A SEARCH FOR FEMALE SEX HORMONES
IN SALMON EMBRYOS OF THE GENUS
ONCORHYNCHUS
by
JAMES. GRANT ROBERTSON

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Zoology

We accept this thesis as conforming to the
standard required from candidates for the
degree of DOCTOR OF PHILOSOPHY

Members of the Department of Zoology

THE UNIVERSITY OF BRITISH COLUMBIA
July, 1954
ABSTRACT

A dialyzing technique was developed to concentrate an estrogen hormone fraction suitable for separation by paper partition chromatography and spectrophotometric assay. Estrogens were not found in sexually differentiating salmon embryos. Small amounts of estriol, estradiol-17β and estrone added to the tissue could not be recovered. However, horse testes assayed by the same technique showed the presence of estradiol-17β and estrone in concentrations of .097 and .143 mg./kg., respectively. The assay of horse testes was carried out on 90 gram lots, whereas the one previous chemical assay was done on 28,000 grams. It is concluded that this technique is very satisfactory for extraction of estrogens from animal gonads, but that hormone added to whole salmon embryos is inactivated by some unknown system.

A partition technique recently developed by F. Mitchell and R. Davies for the extraction of estrogens from human placentae was slightly modified for use with salmon embryos. This method confirmed the negative findings obtained by the dialyzing technique.

On the basis of these experiments, there is no evidence to support the hormonal theory of sex differentiation in fishes.
THE UNIVERSITY OF BRITISH COLUMBIA

Faculty of Graduate Studies

PROGRAMME OF THE

FINAL ORAL EXAMINATION FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

of

JAMES GRANT ROBERTSON

B.Sc. (Manitoba) 1948
M.A. (Brit. Col.) 1951

Monday, July 12, 1954
at 10:00 a.m.
in ROOM 403, Applied Science Bldg.

COMMITTEE IN CHARGE
W.A. Clemens, Chairman

V.C. Brink  E.S. Goranson
I. McT. Cowan  B. Savery
M. Darrach  A.J. Wood
P. Ford

External Examiner - D.R. Idler
PUBLISHED PAPERS


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A search for female sex hormones in salmon embryos of the genus Oncorhynchus

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GRADUATE STUDIES

Field of Study: Zoology

Experimental Zoology - Dr. W. Hoar
Vertebrate Morphogenesis - Dr. P. Ford
Biology of Fishes - Dr. W. Hoar
Limnology - Dr. P. Larkin
Zoological Seminar - Dr. W. Clemens
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Other Studies:

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Biometry - Dr. V. Brink
Biochemistry of Cancer - Dr. M. Darrach
Biochemistry of Antibiotics - Dr. J. Polglaze
3. Positions of the hormones........ 31
4. Elution of the hormones............. 33
5. Elimination of background material 33
6. Absorption curves.................. 33

C. Application to salmon embryo dialysis- 
ates...................................... 35

D. Application to horse testes dialysates 39

VI. A FURTHER SEARCH FOR FEMALE HORMONES IN SALMON 
EMBRYOS........................................ 44

A. The Mitchell-Davies Partition Method.. 44

B. Experiment 1. Recovery of hormone 
added to salmon embryos.................. 47

C. Experiment 2. The failure to detect 
hormones in lyophilized salmon embryos 49

1. The estradiol-estrone fraction .... 49
2. The estriol fraction.................... 51

VII. DISCUSSION................................. 52

VIII. SUMMARY AND CONCLUSION.................. 62

IX. ACKNOWLEDGMENTS.......................... 64

X. LITERATURE CITED.......................... 65
LIST OF TABLES, FIGURES, AND FLOW-SHEETS

TABLE
I. Recovery of 50 micrograms of estradiol-17β by dialysis in a synthetic system...............23
II. The location of free steroids (in centimeters from the starting line) after chromatography....32
III. Concentration of hormones recovered from dialysates of horse testes.......................41
IV. Spectrophotometric assay of the estriol fraction from horse testes........................42
V. Recovery values for hormones added to salmon embryos, Experiment I..........................48
VI. Recovery values for Experiment 2.................50

FIGURE
1. Calibration curve for estradiol-17β in a synthetic system............................................22
2. Apparatus used in dialysis..................................27
3. Absorption spectra showing the elimination of background material (B) from chromatographed estradiol-17β (D), estriol (C), and estrone (E).34
4. Absorption spectra of estradiol-17β (A), estrone (B), and estriol (C), in concentrated sulfuric acid..................................................36
5. Agent stimulators and inhibitors--operation in sex differentiation. (after Witschi, 1950)........54

FLOW-SHEET
I. Preliminary partition system.................................14
II. Partition system in dialysis of embryos...........37
III. Partition system in dialysis of horse testes....40
IV, IVa. Mitchell-Davies partition system slightly modified........................................45,46
A SEARCH FOR FEMALE SEX HORMONES IN SALMON EMBRYOS OF THE GENUS ONCORHYNCHUS

By J. G. Robertson

I. INTRODUCTION

The era of experimental studies on the role of sex hormones in the embryonic sex differentiation of vertebrates commenced with F.R. Lillies' (1917) observations on the freemartin. The freemartin is a female calf that has been transformed into an hermaphrodite. This transformation occurs prenatally, and then only in the presence of a male twin. It is dependent upon a secondary fusion of the two placentae so that blood-borne male hormones can pass from the male to the female twin. There is no freemartin when the twins are of the same sex. The transformation to the freemartin may be so complete that the female develops a testis and male ducts, instead of an ovary and female ducts (Willier, 1934).

The morphological basis of sex differentiation is well worked out. Thus the undifferentiated gonad consists of two distinct zones: an inner medulla, representing the testicular component, and an outer cortex -- the ovarial component. Normally only one of these constituents becomes functional, i.e., forms an ovary or testis (Witschi, 1934). Both constituents may persist into adulthood to give a functional hermaphrodite as in some cyclostomes (Okkelberg, 1921), or the constituents may not be separable, as in
young eels (D'Ancona, 1947). The genital tract has two sets of ducts, which in most vertebrates are simultaneously present and fully formed at some time during development (Burns, 1942, '49). The genital tubercle, located in the urogenital sinus, is the forerunner of both male and female external genitalia.

The experimental basis for the modus operandi of sex differentiation depends upon observations resulting from gonadectomy, graftings of ovarian and testicular tissue, parabiosis, and application of sex hormones. Each approach is designed to determine whether embryonic hormones are essential to the normal or abnormal development of the gonad and its accessory sex structures, i.e. reproduce "Nature's experiment" in the development of the freemartin. Selected examples of these approaches are outlined here.

The removal of the functional left ovary of a chick induced the usually degenerate right ovary to form a testis producing spermatozoa (Benoit, 1923). A prenatal removal of embryonic rabbit ovaries (Jost, 1947) did not inhibit development of the female ducts. However, in testicular castration, the embryonic female ducts continued to develop while the male genital tract failed to do so. According to Burns (1949), the anomaly produced by the ovarectomized female rabbit may be the result of interference from maternal hormones.

Grafting experiments, in which pieces of testis or
ovary were inserted on the chorio-allantoic membrane of a host chick embryo, resulted in a number of the host embryos being modified in the expected male or female direction (Minoura, 1921). These findings could not be confirmed by Willier (1927, *34). Recently, however, Wolff (1947) effected a partial sex reversal in chicks by implanting testicular or ovarian rudiments directly into the body cavity.

Parabiosis, or the uniting of whole organisms so that an exchange between their blood may occur, has resulted in a duplication of the freemartin effect in Amphibia. Thus Burns (1925) joined larval axolotls at a time when the gonads were undifferentiated. He found that both members of a pair were of the same sex, indicating that a reversal of sex must have occurred in a large number of them.

Treatment with hormone preparations has generally effected a modification of sex in vertebrates in line with the above experiments. In fish, Padoa (1939) observed a partial feminization of male trout (*Salmo irideus*) reared in aquaria containing female hormones. Bullough (1940) showed female minnows (*Phoxinus laevis*) developed a testis-like structure after injection of male hormone. However, male fish injected with female hormone showed an injured, but not transformed, testis. Berkowitz (1938, *41) found estrogen caused formation of an ovatestis in the guppy (*Lebistes reticulatus*). In trout (*Salmo trutta*), treatment with male or with female hormone gave conflicting results.
in that similar modifications occurred with either hormone in either sex (Ashby, 1952).

It is interesting that at least in one plant (Melandrium dioecium), mammalian sex hormones shifted the normal and intersexual flowers to the male or female side in accord with the hormone applied (Love and Love, 1940, "45).

According to Burns (1949), the collective results show that hormones function as sex differentiating principles, and are identical with those of adult animals. This thesis has also been developed or supported by Dantchakoff (1950), Wolff (1950), Witschi (1950, "51), and Jost (1953). It is opposed by Moore (1947, "50), principally on the grounds that there is no evidence for the secretion of sex hormones at a time when the gonads are sexually differentiating.

In the animal kingdom, the chemical identity of the natural sex hormones has been worked out only for the mammals. The female hormones have been found to possess the steroid ring system, as do the testicular and adrenocortical hormones. They differ by virtue of their phenolic character and possession of fewer carbon atoms.

