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# Microdetermination of Adrenocortical Steroids by Double Isotope Method. IV.[1] Determination of Corticosteroids in Human Placenta

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Homogenate of the human term placenta was extracted consecutively with ethanol and methanol, and the extract was fractionated with ether–water system into free and conjugated corticosteroids. Fractions obtained by enzymic hydrolysis of conjugates and free fraction were submitted to reverse isotope dilution analysis and the kinds of corticosteroids present in each fraction were identified. Amount of these corticosteroids was determined by the double isotope derivative dilution method in which the carbonyl group in C-3 position alone is derived to thiosemicarbazone-$^{35}$S.

Presence of the following corticosteroids was proved per 1 g (wet weight) of human term placental tissue: 0.136—1.176 μg of free and 0.014—0.099 μg of conjugated 11-deoxycorticosterone, 0.040—0.668 μg of free and 0.079—0.273 μg of conjugated 11-dehydrocorticosterone, 0.026—0.078 μg of free and 0.023—0.037 μg of conjugated corticosterone, 0.016—0.050 μg of free and 0.014—0.031 μg of conjugated cortisone, 0.004—0.045 μg of free and 0.009—0.015 μg of conjugated cortisol, and 0.014 μg of free and 0.011 μg of conjugated aldosterone, with a larger amount than the above of progesterone (0.731—2.370 μg of free and 0.235—0.964 μg of conjugated).

The use of the present method of determination allows separatory determination of free and conjugated corticosteroids, using about one-half of the placenta (250—280 g). Quantitative distribution of corticosteroids in three placenta analyzed here showed a marked individual difference but in all the placental tissues analyzed, the content of 11-deoxycorticosterone and 11-dehydrocorticosterone was higher than that of the three kinds of 11-oxo-17α-hydroxycorticosteroid.

**Keywords**——human term placenta; placental corticoid; microdetermination; double isotope method; thiosemicarbazide sulfur-35; derivative dilution analysis; tritium and sulfur-35 radioactivity

Presence of a kind of corticosteroids in human placenta has been pointed out.[3] Sawasaki, *et al.*[4] confirmed the presence of adrenocortical hormone-like substance in human placental extracts by various biological tests, and named it placental corticoid (PC Substance). $\Delta^4$-3-keto-$C_{21}$-steroids, which behaved like cortisone, cortisol, 11-deoxycortisol, 11-deoxycorticosterone, and corticosterone were found by a paper chromatographic examination of human placental extracts.[5] An aldosterone-like substance was detected by paper chromatography.[6] In the neutral fraction of solvent extracts of 9 kg of human placentas, cortisol, cortisone, 11-dehydrocorticosterone, and aldosterone were determined by the spectrophotometric method.[7] Cortisol and corticosterone were also separated from a fraction by extraction of 6 kg of human placentas by column and paper chromatography, and the both steroids were determined as 17-hydroxycorticosteroids by the Porter-Silber reaction.[8] On the other hand, the

1) Part III: A. Kunugi, U. Matsumoto, and Y. Nagase, *Endocrinol. Jpn.*, **20**, 397 (1973).
2) Location: *1432–1, Horinouchi, Hachioji, Tokyo, 192–03, Japan.*
3) R.H. Johnson and W.J. Haines, *Science*, **116**, 456 (1952); C, DeCourcy, C.H. Gray, and J.B. Lunnon, *Nature* (London), **170**, 494 (1952).
4) C. Sawasaki, M. Kagayama, S. Niinobe, and S. Sugawara, *J. Jpn. Obst. Gynaec. Soc.*, **6**, 265 (1954).
5) H.J. Staemmler, "Probleme der fetalen Endokrinologie," Berlin-Göttingen-Heidelberg, Springer, 1956, p. 185; *idem, Klin. Wochschr.*, **38**, 97 (1960).
6) J.J. Majnarich and R.N. Dillon, *Arch. Biochem. Biophys.*, **49**, 247 (1954).
7) D.L. Berliner, J.E. Jones, and H.A. Salhanick, *J. Biol. Chem.*, **223**, 1043 (1956).
8) C. Sawasaki, *Saishin-Igaku*, **12**, 1187 (1957).

presence of an electrolyte-metabolizing substance was proved in one placenta by using a biological test, and the amount of cortisol, cortisone, and tetrahydrocortisone were determined by the blue tetrazolium method.[9] Menini[10] carried out periodate oxidation of the neutral fraction, obtained by solvent extraction of the human placenta, and detected neutral steroids by gas chromatography. Since corticosteroids with 20,21-ketal side chain were removed as etiocholanic acids by periodate oxidation, he only determined pregnanes and androgens, without referring to corticosteroids.

