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Subcellular location of a 17-20 glycol-splitting enzyme in the swine adrenal

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THE SUBCELLULAR LOCATION OF A 17-20 GLYCOL SPLITTING ENZYME IN THE SWINE ADRENAL

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TABLE OF CONTENTS

THE SUBCELLULAR LOCATION OF A 17-20 GLYCOL SPLITTING ENZYME IN THE SWINE ADRENAL CORTEX

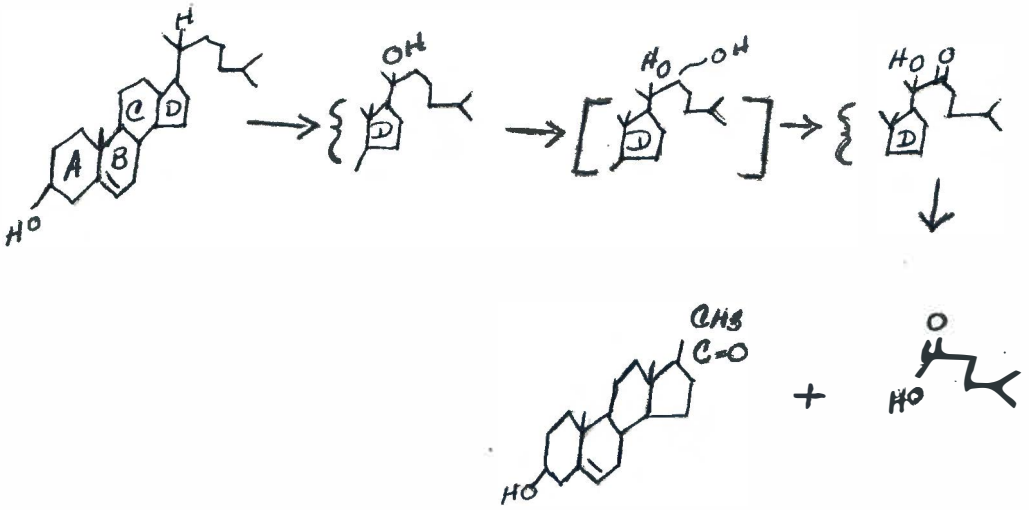
HISTORICAL

At the present time there appears to be two glycol splitting enzymes for steroids. One cleaves 20α, 22ξ-dihydroxycholesterol* to pregnenolone and isocaproaldehyde. The other cleaves pregn-4-ene-17α-20α-diol-3-one** to form androstenedione and acetaldehyde. In 1956 Solomon, et al. (1) incubated cholesterol-4-C^{14} with homogenized bovine adrenal, and obtained, among other products, 20α-hydroxycholesterol and progesterone. These workers did not isolate any material which contained an oxygen atom at carbon-22 when 22α-hydroxy-, 22β-hydroxy-, or 22-ketocholesterol were added as trapping agents. It was also found that reduced nicotinomide adenine dinucleotide phosphate was necessary for the reaction to proceed. In 1961, Shimuzu, et al. (2), incubated 20α-hydroxycholesterol to obtain isocaproic acid, pregnenolone, and a "compound X". This "compound X" on further incubation yielded pregnenolone and isocaproic acid in much greater yield than 20α-hydroxycholesterol. Shimuzu's group had demonstrated earlier that 20α-hydroxycholesterol is converted much more rapidly than cholesterol. In view of their

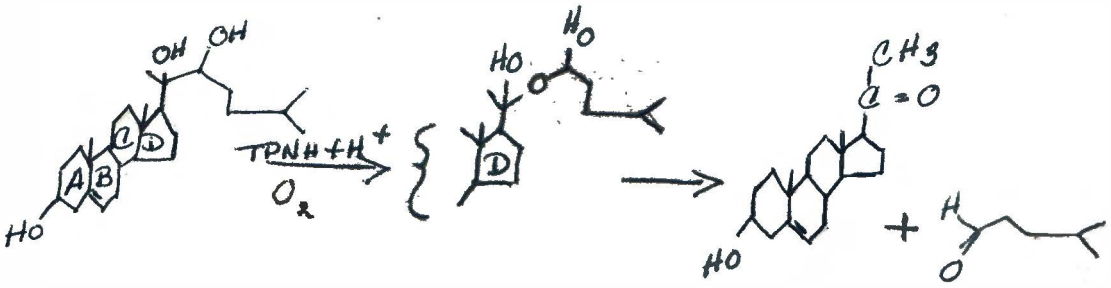
*Henceforth known as dihydrocholesterol

**Henceforth known as pregnendiol

results, they proposed the following pathway for side-chain cleavage:



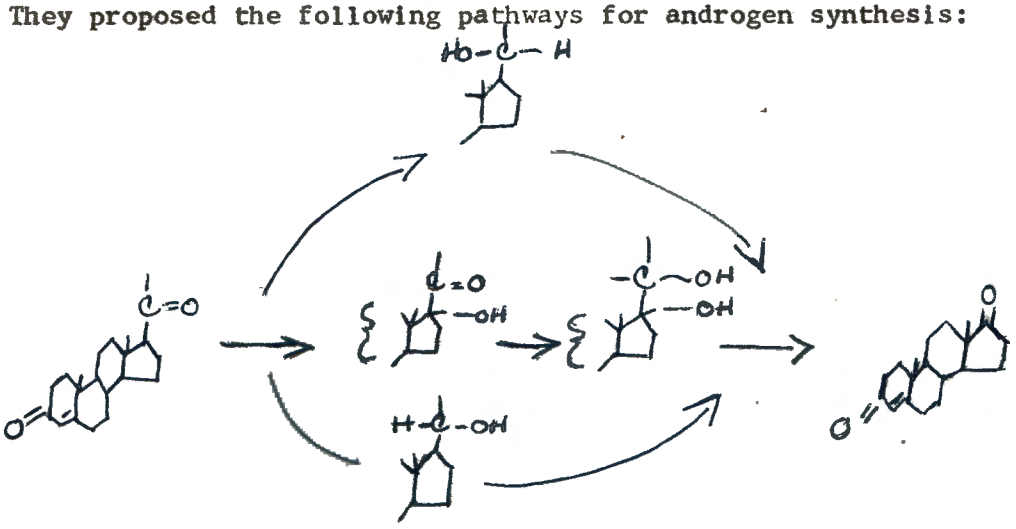
Later that year Shimuzu, Gut, and Dorfman (3) identified "compound X" as dihydrocholesterol, and suggested that 20α-hydroxy-22-keto-cholesterol is an obligatory intermediate in the metabolism of cholesterol to pregnenolone. Constantpoulos and co-workers (4), in 1961, also identified Shimuzu's "compound X" as dihydrocholesterol and gave evidence that the 22-keto derivative is not an intermediate. To accomplish this, two separate enzyme fractions from beef adrenal were used and it was found that reduced pyridine nucleotide was required. Isocaproaldehyde rather than isocaproic acid was trapped. The requirement for reduced pyridine nucleotide suggested that a "mixed function oxygen transferase" oxygenation occurs. The following reaction was proposed:



Further evidence for this reaction was presented by their laboratory (5) in 1966 using doubly labled dihydro, and trapping and counting the isocaproaldehyde produced by the reaction. The H^3/C^{14} ratio in the isocaproaldehyde showed that the 22-keto derivative is not an obligatory intermediate.

Androgenic steroids were first demonstrated in adrenal extracts by von Euw and Reichstein in 1941 (6). In 1958 Davis and Plotz (7) demonstrated <u>in vivo</u> conversion of progesterone to estrogens. Also, in 1958, Lynn and Brown (8) proved that in guinea pig testes the conversion of progesterone to androgens occurs in microsomal fraction and requires TPNH and O_2. Four enzyme systems were studied: (1) Progesterone--> 17α-hydroxyprogesterone, (2) 17α-hydroxyprogesterone--> androstenedione, (3) Androstenedione<--> testosterone, and (4) 17α-hydroxyprogesterone--> pregnendiol. They discovered that the oxidation of 1 mole of progesterone to androstenedione and acetic acid requires ca. 2 M each of O_2 and TPNH, and that oxidation of 17α-hydroxyprogesterone to androstenedione requires ca. 1 M of each. These results led the investigators to suggest that the actual product undergoing

cleavage was 17α-hydroxyprogesterone rather than pregnenediol. Other findings by this group were that the enzymes for 17α-hydroxylation and 17α-20-keto cleavage are located in the microsomal fraction of the cell. The androstenedione reductase and the 17α-hydroxy-20-keto reductase are both found in the soluble fraction. Sweat, et al. (9), in 1960, first demonstrated 17α-hydroxyprogesterone, pregnenediol, and androstenedione in normal human ovaries. They proposed the following pathways for androgen synthesis:



Incubation of pregnenediol, however, yielded no androgen. Dorfman (10) in a summary of 6 pathways for androgen biosynthesis proposed glycol cleavage between 17α-20α-dihydroxycholesterol to form androstenedione. To this date, however, there has been no demonstration of this pathway. In 1964, Ball and Kadis (11) incubated progesterone in sow ovary and obtained pregnenediol, and subsequent incubation did not yield androgenic material. In 1965, Ichii and co-workers (12), incubated pregnenediol with swine adrenal and obtained testosterone. They concluded that formation of androgens

occurred through the action of a desmolase in the swine adrenal. Subsequently, Dominguez has incubated tritiated pregnenediol and carbon-14 labeled 17-hydroxyprogesterone with mouse testes for varying periods of time. It was found that the 17-hydroxyprogesterone is rapidly cleaved to form androgens within the first 15 minutes. As the 17-hydroxyprogesterone-C^{14} disappeared, more was then formed from pregnenediol-H^3, and the appearance of tritiated androgen was observed. These results demonstrate that there is no direct cleavage of pregnendiol to form testosterone or androstenedione in the mouse testis, but that 17-hydroxyprogesterone is an obligatory intermediate. Murota's group (14) has determined that androgen formation occurs mainly in the microsomal fraction of the interstitial cells of the mouse testis. This microsomal fraction is derived predominantly from endoplasmic reticulum of these cells.

It is the purpose of this paper to determine the subcellular location of a 17-20 glycol splitting enzyme in the swine adrenal.

EXPERIMENTAL

17a-hydroxyprogesterone-7-H^3 was obtained from New England Nuclear Corporation. Authentic 17, 20a-dihydroxy-pregne-4-ene-3-one was supplied by the Upjohn Company. Authentic testosterone was obtained from Mann Labs., and authentic testosterone acetate was obtained from Steraloids, Inc.

17,20a-dihydroxy-pregn-4-ene-3-one-7-H^3 was synthesized from 17-hydroxyprogesterone-7-H^3 in the following manner: Sow ovaries were collected immediately after slaughter on dry ice and prepared for incubation by dissecting away all extraneous material, stripping the capsules, and rupturing the cysts. Only those ovaries with four or more corpora lutea were chosen. The tissue was then homogenized in a Waring blendor with three volumes of buffer described by Hayano, et al. (15). The homogenized material was placed in a Spinco Model L ultracentrifuge using a #40 rotor and spun at 144,880 x g for 45 minutes. The supernatant, which contained the soluble fraction of the tissue was preserved in a separate container. The residue was washed three times, and the supernatant was added to the soluble fraction. After centrifugation, 17a-hydroxyprogesterone-7-H^3 was added; and the system was flushed with 95% oxygen-5% carbon dioxide for three minutes and placed in a water-bath shaker at 37° C. After twenty minutes of equillibration, the following cofactors were added in the following ratio----Adenosine triphosphate, 4.0 mM/10 ml of solution, nicotinamide adenine di-

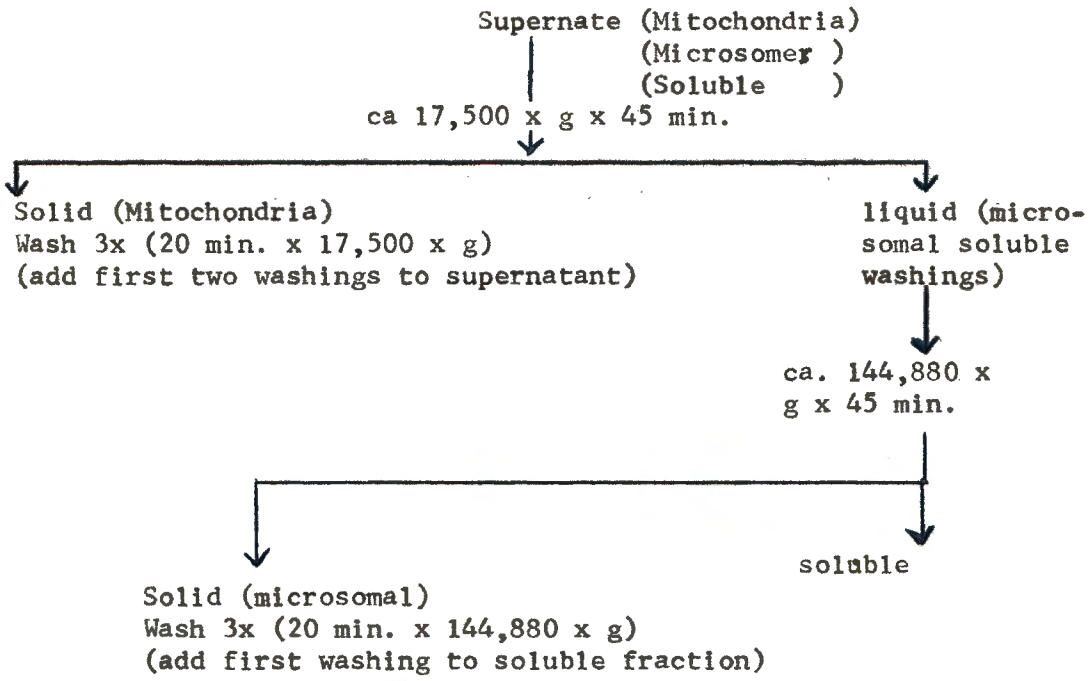
nucleotide, 0.4 mM/ 10 ml, nicotinamide adenine dinucleotide phosphate o.4mM/10 ml, and reduced nicotinamide adenine dinucleotide phosphate 1.0 mgm.