The present study attempts to evaluate the problem by supplying chemical evidence for the embryonic secretion of female sex hormones. Any such evaluation should consider the interference from hormones of maternal origin. This interference would not be expected in animals whose embryonic development occurs outside of the body of the mother, which is the case in most fish. Since the histogenesis of sex
differentiation has been worked out for the chum salmon Oncorhynchus keta, (Robertson, 1953), the same species was selected for this investigation.
II. THE DISTRIBUTION OF FEMALE SEX HORMONES

In the few isolation studies attempted for female hormones, large amounts of source material were used. Thus MacCorquodale, Thayer, and Doisy (1936) aspirated 400 litres of follicular fluid from the graafian follicles of four tons of sow ovaries. After processing this fluid, they obtained 12 milligrams of estradiol-17β. Human and mare pregnancy urine have been the chief source for the isolation of the remaining natural estrogens. These are estrone, estriol, estradiol-17α, equilin, equilenin, and hippulin. Because of the expense and hazards encountered in processing tissues for an isolation of their hormones, most present-day research is carried out entirely on urine. The relative simplicity of this medium and the development of microchemical methods for its assay have resulted in an extensive literature, none of which is conclusive (Heard and Saffron, 1949). The methods do not appear to be applicable to extracts of blood, body tissues, feces or the urine of men (Pincus, 1948). However a counter-current distribution method developed by Engel et al (1950) and Engel (1950) for urine analyses has been helpful to a study of the human placenta (Diczfalusy, 1953).

It is not surprising to find, therefore, the continued use of bioassay methods. Their advantages for detecting estrogenic substances in microgram quantities from relatively crude fractions has greatly outweighed their disadvantages. Two of these are the differential activity of hormones and
the presence of suppressing or augmenting substances in the fractions being assayed (Emmens, 1950; Cohen and Bates, 1952).

The evidence for the presence of female hormones elsewhere in vertebrates is dependent upon the extraction of a phenol fraction that will induce vaginal cornification in the spayed mouse or rat (bioassays). It should be noted that this estrogen activity can also be effected by petroleum and lignite (Doisy, 1934) and a variety of synthetic substances (Emmens, 1950) such as stilbesterol. However, the isolation of estrone from palm kernel extracts, and estriol from female willow flowers (see Fieser and Fieser, 1949) suggests a widespread occurrence of mammalian hormones.

Fellner (1925) was the first to investigate female hormones in fish. He obtained an ovarian extract from an unstated species that would enlarge the rabbit uterus. He states that the aphrodisiac nature of caviar is probably due to the presence of the female hormones indicated by his study. Weismann et al (1937) extracted a phenolic fraction from swordfish (*Xiphias gladius*) ovaries which gave an estrus response in rats. The neutral male fraction arising from his extraction procedure was used as a control. It did not give an estrus response. Donahue (1941) made an alcohol extract of the ovaries from winter flounders. After further processing, the extract was estimated to contain less than ten rat units of estrogen. This is equivalent to less than one microgram of pure estrone.

The only other evidence for female hormones in fish
appears to be that given by Brull and Cuypers (1954). They cautiously claim to have made a chemical detection of folliculin (estrone) and other "phenolsteroids" in an extract of 420 ml. of urine obtained from 25 *Lophius piscatorius*.

On the basis of the studies discussed there can be little doubt that the estrogens are present in adult females of a wide variety of fish, if not all of them.
III. MATERIALS

A. Biological Tissue

Chum salmon (*Oncorhynchus keta*) and sockeye salmon (*O. nerka*) were spawned at Cultus lake in the autumns of 1952 and 1953 and incubated in the University of British Columbia's fish hatchery. The chum salmon were removed for study at two stages in the spring of those years. The first stage, taken three weeks before hatching, represented the period of germ cell multiplication; the second stage was taken at a time when the sexes were well differentiated (yolk just absorbed, two months post-hatching) (Robertson, 1953). No effort was made to collect the sockeye in stages since they were being used as test material. All fish subsisted solely on their yolk sacs.

The fish were used in whole because removal of the embryonic gonads is impractical.

The Alsask Processors Limited, Edmonton, Alberta, deep-froze horse testes and air-expressed them in dry ice to the Vancouver Airport. They were used immediately upon arrival. A second lot of testes were obtained from Mr. Fos Hoy, Newton, B.C.

B. Chemicals

1. Hormones. The crystalline hormones used in this investigation were supplied by the Ciba Company Limited and Ayerst, McKenna and Harrison Limited, Montreal, Canada. It is a pleasure to acknowledge their generosity. The identity and

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1. The nomenclature followed is that of Fieser and Fieser (1949).
purity of these hormones were determined on a Fisher micro-
melting point apparatus after drying over phosphorus pent-
oxide. The melting point ranges found (set in parentheses) 
were in virtual agreement with those of estriol (280-283.5°C.),
estradiol-17β (174-175°C.) and estrone (258-264°C.).

2. Solvents. All solvents used were of reagent grade. The 
hydrocarbons such as petroleum ether, benzene and methyl-
cyclohexane were washed with concentrated sulfuric acid and 
re-distilled. (The petroleum ether was collected at 34-
54°C.). Their derivatives (chloroform, 0-dichlorobenzene, 
nitrobenzene and dichloromethane) were redistilled. Commercial 
grade 95% and absolute alcohol were redistilled except when 
used in making up color reagents. Here the absolute grade 
was refluxed in 2,4-dinitrophenylhydrazine and triple distilled 
(Lappen and Clark, 1951). Methanol, redistilled, was used in 
preference to ethanol. Normal butyl alcohol was redistilled. 
Formamide was made ammonia free by standing over concentrated 
sulfuric acid. Ether was used when peroxide free to 10% acid 
potassium iodide. Ethyl acetate was purified as outlined by 
Vogel (1952).

3. Color reagents. These were made up and used as described 
by Axelrod (1953), Rosenkrantz (1953), and Mitchell and 
Davies (1954).

4. Apparatus. Ground glass equipment was used with water 
lubrication. Distillations in vacuo were serviced by a water 
pump. The chromatographic apparatus consisted of 12 x 24 
inch Corning glass cylinders and standard solvent tray 
assemblies (Cave and Company Limited).
IV. PRELIMINARY CHEMICAL SEPARATION OF AN ESTROGEN FRACTION

A. Partition Methods.

Estrogens may be removed from tissues by a variety of solvents such as ethyl acetate (Kurzrok and Ratner, 1932), ethyl alcohol (Westerfeld; Doisy, et al, 1938), n-butanol (Venning et al, 1937), chloroform (Weismann et al, 1937), and acetone (Szego and Roberts, 1946). It appears, as the Fieser's (1949) state, any organic solvent is suitable.

However where the estrogens are to be removed as a group Mathers (1942) points out that neither benzene or toluene are suitable for extraction of estriol, nor are the petroleum ethers useful for any estrogen. N-butanol is a powerful extractor for both the free and conjugated estrogens, but is not widely used (Emmens, 1950) perhaps because of its high boiling point (118°C.) and difficulties encountered in maintaining it free of butyric acid.

The choice of solvent depends largely on the medium being extracted. For tissues, water miscible solvents such as alcohol are used.

The present study on salmon embryos began with ethyl acetate in order to keep fat contaminants at a minimum level (Deuel, 1950). Subsequent steps in the purification process showed that ethyl acetate extracted powerful emulsifying agents (mainly phospholipids) which interfered at alkali partition levels by forming emulsions. These could not be broken by salting out (NaCl), centrifugation (4,000 r.p.m.,
This difficulty was largely overcome by extracting with acetone and removing the phospholipids. This was done by adding a few drops of ethanol saturated with MgCl₂ (Bush, 1952) to the acetone extract, followed by filtration at -5°C. (Mortin et al, 1952). Subsequent partition with an alkali (N-KOH) gave an emulsion, but this could be broken by centrifuging at 2500 r.p.m, for 15 minutes. Centrifugation, a wasteful process, was replaced by a gentle rolling movement of the separatory funnel containing the immiscible phases, for a minimum of 10 minutes, on each extraction.

It is important to point out that while the partition system may effectively overcome the emulsion problem, it may not effectively remove an estrogen fraction. A study of partition coefficients of estrogens in synthetic systems tabled by Bachman and Petit (1941), Mathers (1942), Friedgood and Garst (1950), and Engel (1950) indicated that the partition of Friedgood and Garst (carbon tetrachloride:ether (18:1)-N-KOH) would effectively place the estrogens in the alkali phase. Moreover, comparatively strong polar substances that contaminate the estrogen fraction could be washed out in an ether extract with 9% NaHCO₃, in a butanol extract with 3M Na₂CO₃, or a combination of these.

Accordingly, Flow-sheet I was formulated and the estrogen fractions at the levels indicated were analysed by paper chromatography. At this time no paper methods had
been devised that would effect a quantitative separation of the female hormones. Since the present hormone fractions were not used in subsequent studies, their further treatment is included here. The principles of paper chromatography are outlined in Section IV, page 28.

B. Early Attempts to Effect An Isolation By Paper Chromatography.

1. Use of Bush's (1952) paper adsorption method.

Stips (2 x 32 cm.) were impregnated with aluminum sulfate and spotted with salmon extracts (Flow-sheet I) to which female hormones were added. In mobile phase systems consisting of benzene-chloroform mixtures, in 3:1, 2:1 and 1:1 ratios (by volume) and benzene-acetone (19:1) no steroid zones were identified by the iodine reaction. The large amount of extraneous biological material hindered resolution. It may be noted that Mitchell and Davies (1954) did not find Bush's method helpful to their studies of placental extracts.

2. Use of Zaffaroni's (1951) paper partition method.

Zaffaroni (1951, '53) impregnated filter paper strips with propylene glycol or fromamide in various concentrations to serve as stationary phases for the resolution of adrenal hormones. Development of the chromatograms was carried out in mobile phases consisting of hexane, benzene and toluene. Attempts were made to use these systems for salmon extracts containing female hormones (Flow-sheet I). Since Bush (1952)
FLOW SHEET I

(1) Lyophilized embryos (50 grams)
Extracted with acetone (2 x 100, 2 x 50 ml.)
in a 12 hour period.

(3) residue discarded.

(2) acetone extract
A few drops MgCl₂ in methanol (saturated) added,
cooled to -5°C. and filtered at -5°C.

