and Fry, Ed., Davis, Philadelphia, 1963, p172.
28. Wimber, D. E., ibid., 1963, p1.
29. Nielson, N. O., Feinendegen, L. E., Bond, V. P., Odartchenko, N., Cottier, H., Proc. Soc. Exp. Biol. and Med., 1964, v116, 498.
30. Quastler, H., in Cell Proliferation, Lamerton and Fry, Ed., Davis, Philadelphia, 1963, p18.

Received March 1, 1965. P.S.E.B.M., 1965, v119.

Steroid Synthesis by Human Fetal Adrenals and Ovaries Maintained in Organ Culture.* (30207)

E. BLOCH,† S. L. ROMNEY, M. KLEIN, L. LIPPIELLO, P. COOPER AND I. P. GOLDRING
(Introduced by Abraham White)
Departments of Gynecology and Obstetrics, Biochemistry, and Surgery, Albert Einstein College of Medicine, Yeshiva University, New York City

Explants of adrenal(1) and ovarian(2) cortex from human fetuses have been maintained in organ culture for varying periods. Steroid synthesis by the adrenal explants declined rapidly with increasing incubation periods; this decline could be partially arrested by the addition of progesterone to the medium(1). The formation of steroids by the ovarian fragments was not studied.

This communication, part of a study on fetal adrenal and gonadal steroidogenesis, describes the synthesis of steroids by human fetal adrenals and ovaries maintained in a similar *in vitro* system.

Materials and methods. The intact amniotic sac of the pregnant human uterus, containing a fetus of 11 weeks gestation (7.5 cm crown-rump length), was obtained at the time of therapeutic interruption and kept on ice for one hour. The fetus was taken from the amniotic sac and the adrenals and ovaries were aseptically removed from the fetus. One gland of each pair was used as a control for chemical analysis and histological examination. The other gland was cut into segments of 2-3 mm^3. Each segment was explanted in organ culture using a siliconized lens paper raft as a modification of the Trowell technique(3). The liquid medium consisted of 70% CMRL 1066 (Connaught Medical Research Labs., Toronto) and 30% horse serum, and included 100 units/ml of a penicillin-streptomycin mixture. Six cultures were prepared from the adrenal gland and two cultures from the ovary. The cultures were incubated at 37°C in a CO_2-air (5:95) saturated atmosphere. After 48 hours incubation, the cultures were grouped and treated as follows:

Group A consisted of 2 adrenal cultures, 2 ovarian cultures and 2 control cultures composed of medium but lacking tissue. *Group B* consisted of 4 adrenal cultures. One-half of the medium of each culture in *Groups A & B* was replaced with an equal volume of medium. The media added to the cultures of *Group A* contained 10 μc (2.22×10^7 dpm) purified 7-^{3}H-progesterone (9.4 $\mu c/\mu$mole, N. E. Nuclear Corp., Boston). Two cultures of *Group B* received 13 I.U. ACTH (ACTHar, Armour & Co.); no other additions were made to the cultures of this group.

After a further 48 hours of incubation, the tissues of the cultures of *Group A* were fixed in 10% neutral buffered formalin for subsequent histological examination, and the media collected and frozen for subsequent analysis. The media of the 2 *ovarian* cultures were combined into one pool. In *Group B*, the change of medium and addition of ACTH was repeated after an additional 48 hours. After a total of 8 days in culture, the tissues were fixed and the media collected and frozen.

The analysis for conversion products of 7-^{3}H-progesterone in the culture media from *Group A* was conducted in the following

* This study was supported by Research Grant HD-00420, NIH, USPHS, and, in part, by the Tobacco Industry Research Committee.

† Research Career Development Investigator, Nat. Inst. of Child Health and Human Development.

TABLE I. Purification of ^{3}H-Cortisol Reisolated from the Media of Fetal Adrenal Segments Maintained in Organ Culture.

