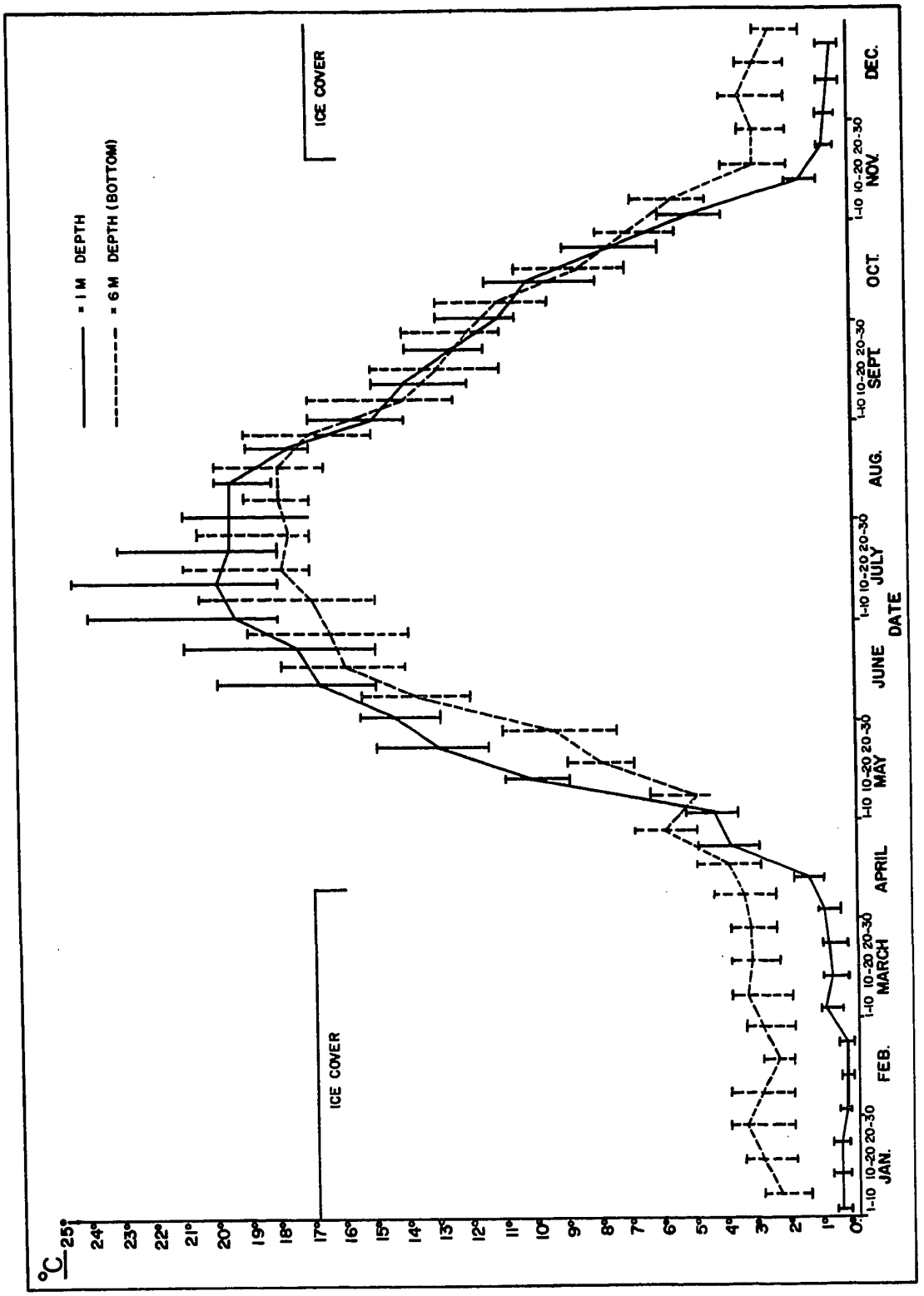
The highest gonadosomatic index in females (Fig.2) was found to be in mid-June,while in males it is in December (Fig.3).

During the present study the fish were caught by gill net,trap net or seine. During the summer months trap-netting was more efficient

FIG. NO. 1

WATER TEMPERATURES IN LAC ST. ANNE.

The temperatures in ^{o}C are given as mean and range values for the years 1963-1966. The plain line represents the temperatures at 1 m depth, the broken line shows the temperatures at 6 m depth, usually coinciding with the bottom of the lake. Data were recorded by staff and graduate students of the Laboratory of Comparative Physiology of the Department of Zoology, University of Alberta, Edmonton.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= 6 M DEPTH (BOTTOM)
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ICE COVER
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JULY
AUG.
SEPT.
OCT.
NOV.
DEC.
DATE

FIG. 1

FIGS. NO. 2,3

SEASONAL CHANGES IN GONADOSOMATIC INDEX OF WHITE SUCKERS IN LAC ST.ANNE DURING 1963-1966

The values are mean and range for 205 females (Fig.2) and 133 males (Fig.3),given as gonadosomatic index X 100. Dotted line represents values for males with unspent gonads. Data were recorded by staff and graduate students of the Laboratory of Comparative Physiology of the Department of Zoology,University of Alberta,Edmonton.

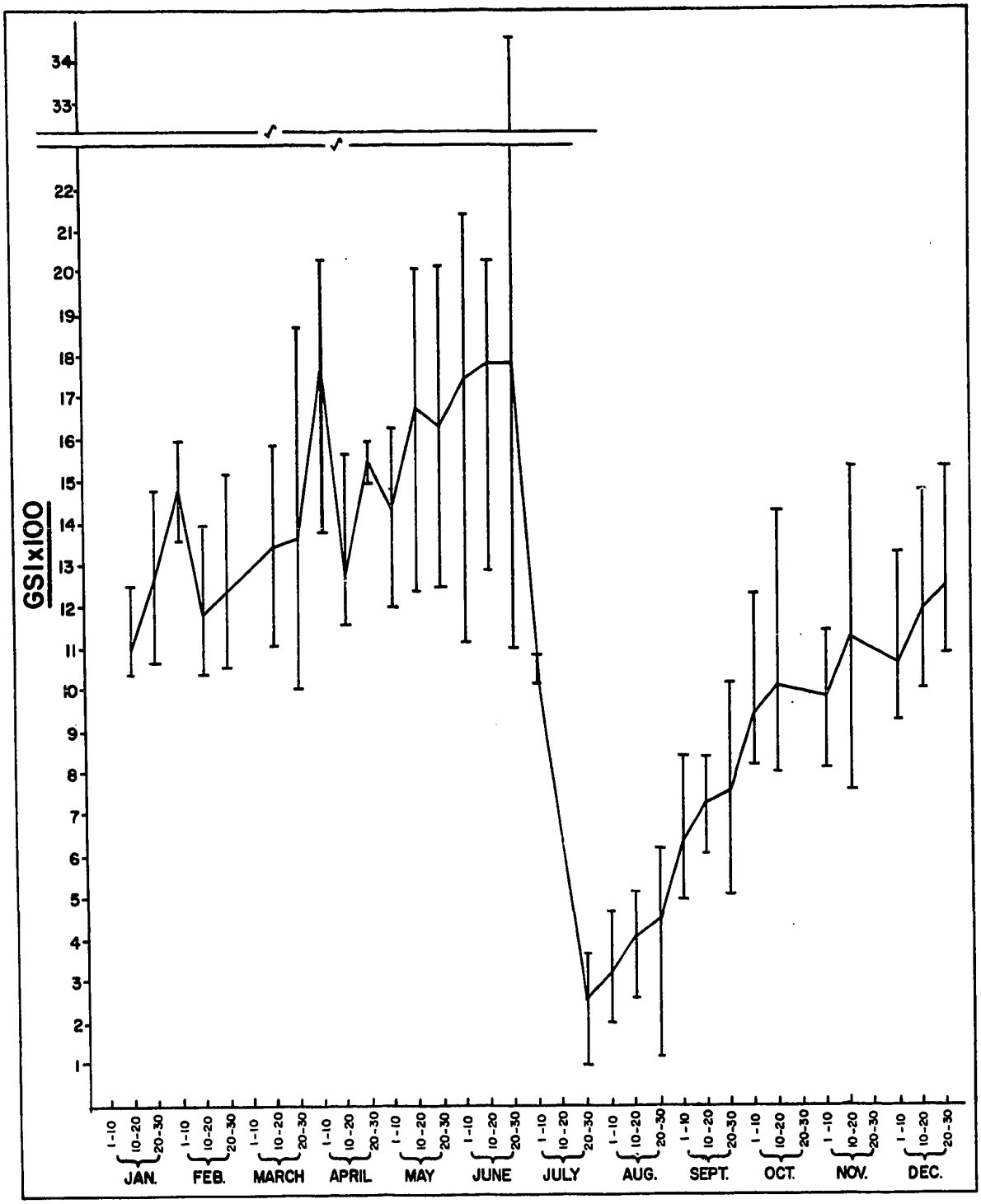
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FIG.2

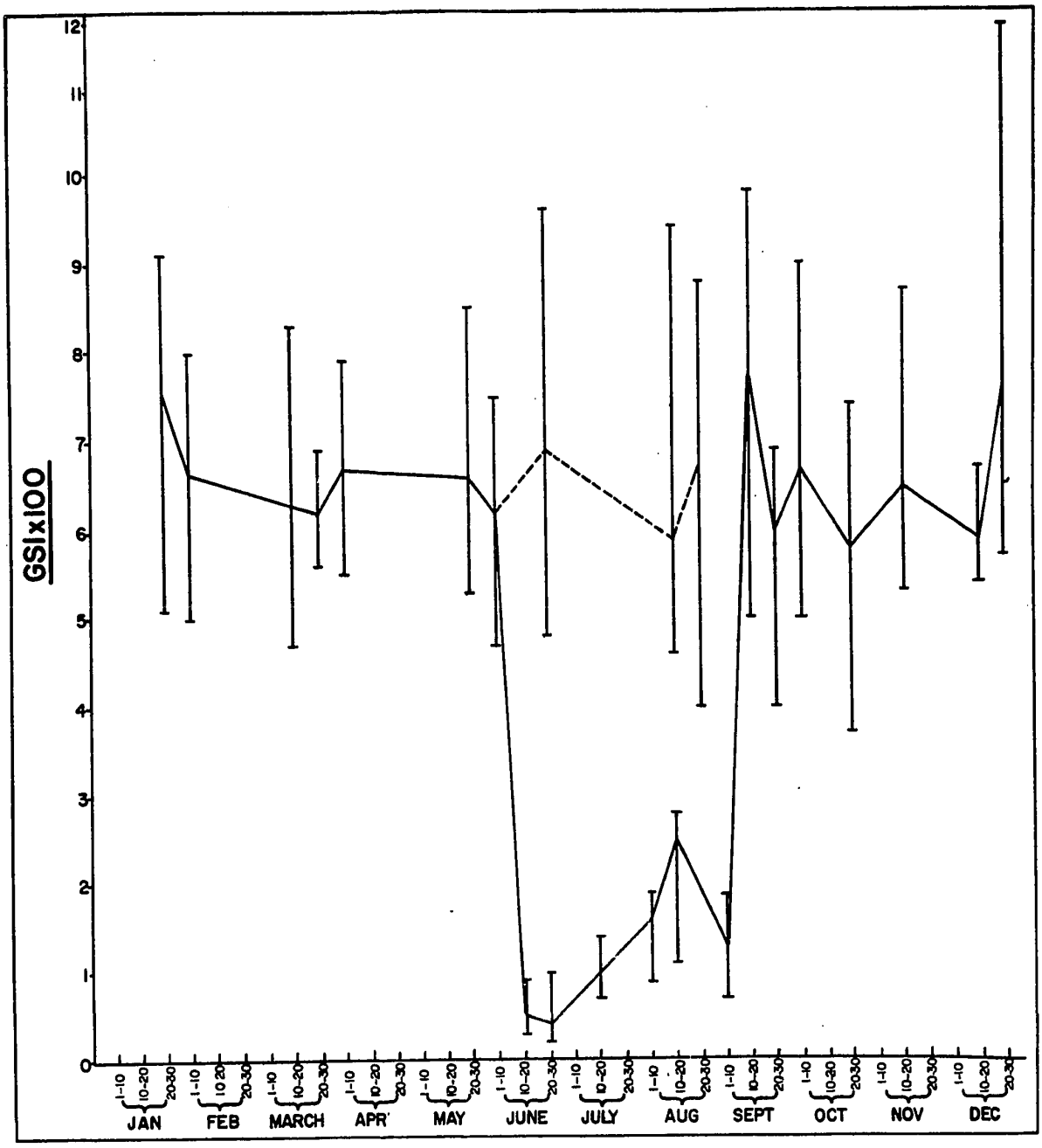
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FIG.3

than gill-netting,while the reverse was true for the winter months. During the spawning season the suckers were caught mainly by seine. As a general observation it may be stated that the fish caught by seine were in best condition,while the trap-netted fish were in better shape than those caught by gill nets.

During the summer,when fishing was done from boats,the suckers were placed,immediately after catching,in metal tanks filled with lake water. After the boats returned to the shore the fish were transferred to 35 gal. metal barrels filled with aerated lake water. On arrival at the university laboratory,the fish were placed in a holding tank of 180 gal.,after being treated with a 1% solution of malachite green as a prophylactic measure against fungus infection. The holding tank was equipped with a pump system allowing the recycling of water by way of a sand filter. The temperature was thermostatically controlled at $10^{o}C$. Although this temperature was lower than the average lake temperature during the summer months,this choice was considered necessary after experiments showed that this was the best temperature to keep fish in healthy condition over longer periods of time. The water in the holding tank was aerated and a steady inflow of dechlorinated fresh water was provided.

During winter,the fish were caught by gill nets lowered through holes drilled into the ice cover. Immediately after freeing them from the gill net,the suckers were placed in 15 gal. plastic containers, filled with lake water and ice. The plastic containers were taken to the field laboratory on toboggans. There the fish were transferred to the same kind of metal barrels as those used in summer and the same procedure was followed. The temperature in the holding tank was $5^{o}C$ during the winter months.

The number of fish in the holding tank was usually 20 - 30. They were kept for periods up to four months before they were used in experiments. Sometimes fishes,that had no treatment and were still in good condition,were returned to the holding tank.

2. Operative procedures.

The experimental procedure and equipment described below were designed by Dr.C.P.Hickman,Jr. and Mr.Brian Hammond in this laboratory.

The fish was lifted from the holding tank by net and anaesthetised in a trough containing 0.5-1.0 g/gal. MS 222 (Tricaine Methanesulfonate Sandoz) and 1% malachite green,for antifungal prophylaxis. It was weighed and placed in an operating box which contained a solution of MS 222 (0.2 g/gal.). The fish was positioned, ventral side up,with the head immersed in water,the tail lying above the water level. With the help of a "Luxo" magnifier with circular fluorescent tube for shadow-free lighting,an 80 cm polyethylene catheter (Clay-Adams PE 200, 1.5 mm in diameter) was introduced into the mesonephric duct. The tube was sutured in place with 4-0 Ethilon monofilament nylon. Using thread,the catheter was secured to the anal fin at two adjacent points. Then the fish was turned on one side and a B-D 18 T special needle (3½") was used to puncture the dorsal aorta or the caudal vein,by introducing it along the last ray of the caudal fin at an angle of 45°. A PE 50 (0.75 mm in diameter) polyethylene tubing of 50 cm length,previously filled with a solution of 250 U.S.P. units/ml Lipohepin (Heparin Sodium Riker),was introduced through the special needle which was removed,leaving the PE 50 tubing (cannula) in situ. The cannula was also secured,with thread,at two adjacent points, to the caudal fin. After the operation,the fish was transferred to a

specially built lucite box. The box was equipped with a pump which introduced aerated,cooled,dechlorinated water from an 80 gal. tank into the front end of the box. Through an opening at the opposite end of the box,water flowed back into the fiberglass tank. The water in the fiberglass tank was maintained thermostatically at a set temperature. There was a constant inflow of fresh,dechlorinated water into the fiberglass tank and a special drain pipe kept the water level constant. This set-up was considered to be an "open system",having a continuous water exchange in the lucite box. However,when the pump was used to recirculate the outflowing water back into the lucite box,without coming in contact with the tank water,the set-up was considered to be a "closed system". In this case the fiberglass tank was used only as a cooling device,keeping the recycling water in the lucite box at a set temperature. The catheter and the cannula were led outside the box through rubber stoppers which closed special openings in the wall of the lucite box. To collect urine,the catheter passed into a Nalgene bottle of 4 oz. placed in a Dewar flask packed with crushed ice and covered with foam rubber to keep the temperature near $0^{o}C$. The outflowing urine was collected for varying periods up to 30 days. The bottles were changed every 6-12 hours. The Nalgene bottles with urine were stored at $-20^{o}C$ in a freezer until the time of processing. Previous observations had shown that there is no qualitative or quantitative biochemical change in urine samples kept at this temperature for periods up to one year.

Substances to be injected were introduced by a 23 G hypodermic needle,attached to a 1 ml Luer glass syringe,through the caudal cannula. After injection the cannula was filled with heparinized saline solution and blocked with a pin.

At termination of an experiment the fish was anaesthetised with MS 222,removed from the lucite box,weighed and scale samples taken from the dorsal fin area for age determination. Blood was withdrawn by heart puncture with a 15 G (1½") hypodermic needle mounted on a 10 ml Luer glass syringe. Afterwards an autopsy was performed. Internal organs and a muscle sample were removed,weighed and stored at -20°C. Later they were freeze-dried and,after attaining steady weight,the tissues were ground with an electric grinder.

3. Reagents and chemicals.

The reagents used in the present study were purified as follows:

Ethyl acetate was washed repeatedly with a saturated solution of sodium bisulfite,with distilled water,a saturated solution of calcium chloride and again with water. Afterwards it was dried over anhydrous calcium chloride,stored in a refrigerator at 4°C for 1-3 hours and finally filtered rapidly through glass wool and distilled. It could be stored for 1-2 months in the refrigerator. Every week the ethyl acetate was checked,for aldehyde and peroxide formation,with acidified potassium iodide solution.

Chloroform was dried over anhydrous potassium carbonate,filtered rapidly through glass wool and distilled immediately prior to use.

Ether was washed with a solution of 50 g ferrous sulfate in 100 ml of 30% sulfuric acid,afterwards with water and distilled over potassium hydroxide pellets,just before use.

Ethyl alcohol (ethanol) and methyl alcohol (methanol) were distilled over potassium hydroxide pellets and then redistilled. They could be kept at room temperature for indefinite periods of time.

Toluene,benzene and ligroin were washed repeatedly with concentrated sulfuric acid (Fisher reagent chemical, sp.gr.at 60°F 1.8407-1.8437) until no chromogenic impurities could be detected. These organic solvents were then washed with distilled water,a saturated solution of sodium carbonate and again with water and afterwards dried over anhydrous calcium chloride. They were distilled, redistilled and stored at room temperature for indefinite periods.

m-Dinitrobenzene was sublimized on a watch-glass placed over a beaker heated on a hot plate.

All other reagents were used without purification. They were purchased as "Spectroline" chemicals from the U.S.Testing Co.,Hoboken, N.J. and were of highest purity. The steroid standards (Table No.1) used in the present investigation were of highest purity and purchased from Vismara Terapeutici ("Vister") Casatenovo Brianza,Italy.

The following tritium labeled steroids were purchased from the New England Nuclear Corp.,Boston,Mass.: cortisone-1,2-^{3}H (SA=6 µc/µg), estradiol-17 β-6,7-^{3}H (SA=20 µc/µg),progesterone-7 α-^{3}H (SA=32 µc/µg) and testosterone-1,2-^{3}H (SA=166 µc/µg). Cortisol-1,2-^{3}H (SA=36 µc/µg) and corticosterone-1,2-^{3}H (SA=158 µc/µg) were purchased from Tracerlab, Boston,Mass. The Nuclear Chicago Corp. provided sodium acetate-1-^{14}C (SA=35.8 mc/mm) and the liquid scintillation reagents.The primary and secondary scintillators were 2,5-diphenyl-oxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) respectively. The scintillator mixture was made up of 0.3 g/l POPOP plus 5 g/l PPO in purified toluene (Nuclear Chicago Bulletin No.11). All dilutions of steroids,labeled and unlabeled, were made up in purified absolute methanol.

TABLE NO. 1

Steroid standards used in the present study,with their trivial and systematic names and the conventionally used abbreviations.

Trivial Name	Systematic Name	Abbreviation
Aldosterone	11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al	-
Androsterone	3α -hydroxy-5α -androstan-17-one	-
Corticosterone	11β,21-dihydroxypregn-4-ene-3,20-dione	B
Cortisol	11β,17α,21-trihydroxypregnene-3,11,20-trione	F
Cortisone	17α,21-dihydroxypregn-4-ene-3,11,20-trione	E
Estradiol	Estra-1,3,5(10)-triene-3,17β-diol	E_2
Estriol	Estra-1,3,5(10)-triene-3,16α,17-triol	E_3
Estrone	3-hydroxyestra-1,3,5(10)-trien-17-one	E_1
Pregnanediol	5β-pregnane-3α,20α-diol	-
Progesterone	Pregn-4-ene-3,20-dione	-
Testosterone	17β-hydroxyandrost-4-en-3-one	-
Urocortisol (Tetrahydro-cortisol)	3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one	THF
Urocortisone (Tetrahydro-cortisone)	3α,17α,21-trihydroxy-5β-pregnane-11,20-dione	THE

4. Methods for extraction and purification of steroids from fish urine.

Frozen urine specimens were thawed at room temperature. The volume and hydrogen ion concentration of the specimens were measured at 25°C. After centrifugation of the urine,for removal of suspended mucous, extraction was performed on 24 hour urine samples or on pooled urine. When the volume of urine to be extracted was more than 150 ml,the urine was concentrated in a flash-evaporator. To prevent foaming,the pH was adjusted,with glacial acetic acid,to 5.0 and a drop of octyl alcohol was added as an antifoaming agent. The temperature during flash-evaporation was maintained at 40°C. After concentration to a volume of 80-120 ml,the pH was readjusted,with N/10 sodium hydroxide,to 7.0 and the urine was extracted for steroids according to a procedure shown in the flow-sheet (Fig.4). At the termination of this procedure there were six extracts or dry residues left. It was assumed that the extracts contained: unconjugated,free steroids in extract # 1; steroids released by enzymatic hydrolysis (β-glucuroniside uronidates) in extract # 2; steroids set free by gentle acid hydrolysis (sulfates) in extract # 3; phenolic steroids of the estradiol-estrone group in extract # 4A; phenolic steroids of the estriol group in extract # 4B and steroids with the 17-oxo structure (androgen catabolites) in extract # 4C. This procedure was rigidly followed for extracts # 1,2 and 3. However,extracts # 4A, 4B and 4C were frequently processed as one single extraction,chromatographic methods being used,for separation, in this case.

In addition to the extraction procedure described above,some urine samples were extracted for estrogens only (according to Finkelstein,1952 and Finkelstein and Goldberg,1957) or for pregnanediol only. The extraction procedure for urinary pregnanediol was my own,

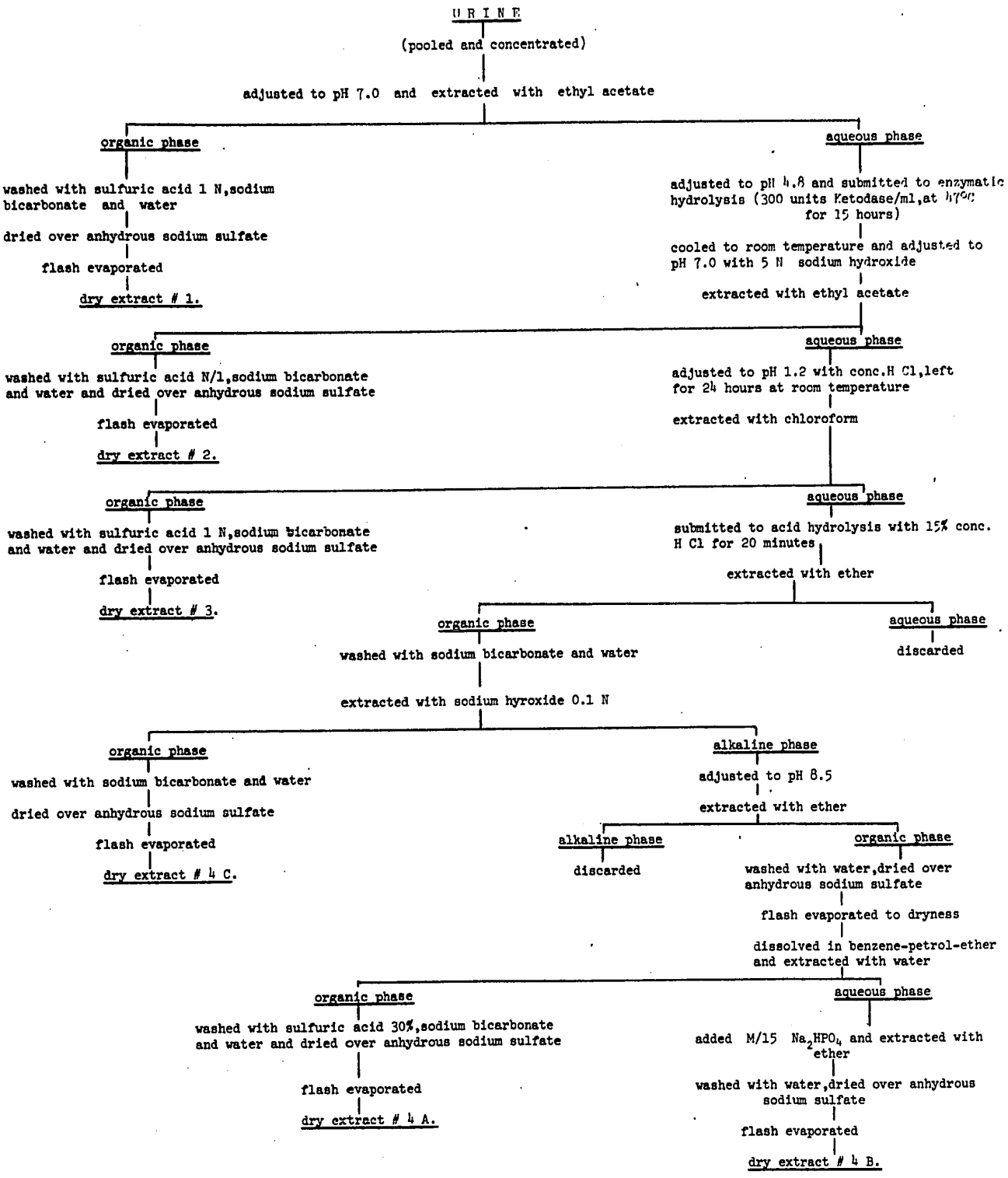


FIG. NO. 4. FLOW-SHEET OF FISH URINE EXTRACTION.

modified after Brown (1955),Jayle and Crepy (1952),Klopper et al. (1955) and Talbot et al.(1943). This extraction was performed as follows:

Thawed urine was adjusted to pH 4.6 with glacial acetic acid. An amount of a 2 M solution of acetate buffer,equal to 10% of the urine volume and 350 units/ml Ketodase (β-glucuronidase from Warner-Chilcott Co.,containing 5000 Fishman units of beef liver β-glucuronidase in 1 ml solution) were added. The mixture was incubated at 37°C for 15 hours. Then the urine was cooled to room temperature and adjusted to pH 13.0 with 5N sodium hydroxide. Extraction was performed once with an equal volume of n-butyl alcohol (distilled over sodium hydroxide and redistilled at 115°C) and twice more with half the volume. The butanol extracts were pooled and washed with a saturated solution of sodium bicarbonate and water. The butanol extract was dried over anhydrous sodium sulfate and filtered through glass wool. Afterwards it was evaporated to dryness in a flashevaporator at 40°C. The residue was moistened with 0.3 ml ethyl alcohol and dissolved in 10 ml benzene. The benzene mixture was transferred to a separatory funnel. The flask with the residue was washed three more times with benzene and the washings were pooled with the first portion in the separatory funnel. Then the benzene solution was oxidized with 20 ml alkaline potassium permanganate (4% potassium permanganate in 1 N sodium hydroxide solution) by shaking for five minutes until the color changed to greyish-brown. The two phases were separated by centrifugation of 10-15 ml portions of the mixture,which were then pooled in the separatory funnel. The benzene solution was separated from the aqueous phase and washed with sodium bicarbonate and water. After drying over anhydrous sodium

sulfate,the benzene solution was filtered through glass wool and flash-evaporated. The residue was taken up with small amounts (3 ml) of benzene and methanol,transferred to a small test tube and evaporated under a stream of nitrogen on a water bath at 40°C. The dry residue was purified by paper chromatography.

The following chromatographic systems were used throughout the present study:

A. Propylene glycol : toluene (Zaffaroni et al.,1950). The paper was impregnated with a mixture of propylene glycol : methanol, 4 : 6 (stationary phase) and the mobile phase was toluene saturated with propylene glycol (1 : 1).

B. Iso-octane : tert-butanol : methanol : water (100 : 45 : 45 : 10). The upper phase was used as the mobile,the lower layer as the stationary phase (Eberlein and Bongiovanni,1955).

C. Toluene : ethyl-acetate : methanol : water (90 : 10 : 50 : 50). The upper layer was the mobile,the lower layer the stationary phase (Bush,1952,1954).

D. Ligroin : benzene : methanol : water (70 : 30 : 80 : 20). The upper layer was the mobile,the lower layer the stationary phase (modified after Bush,1952).

Systems A and D were useful mainly for non-polar compounds,while systems B and C were effective for the separation of polar steroids.

The usual procedure for purification of the residues from the various extractions was the following:

The dry residue was dissolved in 5 drops of methanol and transferred with a Pasteur pipette to a line marked at 12 cm from the upper edge of a Whatman # 2 chromatography paper,cut in the curtain pattern for multiple-strip paper chromatography (Zaffaroni et al.,1950).

Before use,the chromatographic paper was washed with a mixture of water : methanol, 2 : 1, as a descending chromatogram and a second time with ligroin : benzene : methanol, 1 : 1 : 1. The sample strip was 6 cm wide,while the strips for the standard steroids were 1 cm wide. The test tube with the residue was washed twice more,each time with 3 drops of methanol and once with 3 drops of ethyl-acetate and these were also transferred to the chromatogram strip. The steroid standards were streaked on with micropipettes,each standard on a separate strip. There were usually two strips for each standard,one on either side of the sample strip. The amounts of standard steroids used were 2, 5 or 10 µg. The chromatogram was placed in a chromatography jar (Kimax glass) containing the stationary phase,closed hermetically and left over night for equilibration. In the morning the mobile phase was added through a small opening in the lid,closed before and after with a stopper. After the solvent reached the lower edge of the paper (usually 44 cm),the chromatogram was removed from the jar and suspended with stainless steel clips to a string for drying. When dry,the chromatogram was visualized in a darkroom under short,253 nm, and long wave,366 nm, ultraviolet light. The solvent front and,when present, ultraviolet absorbing zones were marked by pencil. Portions, 0.5 cm wide,cut from the standard strips and from the sample strip,were treated each with one of the reagents described in section II,5. The usual procedure was to cut six portions,each 0.5 cm wide,from the sample strip of the first chromatogram. When steroid spots were detected on the treated portions of the sample strip,the corresponding zones were cut out from the remaining,untreated part of the strip and each zone was cut into small sections and introduced into a separate glass stoppered Erlenmeyer flask of 50 ml volume. The zones

corresponding to the detected spots on the standard strips were also cut out and introduced,each into a separate glass stoppered Erlenmeyer flask of 25 ml. Methanol was added for elution and after three hours at room temperature the methanol eluates were transferred to small test tubes. The elution procedure was repeated twice,each time for 15 minutes. The pooled eluates were evaporated under nitrogen at 40°C. The dry residues were dissolved again with methanol and ethyl-acetate,as described above, transferred to a second chromatogram paper (Whatman # 1) and the chromatogram was developed in a second system. Thus the procedure was similar to the first chromatography,with the exception that the sample strips were only 4 cm wide and contained each only one or two compounds,depending on the degree of resolution in the first chromatogram. Again,after drying the chromatogram, 0.5 cm wide strips were cut and treated with the steroid-detecting reagents. After elution of the detected spots,they were transferred to a third chromatogram for further purification. Only rarely was it necessary to use a fourth chromatographic step.

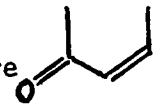
The sequence of chromatogram systems was the following: the first chromatogram was run in system A at 26-27°C; the second chromatogram was developed in system B at 26-27°C and the third was developed in system C at 29-30°C. In the case of non-polar steroids,as e.g. progesterone and 17-ketosteroid standards,system D was used as the third or as a fourth step. For polar steroids,as e.g. cortisol standard,system B at the higher temperature (29-30°C) was used as a fourth step.

For the purpose of comparing the various spots detected on the sample strips,system B was best suited because the R_f values in this system varied only slightly ($<\pm 0.02$).

Temperature control in the chromatography room was achieved by an electric fan for air circulation and an electric hot plate as heat source. With the switch of the hot plate on "low", the room temperature was kept at 26-27°C, with the switch on "medium", the temperature was steady at 29-30°C. A maximum-minimum thermometer gave an accurate record of the range of temperature variation.

5. Methods for detecting steroid compounds on paper chromatograms.

Standard steroids as well as unknown compounds were visualized on paper chromatograms after development. The methods used were considered adequate for establishing some of the biochemical structures characteristic for steroid compounds.

Thus Δ^4-3-oxo-steroids containing structure  in ring A, could be detected by ultraviolet absorption. For this purpose, shortwave (peak emission at 253 nm) ultraviolet light (Mineralight) was used.

Seventeen-oxosteroids (17-KS) with structure  in ring D were detected by the Zimmermann reaction. This was performed as follows: purified m-dinitrobenzene (MDNB) was used to make a 2% solution in ethanol. Just before use, two parts of the MDNB solution was mixed with one part of a 2.5 N solution of potassium hydroxide in ethanol (Bush and Mahesh, 1959). The strips were dipped into the mixture, then placed on filter paper till no wet sheen was seen. Afterwards the strips were placed in an oven at 110°C for 3-5 minutes. The spots were marked immediately because they faded within a short time.

For detection of acetate, a solution of 50 mg Brom-phenol blue in 100 ml water, containing 200 mg citric acid, was used. The paper was sprayed with this solution and if acetate was present, strong blue spots appeared on a yellow background.

Phenolic steroids (estrogens) were detected by Turnbull's blue. A 1% aqueous solution of $K_3Fe(CN)_6$ was mixed in equal volumes, just before use, with a 1% aqueous solution of $FeCl_3$ (Barton et al., 1952). The strips were dipped through the mixture and placed on filter paper. After the blue spots appeared, the strips were washed in acidified water and tap water.

Ketosteroids were detected by the dinitrophenylhydrazine (DNPH) reagent. Three hundred mg 2,4-dinitrophenylhydrazine was dissolved in 44 ml concentrated hydrochloric acid and 50 ml water. Water was added to make 200 ml (Kochakian and Stidworthy, 1952 and Axelrod, 1953). The strips were dipped through a hot solution of the reagent, then held vertically for two minutes until color developed and afterwards washed in acidified water and tap water.

Steroids containing the α-ketol side chain in ring D ($-CO-CH_2OH$) were detected by tetrazolium red reagent(TR). A 0.2% solution of triphenyltetrazolium chloride in water was stored in a brown glass bottle in the refrigerator. Prior to use, two parts of this stock solution were mixed with one part of a 10% solution of aqueous sodium hydroxide (Burton et al., 1951). The strips were dipped through the solution and then placed on filter paper. This reagent was used mainly for detection of those steroids which did not give the alkaline fluorescence reaction, as for example THF and THE. On the other hand, for compounds giving alkaline fluorescence, blue tetrazolium (BT) was preferred, because it could be combined with the alkaline fluorescence detecting method, thus making it unnecessary to use two strips, both reactions being performed on the same chromatogram strip. Besides, BT improved the sensitivity of the alkaline fluorescence by quenching the background fluorescence of the paper.

The combined blue tetrazolium and alkaline fluorescence reactions for detection of α-ketol side chain containing steroids and those containing the Δ^4-3-oxo group,was performed in the following way: a solution of 125 mg blue tetrazolium {3,3'-dianisole-bis{4,4'-(3,5-diphenyl)tetrazolium chloride}} in 100 ml methanol was stored in a brown bottle in the refrigerator. Just prior to use one part of the BT solution was mixed with 9 parts of a 10% solution of aqueous sodium hydroxide. The strips were dipped through the mixture and immediately placed on thick layers of filter paper. If drying on filter paper was omitted the fluorescent spots were dislocated from their original positions. The still humid strips were transferred to a glass plate and covered with a second glass plate. The blue spots were marked and the R_f calculated. After 60 minutes the cover glass was removed and the strips on the supporting glass plate were placed in an oven at 90°C for 20 minutes. After cooling to room temperature the fluorescent spots were observed under long wave (366 nm) ultraviolet light in a darkroom. The fluorescence always extended over a longer surface than the blue spots produced by BT (modified after Bush,1952 and Ayres et al.,1957).

Phosphomolybdic acid (PMA) was used mainly for the detection of 3-hydroxy-steroids and in particular for pregnanediol. Four g of phosphomolybdic acid were dissolved in 100 ml ethanol. The strips were dipped through the solution,blotted between sheets of filter paper and placed in an oven at 80°C for 5 minutes. Afterwards the strips were drawn over a beaker of concentrated ammonium hydroxide,which made the background color fade (Kritchevsky and Kirk,1952).