(5) residue discarded.

(4) acetone filtrate
Evaporated in vacuo and residue transferred with Et₂O (3 x 25 ml.)
to separatory funnel. Et₂O was washed with 9% NaHCO₃ (2 x 25 ml.).

(7) NaHCO₃ phase
Discarded after washing once with 25 ml. HOH which is added to KOH phase.

(6) Et₂O phase
Evaporated in vacuo and CCl₄·Et₂O (18:1, 76 ml.) added and extracted
with N-KOH (5 x 35 ml.).

(9) CCl₄·Et₂O phase
Discarded after washing once with 25 ml. HOH which is added to KOH phase.

(8) KOH phase
Brought to pH 3-4 (HCl) and extracted with Et₂O (4 x 80 ml.).

(11) aqueous phase discarded.

(10) Et₂O phase
Washed with HOH (2 x 25 ml.) which is discarded. Et₂O is evaporated to dryness in vacuo.

(12) estrogen fraction
Transferred in methanol to pyrex tubes for chromatography.
was detecting adrenal steroid zones on paper according to their property of absorbing ultra-violet light at a wavelength of 240 m\(\mu\), the principle was used here for female hormones. These absorb ultra-violet light at 280 m\(\mu\). In the absence of a filter system delivering this wavelength, a mineral lamp with a strong emission band at 226 m\(\mu\) was used. It was observed this light source caused fluorescence (as opposed to absorption) of female hormones in concentration of the order of 100 micrograms per square centimeter on wet paper. Application of this method to the detection of the hormones on paper failed because interfering substances of salmon origin fluoresced much better.

The conundrum arising from these early attempts to separate the female hormones on paper was solved by L.R. Axelrod (1953).

C. **Column chromatography.**

Some carotenoids are found in alkali fractions of fish origin (Bailey, Carter, and Swain, 1952). Since this fraction also contains the estrogens, an attempt was made to eliminate the carotenoids by column chromatography. Stimmel (1946) successfully extracted the estrogen present in late pregnancy urine by using an alumina column. According to the discussion of a paper presented by Bauld (1952) at least two independent investigators (Swyer and Bates) were not able to duplicate Stimmels(1946) results, nor apparently, could Stimmel
duplicate his own findings with another batch of alumina. Bates, however, was successful when Stimmel's original alumina and apparatus were used. Bitman and Sykes (1953) demonstrated that an alkaline celite column would remove estrogens in a synthetic system. This method failed in the presence of chemical contaminants of biological origin when attempted by Mitchell and Davies (1954). An alkaline celite column of different preparation is being used by Heard (1954) to effect purification of estrone in pregnant mare urine.

The present study used Sammuels (1949) method as a working basis. Alumina of 80-200 mesh size was made more retentive by heating at approximately 500°C for 4 hours in covered crucibles between 2 bunsen burners. Glass tubing (1 x 30 cm.) was plugged at one end with 3 cm. of fine glass wool. A glass rod flattened at the tip just sufficient that it would fit the tubing, served to tamp the alumina gently into the column over a distance of 20 cm. The open end of the column was then fitted with a 50 ml. capacity dropping funnel which delivered successive volumes of eluting solvents. The column was washed once with 50 ml. of petroleum ether before use.

An extract was prepared from salmon embryos by evaporating an ether extract (Flow-sheet I, step 3) and suspending the residue in petroleum ether. After adding the suspension to the uppermost surface of the alumina, successive 50 ml. volumes of petroleum ether, 10% chloroform in petroleum ether,
25% chloroform in petroleum ether and finally 50% chloroform in petroleum ether, were run through the column at a flow rate of approximately 1 ml. per minute. The column was kept wet with the solvents at all times. According to Sammuels, estrogens should be present in the final volume. Under the conditions of this experiment, as much as 300 micrograms of added estrogen could not be detected by the Kober (1931) test. It was observed that at least two "carotencoids" remained on the column. They were located as zones immediately below the column head. The first of the zones was red in color and the second, yellow. Their combined distances amounted to approximately 3 centimeters of the 20 centimeter column.

Varying the nature of the eluting solvents by substitution of similar series of benzene-ether and benzene-methanol combinations did not alter the picture. The substitution of the alumina by a silica-celite slurry (2:1 by weight) in petroleum ether also failed to give a Kober detectable estrogen fraction.

The large amount of residue obtained from every fraction eluted from the columns showed that better purification methods were required to effect a recovery of estrogens.

No attempt was made to use these fractions in paper partition systems.

D. Dialysis.

Szego and Roberts (1946) and Roberts and Szego (1946)
were the first investigators to use the principle of dialysis (Thomas, 1934) to recover a steroid hormone fraction. Using bioassay measurements they showed that the sodium salts of estriol, estradiol-17\beta, and estrone dialyzed into water and that the method provided a means of demonstrating estrogenic activity in blood plasma. These results contrasted those of Rakoff, Paschkis, and Cantarow (1943), who found that estrogens in pregnancy serum would not pass through a collodion membrane by ultrafiltration. Zaffaroni and Burton (1953) and Zaffaroni (1953) showed that adrenal hormones would dialyze independently of one another into a 40% methanol solution from citrated blood. The efficiency of their method was greatly increased by adding chloroform which continuously extracted the hormones as they diffused into the outer system.

The conditions for optimum dialysis of female hormones were therefore investigated. Since Szego and Roberts had difficulty in dialyzing free estradiol-17\beta for bioassay, this hormone was used as a standard in the chemical assay to be described.

1. **Synthetic system.**

a) **Dialyzing without stirring.**

Visking sausage casing (diameter 4.2 cm.) was cut into 30 cm. strips and placed in 3 litres of water saturated with the solvents used in dialysis. After \( \frac{1}{2} \) - 1 hour the strips were removed and double-knotted at one end. These were washed twice by filling and emptying the bag with the solution already used. Two washes were made with water, the
second wash being used to check for leaks. Each bag was filled with 10 ml of Gothlin's saline solution, 1 ml methanol containing 50 μg of hormone, and 40 ml of water. The contents were sealed by a double knot at the open end of the casing. The resulting dialyzing bag was immersed in 250 ml of water, a volume which is 5 times greater than that inside the bag. This ratio was suggested by Szego and Roberts (1946). Twenty-five ml of extracting solvent were then added. Butanol was used exclusively at pH values greater than 7, since chloroform may not remove the hormones in this range. After 7 days (168 hours) the dialyzing bag was removed and rinsed with 10-15 ml of water into the dialysate. This was neutralized with HCl (pH 4-5) on alkacid test ribbon and extracted with chloroform (4 x 75 ml).

The contents of the dialyzing bag were emptied into a beaker and the inner casing wall rinsed with 10-15 ml of water into the same beaker. This residuum was treated as above, except that 4 x 25 ml volumes of chloroform were used to extract the aqueous phase.

After evaporating the chloroform in vacuo, the residue was transferred in methanol (3 x 5 ml) by a medicinal dropper to a pyrex tube. Washings were used. The methanol was evaporated in a water bath at 75°C, and the residue dried over phosphorus pentoxide. The hormone content was then assayed by the Kober test as modified by Venning et al, (1937) except that the reagents were used in reduced volumes to make
a final volume of 5 ml. The optical density at 522 m\(\mu\) was read on a Beckman model DU spectrophotometer fitted with a tungsten light source.

b) **Dialyzing with stirring.**

The Visking sausage casing was cut into 50 cm. strips and washed in the manner described except that the casing was double-knotted at a single locus. It was then made 39 cm. long from knot to open end and fixed with linen thread (40 gauge) to the rimmed end of a cut pyrex tube (1.3 x 2.4 cm.) fitted into a bored rubber stopper. This dialyzing bag was placed in an "Exax" 1000 ml. graduate cylinder containing 500 ml. of water. The synthetic medium, consisting of 25 ml. of Gothlin's saline and 155 ml of water, was poured into the casing using a glass funnel. Estradiol was added by pipette. The outside medium was then made up to volume (350 ml. of water and 50 ml. of extracting solvent). A glass rod flattened to a button shape at the tip, and reaching to within 1 cm. of the bottom of the bag served to stir the contents, and thereby decrease the time required for dialysis. The rod was driven by a power stirrer set at its slowest speed.

After 48 hours stirring the casing was removed and rinsed on the outside with approximately 20 ml. of water into the cylinder containing the dialysate. If butanol had been added to the outside medium, the volume was reduced to 500 ml. in vacuo after bringing the aqueous medium to pH 4-5. This procedure removes most of the butanol as a water azeotrope. The remaining fraction was extracted with 4 x 100 ml. of chloroform.
The residuum in the dialyzing bag was removed with washing and extracted with 4 x 50 ml. of chloroform.

c) **Calibration curve for estimation.**

High values obtained in the first group of experiments (numbers 1, 6, 7, 8, 11, Table I) showed the necessity for blanks prepared from dummy runs. A calibration curve was therefore prepared by pooling chloroform extracts of dialysates in 6 dummy runs. After evaporating the chloroform, the residue was dissolved in methanol and divided into 6 portions. Different amounts of hormone were added to 5 of these portions, the sixth serving as a blank. A Kober reaction was carried out on all tubes. The calibration curve obtained is shown in figure 1.

d) **Hormone recoveries in the synthetic system.**

The results from thirty experiments (Table I) showed considerable variation at similar pH values and throughout the pH range attempted. This variation is directly the result of the heterogeneity appearing in blanks run throughout the experiments, and also, therefore, in the hormone runs themselves. Thus a blank of Kober reagents gave the following values for blanks prepared from dummy runs:

