1959

Steroid hormones isolated from an arrhenoblastoma

Michael Floyd Sorrell
University of Nebraska Medical Center

STEROID HORMONES ISOLATED FROM AN ARRHENOBLASTOMA.

Michael F. Sorrell

Submitted in Partial Fulfillment for the Degree of
Doctor of Medicine

College of Medicine, University of Nebraska

April 1, 1959

Omaha, Nebraska

TABLE OF CONTENTS

INTRODUCTION

One of the more interesting tumors of the ovary is the arrhenoblastoma. Rare in occurence, it is characterized by a wide spectrum of histological pattern, an uncertain clinical picture, both as regards malignancy and symptomatology, and the secretion of potent androgenic substances as yet undetermined.

Because of the rarity of the tumor, it would seem fruitless to expand the slowly but persistently enlarging literature on the pathology and clinical aspects. However, if more information were available as regards specific (and probably unique) entities which are secreted by the tumor, it might be possible to understand its mode of secretion as well as devising a urinary method for preoperative diagnosis. In this event, early diagnosis and, therefore, early therapy might culminate in greater therapeutic success.

It is the object of this investigation to analyze the tissue of a "typical arrhenoblastoma" and attempt to isolate from it any steroids that might have secretory significance. To better understand the place of such an analysis, an abbreviated review of available information concerning these tumors is presented.

HISTORY

A. Definitions and classification

In 1903 masculinizing tumors of the ovary were first reported by Pick [1]. He names his tumor "adenoma tubulare testiculare ovarii" because it closely resembled testicular tissue. The first report

in the American literature was by Moats [2] in 1915. He described his tumor as a "fibroblastic sarcoma of embryonic testis." No systematic classification was presented until 1930 when Meyer reported a series of them and gave the tumor its now generally accepted name, the arrhenoblastoma.

Myers' classification [3] is now universally accepted. However, because of the known discrepancy between the histologic architecture and the endocrinological function of these tumors, it would seem advantageous to add to the present classification an additional aspect based on the entity of androgen secretion rather than histologic appearance. In table I such a correlation is presented.

TABLE I

Types	Histolic Appearance	Secretion
1). adenoma tubulare testiculare ovarii	Typical and atypical tubular elements and some solid elements.	Rarely (3 reported cases)
2). intermediate	Typical and atypical tubular elements with more solid elements.	Usually
3). atypical	Least differentiated. Tumors are solid or predominately solid.	Usually

Many early writers included in their case reviews of arrhenoblastomas an adrenal rest tumor of the ovary, the masculinoveblastoma. This tumor differs histologically from the arrhenoblas-

toma. The former is composed of aggregate of lipoid cells, while the latter is made up of small darker stained cells arranged in rudimentary tubules or cords of cells. Although unlike histologically, both tumors give a similar clinical picture.

B. Histogenesis

Meyer [3] proposed a developmental origin in the genital ridge of the embryo, suggesting the arrhenoblastoma aris s from certain undifferentiated cells in the rete ovarii, the anatomic homologue of the testis in the female. Popoff has considered arrhenoblastomas to be of teratomatous origin. Iverson [4] postulates a similar histogenetic origin for the arrhenoblastoma and the masculinovoblastomas.

C. Clinical

The original clinical description of this entity was reported by Bell in 1915, ten years after the original morphopathologic description by Pick.

Since then a host of papers [4, 5, 6, 7, 8, 9, 10, 11, 12] have described individual cases of arrhenoblastoma and their concomitant clinical manifestations. Iverson [4] reviewed the literature up to 1947 and compiled the various clinical manifestations of this tumor out of 94 reported cases (tables II and III). Javert [10] compiled an excellent review of cases up to 1951. A total of 136 authenticated cases have been reported up to 1957.

TABLE II

ETHNOLOGICAL MANIFESTATIONS

Feature	Distribution
	Average age 31.86 years. Range 15-66 years
Race	Hindu, Spanish, Latin American, Caucasian and Negro.
Duration of tumor before diagnosis.	Average 4 years. Range 1-17 years.