A mixture of concentrated sulfuric acid and glacial acetic acid (sulfacetic) was used mainly for the purpose of detecting any presence of cholesterol,but it gave color and fluorescence reactions with some

other steroids. Equal volumes of concentrated sulfuric acid and glacial acetic acid were mixed at a temperature near 0°C and the mixture was stored in the freezer. Before use, the mixture was left at room temperature for 15 minutes and shaken from time to time. The strips were drawn through the mixture and immediately blotted between sheets of filter paper. After five minutes the colored spots were marked and the R_f calculated. The strips were transferred to a clean sheet of filter paper and placed in an oven at 70°C for three minutes. After cooling, the strips were observed under long wave ultraviolet light.

Phosphoric acid was used to detect steroid spots on paper chromatograms because it gives characteristic fluorescence reactions with a series of steroids. Eighteen ml phosphoric acid (A.C.S. ortho-phosphoric acid 85%) was mixed with 6 ml water. After the mixture was allowed to cool, the strips were dipped in the mixture, blotted immediately between thick layers of filter paper and placed on a clean sheet of filter paper in an oven with air current at 85°C for fifteen minutes. After cooling, the strips were observed under short wave (253 nm) and long wave (366 nm) ultraviolet light in a darkroom (modified after Neher and Wettstein, 1951).

6. Experimental procedures.

The experimental work consisted mainly in administration of various compounds to white suckers. For this purpose were used: unlabeled steroids, as well as ^{3}H-labeled steroids, ^{14}C-labeled sodium acetate, ACTH and adrenaline.

Unlabeled steroids were administered by injection. An amount (1 mg) of the selected steroid standard was evaporated under nitrogen

and the crystalline residue in the test tube was dissolved in 0.03 ml ethanol. A mixture of sucker saline : propylene-glycol, 2:1, was added (crystalline steroids being insoluble in water) and well mixed. The solution was injected through the caudal cannula. One ml of saline was used for washing the entire amount into the blood stream. The same procedure was used for injecting labeled steroids with the exception that the tritiated compounds were first diluted so as to get 100 µc in a volume of one ml methanol. The methanol was evaporated under nitrogen in a test tube and the unlabeled (carrier) steroid was dissolved as described above and added to the same test tube. When injecting a fish with labeled steroids or sodium acetate,the urine was usually collected every five minutes in separate test tubes,to detect the appearance of radioactivity,switching after 8-12 hours to the usual method of collecting urine in Nalgene bottles.

Adrenocorticotrop hormone (ACTH) was administered by injection or as continuous infusion into the caudal cannula by means of a syringe pump. Fifteen i.u./Kg body weight were injected every second day or a constant amount (5 i.u./Kg body weight/24 hours) of mammalian ACTH (a solution of one i.u./ml hog ACTH from Parke,Davis and Co.,Ltd.,Ont.) was infused for a given period.

Adrenaline (Epinephrine HCl solution C.S.D.,Parke,Davis & Co., Ltd.,Ont.) was injected through the caudal cannula and washed into the blood stream with sucker saline. The amount of adrenaline injected was 0.5 ml of a 1:1000 solution.

Urine collected from fish treated with unlabeled steroids was processed the same way as that from untreated fish(see section II,4). The urine from fish treated with labeled steroids was processed as follows: after extraction according to the flow-sheet (Fig.4),the dry

residues were chromatographed directly in system B at 26-27°C, on Whatman # 1 chromatographic filter paper, cut in the curtain pattern for multiple-strip paper chromatography, the sample strips being 6 cm wide. After development the sample strips were cut into two equal parts, lengthwise. One half was used for the usual chemical reactions while the second half was cut into 2 cm long portions. Each portion was snipped into small pieces and introduced into separate glass stoppered Erlenmeyer flasks of 25 ml volume. To each flask 2 ml methanol were added and left at room temperature for 3 hours. From each of the eluates 50 µl were taken with a micropipette and transferred to liquid scintillation counting vials. The vials were placed in a freeze-dryer and left for 15 minutes. When complete dryness was achieved, 15 ml of the liquid scintillation mixture (see section II,3) were added to each vial and the vials were tightly closed and shaken till the residues were completely dissolved. They were counted in a model 6860 Mark I liquid scintillation system (Nuclear-Chicago Corp.) at 5°C. Thus the activity curve was obtained, with peaks at the zones where radioactive spots were located on the chromatogram.

Chromatograms, with urinary extracts from fish treated with ^{14}C-sodium acetate, were processed in the same manner as those from fish injected with labeled steroids, with the exception that prior to cutting the second half of the sample strip into 2 cm long portions, the strip was submitted to scanning by an Actigraph gas-flow counter-scanner for chromatogram strips (Nuclear-Chicago Corp.). Counting in the liquid scintillation system was done for 20-40 minutes, each vial being counted three to four times. Tables and graphs were constructed on the basis of mean cpm after subtraction of background counts. Liquid scintillator mixture without added organic material was used as background and

an eluate of a 2 cm portion of a chromatogram strip,run without any material on it,was used as blank.

Blood,withdrawn by heart puncture,was immediately centrifuged and the plasma portion was stored at -20°C for varying periods,up to one month,till it could be processed. For counting,50 µl of thawed plasma was taken and introduced into a liquid scintillation counting vial, freeze-dryed for one hour and then counted in the same way as described above.

Tissues were counted,after reducing them to a fine powder,by suspending one g or,if the total amount was less,the whole powdered organ,in methanol so as to have a suspension of 1:10 (w/v). After three hours at room temperature,the suspension was centrifuged,in a Sorvall RC-2 automatic superspeed refrigerated centrifuge,at 13,000 rpm and 3°C. From the supernatant 50 µl were transferred to a liquid scintillation counting vial and freeze-dryed for 30 minutes. The counting was done as described above,using for blank unlabeled material of the same kind as the labeled one.

III. RESULTS

1. Urinary steroid metabolites in untreated fish.

The initial procedure of urine collection and extraction was limited to 24 hour urine. This approach,however,was unsuccessful because no steroid compounds could be found. After twelve trials, involving eight fish,it was concluded that the amounts excreted during 24 hour periods were too small to be detectable by chemical methods and,therefore,larger volumes of urine would be needed. Thus, urine was pooled from five days and extracted. Four trials,involving four fish,showed that also in this case the amounts of steroid metabolites were insufficient. The next step was to pool urine from 10 days. In this case some steroid compounds could be detected, mainly by phosphoric acid and alkaline fluorescence. Based on these preliminary trials it seemed reasonable to use pooled urine for subsequent extractions and purification. The results are shown as mobilities of the spots detected by color and/or fluorescence on paper chromatograms and grouped as first,second,third and fourth extracts,according to the flow-sheet (Fig.4). I aimed to subdivide each extraction into four categories: males during summer (ms),males during winter (mw),females in summer (fs) and females in winter (fw). The summer months were considered May through October,the winter months

November through March. To make the results comparable with each other, all chromatographic mobilities are given as R_f , R_s and R_T values,on Whatman # 1 filter paper and developed in system B at 26-27°C for eight hours. This was the time necessary for the solvent front to reach the end of the paper (44 cm). The R_f,R_s and R_T values were calculated as follows:

$$R_f = \frac{\text{distance in cm travelled by sample from base line}}{\text{distance in cm from base line to solvent front}}$$

$$R_s = \frac{R_f \text{ of sample}}{R_f \text{ of steroid standard}}$$

$$R_T = R_s \times K \qquad \text{where } K = \frac{\frac{P+D}{2}}{H} = \text{cm/hour travelled by the}$$

steroid standard. P= distance in cm from base line to proximal end of standard spot; D= distance in cm from base line to distal end of standard spot; H= number of hours the chromatogram was developed. The standard steroid used for calculation of the above values,was compound F (cortisol).

The results for the various groups of fish,tabulated in Table No.2, represent steroid compounds detected in groups rather than individuals. Thus,the table includes all compounds detected,without taking into consideration if the urine was pooled from an individual fish or from different fish in the same group. Although this may imply that not all compounds were present in each individual fish,it seemed to be an adequate procedure for comparing groups.

As seen from Table No.2,the highest number of compounds was detected in the female-summer (fs) group,in the second extract. No spots could be detected in the third extract of urine from the female-summer and female-winter (fw) groups. The same held true for the fourth extract in

TABLE NO.2

URINARY STEROID METABOLITES OF UNTREATED FISH

Results are expressed as R_f, R_s and R_T values(see text). Urinary extracts were processed according to flow-sheet (Fig.4).Results represent all compounds detected in each group,i.e. 127 fish-days (6947 ml urine) for the ms group,157 fish-days (10165 ml urine) for the fs group,111 fish-days (3098 ml urine) for the mw group and 151 fish-days (4578 ml urine) for the fw group. All values are given for system B chromatography,developed on Whatman # 1 filter paper,at 26-27°C till the solvent front reached 44 cm. Water temperature was: 10°C in holding tank and 13°C in box for group ms and fs and 5°C in holding tank and box for group mw and fw. (Appendix: fish 1-54 and 58-106).

Abbreviations:

ms= male-summer group; fs= female-summer group; mw= male-winter group; fw= female-winter group; I,II,III,IV= extracts according to flow-sheet. UV= short ultraviolet; BT= blue tetrazolium reaction; NaOH= sodium hydroxide fluorescence reaction; PO_4H_3= phosphoric acid reaction; Phenol= color reaction for phenolic steroids; DNPH= 2,4-dinitrophenyl-hydrazine reaction; c= color; fl= fluorescence.

TABLE NO.2

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Groups and extracts in which the substance was detected.
UV	absorption	0.55	1.19	2.97	ms(I),fs(I)
BT	blue(c)	0.02	0.04	0.10	ms(II),fs(II)
BT	blue(c)	0.07	0.15	0.37	ms(II),fs(II)
BT	blue(c)	0.13	0.28	0.70	fs(II)
BT	blue(c)	0.15	0.32	0.80	fs(II)
BT	blue(c)	0.29	0.63	1.57	ms(II),fs(II)
BT	blue(c)	0.34	0.73	1.82	ms(II),mw(II), fs(II)
NaOH	yellow-green(fl)	0.07	0.15	0.37	ms(I),fs(I), ms(II),fs(II)
NaOH	green(fl)	0.13	0.28	0.70	fs(II)
NaOH	yellow(fl)	0.20	0.43	1.07	fs(I),mw(II)
NaOH	yellow(fl)	0.36	0.78	1.95	ms(II),mw(II), fs(II),fw(II)
NaOH	yellow(fl)	0.45	0.97	2.45	ms(IV),mw(IV)
NaOH	yellow(fl)	0.63	1.36	3.40	ms(IV),mw(IV)
NaOH	yellow(fl)	0.84	1.82	4.55	mw(III)
PO_4H_3	pink(c)	0.01	0.02	0.05	fs(II)
PO_4H_3	yellow-green(fl)	0.15	0.32	0.80	fs(II)
PO_4H_3	purple(c)-orange(fl)	0.17	0.36	0.90	fs(II)
PO_4H_3	purple(c)-white-yellow(fl)	0.30	0.65	1.62	ms(I),fs(I)

TABLE NO.2 (cont'd)

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Groups and extracts in which the substance was detected.
PO_4H_3	green(fl)	0.40	0.86	2.15	mw(II)
PO_4H_3	purple(c)	0.40	0.86	2.15	mw(I),fs(I)
PO_4H_3	white(fl)	0.48	1.04	2.60	fs(I)
PO_4H_3	white(fl)	0.53	1.15	2.87	ms(IV),mw(IV)
PO_4H_3	brown-red(c)-orange(fl)	0.55	1.19	2.97	ms(I),fs(I)
PO_4H_3	purple(c)-violet(fl)	0.63	1.36	3.40	mw(II),fs(II), fw(II)
PO_4H_3	green(fl)	0.63	1.36	3.40	ms(I),ms(IV), mw(IV)
PO_4H_3	green(fl)	0.68	1.47	3.67	fs(I)
PO_4H_3	pink(fl)	0.71	1.54	3.85	mw(IV)
PO_4H_3	olive(fl)	0.79	1.71	4.27	mw(II)
Phenol	blue(c)	0.40	0.86	2.15	fs(II)
Phenol	blue(c)	0.79	1.71	4.27	fs(II)
Phenol	blue(c)	0.95	2.06	5.15	fs(II)
DNPH	orange(c)	0.43	0.93	2.32	fs(I)
DNPH	orange(c)	0.50	1.08	2.70	fs(IV)
DNPH	yellow(c)	0.82	1.78	4.45	mw(IV)
DNPH	orange(c)	0.91	1.97	4.92	mw(I),ms(III), mw(III)

the female-winter group. Thus, during the summer months, the bulk of steroid metabolites in urine of female white suckers is present as glucuroniside uronidate. On the other hand, there is almost a complete lack of urinary steroid metabolites in females, during winter. Only two compounds were found in the second extract, while none were present in extracts I, III and IV. In contrast to this seasonal variation in females, the male group showed a much more uniform distribution of urinary steroid metabolites. There were only slight differences in the number of compounds detected in the four extracts and the same held true for the winter vs. the summer months.

Two important conclusions could be drawn from the results: first, there were no metabolites detected that could have been identified as being equal to any of the standard steroids used and second, there was only one spot (R_f 0.36) encountered with regularity in all four groups and within each group, by system B chromatography, giving positive alkaline fluorescence. This spot separated into two compounds after being rechromatographed in system C, giving R_f values of 0.07 and 0.17.

The chemical structure of these two compounds being unknown to me, I designated them as SP_1 and SP_2. Although the two compounds overlapped in system B, it was possible to distinguish them from each other by the fact that SP_1 gave a positive blue tetrazolium reaction at R_f 0.34, while SP_2 was BT negative. In Table No. 3 are shown the R_f, R_s and R_T values for SP_1 and SP_2 and steroid standards in system B, while Table No. 4 lists the same values in system C chromatography.

As it appeared that the two compounds (SP_1 and SP_2) were the only ones constantly present in all groups and individuals (as far as

TABLE NO.3

The R_f, R_s and R_T values of standard steroids and compounds SP_1 and SP_2, developed in system B (Whatman # 1, 26-27°C) till the solvent front reached 44 cm (8 hours). Abbreviations as in Table No.2. PMA= phospho-molybdic acid reaction; MDNB= meta-dinitrobenzene reaction.

Compound	Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T
SP_1	BT	blue(c)	0.34	0.73	1.82
SP_2	NaOH	yellow(fl)	0.36	0.78	1.95
Aldosterone	NaOH	yellow(fl)	0.42	0.91	2.27
Cortisol	BT	blue(c)	0.46	1.00	2.50
Cortisone	BT	blue(c)	0.59	1.28	3.20
Urocortisol	BT	blue(c)	0.64	1.39	3.47
Estriol	PO_4H_3	orange(fl)	0.67	1.45	3.62
Urocortisone	BT	blue(c)	0.68	1.47	3.67
Corticosterone	BT	blue(c)	0.70	1.52	3.80
Estradiol	PO_4H_3	green(fl)	0.83	1.80	4.50
Estrone	PO_4H_3	yellow(fl)	0.86	1.86	4.65
Androsterone	MDNB	purple(c)	0.89	1.93	4.82
Pregnanediol	PMA	blue(c)	0.91	1.97	4.92

TABLE NO.4

The R_f, R_s and R_T values of standard steroids and compounds SP_1 and SP_2, developed in system C (Whatman # 1, 29-30°C) till the solvent front reached 44 cm (4 hours). Abbreviations as in Table No.3.

Compound	Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T
SP_1	NaOH	yellow(fl)	0.07	0.25	0.75
Estriol	PO_4H_3	orange(fl)	0.12	0.44	1.32
SP_2	NaOH	yellow(fl)	0.17	0.62	1.86
Aldosterone	NaOH	yellow(fl)	0.25	0.92	2.76
Cortisol	NaOH	yellow(fl)	0.27	1.00	3.00
Urocortisol	BT	blue(c)	0.35	1.29	3.87
Cortisone	NaOH	yellow(fl)	0.43	1.59	4.77
Urocortisone	BT	blue(c)	0.45	1.66	4.98
Cortico-sterone	NaOH	yellow(fl)	0.70	2.59	7.77
Estradiol	PO_4H_3	green(fl)	0.79	2.92	8.76
Pregnanediol	PMA	blue(c)	0.87	3.22	9.66
Estrone	PO_4H_3	yellow(fl)	0.90	3.33	9.99
Androsterone	MDNB	purple(c)	0.93	3.44	10.32

tested),it seemed appropriate to investigate some physical characteristics of these compounds.

For the ultraviolet absorption spectra,the two unknown metabolites were extracted from pooled urine and purified by paper chromatography, using system D at 26-27°C as first,system B at 26-27°C as second and system C at 29-30°C as third chromatogram. From the last chromatogram the two zones,corresponding to SP_1 and SP_2,were cut out and eluted with 3 ml methanol each. One ml methanolic eluate of SP_1 and one ml of SP_2 were transferred to spectrophotometer cells. A reference standard, containing 10 µg/ml cortisol in methanol was introduced into a third cell. The ultraviolet spectra were recorded on a Hitachi-Perkin-Elmer spectrophotometer and read against a methanolic eluate of a paper blank. In Fig.5 are shown the ultraviolet spectra of SP_1,SP_2 and cortisol. It can be seen that while cortisol has one strong absorption peak at 242 nm,compounds SP_1 and SP_2 have only very low peaks at the same wavelength. They exhibit however,absorption peaks at 215 nm. Based on the fact that both unknown metabolites gave positive alkaline fluorescence,it had to be assumed that they possess the Δ^4-3-one structure in ring A of the steroid nucleus. Knowing that the basic steroid nucleus does not exhibit any ultraviolet absorption peak and that an isolated bond shows selective absorption in the ultraviolet between 160-206 nm (Ellington and Meakins,1960),it had to be concluded that compounds SP_1 and SP_2 could not have the same structure as the corticosteroids encountered usually in the urine of higher vertebrates,which all exhibit maximum absorption in the ultraviolet between 239-242 nm.

Although it was not possible to elucidate the structure of

compounds SP_1 and SP_2 in the present investigation,two possible explanations are forwarded to account for the discrepancy between the chemical reaction (alkaline fluorescence) and the ultraviolet spectra which seem to exclude each other. One hypothesis would imply that in the molecule there are present specific chemical groupings located on carbon atoms removed from the chromophore,which would account for the large hypsochromic effect (-25 nm) detected in the ultraviolet spectra of the two unknown compounds. This hypothesis would be supported by the chromatographic behaviour,suggesting the presence of more -OH or =O groups in SP_1 and SP_2 than in cortisol. A second hypothesis would assume that the two unknown steroid metabolites possess only a =O group at C-3,without the double bond at C-4. During the alkaline fluorescence reaction dehydration occurs and thus the Δ^4-3-one structure would appear. This assumption is supported by the compounds formed as intermediary steps in steroid synthesis (Johnson,Rogier and Ackerman, 1956 and Johnson,Bannister and Pappo,1956) which have a very similar structure to the one suggested here.

The determination of the absorption spectra in concentrated sulfuric acid was done as follows: an aliquot of one ml methanolic eluate,from each of the two unknown compounds,was transferred to spectrophotometer cells,the methanol was evaporated under a stream of nitrogen and concentrated sulfuric acid was added to the dry residue. The first reading was done (in the Hitachi-Perkin-Elmer 139 u.v.-visible spectrophotometer) after two hours at room temperature,against a paper blank. A solution of 10 µg/ml cortisol in methanol was used as reference standard. A second reading was taken after 24 hours from the time the concentrated sulfuric acid was added.The absorption spectra,

FIG.NO.5

ULTRAVIOLET ABSORPTION SPECTRA OF CORTISOL AND COMPOUNDS SP_1 AND SP_2

The spectra were recorded on a Hitachi-Perkin-Elmer spectrophotometer. The solvent was spectrograde methanol. Data represent mean values from three readings.

FIG.NO.6

ABSORPTION SPECTRA OF CORTISOL AND COMPOUNDS SP_1 AND SP_2 IN CONCENTRATED SULFURIC ACID

The spectra were recorded on a Hitachi-Perkin-Elmer spectrophotometer. The compounds were dissolved in 3 ml sulfuric acid each and left for two hours in the dark,at room temperature. After reading,compounds SP_1 and SP_2 were left for another 22 hours at room temperature,in the dark and then read again. Data represent mean values from three readings.

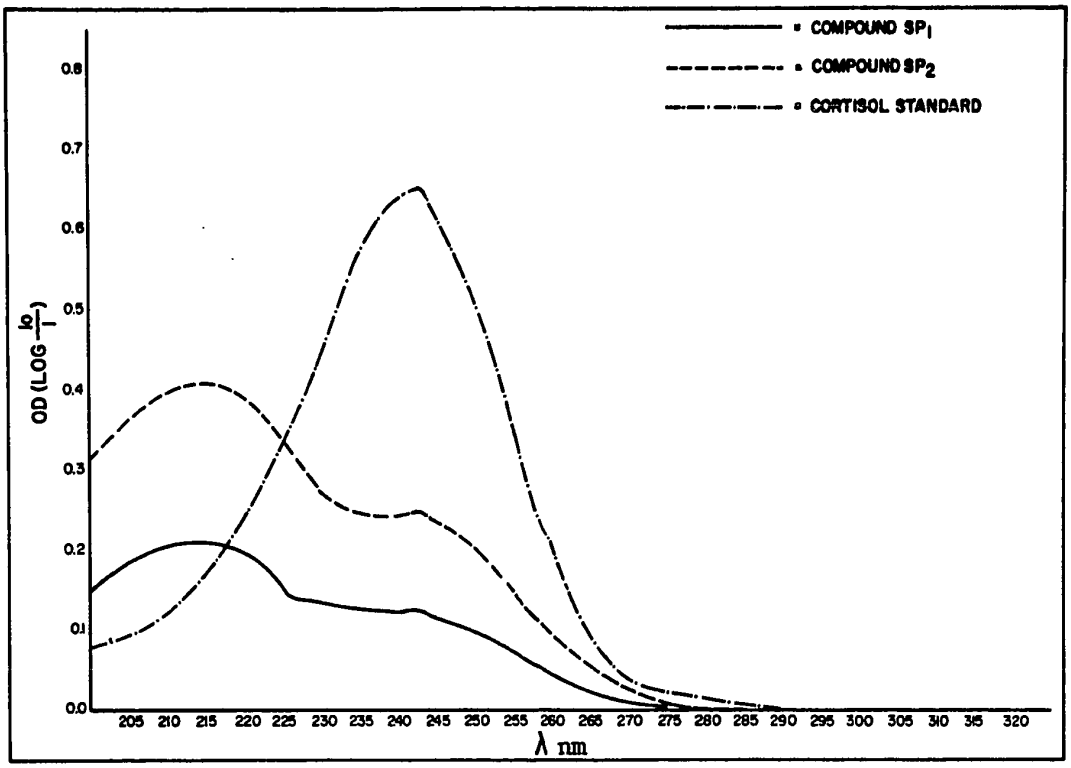

= COMPOUND SP1
= COMPOUND SP2
= CORTISOL STANDARD
OD (LOG Io/I)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320
λ nm

FIG. 5.

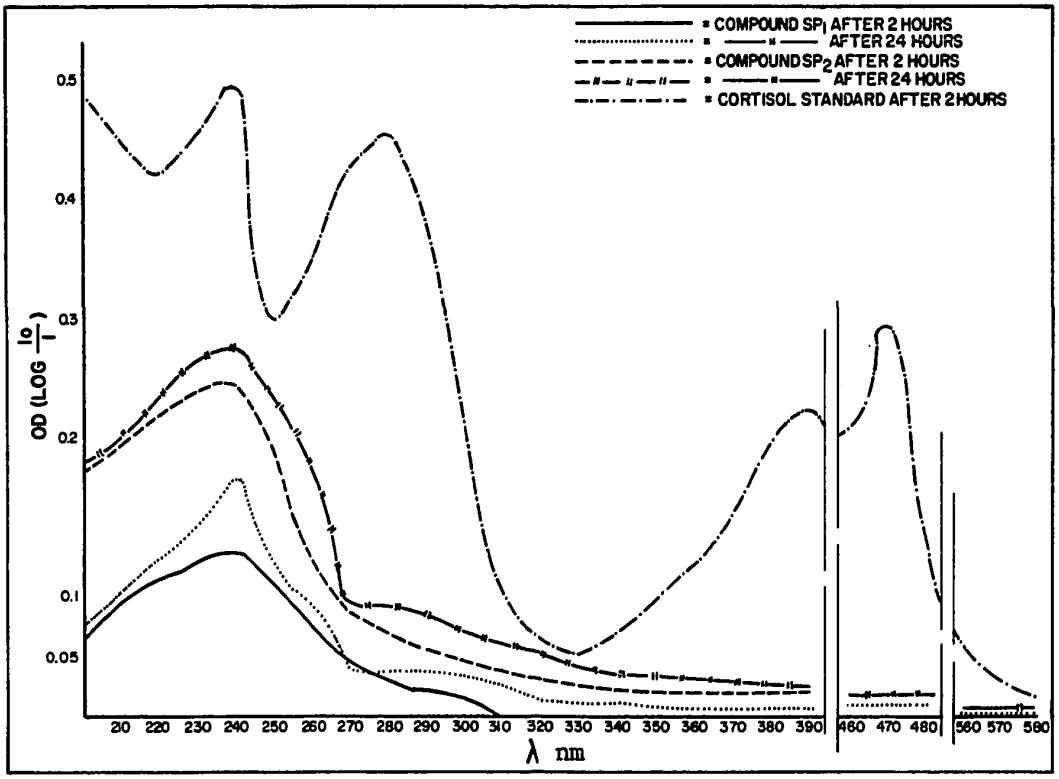

= COMPOUND SP1 AFTER 2 HOURS
= AFTER 24 HOURS
= COMPOUND SP2 AFTER 2 HOURS
= AFTER 24 HOURS
= CORTISOL STANDARD AFTER 2 HOURS
OD (LOG Io/I)
0.05
0.1
0.2
0.3
0.4
0.5
20 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 460 470 480 560 570 580
λ nm

FIG. 6.

shown in Fig.6,exhibited peaks at 240 nm (SP_1 and SP_2) and at 240,280, 390 and 470 nm (cortisol). The fact that in this case the highest intensity was seen at 240 nm for all three compounds,suggested that the hypothesis set forward earlier viz.,that the Δ^4-3-one structure was produced by dehydration during the alkaline fluorescence reaction,was quite possible,because concentrated sulfuric acid is acting too as a dehydrating agent. After 24 hours the spectra were much the same with a change in the height of the 240 nm peaks,suggesting continuous dehydration. Antonucci et al.(1953) and Dorfman (1953) made the observation that a C-11-ketone group produces a hypsochromic effect of 5-13 nm upon the absorption of the Δ^4-3-ketone in concentrated sulfuric acid,parallel to that observed in conventional ultraviolet spectroscopy. Therefore the conclusion was near that there have to be present more substituting chemical groupings than only a C-11-ketone,in compounds SP_1 and SP_2,to explain the disappearance of the less intense peaks at 280,390 and 470 nm which were present in cortisol.

For the elucidation of the fluorometric behaviour of compounds SP_1 and SP_2,one ml of each of the eluates,in parallel with an eluate from a paper blank and with the eluate of 5 µg cortisol,were processed as follows: the eluates were transferred to glass stoppered test tubes. The methanol was evaporated under nitrogen at 40°C,after removal of the stoppers. To the cooled,dry residue was added 1.25 ml of concentrated sulfuric acid for each test tube and the tubes were closed and shaken. After leaving the test tubes over night (20 hours) at room temperature, the sulfuric acid - steroid mixture was transferred to cuvettes and read in an Aminco-Bowman spectrophotofluorometer. The samples and the standard were submitted to various wavelengths of exciting and

TABLE NO.5

FLUORESCENCE SPECTRA OF CORTISOL AND COMPOUNDS SP_1 AND SP_2

The fluorescence intensities (% transmission) are given as uncorrected values, corresponding to one ml methanolic eluate. The dried eluates were treated with 1.25 ml concentrated sulfuric acid each and left for 20 hours in the dark at room temperature. The fluorescence was measured in an Aminco-Bowman spectrophotofluorometer. The amount of cortisol was 5 µg/ml.

Compound	Exciting wavelength in nm	Fluorescence wavelength in nm	Fluorescence intensity recorded as % transmission
sulfuric acid	280	530	2.50
paper blank	280	530	9.00
cortisol	280	530	29.00
SP_1	280	530	9.10
SP_2	280	530	14.00
sulfuric acid	280	540	2.60
paper blank	280	540	8.20
cortisol	280	540	20.34
SP_1	280	540	8.30
SP_2	280	540	11.24
sulfuric acid	325	410	3.00
paper blank	325	410	22.50
cortisol	325	410	49.34
SP_1	325	410	23.12
SP_2	325	410	37.00
sulfuric acid	320	410	2.60
paper blank	320	410	31.50
cortisol	320	410	72.33
SP_1	320	410	32.00
SP_2	320	410	51.20

TABLE NO.5 (cont'd)

Compound	Exciting wavelength in nm	Fluorescence wavelength in nm	Fluorescence intensity recorded as % transmission
sulfuric acid	360	530	2.70
paper blank	360	530	13.00
cortisol	360	530	64.00
SP_1	360	530	14.00
SP_2	360	530	21.60
sulfuric acid	360	540	2.30
paper blank	360	540	10.00
cortisol	360	540	63.00
SP_1	360	540	12.00
SP_2	360	540	18.32
sulfuric acid	435	540	2.30
paper blank	435	540	12.80
cortisol	435	540	45.00
SP_1	435	540	15.00
SP_2	435	540	28.00
sulfuric acid	480	500	3.00
paper blank	480	500	30.00
cortisol	480	500	>100.00
SP_1	480	500	37.20
SP_2	480	500	69.00
sulfuric acid	480	530	3.60
paper blank	480	530	25.00
cortisol	480	530	>100.00
SP_1	480	530	30.60
SP_2	480	530	57.00
sulfuric acid	480	540	3.00
paper blank	480	540	20.00
cortisol	480	540	>100.00
SP_1	480	540	24.00
SP_2	480	540	45.00

fluorescent light and the same was done with the paper blank. The results are recorded in Table No.5. The highest intensity of fluorescence was recorded at 480 nm exciting and 540 nm fluorescent light for cortisol,while the respective wavelengths were 480 nm and 500 nm for compounds SP_1 and SP_2.

According to the Stokes effect,the intensity of fluorescence is a function of the number of absorbed light quanta,irrespective of their wavelength,and the energy distribution of fluorescence,i.e. the fluorescence spectrum,is also independent of the excitation wavelength (Goldzieher,1963). In those instances where various excitation wavelengths produce different fluorescence spectra,it is likely that several molecular species are present. It has to be assumed,therefore, that the difference of 40 nm between the fluorescent peak in cortisol and that in compounds SP_1 and SP_2 is due to the difference in chemical structure.

The last test for checking the relationship between the two unknown compounds and the corticosteroids used as standards,was the recording of the mass spectra of SP_1,SP_2 and cortisol. The data are presented in tabular form,listing the mass number of the peaks and their relative intensities. The latter are usually expressed in relation to the most intense peak of the spectrum,often referred to as the "base peak" (Biemann,1962).

Comparing the mass spectra of cortisol,compound SP_1 and compound SP_2,as shown in Table No.6,it can be seen that there are 17 peaks,common for the three compounds,viz.,at m/e (mass-to-charge ratio) 28,29,31,32, 41,43,55,57,69,95,97,111,123,136,137 and 163. But the relative intensity of these peaks was differing from one compound to the other. Two peaks

TABLE NO.6

MASS SPECTRA OF CORTISOL AND COMPOUNDS SP_1 AND SP_2

Generally only those mass numbers are shown at which peaks were present. The relative height of the peaks is shown as follows:

- = no peak

± = very low

\+ = low

++ = medium

+++ = high

++++ = very high

"Base peaks" were mass numbers 123 and 163 of cortisol and SP_1.

TABLE NO.6

Mass No.	Cortisol	SP_1	SP_2
27	±	+	-
28	++	++	++
29	+	++	++
30	±	+	-
31	++	++	+++
32	±	±	+++
33	-	±	-
34	-	-	-
35	-	-	-
36	-	±	-
37	-	-	-
38	-	-	-
39	±	±	-
40	-	±	-
41	++	++	++
42	±	+	-
43	++	++	++
44	-	++	++
45	-	±	++
46	-	++	-
47	-	-	-
51	-	±	-
52	-	±	-
53	+	+	-
54	-	-	-
55	++	++	++
56	-	±	±
57	±	±	+++
58	-	++	-
59	-	±	-
60	±	±	+
61	-	±	±
62	-	-	-
65	-	±	-
66	-	-	-
67	±	±	-
68	-	-	-
69	±	±	++
70	-	±	+
71	-	±	++
72	-	±	-

TABLE NO.6 (cont'd)

Mass No.	Cortisol	SP_1	SP_2
77	+	±	-
78	±	+	-
79	++	++	-
80	-	+	-
81	±	±	-
82	-	-	-
83	-	±	+
84	-	±	±
85	-	±	++
86	-	±	-
91	++	++	-
92	-	-	-
93	±	±	-
94	-	±	-
95	±	±	±
96	-	±	-
97	±	±	+
98	-	±	-
99	-	±	-
105	++	-	-
106	±	+	-
107	±	++	-
108	±	±	-
109	±	-	-
110	±	±	-
111	±	±	±
112	-	+	-
119	±	±	-
120	-	-	-
121	±	++	-
122	±	±	-
123	++++	++++	+
124	++	++	-
125	±	±	±
126	-	-	+
127	-	±	±
128	-	-	-
131	±	±	-
132	±	-	-
133	±	±	-
134	±	±	-

TABLE NO.6 (cont'd)

Mass No.	Cortisol	SP_1	SP_2
135	+	-	±
136	±	±	±
137	±	±	±
138	-	++	±
139	-	-	±
140	-	-	±
141	-	-	±
145	±	±	-
146	-	-	-
147	±	±	-
148	±	-	-
149	+	-	±
150	-	-	-
151	±	-	±
152	±	-	-
153	±	-	±
154	±	-	±
155	-	-	±
161	±	±	-
162	±	-	-
163	++++	++++	+
164	-	-	-
165	-	-	±
166	-	-	-
167	-	-	-
168	-	-	±
169	-	-	±
189	±	±	-
227	++	+	-
242	±	±	-
268	-	±	-
286	-	±	-
301	++	+	-
331	++	-	-
332	-	-	-
333	-	±	-

(m/e 123 and 163) were of very high intensity in cortisol and SP_1 but of low intensity in SP_2; a high intensity peak was recorded at m/e 32 for SP_2 but was very low in cortisol and SP_1. The following peaks were common only for cortisol and SP_1: m/e 27,30,39,42,53,131,133,134,145,147, 161,189,227,242 and 301,while m/e 135,149,151,153 and 154 were common for cortisol and SP_2 only. Some peaks (m/e 44,45,56 and 138) were present in both SP_1 and SP_2 but lacking in the cortisol spectrum. From these findings it may be concluded that there is greater similarity between cortisol and SP_1 than between SP_1 and SP_2 or cortisol and SP_2. This conclusion was supported also by the fact that SP_1 gave positive blue tetrazolium reaction,indicating the presence of an α-ketol side chain at C-17,which is part also of the cortisol molecule, while SP_2 did not show this reaction and thus,probably lacks the α-ketol group. By analogy with the mass spectrum of cortisone (Fitches, 1962 and Budzikiewicz and Djerassi,1962) it appears that m/e 123 is the result of a fragmentation of ring B of the steroid nucleus,leaving ring A to dominate the consecutive step which leads to the most abundant molecular ion. This peak being present in the same intensity in cortisol as well as in SP_1 it may be assumed that the structure of the molecular ion is the same in both compounds but not in SP_2.

2. Detection of steroid metabolites in extrarenal fluid.

Under the collective name of extrarenal fluid were included all possible routes of excretion,other than the renal pathway,such as the gills (Smith,1929; Sexton and Meyer,1955 and Maetz and Romeu,1964), the fins (Mashiko and Jozuka,1964),the gut and others.

The extrarenal fluid was obtained by making use of a closed-system

set-up,as described in section II,2. Thus,there was a constant volume of water,with no fresh water inflow,cycling from the lucite box into the cooling coils and back into the box. In this way it was possible to collect the extrarenal fluid,mixed with the cycling water,the urine being collected separately through the urinary catheter.

Under closed-system conditions the fish survived only a few days,although the water was well aerated. Looking for an explanation of this fact,water samples were taken 24 hours after the system was closed,but without fish in it,and compared from the point of view of the chemical composition with samples taken 24 hours after a fish was placed in the closed-system.

The results of the chemical analyses showed important differences between the two series of samples (Table No.7). The chemical composition differed greatly,mainly in the content of chloride,copper, iron,phosphate,ammonia and calcium carbonate,which increased significantly after the water was in contact with the fish for 24 hours, and also in the content of fluoride,nitrites and the hydrogen ion concentration which decreased under the same conditions. Other changes consisted in an increase of chromium,manganese,sulfate,calcium,magnesium and total nitrogen as well as an increase in total alkalinity and total hardness. Although it is obvious that the increase of ammonia was a major factor,the other compounds,especially the metal salts,were of no lesser importance (Carpenter,1927 and Doudoroff and Katz,1953) in determining the unfavorable conditions resulting in the death of fish.

Only water,with extrarenal fluid,from fish kept at $5^{o}C$ was used for detection of steroid metabolites. The water was changed every four, six or nine hours and was kept in the freezer at $-20^{o}C$ till processed.