<table>
<thead>
<tr>
<th>pH</th>
<th>System</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>ca 14.0</td>
<td>( \text{Na}_2\text{CO}_3 ) - butanol</td>
<td>0.033</td>
</tr>
<tr>
<td>ca 6.8</td>
<td>( \text{HOH} ) - butanol</td>
<td>0.278</td>
</tr>
<tr>
<td>ca 6.8</td>
<td>( \text{HOH} ) - butanol</td>
<td>0.038</td>
</tr>
<tr>
<td>ca 6.8</td>
<td>( \text{HOH} ) - chloroform</td>
<td>0.185</td>
</tr>
<tr>
<td>ca 6.8</td>
<td>water</td>
<td>0.046</td>
</tr>
<tr>
<td>4.0</td>
<td>( \text{HCl} ) - butanol</td>
<td>0.126</td>
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Figure 1. Calibration curve for estradiol-17β in a synthetic system.
<table>
<thead>
<tr>
<th>No. of Experiment</th>
<th>External Solvent</th>
<th>Duration of Dialysis</th>
<th>Micrograms recovered</th>
<th>Ratio</th>
<th>Percent recovered out</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days</td>
<td>In</td>
<td>Out</td>
<td>In/Out</td>
</tr>
<tr>
<td>1</td>
<td>.3M Na₂CO₃ (pH ca 14) + Butanol</td>
<td>7</td>
<td>14.4</td>
<td>73.9</td>
<td>11.300</td>
</tr>
<tr>
<td>2</td>
<td>Butanol</td>
<td>7</td>
<td>9.9</td>
<td>14.2</td>
<td>1.430</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7</td>
<td>3.5</td>
<td>45.9</td>
<td>13.100</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2</td>
<td>14.0</td>
<td>64.4</td>
<td>4.600</td>
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<tr>
<td>5</td>
<td></td>
<td>2</td>
<td>7.0</td>
<td>32.8</td>
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<td>6</td>
<td>Na₂CO₃:NaHCO₃ (pH 10.1) + Butanol</td>
<td>7</td>
<td>9.7</td>
<td>53.4</td>
<td>5.505</td>
</tr>
<tr>
<td>7</td>
<td>H₃BO₃:NaOH (pH 9.2) + Butanol</td>
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<td>11.5</td>
<td>64.8</td>
<td>5.630</td>
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<td>8</td>
<td>9% NaHCO₃ (pH ca 8.2) + Butanol</td>
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<td>5.8</td>
<td>66.1</td>
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<td>13.4</td>
<td>3.828</td>
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<td>10</td>
<td></td>
<td>7</td>
<td>3.0</td>
<td>36.2</td>
<td>12.067</td>
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<td>11</td>
<td>HOH (pH ca 6.8) + Butanol</td>
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<td>4.5</td>
<td>59.3</td>
<td>13.178</td>
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<td>7.7</td>
<td>33.9</td>
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<tr>
<td>13</td>
<td></td>
<td>7</td>
<td>-</td>
<td>47.2</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>7</td>
<td>-</td>
<td>27.0</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>7</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>2</td>
<td>4.9</td>
<td>49.0</td>
<td>0.100</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>2</td>
<td>-</td>
<td>13.5</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>HOH + Chloroform</td>
<td>7</td>
<td>4.8</td>
<td>27.6</td>
<td>5.750</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>7</td>
<td>4.2</td>
<td>25.9</td>
<td>6.167</td>
</tr>
<tr>
<td>No. of Experiment</td>
<td>External Solvent</td>
<td>Duration of Dialysis</td>
<td>Micrograms recovered</td>
<td>Ratio</td>
<td>Percent recovered out</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>-------</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Days</td>
<td>In</td>
<td>Out</td>
<td>In/Out</td>
</tr>
<tr>
<td>20</td>
<td>HOH + Chloroform: Ether</td>
<td>2</td>
<td>4.0</td>
<td>34.7</td>
<td>8.675</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>2</td>
<td>11.2</td>
<td>46.0</td>
<td>4.107</td>
</tr>
<tr>
<td>22</td>
<td>HOH (pH ca 6.8)</td>
<td>7</td>
<td>4.0</td>
<td>22.0</td>
<td>5.500</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>7</td>
<td>7.7</td>
<td>14.8</td>
<td>1.222</td>
</tr>
<tr>
<td>24</td>
<td>Na3citrate:NaOH:HCl (pH 5.2) + Butanol</td>
<td>7</td>
<td>13.4</td>
<td>35.8</td>
<td>2.672</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>7</td>
<td>7.2</td>
<td>18.3</td>
<td>2.542</td>
</tr>
<tr>
<td>26</td>
<td>K phthalate:HCl (pH 4.1) + Butanol</td>
<td>7</td>
<td>3.4</td>
<td>30.0</td>
<td>8.824</td>
</tr>
<tr>
<td>27</td>
<td>HCl (pH 4.0) +</td>
<td>2</td>
<td>9.8</td>
<td>24.3</td>
<td>2.479</td>
</tr>
<tr>
<td>28</td>
<td>Chloroform: Ether</td>
<td>2</td>
<td>9.0</td>
<td>41.7</td>
<td>4.547</td>
</tr>
<tr>
<td>29**</td>
<td>HOH + chloroform</td>
<td>7</td>
<td>1.4</td>
<td>10.8</td>
<td>7.714</td>
</tr>
<tr>
<td>30**</td>
<td></td>
<td>7</td>
<td>1.3</td>
<td>11.9</td>
<td>9.154</td>
</tr>
</tbody>
</table>

* Blank of chemical reagents only
** Estriol
Where the butanol system could serve as a blank, the chloroform system gave optical densities of .060, .223, and 0. Similarly, when the chloroform system served as a blank, the butanol systems gave densities of .210, 0, 0, .083, and .017. Differences between stirred and unstirred blanks were .068 (against a stirred blank) and .017 and .083 (against an unstirred blank). In face of such an impasse the synthetic runs were discontinued and salmon material introduced.

2. **Biological Systems.**

The rather consistent values obtained in the chloroform-water and chloroform:ether-water systems (experiments 18-21, Table I) suggested the use of this system with salmon material. Since the carbonate-butanol system (experiments 2-5, Table I) appeared to give higher values, it was also included in the test. It may be noted, however that a 7 day run in the two systems and in the absence of dialyzing casing favored the chloroform system. Here a 71% recovery was made from 50 μg. estradiol as compared to 56% from butanol.

Both systems were run in volume ratios suggested by

---

2. Ether was added to chloroform in view of experiments 29, 30, (Table I) which show chloroform does not readily take up estriol.
Zafferoni (1953) for the adrenal steroids. Thus 60 g. of minced salmon embryos in 120 ml. of water were dialyzed under stirring. It was observed that the butanol became yellow with impurities whereas the chloroform-ether system remained colorless.

The procedure finally adopted was as follows. The casing was washed in a water-95% ethanol solution (2:1) and fitted to the apparatus described on page 20 and then 100 ml. of chloroform:ether (20:1) were placed in a graduated cylinder and made up to 850 ml. with water. The tissue to be dialyzed was put through a meat grinder while in the frozen state. Sixty grams of minced tissue was mixed in 120 ml. of water and placed in the dialyzing bag which was then stirred for 48 hours at room temperature, Figure 2. After this time the dialyzing bag was removed, rinsed, and discarded. The dialysate was brought to pH 4-5 and transferred to a separatory funnel. The chloroform:ether already present was used to make an initial extraction. The partition method then used is described in Flow-sheet II. The extra volume of added ether was used to prevent loss of estriol when a washing was made with sodium bicarbonate.

The estrogen fraction obtained by this method was analyzed by paper chromatography (page 35).
Figure 2. Apparatus used in dialysis.
V. PAPER PARTITION CHROMATOGRAPHY OF SALMON EXTRACTS.

A. Introduction.

Paper chromatography is a microchemical method for isolating the components of a complex mixture. It consists of placing the mixture to be chromatographed on strips of filter paper at a measured distance from one end. This end is immersed in a solvent (the mobile phase) which diffuses down the paper. In most steroid hormone studies the paper is previously impregnated with a highly polar solvent or salt (the stationary phase). The chemical compounds making up the residue then move at different rates down the paper according to their molecular differences. The process is regarded as a large number of consecutive partitions of the substances being chromatographed between two phases (Cassidy, 1948). In this respect paper chromatography is not essentially different from ordinary chemical partition methods. Since the success of these chemical methods depend upon the concentration of the substances being purified to the contaminants present (oils etc.) (Engel, 1950) it must also follow that these conditions are limiting factors in paper chromatography. Hence only limited amounts of material can be resolved in a paper system. As these amounts are made small (portioning into lots, spreading the spot on larger widths of paper) the ability to detect the substances being purified becomes a limiting factor (dilution factor). It is because of these principles that preliminary purification methods were used (Section IV).
B. Reproduction of Axelrod's (1953) Method Using Estriol, Estradiol-17\beta, and Estrone.

1) Procedure.

Since this method is briefly outlined by Axelrod (1953), it is enlarged upon here to show how it was used, and to confirm the technique.

A starting line was ruled (2-H pencil) 11 cm. from the edge of a 5 x 46 cm. strip of Whatman No. 1 filter paper. The strip was used as such or divided into 4 columns of 1 centimeter diameter beginning 2 centimeters above the starting line (Burton et al, 1951). After being washed in water 12-24 hours, the strip was transferred to 95% ethyl alcohol for 24 hours. It was dried and impregnated with 50% formamide in methanol (V/V) and blotted between filter paper previously cleaned. The methanol was evaporated in air and the strip laid on a sheet of glass so that the ruled starting line fell over a glass gate 1 cm. high. The gate was constructed of 1 cm. glass rods bent to form a rectangle 4 x 10 cm. The paper strip was covered below the gate with the filter paper used to blot it. A glass box placed (open end down) below the starting line and on the covering paper served to steady the hand when hormone fractions were applied to the starting line by a 1 or 4 microlitre pipette. A stream of air, directed frequently on the starting line, helped to prevent enlargement of the spot. The paper strip was transferred to tanks for development of the chromatogram.