TABLE III

CLINICAL MANIFESTATIONS*

Characteristic	Incidence of signs and symptoms in percent
Amenorrhea	100
Hirsutism	85
Breast Atrophy	70
Clitoral hypertrophy	70
Objective signs, e.g. palpable mass	
Voice change	62
Weight loss	50
Pelvic pain	33
Loss of libido	20

Other signs and symptoms frequently associated with this tumor are, masculine habitus and muscular development, loss of female fat

* Tabulated from Iverson's Data

distribution, acne, and thickening of the skin. Rare cases of psuedohermaphroditism, vaginal atresia, infantile uteri and adnexia have been reported. No one has yet described the sex chromatin pattern but since all reported cases have been unquestionably physiologically female, it is assumed the sex chromatin pattern is female also.

It is generall accepted that the most differentiated of these tumors, the tubulare adenoma, rarely gives rise to the severe masculinizing signs characteristic of the less differentiated tumor.

D. Laboratory

With the undoubtedly marked clinical signs and symptoms of excessive androgenic activity, one might expect laboratory evidence of increased androgen production. Uniquely, this clinical picture is not reflected by elevated 17 ketosteroids. Since the first report of hormone studies by Szathmay [13] in 1937, various investigators have reported normal or upper normal 17 ketosteroid values in the urine.

Everett and Jones [5] reported a case with elevated titers of 17 ketosteroids preoperatively on two 24 hour urine determinations. They demonstrated a fall in titer after surgical removal of the tumor. Control values for the laboratory were not given nor was the method of determination reported. Pregnanediol levels were attemted but no activity could be determined. Dorfman [14] reported a

similar case with elevated 17 ketosteroids preoperatively.

Interestingly enough, bioassay in two cases [9, 15] revealed increased androgenic activity with a demonstrable fall in potency after surgery. This would indicate an androgen that is not degraded to a urinary 17 ketosteroid or an extremely potent androgen unmeasureable by present chemical technique.

Various investigators [4] have attempted to differentiate the masculinovoblastoma and the arrhenoblastoma in the laboratory on the theatrical basis that the masculinovoblastoma will exhibit more of a typical "cushinoid" picture. However, several cases of arrhenoblastomas have been reported which exhibited polycythemia and diabetic glucose tolerance curves. At present, there is no reliable method of differentiation other than histologic.

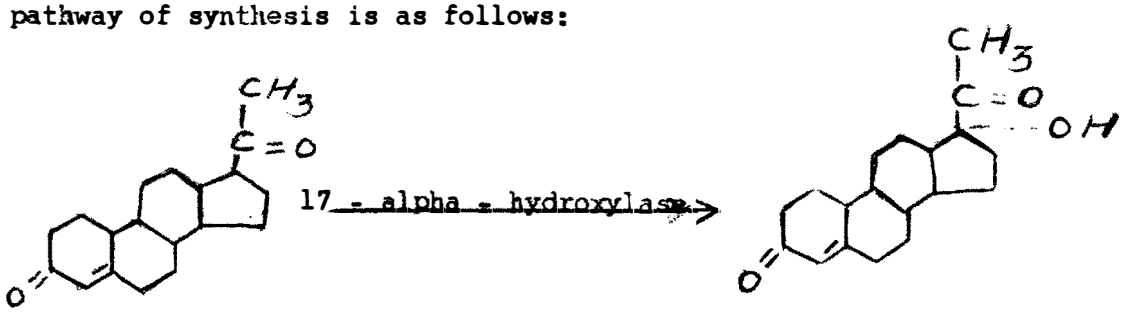
E. Biosynthesis

Three careful laboratory investigations on the hormone enzyme level have been reported. Anliker et al [16] attempted the isolation from a 65 gram arrhenoblastoma. (Total weight of tumor was 110 grams). This tumor was from a 24 year old white female with masculinization but with normal 17 ketosteroid levels. They presented evidence for the presence of androstenedione, androsterone, testosterone and progesterone. However, the amounts isolated were in the range of 2-24 micrograms and the proof of structure based on chromatographic Rf must be considered presumptive at best.

Savard et al [17] incubated a similar tumor with progesterone - C^{14} in fresh human serum. By use of carrier, they isolated 17 alpha-hydroxy-progesterone, 4 androstenedione, testosterone, 20 - alpha hydroxy - 4 -pregnene - 3 - one and 20 beta hydroxy - 4 - pregnene - 3 - one.