Crystallization	Adrenal culture No. 1 Wt (mg)	Adrenal culture No. 1 Specific activity (dpm/μM $\times 10^{-3}$)	Adrenal culture No. 2 Wt (mg)	Adrenal culture No. 2 Specific activity (dpm/μM $\times 10^{-3}$)
After cortisol* addition (calc.)	36.2	16.6 (4.61×10^4 dpm/mg)	37.6	9.5 (2.63×10^4 dpm/mg)
Free alcohol				
First (Cryst.)	11.3	12.2	11.0	4.6
(Supernate)	24.0	17.9	25.6	10.4
Second (Cryst.)	5.99	8.0	5.42	4.5
(Supernate)	3.48	12.5	3.41	6.1
Third (Cryst.)	3.20	7.7	1.79	4.6
(Supernate)	2.10	7.3	3.15	4.9
Acetate†				
First (Cryst.)	.685	7.6	.638	4.5
(Supernate)	.955	7.0	1.90	4.5
Second (Cryst.)	.432	7.9	.219	5.0
(Supernate)	.089	7.6	.262	4.5

* Crystalline carrier cortisol.
† Formed acetate(6) from supernatant of third crystallization; m.pt. (uncorr) = 220-223°C.

fashion: The media from the adrenal, ovarian and sham cultures were extracted exhaustively with dichloromethane and ethyl acetate, with a recovery of 76 to 93% of the added radioactivity. The residues of the extracts were partitioned between hexane and 90% methanol, and the methanolic fractions subjected to paper chromatography in hexane/96% methanol, and, for the ovarian fraction only, in the Bush B_1 solvent system(4).

Results. In the case of the *adrenal* cultures, the zones on the chromatograms corresponding to cortisol and Δ^4-androstene-3,17-dione were eluted and 30.0 to 37.6 mg carrier cortisol‡ or Δ^4-androstene-3,17-dione‡ added to the eluates. Cortisol was crystallized from 3 solvents to constant specific activity (Table I). Conversion to the acetate and recrystallization from ethanol and ethyl acetate-ethanol did not change the specific activities. Cortisol, recovered from the 2 control incubations, was essentially devoid of activity after 2 crystallizations and conversion to the acetate; *i.e.*, the specific activities of the reisolated cortisol acetate were 28 and 97 dpm/μmole. Based on specific activities and corrected for losses prior to carrier addition, approximately 4% and 3% of the substrate progesterone was converted to cortisol by adrenal tissue in these 2 incubations.

Formation of Δ^4-androstene-3,17-dione could not be demonstrated. The radioactivity dissociated from added carrier during consecutive crystallizations, until the specific activities of Δ^4-androstene-3,17-dione reisolated from the 2 adrenal incubations and from the control media did not differ significantly from each other.

Steroid synthesis from endogenous precursors by the adrenal cultures of *Group B* could not be demonstrated, irrespective of ACTH addition. Examination of the extracts of the appropriate media by paper chromatography failed to indicate the presence of steroids with the following functional groups: Δ^4-3-keto, 17-keto, Δ^5-3β-hydroxy, or α-ketolic-C-17 sidechain.

Histological examination of the adrenal explants, cultured for 4 and 8 days, disclosed a well maintained definitive cortex. Pyknotic nuclei and vacuolated cytoplasm in cells of the fetal zone of the adrenal were seen.

In the case of the *ovarian* cultures, 3 radioactive zones corresponding in mobility to

‡ Cortisol (11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione) and androst-4-ene-3, 17-dione, were obtained from commercial sources. 20α-Hydroxypregn 4-ene-3-one was obtained through the generosity of Dr. G. W. Duncan, Upjohn Co., Kalamazoo. All 3 steroids were pure as assessed by paper and thin-layer chromatography and melting points.

progesterone (2.02×10^7 dpm), 17α-hydroxyprogesterone (1.88×10^6 dpm), and 20α-hydroxy-Δ^4-pregnene-3-one (7.2×10^5 dpm), as well as 3 unidentified areas (2.96×10^6 dpm), were eluted from the paper chromatograms. The sum of the individual fractions eluted and corrected for aliquots taken for analyses, represented 64% of the added substrate progesterone.