The reaction proceeded for four hours after which it was stopped by the addition of four volumes of acetone. The mixture was allowed to stand in the cold room overnight. The protein precipitate was filtered off with a coarse fritted funnel and the acetone removed from the protein free filtrate with a rotating vacuum evaporator. Sodium chloride (0.2 gm) was added to the aqueous solution and was extracted with three equal volumes of chloroform. The chloroform was removed in vacuo and the residue was dissolved in 90% methanol, after which the fat was removed by partitioning with Skellysolve B. Additional water was added to the methanol fraction, and the alcohol removed under vacuum. Addition of sodium chloride (0.2 gms), and extraction three times with equal volumes of chloroform, and subsequent evaporation led to a residue which was chromatographed on Whatman No. 1 paper using the technique of Zaffroni (16) with hexane-benzene -1:1 as the mobile phase and formamide as the stationary phase. Prior to chromatography, authentic pregnenediol was added to the residue. An ultraviolet scanner composed of a corning #7-54 ultra-violet transmitting filter and a germicidal lamp was used to locate the 240 mu absorbing steroids on the paper. The absorbing areas were eluded with methanol and rechromatographed in a Zaffroni system

using benzene as the mobile phase. A discrete absorbing area was obtained which had an Rf value of 0.303 corresponding to that of authentic pregnenediol. This absorbing area also showed a discrete radioactive peak on scanning with a Vanguard Model 880-D Dual Channel Autoscanner. The material was eluted with methanol and partitioned between chloroform and water. The chloroform was evaporated and the pregnediol was dissolved in a known volume of ethanol. Radioactivity was determined with a Packard Tri-Carb Liquid Scintillation Counting System Model 314 EX. The steroids were dissolved in 10 ml of a scintillation mixture prepared by dissolving 1 gm of PPO and 0.06 gm of POPOP in 200 ml of toluene. Steroid concentrations were determined by comparison with known concentrations of standard solutions using a Model D.U. Beckman Spectrophotometer at the 240 millimicron peak.

Following the determination of specific activity the pregnenediol was divided into three equal portions to be incubated with the three fractions of swine adrenal.

Swine adrenals were obtained at slaughter on dry ice and kept frozen until use. They were prepared for incubation by stripping away all fat and connective tissue and homogenizing 14 gm in a Waring blendor with three volumes of buffer. The homogenized material centrifuged for ten minutes at 2000 RPM in order to remove the nuclear fraction. The supernatant was spun in the ultracentrifuge as described on the following page:



Following the above flow-sheet the mitochondrial, microsomal, and soluble fractions of the adrenal cortices were obtained. 2,581,220 DPM of pregnenediol were incubated with each fraction: 0.54 gms mitochondria, 0.595 gms microsomal, and 40 ml soluble. The incubation was carried out in the manner described earlier. The mitochondrial and microsomal fractions were reacted for 1½ hours, while the soluble fraction was reacted for a three-hour time period. Each incubation was terminated with four volumes of acetone and placed in the cold room overnight. The soluble fraction was treated as described earlier. Partitions between 90% methanol and Skellysolve B were not done

with the microsomal and mitochondrial fractions. The three fractions were chromatographed first in the hexane-benzene system and then in benzene. Prior to chromatography in the benzene system, 0.2 mgm of standard testosterone were added to each fraction to act as a carrier, and afterwards Rf's* and specific** activities were determined. Following this, ½ of the suspected testosterone from the soluble fraction was acetylated to tesotsterone acetate with pyridine and acetic anhydride. Also the entire amounts of suspected tesotsterone from the microsomal and mitochondrial fractions were acetylated. After acetylation the steroids were partitioned between ethyl acetate and water and chromatographed---in the hexane system, partitioned between chloroform and water, and measured in the Scintillation counter and spectrophotometer.

The results of all measurements and calculations follow.