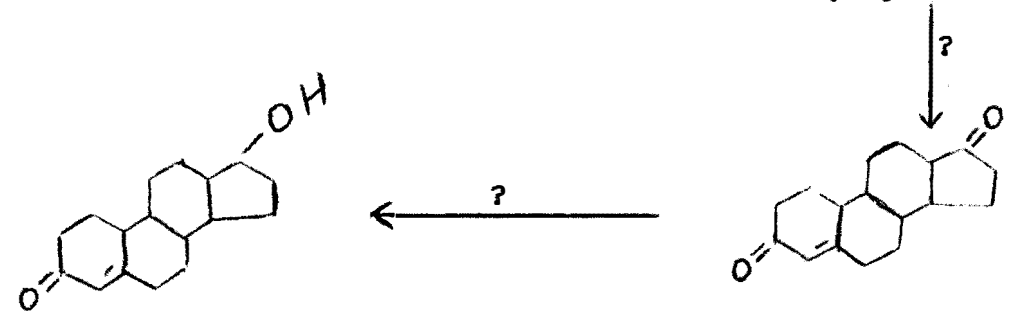
Weist et al [18] incubated a virilizing arrhenoblastoma from a 20 year old woman with progesterone - 4 - C^{14}. 17 - hydroxy progesterone, androstenedione and two more polar substances which were not identified were isolated.

In normal testes (Savard et al [14]) and ovaries (Solomon et al [20]) incubations have resulted in the isolation of a similar series of steroids. These findings have led to the postulate that the pathway of synthesis is as follows:



progesterone

17 - alpha - hydroxy - progesterone



testosterone

androstenedione

The exact mechanisms of reaction are not well-known but Slaunwhite et al postulated that a series of dehydrations and rearrangements by hydrations leads to the formation of androstenedione and/or testosterone. However, the data of Lynn would indicate the presence of a potent oxidase system which employs molecular oxygen. Salhanick and Kadis [21] have suggested that their experiments indicate the formation of the 17-hydroxyl peracetate which decomposes spontaneously to androstenedione. However, most of these authors are impressed with the possibility that these substances "do not account in a quantitative way for the tumors virilizing properties [17]." This concept will be elaborated upon in the discussion.

MATERIALS AND METHODS

A. Tumor

The aliquot of tumor was removed from a 22 year old white female who exhibited amenorrhea of 15 months duration and hirsutism. 50 mg. challenge dose of progesterone did not produce menstruation. FSH levels were negligible. 17 ketosteroids were within normal range. Pathologic examination revealed an arrhenoblastoma of the ovary. 17.8 grams of tissue was available for extraction. This had been deep-frozen since the time of surgery, but sufficient opportunity for minor oxidations had occurred. For this reason, incubation studies were not attempted.

B. Laboratory Methods

All solvents were purified by standard procedures and by distillation prior to use. All evaporations involving more than 10 ml were carried out in vacuo. When quantities less than 10 ml were to be evaporated, the solvent was blown off by a fine jet of nitrogen. Acetylation was carried out in pyridine and acetic anhydride (1:4) at room temperature. The Zimmerman reaction for the estimation of 17 - ketosteroids was the routine clinical procedure in which m - dinitrobenzene in 0.1 ml KOH in Ethanol was employed.

C. Extraction of Tumor

The aliquot of tumor available for extraction was homogenized in a waring blendor and extracted three times with a total of 4 liters of Acetone at 50° C. Two fractions were obtained: The aqueous acetone fraction, which was evaporated in vacuo, and the protein residue which was hydrolized with one liter of 5 percent NaOH for 2 days at room temperature.

D. Acetone Fraction

The aqueous residue from the acetone fraction was extracted quantitatively with chloroform. After the chloroform was removed in vacuo, the fatty residue was partitioned between hexan, which was discarded, and 75 percent methanol, which was concentrated in vacuo. The aqueous residue from the methanol was extracted with chloroform and dried for column chromatography. The final weight

of the yellow oil was 221 mg.

E. Column Chromatography

The silica gel (100-300 mesh) was washed extensively with acetone, ethanol, and ether and activated by heating at 120° C for one hour. A column 15 x 120 mm was prepared with 10 grams of silica gel in a hexane slurry. The oil was placed on the surface of the column by dissolving it in 5 cc of chloroform. The details of the solvent system and residue are shown in table IV.