The tanks contained 250-300 ml. of the developing solvent which permeated a filter paper lining. A further
100 ml. of the developing solvent, saturated with the stationary phase, was added to troughs suspending the paper strip into the tank. Sealing was effected by a glass cover made air-tight with a paste of starch in glycerol and a heavy weight.

Three tanks were used and for each, the condition of the developing solvent was maintained by replacement. The methylcyclohexane-formamide system (hereafter referred to as tank I) served to make 24 hour runs at 20±2°C on paper just spotted. This removes inert impurities from the hormone fractions. Chromatograms containing estradiol-17β and estrone were then transferred for a 12 hour run in the o-dichlorobenzene-formamide system (hereafter referred to as tank II). Paper containing the estriol fraction was transferred from tank I for resolution in a dichloromethane-formamide system (tank III). The running time here was 10 hours.

On removal, the chromatograms were dried by a warm air fan (30°C.) approximately 10 hours and then by a cold fan (room temperature) for 10-20 hours. The chromatograms were considered dry when samples of the paper from above the origin did not char in 15% fuming sulfuric acid. This is a very important test and devised in the present investigation to overcome negative results obtained with color indicators used when the paper only appeared to be dry.

2) Detection of estrogens.

Axelrod lists 7 color reagents for the detection of
the hormones. Of these, 15% fuming sulfuric acid, benzoyl chloride-zince chloride, and nitrous acid-mercuric nitrate (Millon's reagent modified) give consistent results (and were therefore used in this study). The phenolsulfonate-phosphoric acid test was not attempted. Rosenkrantz (1953) antimony trichloride in nitrobenzene was found very useful. The most sensitive indicator was Folin and Ciocalteau's phenol test as modified for paper chromatography by Mitchell and Davies (1954). This test was not available until very late in these studies.

3) Position of the hormones.

The positions of estriol, estradiol-17β and estrone, and a mixture of these are shown (Table II) after 24 hours in tank I. It is clear that these hormones do not stay exactly on the starting line as claimed by Axelrod. Attempts to adjust this observation by chromatography in petroleum ether, cyclohexane, and cyclohexane-methylcyclohexane (1:1, V/V) did not alter the picture. Identical positions also were found when undiluted formamide was used as the stationary phase.

Observations made on a mixed chromatogram carried over and developed in tank II (12 hours) showed estradiol-17β and estrone to move in accord with migration rates expected of this system. However estriol did not remain stationary as suggested by Axelrod. Estriol transferred from tank I to tank III for development gave the results defined by Axelrod.
Table II. The location of free steroids (in centimeters from the starting line) after chromatography.

<table>
<thead>
<tr>
<th>Development in</th>
<th>Formamide-methyl-cyclohexane (Tank I)</th>
<th>Formamide-O-dichlorobenzene (Tank II)</th>
<th>Dichloromethane (Tank III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting line</td>
<td>0.0-0.5 cm.</td>
<td>8.0-12.1 cm.</td>
<td>3.7-6.9 cm.</td>
</tr>
<tr>
<td>Estriol</td>
<td>1.3-4.3 cm.</td>
<td>14.5-19.5 cm.</td>
<td></td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>1.4-4.7 cm.</td>
<td>34.4-40.2 cm.</td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>1.4-4.6 cm.</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Mixture</td>
<td>1.5-4.8 cm.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (hours)</td>
<td>24</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixture of hormones after development in Tank I.

Estriol after development in Tank I.
4) **Elution of the hormones.**

After detection of the spots on measured 1-5 mm. strips cut from the chromatogram, the hormones remaining on the paper were eluted with methanol (2 x 5 ml., 2 x 1 hour) into pyrex tubes. The eluates were transferred with a medicinal dropper to sintered glass filters (medium porosity) and the filtrates collected in pyrex tubes. Washings were made throughout this transfer. The methanol was concentrated to 5 ml. and the amount of hormone determined in a Beckman DU spectrophotometer having an ultraviolet light source. Blanks and standards were prepared in the same way.

5) **Elimination of background material.**

Despite previous washing of the filter paper, impurities of paper origin were present in the hormone eluates. These interfered with optical density values. This difficulty was overcome by preparing a blank from the paper being chromatographed (Axelrod, 1953). The results obtained in the present study are shown in figure 3.

A small variation at the absorption maximum is apparent (curves C, D, E). This is the result of the wide margin of error introduced by using small amounts of hormone (10 μg./ml.). According to Friedgood and Garst (1950) ultraviolet measurements should not be attempted when hormone concentrations are less than 15 μg./ml.

6) **Absorption Curves.**

Umberger and Curtis (1948) used a sulfuric acid reaction to identify individual estrogens. Specific absorption curves were shown for each estrogen when measured in the
Figure 3. Absorption spectra showing the elimination of background material from chromatographed estradiol-17β (D), estriol (C), and estrone (E). Curve A is estradiol-17β against pure methanol. Curve B is a prepared blank against pure methanol.
400-550 mµ. range. Axelrod modified the method by eliminating the heat period of 12 minutes. The hormones were stood in sulfuric acid for one and three quarter hours (minimum) in the dark. The absorption curves were then determined at wavelengths from 220-600 mµ.

The present results from chromatographed estrogens are shown in Figure 4. Estriol gave a sharp peak at 310 mµ., in agreement with reported values. However a small peak heretofore recorded at 452 mµ. was evident at 440 mµ. in this study. Estradiol-17β gave absorption maxima at 310, 370, 430, and 452 mµ. Only the peak of 310 agrees with Axelrod's result. However those at 430 and 452 were obtained by Umberger and Curtis. Estrone showed maxima at 300 and 450 in agreement with previous findings.

Since it is well known that both the brand and specific gravity of sulfuric acid as well as the concentrations of hormone affect the findings (Umberger and Curtis, 1948) it is concluded that serviceable results were obtained from the 6 µg./ml. (as opposed to Axelrod's 60-80 µg./ml.).

C. Application to salmon Embryo Dialysates.

Minced salmon embryo (60 g.) containing 100 µg. quantities of added estriol, estradiol-17β, and estrone was dialyzed and the extract (Flow-sheet II) examined chromatographically. After 24 hours development in tank I, a yellow zone of impurities occupied a position similar to a mixture of estrogens in a pure system. Color tests made
Figure 4. Absorption spectra of estradiol-17β (A), estrone (B), and estriol (C), in concentrated sulfuric acid.
FLOW SHEET II

(1) dialysate
Brought to pH 4-5 and extracted
with CHCl₃, Et₂O (20 : 1) (6 x 100 ml.).

(2) CHCl₃, Et₂O
100 ml. Et₂O added and
washed with 9% NaHCO₃
(2 x 100 ml.) and then
with HOH (2 x 100 ml.).

(3) aqueous phase discarded.

(4) CHCl₃, Et₂O
Evaporated to dryness
in vacuo.

(6) aqueous phase discarded.

(5) total estrogen fraction
Transferred in methanol
to pyrex tubes for
chromatography.
at this stage did not indicate that hormones were present on either side of the yellow zone.

A similar run was made on a second lot except that it was carried over for further development in tank II. The yellow zone noted previously had migrated 2-3 cm. more down the paper. Color tests made on sample strips failed to indicate any one of the hormones. In another run areas before, after, and including, the yellow zone were eluted and examined spectrophotometrically at 270-300 m\(\mu\) with negative results. These were confirmed by the Kober test made on the eluates.

This failure suggested that larger amounts of hormone would have to be added if a recovery was to be effected. However, since the present levels being used were 5 times greater than any known source of female hormone, further recovery experiments were regarded as being of no value to the present study. On this basis it may also be considered that dialysis is not an effective method for a chemical assay of hormones in biological tissue. This generalization, however, is unwarranted in view of the organ systems (liver, kidney, blood etc.) present in the fish embryos. Such systems in mammals are believed to contain substances which destroy or inactivate as much as 90% of added estrogen (Pearlman, 1948; Paschkis and Rakoff, 1950; Fishman, 1951). Accordingly the method was evaluated by assaying estradiol-17\(\beta\) and estrone present in horse testes.
D. **Application to Horse Testes Dialysates.**

Horse testes were selected for assay partly because of the large amounts of estradiol-17β and estrone known to be present, and partly because only one chemical assay had been made previously (Beall, 1940). Like most isolation studies of hormone in tissue, Beall used a large amount of testes (28 kg.) from which the hormones were extracted with 280 litres of alcohol. After purification he obtained 210 μg. of estradiol-17β and 360 μg. of estrone per kilogram of tissue. These values suggested that the dialyzing technique would yield results on approximately 100 g. of horse testes.

Three 60 g. lots of minced testes from 3 different horses were dialyzed and extracted for separation by paper chromatography (Flow-sheet III).

The estradiol-estrone fraction was portioned into two lots from 20 ml. of methanol. One lot was tested by the Kober reaction (which was positive) and the other, chromatographed. A sample strip from the chromatogram was tested for the presence of hormones by the Mitchell-Davies (1954) phenolic test, now available. Two zones reacted to the test. They did not occupy positions, however, similar to pure estradiol-17β and estrone run in parallel. It is well known that biological impurities alter migration rates (Rosenkrantz, 1953) and it is presumed that this explanation of observed results applied.

The zones remaining on the chromatogram were eluted and assayed spectrophotometrically for the presence of the
FLOW SHEET III

(1) 3 x 60 g. minced horse testes.

(2) dialysate
Brought to pH 4-5 and extracted with
CHCl₃:Et₂O (20:1) (6 x 100 ml.).

(4) aqueous phase discarded.