To the eluate containing 20α-hydroxy-Δ^4-pregnene-3-one, 550 μg of this steroid were added as crystalline carrier,‡ and the mixture subjected to two sequential developments on paper chromatograms in the solvent systems hexane/96% methanol(4) and heptane-propylene glycol(5). The eluate from the second chromatogram contained 212 μg of 20α-hydroxy-Δ^4-pregnene-3-one with a specific activity of 1.21×10^5 dpm/μmole.

Acetylation(6) of two-thirds of the eluate yielded 103 μg 20-acetoxy-Δ^4-pregnene-3-one with a specific activity of 1.08×10^5 dpm/μmole. CrO_3 oxidation(7) of the remaining one-third of the eluate yielded 27 μg of progesterone with a specific activity of 1.12×10^5 dpm/μmole. Both derivatives were identified by their mobility upon paper chromatography in the solvent system hexane/96% methanol. 20α-Hydroxy-Δ^4-pregnene-3-one was considered to have been purified to constant specific activity; allowing for losses prior to carrier steroid addition, about 1.5% of the substrate progesterone was reduced to the carbon-20α-hydroxy isomer.

Pyknotic nuclei and vacuolated cytoplasm in the cells of the ovarian medulla were seen; the cortex of the ovary appeared to be well maintained.

Discussion. The results demonstrate that human fetal adrenal tissue retained its capacity to convert progesterone to cortisol during a minimum of a 2-day, and a maximum of a 4-day, culture period. The inference is drawn that adrenal 17α-, 11β- and 21-hydroxylases are, at least to some extent, stable under the experimental conditions employed. This finding is similar to that reported by Villee(1), wherein fetal adrenals hydroxylated progesterone after a 24-hour culture period, but lost this ability after a 5-day culture period. The synthesis of cortisol from acetate(7) and progesterone(8) has been previously demonstrated during incubation with human fetal adrenal tissues.

Endogenous steroid synthesis by fetal adrenals could not be demonstrated in our experiments. Using post-natal adrenal tissue, Lanman reported 260 μg dehydroepiandrosterone equivalents of Zimmermann reaction material in extracts of media from adrenals obtained from human infants and kept in culture for 6 to 17 days(9).

20α-Hydroxy-Δ^4-pregnene-3-one was isolated from the media of the ovarian cultures. The isolated steroid probably represents a conversion product from the substrate 7-^{3}H-progesterone, although formation from a trace contamination in the substrate cannot be entirely excluded. The substrate progesterone was shown to contain less than 0.1% of the carbon-20α reduction product. 20α-Hydroxy-Δ^4-pregnene-3-one has been isolated from fetal ovarian homogenates incubated with 4-^{14}C-progesterone in phosphate buffer (6).

Summary. Adrenal tissue from an 11-week-old human fetus, maintained in organ culture for 4 to 8 days, converted 7-^{3}H-progesterone to cortisol. Steroid synthesis from endogenous precursors, both with and without added ACTH in the culture medium, could not be demonstrated. The definitive cortex of the adrenal was histologically well maintained for 8 days in culture, while the fetal zone showed evidence of extensive degeneration. Fetal ovarian tissue under the same *in vitro* conditions, formed 7-^{3}H-20α-hydroxy-Δ^4-pregnene-3-one. The results demonstrate the capacity for steroid synthesis by adrenal and ovarian tissue from a human fetus maintained in organ culture.

Addendum. While this report has been "in press," Stark *et al*(10) reported the synthesis of corticosteroids, including cortisol, by adrenal glands from human fetuses, 18-36 cm body length, maintained in organ culture for 25 days or less. Corticosteroid synthesis was maximal during the 3rd to 8th day of culture, and was stimulated by ACTH.

