[20] L. H. MUSCHEL, W. F. CAREY, L. S. BARON, *J. Immunol.*, 82 (1959) 38.
[21] P. MITCHELL AND J. MOYLE, *J. Gen. Microbiol.*, 16 (1957) 184.
[22] T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
[23] A. G. MARR AND E. H. COTA-ROBLES, *J. Bacteriol.*, 74 (1957) 79.
[24] E. H. COTA,ROBLES, A. G. MARR AND E. H. NILSON, *J. Bacteriol.*, 75 (1958) 243.
[25] E. LEIFSON, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 20 (1954) 102.
[26] T. ASAI AND K. SHODA, *J. Gen. Appl. Microbiol.*, 4 (1958) 289.
[27] T. ASAI, *J. Agr. Chem. Soc. Japan*, 11 (1935) 686.
[28] A. H. STOUTHAMER, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 25 (1959) 241.

Biochim. Biophys. Acta, 40 (1960) 277–289

THE SYNTHESIS AND METABOLISM OF PROGESTERONE IN THE HUMAN AND BOVINE OVARY

MAX L. SWEAT, DAVID L. BERLINER*, MELVIN J. BRYSON, CHARLES NABORS JR., JACK HASKELL AND EMIL G. HOLMSTROM

Department of Obstetrics and Gynecology and Department of Anatomy, University of Utah College of Medicine, Salt Lake City, Utah (U.S.A.)

(Received September 8th, 1959)

SUMMARY

1. The biosynthesis of progesterone from [1-^{14}C]acetate has been demonstrated.

2. By means of *in vitro* incubations of [4-^{14}C]progesterone and [4-^{14}C]androstenedione in human ovarian tissue, a continuous spectrum of intermediate compounds leading to the synthesis of estrogens has been demonstrated. Presumptive evidence has been obtained for the following products: 6β-hydroxyprogesterone, pregnanedione, allopregnanedione, 20α-hydroxypregnene-3-one, 20β-hydroxypregnene-3-one, 17-hydroxyprogesterone, 17α-20ξ-dihydroxypregnene-3-one, 20ξ-hydroxypregnane-3-one, androstenedione, estrone, and estradiol-17β.

INTRODUCTION

Although it has been assumed that the early steps of progesterone biosynthesis follow the same general pathway as the synthesis of cholesterol[1,2], direct evidence for its synthesis from simple carbon compounds in the ovary has not been heretofore available. The studies reported in this paper demonstrate the *in vitro* biosynthesis of progesterone from [1-^{14}C]acetate incubated with corpora luteal tissue. The subsequent course of progesterone metabolism in the human ovary has been followed by the *in vitro* incubation of [4-^{14}C]progesterone and [4-^{14}C]androstenedione with ovarian stromal and corpora luteal tissue. Seven products have been characterized. The nature of the products is such as to suggest a relatively detailed metabolic scheme in the transformation of progesterone to androstenedione and estrogens.

* Key investigator, American Cancer Society.

EXPERIMENTAL

Minced human stromal or luteal tissue[1] (0.5–1.0 g) or bovine corpora luteal tissue (5–6 g) was incubated with chromatographically pure radioactive compounds (human tissue: 10,000 counts/min-[4-^{14}C]progesterone, specific activity $1.07 \cdot 10^7$ counts/min/mg; 10,000 counts/min-[4-^{14}C]androstenedione, specific activity $1.11 \cdot 10^7$ counts/min/mg; 10,000 counts/min-[4-^{14}C]testosterone, specific activity $1.1 \cdot 10^7$ counts/min/mg—bovine tissue: 1,000,000 counts/min-[1-^{14}C]sodium acetate, specific activity 30.5 μC/mg) in a buffer medium (0.05 *M* sodium–potassium phosphate, pH 7.3 and 0.004 *M* magnesium chloride) at 37° for 2 h (bovine tissue, 4 h). The incubations were terminated by addition of 3 volumes of acetone which was subsequently removed by vacuum distillation. The residue from a chloroform extract of the water solution was dissolved in a few drops of chloroform–methanol (1 : 1) mixture and chromatographed in ZAFFARONI's[3] formamide systems. The radioactive areas were located by means of an automatic strip feed counter[4] and analyzed by methods heretofore described[5].

