

Studies in Steroid Metabolism

VIII. Steroidogenesis in a Heterologous Tumor Transplant*

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SUMMARY

From a heterologously transplanted embryonal carcinoma of the testis in the cheek pouch of the golden hamster (Deac 3 tumor) 182 μ g. of testosterone was isolated. Evidence for this identification was obtained through spectroscopic procedures, mixed chromatograms and carrier dilution experiments. Progesterone was tentatively identified by mixed paper chromatography and ultraviolet spectra.

Relatively little is known concerning the maintenance of biochemical integrity of tissue after it has been heterologously transplanted. The possibility of tissue enzyme adaptation to the new host following such a transplant is not easily ruled out. This subject is of particular interest in view of the use of human tumor transplants in rodents as a screening method for antitumor agents.

A recent report from this laboratory (3, 6) described the biosynthesis of androgens, estrogens, and progesterone from a testicular tumor, following incubation of the excised tissue with labeled acetate. By use of a similar procedure, it was possible to demonstrate the formation of testosterone and possibly progesterone, but not estrogen, from a heterologous transplant of a similar tumor (4) which had been retransplanted 21 times.

EXPERIMENTAL AND RESULTS

Incubation.—A freshly excised piece of Deac III tumor (4) weighing 0.73 gm., which had been retransplanted 21 times into cortisone-treated hamsters, was sliced in a hand microtome. The slices were incubated in 25 ml. of Krebs-Ringer phosphate buffer at pH 7.4, containing 50,000 units of penicillin, 6000 units of chorionic gonadotrophin,¹ and 4 mg. of 1-C¹⁴-acetate (specific activity 1 mc/mmmole) for 3 hours at 37.5° C.

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¹ The chorionic gonadotrophin (Antuitrin-S) was generously donated by Parke, Davis & Co.

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Extraction.—The contents of the flask were homogenized with 20 parts of a solution of 2 parts of chloroform and 1 part of methanol. The mixture was filtered, and one-fifth of its volume of water was added. The lower phase was separated and washed with water. The aqueous layers were twice extracted with chloroform, and the extract was washed with water. The combined organic phases were taken to dryness *in vacuo*, following withdrawal of a 1/1000th aliquot. This small portion was dried on a planchet and, after the radioactivity was measured in a windowless gas-flow counter (Tracerlab SC 50A), was shown to contain 48 counts/min.

This and all subsequent samples were counted at infinite dilution to an accuracy of ± 5 per cent.

Preliminary purification.—The dried residue was partitioned between ligroin and 90 per cent methanol with eight transfers utilizing 50 ml. of solvent in each phase. Following determination of radioactivity in each fraction the contents of the funnels numbered 6 through 8 (14,500 counts/min) were taken to dryness, and the residue was partitioned with eight transfers between toluene and 1 N sodium hydroxide. The lower phase from each funnel was neutralized and extracted with chloroform. The organic residues from each individual tube were combined and their radioactivity was determined. The acidic fraction (funnels 6–8) contained 783 counts/min, and the neutral fraction (funnels 0–3) contained 8349 counts/min.

NEUTRAL FRACTION

Testosterone.—The contents of funnels 0–3 were combined and evaporated to dryness. The residue was redissolved in 2 ml. of methanol and divided in half. One ml. was applied to a paper strip

15 cm. wide which was previously impregnated with propylene glycol. The paper chromatogram was developed with ligroin (5) for 18.5 hours. The run-off containing 325 counts/min was collected in a beaker. The chromatogram was dried in an oven at 70° C., and, following inspection under ultraviolet light, a 1-cm. strip was cut from the edge and treated with Zimmermann's reagent. There appeared a strong UV absorbing zone approximately 5–6 cm. from the origin which gave an intense blue color on staining. The radioactive area on the strip was determined with a windowless gas-flow paper strip scanner, and it appeared coincidental with the UV absorbing zone. The total estimated radioactivity, based on the 1-cm. strip amounted to approximately 2000 counts/min.

The area of the chromatogram visible under UV light was then cut from the strip, eluted with absolute ethanol, and evaporated to dryness *in vacuo*. The residue was dissolved in methanol,

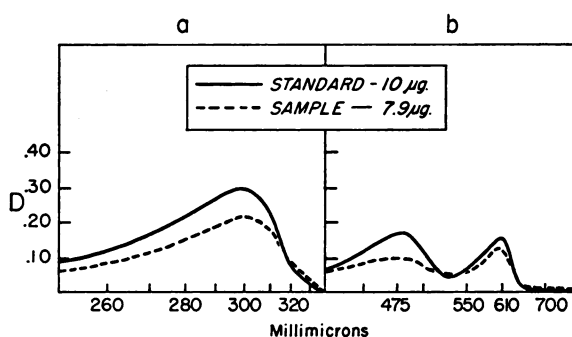


CHART 1.—*a*) Sulfuric acid spectra of:
solid line — = testosterone.
broken line -- = tumor extract.
b) Allen spectra (sulfuric acid and methanol) of:
solid line — = testosterone
broken line -- = tumor extract.

filtered, and examined in a Beckmann model DK-2 recording spectrophotometer. The solution showed an absorption maximum at 240 $m\mu$, which, compared with a standard of authentic testosterone, corresponded to 79 μg . of material. One-tenth of this extract was taken to dryness in a test tube and allowed to react with 4 ml. of sulfuric acid (7) for 2 hours. An ultraviolet spectrum taken at that time is shown in Chart 1a. Another such portion was treated similarly with a mixture of sulfuric acid and methanol (1), and the resulting spectrum is shown in Chart 1b.