TABLE IV

CHROMATOGRAPHY OF ACETONE EXTRACT ON SILICA GEL

Fraction Number	Solvent	Volume	Weight of material	Comment
		ml.	mg.	
1.	Hexane	100	84.4	14 hours
2.	Hexane-Acetone, 10:1	100	87.6	4.5 hours
3.	Hexane-Acetone, 1:1	100	31.0	2 hours
4.	Acetone	100	7.8	1 hour
5.	Acetone-Ethanol, 20:1	100	6.3	1 hour
6.	Acetone-Ethanol, 4:1	100	17.8	1 hour
7.	Ethanol	100	23.5	-
8.	Glacial acetic	100	-	stored

The resultant solutions were evaporated in vacuo, reconstituted with ethanol and spectrophotometric curves obtained.

F. Reextraction of Tumor Residue after Alkali Hydrolysis

It is often difficult to extract steroids from crude tissue, and whether this is a reflection of "protein binding" or inadequate mechanical procedures is difficult to demonstrate. However, it is known that alkali digestion renders the material readily extractable with ether (22). It is often not desirable for a first step because of the degredative action of the alkali on the steroids. Progesterone and many estrogenic compounds, however, are stable to alkali, and for this reason, the protein residue was hydrolized in 1 liter of 5 percent NaOH for 48 hours at room temperature, and then extracted with ether. The solution was then evaporated in vacuo. This fraction has not yet been explored. However, on the basis of past experience, it is anticipated that a larger yield of steroids will be obtained if the dihydroxy acetone side-chain is not present.

G. Paper Chromatography

Isolation of the individual steroids was accomplished by paper chromatography according to the methods of Zaffaroni [23]. Each of the fractions eluted from the column (supra vide) were rechromatogramed on paper in the appropriate solvent systems. These were determined on the basis of polarity. A diagrammatic

representation of these chromatograms are presented in the next series of tables. Whatman #1 paper was used for all chromatograms.

TABLE V

CHROMATOGRAM OF COLLUMN FRACTION # 1 MOBILE PHASE: HEXANE SYSTEM

Area	Rf	Characteristics in ultraviolet	Absorption maxima
1 - 1	.01	origin	-
1- 2	.41	not determined	-
1 - 3	.92	fat	-

TABLE VI

CHROMATOGRAM OF COLUMN FRACTION # 2 MOBILE PHASE: HEXANE

Area	Rf	Characteristics in ultraviolet	Absorption maxima
2 - 1	.02	origin	-
2 - 2	.06		240
2 - 3	.11		240
2 - 4	.26		240
2 - 5	.36		230
2 - 6	.51		230
2 - 7	.60	not determined	
2- 8	.81	fat	-

TABLE VII

CHROMATOGRAM OF COLUMN FRACTION # 3 MOBILE PHASE: HEXANE-BENZENE

Area	Rf	CHARACTERISTICS IN ULTRAVIOLET	Absorption maxima
3 - 1	.01	origin	-
3 - 2	.03	fluorescent - purple	265 -270
3 - 3	.11	Absorbent	240
3 - 4	.24	blank	-
3 - 5	.34	questionable absorbence	230
3 - 6	.44	Absorbent	230
3 - 7	.68	silver - blue fluorescence	230 -275
3 - 8	.86	fat	-

TABLE VIII

CHROMATOGRAM OF COLUMN FRACTION # 4 MOBILE PHASE: HEXANE-BENZENE

Area	Rf	Characteristics in ultraviolet	Absorption maxima
4 - 1	.02	origin	-
4-2	.07	doubtful absorbence	290
4 - 3	.10	purple fluorescence	290
4 - 4	.20	questionable absorption	250-270 shoulder
4 - 5	.50	blank	-
4 - 6	.40	fat	-

TABLE IX

CHROMATOGRAMS OF COLUMN FRACTION # 5 MOBILE PHASE: HEXANE-BENZENE

Area	Rf	Characteristic in ultraviolet	Absorption maxima
5 - 1	.02	origin	-
5 - 2	.08	questionable absorption	230-280
5 - 3	.11	purplish fluorescence	280
5 - 4	.21	questionable absorption	260
5 - 5	.53	blank	-
5 - 6	.9	fat	-