TABLE NO.7

CHEMICAL ANALYSIS OF WATER

Three samples of water,after recycling for 24 hours,from "closed-system" boxes,were taken before placing fish in them and three samples (three different fish) of water were collected after the fish were kept in the water for 24 hour periods. The data are mean values,the range being ±0.02.

Substance or parameter	Closed-system, recycling water, without fish.	Closed-system, recycling water, after keeping in it fish for 24 hours.
CHLORIDE	9.08 ppm	40.86 ppm
CHROMIUM	0.015 ppm	0.12 ppm
COPPER	0.04 ppm	0.83 ppm
IRON	0.03 ppm	0.43 ppm
FLUORIDE	1.58 ppm	0.15 ppm
MANGANESE	0.15 ppm	0.30 ppm
SILICON	4.50 ppm	4.00 ppm
SULFATE	42.00 ppm	58.00 ppm
ORTHOPHOSPHATE	0.10 ppm	0.35 ppm
METAPHOSPHATE	0.05 ppm	1.15 ppm
TOTAL PHOSPHATE	0.15 ppm	1.50 ppm
NITRITES	0.163 ppm	0.037 ppm
AMMONIA	0.00 ppm	>5.00 ppm
TOTAL NITROGEN	0.00 ppm	0.15 ppm

TABLE NO.7 (cont'd)

Substance or parameter		Closed-system, recycling water, without fish.	Closed-system, recycling water, after keeping in it fish for 24 hours.
CALCIUM		43.00 ppm	53.00 ppm
MAGNESIUM		12.00 ppm	20.00 ppm
TOTAL HARDNESS		55.00 ppm	73.00 ppm
CALCIUM CARBONATE		0.00 ppm	3.50 ppm
TOTAL ALKALINITY		32.00 ppm	180.00 ppm
pH		8.8	7.0
TURBIDITY		0	55
TRANSMITTANCE	filtered	100 %	91 %
	shaken	100 %	86 %
COLOR		0	135

The water,with extrarenal fluid,was processed,in the same way as urine, for the extraction ,purification and detection of steroid metabolites. The main purpose was to establish the qualitative presence or absence of any steroid-like compounds in the extrarenal fluid. Table No.8 lists the R_f,R_s and R_T values of the metabolites detected in the four extracts from water-extrarenal-fluid pooled from 15 fish-days. The results show the presence of only few steroid metabolites in the extrarenal fluid. Four compounds were detected as free steroids,two were glucuroniside uronidates,while three compounds were detected after acid hydrolysis. The third extract did not yield any compounds.All the detected compounds differed from the steroid metabolites found in urine of untreated suckers and neither of them could be identified with the reference standards used. Thus,it appeared that although some steroid-like substances were excreted by pararenal routes,they were different from those excreted by the renal pathway.

3. Urinary steroids detected after administration of ACTH.

The investigations of Rasquin (1951),Rasquin and Hafter (1951 a,b) and Rasquin and Atz (1952) showed that the interrenal tissue of normal or hypophysectomized teleost fish (Astyanax)could be stimulated with mammalian ACTH. The response resembled that elicited by fish pituitary preparations. These investigations were confirmed by Chavin (1954,1956) in the goldfish,Carassius auratus.Fontaine and Hatey (1953) studied the response of the interrenal of teleost fish to ACTH stimulation in relation to temperature. At 16°C, the atrophied interrenal of the hypophysectomized eel was restored to normal weight one hour after injection of mammalian ACTH. But the same treatment had

TABLE NO.8

STEROID METABOLITES DETECTED IN EXTRARENAL FLUID

The R_f, R_s and R_T values of steroid metabolites detected in extrarenal fluid from 15 fish-days. Temperature of water was 5°C. Water was changed every four, six or nine hours and stored in freezer at -20°C. (Appendix: 62 - 3 days, 86 - 4 days, 96 - 2 days, B 3 - first four days, B 5 - 2 days). Abbreviations as in Table No.2.

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extracts in which the compound was detected
NaOH	green(fl)	0.07	0.15	0.37	I
NaOH	yellow(fl)	0.11	0.23	0.57	II
NaOH	green(fl)	0.15	0.32	0.80	II
NaOH	yellow(fl)	0.50	1.08	2.70	IV
Phenol	blue(c)	0.48	1.04	2.60	I
DNPH	orange(c)	0.52	1.13	2.82	IV
DNPH	orange(c)	0.63	1.36	3.40	I
DNPH	yellow(c)	0.64	1.39	3.47	IV
DNPH	yellow(c)	0.65	1.41	3.52	I

no effect at 6°C.

Based on the reports cited above,it seemed reasonable to try to stimulate production of steroids in vivo by administration of mammalian ACTH. For this purpose,suckers of both sexes were injected each with 15 i.u./Kg body weight hog ACTH,every second day,for periods up to eight days,or they were infused with 5 i.u./Kg body weight/24 hours of the same preparation,up to 10 days. Urine was collected and pooled over the entire holding period and extracted. The dry residues from the four extractions were stored in a freezer at -20°C till the time of chromatographic purification. Extractions and chromatographic purification were done as described for urine from untreated fish (see section II,4). Table No.9 lists the R_f,R_s and R_T values of urinary steroid metabolites detected after ACTH treatment of fish kept at 13°C.

Comparing Table No.2 with Table No.9, it may be observed that there are steroid-like compounds which,although not detected in urine of untreated fish,appeared after ACTH stimulation. Such compounds are e.g. R_f 0.11, 0.24, 0.39 and 0.42, all being detected as glucuroniside uronidates giving positive alkaline fluorescence. R_f 0.21 and 0.26, also glucuroniside uronidates,gave positive fluorescence reactions with phosphoric acid. On the other hand,some compounds present in untreated fish in one or two extracts were now detected in three or even all four extracts. For example R_f 0.07,detected in untreated fish as free steroid and as glucuroniside uronidate,was present,after ACTH treatment,in all four extracts. Furthermore there were double the number of DNPH positive compounds after ACTH stimulation than without treatment. Thus,ACTH administration was followed by the appearance of some urinary steroid metabolites not detected in untreated fish.

TABLE NO.9

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH ACTH

The R_f, R_s and R_T values of steroid metabolites detected in urine of fish injected or infused for periods up to ten days with ACTH.(For treatment of fish and processing of urine see text). Values are given for system B chromatography, developed on Whatman # 1 filter paper, at 26-27°C, till the solvent front reached 44 cm. Abbreviations as in Table No.2. M = male group; F = female group.

(Appendix: A 1,A 2,B 6,B 7,E 6,E 8,E 9,F 2,F 6,G 10,H 2,H 3).

TABLE NO.9

Method of detection	color(c) or fluorescence	R_f	R_s	R_T	Groups and extracts in which the compound was detected
UV	absorption	0.42	0.91	2.27	F(II)
BT	blue(c)	0.07	0.15	0.37	F(II,III,IV) M(II,III,IV)
BT	blue(c)	0.15	0.32	0.80	F(II)
BT	blue(c)	0.34	0.73	1.82	F(II),M(II)
BT	blue(c)	0.59	1.28	3.20	F(IV),M(IV)
BT	blue(c)	0.93	2.02	5.05	M(III)
NaOH	yellow-green(fl)	0.07	0.15	0.37	F(I,II,III,IV), M(I,II,III,IV)
NaOH	yellow-green(fl)	0.11	0.23	0.57	F(II)
NaOH	yellow(fl)	0.24	0.52	1.30	F(II),M(II)
NaOH	yellow(fl)	0.36	0.78	1.95	F(II),M(II)
NaOH	yellow(fl)	0.39	0.84	2.10	M(II)
NaOH	yellow(fl)	0.42	0.91	2.27	F(II),M(II)
NaOH	yellow(fl)	0.45	0.97	2.42	M(IV)
NaOH	yellow(fl)	0.63	1.36	3.40	M(IV)
NaOH	yellow(fl)	0.72	1.56	3.90	M(IV)
PO_4H_3	yellow-green(fl)	0.15	0.32	0.80	F(II)
PO_4H_3	purple(c)-brick-red(fl)	0.21	0.45	1.12	F(II),M(II)
PO_4H_3	violet(fl)	0.26	0.56	1.40	F(II)
PO_4H_3	purple(c)	0.40	0.86	2.15	M(I)
PO_4H_3	green(fl)	0.40	0.86	2.15	M(II)

TABLE NO.9 (cont'd)

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Groups and extracts in which the compound was detected
PO_4H_3	white(fl)	0.53	1.15	2.87	M(IV)
PO_4H_3	orange(fl)	0.55	1.19	2.97	M(I)
PO_4H_3	pink-purple(c)	0.61	1.32	3.30	F(II)
PO_4H_3	violet(fl)	0.63	1.36	3.40	F(II)
PO_4H_3	green(fl)	0.63	1.36	3.40	M(IV)
PO_4H_3	green(fl)	0.68	1.47	3.67	F(I)
Phenol	blue(c)	0.79	1.71	4.27	F(II)
DNPH	orange(c)	0.50	1.08	2.70	F(IV)
DNPH	orange(c)	0.60	1.30	3.25	F(I),M(I)
DNPH	orange(c)	0.72	1.56	3.90	F(I),M(I)
DNPH	yellow(c)	0.82	1.78	4.45	F(IV)
DNPH	yellow(c)	0.85	1.84	4.60	M(IV)
DNPH	yellow(c)	0.88	1.91	4.77	F(I),M(I)
DNPH	yellow(c)	0.90	1.95	4.87	F(III)
DNPH	orange(c)	0.91	1.97	4.92	M(I,III)

The mechanism of action of ACTH in suckers however,remains open for discussion. One possibility is,of course,that ACTH stimulates the interrenal tissue to produce steroids. But the results obtained in the present investigation do not necessarily point that way. It is equally possible that ACTH affects only the excretory pathway,causing the appearance of new urinary steroid catabolites by shifting their excretion from a pararenal to the renal route. Another possibility would be that ACTH is opening new catabolic pathways,inducing the appearance of different catabolites from the same parent compounds, without affecting the secretory tissue per se.

4. Urinary metabolites detected after administration of steroids.

Comparing the urinary metabolites,detected after treatment with various steroid compounds,with those found in urine of untreated fish (see Table No.2),three categories of metabolites could be established: (a) steroid compounds detected in the urine of both untreated and steroid-treated fish, (b) compounds found only in urine of untreated animals but missing from the urine of treated fish and (c) compounds found in urine of treated fish only.

The urinary catabolites detected in female suckers,treated with estradiol-17 β,as described in section II,6, are listed in Table No.10. In the first extract could be detected nine compounds,as free steroids, which were not found in untreated fish. One of these, R_f 0.46 was identified as cortisol,based on chromatographic mobility,reactions with blue tetrazolium,phosphoric acid and sodium hydroxide and ultraviolet absorption (λ_{max}= 242 nm). After Ketodase hydrolysis ten new spots were detected. Compounds SP_1 and SP_2 were present as in

TABLE NO.10

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH ESTRADIOL-17β

The R_f,R_s and R_T values of urinary steroid metabolites detected in urine of fish injected with 1 mg estradiol-17β. The extracts were processed according to the flow-sheet (Fig.4) and the values are given for system B chromatography,developed on Whatman # 1 paper,at 26-27°C till the solvent front reached 44 cm. Abbreviations as in Table No.2.

E= extract processed according to Finkelstein (1952).

(Appendix: A 9,B 8,C 9,I 10,L 1).

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extracts in which the compound was detected
BT	blue(c)	0.18	0.39	0.97	II
BT	blue(c)	0.46	1.00	2.50	I
BT	blue(c)	0.92	2.00	5.00	II
BT	blue(c)	0.93	2.02	5.05	IV
BT	blue(c)	0.96	2.08	5.20	II
NaOH	yellow-white(fl)	0.00	base line		II
NaOH	yellow-green(fl)	0.11	0.23	0.57	E,I,II
NaOH	yellow(fl)	0.36	0.78	1.95	II
NaOH	yellow(fl)	0.46	1.00	2.50	I
PO_4H_3	pink(c)	0.01	0.02	0.05	II
PO_4H_3	purple(c) orange(fl)	0.24	0.52	1.30	II
PO_4H_3	olive(fl)	0.29	0.63	1.57	E,I

TABLE NO.10 (cont'd)

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extracts in which the compound was detected
PO_4H_3	violet(fl)	0.41	0.89	2.22	II
PO_4H_3	brown(fl)	0.44	0.95	2.37	E,I
PO_4H_3	green(fl)	0.46	1.00	2.50	I
PO_4H_3	olive(fl)	0.52	1.13	2.82	II
PO_4H_3	dark blue(fl)	0.59	1.28	3.20	II
PO_4H_3	light blue(fl)	0.74	1.60	4.00	II
PO_4H_3	blue-white(fl)	0.91	1.97	4.92	E,I
DNPH	orange(c)	0.00	base line		I
DNPH	orange(c)	0.51	1.10	2.75	I
DNPH	orange(c)	0.65	1.41	3.52	E,I
DNPH	orange(c)	0.74	1.60	4.00	E,I

untreated fish.On the other hand,estradiol inhibited the appearance of a series of metabolites present in urine of untreated animals.

In Table No.11 are listed urinary metabolites detected in female fish after treatment with progesterone. In the first extract nine new compounds could be visualized. One of these, R_f 0.24, showed positive blue tetrazolium reaction,gave fluorescence with phosphoric acid and sodium hydroxide and,thus,was suggestive of a hydroxilated metabolite of cortisol. In the second extract 17 new compounds were detected. One of the newly detected spots, R_f 0.68 had characteristics which suggested its identity with urocortisone (THE). Significant was the appearance of compounds giving positive reaction with phosphomolybdic acid,i.e. 3-hydroxy-steroids. This demonstrated the ability of female suckers to metabolize progesterone. The third extract yielded five spots which were not found in untreated fish. However,a great number of compounds,present in urine of untreated fish,were missing after progesterone treatment. Among the compounds missing in the treated fish were also SP_1 and SP_2.

The urinary steroid metabolites detected in male fish after administration of testosterone are listed in Table No.12. In the first and third extracts two new compounds could be detected of which one appeared to be a direct catabolite of testosterone,showing positive reactions with MDNB and DNPH and a mobility (R_f 0.85) somewhat more polar than androsterone. In the second extract only this same compound, R_f 0.85, could be seen. Four new spots were detected in the fourth extract,one being R_f 0.85. Most of the metabolites present in urine of untreated fish were missing. I concluded,therefore,that testosterone has a similar effect in suckers as in higher vertebrates,i.e.

TABLE NO.11

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH PROGESTERONE

The R_f, R_s and R_T values of urinary steroid metabolites detected in urine of fish injected with 1 mg progesterone. The extracts were processed according to the flow-sheet (Fig.4) and values are given for system B chromatography, developed on Whatman # 1 paper, at 26-27°C, till the solvent front reached 44 cm. Abbreviations as in Table No.2.

Sulfacetic = reaction with sulfuric acid - acetic acid reagent.

* = color appeared before heating. (Appendix: A 7, A 8, D 1, D 2, M 5).

TABLE NO.11

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH PROGESTERONE

The R_f, R_s and R_T values of urinary steroid metabolites detected in urine of fish injected with 1 mg progesterone. The extracts were processed according to the flow-sheet (Fig.4) and values are given for system B chromatography, developed on Whatman # 1 paper, at 26-27°C, till the solvent front reached 44 cm. Abbreviations as in Table No.2.

Sulfacetic = reaction with sulfuric acid - acetic acid reagent.

* = color appeared before heating. (Appendix: A 7, A 8, D 1, D 2, M 5).

TABLE NO.11

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extracts in which the compound was detected
BT	blue(c)	0.01	0.02	0.05	II
BT	blue(c)	0.02	0.04	0.10	I,III
BT	blue(c)	0.11	0.23	0.57	I,II
BT	blue(c)	0.24	0.52	1.30	I
BT	blue(c)	0.25	0.54	1.35	II
BT	blue(c)	0.48	1.04	2.60	I
BT	blue(c)	0.68	1.47	3.67	II
BT	blue(c)	0.90	1.95	4.87	II,III
NaOH	white(fl)	0.01	0.02	0.05	II
NaOH	yellow-green(fl)	0.07	0.15	0.37	I,III
NaOH	yellow-green(fl)	0.11	0.23	0.57	III
NaOH	yellow(fl)	0.16	0.34	0.85	II
NaOH	yellow(fl)	0.24	0.52	1.30	I
PO_4H_3	pink(c)	0.01	0.02	0.05	II
PO_4H_3	purple(c)	0.12	0.26	0.65	II
PO_4H_3	pink(fl)	0.14	0.30	0.75	II
PO_4H_3	yellow-green(fl)	0.24	0.52	1.30	I
PO_4H_3	purple(c)	0.39	0.84	2.10	II
PO_4H_3	orange-brown(c)	0.43	0.93	2.32	I
PO_4H_3	violet(fl)	0.53	1.15	2.87	II
PO_4H_3	brown(c)	0.61	1.32	3.30	II

TABLE NO.11 (cont'd)

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extracts in which the compound was detected
PO_4H_3	brown(c)	0.80	1.73	4.32	II
PMA	blue(c)	0.31	0.67	1.67	II
PMA	blue(c)	0.57	1.23	3.07	II
PMA	blue(c)	0.61	1.32	3.30	I
PMA	blue(c)	0.71	1.54	3.85	II
PMA	blue(c)	0.86	1.86	4.65	II
PMA	blue(c)	0.91	1.97	4.92	III
PMA	brown-red(c)	0.91	1.97	4.92	II
DNPH	orange(c)	0.00	base line		II
DNPH	orange(c)	0.35	0.76	1.90	I
DNPH	orange(c)	0.43	0.93	2.32	I
DNPH	orange(c)	0.51	1.10	2.75	I
DNPH	yellow(c)	0.77	1.67	4.17	I
DNPH	yellow(c)	0.90	1.95	4.87	III
Sulfacetic	cherry-red(c)*	0.01	0.02	0.05	II
Sulfacetic	green(fl)	0.02	0.04	0.10	I
Sulfacetic	orange(fl)	0.13	0.28	0.70	II
Sulfacetic	purple(c),pink(fl)	0.14	0.30	0.75	II
Sulfacetic	pink(c),violet(fl)	0.42	0.91	2.27	II
Sulfacetic	yellow-brown(c)	0.43	0.93	2.32	I
Sulfacetic	blue(fl)	0.50	1.08	2.70	II
Sulfacetic	pink(c)*	0.70	1.52	3.80	II

TABLE NO.12

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH TESTOSTERONE

The R_f, R_s and R_T values of steroid metabolites detected in urine of fish injected with 1 mg testosterone. The extracts were processed according to the flow-sheet (Fig.4). The values are given for system B chromatography, developed on Whatman # 1 paper, at 26-27°C till the solvent front reached 44 cm. Abbreviations as in Table No.2. (Appendix: A 4, A 5, C 5, C 7, C 8).

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extract in which the compound was detected
BT	blue(c)	0.02	0.04	0.10	I,III
BT	blue(c)	0.39	0.84	2.10	IV
NaOH	yellow(fl)	0.48	1.04	2.60	IV
NaOH	yellow(fl)	0.70	1.52	3.80	IV
DNPH	orange(c)	0.02	0.04	0.10	I
DNPH	orange(c)	0.85	1.84	4.60	I,II,III,IV
MDNB	brown-violet(c)	0.85	1.84	4.60	I,II,III,IV

suppression of steroidogenesis (Robson,1936,1937 and 1938 a, b). I also concluded that R_f 0.85 is a catabolite of testosterone.

In Table No.13 are listed the compounds detected in urine of suckers treated with cortisol. The results showed no differences between the sexes,which led to the conclusion that this corticosteroid was an inhibitor of the sex-specific steroids,either by way of direct antagonism or via the pituitary. The first extract yielded two very polar spots. All compounds present in urine of untreated fish were missing. In the second extract four new compounds were detected. Three of these were more polar than cortisol. Most of the metabolites seen in urine of untreated fish were missing,including compounds SP_1 and SP_2. In the third extract one new compound was detected,while in the fourth extract no spots were found. It seems,therefore,that cortisol is acting as a potent suppressor of urinary steroid metabolites and,possibly,even of the parent steroids themselves.

As in the case of cortisol,there were found no differences between the urinary steroid metabolites of male and female suckers, after administration of corticosterone (Table No.14). In the first extract four new compounds were seen. In the second extract six new spots were detected. Of these,four were more polar than cortisol. Only two compounds were common for untreated and treated fish alike, while all the others were missing in urine of corticosterone treated fish,including compounds SP_1 and SP_2. In the third extract three new spots were seen. Thus,it appeared that corticosterone,although suppressing most of the steroid metabolites present in urine of untreated fish,is not as strong an inhibiting agent as testosterone or cortisol.

TABLE NO.13

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH CORTISOL

The R_f,R_s and R_T values of steroid metabolites detected in urine of three females and four males injected with 1 mg cortisol each. The results were the same for both sexes. The extracts were processed according to the flow-sheet (Fig.4). The values are given for system B chromatography,developed on Whatman # 1 paper,at 26-27°C, till the solvent front reached 44 cm. Abbreviations as in Table No.2. *=before heating. (Appendix: D 3,E 4,F 1,F 4,H 1,L 2,P 2).

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extract in which the compound was detected
BT	blue(c)	0.01	0.02	0.05	III
BT	blue(c)	0.02	0.04	0.10	II
BT	blue(c)	0.15	0.32	0.80	I
BT	blue(c)	0.93	2.02	5.05	III
NaOH	yellow(fl)	0.01	0.02	0.05	II
NaOH	green(fl)	0.27	0.58	1.45	II
PO_4H_3	pink(c)*	0.01	0.02	0.05	II
PO_4H_3	purple(c), purple(fl)	0.54	1.17	2.92	II
DNPH	orange(c)	0.00	base line		I,II

TABLE NO.14

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH CORTICOSTERONE

The R_f,R_s and R_T values of steroid metabolites detected in urine of three males and three females,injected with 1 mg corticosterone each. The results were the same for both sexes. The extracts were processed according to the flow-sheet (Fig.4) and the values are given for system B chromatography,developed on Whatman # 1 paper,at 26-27^{o}C,till the solvent front reached 44 cm. Abbreviations as in Table No.11. (Appendix: C 6,D 5,D 6,D 8,E 7,F 3).

TABLE NO.14

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extract in which the compound was detected
BT	blue(c)	0.01	0.02	0.05	II
BT	blue(c)	0.02	0.04	0.10	III
BT	blue(c)	0.84	1.82	4.55	II
BT	blue(c)	0.93	2.02	5.05	III
NaOH	yellow-green(fl)	0.05	0.15	0.35	I,II,III
NaOH	yellow-green(fl)	0.11	0.23	0.57	III
NaOH	yellow(fl)	0.17	0.37	0.90	II
NaOH	yellow(fl)	0.24	0.52	1.30	I
Sulfacetic	cherry-red(c)	0.01	0.02	0.05	II
Sulfacetic	purple(c),red(fl)	0.15	0.32	0.80	II
Sulfacetic	purple(c), violet(fl)	0.48	1.84	2.60	II
DNPH	orange(c)	0.00	base line		I,II
DNPH	yellow(c)	0.17	0.36	0.90	II
DNPH	orange(c)	0.37	0.80	2.00	I
DNPH	orange(c)	0.43	0.93	2.32	I
DNPH	yellow(c)	0.68	1.47	3.64	I
DNPH	orange(c)	0.91	1.97	4.92	III

The urinary steroid catabolites detected after treatment with cortisone are listed in Table No.15. Again no differences were observed between the sexes. Nine compounds present in urine of untreated fish were missing in the first extract after cortisone treatment. However, eight compounds were detected which had not been seen in urine of untreated suckers and two of these had the R_f values of 0.34 and 0.36,i.e. compounds SP_1 and SP_2. This I considered to be an important finding, because in untreated fish these two compounds were never detected as free steroids. In the second extract,compounds SP_1 and SP_2 were present both in the untreated and treated animals. In this extract it was possible to detect six new spots,while fourteen compounds,present in the untreated fish,were absent. In the third extract compounds SP_1 and SP_2 could again be detected. Thus,the main characteristic of the urinary steroid metabolites,detected after cortisone administration, was the appearance of compounds SP_1 and SP_2,not only as glucuroniside uronidates,as was the case in untreated fish,but also as free steroids and after solvolysis in the third extract.

As a more general conclusion it might be said that the treatment of white suckers with steroid hormones resulted in changes in the quality and quantity of urinary steroid metabolites. The changes differed with the specific steroid injected. It has to be emphasized that,although all three corticosteroids used had an inhibitory effect upon the number of metabolites present compared to untreated fish,there was one significant difference between cortisol and corticosterone on one hand and cortisone on the other,in that cortisol and corticosterone suppressed completely the excretion of compounds SP_1 and SP_2,cortisone,on the contrary,induced the appearance of these two compounds as free steroids and as sulfate ester conjugates,besides the glucuroniside uronidate

TABLE NO.15

URINARY STEROID METABOLITES DETECTED AFTER TREATMENT WITH CORTISONE

The R_f,R_s and R_T values of steroid metabolites,detected in urine of four males and four females,injected with 1 mg cortisone each. The results were the same for both sexes. The extracts were processed according to the flow-sheet (Fig.4). Values are given for system B chromatography,developed on Whatman # 1 paper,at 26-27°C,till the solvent front reached 44 cm. Abbreviations as in Table No.11. (Appendix: D 4,F 7,F 9,F 10,G 9,I 9,M 1,N 8).

TABLE NO.15

Method of detection	color(c) or fluorescence(fl)	R_f	R_s	R_T	Extract in which the compound was detected
BT	blue(c)	0.02	0.04	0.10	II,III
BT	blue(c)	0.14	0.30	0.75	I,II
BT	blue(c)	0.34	0.73	1.82	I,II
BT	blue(c)	0.88	1.90	4.75	II
BT	blue(c)	0.93	2.02	5.05	III
NaOH	yellow-white(fl)	0.00	base line		II
NaOH	yellow-green(fl)	0.11	0.23	0.57	I,II
NaOH	yellow(fl)	0.36	0.78	1.95	I,II,III
Sulfacetic	cherry-red(c)	0.01	0.02	0.05	II
DNPH	golden-yellow(c)	0.00	base line		I,II
DNPH	yellow(c)	0.36	0.78	1.95	II
DNPH	orange(c)	0.46	1.00	2.50	I
DNPH	yellow(c)	0.54	1.17	2.92	I
DNPH	yellow(c)	0.64	1.38	3.45	I

form,as they were detected in the urine of untreated fish.

Regarding the group of "sex" steroids,it can be said that while testosterone is mainly a strong inhibitor of urinary steroid metabolites, estradiol was stimulating the formation of a number of new metabolic compounds,similar to the number of compounds it suppressed. Progesterone, on the other hand seems to act quite contrary to testosterone by stimulating the appearance of a greater number of new steroid metabolites than the number it suppressed. Nevertheless,progesterone too suppressed the excretion of compounds SP_1 and SP_2,as did testosterone, corticosterone and cortisol.

5. Experiments with labeled steroids.

A. Detection of urinary metabolites.

The technique used for administration of labeled steroid compounds and to detect radioactive zones on chromatograms was described in part II,6.

Female suckers were injected with one mg estradiol-17 β and 100 μc estradiol-17 β- ^3H and the pooled urine was submitted to extraction and purification. The results of the detected radioactivity in the four extracts are given in Figs.7,8,9 and 10.

In the first extract the only noteworthy activity was at the baseline which gave a DNPH positive reaction,indicating the presence of a ketosteroid. In the second extract (Fig.8) the 0-2 cm zone of the chromatogram,including the baseline and R_f 0.01 and 0.02,gave fluorescence reactions with phosphoric acid and sodium hydroxide and a color reaction with phosphoric acid. The activity was much higher

FIG.NO.7

URINARY STEROID METABOLITES DETECTED AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

Four female suckers were injected with 100 μc estradiol-17β-^{3}H plus 1 mg estradiol carrier each and the urine was pooled and extracted. The results are based on R_f values for system B chromatography, developed on Whatman # 1 paper, at 26-27°C. (Appendix: I 10, L 1, L 9, N 4).

First extract

Abbreviations: B.L.= base line; S.F.= solvent front; Ald.=aldosterone; F = cortisol; E = cortisone; B = corticosterone; THF = urocortisone; THE = urocortisol; E_3= estriol; E_2= estradiol; E_1= estrone; Andr.=androsterone; Pdiol.= pregnanediol.

FIG.NO.8

URINARY STEROID METABOLITES DETECTED AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

Second extract

Explanations and abbreviations as in Fig.No.7.

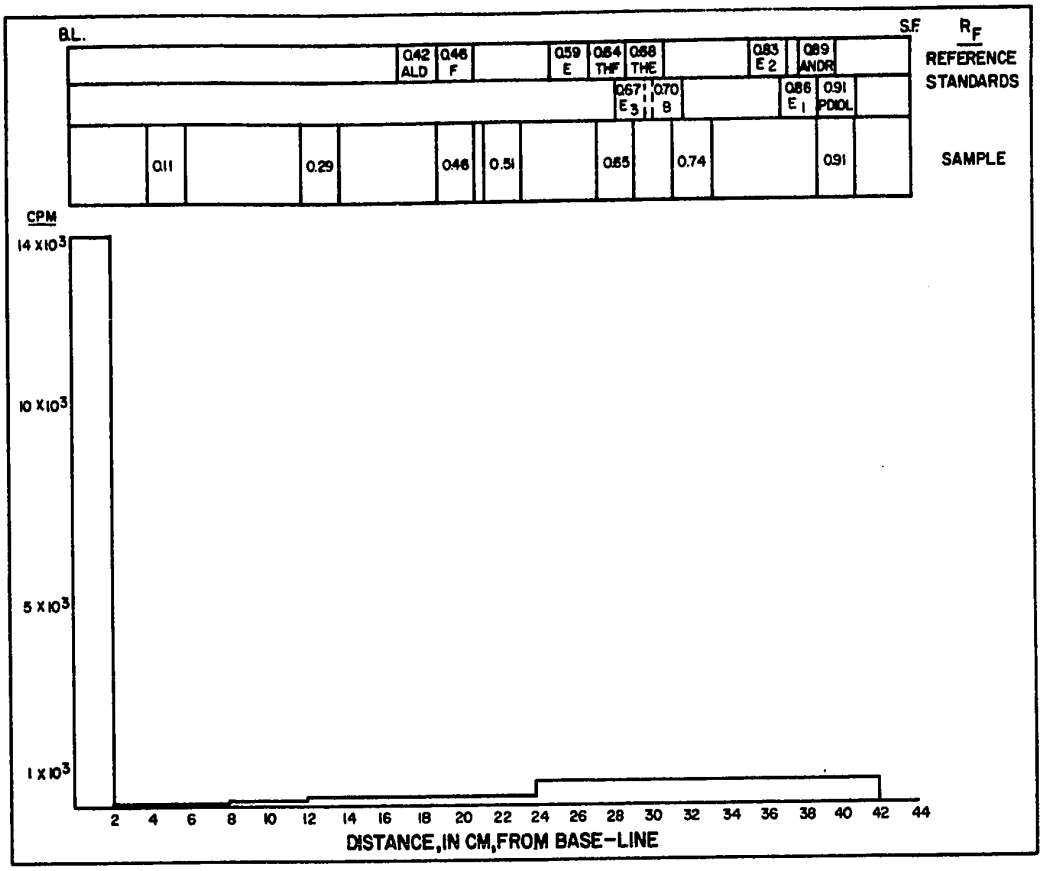

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E 2
0.89 ANDR
0.67 E 3
0.70 B
0.86 E 1
0.91 PDIOL
REFERENCE STANDARDS
0.11
0.29
0.46
0.51
0.65
0.74
0.91
SAMPLE
CPM
14 X10³
10 X10³
5 X10³
1 X10³
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 7

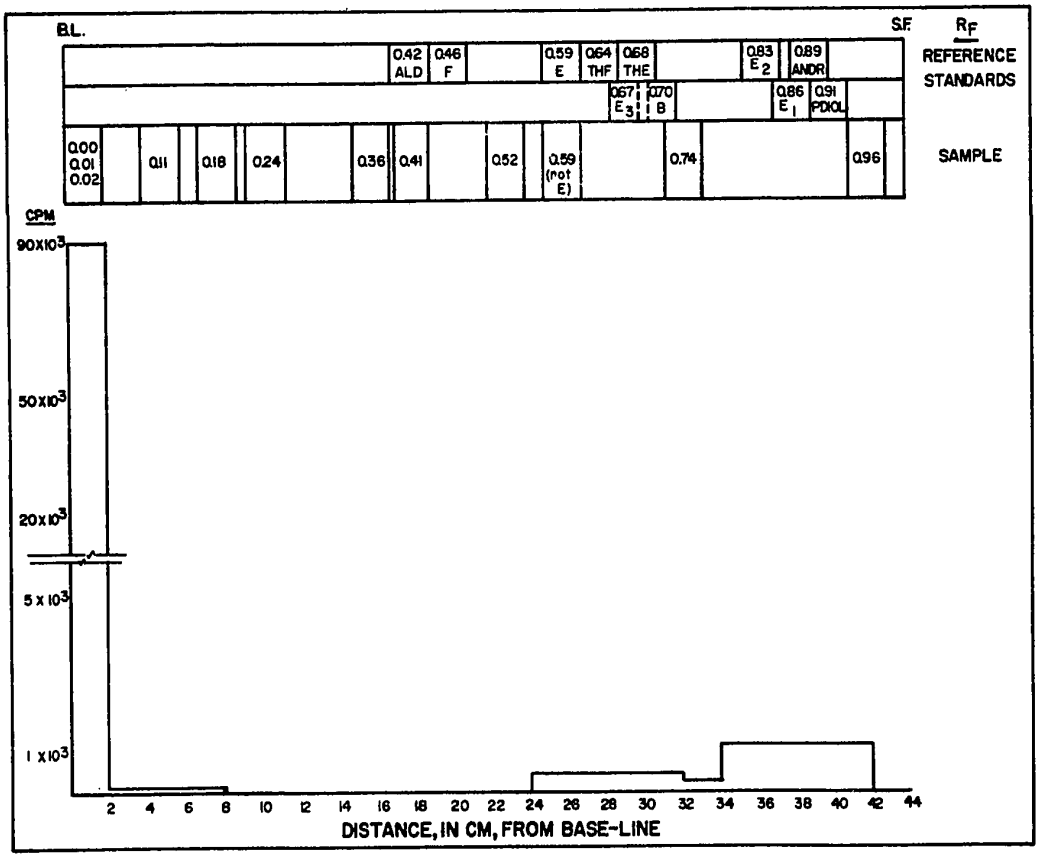

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E 2
0.89 ANDR
0.67 E 3
0.70 B
0.86 E 1
0.91 PDIOL
REFERENCE STANDARDS
0.00 0.01 0.02
0.11
0.18
0.24
0.36
0.41
0.52
0.59 (not E)
0.74
0.96
SAMPLE
CPM
90X10³
50X10³
20X10³
5 X10³
1 X10³
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 8

in the second extract than in the first at the same site. Therefore, the possibility of a relationship between the relatively high radioactivity peak at the baseline and the presence of estradiol catabolites cannot be excluded. However, only very weak radioactivity could be detected in the regions corresponding to the estriol, estrone and estradiol reference standards. In the third urinary extract, of estradiol-17 β-^{3}H treated fish (Fig.9), one relative peak was detected, corresponding to the R_f of estradiol and estrone reference standards, although it was not possible to detect any spots by chemical means. In the fourth extract (Fig.10) there was a fairly wide zone of activity, between 20 - 38 cm, with a relative peak corresponding to the R_f of estriol, urocortisone and corticosterone reference standards but not to chemically detectable spots. Thus, it appears that, although some of the injected tritium was recovered in the urinary extracts, the activity was very weak and even the relative peaks were not corresponding to chemically detectable metabolites of the three estrogens used as reference standards. If we assume that the activity, detected in the third extract at the site of the estradiol and estrone reference standards and in the fourth extract at the R_f of estriol standard, shows in fact the presence of these compounds, we have to conclude that suckers possess the enzymatic mechanism needed for the production of metabolites from estradiol, but that the three estrogens used as reference standards are not the normal products of estrogen metabolism in these fish. There have to be different catabolic pathways and, possibly, different excretory pathways for the products of estrogen metabolism.

Figs.11,12,13 and 14 show the results of the four extracts from pooled urine of female suckers injected with 100 μc progesterone-^{3}H plus

FIG.NO.9

URINARY STEROID METABOLITES DETECTED AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

Third extract

Explanations and abbreviations as in Fig.No.7.

FIG.NO.10

URINARY STEROID METABOLITES DETECTED AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

Fourth extract

Explanations and abbreviations as in Fig.No.7.