A third portion of the remaining extract was compared to authentic testosterone in a mixed chromatogram using the ligroin-propylene glycol system. No separation of the extract from testosterone occurred.

Infrared spectroscopy.—The remaining seven-tenths of this first half of the extract was chromatographed on an aluminum oxide column by successively eluting the column with benzene and 5 per cent methanol in benzene. A white semi-crystalline material was obtained which was transferred with chloroform to a small amount of finely ground optical grade potassium bromide. After evaporation of the solvent *in vacuo* followed by thorough mixing a micro window was produced by pressing the mixture in a die at 12 tons per square inch. An infrared spectrum of this material was compared to a similarly prepared sample of testosterone, and, as shown in Chart 2, the two curves are identical.

Carrier dilution experiments.—The second portion of the original extract was chromatographed

TABLE 1
CARRIER DILUTION OF TESTOSTERONE FROM EXTRACT

Crystallization	Counts/min/mg crystals	Counts/min/mg mother liquor
Testosterone:		
Second	194	
Third	160	157
Fourth	158	164
2,4-dinitrophenyl-hydrazone:*		
Second	29	30
Calc. as free steroid and corrected for dilution	151	156

* Before preparing the derivative the remaining material (3.1 mg.) was further diluted with 6.82 mg. of testosterone.

on paper exactly like the first portion, and the UV absorbing zone was eluted. To this was added 10.1 mg. of testosterone. The mixture was recrystallized until the crystals and the mother liquor attained constant specific activity. The remaining 3.1 mg. of testosterone was further diluted to 9.92 mg., and the 2,4-dinitrophenylhydrazone was prepared. The derivative also was recrystallized to constant specific activity. The results of this work are shown in Table 1.

Low polarity material.—The run-off from the first paper chromatogram (325 counts/min) was rechromatographed in propylene glycol-ligroin for 3.5 hours, along with a strip containing authentic progesterone. The area corresponding to progesterone showed weak UV absorption and gave a blue Zimmermann color. This zone was eluted and was shown to contain 304 counts/min. After elution from the planchet the extract was divided into two parts. One of these was compared

to authentic progesterone in a mixed chromatogram in the same system as before. No separation occurred between the extract and progesterone. The second part was dissolved in methanol. This showed an absorption in the UV region at $240\text{ m}\mu$, corresponding to approximately $15\text{ }\mu\text{g.}$ of progesterone. A sulfuric acid spectrum of this portion yielded no significant results because of the very low extinction coefficient.

PHENOLIC FRACTION

The material from funnels 6 through 9, following partitioning between toluene and base, was combined and washed with water. One-half of the extract was applied to a paper chromatogram which was developed in toluene-propylene glycol. Following treatment with ferric chloride-ferricya-

Perhaps the most interesting finding in the present study is the high concentration of testosterone isolated from the tumor tissue. If the $182\text{ }\mu\text{g.}$ of this steroid had been formed during incubation, the final product should have contained several millions of counts of radioactivity. Since only approximately 4000 counts/min were detected, it must be assumed that a large amount of testosterone was trapped in the cells of this well vascularized tumor. It is unlikely that such a large quantity of steroid could have been formed during the incubation form unlabeled acetate. No reason can be given at this time for the apparent inability of the endogenous hormone to enter the host animals' blood stream.

It is of course impossible to draw any firm conclusions—particularly relating to differences—

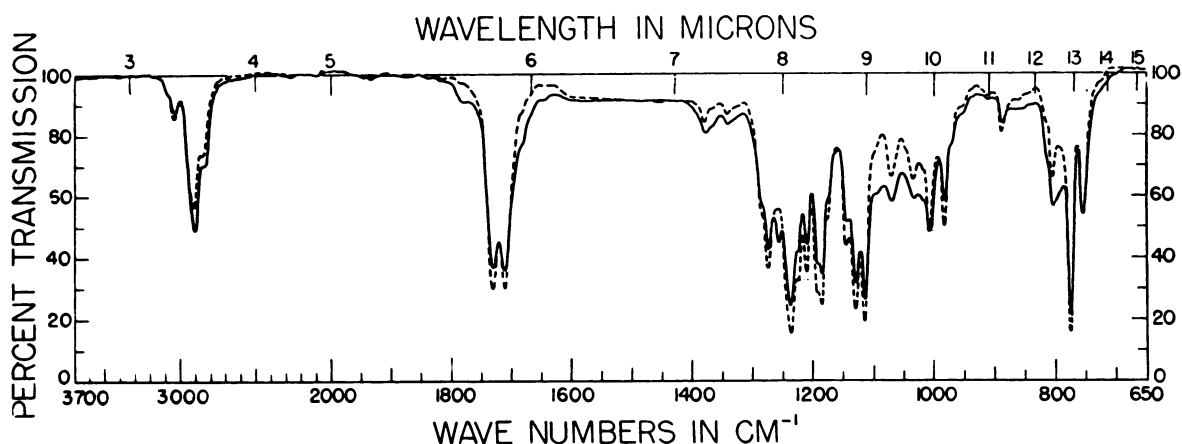


CHART 2.—Infrared spectra of the tumor extract (solid line) and testosterone (broken line). Both samples were prepared as

micro KBr windows and were recorded on a Perkin-Elmer model 21 double-beam infrared spectrophotometer.