Preparation of [4-^{14}C]androstenedione

This radioactive compound was prepared by dissolving 500,000 counts/min of testosterone in 15 drops of glacial acetic acid to which one small dry crystal of chromium trioxide was added. The mixture was shaken thoroughly in a glass stoppered tube and allowed to stand overnight at room temperature. The following morning 7 ml of water were added to the mixture, immediately after which the steroid was extracted with ethyl acetate. The ethyl acetate was removed with a stream of nitrogen and the residue was dissolved in methanol–chloroform (1 : 1) for chromatography on the hexane–benzene system. The androstenedione area was eluted from the paper with methanol and dried for use in the experiment.

ZIMMERMAN *reaction[6] for detection of ketonic carrier compounds*

A thin longitudinal strip of the chromatogram was dipped in a mixture of two volumes of *m*-dinitrobenzene (1 % in methanol) and one volume of freshly prepared 15 % potassium hydroxide and dried on a medium warm glass plate, to develop the color. (3 keto compounds gave a blue violet color; 17 keto compounds gave a violet color and 20 keto groups gave a brown purple color.)

Ferric chloride test[7] for detection of carrier estrogens

A longitudinal strip or sample of the chromatogram was dipped in a mixture consisting of equal volumes of 1 % ferric chloride ($FeCl_3$) and 1 % $K_3Fe(CN)_6$ and subsequently washed in 0.1 *N* hydrochloric acid. Excess hydrochloric acid was removed by washing in tap water and the chromatogram was allowed to dry.

Reduction of ketonic groups

2 ml of a saturated solution of $LiAlH_4$ in anhydrous diethyl ether were added to the dry steroid, mixed thoroughly and allowed to stand at room temperature for 5 min. 3–4 ml of distilled water was then added to stop the reaction and decompose the excess $LiAlH_4$. The reduced steroid was then extracted from the solution with ethyl acetate which was removed by evaporation under a stream of nitrogen.

20α-and 20β-hydroxypregnene-3-one were generously supplied by Dr. W. KLYNE

(Post Graduate Medical School, London). 6β-hydroxyprogesterone was a gift from Syntex, S.A.. The other steroids were obtained from commercial sources.

RESULTS

Biosynthesis of progesterone

Fig. 1 is a tracing of the radiochromatograph obtained from an incubation of bovine corpora luteal tissue with [2-^{14}C]acetate.

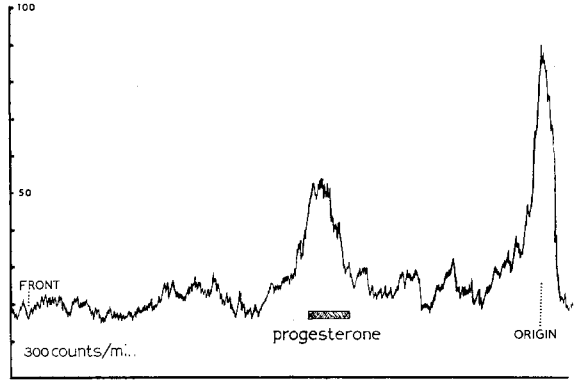

Fig. 1. Biosynthesis (bovine corpora luteal tissue) of progesterone from [1-^{14}C]acetate. The crosshatched area represents non-radioactive (carrier) progesterone (detected by u.v. absorption).

The peak at the mid-portion of the chromatogram was eluted and rechromatographed in the hexane–formamide and heptane–formamide systems with carrier progesterone. In both systems the radioactive peak migrated at the same rate as the carrier compound. The specific activities of the two chromatograms from two solvent systems were shown to be the same (hexane–formamide, 2.04 counts/min/mg; heptane–formamide, 2.06 counts/min/mg. 93 and 85 μg respectively of the 100 μg progesterone originally added were recovered from the chromatogram). The compound was shown to be not acetylatable. Three consecutive crystallizations of the mixture of progesterone and the unknown radioactive compound from two solvents (heptane and hexane) exhibited the same specific activities:

	Crystallization	*Specific activity*
1)	(Hexane)	53.0 counts/min/mg
2)	(Heptane)	57.1 counts/min/mg
3)	(Heptane)	56.3 counts/min/mg

Identification of the other radioactive peaks was not carried out in these experiments.