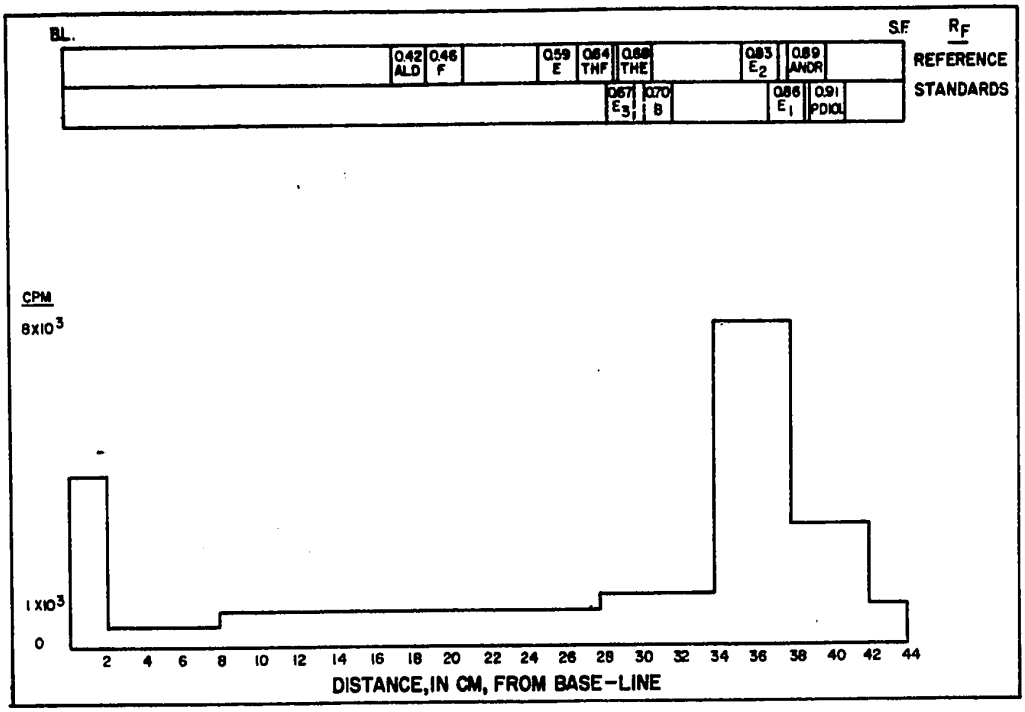

BL.
S.F.
R_F
REFERENCE STANDARDS
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
CPM
8X10³
1X10³
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.9

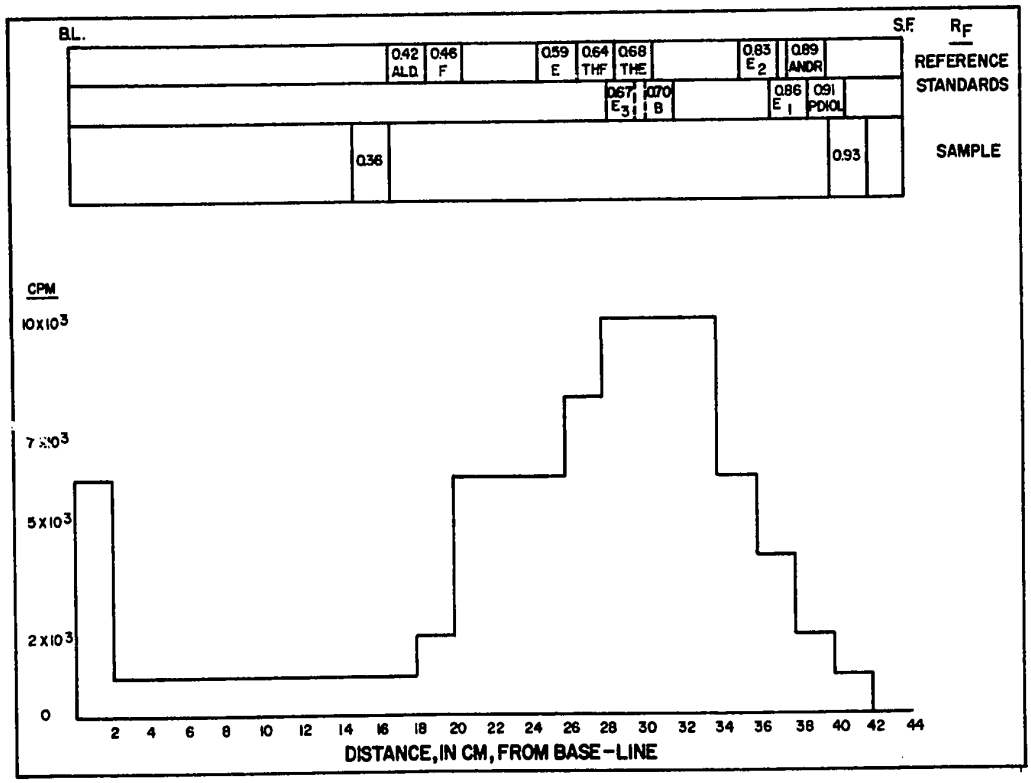

BL.
S.F.
R_F
REFERENCE STANDARDS
SAMPLE
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
0.36
0.93
CPM
10X10³
7X10³
5X10³
2X10³
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.10

carrier. In the first extract (Fig.11) the overall activity was quite low,the highest relative peak reaching only about 6,000 cpm. This particular peak was in the zone of compound R_f 0.24 which,due to its positive alkaline fluorescence,phosphoric acid fluorescence and a positive blue tetrazolium reaction,was considered to be a cortisol catabolite. In the second extract (Fig.12) the highest peak was in the region of compounds R_f 0.42, 0.50 and 0.53,which coincided with the R_f of standard cortisol (R_f 0.46). Two smaller peaks were detected at the location of compounds R_f 0.02 and 0.25. In the third extract (Fig.13) there were two zones of relative activity: one exhibited a peak at the site of R_f 0.02 while the second zone was located near the solvent front and corresponded to a phosphomolybdic acid positive spot with the R_f of pregnanediol. Although the activity of the latter was only about 4,500 cpm,it seemed noteworthy that the presence of pregnanediol was demonstrable chemically as well as by radioactive means. In the fourth extract (Fig.14)one prominent peak was present,corresponding to the R_f of standard pregnanediol (R_f 0.91) but chemically it was not possible to detect any positive spot at that location.

In conclusion it may be said that,except for the third extract, there was a marked discrepancy between the chemical findings and the radioactive peaks. Whereas chemically there were detected phosphomolybdic acid positive spots in the first,second and third extracts and,moreover these were detected only after progesterone administration,radioactivity corresponding to these PMA positive spots was detected only in the third extract. Paradoxically,in the fourth extract,where no PMA positive spots were detected chemically,the radioactivity was highest at the location of standard pregnanediol.

FIG.NO.11

URINARY STEROID METABOLITES DETECTED AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

Five female suckers were injected with 100 µc progesterone-^{3}H plus 1 mg progesterone carrier each and the urine was pooled and extracted.The results are based on R_f values for system B chromatography,developed on Whatman # 1 paper,at 26-27°C.(Appendix: M 2,M 5,M 9,M10,N 3).

First extract

Abbreviations as in Fig.No.7.

FIG.NO.12

URINARY STEROID METABOLITES DETECTED AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

Second extract

Explanations as in Fig.No.11. Abbreviations as in Fig.No.7.

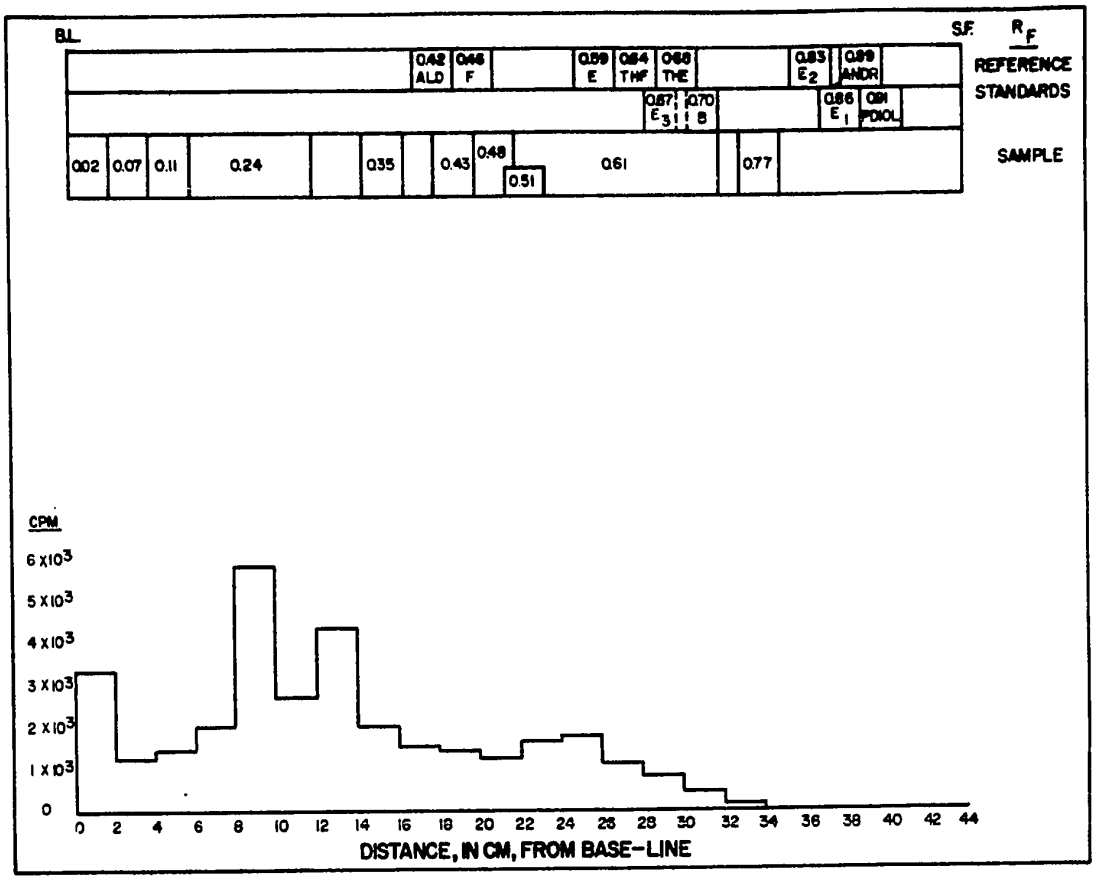

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
REFERENCE STANDARDS
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
0.02
0.07
0.11
0.24
0.35
0.43
0.48
0.51
0.61
0.77
SAMPLE
CPM
6 X10³
5 X10³
4 X10³
3 X10³
2 X10³
1 X10³
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.11

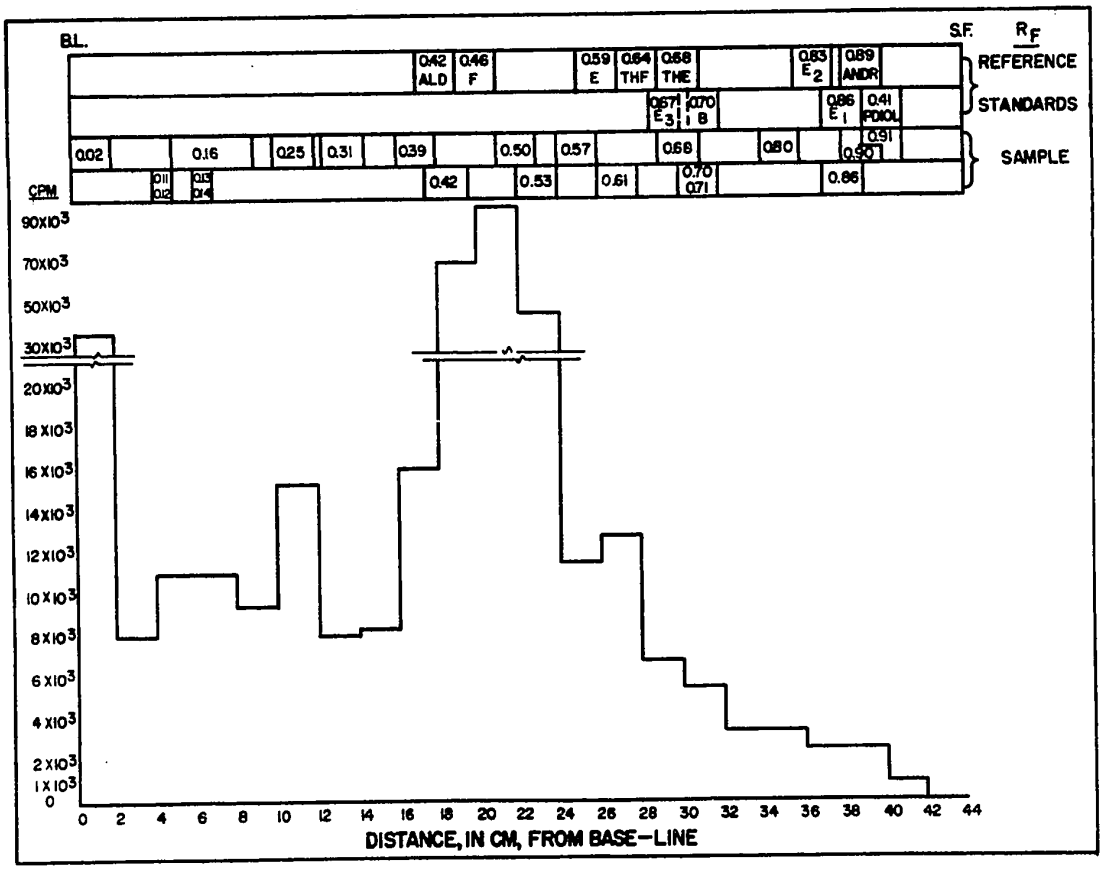

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
REFERENCE STANDARDS
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
0.02
0.16
0.25
0.31
0.39
0.50
0.57
0.69
0.80
0.90
0.91
SAMPLE
CPM
0.11
0.12
0.13
0.14
0.42
0.53
0.61
0.70
0.71
0.86
90X10³
70X10³
50X10³
30X10³
20X10³
18 X10³
16 X10³
14X10³
12X10³
10 X10³
8X10³
6X10³
4 X10³
2X10³
1X10³
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.12

FIG.NO.13

URINARY STEROID METABOLITES DETECTED AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

Third extract

Explanations as in Fig.No.11. Abbreviations as in Fig.No.7.

FIG.NO.14

URINARY STEROID METABOLITES DETECTED AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

Fourth extract

Explanations as in Fig.No.11. Abbreviations as in Fig.No.7.

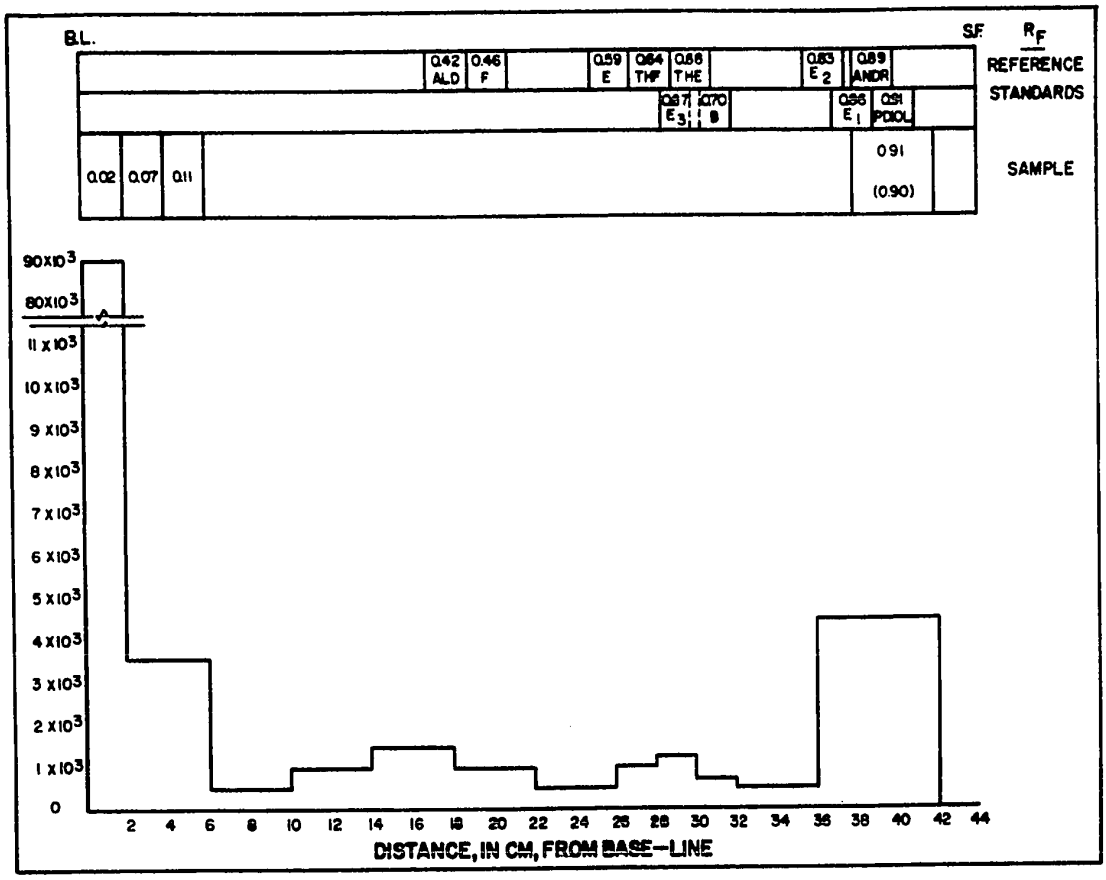

B.L.
S.F.
R_F
REFERENCE STANDARDS
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.87 E_3
0.70 B
0.86 E_1
0.91 PDIOL
SAMPLE
0.02
0.07
0.11
0.91
(0.90)
90x10³
80x10³
11 x10³
10 x10³
9 x10³
8 x10³
7 x10³
6 x10³
5 x10³
4 x10³
3 x10³
2 x10³
1 x10³
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.13

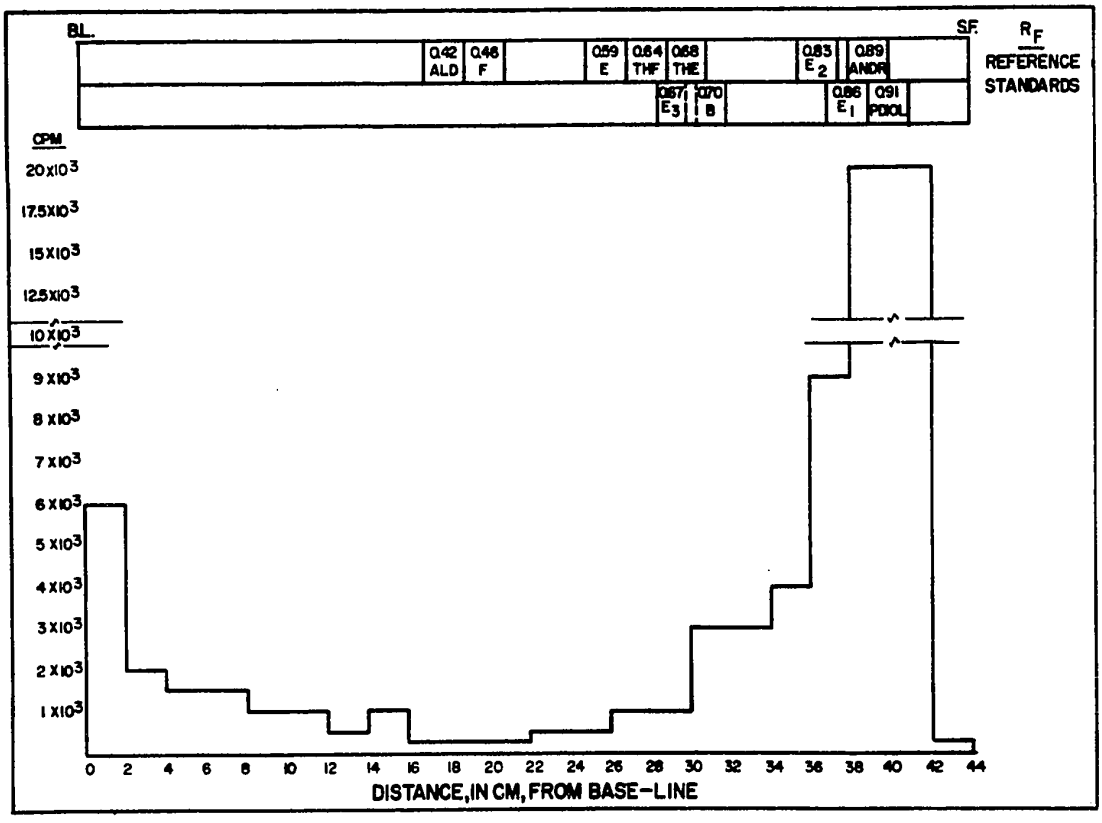

B.L.
S.F.
R_F
REFERENCE STANDARDS
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.87 E_3
0.70 B
0.86 E_1
0.91 PDIOL
CPM
20x10³
17.5x10³
15x10³
12.5x10³
10x10³
9 x10³
8 x10³
7 x10³
6 x10³
5 x10³
4 x10³
3 x10³
2 x10³
1 x10³
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.14

The results,showing radioactivity detected on the chromatograms of the four extracts of sucker urine from males,injected with 100 μc testosterone- ^{3}H plus carrier,are recorded in Figs. 15,16,17 and 18. In Fig.15 it can be seen that in the first extract there was one high peak at R_f 0.02,corresponding to a compound giving positive reactions with both blue tetrazolium and DNPH. In the second extract (Fig.16) two peaks were detected both of low activity: one between 0-2 cm,yielding no chemically positive spot and a second one corresponding to R_f 0.85. In the third extract (Fig.17) the activity was quite low all over the chromatogram,but two relative peaks could be observed. One was between 2-8 cm,where no chemically detectable spot was found,and the second corresponding to R_f 0.85. There were three peaks of medium activity present in the fourth extract (Fig.18). These peaks corresponded to compounds R_f 0.48, 0.70 and 0.85. Of these R_f 0.48 and 0.70 gave positive alkaline fluorescence.

Thus,it appeared that the main catabolite of testosterone was compound R_f 0.85. This compound gave positive color reaction with DNPH and also a brown-violet color with MDNB. It was not detected in urine of untreated fish or in fish treated with steroids other than testosterone. It was the only compound to give a positive MDNB reaction. The highest activity of R_f 0.85 was present in the fourth extract, indicating that it was excreted mainly as a sulfate ester.

Radioactive peaks,found in urinary extracts from fish of both sexes injected with cortisone-^{3}H plus carrier,are shown in Figs.19,20 and 21. In the first extract (Fig.19) there was one very high peak corresponding to R_f 0.34 and 0.36 (SP_1 and SP_2). In the second extract this peak was more than four times higher than in the first extract,

FIG.NO.15

URINARY STEROID METABOLITES DETECTED AFTER TESTOSTERONE-^{3}H AND CARRIER TESTOSTERONE ADMINISTRATION

Five male suckers were injected with 100 μc testosterone-^{3}H plus 1 mg testosterone carrier each and the urine was pooled and extracted. The results are based on R_f values for system B chromatography, developed on Whatman # 1 paper, at 26-27°C. (Appendix: M 8, N 2, N 7, N 9, P 3).

First extract

Abbreviations as in Fig.No.7.

FIG.NO.16

URINARY STEROID METABOLITES DETECTED AFTER TESTOSTERONE-^{3}H AND CARRIER TESTOSTERONE ADMINISTRATION

Second extract

Explanations as in Fig.No.15. Abbreviations as in Fig.No.7.

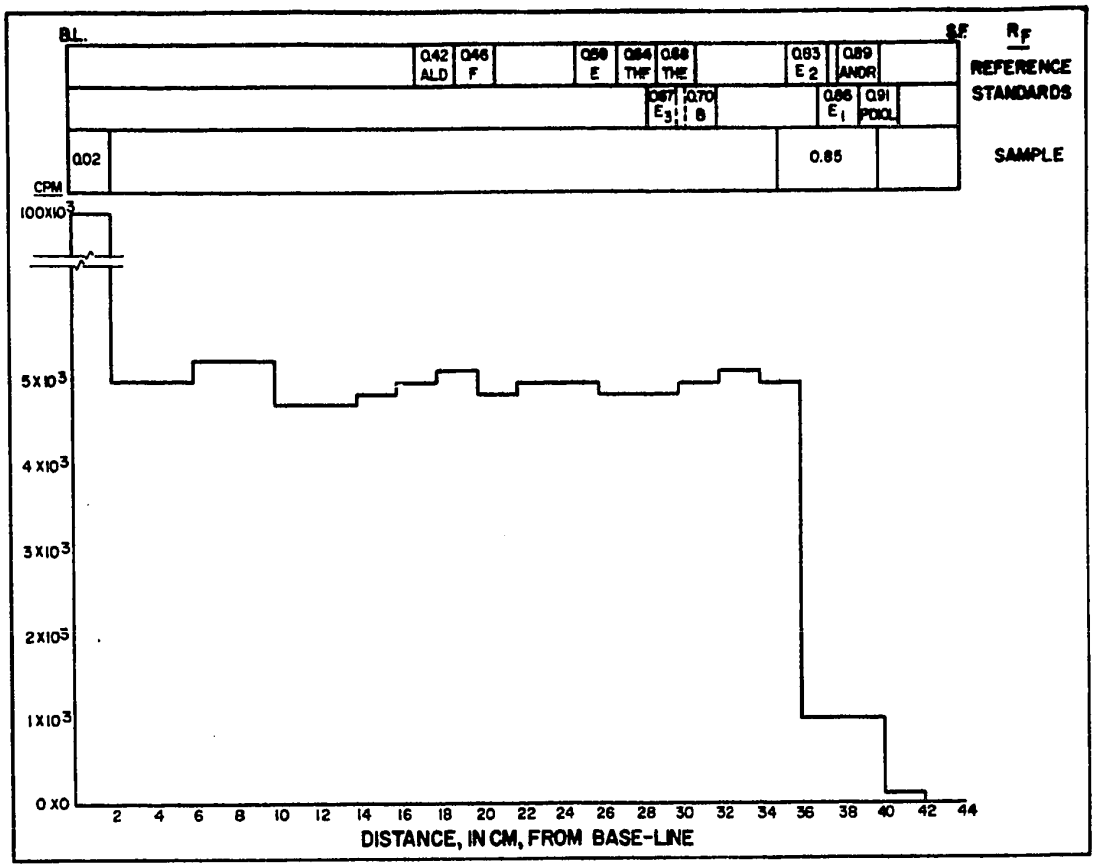

B.L.
S.F
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
REFERENCE STANDARDS
0.02
0.85
SAMPLE
CPM
100X10³
5X10³
4X10³
3X10³
2X10³
1X10³
0 X0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.15

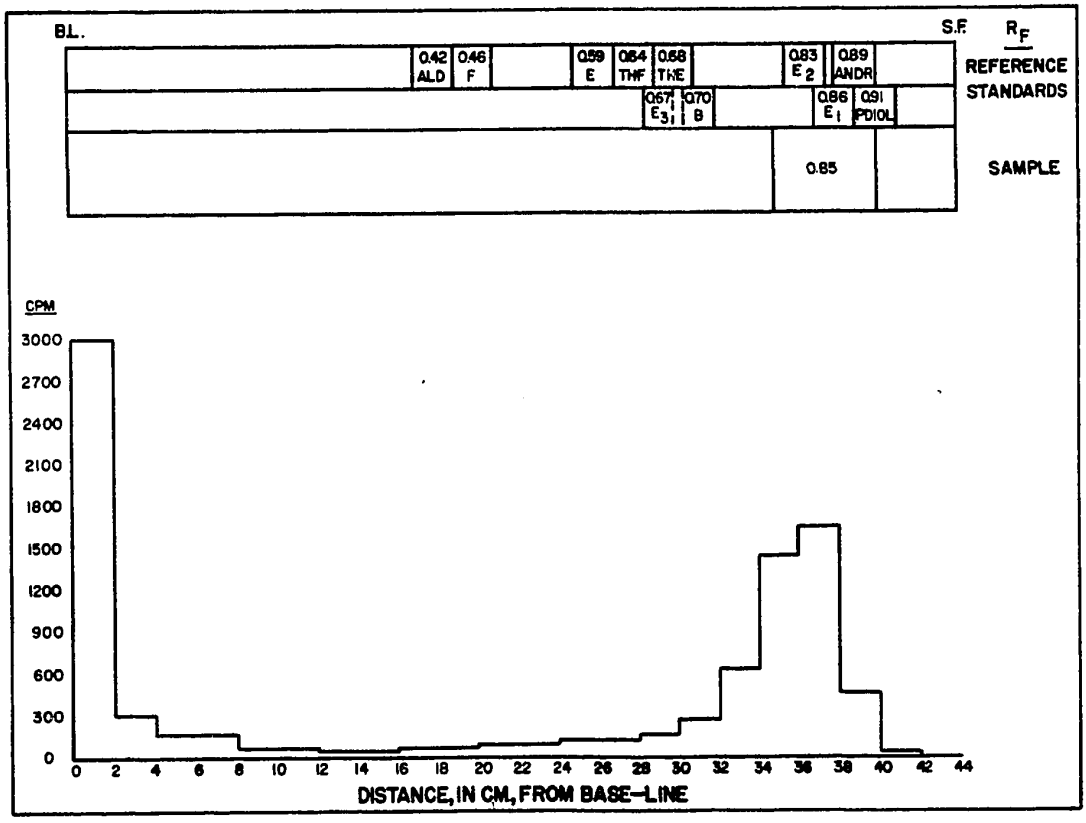

B.L.
S.F
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
REFERENCE STANDARDS
0.85
SAMPLE
CPM
3000
2700
2400
2100
1800
1500
1200
900
600
300
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.16

FIG.NO.17

URINARY STEROID METABOLITES DETECTED AFTER TESTOSTERONE-^{3}H AND CARRIER TESTOSTERONE ADMINISTRATION

Third extract

Explanations as in Fig.No.15. Abbreviations as in Fig.No.7.

FIG.NO.18

URINARY STEROID METABOLITES DETECTED AFTER TESTOSTERONE-^{3}H AND CARRIER TESTOSTERONE ADMINISTRATION

Fourth extract

Explanations as in Fig.No.15. Abbreviations as in Fig.No.7.

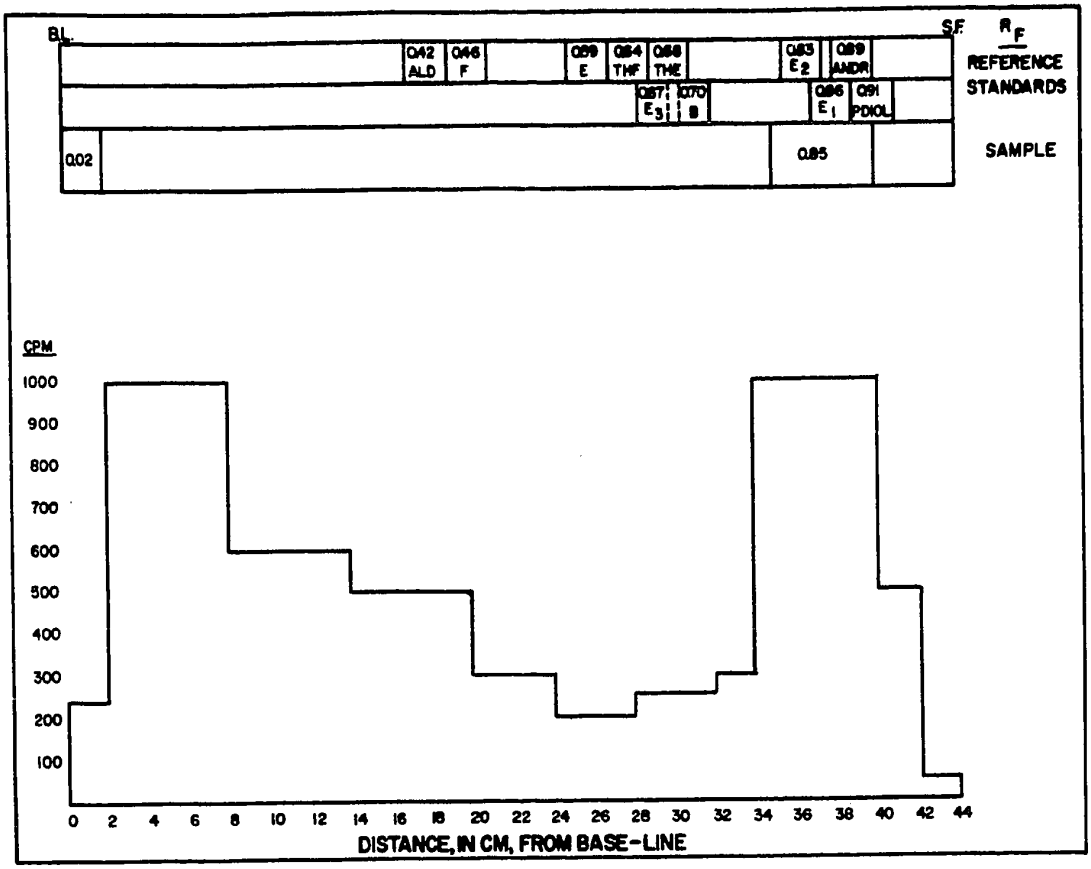

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.66 THE
0.83 E_2
0.89 ANDR
0.87 E_3
0.70 B
0.86 E_1
0.91 PDIOL
REFERENCE STANDARDS
0.02
0.85
SAMPLE
CPM
1000
900
800
700
600
500
400
300
200
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.17

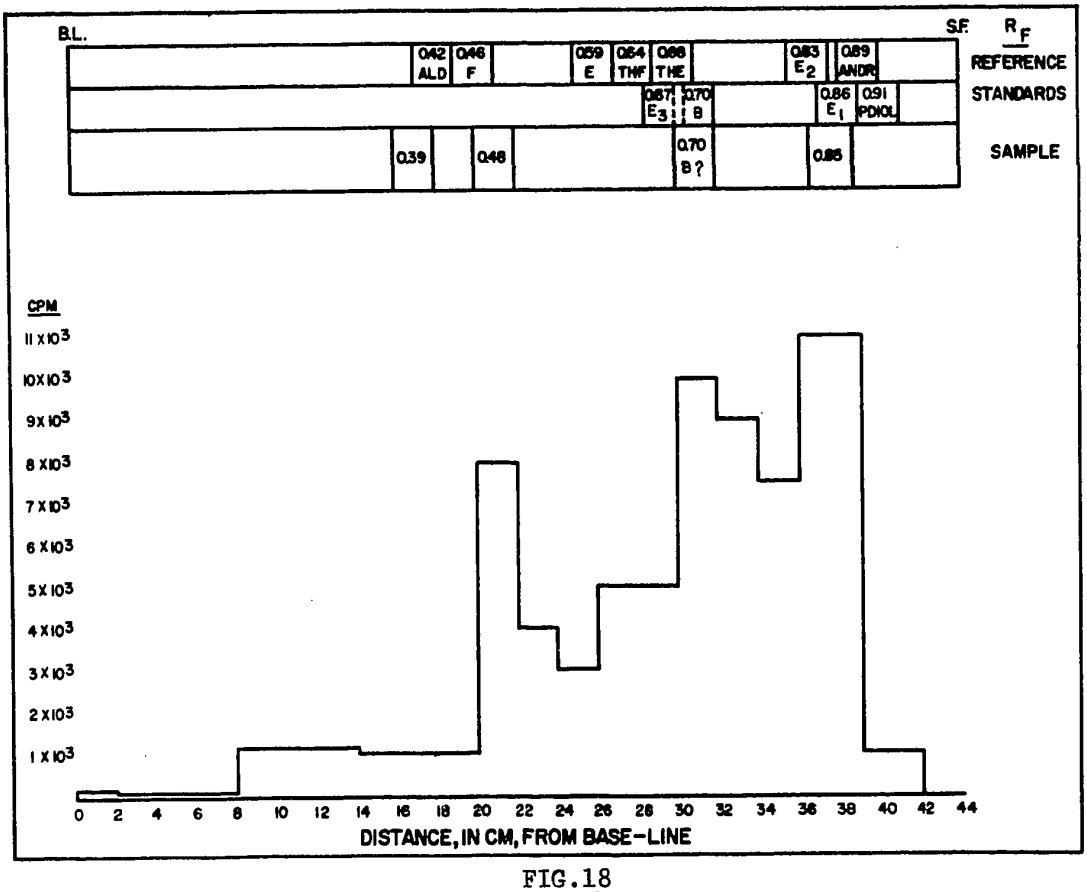

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.66 THE
0.83 E_2
0.89 ANDR
0.87 E_3
0.70 B
0.86 E_1
0.91 PDIOL
REFERENCE STANDARDS
0.39
0.46
0.70 B?
0.85
SAMPLE
CPM
11 X 10³
10 X 10³
9 X 10³
8 X 10³
7 X 10³
6 X 10³
5 X 10³
4 X 10³
3 X 10³
2 X 10³
1 X 10³
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.18

exhibiting by far the highest radioactivity found during the present study,i.e. 3×10^6 cpm compared to a maximum of 10×10^4cpm found in urinary extracts from fish treated with other ^{3}H-labeled steroids,as for example testosterone-^{3}H. In the third extract (Fig.21) the highest activity was detected at R_f 0.36 (R_f 0.34 not giving the blue tetrazolium reaction in this case).

After rechromatographing the active zones from system B (10-22 cm) on system C chromatograms it was possible to demonstrate that the two compounds,SP_1 and SP_2,were direct catabolites of cortisone. In each of the three chromatograms there were two peaks,corresponding to R_f 0.07 (SP_1),and R_f 0.17 (SP_2) respectively. The highest activity was in the second extract,indicating that the two compounds were excreted mainly as glucuroniside uronidates,although they were present also in the first and third extracts,which was in contrast with the findings in untreated fish,where the two compounds were never detected as free steroids or as sulfate esters(Figs. 22,23,24).

B. Appearance rate of urinary radioactivity in fish treated with ^{3}H-labeled steroids.

During the experiments reported above (part III,5,A) I considered it worthwhile to record the period of time elapsing between the moment of injection of the ^{3}H-labeled steroids and the appearance of radioactivity in the urine. For this purpose urine was collected in separate test tubes,changing them every five minutes and correcting for collection delay time. This fraction collection was usually stopped after 5-8 hours and urine collection was continued the usual way,in Nalgene bottles, for the chemical and isotope detection of metabolites. From each test

FIG.NO.19

URINARY STEROID METABOLITES DETECTED AFTER CORTISONE-^{3}H AND CARRIER CORTISONE ADMINISTRATION

Three male and two female suckers were injected with 100 µc cortisone-^{3}H plus 1 mg cortisone carrier each and the urine was pooled separately for the two sexes and extracted. The results are based on R_f values for system B chromatography, developed on Whatman # 1 paper, at 26-27°C. (Appendix: I 9, L 4, L 5, M 1, N 8).