TABLE X

CHROMATOGRAMS OF COLUMN FRACTION # 6 MOBILE PHASE: BENZENE-CHLOROFORM

Area	Rf	Characteristics in ultraviolet	Absorption maxima
6 - 1	.04	origin	-
6 - 2	.20	questionable absorption	260
6 - 3	.29	blue fluorescence	280-290
6 - 4	.53	blank	-
6 - 5	.65	absorption	260
6 - 6	.88	fat	-

TABLE XI

CHROMATOGRAMS OF COLUMN FRACTION # 7 MOBILE PHASE: BENZENE-CHLOROFORM

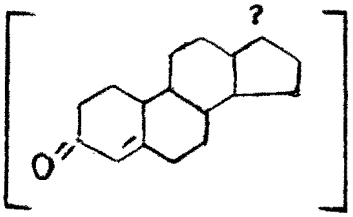
Area	Rf	Characteristics in ultraviolet	Absorption maxima
7 - 1	.03	origin	-
7 - 2	.14	questionable absorption	?
7 - 3	.24	blue fluorescence	250-280 (shoulder)
7 - 4	.45	blank	-
7 - 5	.74	absorption	260
7 - 6	.91	fat	-

From resultant chromatograms, certain substances were pooled on basis of approximate similarity of Rf and/or ultraviolet maxima. From the final pools, it was possible to separate a large number of unknown compounds. Since it was apparent at the start that the most interesting substances would not be in the progesterone, 17 - alpha - hydroxy - progesterone, androstenedione series, these areas were not explored. The most interesting substance (compound #3) was then studied extensively to demonstrate its presence and its possible structure.

H. Characterization of compound 33:

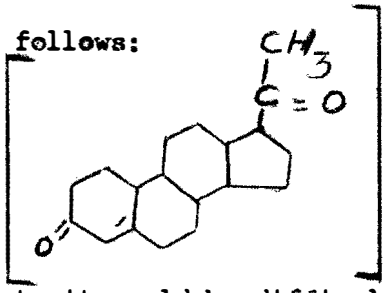
This substance was first detected after the third eluate of the column was chromatographed in the hexane - benzene system. It had an

Rf of .11. On the basis of the solubility in the extracting solvents, its elution from the column with hexane - acetone and its Rf, it was apparent that the substance was a steroid with threee oxygen functions. Ultraviolet analysis revealed a peak of maximal absorption at 240 mu which is characteristic of the alpha - beta unsaturated ketone. Since practically all known natural steroids with this conjugation have it in the A ring, it was possible to predict the structure, as follows:



+ 2 " O "

It can be seen that the side-chain remained unknown. The Zimmerman reaction was performed on a micro scale and the absorption maxima determined to be at 490 mu. Since most 17 - ketones have absorption maxima at 520 (24), it was apparent that an intact side-chain was present and it was a 20 - ketone. The presumptive structure was then as follows:



+ 1 " O "

As is evident, it would be difficult to place the additional oxygen. The unnatural positions would seem to be eliminated. These would include positions 1, 2, 8, 9, 12 and 15. Positions 4 and 6 are eliminated because there is no additional conjugation to the

delta - 4, 3 - ketone demostrable by ultraviolet analysis unless position 6 carried the oxygen as a hydroxyl group. This leaves available for the hydroxyl group positions 6, 7, 11, 14 and 16. Ketones might be placed at 7, 11 or 16. Either hydroxyls or aldehydes might be located at the angular 18 or 19 methyl groups.

A series of steroids with the hydroxyl group in the various possible sites were chromatogramed to determine which might have similar Rf's. The comparative Rf's are presented in table XII.