Metabolism of progesterone

The chromatographic spectra of progesterone metabolism in the human ovary are shown in Fig. 2. The initial spectrum is a tracing of a radio-chromatograph obtained after the incubation of 10,000 counts/min [4-^{14}C]progesterone in 0.5 g of

human corpora luteal tissue. The subsequent spectra were obtained from composite extracts from incubations of both stromal and corpora luteal tissue. Essentially the same compounds have been found in both types of tissue. The figure also shows the sequence in which the various radioactive areas were resolved for analysis. The following is presumptive evidence for the identity of the isolated compounds:

1. *6β-hydroxyprogesterone*: The radioactivity remaining at the origin in the original hexane–formamide chromatogram was rechromatographed in the hexane–benzene system with the carrier compound (non-radioactive 6β-hydroxyprogesterone) and found to migrate at the same rate. The two compounds also positioned themselves in the same area in the benzene system. The acetate of both compounds ran identically in the hexane–benzene system. Mild oxidation of the radioactive compound resulted in a derivative which migrated identically with 6-keto-progesterone.

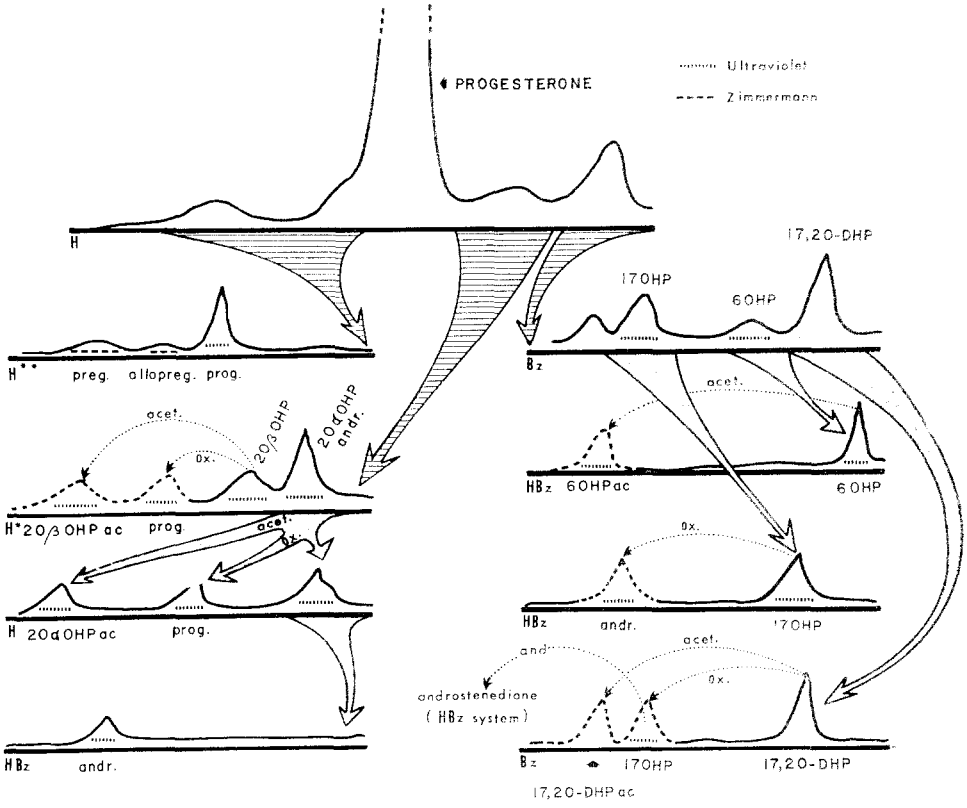

Fig. 2. Chromatographic spectra of progesterone products in the human ovary and the sequence of analysis. The wide portions of the hatched arrows represent the area of the chromatogram eluted. The arrows point to subsequent treatment of the eluted radioactive material. The following abbreviations are employed: H, chromatography to front in hexane system; H*, chromatography in hexane dropped 1 h from front; H**, chromatography in hexane dropped 1–1.5 h from front; Bz, chromatography to front in benzene system; HBz, chromatography to front in hexane–benzene system; prog., progesterone; preg., pregnanedione; allopreg., allopregnanedione; 20α-OHP, 20α-hydroxypregnene-3-one; 20βOHP, 20β-hydroxypregnene-3-one; 6βHP, 6β-hydroxyprogesterone; 17OHP, 17α-hydroxyprogesterone; 17,20-DHP, 17α, 20ξ-dihydroxypregnene-3-one; andr., androstenedione; ac, acetate ester; ox, oxidation; acet., acetylation. The dotted lines represent areas in which the carrier compounds were detected with either the Zimmermann reaction or u.v. absorption. A carrier compound was not available for the 17, 20-DHP compound.

2. *Pregnanedione*: Initial and secondary chromatography in the hexane system proved that the radioactive compound migrates identically with the index carrier (R_F 0.6–0.8). Reduction of the combined compounds gave a more high polar compound (probably monohydroxy compounds, as they were of intermediate polarity) which ran identically with the reduced carrier. Upon acetylation of the reduced compounds (carrier and radioactive), the radioactive and non-radioactive compounds were found in the identical area of the chromatogram. The original compound did not form an acetate ester.