First extract

Abbreviations as in Fig.No.7.

FIG.NO.20

URINARY STEROID METABOLITES DETECTED AFTER CORTISONE-^{3}H AND CARRIER CORTISONE ADMINISTRATION

Second extract

Explanations as in Fig.No.19. Abbreviations as in Fig.No.7.

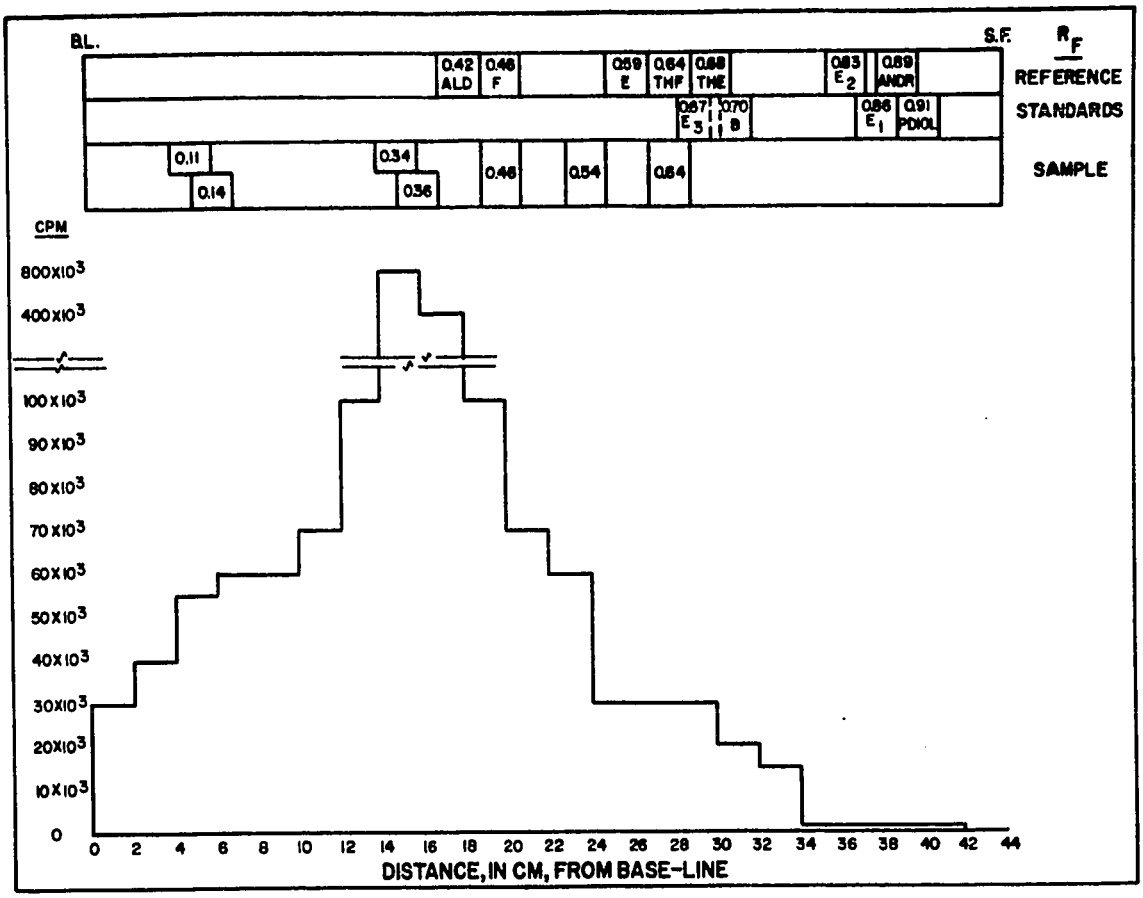

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
REFERENCE STANDARDS
0.11
0.14
0.34
0.36
0.46
0.54
0.64
SAMPLE
CPM
800X10³
400X10³
100X10³
90X10³
80X10³
70X10³
60X10³
50X10³
40X10³
30X10³
20X10³
10X10³
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 19

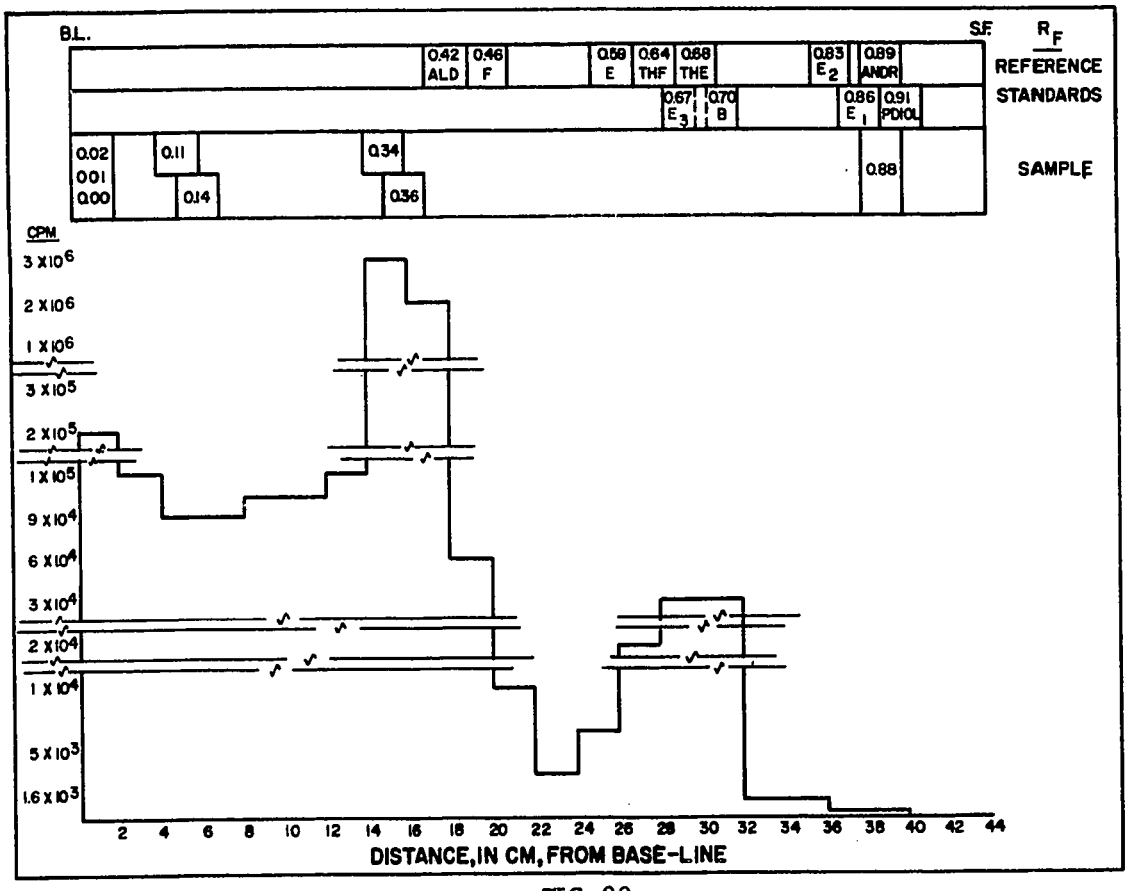

B.L.
S.F.
R_F
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E_2
0.89 ANDR
0.67 E_3
0.70 B
0.86 E_1
0.91 PDIOL
REFERENCE STANDARDS
0.02
0.01
0.00
0.11
0.14
0.34
0.36
0.88
SAMPLE
CPM
3 X10⁶
2 X10⁶
1 X10⁶
3 X10⁵
2 X10⁵
1 X10⁵
9 X10⁴
6 X10⁴
3 X10⁴
2 X10⁴
1 X10⁴
5 X10³
1.6 X10³
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 20

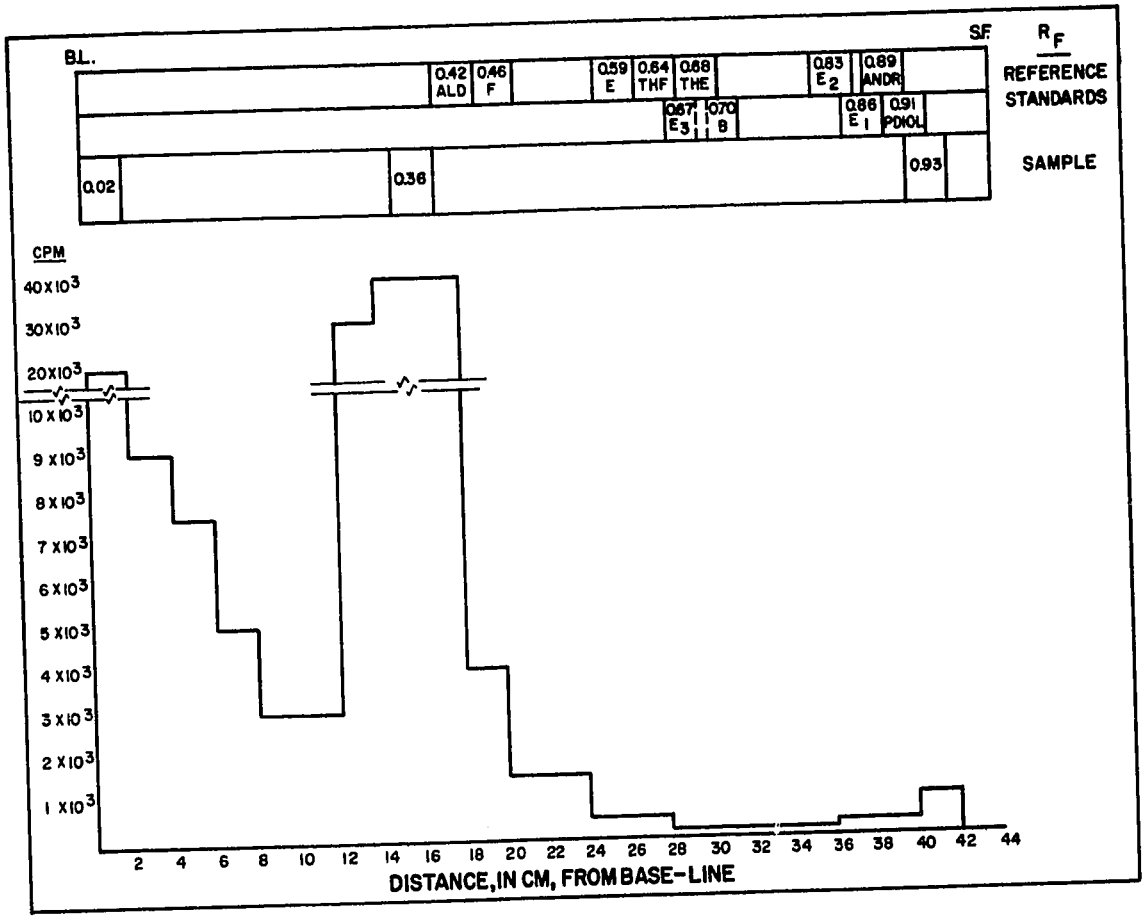


FIG.21

FIG.NO.21

URINARY STEROID METABOLITES DETECTED AFTER CORTISONE-^{3}H AND CARRIER CORTISONE ADMINISTRATION

Third extract

Explanations as in Fig.No.19. Abbreviations as in Fig.No.7.

FIG.NO.22

URINARY STEROID METABOLITES DETECTED AFTER CORTISONE-^{3}H AND CARRIER CORTISONE ADMINISTRATION

First extract

Portion 10-22 cm from system B rechromatographed on system C (Whatman # 1 paper,29-30^{o}C). Abbreviations as in Fig.No.7.

FIG.NO.23

URINARY STEROID METABOLITES DETECTED AFTER CORTISONE-^{3}H AND CARRIER CORTISONE ADMINISTRATION

Second extract

Portion 12-20 cm from system B rechromatographed on system C(Whatman # 1 paper,29-30^{o}C). Abbreviations as in Fig.No.7.

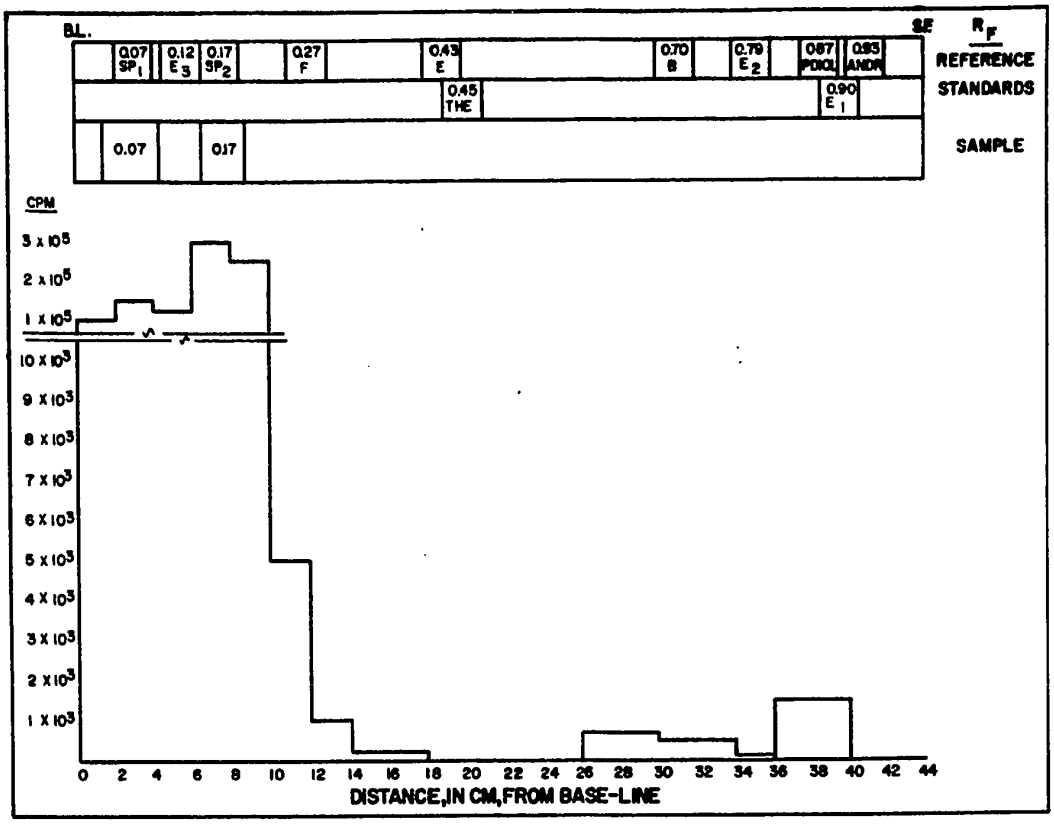

BL.
SF
R_F
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PIXL
0.93 ANDR
REFERENCE STANDARDS
0.45 THE
0.90 E1
0.07
0.17
SAMPLE
CPM
3 x 10^5
2 x 10^5
1 x 10^5
10 x 10^3
9 x 10^3
8 x 10^3
7 x 10^3
6 x 10^3
5 x 10^3
4 x 10^3
3 x 10^3
2 x 10^3
1 x 10^3
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.22

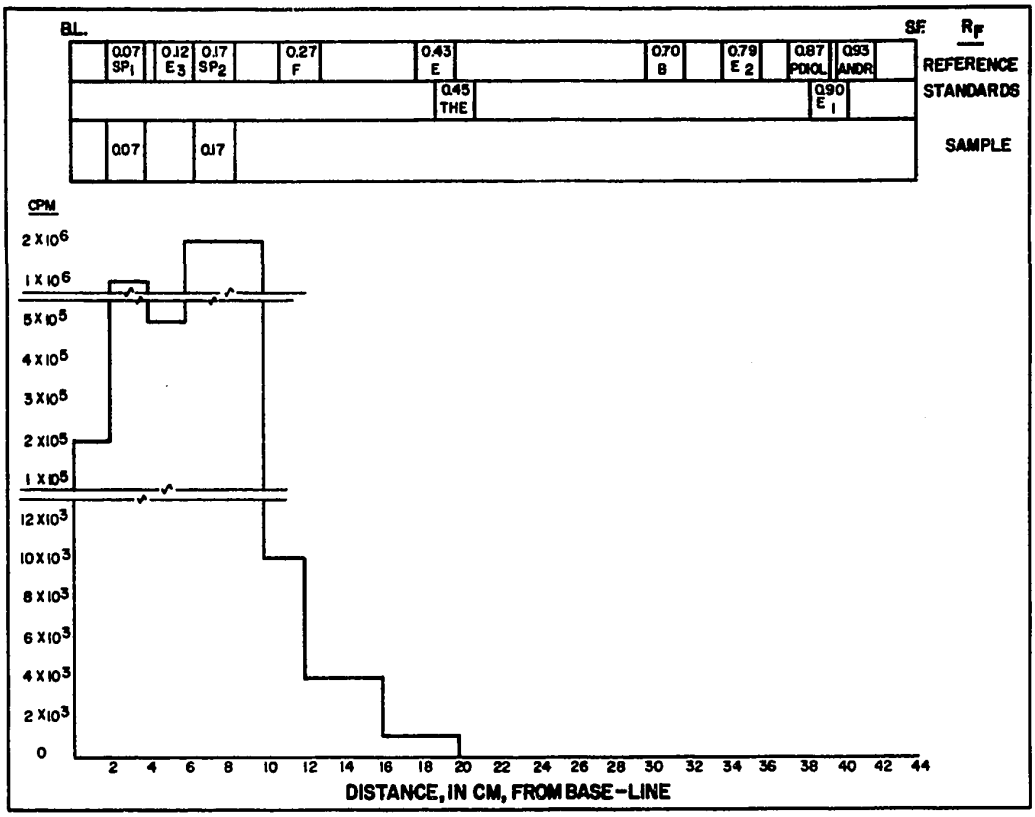

BL.
SF
R_F
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PIXL
0.93 ANDR
REFERENCE STANDARDS
0.45 THE
0.90 E1
0.07
0.17
SAMPLE
CPM
2 X 10^6
1 X 10^6
5 X 10^5
4 X 10^5
3 X 10^5
2 X 10^5
1 X 10^5
12 X 10^3
10 X 10^3
8 X 10^3
6 X 10^3
4 X 10^3
2 X 10^3
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG.23

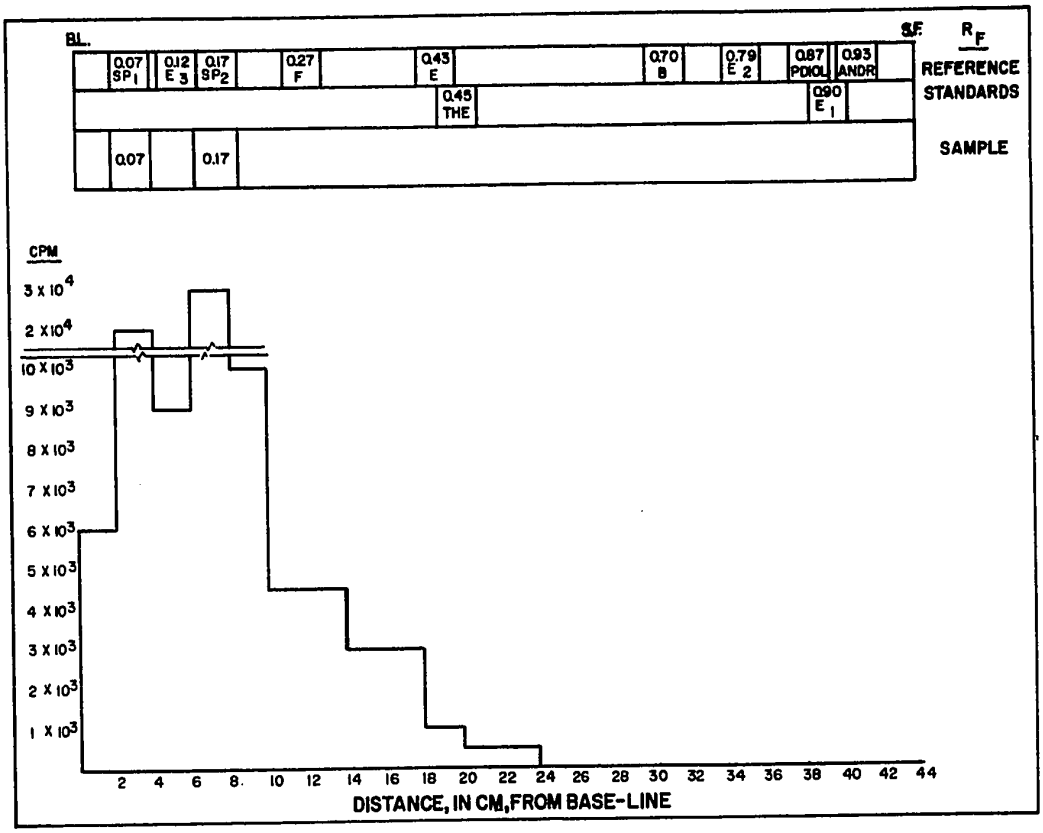


FIG.24

FIG.NO.24

URINARY STEROID METABOLITES DETECTED AFTER CORTISONE- H AND CARRIER CORTISONE ADMINISTRATION

Third extract

Explanations as in Fig.No.23. Abbreviations as in Fig.No.7.

tube,after measuring the excreted volume, 50 µl were taken for liquid scintillation counting.

In fish injected with estradiol-17β-^{3}H plus carrier,the radioactivity appeared in urine 30 minutes after the time of injection (corrected time)(Fig.25). The activity increased rapidly the first 30 minutes and then at a lower rate for another 90 minutes. At this time,i.e. two hours after the time of injection,the activity reached a plateau lasting for about 20 minutes,afterwards decreasing slowly.

In Fig.26 is shown the activity curve of urine samples collected after administration of progesterone-^{3}H plus carrier. Radioactivity appeared in urine 100 minutes after the time of injection. The highest peak was reached after three hours from the time of injection and was followed by a slow decrease in activity.

When testosterone-^{3}H was injected with carrier,the radioactivity appeared after 15 minutes (corrected time) and a peak was reached after two hours (Fig.27).

Besides the data recorded from the experiments described above, different experiments were undertaken to elucidate if ACTH,adrenaline and the presence or absence of carrier steroid would play a role in the appearance rate of urinary radioactivity after administration of ^{3}H-labeled steroids to suckers.

Thus, 100 µc progesterone-^{3}H without added carrier,was injected five minutes after the administration of 15 i.u. ACTH/Kg body weight. The activity curve,shown in Fig.28,exhibited a shorter latent period of appearance than in the case of progesterone-^{3}H with carrier(without ACTH). On the other hand,the peak reached in one hour and 40 minutes was of much lower activity. Because the result could be due to the fact

FIG.NO.25

URINARY RADIOACTIVITY AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

The results are mean values from three female suckers injected with 100 μc estradiol-^{3}H and 1 mg estradiol carrier each.(Appendix: I10,L 6,N 4).

FIG.NO.26

URINARY RADIOACTIVITY AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

The results are mean values from four female suckers injected with 100 μc progesterone-^{3}H and 1 mg progesterone carrier each. (Appendix: M 5,M 9,M10,N 3).

FIG.NO.25

URINARY RADIOACTIVITY AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

The results are mean values from three female suckers injected with 100 μc estradiol-^{3}H and 1 mg estradiol carrier each.(Appendix: I10,L 6,N 4).

FIG.NO.26

URINARY RADIOACTIVITY AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

The results are mean values from four female suckers injected with 100 μc progesterone-^{3}H and 1 mg progesterone carrier each. (Appendix: M 5,M 9,M10,N 3).

FIG.NO.25

URINARY RADIOACTIVITY AFTER ESTRADIOL-17β-^{3}H AND CARRIER ESTRADIOL ADMINISTRATION

The results are mean values from three female suckers injected with 100 μc estradiol-^{3}H and 1 mg estradiol carrier each.(Appendix: I10,L 6,N 4).

FIG.NO.26

URINARY RADIOACTIVITY AFTER PROGESTERONE-^{3}H AND CARRIER PROGESTERONE ADMINISTRATION

The results are mean values from four female suckers injected with 100 μc progesterone-^{3}H and 1 mg progesterone carrier each. (Appendix: M 5,M 9,M10,N 3).

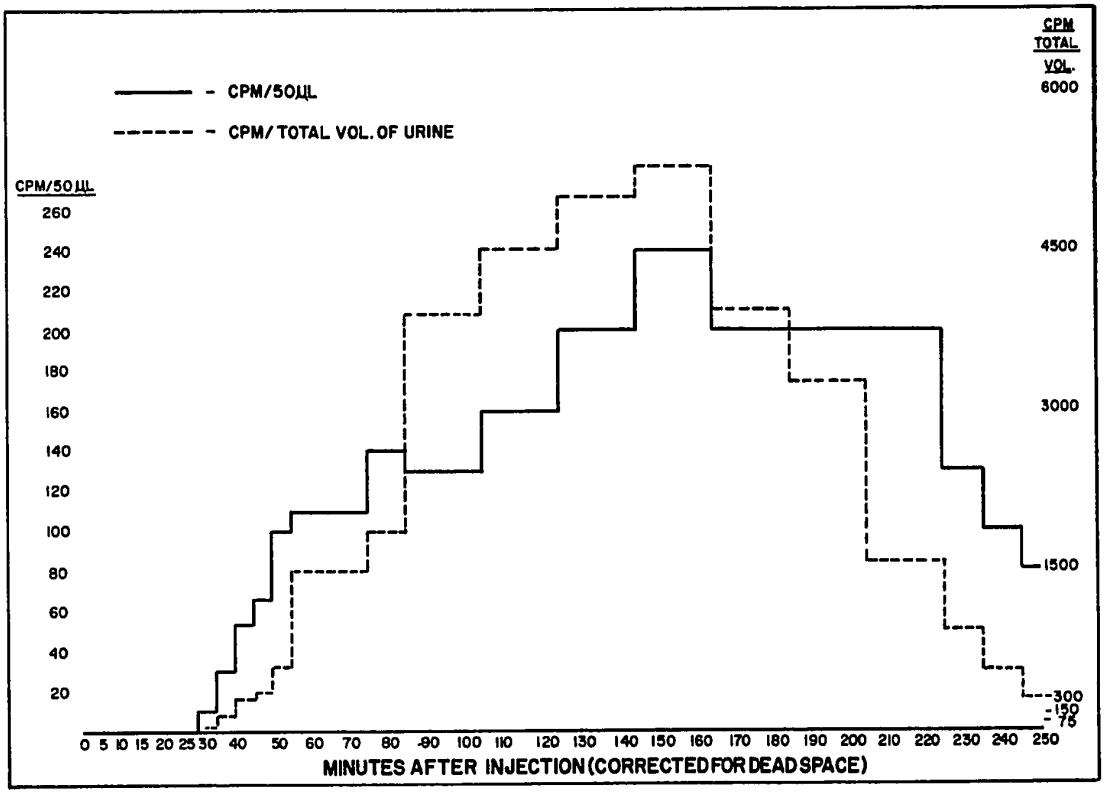

CPM TOTAL VOL.
6000
4500
3000
1500
300
150
75
— CPM/50µL
------ CPM/ TOTAL VOL. OF URINE
CPM/50 µL
260
240
220
200
180
160
140
120
100
80
60
40
20
0 5 10 15 20 25 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250
MINUTES AFTER INJECTION (CORRECTED FOR DEAD SPACE)

FIG.25

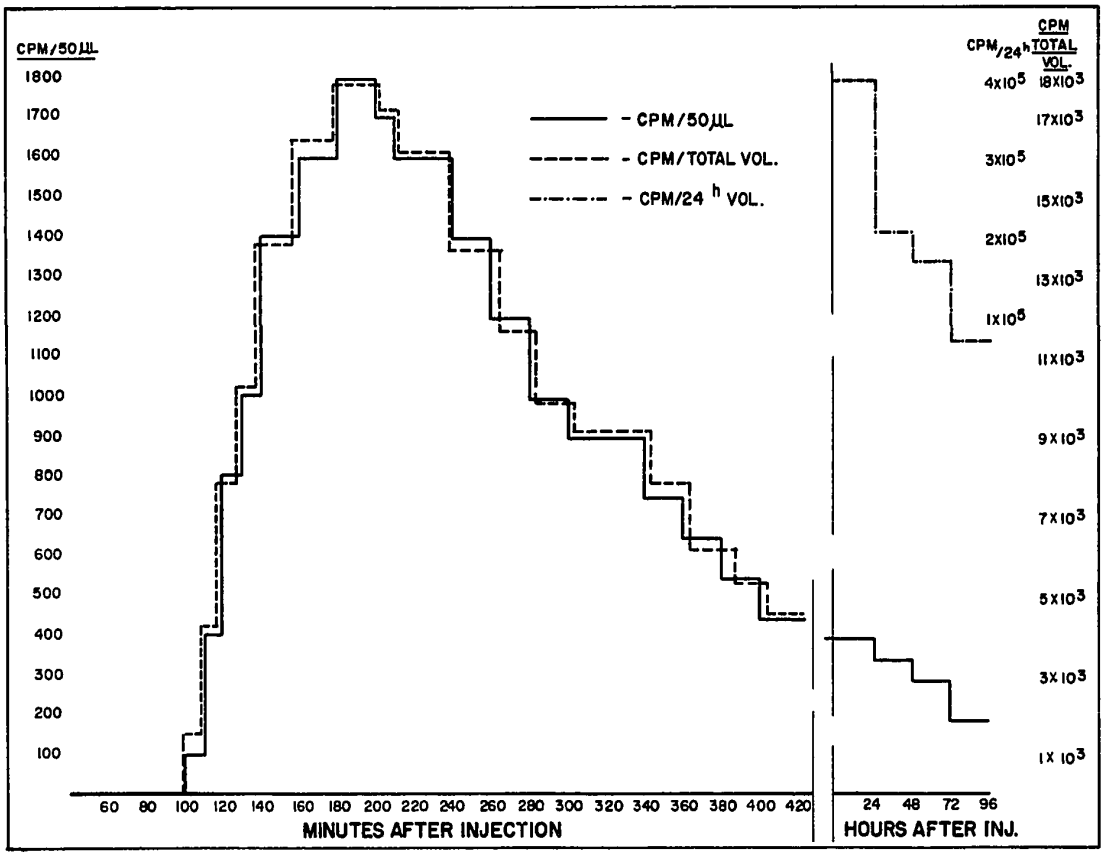

CPM/50µL
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
— CPM/50µL
------ CPM/TOTAL VOL.
-·-·-·- CPM/24 h VOL.
CPM/24h
4x10^5
3x10^5
2x10^5
1x10^5
CPM TOTAL VOL.
18X10^3
17X10^3
15X10^3
13X10^3
11X10^3
9X10^3
7X10^3
5X10^3
3X10^3
1X10^3
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420
MINUTES AFTER INJECTION
24 48 72 96
HOURS AFTER INJ.

FIG.26

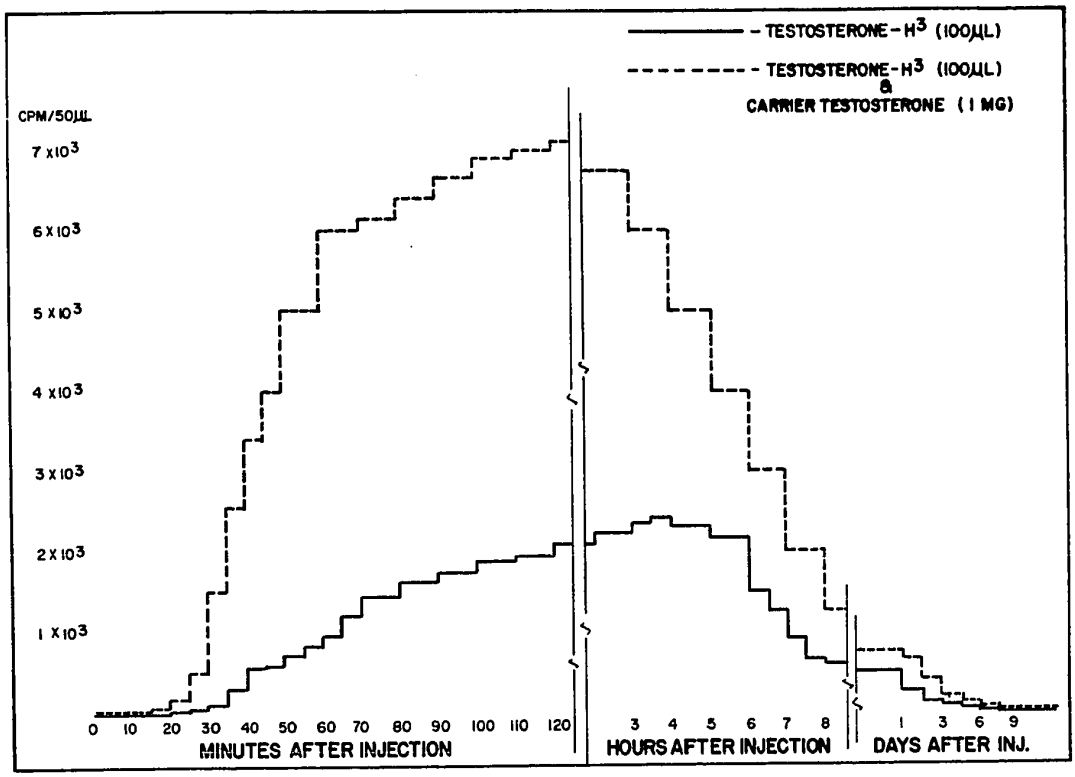


FIG.27

FIG.NO.27

URINARY RADIOACTIVITY AFTER ADMINISTRATION OF TESTOSTERONE-^{3}H AND AFTER TESTOSTERONE-^{3}H PLUS CARRIER TESTOSTERONE ADMINISTRATION

The results are mean values from two male suckers injected with 100 µc testosterone-^{3}H each and from two male suckers injected with 100 µc testosterone-^{3}H plus 1 mg testosterone carrier each.

(Appendix: M 8,N 5,P 1,P 3).

that no carrier progesterone was present, another experiment was done, injecting progesterone-^{3}H without carrier (Fig.29). In this case the radioactivity appeared in urine 100 minutes after the time of injection and a peak was reached after two hours and 50 minutes. After four hours and 25 minutes from the time of injection, 15 i.u. ACTH/Kg body weight were injected to the same fish. Five minutes later the activity started to increase rapidly, reaching a peak in ten minutes. The activity of this peak was almost double than the first peak. After a plateau of 20 minutes the activity decreased slowly.

With the aim to find out if the above results were particular for progesterone or if they were common to all steroids, I injected 100 μc testosterone-^{3}H without carrier and compared the results of urinary radioactivity with those found in the fish injected with testosterone-^{3}H plus carrier steroid (Fig.27). It can be seen that in both cases the urinary radioactivity appeared almost at the same time but peak activity was reached later in the fish without added carrier. On the other hand the peak was much higher in the case of added carrier testosterone (7,000 cpm vs. 2,000 cpm).

It could be concluded therefore that:

a. the time elapsing between the injection of a labeled steroid and the appearance of urinary radioactivity seems to be a characteristic feature of the particular steroid, due, probably, to the specific catabolic processes it has to undergo, and the same could be said about the time the peak urinary activity is reached.

b. the intensity of the urinary radioactivity peak seems to be dependent on the presence or absence of carrier steroid, its presence having an intensifying effect upon the hight of activity.

FIG.NO.28

URINARY RADIOACTIVITY AFTER PROGESTERONE-^{3}H AND ACTH ADMINISTRATION

The results are mean values from two female suckers injected with 100 µc progesterone-^{3}H and 15 i.u.ACTH/Kg body weight each. (Appendix: M 4,M 6).

FIG.NO.29

URINARY RADIOACTIVITY AFTER PROGESTERONE-^{3}H ADMINISTRATION,FOLLOWED BY INJECTION OF ACTH

The results are given in cpm/50 µl urine for a female sucker injected with 100 µc progesterone-^{3}H,followed after 4 hours and 25 minutes by an injection of 15 i.u. ACTH/Kg body weight. (Appendix: M 7).