*Rf -- Distance from starting line of steroid/distance of solvent.

** -- DPM (disintigrations/minute)/milligrams of steroid

RESULTS AND DISCUSSION

17,20a-dihydroxy-pregn-4-ene-3-one-7-H^3 prior to incubation.
2,581,220 DPM
0.513 mgm
S.A.* 5.03×10^6

Following incubation.

1st chromatogram Hexane-Benzene

	Soluble	Mitochondria	Microsomes
Testosterone	43,338 DPM	9,708	41,382
Diol	560,834 DPM	-----	-----

2nd chromatogram Benzene

	Soluble	Mitochondria	Microsomes
Testosterone	23,250 DPM	55,620 DPM	6,594 DPM
	.175 mgm	.156 mgm	.169 mgm
	132,800 S.A.*	365,500 S.A.	39,018 S.A.
Diol	78,748 DPM	529,600 DPM	2,047,420 DPM

3rd chromatogram Hexane-Benzene

	Mitochondria	Microsomes
	Rf 0.65	Rf 0.63
Testosterone	2,200 DPM	7,937 DPM
	.107 mgm	.125 mgm
	20,560 S.A.	63,490 S.A.

4th chromatogram Hexane

	Soluble(½ total)	Mitochondria	Microsomes (both total)
Testosterone	11,508 DPM	3,304 DPM	2,516 DPM
	.1586 mgm	.116 mgm	.148 mgm
	72,550 S.A.	28,360 S.A.	16,980 S.A.

**Conversion	0.92%	0.13%	0.09%

*S.A. = specific activity = DPM (disintigrations/minute)/ milligrams of steroid.
** = % conversion = DPM testo ac/DPM Diol used

DISCUSSION OF RESULTS

The above results are clearly inadequate to yield any definite conclusions. There are, however, reasons to suggest that the side chain is removed from the diol to form testosterone by enzymes in the soluble fraction. The mitochondrial and microsomal fractions contained much more unconverted diol following incubation and purification than the soluble fraction. DPM of the diol in the mitochondrial fraction and the microsomal fraction were not determined after the first chromatography in Hexane-benzene because it was felt that there were too many impurities as demonstrated by the soluble fraction. Like Ichii, et al. (12), it was impossible to purify the diol sufficiently for measurement by ultraviolet spectrophotometry; therefore, it was impossible to obtain specific activities. Following conversion of purified testosterone to testosterone-acetate the radioactivity and specific activity were much greater in the soluble fraction than in the microsomal or mitochondrial fractions.

It is obvious that there are many inadequacies in this experiment. The specific activities varied greatly following each chromatography. The testosterone and/or testosterone-acetate should have been crystalized and the melting points determined. For various reasons, such as non-reaction on incubation, attempts to repeat the experiment in order to obtain additional and more accurate data were unsuccessful.

BIBLIOGRAPHY

1. Solomon, S., Levinton, P., and Lieberman, S. Rev. Can. Biol., 15:282, 1956.

2. Shimuzu, K., Hayano, M., Gut, M., and Dorfman, R. I. J. Biol. Chem., 236:695, 1961.

3. Shimuzu, K., Gut, M., and Dorfman, R. I. J. Biol. Chem., 237:699, 1962.

4. Constantopoulos, G., Satoh, P. S., and Tchen, T. T. Biochem. and Biophys. Research Communications, 4:460, 1961.

5. _____. Biochemistry, 5:1646, 1966.

6. von Euw, J., and Reichstein, T. Helv. Chim. Acta, 24:, 1941.

7. Davis, M. E., and Plotz, J. E. Am. J. Ob. & Gyn., 79:939, 1958.

8. Lynn and Brown. J. Biol. Chem., 232:, 1958.

9. Sweat, M. L., Berliner, D. L., Bryson, M. J., Nabors, C., Haskell and Holstrom. Biochim, et Biophys. Acta, 40:289, 1960.

10. Dorfman, R. I. Pituitary-Ovarian Endocrinology, Holden-Day, San Francisco, 1963.

11. Ball and Kadis. Steroids, 4:533, 1964.

12. Ichii, S., Kobayashi, S., and Matsuba, M. Steroids, 5:123, 1965.

13. Dominguez, O.V. Steroids 7:433 1966

14. Murota, S., Shikita, M., and Tamaoki, B. Steroids, 5:409, 1965.

15. Hayano, M., Lindberg, M. C., Wiener, M., Rosenkrantz, H., and Dorfman, R. I. Endocrinology, 55:326, 1954.

16. Zaffroni, A. Rec. Progr. Hormone Res., 8:51, 1953.