(3) CHCl₃:Et₂O
100 ml. Et₂O added and
washed with 9% NaHCO₃
(2 x 100 ml.) and then
with HOH (2 x 100 ml.).

(6) aqueous phases discarded.

(5) CHCl₃:Et₂O
Pooled, in vacuo to dryness;
2 ml. ethanol and
250 ml. benzene added
(in order). Extracted
with Et₂O (4 x 200 ml.).

(9) aqueous phase
Extracted with Et₂O
(4 x 150 ml.).

(7) benzene phase
Evaporated in vacuo to
dryness.

(10) aqueous phase discarded

(8) estradiol and estrone
fraction
In vacuo to dryness
Transferred in methanol
tube for chromatography.

(12) estriol fraction
Transferred to pyrex
tube in methanol for
chromatography.
phenol group (present in all female hormones and in many other substances) already indicated by the Mitchell-Davies test. No such group was observed. These contradictions, suggested that background material obscured the absorption peak expected at 280 m\(\mu\). A Kober test was therefore applied to each eluate. Each gave a clear, deep pink color, a positive reaction.

The concentrations of the Kober positive material in the eluates were measured against the chromatographed blank, estradiol-17\(\beta\), and estrone by the method of Venning et al (1937). The results are shown in Table III. Small amounts of hormone (in order of 10 \(\mu\)g./90 grams of testes) were successfully extracted.

Table III. Concentration of hormones recovered from dialysates of horse testes.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Wavelength</th>
<th>Difference</th>
<th>(\mu)g.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>420</td>
<td>522</td>
<td>522-420</td>
</tr>
<tr>
<td>Blank</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Estradiol standard</td>
<td>.089</td>
<td>.718</td>
<td>.629</td>
</tr>
<tr>
<td>Estrone standard</td>
<td>.036</td>
<td>.585</td>
<td>.549</td>
</tr>
<tr>
<td>Estradiol horse</td>
<td>.209</td>
<td>.316</td>
<td>.107</td>
</tr>
<tr>
<td>Estrone horse</td>
<td>.211</td>
<td>.348</td>
<td>.157</td>
</tr>
</tbody>
</table>

The possibility remained that the substances identified as estradiol-17\(\beta\) and estrone in these horse testes might be male hormone despite the chemical differences between male and female hormones. Hence androsterone and testosterone were chromatographed in concentrations (20 \(\mu\)g.) twice that of the estrogens recovered. No trace was found by the Mitchell-Davies test. A Kober test made on 20 \(\mu\)g. amounts of crystalline
androsterone and testosterone gave pale orange and violet colors, respectively. The pink color of the estrogens did not, therefore, develop.

In resume, the evidence identifying estradiol-17β and estrone in the present study is:

1. Both hormones were isolated from horse testes by Beall (1940).

2. Both hormones were located on a paper partition system designed for female hormones.

3. The Mitchell-Davies test (1954) requires a phenol group if the substances under test are in micro-quantities. The female hormones recovered satisfied these conditions.

4. The Kober test (Kober, 1931; Brown, 1952; Bauld, 1954) requires an oxygen group at the 17 position and an available H at the 16 position (Bates, 1952). Such positions are typical of sex hormones.

5. Male hormones did not give the Kober colors.

The total estriol fraction from 180 g. of horse testes was run on a 1 cm. strip (page 29). After development in tanks I and III, a Mitchell-Davies-positive zone was evident close to the starting line. The zone however did not give a Kober color. Furthermore, measurements were not possible against a blank and control run of estriol (Table IV).

Table IV. Spectrophotometric assay of the estriol fraction from horse testes.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Wavelength</th>
<th>Difference</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>420</td>
<td>522</td>
<td>522-420</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estriol standard</td>
<td>.013</td>
<td>.348</td>
<td>.335</td>
</tr>
<tr>
<td>&quot;Mitchell-Davies&quot;</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Zone</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Thus, no estriol was found, an observation in accord with Beall's (1940) results.

Finally, the entire experiment was repeated on a similar amount of horse testes. These were from a different source, and of much smaller size (approximately 150 g. per testicle as compared to 330 g. in the previous experiment). In this test the dialysate extract was separated into male and female fractions using the KOH/CCl₄:Et₂O partition (Flow-sheet I). The estrogen portion, however, was not separated into estradiol-estrone and estriol fractions.

The male fraction did not respond to Kober's test.

The entire female fraction from 180 g. of minced tissue was chromatographed (as compared with 90 g. previously). Only the estradiol zone was located by the phenol test, and this appeared to be in much lower concentration than found earlier. It was eluted and rechromatographed for further purification in order to obtain its absorption curve in sulfuric acid. The material could not, however, be located after this procedure.

This failure to reproduce the previous results could have been caused by:

1. The smaller testes from probably younger horses.

2. Storage period of the testes which was at least two weeks longer than those first used. For placentae, Mitchell and Davies (1954) point out processing should occur within 30 minutes of delivery.

3. Estrone may not have been present in the testes. For example, Diczfalussy (1953) found estradiol-17β in only 2 of 6 placenta examined.
VI. A FURTHER SEARCH FOR FEMALE HORMONES IN SALMON EMBRYOS.

A. The Mitchell-Davies Partition Method.

Because of the apparent or possible destruction of hormones when salmon embryos were being dialyzed, use was made of a technique recently developed by Mitchell and Davies (1954) for the recovery of estrogens in placentae. This method, based on previous studies by Engel (1950), offered the further advantage of recovering conjugated and protein-bound estrogens. Their paper partition analysis was not attempted in view of Axelrod's paper method already worked out.

Their method as applied to salmon embryos is shown in Flow-sheets IV and IVa. It will be noticed that an extra step is included just prior to the alkali partition (step 20). This was found necessary because of the emulsion formed when a 10 ml. aliquot of EtgO:CCl₄ was partitioned with 5 ml. of N-KOH (experiment 2). Moreover, the N-NaOH used by Mitchell and Davies was replaced by N-KOH in order to effect a better partition (Friedgood and Garst, 1950). The extraction of the hormones from an alkaline solution at pH 9 (Engel, 1950) is the most outstanding feature of the method. This was done with a Beckman pH meter in the present experiments, but "hydrion" paper was used by Engel and presumably by Mitchell and Davies. Under these conditions the entire procedure was carried through without interference by emulsions.
FLOW SHEET IV

(1) Minced embryos (400 grams)
   (Lyophilized embryos (220 grams)
   Extracted with 80% (V/V) ethanol
   (2 x 450 ml.) and 95% (V/V)
   ethanol (2 x 400 ml.).

(2) ethanol extract
   Cooled at 4°C, 12 hours,
   filtered at 4°C.

(3) residue
   Extracted with n-butanol (3 x 400 ml.).

(7) residue
   (4) ethanol extract
   Pooled with
   fraction A

(5) butanol extract
   Pooled with
   fraction B.

(6) Combined and evaporated to dryness in vacuo. Suspended in
   400 ml. water; extracted with 4 x
   200 ml. Et₂O.

(8) residue fraction B
   Fraction A added and
   made up to 700 ml. with
   H₂O and 700 ml. 5% (W/V)
   NaOH added. Stood at
   room temperature 24
   hours and made acid with
   concentrated H₂SO₄.
   Divided into two portions
   and each extracted with
   4 x 200 ml. Et₂O. Combine
   Et₂O.

(9) aqueous phase
   (10) Et₂O extract
   Brought to 425 ml. "free"
   with H₂O; boiled;
   75 ml. concentrated
   HCl added; refluxed
   40 minutes; cooled;
   Extract with Et₂O
   (4 x 200 ml.).

(11) Et₂O (14) aqueous extract
     estrogen fraction (1).

(12) Et₂O extract "Conjugated"
     estrogen fraction (2).

(13) aqueous phase
discarded.

Estrogen fractions 1, 2, 3 treated as outlined in Flow sheet IVa.
FLOW SHEET IVa

(15) Fractions 1, 2, 3 combined and evaporated in vacuo to 800 ml; washed with 9% (W/V) NaHCO₃ (3 x 80 ml).

(16) Et₂O extract
Removed Et₂O in vacuo. 800 ml. acetone and a few drops MgCl₂ in alcohol (saturated) added. Cooled to 0°C. and filtered at this temperature.

(17) aqueous phase discarded.

(18) acetone filtrate
Evaporated in vacuo; a solution of 600 ml. Et₂O and 30 ml. CCl₄ is added. Extracted with N-KOH (6 x 100 ml).

(19) residue discarded.

(20) aqueous phase
Washed once with 100 ml. Et₂O which is discarded. Aqueous phase brought to exactly pH 9 with H₂SO₄ using 6 gr. Na₂CO₃ as a buffer. Extracted with Et₂O (6 x 200 ml).

(21) Et₂O:CCl₄ discarded.

(22) Et₂O extract
In vacuo to dryness; 4 ml. ethanol and 500 ml. benzene added (in order). Extracted with HOH (5 x 150 ml).

(23) aqueous phase discarded.

(24) benzene phase
Evaporated in vacuo just sufficient to remove benzene (no odor).

(25) residue
Dissolved in 25 ml. 90% methanol and washed with 25 ml. petroleum ether; ether fraction back-extracted with 3 x 25 ml. 90% methanol and the pooled methanol evaporated to dryness in vacuo.

(26) Estradiol-estrone fraction (4)
Transferred in methanol to pyrex tube for chromatography.

(27) aqueous phase
Extracted with Et₂O (5 x 150 ml.).

(28) aqueous phase discarded.

(29) Et₂O phase
Evaporated to dryness and treated as in fraction (4) except that 4 times the volumes of methanol and petroleum ether are used.