3. *Allopregnanedione*: This compound was not found in all incubations. However, in some of the chromatographs a shoulder was observed on the low polar side of the progesterone peak. Lengthening the time of chromatography (dropping of the solvent for one hour) resulted in resolution of this compound from progesterone. The compound migrated at an identical rate with allopregnane carrier and would not acetylate.

4. *20α-hydroxypregnene-3-one*: This compound migrated slightly from the origin in hexane and was resolved by dropping the solvent for several hours. It ran identically with the index compound in both the hexane and hexane–benzene systems. Oxidation converted it to progesterone. Acetylation yielded a derivative which ran identically with the acetate of the carrier compound.

5. *20β-hydroxypregnene-3-one*: This compound migrated midway between the origin and progesterone area in the hexane system. It conformed to the carrier compound in both hexane and hexane–benzene systems. Progesterone was formed upon oxidation. When the combined area containing the index compound and the radioactive compound was acetylated both migrated to the front of the hexane chromatographic system.

6. *17-hydroxyprogesterone*: This compound had an R_F of approx. 0.3 in hexane–benzene and 0.6 in the benzene systems. In both systems it ran identically with the index compound. Oxidation converted it to androstenedione. It did not form an acetate ester.

7. *17α–20ξ-dihydroxypregnene-3-one*: In benzene the radioactivity corresponding to this compound exhibited an R_F of 0.25; in chloroform, an R_F of 0.6. Its acetate migrated between the front and 17-hydroxyprogesterone in the benzene system (R_F 0.9). The acetate had an R_F in the hexane–benzene system of 0.5. Mild oxidation gave as products, 17-hydroxyprogesterone and androstenedione. The observations that this compound forms an acetate which migrates at a slightly faster rate than 17-hydroxyprogesterone, and yields 17-hydroxyprogesterone and androstenedione upon mild oxidation is strong evidence for the structure listed. As 20α-hydroxypregnene-3-one is the major product in ovarian progesterone metabolism, it is likely that the compound is 17α,20α-dihydroxypregnene-3-one.

8. *20ξ-hydroxypregnane-3-one*: The R_F of this compound in the hexane–formamide system is almost identical to that of progesterone. Upon acetylation of the progesterone peak, two products are noted: Unchanged progesterone and an acetylated compound which migrates to the front. When the compound is hydrolyzed to remove the acetate group and subsequently oxidized (mild), a derivative is observed which migrates at the same rate as pregnanedione. As the reduction of the 20 ketone to a 20α-hydroxy group seems to dominate in the human ovary, this compound is thought to be 20α-hydroxypregnane-3-one.