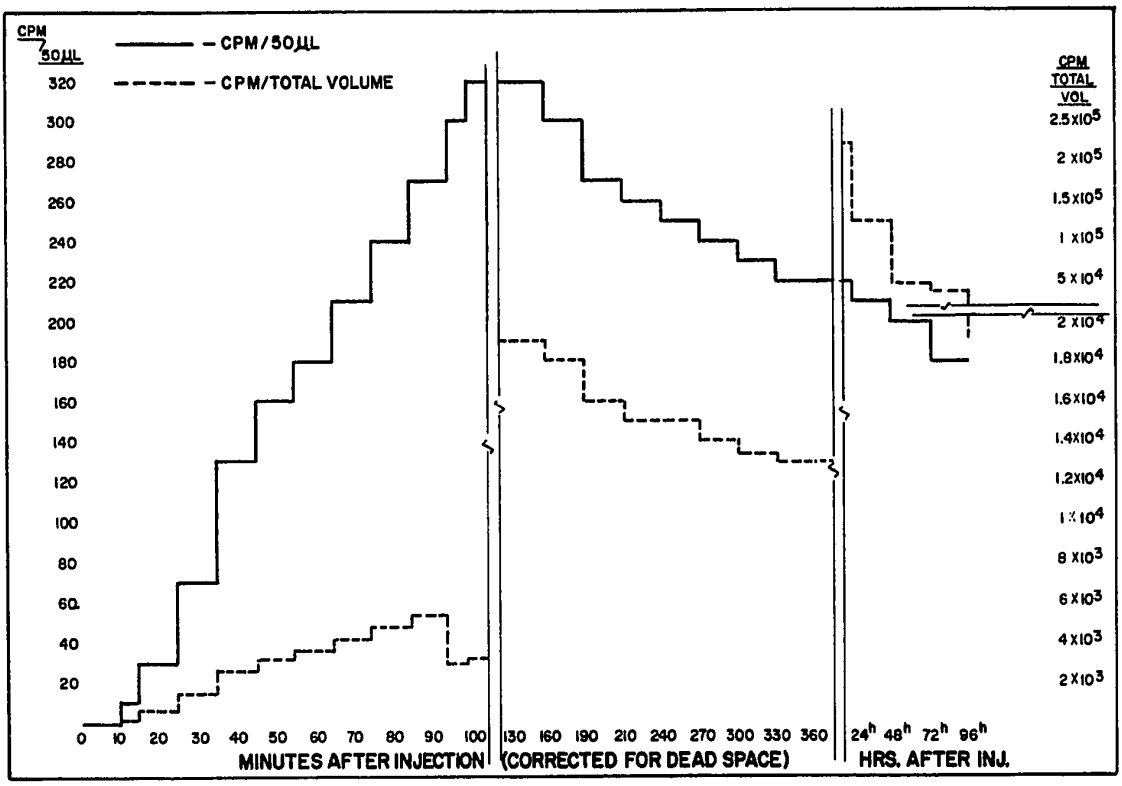

CPM/50µL
CPM/50µL
CPM/TOTAL VOLUME
CPM TOTAL VOL
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
2.5x10^5
2 x10^5
1.5x10^5
1 x10^5
5 x10^4
2 x10^4
1.8x10^4
1.6x10^4
1.4x10^4
1.2x10^4
1 x10^4
8 x10^3
6 x10^3
4 x10^3
2 x10^3
0 10 20 30 40 50 60 70 80 90 100 130 160 190 210 240 270 300 330 360 24h 48h 72h 96h
MINUTES AFTER INJECTION (CORRECTED FOR DEAD SPACE) HRS. AFTER INJ.

FIG.28

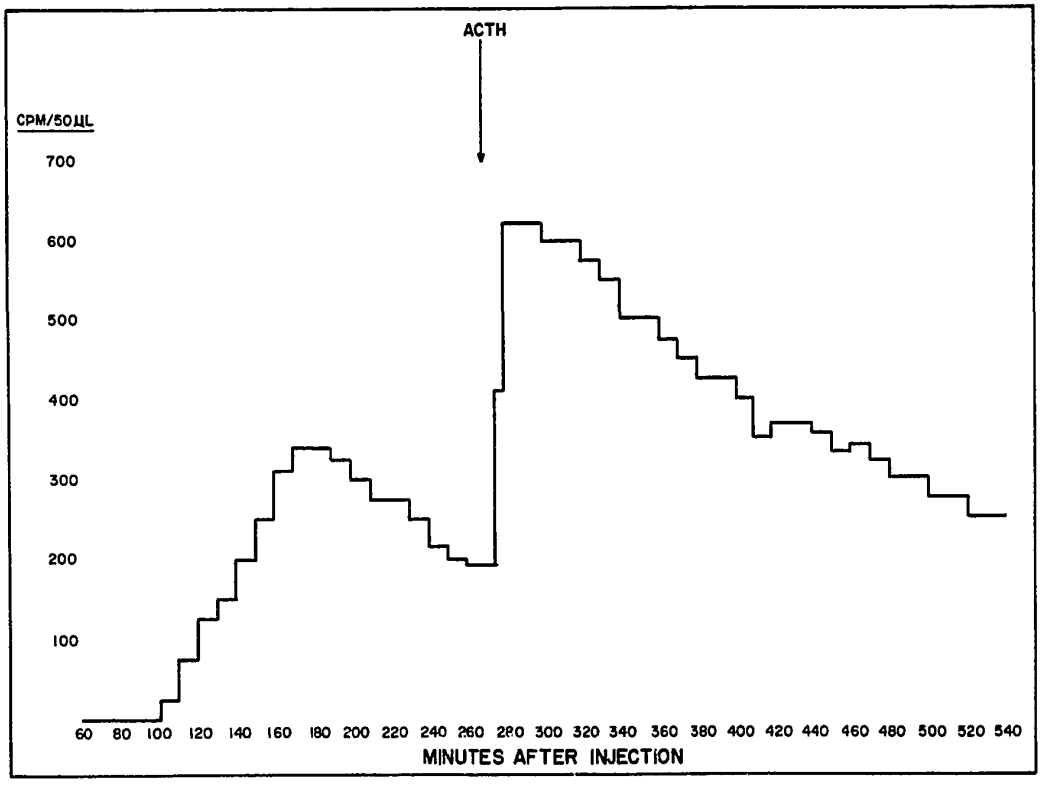

ACTH
CPM/50µL
700
600
500
400
300
200
100
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 520 540
MINUTES AFTER INJECTION

FIG.29

c. ACTH,injected simultaneously with the labeled steroid was reducing the latent time of appearance of urinary radioactivity.

d. ACTH increased the intensity of the peak activity but only when injected after the time a first activity peak was reached.

Based on the assumption that ACTH could,possibly,act mainly as a stressor,I tried to duplicate the results obtained with ACTH by injecting adrenaline.

Thus,24 hours after injecting 100 μc progesterone-^{3}H plus one mg carrier progesterone,0.5 ml of a 1:1000 solution of adrenaline were administered through the caudal vein cannula. The results are shown in Fig.30. Urinary radioactivity decreased rapidly during the first 50 minutes,to ¼ of the value recorded before the injection. Afterwards the slope became less steep but reached a minimum,i.e. no detectable radioactivity at about 3½ hours. One hour later,i.e. 270 minutes after the injection,radioactivity appeared again in the urine and started to increase slowly,reaching almost the same intensity as before the adrenaline injection,at about ten hours after treatment. The urine flow rate had a fairly parallel curve to the radioactivity with the difference that urine volume decreased at a slower rate than radioactivity. On the other hand,after the urinary radioactivity started to increase again,the urine flow rate lagged behind,staying at a low level for more than eight hours.

Thus,it was obvious that the effect of adrenaline upon urinary radioactivity in suckers was completely different and even contrary to the effect of ACTH and therefore it could be concluded that ACTH was not acting as a stressor in this case.

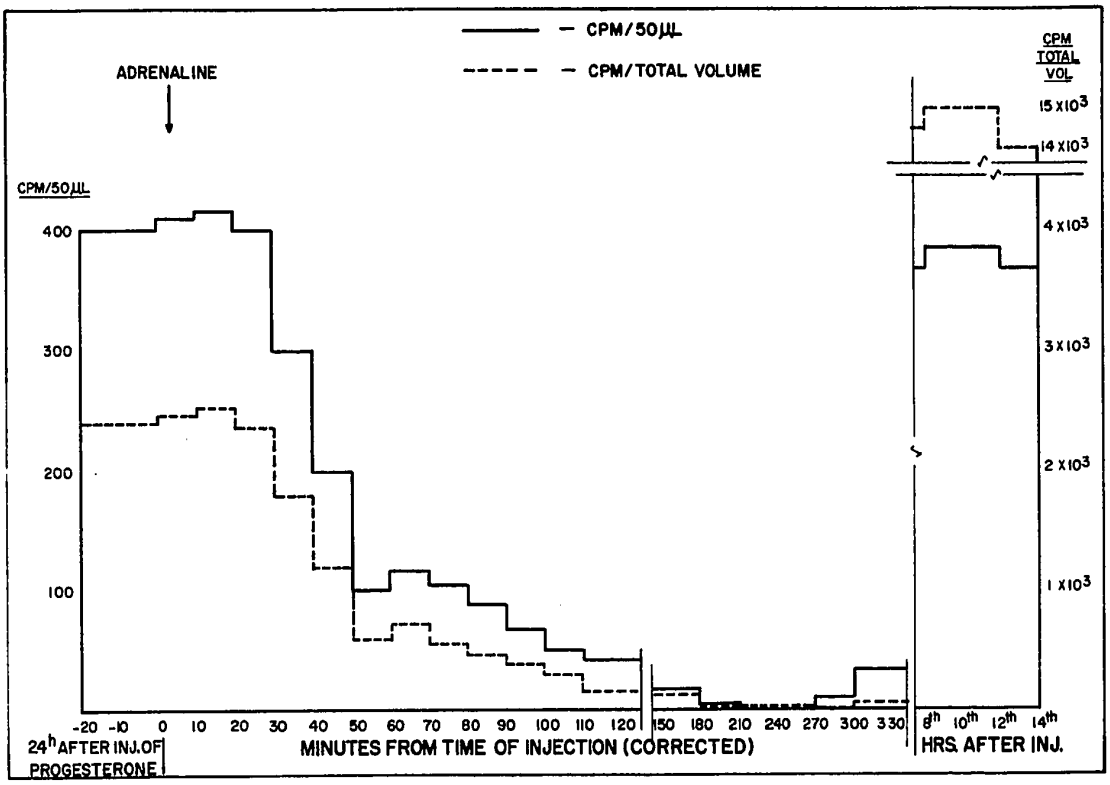


FIG.30

FIG.NO.30

URINARY RADIOACTIVITY AFTER INJECTION OF PROGESTERONE-^{3}H PLUS PROGESTERONE CARRIER FOLLOWED BY ADRENALINE ADMINISTRATION

Two female suckers were injected with 100 µc progesterone-^{3}H and 1 mg progesterone carrier each. Twenty four hours later 0.5 ml of a solution of 1:1000 adrenaline was injected into each of the two fish. (Appendix: M 3,N 6).

6. Experiments with ^{14}C-labeled sodium acetate.

Assuming that the metabolic pathways in fish are similar to those in higher vertebrates,I injected labeled sodium acetate to suckers, expecting to recover ^{14}C,from sodium acetate,incorporated in urinary steroid catabolites.

After administration of 80 μc of sodium acetate-^{14}C,urine was collected,pooled,extracted and purified as described in section II,6. The results are shown in Figs.31,32,33 and 34. In the first extract (Fig.31) two radioactive peaks were detected.The first one,at 28-30 cm from the base-line had no corresponding reference standard or chemically detectable spots. The second,showing higher activity,was located at R_f 0.93,a compound giving a positive DNPH reaction. In the second extract (Fig.32) two relative peaks were detected. One corresponding to compound SP_1 and the other corresponding to SP_2. The third extract (Fig.33) exhibited only a low relative peak,corresponding to the R_f of standard estriol,but there was no chemically detectable spot at that location. In the fourth extract (Fig.34) there was only very low activity detectable,concentrated in the lower half of the chromatogram(18-44 cm) and corresponding to a compound giving positive alkaline fluorescence with R_f 0.50 and a DNPH positive compound (R_f 0.63). Thus,it may be said that although there was some activity to be found in all four extracts,this activity was less than 0.01% of the injected amount. This suggested that the excretion of steroid catabolites, originating from acetate,is very low in urine,and that,therefore,it had to be concluded that either there is another excretory route for these catabolites or that acetate plays a very minor role in steroid biosynthesis in these fish.

FIG.NO.31

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION

Two female suckers were injected with 80 µc sodium acetate- ^{14}C each. The results are based on R_f values for system C chromatography, developed on Whatman # 1 paper, at 29-30°C. (Appendix: E 3, E10).

First extract

Abbreviations: B.L.= base line; S.F.= solvent front; B = corticosterone; E = cortisone; F = cortisol; E_1= estrone; E_2= estradiol; E_3= estriol; THE = urocortisone; Pdiol.= pregnanediol; Andr.= androsterone; SP_1= compound SP_1; SP_2= compound SP_2; DNPH = dinitrophenylhydrazine reaction; BT = blue tetrazolium reaction; NaOH = alkaline fluorescence reaction.

FIG.NO.32

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE- ^{14}C ADMINISTRATION

Second extract

Explanations and abbreviations as in Fig. No. 31.

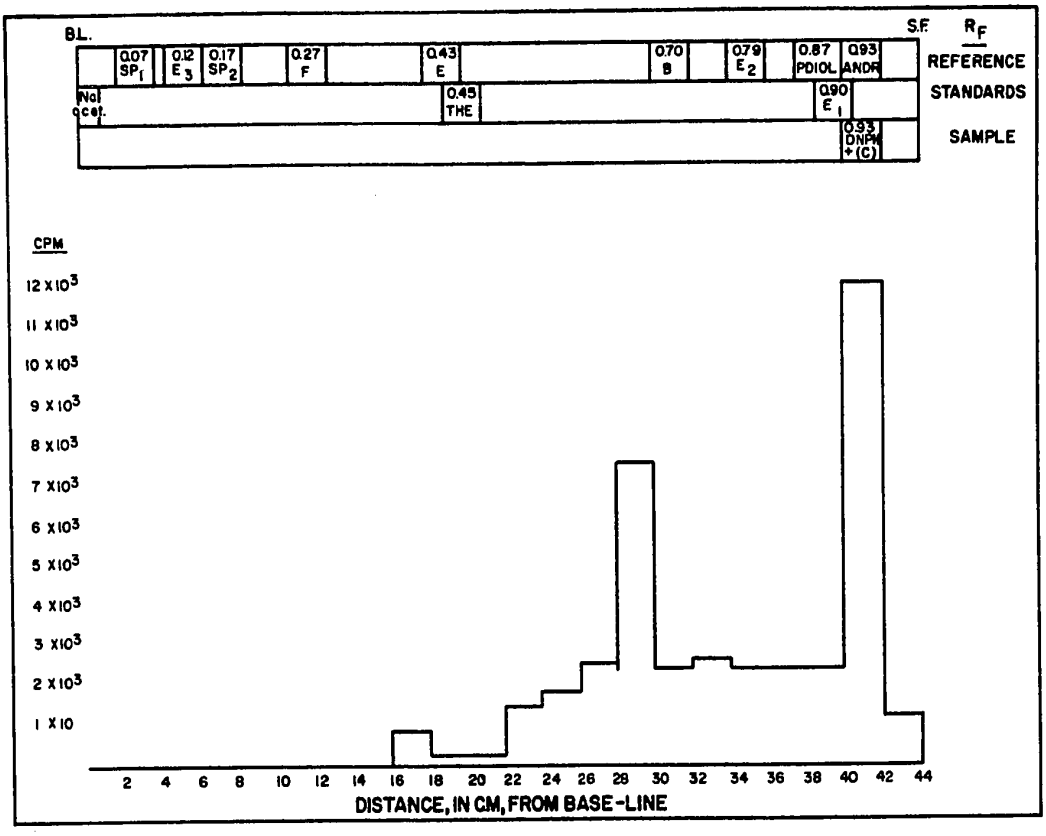

B.L.
S.F
R_F
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PDIOL
0.93 ANDR
REFERENCE STANDARDS
Nal acet.
0.45 THE
0.90 E1
0.93 DNPH + (C)
SAMPLE
CPM
12 X10³
11 X10³
10 X10³
9 X10³
8 X10³
7 X10³
6 X10³
5 X10³
4 X10³
3 X10³
2 X10³
1 X10
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 31

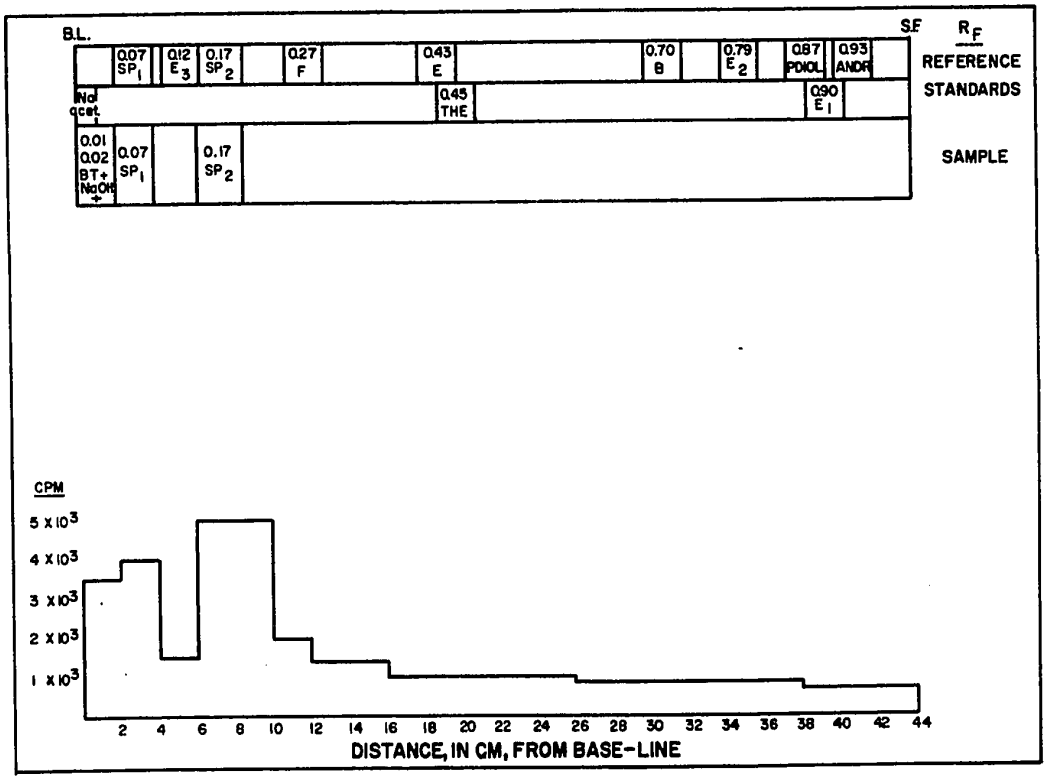

B.L.
S.F
R_F
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PDIOL
0.93 ANDR
REFERENCE STANDARDS
Nal acet.
0.45 THE
0.90 E1
0.01 0.02 BT + NaOH +
0.07 SP1
0.17 SP2
SAMPLE
CPM
5 X10³
4 X10³
3 X10³
2 X10³
1 X10³
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 32

FIG.NO.33

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION

Third extract

Explanations and abbreviations as in Fig.No.31.

FIG.NO.34

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION

Fourth extract

Explanations and abbreviations as in Fig.No.31.

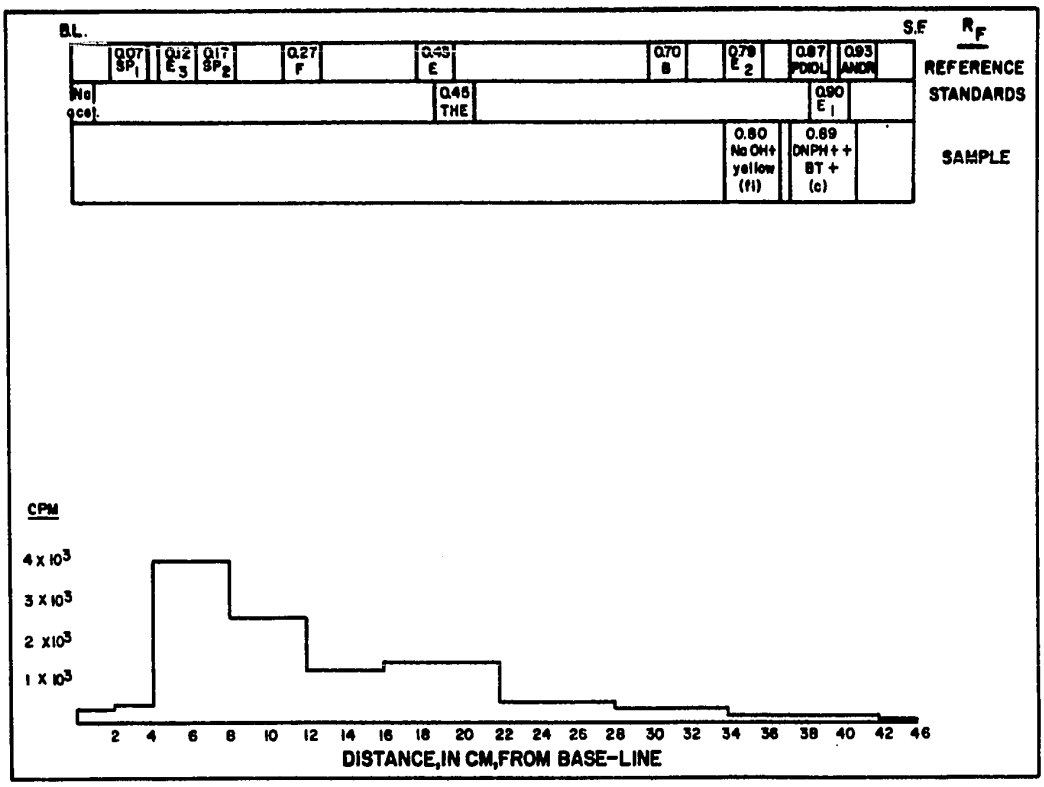

B.L.
S.F
R_F
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PDIOL
0.93 ANDR
REFERENCE STANDARDS
Na acet.
0.45 THE
0.90 E1
0.80 Na OH+ yellow (fl)
0.89 DNPH++ BT + (c)
SAMPLE
CPM
4 X 10³
3 X 10³
2 X10³
1 X 10³
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 46
DISTANCE, IN CM, FROM BASE-LINE

FIG. 33

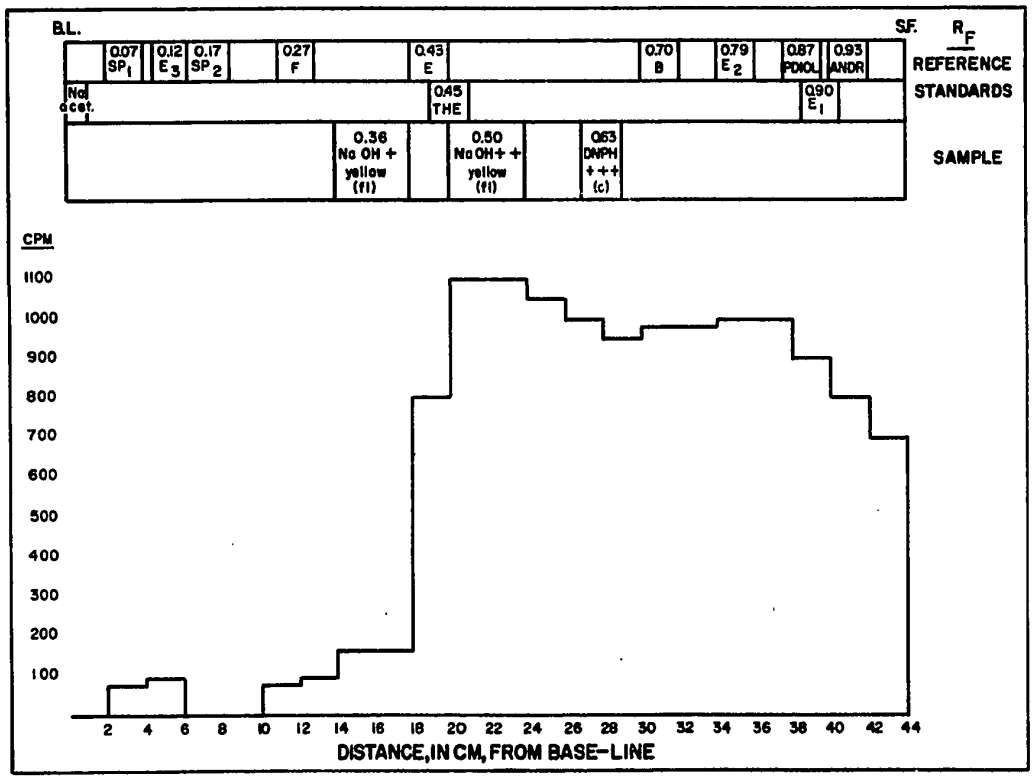

B.L.
S.F.
R_F
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PDIOL
0.93 ANDR
REFERENCE STANDARDS
Na acet.
0.45 THE
0.90 E1
0.36 Na OH + yellow (fl)
0.50 Na OH++ yellow (fl)
0.63 DNPH +++ (c)
SAMPLE
CPM
1100
1000
900
800
700
600
500
400
300
200
100
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 34

Based on the finding that ACTH had a stimulatory effect upon the radioactivity excreted after administration of labeled steroids,I thought that it may have a similar effect when injected after treatment with sodium acetate-^{14}C.

In Figs.35,36,37 and 38 are shown the steroid catabolites detected in sucker urine after administration of sodium acetate-^{14}C,followed by ACTH infusion. In the first extract (Fig.35) a radioactive peak was detected at the location corresponding to the R_f of SP_1 and SP_2,used in this case as reference standards,although no chemically detectable spot could be seen. One smaller peak was at R_f 0.88,corresponding to a compound giving a positive DNPH reaction. In the second extract (Fig.36) a prominent peak was detected at the location of SP_1 and SP_2 which were detected also chemically on the chromatogram strip. The third extract (Fig.37) exhibited only one,not very active,peak corresponding to R_f 0.09,a compound giving a positive BT reaction. The fourth extract exhibited only low activity with two relative peaks,one corresponding to R_f 0.07,a BT positive compound and the second at R_f 0.45,a compound showing alkaline fluorescence.

To make sure that the activity peak in the second extract was in fact due to the presence of compounds SP_1 and SP_2,the eluate of this portion of the chromatogram was rechromatographed in system C. The result is shown in Fig.39. Two active peaks could be clearly distinguished: one corresponding to R_f 0.07 (SP_1) and a second one at R_f 0.17 (SP_2).

Comparing the results obtained after administration of sodium acetate-^{14}C alone with those obtained after sodium acetate-^{14}C plus ACTH,it can be stated that ACTH had a stimulatory effect upon the

FIG.NO.35

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION FOLLOWED BY ACTH INFUSION

A female sucker was injected with 80 µc sodium acetate-^{14}C and two days later was started an infusion of 5 i.u. ACTH/Kg body weight/24 hours, for 5 days. The pooled urine from 30 days was extracted and purified. The results are based on R_f values for system B chromatography, developed on Whatman # 1 paper, at 26-27°C. (Appendix: E 8).

First extract

Abbreviations as in Fig.No.31.

FIG.NO.36

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION FOLLOWED BY ACTH INFUSION

Second extract

Explanations and abbreviations as in Fig.No.35.

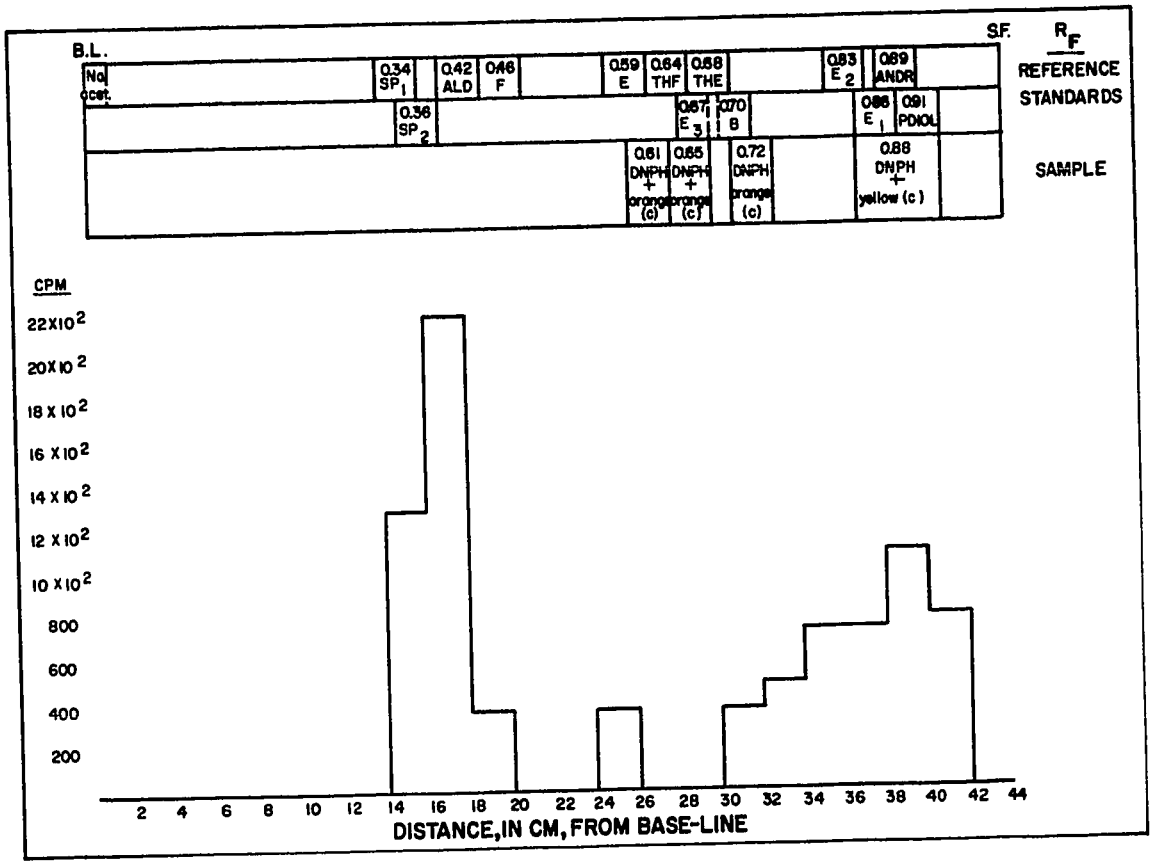

B.L.
S.F.
R_F
REFERENCE STANDARDS
SAMPLE
Na acet.
0.34 SP1
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E2
0.89 ANDR
0.36 SP2
0.67 E3
0.70 B
0.86 E1
0.91 PDIOL
0.61 DNPH + orange (c)
0.65 DNPH + orange (c)
0.72 DNPH orange (c)
0.88 DNPH + yellow (c)
CPM
22X10²
20X10²
18X10²
16X10²
14X10²
12X10²
10X10²
800
600
400
200
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 35

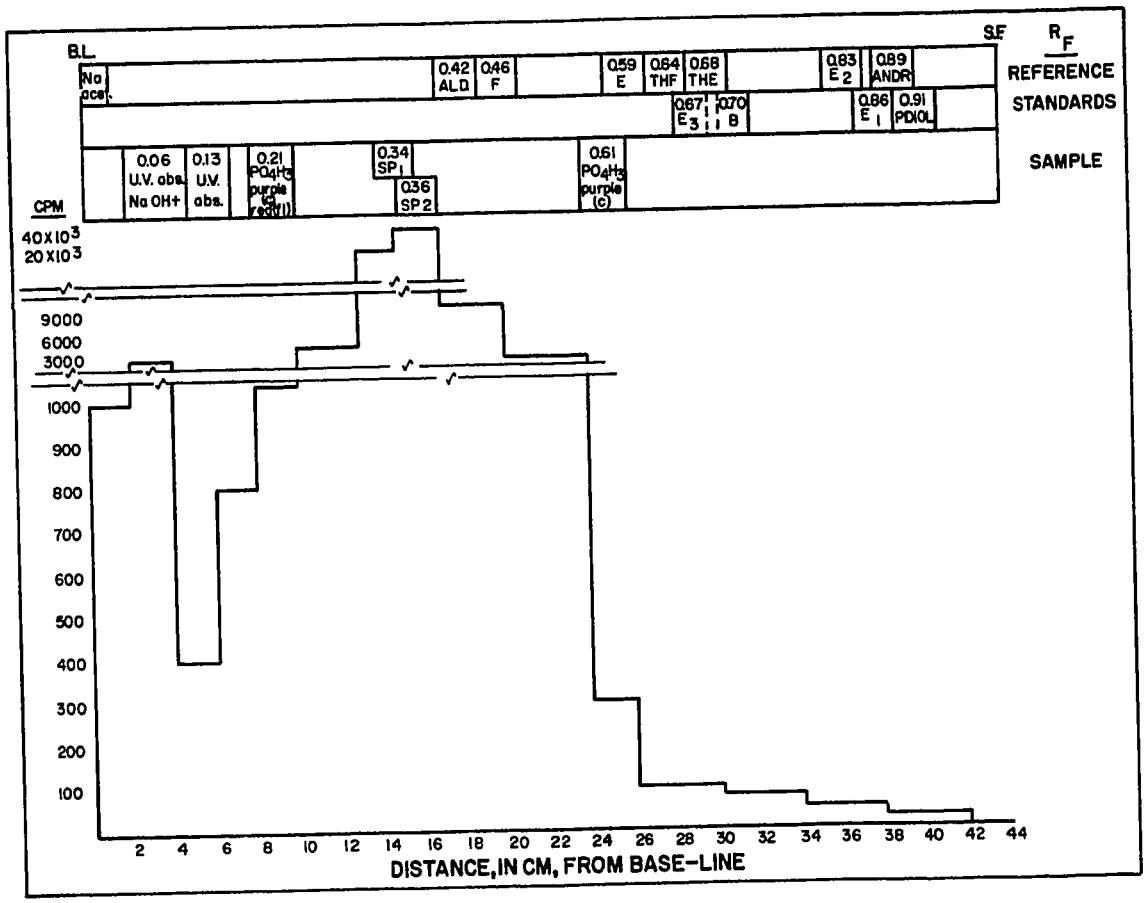

B.L.
S.F
R_F
REFERENCE STANDARDS
SAMPLE
Na acet.
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E2
0.89 ANDR
0.67 E3
0.70 B
0.86 E1
0.91 PDIOL
0.06 U.V. abs. Na OH+
0.13 U.V. abs.
0.21 PO4Hs purple (c)
0.34 SP1
0.36 SP2
0.61 PO4Hs purple (c)
CPM
40X10³
20X10³
9000
6000
3000
1000
900
800
700
600
500
400
300
200
100
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 36

FIG.NO.37

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION FOLLOWED BY ACTH INFUSION

Third extract

The results are based on R_f values for system C chromatography, developed on Whatman # 1 paper, at 29-30°C. Explanations and abbreviations as in Fig.No.35.

FIG.NO.38

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION FOLLOWED BY ACTH INFUSION

Fourth extract

Explanations and abbreviations as in Fig.No.35.

