Phytochemistry, 1969, Vol. 8, pp. 1215 to 1219 Pergamon Press. Printed in England.

THE METABOLISM OF PROGESTERONE BY PLANT MICROSOMES

S. J. STOHS

College of Pharmacy, University of Nebraska, Lincoln, Nebraska 68508, U.S.A.

(*Received* 28 *December* 1968, *in revised form* 18 *February* 1969)

Abstract—Microsomes isolated either from *Cheiranthus cheiri* or *Dioscorea deltoidea* converted 4-^{14}C-progesterone into a single metabolite, 5α-pregnane-3,20-dione, in the presence of a NADPH generating system. The maximum conversion occurs after approximately three hr incubation. The reaction does depend directly upon the amount of enzyme added, and the reaction has an optimum pH of approximately 7.

INTRODUCTION

THE BIOSYNTHETIC sequence for steroid production from acetate through mevalonate to cholesterol is well documented in animal and bacterial systems.[1] The formation of more complex plant steroids as cardenolides and certain steroidal alkaloids is believed to proceed from cholesterol via pregnenolone, and progesterone,[2,3] although many intermediate steps are incompletely understood.

Mevalonic acid-2-^{14}C has been shown to be incorporated into cholesterol and the sapogenins kryptogenin and diosgenin by *Dioscorea* seedlings.[4] Similarly, ^{14}C-cholesterol is incorporated into kryptogenin and diosgenin.[5] The conversion of cholesterol to pregnenolone has been reported in leaves of *Haplopappus heterophyllus*[6] and *Digitalis purpurea*.[7] Several laboratories have subsequently demonstrated the conversion of pregnenolone to progesterone employing leaves from *Holarrhena floribunda*[8] and *D. lanata*.[9] The incorporation of pregnenolone into cardenolides has been adequately demonstrated in several laboratories,[9–11] while similar results have been recently observed for the incorporation of progesterone into cardenolides of *Digitalis*[10–12] and *Strophanthus*.[13,14]

We have undertaken a study of the metabolism of ^{14}C-progesterone by microsomes from cardenolide and sapogenin producing plants grown in suspension culture. The author is unaware of any report in the literature dealing with the use of plant microsomes in the study

[1] I. D. FRANTZ and G. J. SCHROEPFER, *Ann. Rev. Biochem.* **36**, 656 (1967).
[2] E. HEFTMANN, *Am. Perfumer Cosmet.* **82**, 47 (1967).
[3] E. HEFTMANN, *Lloydia* **30**, 209 (1967).
[4] R. D. BENNETT, E. HEFTMANN, W. H. PRESTON and J. R. HAUN, *Archs Biochem. Biophys.* **103**, 74 (1963).
[5] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 577 (1965).
[6] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).
[7] E. CASPI, D. O. LEWIS, D. M. PLATAK, K. U. THIMANN and A. WINTER, *Experientia* **22**, 506 (1966).
[8] R. D. BENNETT and E. HEFTMANN, *Science* **149**, 652 (1967).
[9] E. CASPI and D. O. LEWIS, *Science* **156**, 519 (1967).
[10] R. TSCHESCHE and G. LILLIENWIESS, *Z. Naturf.* **196**, 265 (1964).
[11] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **6**, 1521 (1967).
[12] R. D. BENNETT, H. H. SAUER and E. HEFTMANN, *Phytochem.* **7**, 41 (1968).
[13] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **7**, 1543 (1968).
[14] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **8**, 69 (1969).

of plant steroid metabolism. Several reports do exist with regard to steroid metabolism by microsomes from fungi and bacteria,[15] and the literature is replete with investigations of drug and steroid transformations by microsomes from animal tissues.

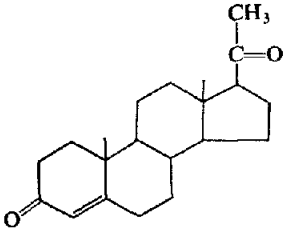
RESULTS

Progesterone-4-^{14}C was incubated with microsomes from either *Dioscorea deltoidea* or *Cheiranthus cheiri* var. Goliath for 2 hr. The incubation mixture was extracted with CH_2Cl_2 which was subsequently examined by TLC.