9. *Androstenedione*: In the initial chromatography with hexane, this compound

migrates slightly in front of the origin. In the hexane–benzene system it has an R_F of 0.65. It migrates identically in both systems with the index compound and does not form an acetate.

Only small radioactive areas corresponding to the following compounds were observed in chromatographs from the progesterone incubations. However, when [4-^{14}C]androstenedione was employed as the substrate, radioactive areas of sufficient magnitude to acetylate and rechromatograph (Fig. 3) were obtained.

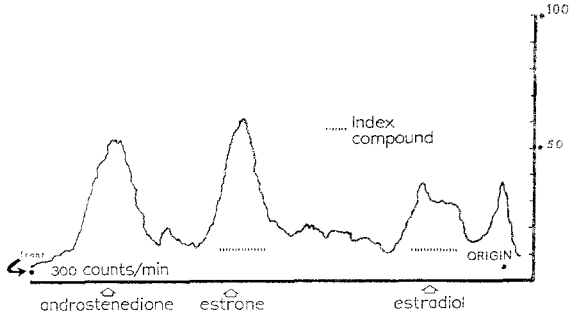

Fig. 3. Tracing of a radiochromatograph demonstrating the synthesis of estradiol and estrone from [4-^{14}C]androstenedione (ovarian stromal or luteal tissue).

10. *Estrone*: In both the hexane–benzene and benzene systems the radioactive compound migrates identically with index estrone. The acetylated derivative has an R_F of about 0.65 in hexane–formamide and migrates with estrone acetate.

11. *Estradiol-17β*: The radioactivity of this compound migrates at the same rate as index estradiol-17β chromatographed in the benzene and chloroform systems. Acetylation forms an acetate which runs with the front in hexane.

12. *Origin–peak* (Fig. 3): The radioactive compound in this peak has not been studied.

DISCUSSION

Fig. 4 depicts possible steps in the conversion of progesterone to androstenedione and the estrogens. All products listed in the figure which precede the step of aromatization can be readily demonstrated in a continuous spectrum after [4-^{14}C]progesterone is incubated in human corpora luteal or stromal tissue. Only two of the products, 20α-hydroxypregnene-3-one and 20β-hydroxypregnene-3-one have been heretofore isolated from normal human ovaries[8]. Both 17-hydroxyprogesterone and androstenedione have been reported as products of progesterone metabolism in tissue from an arrhenoblastoma[9] and polycystic (Stein-Leventhal) ovaries[10]. The latter investigators[10] also reported tentative identification of testosterone, but several studies[11] with polycystic ovaries in this laboratory have revealed only those products listed in Fig. 4.

In the current experiments only traces of estrone and estrodiol have been observed after incubation with [4-^{14}C]progesterone. However, the formation of both estrone and estradiol were readily demonstrated when [4-^{14}C]androstenedione was incubated as a precursor (Fig. 3). The compound/s remaining at the origin (Fig. 3) has not been identified. However, its failure to form an acetate ester indicates it is not estriol.

The metabolism of progesterone in the human ovary seems similar to that in experiments. HEARD *et al.*[18] and WEST *et al.*[19] have demonstrated the *in vivo* conversion of testosterone to estrogens. It has been shown in this investigation as well as in investigations by others[20,21] that testosterone can be converted to estrogens in the human ovary. Placental tissue also can carry out this conversion[22] as well as other conversions[23]. The present experiments suggest that androstenedione is the dominating precursor in the course of synthesis of the estrogens. The synthesis of both estrone and estradiol can be demonstrated from this precursor (Fig. 3).