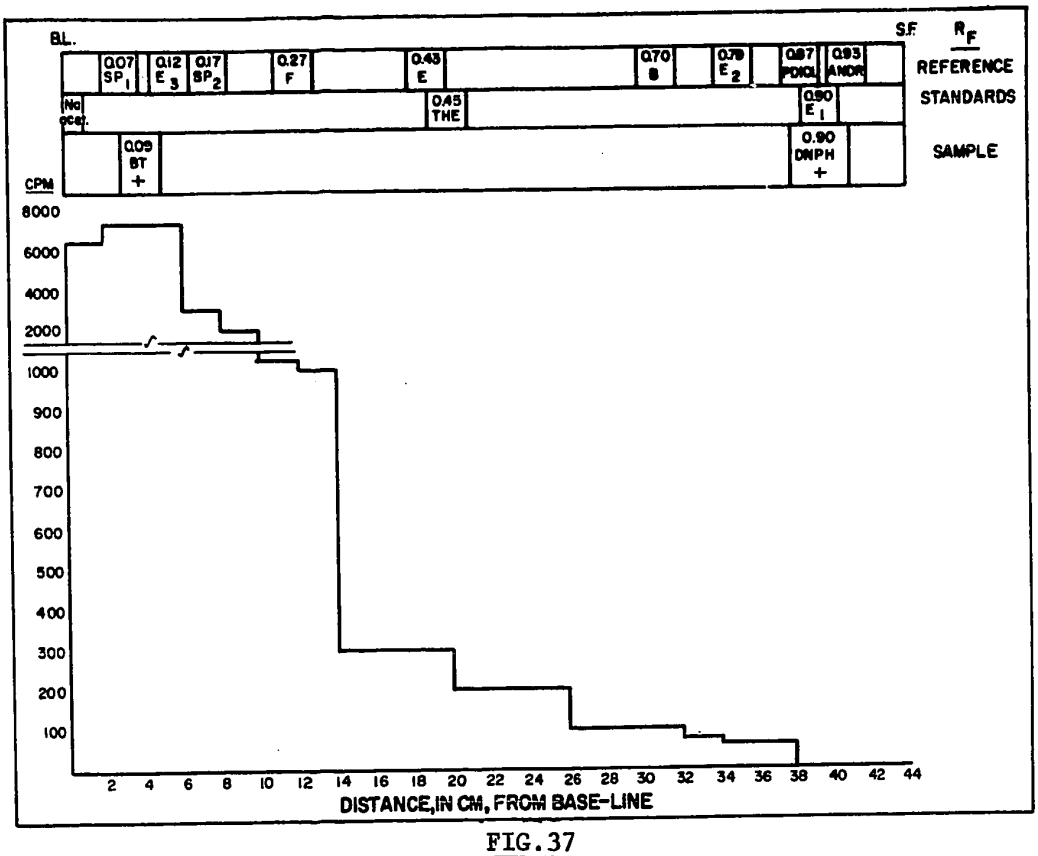

B.L.
S.F
R_F
REFERENCE
STANDARDS
SAMPLE
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.78 E2
0.87 PDIOL
0.93 ANDR
No ocat.
0.45 THE
0.90 E1
0.09 BT +
0.90 DNPH +
CPM
8000
6000
4000
2000
1000
900
800
700
600
500
400
300
200
100
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 37

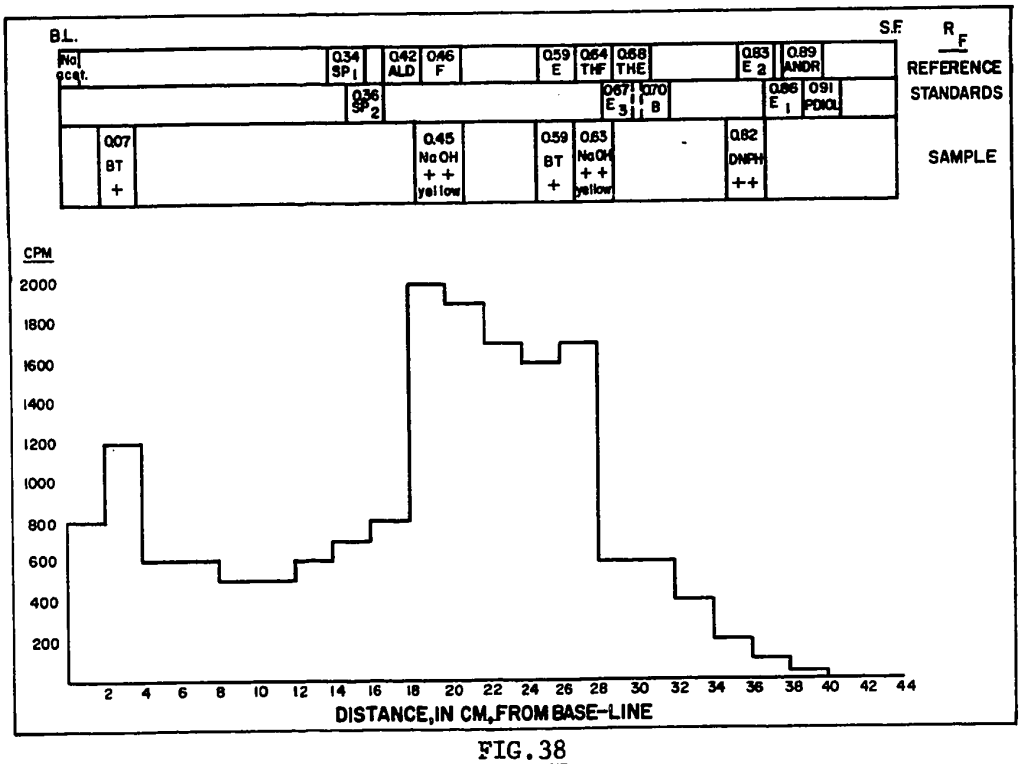

B.L.
S.F
R_F
REFERENCE
STANDARDS
SAMPLE
No ocat.
0.34 SP1
0.42 ALD
0.46 F
0.59 E
0.64 THF
0.68 THE
0.83 E2
0.89 ANDR
0.36 SP2
0.67 E3
0.70 B
0.86 E1
0.91 PDIOL
0.07 BT +
0.45 NaOH ++ yellow
0.59 BT +
0.63 NaOH ++ yellow
0.82 DNPH ++
CPM
2000
1800
1600
1400
1200
1000
800
600
400
200
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 38

appearance of steroid catabolites in urine of injected suckers as,for example,the detection of compounds SP_1 and SP_2 in the first extract,in contrast to fish treated with sodium acetate- ^{14}C alone,where these compounds were detected only in the second extract. ACTH had also an effect upon the amount of catabolites excreted,as could be seen in the second extract where the radioactivity was almost ten times higher for compounds SP_1 and SP_2 after ACTH administration.

Being given the possibility that ACTH acted as a foreign peptide rather than having a specific action,a control experiment was done, injecting TSH instead of ACTH. After administration of sodium acetate labeled with ^{14}C,urine was collected in separate test tubes,changing them every five minutes. Radioactivity appeared after 20 minutes (correcting for collection delay) and increased in intensity until it reached a plateau,55 minutes after injection (Fig.40). Forty minutes later,i.e. 95 minutes after administration of labeled sodium acetate, 10 i.u. lyophilized thyrotrophin (Thyrotron,Nordic Biochemicals Ltd.) were injected by way of the caudal vein cannula. The radioactivity in the urine did not change in intensity and the plateau remained the same. Two hours and 20 minutes later,i.e. four hours after injection of sodium acetate,10 i.u. ACTH were injected into the circulation. After five minutes the urinary radioactivity started to increase reaching a level of intensity almost double than the previous plateau.After one hour the urinary radioactivity started to decrease slowly.

This experiment demonstrated that ACTH was not acting as a foreign peptide but rather had a specific activity upon the amount of radioactivity excreted by the renal pathway.

FIG.NO.39

URINARY STEROID METABOLITES DETECTED AFTER SODIUM ACETATE-^{14}C ADMINISTRATION FOLLOWED BY ACTH INFUSION

Second extract

Portion 10-24 cm from system B, rechromatographed on system C, developed on Whatman # 1 paper, at 29-30°C. Abbreviations as in Fig.No.31.

FIG.NO.40

URINARY RADIOACTIVITY AFTER SODIUM ACETATE-^{14}C, TSH AND ACTH ADMINISTRATION

A female sucker was injected with 80 µc sodium acetate-^{14}C. One hour and 35 minutes later 10 i.u. of TSH were injected. Four hours from the time sodium acetate had been administered, 10 i.u. ACTH were injected to the same fish. Results are given in cpm/50 µl urine.

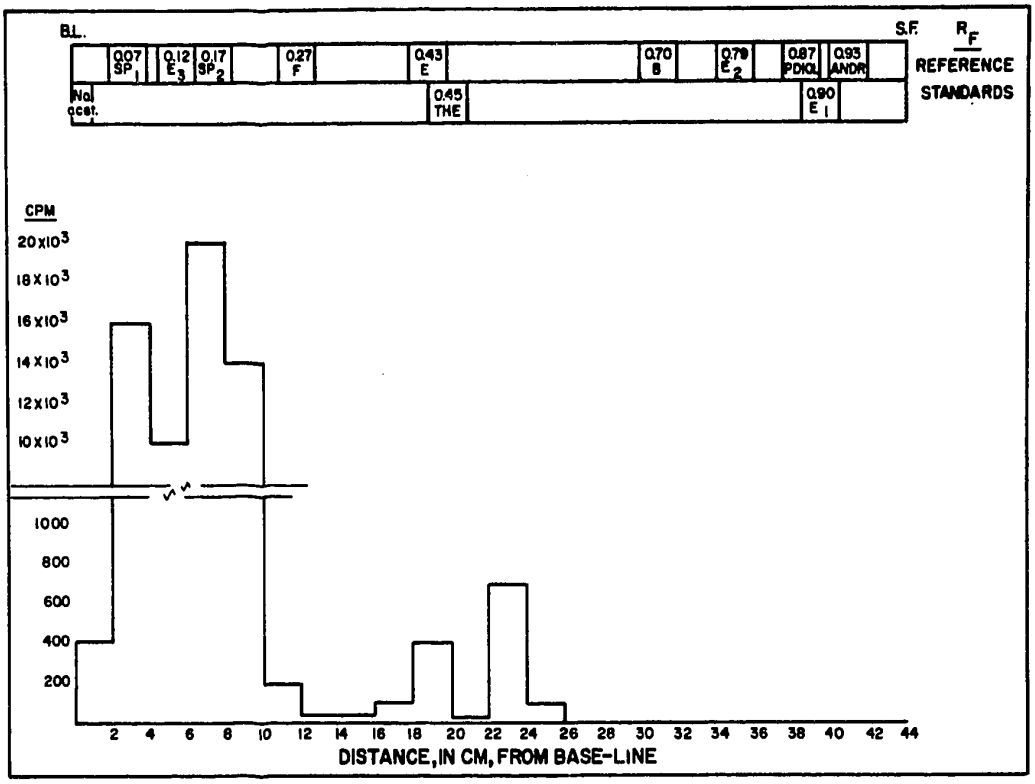

BL.
S.F.
R_F
REFERENCE STANDARDS
0.07 SP1
0.12 E3
0.17 SP2
0.27 F
0.43 E
0.70 B
0.79 E2
0.87 PDXL
0.93 ANDR
No acet.
0.45 THE
0.90 E1
CPM
20x10³
18x10³
16x10³
14x10³
12x10³
10x10³
1000
800
600
400
200
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
DISTANCE, IN CM, FROM BASE-LINE

FIG. 39

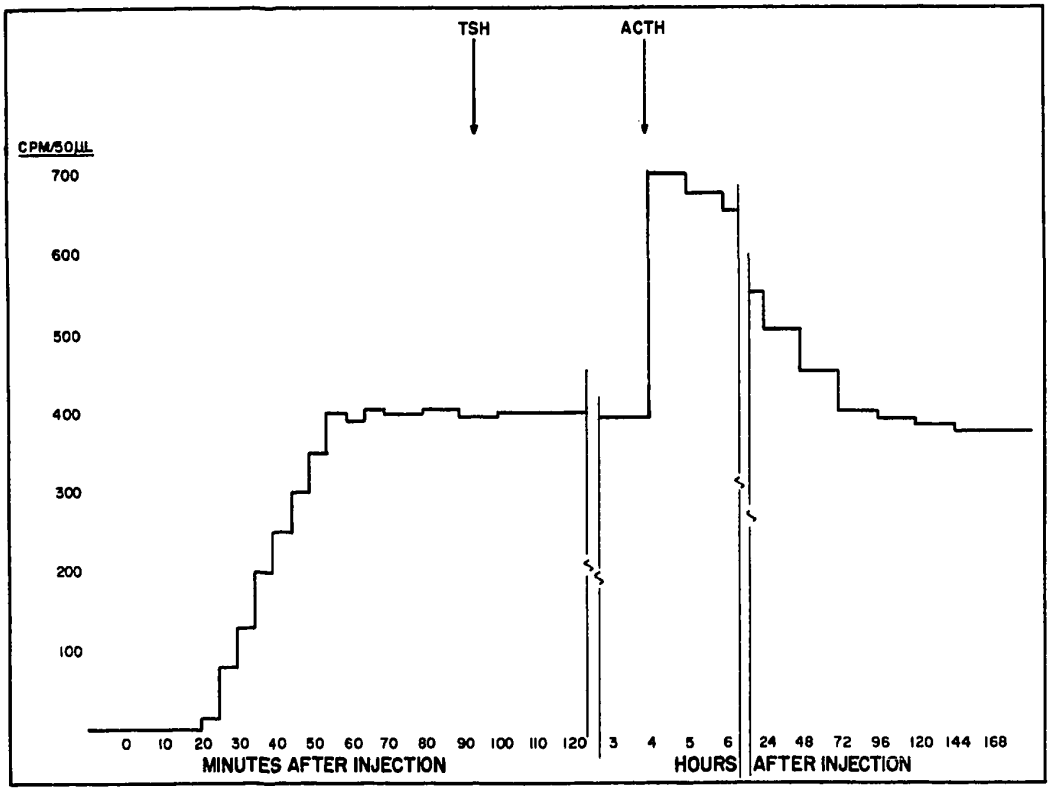

TSH
ACTH
CPM/50µl
700
600
500
400
300
200
100
0 10 20 30 40 50 60 70 80 90 100 110 120 3 4 5 6 24 48 72 96 120 144 168
MINUTES AFTER INJECTION
HOURS AFTER INJECTION

FIG. 40

7. Tissue radioactivity after administration of ^{3}H-labeled steroids.

Fishes used for detection of tissue radioactivity were killed 5-8 days after the injection of a particular, ^{3}H-labeled, steroid, by collecting the blood by heart puncture. The organs were cut out and dryed to constant weight. The tissues were processed as described in section II,6. The results (Table No.16) were studied in two ways: first by comparing the activity in different tissues of animals treated by the same compound and, secondly, by comparing the activity in the same kind of tissue of animals treated with different compounds. The various tissues having differing bulk in the body, the results were given as cpm/g tissue, thus expressing the concentration rather than the total radioactivity.

In fish treated with estradiol-17β-^{3}H plus carrier estradiol, the highest activity was found in the bile, medium activity was detected in the liver, gut and spleen, while low activity was present in the head kidneys, gills and gonads. Muscle tissue had no activity at all. This suggested that the major part of estrogen metabolites were concentrated in the entero-hepatic region and that the renal and branchial routes were certainly not the main excretory pathways for phenolic steroids. The same appeared to hold true also for fish treated with: progesterone-^{3}H, testosterone-^{3}H and cortisone-^{3}H, each with its respective carrier steroid.

A comparison of the same kind of tissue, in fish treated with different compounds, showed that the bile was most active after the administration of estradiol-17β-^{3}H, the liver and gut had highest activity after testosterone-^{3}H while the spleen was most active after

TABLE NO.16

TISSUE RADIOACTIVITY OF FISH TREATED WITH ^{3}H-LABELED STEROIDS

The fish were injected with 100 µc of ^{3}H-labeled steroid with or without added carrier steroid (1 mg),or with 15 i.u. ACTH/Kg body weight. The fish were killed 5-8 days or one hour after the treatment. H.K.=head kidneys. (Appendix: Estradiol-^{3}H+carrier: L 6,L 9,N 4;

Progesterone-^{3}H+carrier: M 2,M10,N 3;

Testosterone-^{3}H+carrier: N 2,N 7,N 9;

Cortisone-^{3}H+carrier: L 3,L 4,L 5,L 8;

Progesterone-^{3}H + ACTH: M 4,M 6,M 7;

Testosterone-^{3}H: N 1,N 5;

Cortisone-^{3}H: P 5;

Cortisone-^{3}H + ACTH: P 6).

TABLE NO.16

Treatment	Radioactivity cpm(x 10^3)/g tissue									cpm(x 10^3) /50 µl
5-8 days after treatment	Bile	Liver	Gut	Spleen	H.K.	Kidneys	Gills	Gonads	Muscle	Plasma
Estradiol-^{3}H+ Estradiol	5540 5420-5660	294 272-316	187 151-223	118 110-126	10 9 -11	10 8 -12	2 1.5-2.5	5 3-7	0	-
Progesterone-^{3}H +Progesterone	900 848-952	78 70-86	260 245-275	30 28-32	6 5.6-6.4	7 6.8-7.2	3 2.9-3.1	2 1.8-2.2	3 2.8-3.2	-
Testosterone-^{3}H +Testosterone	720 717-724	320 315-326	1004 989-1023	1 0.8-1.2	4 3-5	4 3.5-4.5	2 1.8-2.3	4 3.9-4.1	1 0.9-1.1	74 73-75
Cortisone-^{3}H +Cortisone	1700 1648-1738	28 26-30	337 311-370	13 12-14	37 35.5-38	6 5-7	37 35.6-38.6	2 1.7-2.1	1 0.9-1.0	20 17-23.5
Progesterone-^{3}H +ACTH	383 378-388	61 58-64	226 200-252	38 32-44	25 22-28	43 39-47	40 35-45	17 14-20	23 22-24	28 27.8-28.2
Testosterone-^{3}H	900 851-949	62 60-64	265 259-271	37 33-41	22 20-24	21 20-22	10 9.5-10.5	8 6-10	10 9 -11	78 77-79
One hour after treatment										
Cortisone-^{3}H	180	1110	75	147	136	472	180	10	34	336
Cortisone-^{3}H +ACTH	195	870	89	106	148	471	180	11	30	270

estradiol-^{3}H treatment. It appeared that the concentration of a particular steroid in the kidneys was higher without added carrier than with carrier steroid and that ACTH administration resulted in a shift from excretion by the entero-hepatic pathway to the renal route.

From the point of view of total activity,muscle tissue was most important,because even small concentrations sum up to appreciable amounts due to the bulk of this tissue in the body. In the case of estradiol-^{3}H, no radioactivity could be detected in muscle,suggesting a rapid turn-over and excretion of this steroid,none being stored in muscle tissue. This was supported also by the high activity found in the bile.The presence of carrier steroid seemed to prevent muscle storage. This could be explained partly by the shift from the branchio-renal excretory pathway to the entero-hepatic pathway,partly by the fact that excretion was much intensified over the excretory rate without carrier steroid. On the other hand,ACTH seemed to favor muscle storage of steroids.

If we consider the radioactivity detected in the various tissues after a short-term experiment,i.e. 60 minutes after treatment with cortisone-^{3}H,it appeared that the highest concentration was present in the liver and the kidneys,while the activity in bile and gut was relatively low. The plasma concentration was much higher than in the long-term experiments and the same was true for the gills and the head kidneys. This seemed to suggest that although the catabolic processes were localized in the liver,the metabolites were excreted by the branchio-renal pathway,for the first hour. In time, the excretory pathway was shifted and the entero-hepatic route became predominant.

IV. DISCUSSIONS

According to the classical definition,hormones are biologically active compounds produced in endocrine structures,from which they are released directly into the blood stream. Or,by other words,hormones are chemical compounds produced by one tissue with the primary function of exerting a specific effect of functional value on another tissue. But the various definitions have to be considered critically,because they exclude metabolites,formed outside the glands or tissues of origin, many of which still possess biological activity. It is therefore quite difficult to distinguish sharply between "true" hormones and metabolites which may well arise in the blood and exercise important regulatory functions in tissue metabolism without being "true" hormones.

In this connection and on the basis of our present knowledge, even the usual classification of steroid hormones into male and female gonadal hormones and adrenal hormones is not correct,because each of these hormones could originate in the adrenals,the ovaries or the testes. Thus,the only unifying characteristic of the mentioned hormones is their steroid nature.

The general purpose of the steroid hormones is not to maintain a constant internal medium against environmental changes but to cause

definite changes in the internal biochemical processes which would be beneficial to the continued existence of the species. To relate the secretion of these hormones to a need,which requires changes in the internal medium rather than constancy,the vertebrates have developed a control system of steroid output by releasing pituitary "trophic" hormones which are under the direct command of the hypothalamic releasing factors and,thus,under the influence of the central nervous system and,implicitly,the external environment.

Steroids in fish are produced mainly by two tissues: the gonads and the adrenocortical tissue,known in fish as interrenal gland. But there is as yet no agreement concerning the exact locus of steroid hormone production.

The interrenal tissue of teleosts varies considerably not only amongst species but also in individuals of the same species. In Catostomus,areas of interrenal tissue are scattered randomly throughout the lymphoid tissue of the head kidneys with the bigger islets found around the branches of the cardinal veins. The interrenal cells contain large,rounded,basophilic nuclei with acidophilic cytoplasm and may be readily distinguished from the smaller lymphoid cells with basophilic cytoplasm. The head kidneys of the white sucker vary in size, ranging between 1-3 g (for one half),the two halves(one from either side) being sometimes equal and sometimes unequal in size. I found that the head kidney/somatic index(x 1000),based on the average net weight of one half head kidney,was fairly constant (1.7 ± 0.2) for both males and females and not subject to seasonal variations.

The most comprehensive review of the distribution and form of the teleost interrenal gland is that of Nandi(1962).

Until recently it was assumed that the teleost interrenal does not possess the properties thought to be characteristic of the eutherian adrenocortical tissue(Rasquin,1951). However,Chavin and Kovacevic(1961) reinvestigated these negative findings,which seemed at variance with the reported physiological activity of this tissue,and found that the histochemical reactions of the interrenal cells in the goldfish, Carassius auratus,are comparable to those of the zona fasciculata and zona reticularis of the mammalian adrenal cortex. They showed that exposure to high temperature had a marked effect on both the cytoplasm and the nucleus of the interrenal cells of the goldfish,producing a state of exhaustion in large areas of the tissue.

For many years the problem if and what kind of gonadal steroids are present in fish was a controversial subject. There was only circumstantial evidence to indicate that the lower vertebrates were not much different,in this respect,from the more evolved ones. Knowles(1939) investigated the effect of injected testosterone,estrone and mammalian anterior lobe pituitary extract into immature adult lampreys and ammocoete larvae. He found that whereas sex steroids were more effective in the adults,in the ammocoetes the pituitary extracts had a greater effect in inducing changes in secondary sexual characters. Botticelli et al.(1963) identified estradiol,estrone and progesterone in the ovaries of Petromyzon. Chieffi and Lupo(1961) found testosterone, androstenedione,progesterone and estradiol in the testes of Scyliorhynus stellaris and Simpson,Wright and Gottfried(1963) detected androsterone, androstenedione,dehydroepiandrosterone,pregnenolone and progesterone in the semen of Squalus acanthias. An unusual finding was that the semen of this fish contained a high concentration of deoxycorticosterone.

Wotiz et al.(1960) showed that estrone and progesterone occur in the ovaries of Squalus suckleyi. Simpson,Wright and Hunt(1963) found that over 75% of the hormone content,i.e. estradiol and estrone,of the ovary of Scyliorhynus caniculus is localized within the eggs. The concentration of hormones in the eggs remains constant during maturity and thus,the total amount increases as the ovary develops. These workers (Simpson, Wright and Hunt,1963) have also found free estradiol and estrone in the blood plasma of this elasmobranch. Chieffi and Lupo (1963) have reported the finding of estradiol,estriol and progesterone in the ovaries of Torpedo marmorata. But it was only very recently that testosterone was isolated from the blood of elasmobranchs by Idler and Truscott (1966). They succeeded to isolate and identify testosterone in the blood of Raja radiata and R.ocellata.

The endocrinology of the gonadal hormones in teleosts was reviewed by Hoar (1957,1965),by Pickford and Atz (1957),by Dodd (1955, 1960),by Forbes (1961) and by Bern and Nandi (1964). All these reviews contain data which demonstrate unequivocally the dependence of the sex characters of teleosts on the gonads.

The first indications that teleost testes produce androgens were those of Hazleton and Goodrich (1937) and Potter and Hoar (1954) who prepared extracts of salmon testes which showed male hormone activity when bioassayed. Chieffi (1962) and Chieffi and Botte (1962) identified estriol,estrone,estradiol and progesterone but not testosterone in the teleost Morone labrax. Idler and his co-workers subjected the sockeye salmon,Oncorhynchus nerka,and other species of salmon to a thorough biochemical study,identifying testosterone in the testes and plasma, correlating the hormone titer with the state of maturity of the fish

(Idler and Tsuyuki,1959; Grajcer and Idler,1961,1963; Idler et al.,1961 b). A very important finding was the isolation of 11-ketotestosterone from post spawned male sockeye salmon by Idler et al.(1960 b). The identification of this steroid,with characteristics which may be indistinguishable from those of corticosterone,resolved many of the apparent contradictions which arose during investigations concerning the plasma steroids in teleosts.

In the past,the chemical nature of the female hormones in fish was uncertain (Hoar,1955; Pickford and Atz,1957; Emmens,1959). But more recent work has demonstrated the steroid nature of the ovarian products of fish and their essential similarity to those of mammals. The mature ova of the cod,Gadus calarias,have been shown,by Gottfried et al.(1962), to contain estradiol and estrone while none occured in immature ova. Estrone,estradiol and estriol have been demonstrated in the ovaries of the conger eel (Lupo and Chieffi,1963). Progesterone has been identified in teleost fish by Lupo and Chieffi (1963). Reinboth (1962 a, b) and Reinboth and Simon (1962) studied the endocrinology of hermaphroditic fishes. The results indicated an interplay of pituitary and gonadal hormones in the differentiation of their gonads and the timing of reproduction. To elucidate the biochemical aspects of this work, Reinboth,Callard and Leathem (1965) subjected ovarian tissue from the amphisexual teleost Centropristes striatus to incubation with ^{14}C-labeled progesterone or testosterone. The results showed that with progesterone-^{14}C as precursor,only pregnane-3,20-dione and progesterone itself was obtained. After incubation with testosterone-^{14}C, several compounds were observed in the alkaline as well as in the nonphenolic fractions. Investigation of the nonphenolic fraction revealed the

presence of: 17β-ol-5β-androstane-3-one, 5β-androstane-3α,17β-diol and 5-androstane-3,17-dione.

Although the present information on the steroid content of adrenocortical tissue in higher vertebrates is extensive,there has been done but little work on extracts obtained from fish. Fontaine and Hatey (1954), Bondy et al.(1957) and Phillips and Chester-Jones (1957) demonstrated the presence of glucocorticoids in fish blood. In 1959, Fontaine and Leloup-Hatey provided direct evidence for the presence of adrenocorticosteroids in the teleostean interrenal tissue. They found that the interrenal of salmon contains significant amounts of corticosteroids,cortisol being present in larger amounts than corticosterone. Phillips,Holmes and Bondy (1959) extracted and analyzed almost 9000 ml of blood from 364 male sockeye salmon. They found corticosterone, cortisone and cortisol,in this decreasing order,and believed to have detected also small amounts of aldosterone. Searching for evidence that these corticosteroids originated in the cells of the interrenal, Phillips and Mulrow (1959) incubated head kidney of Fundulus heteroclitus in media containing added tritiated progesterone. They found that the interrenal tissue of killifish possesses enzyme systems enabling this tissue to convert progesterone into cortisol,cortisone and aldosterone.

Idler,Ronald and Schmidt (1959) isolated besides cortisone and cortisol also 17-hydroxyprogesterone from plasma of prespawning sockeye salmon. In a similar way Idler,Fagerlund and Ronald (1960) identified pregn-4-ene-17α,20β-diol-3-one from sockeye salmon plasma. Five species of teleosts were studied by Nandi and Bern (1960) who found that the interrenal tissue of Salmo gairdnerii produced mainly cortisol and small

amounts of cortisone and corticosterone while Mugil cephalus yielded cortisol and cortisone. Anoplopoma fimbria produced only cortisol, Tilapia mossambica only cortisone and no corticosteroids could be detected in Bodianus bilunulatus, despite the use of adequate volumes of interrenal tissue. Idler, Schmidt and Bitners (1961) succeeded to isolate and identify adrenosterone in sockeye salmon plasma and later also 20β-dihydrocortisone, from the same species (Idler, Schmidt and Ronald, 1962). Bern et al. (1962) identified corticosterone, cortisol and aldosterone in the media of incubated interrenal tissue from three elasmobranch species. Corticosterone and cortisol were detected in a large volume of serum from the atlantic hagfish, Myxine glutinosa, but no aldosterone was found (Phillips et al., 1962).

Sex-dependent differences in amounts of corticosteroids in various species of fish could be demonstrated by Phillips (1959). For instance in the lamprey, Petromyzon marinus, the amount of cortisol was almost double in the male than in the female, but corticosterone was somewhat higher in the female. In the bull shark, Carcharinus leucas, on the contrary, the amount of cortisol was three times higher in the female than in the male, while the amount of corticosterone was equal in both sexes. In the white sucker, Catostomus commersonii, there was more than double the amount of cortisol in the male than in the female while the situation was reversed in the case of corticosterone. In the long nosed sucker, Catostomus catostomus, on the other hand, the amount of cortisol was only slightly higher in the male but there was three times more corticosterone in the male than in the female. A similar sex-dependent difference was shown by Idler, Freeman and Truscott (1964) for cortisol in Atlantic salmon plasma, the amount in the male being three times higher, but in

contrast to the findings of Phillips (Phillips,1959),they were able to demonstrate that the major plasma steroids are the 17-hydroxysteroids with a dihydroxyacetone structure in the side chain,i.e. cortisol and cortisone. There was no indication of the presence of large quantities of steroids lacking the hydroxyl group at C-17.

Butler (1965) reported results obtained by incubating interrenal tissue of Anguilla and Conger with ^{14}C labeled progesterone. Anguilla interrenal tissue produced cortisol,cortisone and 11-deoxycorticosterone (DOC),while Conger eel interrenal yielded cortisone and corticosterone. No aldosterone was detected in either species.

In Eutheria steroid biosynthesis starts with acetate which is converted,by the liver,into cholesterol. As a general rule there is very little squalene or other sterol precursors of cholesterol in the liver. An exception to this rule is found in the shark and related species of fish (Heller et al.,1957). Shark liver contains huge amounts of squalene in the fall. By contrast,the liver in early spring is smaller and contains only a small fraction of squalene found in the fall. But nothing is known about the fate of this squalene and how it is utilized by the animal. Considering the potential amount of energy that the total oxidation of squalene could produce,it must be concluded that the metabolism of squalene via lanosterol and cholesterol would be very inefficient for energy production,and it seems more likely that this large reserve of squalene is degraded by reactions which do not result in the formation of cyclic sterols.

Whether cholesterol itself is an obligatory intermediate in the biosynthesis of steroids is still subject to debate. Although cholesterol has been shown to serve as precursor of steroid hormones (Zaffaroni et

al.,1951; Werbin et al.,1957; Ungar and Dorfman,1953),and enzyme systems which split isocaproic acid from cholesterol,leaving the C_{21} steroid pregn-5-ene-3β-ol-20-one(i.e. pregnenolone),have been demonstrated in the steroid producing tissues (Staple et al.,1955; Lynn et al.,1954), the question is whether this is the only possible sequence of reactions. Certainly pregnenolone can give rise to all of the steroids,but is it not possible that there may be another compound which may be converted to steroids without involving cholesterol? Hechter et al. (1953) perfused bovine adrenal glands with blood containing either acetate-1-^{14}C or cholesterol-4-^{14}C and isolated cortisol from the perfusate. When acetate was the labeled precursor,cortisol had a much higher specific activity than either the plasma or tissue cholesterol. When labeled cholesterol was perfused,however,the specific activity of cortisol was lower than that of any of the cholesterol fractions. On the other hand, the problem can be taken further backward by discussing whether acetate is necessary for steroid biosynthesis. At present it becomes more and more accepted that there is more than one pathway in steroid biosynthesis and that compounds like zymosterol (Schwenk et al.,1955),desmosterol and 24,25-dihydroxyzymosterol are more likely to be the precursors of steroids than cholesterol.

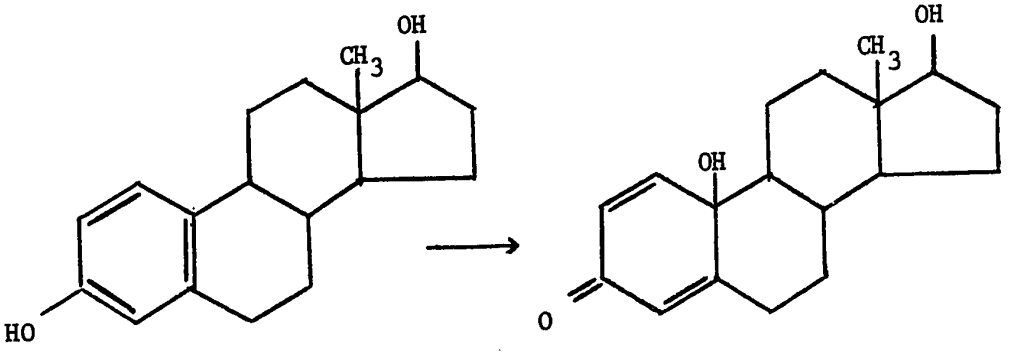
In the light of the reports mentioned above,it appears that the present study demonstrates the fact that,although acetate could be used by the white sucker for steroid biosynthesis,nevertheless it is treated almost as an indifferent compound from the point of view of steroid metabolism. This conclusion was supported by the work of Schwenk et al. (1955),implying that there is a much lower rate of sterol formation from acetate in fish than in mammals,and also by the paper of Blondin

et al. (1966) who demonstrated that mevalonate is more specifically used in fish for sterol synthesis than is acetate; the yields of non-saponifiable materials from incubation of freshwater bass liver homogenates with mevalonic acid-^{14}C were almost double at 35°C than at 25°C, and the incorporation of ^{14}C from mevalonic acid-^{14}C into lipid material was ten times less with fish than with mammalian (rat) liver homogenates. The temperature dependence of the conversion rate of mevalonic acid into sterols suggests strongly that the poikilothermic nature is the primary cause of different biosynthetic pathways in fish. It seems that the low environmental and body temperatures, optimal for many fishes, including the white sucker, makes it necessary to use a different biosynthetic pathway, capable of a relatively high production rate of steroids even at temperatures which would reduce to practically zero the steroid output by the biosynthetic pathways of homeotherms. Sandor et al. (1966) was able to show that incubation of eel, Anguilla anguilla, head kidneys with progesterone-^{14}C, yielded cortisol and cortisone but no identifiable corticosterone. No corticosteroids could be isolated after incubation of the same material with sodium acetate-^{14}C. By incubating the eel head kidney preparation with corticosterone-^{3}H, they obtained labeled cortisol, a relation which was never encountered in higher vertebrates. Furthermore they postulated the tentative pathway: pregnenolone → 17α-hydroxypregnenolone → 17α-hydroxyprogesterone → cortisol → cortisone, showing that progesterone is not an obligatory intermediary in fish. These findings are in accordance with the results of the present study, with the exception that in the white sucker the biosynthetic step cortisol → cortisone could not be demonstrated, because compounds SP_1 and SP_2, proven catabolites of cortisone, could not be

detected after cortisol administration,implying that cortisol,in this case,was not converted into cortisone. Idler and Truscott (1963) reported the probable biosynthetic pathway of 11-ketotestosterone in the sockeye salmon. This steroid,which had not been isolated from any living source before the mentioned report,was shown to originate from 17α-hydroxyprogesterone via androstenedione and testosterone or via Reichstein's substance S (17α-hydroxy-11-deoxycorticosterone) and cortisol. This indicates that there are biosynthetic pathways in fish which may be very different from those in the Eutheria.

Some of the major reactions carried out by the liver are the reduction of the Δ^4-3-one group and the conjugation of steroids as glucuroniside uronidates and sulfate esters. No such conjugation occurs in hepatectomized or eviscerated animals (Berliner and Wiest,1955; Berliner et al.,1958). The liver is the major site of both the changes in the steroid molecule and its combination with acidic groups. Steroids, therefore,leave the liver in more water-soluble form and are more easily eliminated from the body. In this organ the major reactions are reductive,particularly in ring A of the steroid nucleus. The phenolic hydroxyl of estradiol readily conjugates with glucuronic acid,although the benzene ring usually cannot be reduced by hepatic enzymes of higher vertebrates. But it has to be assumed that in the white sucker the benzene ring underwent reduction,on the ground that after a pharmacologic dose of estradiol-17 β no phenolic compounds could be detected in the urine,although some were seen in the urine of untreated fish. The possible catabolic pathway of estradiol-17β in suckers could be the following: enzymically activated oxygen attacks the phenolic hydroxyl group; the resulting phenoxy radical of estradiol may react with a

suitable acceptor. Interaction with a hydroxyl radical leads to a hydroxylated product. Thus the urinary metabolite could be a compound like 17β-hydroxy-estra-p-quinol (Hecker and Zayed,1961).



ESTRADIOL - 17β

17β -HYDROXY-ESTRA-p-QUINOL

On the reduction of the Δ^4-double bond the 5α and 5β isomers of the various steroids are formed. Apparently there is a whole series of highly specific Δ^4-5β-hydrogenases in the supernatant obtained after high-speed centrifugation of liver homogenates (Tomkins,1956) and Δ^4-5α-hydrogenases in the liver microsomes (McGuire and Tomkins,1959). This fact could explain the rather unusual characteristics of compounds SP_1 and SP_2,assuming that the Δ^4-3-one structure in ring A of cortisone is reduced to a 5α or β-3-one form. Because of the high specificity of the respective hydrogenase even as closely related a steroid as cortisol would not be reduced (Tomkins,1956). On the other hand,it is doubtful if sucker tissues contain the 11 -hydroxysteroid dehydrogenase,another microsomal enzyme,which usually catalyzes the readily reversible reaction: cortisol $\rightleftarrows$ cortisone (Hurlock and Talalay,1959). The problem of the immediate precursor of cortisone,in suckers,remains open for investigation. It could not be progesterone because this compound did

not yield detectable amounts of the two main urinary catabolites,i.e. SP_1 and SP_2,after intravenous administration.

On the basis that aldosterone was never detected in vivo in fish blood and no compound could be found in sucker urine to suggest the presence of aldosterone or one of its catabolites,the hypothesis is put forward that 18-hydroxy-Δ^4-3-one and/or 19-hydroxy-Δ^4-3-one steroids might represent the metabolites of a compound with mineralocorticoid activity which could substitute for aldosterone in fish. This view is substantiated by the work of Ulick and Kusch (1960) who found 18-hydroxy-corticosterone in the bullfrog adrenals and were able to extract it from the adrenal tissue of Rana catesbiana after incubation with progesterone.

It is possible that many of the unanswered questions in fish steroid metabolism will be resolved by studying the steroid conjugates not as end-products but as biosynthetic precursors. Evidence,that steroid sulfates may be further metabolized as the conjugated steroid, was presented by Baulieu et al. (1963). Lebeau and Baulieu (1963) investigated the sulfo-conjugation of corticosteroids by the adrenal gland,while Adams (1963) was able to show that cell-free adrenal extracts can induce sulfo-conjugation in estrogens and androgens. Thus,a new approach was opened,showing that steroids can be secreted as conjugates and may undergo a completely different metabolic process than as free steroids.

The routes by which the major portions of the steroid metabolites are excreted,differ among animal species. The carnivores,herbivores and rodents excrete most of the steroid metabolites by way of the bile and, apparently,the same holds true for fish,as indicated by the present

investigation. The kidney,although glomerular in structure,is not used as the main pathway for steroid metabolite excretion. It seems that the kidney of freshwater teleosts is rather an osmoregulatory organ with the main task to rid the body of excess water while minimizing salt loss, than an excretory organ for wastes and metabolic products. This view is substantiated by Fromm (1963) who reported that although starved rainbow trout excreted an average of 136 mg nitrogen/kg body weight/day, only 3% of the total waste nitrogen was excreted via the kidneys.