(30) estriol fraction (5)
Transferred in methanol to pyrex tube for chromatography.
B. **Experiment I. Recovery of Hormone Added to Salmon Embryos.**

400 g. of minced salmon embryos were used to test the magnitude of recovery. Mitchell and Davies (1953) obtained average recoveries in placentae of 27% (estriol) and 13% (estradiol and estrone). Accordingly, it was estimated that 200 g. each of added estriol, estradiol-17β and estrone would provide sufficient hormone for assay after a paper partition separation. The hormones were added to the salmon at the 80% ethanol level (Mitchell and Davies, 1954) and processed. At the stage (step 6) where the material is brought to near dryness, it was accidently charred, resulting in an unknown loss of hormone and other chemicals of tissue origin. The damage done was quite evident at the alkali partition since an acetone step was not needed to avoid an emulsion. It became apparent that there would be insufficient hormone to carry out the remaining steps. Rather than discontinue the labor already involved another 100 µg. of each hormone was added (ether phase, step 20).

The estriol and estradiol-estrone fractions were dried over phosphorus pentoxide and chromatographed. Because of the large amount of residue in the latter fraction, only half of its volume was used.

The developed chromatograms showed amber zones just off the starting line of both estriol and estradiol-estrone fractions. Sample strips were recovered from each chromatogram and examined for hormones by the Mitchell-Davies phenol test.
Two zones appeared in addition to the amber zones previously noted. It was evident that estriol and estradiol-17β did not advance much beyond their respective amber zones. The area marking the advance was eluted for assay. Contrary to these findings, estrone was well isolated. It also was eluted for assay (Table V).

Table V. Recovery values for hormones added to salmon embryos, Experiment 1.

<table>
<thead>
<tr>
<th>Material</th>
<th>μg. added</th>
<th>Wavelength</th>
<th>Difference</th>
<th>Recoveries</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>420</td>
<td>522</td>
<td>522-420</td>
<td>μg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>μg.</td>
</tr>
<tr>
<td>Blank</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Estradiol</td>
<td>-</td>
<td>.036</td>
<td>.585</td>
<td>.549</td>
<td>52.1</td>
</tr>
<tr>
<td>standard</td>
<td>-</td>
<td>.089</td>
<td>.718</td>
<td>.629</td>
<td>52.1</td>
</tr>
<tr>
<td>Estrone</td>
<td>-</td>
<td>.266</td>
<td>.332</td>
<td>.066</td>
<td>5.5</td>
</tr>
<tr>
<td>standard</td>
<td>-</td>
<td>.094</td>
<td>.309</td>
<td>.215</td>
<td>30.4</td>
</tr>
<tr>
<td>Embryo</td>
<td>300</td>
<td>.013</td>
<td>.348</td>
<td>.335</td>
<td>22.9</td>
</tr>
<tr>
<td>Estradiol</td>
<td>300</td>
<td>.016</td>
<td>.111</td>
<td>.095</td>
<td>6.5</td>
</tr>
<tr>
<td>Embryo</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It will be recalled that this experiment was charred at a critical stage and that an extra 100 μg. of each hormone was added late in the experiment. Accordingly the percent recoveries of 3.66 and 13.60 for estradiol and estrone, respectively, have little meaning. The small recovery made of estradiol when compared to estrone was caused by the failure
of estradiol to separate completely from adjacent impurities (the amber zone). An indication of the interference is shown by the high density value of estradiol when compared to estrone at 420 m\(\mu\). (Table IV). This explanation is also applicable to the estriol recovery. All recoveries indicate a great loss in processing the 100 \(\mu\)g. quantities of each hormone added to step 20 (Flow-sheet IVa).

C. Experiment 2. The Failure to Detect Hormones in 220 g. of Lyophilized Salmon Embryos.

This experiment differed from the preceding one in that no hormone was added, charring did not occur, and acetone was necessary for a clean alkali partition (Flow-sheet IV, IVa).

The residue representing the estradiol-estrone fraction was enormously large compared with that found in the previous experiment. A similar fraction but of placental origin weighed 2-10 mg. (Mitchell and Davies, 1954). In the present case it was at least of 1 ml. volume after drying over phosphorus pentoxide. The estriol fraction was minute and not noticeably larger than that found in experiment 1.

1. The estradiol-estrone fraction.

This fraction was made up to 10 ml. in methanol and two 1 ml. aliquots were taken for chromatography. Forty micrograms each, of estradiol-17\(\beta\) and estrone were added to aliquot 1. Both aliquots were then chromatographed on separate strips.

The chromatograms showed the same amber zones found for this fraction in experiment 1. The Mitchell-Davies test
on sample strips showed an additional 2 zones only for aliquot 1 which contained the added hormones. The amber zones from both papers, and the hormone zones were eluted and Kober tested. Only the hormone zones gave a positive reaction. The spectrophotometer readings are shown in Table VI.

Table VI. Recovery values for Experiment 2.

<table>
<thead>
<tr>
<th>Material</th>
<th>Wavelength</th>
<th>Difference</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>420</td>
<td>522</td>
<td>522-420</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol standard</td>
<td></td>
<td></td>
<td>Lost in manipulation</td>
</tr>
<tr>
<td>Estrone standard</td>
<td>.095</td>
<td>.699</td>
<td>.604</td>
</tr>
<tr>
<td>Amber zone,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aliquot 1</td>
<td>1.170</td>
<td>.860</td>
<td></td>
</tr>
<tr>
<td>Amber zone,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aliquot 2</td>
<td>.655</td>
<td>.509</td>
<td></td>
</tr>
<tr>
<td>40 g. Estradiol*</td>
<td>.245</td>
<td>.344</td>
<td>.099</td>
</tr>
<tr>
<td>40 g. Estrone*</td>
<td>.194</td>
<td>.480</td>
<td>.286</td>
</tr>
</tbody>
</table>

* Added to residue being chromatographed.

Aliquot 1 containing 40 µg. each of added estradiol-17 and estrone gave percent recoveries of 20.5 and 59.1, respectively. These values are in accord with the interfering materials whose density values are shown at wavelength 420, Table VI. The optical densities obtained from the eluted amber zone showed an absence of hormone.

The amber zone of aliquot 2 origin was also Kober negative and no hormone could be detected spectrophotometrically. No other zones appeared in this aliquot.

The recovery obtained from 40 µg. of added estradiol in aliquot 1, suggested that 20 µg. would be the minimum limit for assay. If this were so, then 10 x 20 µg. would be required
for an assay of the total estradiol-estrone fraction. If
the Mitchell-Davies average recovery value of 13% from the
estradiol-17β added to placentae could be extrapolated to
fish embryos, then the amount of free estradiol needed for
a chemical assay of these embryos, would be 200 x 100/13,
\(\text{i.e. } 3.75 \text{ mg./kg.}\). This value is at least 10 times greater
than any other value reported for biological tissue. Accord­
ingly, it indicates the extreme improbability of detecting
hormones in salmon embryos.

2. The estriol fraction.

A chromatogram of this fraction showed a single zone,
the amber zone. This was eluted and found to be Kober
negative.

The results show that the hormones under study by
this method, could be recovered only in those fractions
containing added hormone. At the hormone levels required
to make this detection, there was an absence of these
hormones in salmon embryos.
VII. DISCUSSION

The basic pattern of the undifferentiated gonad in vertebrates consists of an inner medullary (testicular) zone and an outer cortical (ovarial zone). Germ cells migrate into these areas after which they may be induced to form male or female cells in their respective areas, and irrespective of their genetic constitution. Thus, in Amphibia, the cortex appears before the medulla in the undifferentiated gonad. Germ cells migrating into this area become female cells. Later the medullary area differentiates and germ cells migrating into this area become male cells. Both male and female cells are therefore present at the same time. Further differentiation results in a loss of one or the other of these areas. When this occurs the initial hermaphrodite becomes male or female.

The sex reversal effected by high temperature rearings and parabiotic studies of frogs (Witschi, 1929, '34), provided evidence that inductor substances were essential components of the different areas. Witschi called these inductors medullarin and corticin. Corticin suppresses the action of medullarin and stimulates cortical differentiation so that female cells form. Similarly medullarin opposes the action of corticin and induces the formation of male cells. Since both inductors are present simultaneously, the genetic constitution normally determines the outcome of sex. Thus in a genetically determined female, corticin normally would
have more inductive force than medullarin. Recently (Witschi, 1950) restated his theory to conform to a hormonal theory of sex differentiation (Figure 5). Apparently Witschi does not identify the inductor substances with sex hormones.

Burns (1949) insists that inductors and hormones are identical, since the differentiation effect ascribed to the inductors may also be closely simulated with hormones. Moore (1947) however, found that androgen and estrogen treatment of young opossum did not modify the ovary or testes, nor did ovariectomy prevent the differentiation of the genital tract. From this, his many other experiments on the opossum, and an evaluation of the literature, Moore (1947) concluded that "the most acceptable evidence for the control of sex differentiation in vertebrates rests upon the concept of the operation of genetic factors", while admitting (Moore, 1950) "that no one has provided a more acceptable explanation (for the freemartin) than that originally given (by Lillie, 1917)". Moore's arguments are based principally on the widespread failure to obtain an experimental reversal of sex in the gonads of young mammals, and the lack of evidence for a hormone secretion from foetal gonada consistent with their differentiation and that of the genital tract. However, Burns (1950) found that female hormone would induce a sex reversal (indicated by cortical differentiation) in the testis of the opossum. Castration and grafting experiments on the rabbit and the rat, respectively, lead Jost (1950, '53) to
Figure 5. Agent stimulators and inhibitors operating in sex differentiation. (Witschi, 1950). c, cortesin; m, medullarin.
ascribe to the foetal testis the role of androgenic hormone production, and that these androgens directed sex differentiation.