The significance of the formation of pregnanedione, allopregnanedione, 6β-hydroxyprogesterone and 20ξ-hydroxypregnane-3-one (latter compound not included

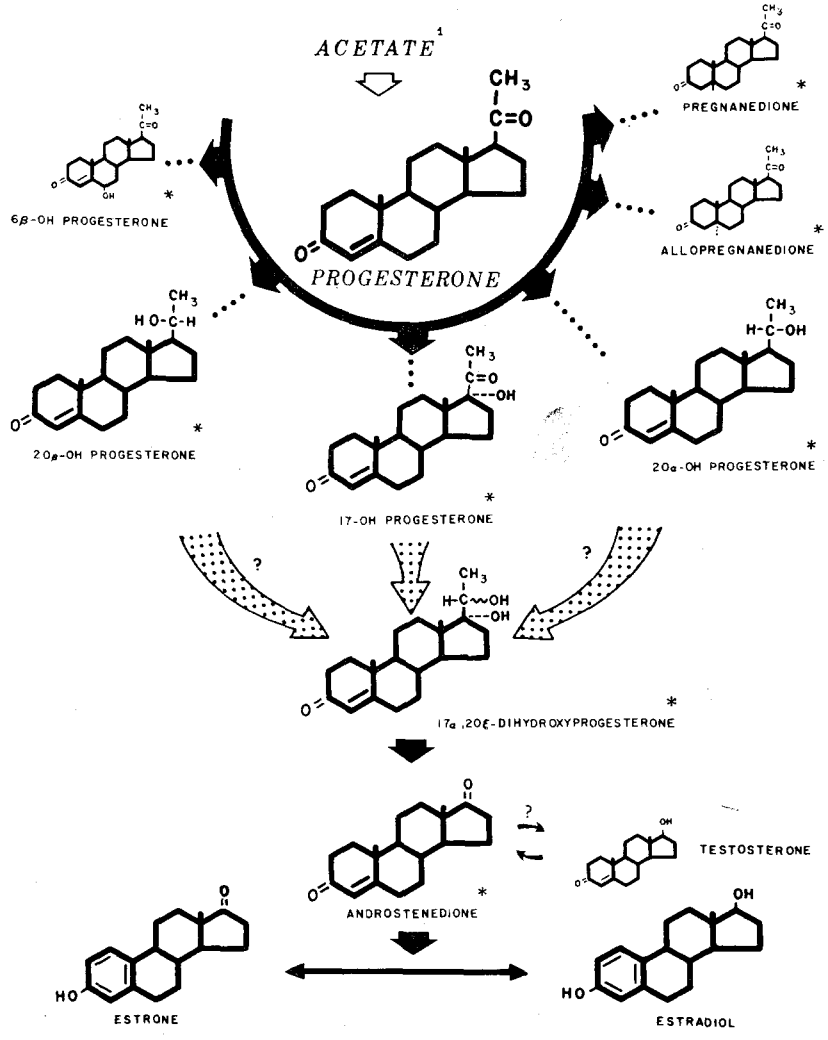

Fig. 4. The metabolism of progesterone in the human ovary (proposed sequence of biosynthesis).
[1] The conversion of acetate to progesterone was demonstrated only in bovine corpora lutea.
* Compounds isolated after incubation of [4-^{14}C]progesterone in human ovarian tissue. The estrogens were isolated after incubation of androstenedione.

in Fig. 4) is not known. These compounds may be associated with the mechanism of other species. SOLOMON *et al.*[12] have identified androstenedione, and 17-hydroxyprogesterone after the *in vitro* incubation of progesterone in bovine ovarian tissue. Two laboratories[13,14] have reported 20β-hydroxypregnene-3-one to be a product of progesterone metabolism in the bovine ovary. A similar pattern of progesterone metabolism to that observed in the ovary appears to occur in the rat and guinea pig testis[15]. The overall synthesis of estrogens from [^{14}C]acetate incubated with ovarian tissue has been reported by RABINOWITZ AND DOWBEN[16]. However, HOLLANDER AND HOLLANDER[17] were unsuccessful in demonstrating these conversions in similar action of progesterone or may be decomposition products in the course of the breakdown of progesterone for conjugation and elimination from the organism.