nide reagent no color was visible on the paper strip. No radioactivity was detectable either, when the strip was scanned in the flow counter.

The second half of the phenolic fraction was subjected to bioassay by the method of Astwood (2). Neither water imbibition (after 6 hours) nor uterine growth (after 30 hours) was detected with this material when it was distributed among six test animals.

DISCUSSION

The use of transplanted tumors as a research tool has been discussed by Patterson *et al.* (4). These authors pointed out that the problems arising from a study of heterologously transplanted tumors in relation to spontaneous neoplasms need much further clarification from the clinical, biological, and chemical points of view. Therefore, the existence of an endocrine tumor in the cheek pouch of the golden hamster quite naturally raises the question of hormone biosynthesis.

regarding the comparison of a spontaneous neoplasm from one patient with the transplanted tumor tissue from a different donor, though the nature and pathology of the two tumors may be quite similar.

In Table 2 are shown comparative data regarding the incorporation of labeled acetate into the neutral fraction and into individual steroids for the embryonal tumor previously reported on and the tissue from the present study. The transplant incubation resulted in less than one-eighth of the radioactivity incorporated into the organic phase as compared with that in the earlier study, while its substrate contained 25 times the amount of radioactive material. Nevertheless, the total number of counts incorporated into the testosterone is almost double. Assuming capacity for the production of steroids for both tissues of an equal order one could expect quantities of estrogen and progesterone well above the lower limits of sensi-

tivity of the methods used. The formation of a progesterone-like substance is in keeping with the results from the embryonal tumor, but if it was progesterone its activity was only one-tenth that of testosterone.

In contrast, the present tumor gave no evidence for the formation of estrogens either prior to or during incubation, as evidenced by the lack of a recognizable radioactive estrogen fraction as well

as the lack of biological activity.

The inability to detect estrogens from the transplant cannot be assigned any significance at this time. This difference may be due to any of several factors, such as differences in the original tumors, changes in the tumor following transplantation, or the differential ability of various steroids to enter the hosts' blood stream.

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REFERENCES

- ALLEN, W. M.; HAYWARD, S. J.; and PINTO, A. A Color Test for Dehydroisoandrosterone and Closely Related Steroids of Use in the Diagnosis of Adrenocortical Tumors. *J. Clin. Endocrinol.*, **10**:54-70, 1950.
- ASTWOOD, E. B. A Six-hour Assay for the Quantitative Determination of Estrogen. *Endocrinology*, **23**:25-31, 1938.
- DAVIS, J. W.; LYONS, M. K.; LEMON, H. M.; and WOTIZ, H. Steroid Biosynthesis by Testicular Embryonal Carcinoma Tissue. *Boston M. Quart.*, **6**(3):2-6, 1955.
- PATTEBSON, W. B.; PATTERSON, H. R.; and CHUTE, R. N. Transplantable Human Cancers. *Cancer*, **10**:1281-92, 1957.
- SAVARD, K. Paper Partition Chromatography of C₁₉- and C₂₁-Ketosteroids. *J. Biol. Chem.*, **202**:457-77, 1953.
- WOTIZ, H. H.; DAVIS, J. W.; and LEMON, H. M. Steroid Biosynthesis by Surviving Testicular Tumor Tissue. *J. Biol. Chem.*, **216**:677-87, 1955.
- ZAFFARONI, A. Absorption Spectra of Sulfuric Acid Chromogens Obtained from Adrenal Steroids and Related Products. *J. Am. Chem. Soc.*, **72**:3828, 1950.

TABLE 2

RADIOACTIVITY OF STEROIDS OBTAINED FROM INCUBATIONS OF AN EMBRYONAL TUMOR (6) AND FROM A TUMOR TRANSPLANT

STEROID	COUNTS/MIN OF RADIOACTIVITY OF STEROID FROM	
	Embryonal tumor*	Transplant†
Testosterone	2530	~4000
Androstenedione	2350	0
Progesterone	2020	~ 300‡
Estradiol	575	0
Estrone	1050	0
Per cent incorporation in organic phase	0.74	0.096

* Incubation: 0.8 gm. tissue, 2 μ c. (0.16 mg.) acetate-1-C¹⁴, 3 hr. at 37° C.

† Incubation: 0.73 gm. tissue, 50 μ c. (4 mg.) acetate-1-C¹⁴, 3 hr. at 37° C.

‡ For the sake of comparison the progesterone-like substance is assumed to be progesterone.