TABLE 1.

System	% Progesterone converted to 5α-pregnane-3,20-dione by *Cheiranthus* microsomes
Complete NADPH generating system + microsomes (9 mg protein)	12·4%
No microsomes	0
Heated microsomes	0
NADPH	4·0%
NADH	0
12,500 × *g* Supernatant (7·5 ml)	3·6%

For the results in Table 1, microsomes were employed which were isolated from chlorophyllous *C. cheiri* plantlets growing in aseptic suspension cultures and are known to produce unknown steroids. In the presence of microsomes and a NADPH generating system, 4-^{14}C-progesterone (I) was converted to a single metabolite to the extent of 12·4 per cent



(I) Progesterone

of the recovered radioactivity during the 2-hr incubation. When no microsomes or heated microsomes were used, no metabolite of progesterone was detected by the combined techniques of TLC and liquid scintillation counting. When the NADPH generating system was replaced with NADH, again no metabolite of progesterone was detected. The replacement of the NADPH generating system with 3·0 mg of NADPH could not completely satisfy the coenzymatic requirement. It is apparent that a continuous source of NADPH is required, and the requirement for the reduced coenzyme can not be met by NADH. The use of the 12,500 × *g* supernatant fraction in place of microsomes resulted in only a 3·6 per cent conversion of progesterone to the metabolite.

For the results in Table 2, microsomes were isolated from *D. deltoidea* undifferentiated tissue suspension cultures known to produce approximately 1% diosgenin, and other sterols

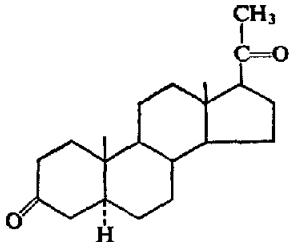
[15] M. H. J. ZUIDWEG, *Biochim. Biophys. Acta* **152**, 144 (1968).

TABLE 2.

System	% Progesterone converted to 5α-pregnane-3,20-dione by *Dioscorea* microsomes
Complete NADPH generating system + microsomes (9 mg protein)	11·9%
No microsomes	0
Heated microsomes	0
NADH	0·1%

and sapogenins. As for *C. cheiri* microsomes, a single metabolite was formed from progesterone in the presence of a NADPH generating system. Again the NADPH generating system could not be replaced by NADH and no conversion product resulted in the absence of microsomes or in the presence of microsomes heated for 10 min in a boiling water bath.

The metabolite of 4-^{14}C-progesterone was chromatographed with a series of reference steroids on thin-layer plates of Absorbosil-1 developed in cyclohexane-ethylacetate (1:1). The R_f of 5α-pregnane-3,20-dione (II) most closely agreed with that of the progesterone metabolite. The metabolite was co-chromatographed with 5α-pregnane-3,20-dione (II) on



(II) 5α-Pregnane-3,20-dione

Adsorbosil-1 plates in two additional solvent systems (See Table 3) with identical R_fs being obtained. Further evidence that the metabolite was indeed 5α-pregnane-3,20-dione (II) was obtained by isolating the metabolite using TLC and crystallizing it to a constant specific activity following dilution with non-radioactive 5α-pregnane-3,20-dione (see Table 4). The 5α-pregnane-3,20-dione was recrystallized three times from 95% ethanol.