The objective of the present study was to show the presence of female sex hormones in salmon embryos. Because of the suggestiveness of the literature on sex differentiation, it appeared that a positive assay would provide the evidence that sex hormones were the factors of sex differentiation, if the hormone could be shown in the undifferentiated gonad. An approach was made by using chemical assay methods based on the recovery of added mammalian hormones. This would provide proof both for the fractionation procedures being used and the similarity of fish hormones to those of mammals.

The results, discussed progressively in respect to the literature and methods being attempted, showed great difficulties at that point where the phenolic estrogen fraction is separated from the neutral steroids. The emulsions which occur at this alkali partition have been an obstacle even for the initial extraction of urine (Bachman and Pettit, 1941). Centrifugation has largely overcome this difficulty but, as suggested by Diczfalusy (1953), microgram quantities of estrogen might be discarded in micelles formed from some impurity at the interphase between immiscible solvents. It is not surprising to find therefore, that an extraction procedure such as that worked out in a synthetic system (Friedgood and Garst, 1951) has no real application
in the presence of biologically complex material such as salmon embryos with their attached yolk sacs. Centrifugation and other methods used to break emulsions, did not effect a separation between a carbon tetrachloride: ether - N-KOH partition. This problem was overcome by removing most of the emulsion-promoting contaminants, such as the phospholipids, with MgCl₂ and chilled acetone.

As thorough as may have been the extraction procedures in the partition systems used to concentrate an estrogen fraction, this fraction nonetheless was made up of a large mass of material. Separation of 100 µg, each of female hormones added to this mass was not possible by the paper adsorption method of Bush (1952).

Attempts were made to concentrate an estrogen fraction by column chromatography. In view of failures (Bauld, 1952) experienced with an aluminum column method (Stimmel, 1946) this procedure was not attempted. A method suggested by Samuels (1947) was used and was found to be unsatisfactory for color detection (Kober, 1931) of comparatively large amounts of added estrogen. Replacement of the solvent systems, and/or of the alumina by a silica-celite slurry, did not alter the findings. It may be noted that an alkaline-celite column developed by Bitman and Sykes (1953) for extracting estrogens, failed when used with biological material (Mitchell and Davies, 1954).

The success obtained by Zaffaroni (1953) in dialyzing
adrenocortical hormones suggested that dialysis would effectively concentrate a female hormone fraction. Moreover Szego and Roberts (1946) and Roberts and Szego (1946) had previously used this principle to demonstrate estrogen activity in blood plasma. The uncertainty that this method was suited to a chemical assay prompted a study of the conditions necessary for a maximum yield of estrogens. The results from a synthetic system were difficult to interpret because, apparently, of the heterogeneity of the Visking dialyzing casing. This heterogeneity interfered with optical density values obtained in both blank and hormone dialysates. However a choice seemed evident between the butanol-\(0.3M \text{Na}_2\text{CO}_3\) system and the chloroform:ether-water system. By a test run on biological material, the latter system offered the advantage of a cleaner extract, and was therefore adopted.

After further purification of the extract a paper partition analysis (Axelrod, 1953) was carried out on the residue. The results showed that 100 \(\mu\)g. each of added hormones could not be detected on the paper chromatogram or empirical eluates cut from it. Since the concentration of the added hormones represented 5 times the values reported as detectable by other methods for estrogens from other tissue sources (Beall, 1940), dialysis of salmon embryos did not appear to warrant further study. The apparent failure could be the result of inactivation or destruction of the hormone by unknown inactivating systems (Fishman, 1951) or perhaps
by a binding of the hormones to salmon proteins (Eik-Nes et al, 1954). However, for blood plasma, dialysis is believed to release 65% of the protein bound hormone (Szego and Roberts, 1946).

Accordingly, the dialytic method developed was evaluated by assaying the estrogen content of horse testes. In the one previous chemical assay, Beall (1940) isolated crystalline estradiol-17β (.210 mg./kg.) and estrone (.360 mg/kg.) from 28,000 grams of tissue. The values obtained from a 90 g. lot in the present study showed estradiol and estrone in concentrations of .097 and .143 mg./kg., respectively. The smaller values obtained here for estradiol and estrone may be more apparent than real. Physiological variation such as that known to occur in placentae (Diczfalusy, 1953) may have affected the determination. The intensity of the Kober color did not suggest that much interference would be effected by the correction readings made at 420 µm. (Yenning, et al, 1937). This possibility did not prove to be so, especially in the case of estradiol. Thus better results might have been obtained by application of other colormetric procedures (Haslewood, 1950) for a Kober quantation.

Estriol, which has been isolated only from the human placenta (Pincus and Pearlman, 1943), was not found in the horse testes.

An attempt to duplicate these results on a second lot of testes was unsuccessful. Here the entire estradiol-
estrone fraction from a 180 g. lot of tissue was chromatographed. Estradiol was located by the phenol test (Mitchell and Davies, 1954), but estrone did not appear. The small amount of estradiol, suggested by the color reaction, was eluted and rechromatographed in order to purify it for a determination of its absorption curve in sulfuric acid. It could not be located on a second chromatogram. These results suggested that storage of the testes may have destroyed some of the hormones (Mitchell and Davies, 1954) recommend processing placental tissue immediately on delivery from the mother) or that, in the case of estrone, it was not present. Thus Diczfalussy (1953) found estradiol in only two of six placentae examined. Since the testicles were apparently from younger horses, this too, may have influenced the results.

Since the dialytic methods used were not adapted for recovering conjugated and protein-bound estrogen fractions, and because of an apparent destruction of hormone added to salmon embryos being dialyzed, a recently developed procedure for extraction of hormones from placentae (Mitchell and Davies, 1954) was attempted. A recovery experiment from 200 μg. each, of the hormones added to minced tissue, was spoiled by charring. The remaining experiment showed the difficulties previously noted in partition analyses, viz., the final fractions were enormous in comparison with their possible hormone content. This content however was not evident when the estradiol-estrone fraction was chromatographed in fractions
of .1 volume. This volume represented the maximum load the chromatography paper could handle and be consistent with a starting line spot no wider than .5 cm. (Axelrod, 1953).

The estriol fraction was chromatographed in toto. No estriol was found.

A test recovery was made on an aliquot of the estradiol-estrone fraction. By using Mitchell and Davies' (1954) average recovery value of 13% for estradiol-17β added to placentae, it was estimated that the amount of estradiol needed for chemical assay of salmon embryos was 3.75 mg./kg. tissue. The magnitude of this quantity with respect to values found in biological tissue, suggested the extreme improbability of detecting hormones in salmon embryos.

It must be concluded, therefore, that until more sensitive chemical methods are developed, direct support of the hormone theory of sex differentiation cannot be found in fish.

The dialyzing method developed here could be refined by first inactivating the enzymes or bacteria that appear to destroy the added hormone. This might be effected by a preliminary heating of the tissue in a saline solution, or better, by dialyzing near 0°C. The pooling of extracts of dialysates would compensate for the comparatively small amount of starting material that can be managed by this method. The dialysate too, may be more profitably treated by a preliminary enzyme (such as glucuronidase) or acid hydrolysis of its residue. It does not seem at all probable that the partition method of Mitchell and Davies offers any
advantage because of the large amount of material in the final residue. However, the Mitchell-Davies paper partition technique (not used in this study) appears to be easier and quicker than that of Axelrod's.

The chemical methods applied to salmon embryos do not represent all of the techniques available for micro-chemical studies. Methods involving counter-current distribution, paper electrophoresis, polarography, and fluorimetry are being worked out, and while these are still unsatisfactory for most purposes, their further refinement may make possible a more profitable study of hormone secretion in embryos.
The problem of sex hormone secretion during embryogenesis of vertebrate animals was approached by a chemical search for female hormones in salmon embryos.

After preliminary experimentation, a dialyzing technique was developed to concentrate an estrogen fraction suitable for separation by paper partition chromatography and spectrophotometric assay. Estrogens were not found in sexually differentiating salmon embryos. Small amounts of estriol, estradiol-17\(\beta\) and estrone added to the tissue could not be recovered. However horse testes assayed by the same technique showed the presence of estradiol-17\(\beta\) and estrone in concentrations of .097 and .143 mg./kg., respectively. The assay of horse testes was carried out on 90 gram lots, whereas the one previous chemical assay was done on 28,000 grams. It is concluded that this technique is very satisfactory for extraction of estrogen from animal gonads but hormone added to whole salmon embryos is inactivated by some unknown system.

A partition technique recently developed by F. Mitchell and R. Davies for the extraction of estrogens from human placentae was slightly modified for use with salmon embryos. This method confirmed the negative findings obtained by the dialyzing technique.

On the basis of these experiments on fish, no evidence could be obtained in support of the hormonal theory of sex
differentiation. This is not a withdrawal from support of the concept, but rather indicates that a good deal of refinement in chemical assay procedure will be necessary before a proper consideration of the problem can be given.
ACKNOWLEDGMENTS

The author gratefully acknowledges the opportunity to carry out this research provided by Dr. W.A. Clemens, Director of the Fisheries Institute, and former Head of the Department of Zoology, the University of British Columbia.

The program was developed under the supervision of Dr. W.S. Hoar, Professor of Fisheries and Zoology, and suggestions by, Dr. M. Darrach, Head of the Department of Biochemistry, the University of British Columbia. I am deeply grateful to them for their help.

I wish to thank Dr. N. Carter, Director of the Pacific Fisheries Experimental Station, the Fisheries Research Board of Canada, for making available their lyophilizing apparatus, and Dr. D. Idler of that station, for his suggestion of the silica-celite column used in the preliminary experiments.

Dr. A.W. Matthews, Dean of the Faculty of Pharmacy, kindly allowed me to use their Beckman DU spectrophotometer.

Miss M. Nagai, Miss D. Timberley, and Mr. A. Beach assisted with the typing and preparation of the illustrations.
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