1. Villee, D. B., 46th Meeting of Endocrine Society, San Francisco, 1964, v160. (Abstract)

2. Gaillard, P. J., Int. Rev. Cytol., 1953, v2, 356.

3. Trowell, O. A., Exp. Cell Res., 1954, v6, 246.
4. Bush, I. A., Recent Prog. Hormone Res., 1954, v9, 321.
5. Rubin, B. L., Dorfman, R. I., Pincus, G., ibid., 1954, v9, 213.
6. Bloch, E., Endocrinology, 1964, v74, 833.
7. Bloch, E., Benirschke, K., J. Biol. Chem., 1959, v234, 1085.
8. Villee, D. B., Engel, L. L., Loring, J. M., Villee, C. A., ibid., 1961, v69, 354.
9. Lanman, J. T., Silverman, L. M., ibid., 1957, v60, 433.
10. Stark, E., Gyevai, A., Szalay, K., Acs, Z., Canadian Journal Physiology and Pharmacology, 1965, v43, 1.

Received March 3, 1965. P.S.E.B.M., 1965, v119.

Effect of Adrenal Steroids upon Plasma Volume of Intact and Adrenalectomized Dogs.* (30208)

W. W. SWINGLE AND A. J. SWINGLE
Section of Adrenal Physiology, Bureau of Research in Neurology and Psychiatry,† Princeton, N. J.

These experiments concern (a) the role of adrenal steroids in inducing alterations of the plasma volume of intact and adrenalectomized dogs following introduction by gavage of 250 ml of tap water or 0.9% NaCl into the gastrointestinal tract and (b) the effect of these steroids upon the plasma volume of intact animals subjected to prolonged food and water deprivation.

Methods. Fourteen adrenalectomized and 18 intact dogs were used. The adrenals had been removed for periods varying from several months to several years. During these intervals the animals were studied through cycles of insufficiency and recovery on substitution therapy. When used in the present experiments they were strong, vigorous animals, at peak weight and with normal blood chemistry, plasma volumes and arterial pressures. They were maintained by daily i.m. injections of 1 mg of desoxycorticosterone acetate in oil (DCA) plus 1 g of NaCl in their daily ration of fresh, ground, beef heart and puppy kibble. Before starting an experiment, steroid and salt supplements were withdrawn and control readings obtained for blood concentration and plasma volume. Food was withheld for the ensuing 24 hours, but unless otherwise stated, access to water was permitted to the time of steroid injection.

The free alcohols of the various steroids used were solubilized for i.v. injection by the method previously described(1). Saline was administered by stomach tube and followed within 5 minutes by i.v. injection of steroid. One and one-half hours after the last injection, blood samples were drawn. The 1.5-hour waiting period was arbitrarily chosen but since it proved satisfactory it was adhered to in all later experiments. No anesthesia was used at any time; the dogs were trained to remain quiet and relaxed during the necessary manipulations attending i.v. injections and blood sampling. The plasma volume method was that recommended by Gregersen and Stewart(2) and Chien and Gregersen(3). It has been utilized and adequately described by the present writers(4).

Results. Plasma volume changes following stomach tube administration of tap water or 0.9% saline to intact dogs. Nine dogs were used: 4 for testing effects of tap water and 5 for similar studies of saline. After giving the fluid the animals were placed in metabolism cages for 1.5 hours and then sampled at termination of this interval. The essential data were averaged (Table I A1). The plasma volume of dogs receiving tap water showed no appreciable alteration nor was any fluid voided during the test. The 5 animals given 250 ml of 0.9% saline responded quite differently. Plasma volume increased by an

* Acknowledgement is made for financial and other valuable aid: Ciba Pharmaceutical Products Co., Schering Corp., Lederle Laboratories, National Science Foundation, and Nat. Inst. of Arthritis and Metab. Diseases, USPHS.

† Located at New Jersey Neuro-Psychiatric Inst.