TABLE XII

HYDROXYLATED STEROIDS

Compound tested	Hexane-Benzene system	Benzene system
	Rf	Rf
6 - beta - hydroxy - progesterone	0.37	-
11 - alpha - hydroxy - progesterone	0.14	0.38
11 - beta - hydroxy - progesterone	0.29	0.57
17-alpha - hydroxy - progesterone	0.48	-
16 - alpha - hydroxy - progesterone	0.09; 0.13	0.24
19 - alpha - hydroxy - progesterone	0.10	0.27
Compound 33	0.11; 0.10	-

Utilizing data from the above table it would seem feasable that the unknown compound might be either the 16 or the 19 hydroxylated substance. The 14 - hydroxylated derivative is neither known nor a-

vailable from synthetic sources for comparison; however, the possibility of compound 33 being this substance appears unlikely because tertiary hydroxyl derivatives do not exhibit the polarity of this substance. Similarly, a ketonic derivative appears unlikely because they also are markedly less polar. For example, 11 - keto - porgesterone proved to have an Rf of 0.55 and 6 - keto - progesterone an Rf of 0.71.

To confirm or rule out the presence of the hydroxyl grouping, a micro - acetylation was carried out on a small aliquot of compound 33, a 19 - hydroxy - progesterone and a 16 - hydroxy - progesterone. Whereas the known acetate proved , have almost identical Rf's of 0.78, the unknown substance was not visualized in this area. Interestingly enough, this indicated that compound 33 may not have an hydroxyl group capable of being acetylated.

At about this time, Wiest et al [18] published the isolation of two unknown substances from an arrhenoblastoma. No data was available on either of these substances except that substance I_2 had an Rf of 0.36 in benzene - chloroform (4:1) - formamide. This would correspond to our Rf of about 0.25 in benzene - formamide.

Present investigations are aimed at completion of the identification of this substance. Presumably, oxidation of the aldehyde will yield a more polar acid. This finding will confirm the presence of the third oxygen on either 18 or 19 methyl group.

Additional investigation will be directed at determining whe-

ther the unknown compound has biological activity. This activity will be determined for both, androgenic and progestational potency. However, such an investigation is outside the scope of this thesis.

DISCUSSION

The problem confronting the investigation of an arrhenoblastoma is the is the heretofore unexplained endocrinological basis for the severe masculinization manifested by most tumors. Two possible explanations present themselves: First, the tumor may have an increased rate of synthesis of the natural androgenic hormones. Thus, there would be increased production of 17 - hydroxy - progesterone, androstenedione, and testosterone. If this were the case, it should be possible to easily establish a diagnosis by the presence of increased urinary levels of pregnanetriol and 17 - ketosteroids.

This possibility seems unlikely because the 17 - ketosteroids are not increased. However, if one assumed that the biological limit insofar as masculinization of the patient is concerned is approximately 300 mg equivalents of testosterone per month, this would mean that the tumor would need secrete only 10 mgs per day. Allowing for greater biological efficiency in the production and function of the substance and allowing for a large excretory component being unmeasured in the bile (Sandberg et al), it is apparent that this type of increased secretion might never be reflected by increased 17 ketosteroids except in a few isolated instances where an inordinate amount of hormone was excreted. Patients suffering from an arrhenoblastoma usually exhibit a greater degree of maculinization, by far, than those patients with similar clinical virilizing syn-

dromes (e.g. adrenogenital syndrome) that conversely are characterized by increased 17 ketosteroid levels.

Finally, the demonstration that the urine from patients with an arrhenoblastoma has large amounts of biological activity in the presence of normal 17 ketosteroids, tend further, to support the hypothesis of a new hormone. Addition support for this theory is now evident on the basis that a new hormone has been isolated from the arrhenoblastoma. Whether this hormone be characterized or not, it is certain that it is an unusual substance. Indeed, the Wiest group considered it so unusual, they did not attempt identification, other than to report its Rf. It is hoped that bioassay will confirm our impression of androgenic activity.

Much work remains to be done on the remaining fractions. While their biological activity remains obscure, they are now sufficiently pure, that characterization of several other hormones should be possible.

SUMMARY

Steroids were extracted and one compound partially characterized from a masculinizing arrhenoblastoma of the ovary. Methods of isolation and identification are described in detail. The unknown compound is thought to be either a hydroxy or aldehyde derivative of progesterone. The significance of these findings is discussed. A proposed scheme of biosynthesis of androgens is also discussed in detail.

******

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. E. G. Holmstrom for the tumor and clinical information associated with this case, to Dr. Barney Kadis and Connie Harris for much invaluable technical advice and to Dr. H. A. Salhanick for giving so generously of his time, interest, and facilities in support of this investigation.