MEYER[24] has made the interesting observation that 19-hydroxyandrostenedione can be converted to estrone in the bovine ovary. 19-hydroxylation is known to occur biologically[25,26] and it is possible that this compound is an intermediate in the process of aromatization. It is also possible that a pathway of estrogen synthesis exists in which 19-hydroxylation precedes the cleavage of the side chain.

ACKNOWLEDGEMENTS

The technical assistance of S. JUTILA and C. SLOTBOOM is gratefully acknowledged. This investigation was supported by USPHS grants RG-4207 and A-1803.

REFERENCES

1 K. BLOCH, *Vitamins and Hormones*, Vol. 15, Academic Press, New York, 1957, p. 119.
2 G. POPJAK, *Ann. Rev. Biochem.*, 27 (1958) 533.
3 A. ZAFFARONI, *Recent Progress in Hormone Research*, Vol. 8, Academic Press, New York, 1953, p. 51.
4 D. L. BERLINER, O. V. DOMINGUEZ AND G. WESTENSKOW, *Anal. Chem.*, 29 (1957) 1797.
5 D. L. BERLINER AND H. A. SALHANICK, *Anal. Chem.*, 28 (1956) 1608.
6 W. ZIMMERMANN, *Z. physiol. Chem. Hoppe Seyler's*, 233 (1935) 257.
7 G. M. BARTON, R. S. EVANS AND J. A. F. GARDNER, *Nature*, 170 (1952) 249.
8 J. ZANDER, T. R. FORBES ,VON MÜNSTERMANN AND R. NEHER, *J. Clin. Endocrinol. and Metabolism*, 18 (1958) 337.
9 W. G. WIEST, J. ZANDER AND E. G. HOLMSTROM, *J. Clin. Endocrinol. and Metabolism*, 19 (1959) 297.
10 V. J. O'DONNELL AND J. G. MCCAIG, *Biochem. J.*, 71 (1959) 9 P.
11 Unpublished observation of the authors.
12 S. SOLOMON, R. V. WIELE AND S. LIEBERMAN, *J. Am. Chem. Chem. Soc.*, 78 (1956) 5453.
13 J. GORSKI, O. V. DOMINGUEZ, L. T. SAMUELS AND R. E. ERB, *Endocrinology*, 62 (1958) 234.
14 M. HAYANO, M. C. LINDBERG, M. WIENER, H. ROSENKRANTZ AND R. DORFMAN, *Endocrinology*, 55 (1954) 326.
15 W. S. LYNN JR. AND R. H. BROEN, *J. Biol. Chem.*, 232 (1958) 1015.
16 J. L. RABINOWITZ AND R. M. DOWBEN, *Biochim. Biophys. Acta*, 16 (1955) 98.
17 N. HOLLANDER AND V. P. HOLLANDER, *J. Biol. Chem.*, 233 (1958) 1097.
18 R. D. H. HEARD, P. H. JELLINEK AND V. J. O'DONNELL, *Endocrinology*, 57 (1955) 209.
19 C. D. WEST, B. L. DAMAST, S. D. SARRO AND O. H. PEARSON, *J. Biol. Chem.*, 218 (1956) 409.
20 B. BAGGETT, L. L. ENGEL, K. SAVARD AND R. I. DORFMAN, *J. Biol. Chem.*, 221 (1956) 931.
21 H. H. WOTIZ, J. W. DAVIS, H. M. LEMON AND M. GUT, *J. Biol. Chem.*, 222 (1956) 487.
22 K. J. RYAN, *J. Biol. Chem.*, 234 (1959) 268.
23 D. L. BERLINER AND H. A. SALHANICK, *J. Clin. Endocrinol. and Metabolism*, 16 (1956) 903.
24 A. S. MEYER, *Biochim. Biophys. Acta*, 17 (1955) 441.
25 A. ZAFFARONI, V. TRONCOSO AND M. GARCIA, *Chem. and. Ind.*, (1955) 534.
26 M. HAYANO AND R. I. DORFMAN, *Arch. Biochem. Biophys.*, 55 (1955) 289.