Most investigators,speculating on the mode of action of steroids, whether they believe the effect to be achieved by the activation of an enzyme,by an alteration in the permeability of a membrane or by serving as a coenzyme in some particular reaction,have emphasized the binding of the steroid to a protein either as an essential part of the mechanism of action of the hormone or as an essential preliminary step in that action. Daughaday (1956) and Bush (1957) demonstrated independently the presence in human plasma of a specific corticosteroid-binding globulin, different from albumin. Slaunwhite and Sandberg (1959) obtained experimental data which could be interpreted only with the assumption of a special plasma protein,"transcortin",complexing with corticosteroids more firmly than albumin does (Sandberg and Slaunwhite,1959).

For many years the concept has prevailed that protein binding in the blood would be a mechanism of transport. This idea would be valid for blood constituents that are insoluble unless associated with plasma proteins. Such limitation,however,does not apply to steroid hormones at physiological concentrations. A rather better concept may be the interpretation that serum protein binding of steroid hormones would constitute a regulatory function,providing a buffer system,to control

the chemical activity,i.e. the effective concentration of the free steroid hormone (Westphal,1955).

The regulatory and control mechanisms of steroid function and metabolism may be grouped into:

1. the hypothalamo-pituitary axis,comprising:
    a. environmental factors,affecting the hypothalamus and its pituitary hormone releasing factors;
    b. pituitary trophic hormones.
2. the interaction of steroids with various biochemical compounds.
    a. with other steroid compounds;
    b. with non-steroid hormones;
    c. with non-hormonal compounds,such as proteins,enzymes,vitamins.

At present our knowledge about the mechanism of action of the sequential chain: environmental factors → hypothalamus → pituitary is still in a very rudimentary stage and the available data are very fragmentary. Although anatomically there is strong evidence for a physiological link between the hypothalamus and the pituitary,there are no direct experimental data available for fish. The only observation in this field concerns the dogfish,Scyliorhinus caniculus,in which Mellinger (1964) showed that gonadotrophic function is completely independent of the hypothalamo-adenohypophysial connections. The sexual cycles of the dogfish,however,have no definite seasonal character either. On the other hand,seasonal variations in the aldehyde-fuchsin positive neurosecretion of the lateral tuberal nucleus have often been described in correlation with the sexual cycle (Scharrer and Scharrer,1963).

It has been shown that biological rhythms in animals are under direct control of the hypothalamus,whether it concerns the 24 hour

biological clock (Richter,1965) or seasonal rhythms (Baggerman,1957). But most of the work has been done on the pituitary - target tissue axis,concluding from the experimental results,by extrapolation,on the role of the hypothalamus in the environment → hypothalamus → pituitary → → target tissue sequencial chain.

In teleosts it is well known that hypophysectomy inhibits the development of the gonads,e.g. in Chasmichthys gulosus (Egami,1960), and that administration of pituitary extracts from fishes accelerates the growth of the gonads. Seasonal changes in the cytological features of the adenohypophysis,which parallel the seasonal gonadal cycle,also are well established in several species of fish.

It was shown that light and/or temperature are the major factors in regulating the sexual cycles in most animals. In Oryzias latipes and in Misgurnus anguillicaudatus it has been reported that the atrophic winter ovaries are stimulated to develop if the fishes are kept at higher temperatures. This ovarian development can be inhibited by implantation of estrogen pellets (Egami and Ishii,1962). Egami and Ishii (1962) demonstrated that reserpine,a hypothalamic depressor, counteracted the inhibitory effect of estrogen on the ovaries,if applied concurrently,and if given alone it actually stimulated the growth of the ovaries.This suggests that the hypothalamic centre exerts an inhibitory effect upon the release of gonadotrophins from the pituitary and that the inhibition is enhanced by estrogens or low temperature. The inhibitory effect of estrogen on the secretion of gonadotrophins has also been observed in Gambusia (Ishii,1961).

Temperature is a major factor in the control of biological mechanisms in teleosts. That this is connected with steroid metabolism

was suggested by the observations of Robertson et al.(1963). Immature rainbow trout,treated with cortisol,died much more rapidly and with smaller doses when the water temperature was raised from 13° to 17°C. Scherr (1952) found a similar behavior in cortisone treated mice. At -5°C all mice survived while at 35°C they suffered a high mortality. This effect can be explained by the findings of Freeman and Idler (1966) in post-spawned female Atlantic salmon. They could demonstrate that with increasing temperature (from 4° to 30°C) there was a lowering of the transcortin-bound cortisol level in plasma. They found an identical curve also in ripe female Atlantic cod. The same results were reported also for mammals. Bush (1957) and Sandberg and Slaunwhite (1959) showed that at 4°C and within the range of physiological corticosteroid concentrations,the binding of transcortin in plasma is virtually complete. But with rising temperature the percentage of unbound cortisol increases. At 37°C as much as 10% and more may be dissociated under these conditions. If protein binding renders corticoids biologically inactive (Sandberg and Slaunwhite,1959),as was shown through the prevention of the effects of glucocorticoids on liver glycogen deposition (Slaunwhite et al.,1962) by transcortin,then it can be easily understood that high temperatures,releasing free corticosteroids, will enhance the amount of active steroids in the plasma. Low temperatures, on the contrary,will remove,by protein-binding,the active steroids and make them unavailable for the usual metabolic processes (Sandberg and Slaunwhite,1963).

Most of the work done on the interplay of pituitary trophic hormones with the hormones of the peripheral target tissues was performed in Eutheria and until recently it was assumed that the data

obtained from higher vertebrates would apply also to the anamniota. But as more and more investigators probed into the endocrine processes taking place in poikilotherms,and particularly in fish,it became clear that the analogies between the endocrine patterns in fish and those in Eutheria were more superficial than assumed earlier. One striking example was the finding that the incubation of interrenals,from the catfish Heteropneustes fossilis,with ACTH yielded predominantly cortisol whereas incubation with luteinizing hormone (LH) produced mainly deoxycorticosterone(DOC) (Sundararaj and Goswami,1966). Sundararaj and Goswami (1966) tested various hormones on hypophysectomized female gravid catfish and showed that LH,DOC and cortisol proved to be potent ovulating agents. They suggested that DOC induces ovulation under the stimulation of LH. That DOC plays an important role in the reproductive endocrinology of fish was supported by the finding of high concentrations of this steroid in the semen of dogfish (Simpson et al.,1963 a). Another example of basic differences between the functions of pituitary trophic hormones in fish and higher vertebrates was reported by Klicka (1965) who demonstrated that ACTH increased the metabolic rate in goldfish whereas TSH had no influence at all or even decreased the basal metabolic rate in this species. On the other hand,Fontaine and Hatey (1953) and Olivereau and Fromentin (1954) reported a decrease in cortical cell size and a loss of granulation in the interrenal of Anguilla anguilla after hypophysectomy. Mammalian ACTH had a restorative action and induced hypertrophy. Similar results were obtained by Chavin in the goldfish Carassius auratus (Chavin,1956). After injections of ACTH (2 i.u./day for 9 days) the interrenal tissue of both the intact and hypophysectomized fish was greatly thickened,whereas after injection of adrenal cortical

extract there was no change from the normal.

An intermingling of chromaffin (adrenomedullary) and interrenal tissue in teleost fish has been noted by several workers. Nandi (1962) examined the distribution and histology of chromaffin tissue in over 125 species of teleosts and found that the cells are closely associated with the postcardinal veins in the region of the head kidney. Usually they are located between the lumen of the veins and the interrenal cells (Nandi,1961).This intermingling of chromaffin and interrenal cells seems not to be limited to the anatomical aspect but appears to extend into the functional aspect as well. Mahon et al. (1962) reported that ACTH,injected for 2-7 days,produced interrenal hyperplasia in goldfish. This hyperplasia was even more marked in the chromaffin tissue than in the interrenal cells. On the other hand,the interrenal cells of fish exposed to heat (34^{o}C) showed greater changes than those treated with ACTH,while the chromaffin tissue was only slightly affected (Rasquin,1951; Slicher,1961).This seems to suggest that the main target for ACTH in fish is the chromaffin tissue. Thus,ACTH may have in fish a different function from that in higher vertebrates.In the present work one of these functions was recognized as the control over the excretory pathway of steroid metabolites. But if ACTH has functions in fish which differ from those in the Eutheria,then is it not possible that another compound,such as adrenaline,may have in fish a similar effect as ACTH has in higher vertebrates? That this could be true was shown by Carballeira et al.(1965) who reported the following: enzymatic transformations of labeled steroid precursors lead to biosynthesis of corticosteroids in vitro with pure chromaffin tissue; NAD promotes a greater accumulation of progesterone converted from

pregnenolone than in the cortex and a hydroxylating action at C-17,11 and 21 of progesterone. Smith and Matthews (1942) found a decreased oxygen consumption after adrenaline injection in Girella nigricans, a fact which contrasts with the effect of adrenaline on oxygen consumption in mammals. This and the fact that adrenaline produces a marked dilatation of the gill vessels of fish (Keys and Bateman,1932) appears to indicate that this compound has effects in fish which are different and sometimes even the opposite from those in Eutheria. The hypothesis that the chromaffin tissue in fish might have an activity comparable to that of the adrenal cortex in higher vertebrates cannot,therefore,be rejected offhand.

Although it is true that,in general,the steroid hormones detected in tissues and plasma of teleost fish are very similar to those found in higher vertebrates,it has to be accepted that their functions and metabolic pathways are different. It would be highly speculative to even attempt to give an explanation for these differences,but the idea that evolutionary isolation of this group could account for many of the unusual features in teleost endocrinology is very tempting. Particularly if we contemplate the fact that there are no steroids to be found in Protochordata whereas in Amphibia steroid endocrinology is very similar to that in higher vertebrates. Much more research is needed in the field of fish endocrinology,and particularly steroid metabolism, to elucidate this important problem.

V. CONCLUSIONS

1. Urinary steroid catabolites in the white sucker are excreted in very small amounts. They could be detected, chemically, only in urine pooled from more than ten days.

2. Male suckers showed only slight seasonal variation in the number of urinary steroid catabolites and the form under which they were excreted. Females, however, showed marked seasonal variations: the highest number of urinary steroid compounds were detected, as glucuroniside uronidates, during summer, whereas there was almost a complete lack of urinary steroid catabolites during winter.

3. Only two, very polar, steroid catabolites were constantly found in urine of untreated suckers. These compounds were detected as glucuroniside uronidates and have been named SP_1 and SP_2.

4. The hypothesis is put forward that compounds SP_1 and SP_2 have a 3-one structure in ring A which, during assays, becomes dehydrated to the Δ^4-3-one form. The main difference between the two compounds is the presence of an α-ketol side chain in SP_1.

5. a. The administration of progesterone, testosterone, cortisol or corticosterone suppressed the excretion of compounds SP_1 and SP_2.

Cortisone,on the other hand,caused the appearance of the two compounds as free steroids and as sulfate esters,besides the glucuroniside uronidate form.

b. Compounds SP_1 and SP_2 are direct catabolites of cortisone.

5. The excretory pattern of steroid catabolites by white suckers, after the administration of pharmacological doses of steroids, showed two phases: an immediate,short-term phase,involving mainly the branchio-renal route and a later,protracted phase, involving the entero-hepatic pathway.

6. a. Estradiol treatment did not yield phenolic catabolites in urine. It is,therefore,suggested that white suckers possess the enzymatic potential to alter the phenolic structure of estrogens.

b. Estradiol treatment caused the appearance of free urinary cortisol in female suckers.

7. Progesterone administration resulted in the appearance of urinary compounds,giving a positive reaction with phosphomolybdic acid, in female suckers.

8. Testosterone administration caused in male fish the appearance of a urinary compound giving a positive Zimmermann reaction.

9. a. ACTH administration resulted in the appearance of some urinary steroid catabolites which were not detected in untreated fish.

b. ACTH seemed to change the pattern of steroid conjugation by causing the appearance of the free compound and sulfate esters besides the glucuronic acid linked form,present in urine of untreated fish.

c. ACTH caused a more rapid onset and a higher rate of renal excretion of steroid catabolites, shifting the excretory pathway from the entero-hepatic to the renal route.

d. ACTH, injected simultaneously with ^{3}H-labeled steroids, reduced markedly the latent period of appearance of urinary radioactivity but did not affect the intensity. However, when injected after the urinary activity peak was already reached, ACTH administration resulted in a significant increase in urinary radioactivity.

e. ACTH, injected after administration of sodium acetate-^{14}C, had a stimulatory effect upon the qualitative and quantitative appearance of ^{14}C-labeled urinary steroid catabolites.

10. The latent period of appearance of urinary radioactivity, after the administration of labeled steroids, as well as the time till the activity peak is reached, is a characteristic feature of the particular steroid injected due, probably, to the specific catabolic processes involved.

11. Carrier steroid, administered simultaneously with the labeled compound, did not alter the duration of the latent period of appearance of urinary radioactivity but increased significantly the intensity.

12. The administration of carrier steroid, together with the labeled compound, reduced markedly tissue storage and caused a higher concentration of radioactivity in the gut and a lower concentration in the kidney than in the case of labeled steroid alone.

13. Acetate seems to play, in the white sucker, only a minor role in steroid biosynthesis.

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A P P E N D I X

List of white suckers used in the present investigation.

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
1	F	4	1173	10	13	3 June 1963	7 June 1963	None	D
2	M	3	741	10	13	3 June	4 June	None	D
3	M	4	1104	10	13	5 June	9 June	None	D
4	F	3	690	10	13	8 June	17 June	None	K
5	F	4	1043	10	13	10 June	24 June	None	K
6	M	3	841	10	13	18 June	24 June	Last 2 days in closed system	D
7	F	4	1158	10	13	25 June	1 July	None	D
8	F	4	1114	10	13	25 June	28 June	None	D
9	F	4	1011	10	13	29 June	1 July	None	D
10	M	3	950	10	13	2 July	11 July	None	K
11	M	4	939	10	13	2 July	14 July	None	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
12	F	4	1226	10	13	12 July 1963	16 July 1963	None	K
13	F	3	986	10	13	15 July	21 July	None	D
14	F	4	1045	10	13	17 July	26 July	Last 3 days in closed system	D
15	F	3	895	10	13	22 July	8 Aug.	None	K
16	M	4	814	10	13	27 July	5 Aug.	None	K
17	F	4	863	10	13	1 Aug.	4 Aug.	None	D
18	F	4	1019	10	13	5 Aug.	6 Aug.	None	D
19	F	4	872	10	13	6 Aug.	10 Aug.	None	K
20	F	4	1041	10	13	7 Aug.	14 Aug.	None	K
21	M	4	902	10	13	9 Aug.	28 Aug.	Last 3 days in closed system	D
22	F	3	1382	10	13	11 Aug.	12 Aug.	None	D
23	F	4	956	10	13	13 Aug.	14 Aug.	None	D
24	F	4	1014	10	13	15 Aug.	18 Aug.	None	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
25	M	3	931	10	13	15 Aug.1963	24 Aug.1963	None	K
26	M	3	848	10	13	19 Aug.	25 Aug.	None	K
27	M	3	867	10	13	25 Aug.	26 Aug.	None	D
28	M	4	1010	10	13	26 Aug.	31 Aug.	None	D
29	F	4	1125	10	13	27 Aug.	30 Aug.	None	D
30	F	4	1174	10	13	29 Aug.	31 Aug.	None	D
31	F	5	1385	10	13	1 Sept.	3 Sept.	None	D
32	F	4	1219	10	13	3 Sept.	4 Sept.	None	D
33	M	3	1000	10	13	3 Sept.	17 Sept.	None	K
34	F	4	1182	10	13	5 Sept.	18 Sept.	None	K
35	F	4	1277	10	13	14 Sept.	17 Sept.	None	D
36	F	4	1200	10	13	18 Sept.	21 Sept.	None	D
37	F	4	1213	10	13	18 Sept.	24 Sept.	None	K
38	M	3	769	10	13	22 Sept.	25 Sept.	None	D
39	F	4	1176	10	13	22 Sept.	25 Sept.	None	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T^{o} in ^{o}C	Lucite box T^{o} in ^{o}C	Experiment started	Experiment stopped	Treatment	Died or killed
40	F	4	1637	10	13	25 Sept.1963	26 Sept.1963	None	D
41	M	3	1100	10	13	26 Sept.	28 Sept.	None	D
42	M	4	1003	10	13	26 Sept.	29 Sept.	None	D
43	F	4	1380	10	13	27 Sept.	30 Sept.	None	D
44	F	4	1266	10	13	29 Sept.	2 Oct.	None	D
45	M	4	996	10	13	30 Sept.	7 Oct.	None	K
46	M	3	1001	10	13	3 Oct.	6 Oct.	None	D
47	M	3	975	10	13	4 Oct.	23 Oct.	None	K
48	F	4	1390	10	13	7 Oct.	9 Oct.	None	D
49	F	3	1318	10	13	8 Oct.	11 Oct.	None	D
50	F	4	1108	10	13	10 Oct.	12 Oct.	None	D
51	F	3	1283	10	13	12 Oct.	14 Oct.	None	D
52	F	3	1081	10	13	13 Oct.	2 Nov.	From 24 Oct.grad. decreased T^{o} to $5^{o}C$.	K
53	F	4	1067	10	13	15 Oct.	18 Oct.	None	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
54	F	3	933	10	13	19 Oct.1963	23 Oct.1963	None	K
55	F	3	1182	10	13-9	24 Oct.	27 Oct.	Grad.decrease of T°.	D
56	M	3	939	10	13-7	24 Oct.	30 Oct.	Grad.decrease of T°.	K
57	F	3	1150	8	9-5	28 Oct.	31 Oct.	Grad.decrease of T°.	D
58	F	3	1627	5	5	1 Nov.	6 Nov.	None	K
59	F	3	1182	5	5	3 Nov.	9 Nov.	None	D
60	F	3	1210	5	5	7 Nov.	10 Nov.	None	D
61	F	4	1635	5	5	10 Nov.	12 Nov.	None	D
62	F	5	1274	5	5	11 Nov.	17 Nov.	Last 3 days in closed system	D
63	M	3	1034	5	5	13 Nov.	16 Nov.	None	D
64	M	3	1131	5	5	17 Nov.	3 Dec.	None	K
65	M	3	1333	5	5	18 Nov.	27 Nov.	None	K
66	F	3	1596	5	5	28 Nov.	3 Dec.	None	D
67	F	3	1136	5	5	11 Dec.	23 Dec.	None	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° °C in	Lucite box T° °C in	Experiment started	Experiment stopped	Treatment	Died or killed
68	F	4	1439	5	5	11 Dec.1963	15 Dec.1963	None	D
69	M	3	1060	5	5	16 Dec.	27 Dec.	None	K
70	F	3	1137	5	5	24 Dec.	27 Dec.	None	D
71	F	3	1184	5	5	28 Dec.	29 Dec.	None	D
72	F	4	1103	5	5	28 Dec.1963	1 Jan.1964	None	K
73	M	3	1125	5	5	30 Dec.	3 Jan.	None	K
74	F	4	1168	5	5	2 Jan.	6 Jan.	None	K
75	M	3	921	5	5	4 Jan.	7 Jan.	None	D
76	F	4	1166	5	5	7 Jan.	8 Jan.	None	D
77	F	3	1114	5	5	8 Jan.	13 Jan.	None	K
78	M	3	1080	5	5	9 Jan.	12 Jan.	None	D
79	M	3	1165	5	5	13 Jan.	15 Jan.	None	D
80	F	3	1130	5	5	14 Jan.	15 Jan.	None	D
81	M	3	932	5	5	15 Jan.	26 Jan.	None	K
82	F	4	1056	5	5	16 Jan.	25 Jan.	None	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
83	M	4	918	5	5	16 Jan.1964	20 Jan.1964	None	K
84	F	3	1081	5	5	21 Jan.	31 Jan.	None	K
85	F	3	1250	5	5	26 Jan.	1 Feb.	None	D
86	M	3	1004	5	5	27 Jan.	3 Feb.	Last 4 days in closed system	D
87	F	3	1169	5	5	1 Feb.	12 Feb.	None	K
88	M	3	967	5	5	2 Feb.	4 Feb.	None	D
89	F	4	1310	5	5	4 Feb.	24 Feb.	None	K
90	M	3	1015	5	5	5 Feb.	7 Feb.	None	D
91	F	3	1217	5	5	8 Feb.	11 Feb.	None	D
92	F	4	1335	5	5	12 Feb.	13 Feb.	None	D
93	M	4	1191	5	5	13 Feb.	26 Feb.	None	K
94	F	3	1088	5	5	14 Feb.	16 Feb.	None	D
95	F	3	1026	5	5	17 Feb.	26 Feb.	None	K
96	M	4	947	5	5	27 Feb.	3 March	Last 2 days in closed system	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T^{o} in ^{o}C	Lucite box T^{o} in ^{o}C	Experiment started	Experiment stopped	Treatment	Died or killed
97	M	3	1013	5	5	27 Feb.1964	1 March 1964	None	D
98	F	4	1343	5	5	2 March	5 March	None	D
99	M	4	1009	5	5	4 March	6 March	None	D
100	F	4	1042	5	5	6 March	10 March	None	K
101	M	3	933	5	5	7 March	12 March	None	D
102	M	3	764	5	5	11 March	12 March	None	D
103	F	4	1412	5	5	13 March	27 March	None	K
104	M	3	947	5	5	13 March	15 March	None	D
105	M	3	877	5	5	16 March	20 March	None	K
106	M	4	804	5	5	21 March	26 March	None	D
107	M	3	878	5	5-18	1 Oct.	24 Oct.	Grad.increase of T^{o}.	K
108	F	3	1109	5	5-18	1 Oct.	24 Oct.	Grad.increase of T^{o}.	K
109	M	3	982	5	5-16	1 Oct.	21 Oct.	Grad.increase of T^{o}.	D
110	F	3	1440	5	5-18	26 Oct.	16 Nov.	Grad.increase of T^{o}.	K
111	M	3	886	5	5-18	26 Oct.	16 Nov.	Grad.increase of T^{o}.	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
112	F	4	1135	5	5-17	26 Oct.1964	13 Nov.1964	Grad.increase of T°.	D
113	M	3	1019	5	5-13	17 Nov.	13 Nov.	Grad.increase of T°.	K
114	F	4	1256	5	5-13	17 Nov.	1 Dec.	Grad.increase of T°.	K
115	M	3	1019	5	5-13	17 Nov.	2 Dec.	Grad.increase of T°.	K
A 1	M	3	874	5	13	2 Dec.	25 Dec.	inj.15 i.u.ACTH/Kg body wt./48 h.,6-14 Dec.	K
A 2	F	3	1302	5	13	2 Dec.	22 Dec.	inj.15 i.u.ACTH/Kg body wt./48 h.,6-14 Dec.	K
A 3	M	3	1115	5	13	23 Dec.	30 Dec.	inj.15 i.u.ACTH/Kg body wt.,once on 23 Dec.	K
A 4	M	4	1005	5	13	2 Jan.1965	25 Jan.1965	inj.1 mg testosterone	K
A 5	M	4	951	5	13	2 Jan.	21 Jan.	inj.1 mg testosterone	K
A 6	M	3	1063	5	13	22 Jan.	26 Jan.	inj.1 mg testosterone	K
A 7	F	4	1387	5	13	26 Jan.	18 Feb.	inj.1 mg progesterone	D-fungus
A 8	F	4	1257	5	13	27 Jan.	15 Feb.	inj.1 mg progesterone	D-fungus

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
A 9	F	3	1259	5	13	1 Feb.1965	20 Feb.1965	inj.1 mg estradiol	D-fungus
A10	F	3	1123	5	13	16 Feb.	20 Feb.	inj.1 mg progesterone	K
B 1	F	4	1150	5	13	19 Feb.	26 Feb.	inj.1 mg estradiol	K-fungus
B 2	F	4	1175	5	13	21 Feb.	28 Feb.	inj.1 mg estradiol	K
B 3	F	3	1205	5	5	1 March	8 March	closed system	D
B 4	M	3	950	5	5	1 March	4 March	closed system	D
B 5	F	3	1125	5	5	5 March	7 March	closed system	D
B 6	M	3	875	5	13	9 March	18 March	inj.15 i.u.ACTH/Kg body wt./48 h.,13-17 March	D
B 7	M	4	1015	5	13	9 March	20 March	inj.15 i.u.ACTH/Kg body wt./48 h.,13-19 March	K
B 8	F	4	1175	5	13	12 March	31 March	inj.1 mg estradiol	K-fungus
B 9	F	4	1283	5	13	19 March	31 March	inj.1 mg cortisol	D-fungus
B10	F	4	1394	5	13	21 March	25 March	inj.1 mg cortisone	K
C 1	M	3	1082	5	13	26 March	31 March	inj.1 mg testosterone	K
C 2	F	4	1290	10	13	1 May	7 May	inj.1 mg cortisol	D-fungus

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° °C	Lucite box T° °C	Experiment started	Experiment stopped	Treatment	Died or killed
C 3	F	3	1127	10	13	2 May 1965	7 May 1965	inj.1 mg progesterone	K
C 4	M	3	840	10	13	8 May	13 May	inj.1 mg corticosterone	K
C 5	M	4	1040	10	13	8 May	17 May	inj.1 mg testosterone	K
C 6	M	4	1000	10	13	14 May	1 June	inj.1 mg corticosterone	D
C 7	M	2	763	10	13	18 May	6 June	inj.1 mg testosterone	D
C 8	M	3	771	10	13	2 June	25 June	inj.1 mg testosterone	D
C 9	F	4	1063	10	13	7 June	21 June	inj.1 mg estradiol	K-fungus
C10	F	3	1139	10	13	22 June	30 June	inj.1 mg estradiol	K-fungus
D 1	F	3	1096	10	13	26 June	19 July	inj.1 mg progesterone	D-fungus
D 2	F	3	1021	10	13	1 July	20 July	inj.1 mg progesterone	K-fungus
D 3	M	3	1107	10	13	20 July	8 Aug.	inj.1 mg cortisol	D-fungus
D 4	M	3	900	10	13	21 July	11 Aug.	inj.1 mg cortisone	K-fungus
D 5	F	3	1116	10	13	9 Aug.	28 Aug.	inj.1 mg corticosterone	K
D 6	M	4	990	10	13	12 Aug.	31 Aug.	inj.1 mg corticosterone	K
D 7	M	3	752	10	13	15 Sept.	20 Sept.	inj.1 mg corticosterone	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
D 8	M	3	813	10	13	16 Sept.1965	24 Sept.1965	inj.1 mg corticosterone	K
D 9	F	5	1423	10	13	21 Sept.	26 Sept.	inj.1 mg corticosterone	K
D10	F	3	1175	10	13	25 Sept.	30 Sept.	inj.1 mg corticosterone	K
E 1	F	4	1122	10	13	27 Sept.	9 Oct.	inj.1 mg corticosterone	D
E 2	M	4	1009	10	13	1 Oct.	6 Oct.	inj.1 mg cortisol	K
E 3	F	4	1126	10	13	9 Oct.	28 Oct.	inj.80 µc Na acetate-^{14}C	K
E 4	F	3	1194	10	13	10 Oct.	29 Oct.	inj.1 mg cortisol	D-fungus
E 5	F	3	1157	10	13	10 Oct.	30 Oct.	inj.80 µc Na acetate-^{14}C, 10 i.u.TSH,10 i.u.ACTH	K
E 6	M	3	989	10	13	29 Oct.	8 Nov.	inj.15 i.u.ACTH/Kg body wt./48 h.,2-8 Nov.	D
E 7	F	3	1275	5	13	1 Nov.	20 Nov.	inj.1 mg corticosterone	K
E 8	F	3	1167	5	13	1 Nov.	30 Nov.	inj.80 µc Na acetate-^{14}C, inf.5 i.u.ACTH/Kg body wt./24 h.,3-8 Nov.	K
E 9	F	4	1203	5	13	9 Nov.	30 Nov.	inf.5 i.u.ACTH/Kg body wt./24 h.,13-23 Nov.	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
E10	M	3	1026	5	13	21 Nov.1965	10 Dec.1965	inj.80 µc Na acetate-^{14}C	K
F 1	F	3	1196	5	13	1 Dec.	18 Dec.	inj.1 mg cortisol	D-fungus
F 2	M	3	1002	5	13	3 Dec.	26 Dec.	inf.5 i.u.ACTH/Kg body wt./24 h.,7-16 Dec.	K
F 3	F	3	1334	5	13	11 Dec.	25 Dec.	inj.1 mg corticosterone	K
F 4	M	3	1026	5	13	19 Dec.	30 Dec.	inj.1 mg cortisol	K-fungus
F 5	M	3	830	5	13	26 Dec.	31 Dec.	inj.1 mg cortisol	K
F 6	F	4	1200	5	13	27 Dec.1965	4 Jan.1966	inj.15 i.u.ACTH/Kg body wt./48 h.,29 Dec.-4 Jan.	D
F 7	M	3	933	5	13	2 Jan.	16 Jan.	inj.1 mg cortisone	K-fungus
F 8	F	3	1338	5	13	2 Jan.	6 Jan.	inj.1 mg cortisone	K
F 9	F	3	1349	5	13	5 Jan.	21 Jan.	inj.1 mg cortisone	K-fungus
F10	F	4	1243	5	13	7 Jan.	31 Jan.	inj.1 mg cortisone	K-fungus
G 1	F	3	1251	5	13	17 Jan.	21 Jan.	inj.0.5 ml of a sol. 1:1000 adrenaline	K
G 2	F	4	1178	5	13	22 Jan.	26 Jan.	inj.0.5 ml of a sol. 1:1000 adrenaline	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
G 3	M	3	877	5	13	22 Jan.1966	26 Jan.1966	inj.0.5 ml of a sol. 1:1000 adrenaline	K
G 4	M	4	1122	5	13	27 Jan.	29 Jan.	inj.0.5 ml of a sol. 1:1000 adrenaline	K
G 5	F	3	1300	5	13	27 Jan.	31 Jan.	inj.0.5 ml of a sol. 1:1000 adrenaline	K
G 6	M	3	851	5	13	30 Jan.	31 Jan.	inj.0.5 ml of a sol. 1:1000 adrenaline	K
G 7	F	4	1150	5	13	1 Feb.	9 Feb.	inj.15 i.u.ACTH/Kg body wt.,once on 1 Feb.	K
G 8	F	3	1175	5	13	2 Feb.	13 Feb.	inj.1 mg cortisol	K-fungus
G 9	M	3	1124	5	13	2 Feb.	16 Feb.	inj.1 mg cortisone	D-fungus
G10*	M	3	989	5	13	10 Feb.	11 March	inf.5 i.u.ACTH/Kg body wt./24 h.,14-23 Feb.	K
H 1	M	4	1108	5	13	14 Feb.	26 Feb.	inj.1 mg cortisol	K-fungus
H 2*	F	4	1205	5	13	17 Feb.	12 March	inf.5 i.u.ACTH/Kg body wt./24 h.,21 Feb-2 March	D

* Fish No.G10 and H 2 were infused by the same pump and,therefore,were given 5 i.u.ACTH/24 hours,between 21-23 Feb.

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
H 3	F	4	1375	5	13	27 Feb.1966	7 March 1966	inj.15 i.u.ACTH/Kg body wt.,once on 28 Feb.	K
H 4	M	3	988	5	13	8 March	15 March	inj.15 i.u.ACTH/Kg body wt.,once on 8 March	K
H 5	M	3	1089	5	13	12 March	20 March	inj.10 i.u.TSH	K
H 6	F	4	1301	5	13	13 March	20 March	inj.10 i.u.TSH	K-fungus
H 7	F	4	1350	5	13	16 March	23 March	inj.10 i.u.TSH	K
H 8	M	3	939	5	13	21 March	29 March	inj.10 i.u.TSH	K-fungus
H 9	M	3	760	5	13	24 March	31 March	inj.10 i.u.TSH	K
H10	F	3	1285	5	6-12	1 April	7 April	Grad.increase of T°	D
I 1	F	3	1210	5	6-12	1 April	7 April	Grad.increase of T°	K
I 2	M	3	803	5	6-12	1 April	7 April	Grad.increase of T°	K
I 3	F	4	1228	5	5-11	8 April	14 April	Grad.increase of T°	D
I 4	M	3	887	5	5-11	8 April	14 April	Grad.increase of T°	K
I 5	M	3	711	5	5-12	8 April	15 April	Grad.increase of T°	K
I 6	F	4	1039	5	5-12	16 April	23 April	Grad.increase of T°	D

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T^{o} in ^{o}C	Lucite box T^{o} in ^{o}C	Experiment started	Experiment stopped	Treatment	Died or killed
I 7	F	4	1233	5	5-13	16 Apr.1966	24 Apr.1966	Grad.increase of T^{o}	K
I 8	F	4	1258	5	5-13	16 April	24 April	Grad.increase of T^{o}	K
I 9	F	3	1383	10	13	25 April	14 May	inj.100 µc cortisone-^{3}H +1 mg cortisone	K-fungus
I10	F	3	1187	10	13	25 April	9 May	inj.100 µc estradiol-^{3}H + 1 mg estradiol	K-fungus
L 1	F	4	1212	10	13	25 April	14 May	inj.100 µc estradiol-^{3}H + 1 mg estradiol	K-fungus
L 2	F	4	1305	10	13	10 May	29 May	inj.1 mg cortisol	K
L 3	F	4	1185	10	13	15 May	22 May	inj.100 µc cortisone-^{3}H + 1 mg cortisone	K
L 4	M	4	1070	10	13	15 May	22 May	inj.100 µc cortisone-^{3}H + 1 mg cortisone	K-fungus
L 5	M	3	925	10	13	23 May	30 May	inj.100 µc cortisone-^{3}H + 1 mg cortisone	K-fungus
L 6	F	3	1130	10	13	23 May	30 May	inj.100 µc estradiol-^{3}H + 1 mg estradiol	K
L 7	F	4	1288	10	13	30 May	3 June	inj.1 mg cortisol	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
L 8	F	4	1246	10	13	31 May 1966	7 June 1966	inj.100 µc cortisone-^{3}H + 1 mg cortisone	K-fungus
L 9	F	4	1251	10	13	31 May	7 June	inj.100 µc estradiol-^{3}H + 1 mg estradiol	K
L10	F	4	1245	10	13	4 June	8 June	inj.1 mg cortisol	K
M 1	M	3	885	10	13	8 June	27 June	inj.100 µc cortisone-^{3}H + 1 mg cortisone	K-fungus
M 2	F	4	1187	10	13	8 June	12 June	inj.100 µc progesterone-^{3}H + 1 mg progesterone	K
M 3	F	4	1091	10	13	9 June	10 June	inj.100 µc progesterone-^{3}H + 1 mg progesterone,0.5 ml adrenaline 1:1000	K
M 4	F	4	1274	10	13	11 June	15 June	inj.100 µc progesterone-^{3}H + 15 i.u.ACTH/Kg body wt.	K
M 5	F	4	1154	10	13	13 June	1 July	inj.100 µc progesterone-^{3}H + 1 mg progesterone	D-fungus
M 6	F	3	1133	10	13	16 June	20 June	inj.100 µc progesterone-^{3}H + 15 i.u.ACTH/Kg body wt.	K
M 7	F	3	1148	10	13	21 June	25 June	inj.100 µc progesterone-^{3}H & later 15 i.u.ACTH/Kg body wt.	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
M 8	M	3	845	10	13	26 June 1966	20 July 1966	inj.100 µc testosterone-^{3}H + 1 mg testosterone	K
M 9	F	4	1265	10	13	28 June	15 July	inj.100 µc progesterone-^{3}H + 1 mg progesterone	D-fungus
M10	F	4	1218	10	13	2 July	6 July	inj.100 µc progesterone-^{3}H + 1 mg progesterone	K
N 1	M	3	830	10	13	7 July	11 July	inj.100 µc testosterone-^{3}H	K
N 2	M	3	884	10	13	12 July	16 July	inj.100 µc testosterone-^{3}H + 1 mg testosterone	K
N 3	F	4	1189	10	13	16 July	20 July	inj.100 µc progesterone-^{3}H + 1 mg progesterone	K
N 4	F	3	1172	10	13	17 July	24 July	inj.100 µc estradiol-^{3}H + 1 mg estradiol	K
N 5	M	3	889	10	13	21 July	25 July	inj.100 µc testosterone-^{3}H	K
N 6	F	3	1219	10	13	21 July	25 July	inj.100 µc progesterone-^{3}H + 1 mg progesterone,0.5 ml adrenaline 1:1000	K
N 7	M	3	726	10	13	26 July	30 July	inj.100 µc testosterone-^{3}H + 1 mg testosterone	K

Fish No.	Sex	Age in years	Weight in g. at the end	Holding tank T° in °C	Lucite box T° in °C	Experiment started	Experiment stopped	Treatment	Died or killed
N 8	F	3	1217	10	13	27 July 1966	14 Aug.1966	inj.100 µc cortisone-^{3}H + 1 mg cortisone	K-fungus
N 9	M	3	947	10	13	31 July	4 Aug.	inj.100 µc testosterone-^{3}H + 1 mg testosterone	K
N10	M	3	985	10	13	31 July	4 Aug.	inj.1 mg cortisol	K
P 1	M	3	961	10	13	5 Aug.	14 Aug.	inj.100 µc testosterone-^{3}H	K
P 2	M	3	1000	10	13	10 Aug.	27 Aug.	inj.1 mg cortisol	K-fungus
P 3	M	3	987	10	13	15 Aug.	27 Aug.	inj.100 µc testosterone-^{3}H + 1 mg testosterone	K
P 4	F	3	1166	10	13	15 Aug.	27 Aug.	inf.5 i.u.ACTH/Kg body wt./24 h.,19-27 Aug.	K
P 5	F	4	1213	10	13	killed one hour after injection		inj.100 µc cortisone-^{3}H	K
P 6	F	4	1198	10	13	killed one hour after injection		inj.100 µc cortisone-^{3}H + 15 i.u.ACTH/Kg body wt.	K