BIBLIOGRAPHY

1. Pick, L., Uber Adenome der mannlichen und weiblichen Keimdruse bei Hermaphroditismus verus und spurius. Nebst Bemerkungen uber das endometriumahnliche Adenom am inneren weiblichen Genitals. Berl. blin. Wchnschr. 42: 502, 1905.

2. Moots, C. W., Lateral partial glandular hermaphroditism. Am. J. Obst. and Gynec. 1: 864, 1921.

3. Meyer, R,. Pathology of some special ovarian tumors and their relation to sex characteristics. Am. J. Ostet. and Gynec. 22: 697, 1931.

4. Iverson, L., Masculinizing tumors of the ovary. Surgery, Gynec. and Obst. 84: 213, 1947.

5. Jones, G. S. and Everett, H. S., Arrhenoblastoma of the ovary, with a report of 2 cases. Am. J. Obst. and Gynecol. 52: 614, 1946.

6. Riley, C. L. and Murphy, C. H., Arrhenoblastoma of the ovary. Am. J. Obst. and Gynecol. 62: 369, 1951.

7. Wachs, C. S. and Weber, L. L., Arrhenoblastoma of the ovary. Am. J. of Obstet. and Gynecol. 61: 212, 1951.

8. Dockerty, M. B., Ovarian Neoplasms; a collective review of the recent literature. Internat. Abstr. Surg. 81: 179, 1945.

9. Pederson, Jørgen, Virilizing ovarian tumors. J. Clin. Endo., 7: 115, 1947.

10. Javert, C. I., and Finn, W. F., Arrhenoblastoma. Cancer. 4: No. 1, January, 1951.

11. Nelson, H. B., Calderwood, G. C., and Cohen, M., Malignant arrhenoblastoma of the ovary. Am. J. of Obstet. and Gynecol. 73: 1115, 1957.

12. Krack, F. and Wolfermann, J. J., Arrhenoblastoma of the ovary. Ann. Surg. 114: 78, 1941.

13. Szathmay, Z., Hormonuntersuchungen bei Arrhenoblastoma. Arch. F. Gynak, 164: 478, 1937.

14. Dorfman, R. I. and Shipley, R. A., Androgen, New York, John

Wiley and sons, INC. 1956. pp. 267.

15. Kanter, A. E. and Klawans, A. H., Arrhenoblastoma of the ovary. Am. J. Cancer. 40: 474, 1940.

16. Anliker, R., Rohr, O., and Ruzicka, L., Uber den Nachweis von androgenen Hormonen in einem virilischierenden Ovarial tumor, Ann. d. Chem. (Liebig's) 602: 109, 1957.

17. Savard, K., Dorman, R., Gakrilove, J. and Soffer, L., Androgen formation in vitro by human ovarian tumor tissue. Abstract No. 133, Program of the 39th Meeting of the Endocrine Society, New York, N. Y., May 30 - June 1, 1957.

18. Wiest, W. G., Zander, Josef and Holmstom, E. G., Metabolism of Progesterone - 4 - C^{14} by an arrhenoblastoma. J. Clin. Endo. and Metab. 19: 297, 1959. (March).

19. Savard, K. and Dorfman, R., Biosynthesis of androgens from Progesterone by human testicular tissue in vitro. J. Clin. Endo. and Metab. 16: 1629, 1956.

20. Solomon, S., Wiele, R. V., and Lieberman, S., The in vitro synthesis of 17 - alpha - hydroxy progesterone and delta - 4 - androstene - 3, 17 - dione from progesterone by bovine ovarian tissue. J. of A. C. S. 78: 5433, 1956.

21. Salhanick, H. A., and Kadis, B. Personal communication to the author.

22. Noall, M. W., Salhanick, H. A., Neher, G. M., and Zarrow, M. X., Methods for the isolation of progesterone from the human placenta. J. Biol. Chem. 201: 321, 1951.

23. Zaffaroni, A., A micromethod for the analysis of adrenocortical steroids, Recent Progr. Hormone Res. 8: 51, 1953.

24. Wilson, Hildegard, Chromogenic values of various ketosteroids in a micromodification of the Zimmerman reaction. Comparison with the micro method.