Having determined the nature of the metabolite of progesterone, several basic properties of the system were further investigated. Over the range of enzyme concentrations investigated,

TABLE 3. CO-CHROMATOGRAPHY OF PROGESTERONE METABOLITE WITH 5α-PREGNANE-3,20-DIONE

Solvent system	R_f values		
	Progesterone	Metabolite	5α-Pregnane-3,20-dione
$CHCl_3$–EtoAc (4:1)	0·65	0·79	0·80
Cyclohexane–EtoAc (1:1)	0·50	0·67	0·65
CH_2Cl_2–MeOH (48:1)	0·76	0·90	0·90

TABLE 4. RECRYSTALLIZATION OF 5α-PREGNANE-3,20-DIONE BY ISOTOPE DILUTION TO CONSTANT SPECIFIC ACTIVITY

Recrystallization	Counts/min/mmole ($\times 10^3$)	Disintegrations/min/mmole ($\times 10^4$)
First	4·25	1·30
Second	4·27	1·32
Third	4·24	1·31

the amount of 5α-pregnane-3,20-dione (II) formed from progesterone (I) in 2 hr varies directly with the amount of enzyme present. The time-course of the reaction indicates that under our experimental conditions, maximum conversion occurs after incubating the reaction mixture for 3 hr. Although all our experiments were conducted at pH 7·4, a study of the reaction at different pHs indicated that optimal formation of 5α-pregnane-3,20-dione occurs at approximately pH 7·0.

DISCUSSION

Graves and Smith[16] first reported the conversion of progesterone to 5α-pregnane-3,20-dione. These investigators employed several plant species in suspension culture, including *Nicotiana*, *Solanum*, *Atropa* and *Digitalis*. The *Digitalis* species were reported to produce no cardenolides. The cultures were incubated up to 14 days with the substrate. Bennett *et al.*[12] have recently administered 4-^{14}C-progesterone to a *D. lanata* plant and harvested the plant after 5 weeks. Upon examination of the steroidal fraction, they found that 5α-pregnane-3,20-dione was the metabolite with the greatest specific activity. The metabolite 5β-pregnane-3,20-dione had a specific activity of only approximately one-tenth that of the 5α-pregnane-3,20-dione. Interestingly, no 5α-cardenolides have been found in *D. lanata* although evidence is accumulating indicating that C19-C21 steroids as progesterone may be intermediates in the biosynthesis of cardenolides and certain steroidal alkaloids.[10–14] Caspi *et al.*[17] have established that the biosynthetic route from progesterone via deoxycorticosterone to cardenolides was of no significance.

Our results in conjunction with those previously cited suggest that 5α-pregnane-3,20-dione may indeed by the first stable intermediate in the conversion of progesterone to the more complex plant steroids.

EXPERIMENTAL

Plant Tissue Cultures

Plant tissue suspension cultures of unorganized *Dioscorea deltoidea* and organized, pigmented plantlets of *Cheiranthus goliath* were grown on modified Skoog and Murishige's medium containing 0·1 ppm 2,4-dichloroacetic acid.[18] The cultures were transferred routinely at four week intervals. The *Cheiranthus* cultures were grown under continuous low intensity light (3,230–5,380 lux). The *D. deltoidea* cultures are known to produce diosgenin and other steroids,[18] while the suspension cultures of *C. cheiri* have been shown to produce unidentified steroids and Raymond positive compounds (unpublished results).

[16] J. M. H. GRAVES and W. K. SMITH, *Nature* **214**, 1248 (1967).
[17] E. CASPI, J. A. F. WICKRAMASINGHE and D. O. LEWIS, *Biochem. J.* **108**, 499 (1968).
[18] B. KAUL and E. JOHN STABA, *Lloydia* **31**, 171 (1968).

Microsome Isolation

30 g tissue was homogenized in 35 ml of 0·25 M sucrose + 0·05 M tris chloride + 0·005 M $MgCl_2$ + 0·025 M KCl, pH 7·4, at 4° in a Sorvall Omni-Mix Homogenizer operated at maximum speed for 1 min. The homogenate was filtered with vacuum through four layers of cheese cloth, and the filtrate was centrifuged at 12,500 × *g* for 15 min in a Sorvall RC-2B refrigerated centrifuge. The pellet was discarded and the supernatant fraction was centrifuged at 100,000 × *g* for 1 hr in a Spinco Model L Ultracentrifuge. The resulting microsomal pellet was suspended in 0·01 M potassium phosphate buffer, pH 7·4. Protein was determined by the Lowry method.[19] Unless otherwise specified, microsomes equivalent to approximately 9 mg protein were added to each incubation flask.

Incubation Mixture

Progesterone-4-^{14}C having a specific activity of 36·1 mc/mM (114 μc/mg) was obtained from Nuclear-Chicago, and was shown by TLC to have greater than 99 per cent purity. The standard reaction mixture in a 10 ml volume contained: microsomes (9 mg protein), 0·05 M phosphate buffer pH 7·4, 3·0 mg $NADP^+$, 14 mg glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase (Cal-Biochem), 0·005 M $MgCl_2$, 0·01 M KCl, 0·01 M NaCl, and 0·20μc 4-^{14}C-progesterone. When 3 mg NADH or NADPH were used, the NADPH generating system of glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were omitted. The reaction mixtures were incubated in 25 ml Erlenmeyer flasks at 30° with agitation, and aerated with 95% O_2–5% CO_2. An incubation time of 2 hr was routinely used unless otherwise specified.

Extraction

The entire contents of each reaction flask were poured into 40 ml CH_2Cl_2, the incubation flask being washed out with 1 ml of water which was added to the CH_2Cl_2. The mixtures were stoppered and placed on a shaker for 1 hr facilitating extraction of progesterone and its metabolite(s) from the aqueous phase. The resulting emulsion was centrifuged to separate the phases and the upper aqueous phase removed by pipet and discarded. The organic phase was dried and evaporated to dryness *in vacuo*. The residue was transferred to a 3 dram vial with three 1 ml portions of CH_2Cl_2, evaporated to dryness in N_2 and the residue redissolved in 1·0 ml CH_2Cl_2. This solution was examined by TLC.

Thin-Layer Chromatography

Thin-layer plates of Adsorbosil-1 (Applied Science Laboratories, Inc., State College, Pa.) were employed. The CH_2Cl_2 extracts and a standard 4-^{14}C-progesterone solution were applied in 100 μl amounts. Each 100 μl aliquot applied contained approximately 30,000 cpm. The plates were developed in cyclohexane-ethyl acetate (1:1). The developed extracts were cut into eighteen to twenty 1 cm × 2 cm areas with a razor blade, each of which was placed into a liquid scintillation counting vial and toluene counting solution added.[20] Occasionally, 0·5 cm × 2 cm sections were cut when poorer separation of radioactive components resulted. The samples were counted in a Beckman LS-100 liquid scintillation counter. When it had been unequivocally determined that 5α-pregnane-3,20-dione was the metabolite of progesterone, non-radioactive 5α-pregnane-3,20-dione and progesterone was co-chromatographed with the extracts and visualized by spraying with 0·05% rhodamine 6G in methanol and examining the plates under u.v. light.

Identification of 5α-Pregnane-3,20-Dione

The metabolite of 4-^{14}C-progesterone, produced in the presence of microsomes from either *Dioscorea* or *Cheiranthus* in the presence of an NADPH generating system, was shown to have R_f s identical to 5α-pregnane-3,20-dione (II) when chromatographed on thin-layer plates in three solvent systems (see Table 3). Standard compounds were located both by spraying with rhodamine 6G or 50% H_2SO_4 char. Location of radioactivity was determined by liquid scintillation. This metabolite was subsequently isolated by TLC, extracted from the Adsorbosil-1 with CH_2Cl_2 using a Soxhlet, and recrystallized three times from 95% ethanol with non-radioactive 5α-pregnane-3,20-dione. The specific activity was determined following each recrystallization. Aliquots containing not less than 1000 cpm above background were counted for 20 min, giving a two sigma statistical error of not more than 3 per cent.

Acknowledgements—The author gratefully acknowledges the technical assistance of Mr. Lester Reinke and Mr. Thomas Romanek. The author also thanks Dr. E. John Staba for his helpful suggestions and interest in this work. These investigations were supported in part by research grant GM-13440-02 from the Public Health Service, National Institutes of Health.

[19] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
[20] R. J. Herberg, *Anal. Chem.* **32**, 42 (1960).