As described above, data on the analysis of corticosteroids in human placenta have been controversial, primarily due to the technical difficulties. In the present series of work, we carried out determination of corticosteroids in one-half of human term placenta, in order to find the individual difference, by identification with the reverse isotope dilution analysis together with thin-layer chromatography (TLC), and determining the amount using the double isotope derivative dilution method in which only the carbonyl group at C-3 position is derived to thiosemicarbazone-$^{35}S$, as reported in our preceding paper.[11]

## Materials and Method

**Reagents**——The following labeled steroids were purified by TLC on a plate of silica gel (Camag, DF-5), with a solvent system of $CHCl_3$–EtOH (9: 1, v/v): Cortisol [1,2-$^3H$], specific activity, 20 Ci/mmol (Radio-

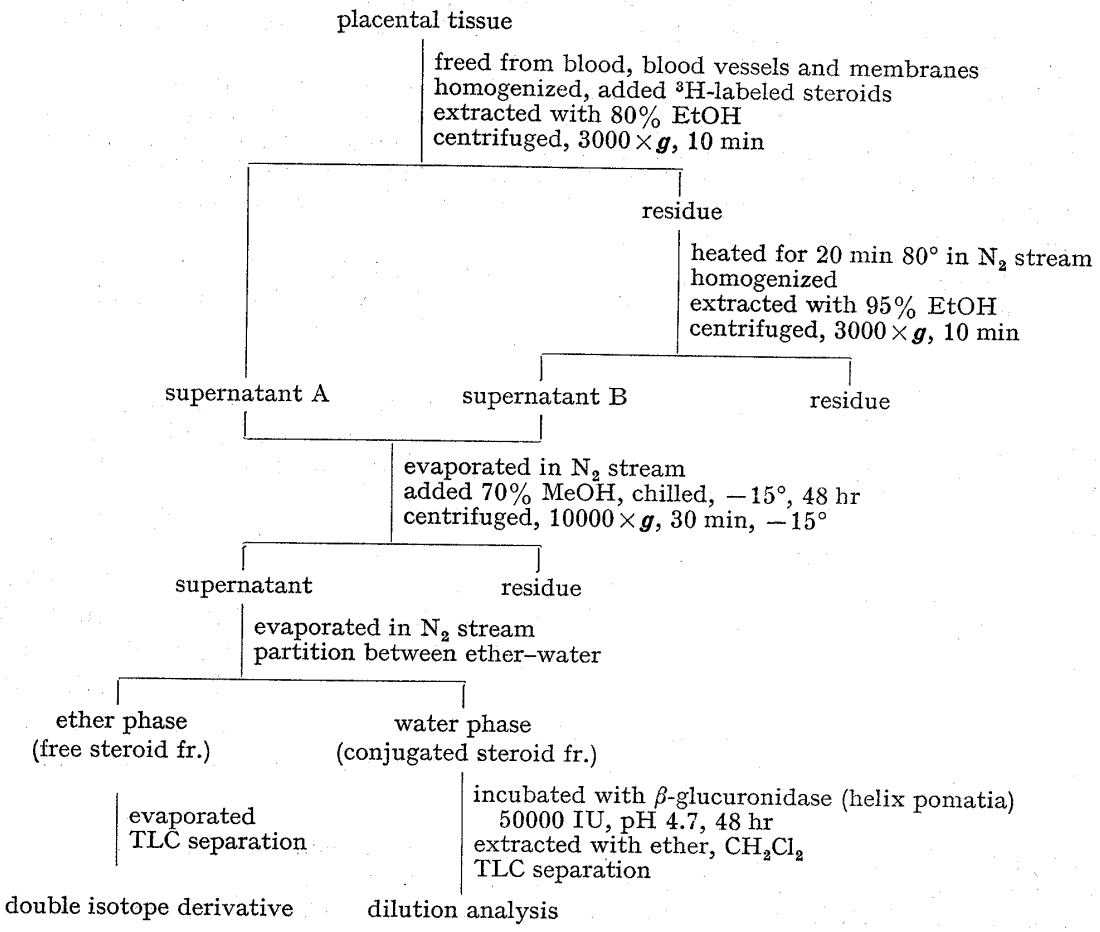


Chart 1

9) M. Suzuki, *J. Jpn. Obst. Gynaec. Soc.*, **10**, 1679 (1958).
10) E. Menini, "The Gas Liquid Chromatography of Steroids," ed. by J.K. Grant, Cambridge University Press, Cambridge, 1967, p. 78.
11) A. Kunugi, U. Matsumoto, and Y. Nagase, *Endocrinol. Jpn.*, **20**, 23 (1973).

chemical Centre, Amersham, England); corticosterone[1,2-$^3$H], specific activity, 10 Ci/mmol (Radiochemical Centre); cortisone[1,2-$^3$H], specific activity, 53 Ci/mmol (Radiochemical Centre); aldosterone[1,2-$^3$H], specific activity, 17 Ci/mmol (Radiochemical Centre); 11-deoxycorticosterone[1,2-$^3$H], specific activity, 30 Ci/mmol (New England Nuclear, Boston, U.S.A.); progesterone[1,2-$^3$H], specific activity, 33.5 Ci/mmol (Radiochemical Centre). Thiosemicarbazide-$^{35}$S was synthesized by the same method as described in our previous paper.[12] $\beta$-Glucuronidase/aryl sulfatase (*Helix pomatia*, activity, 650000 IU/g) (C.F. Böhringer u. Söhne GmbH, Mannheim, Germany). Acetate buffer solution (pH 4.70) was prepared by mixing 91 ml of 0.2 M AcOH and 109 ml of 0.2 M AcONa. Hydrophobic liquid scintillator was prepared by dissolving 4 g of PPO and 0.1 g of dimethyl-POPOP in 1 l of toluene. Hydrophilic liquid scintillator was prepared by dissolving 4 g of PPO, 0.4 g of dimethyl-POPOP, and 100 g of naphthalene in 1 l of dioxane–toluene–methyl cellosolve (15: 3: 2, v/v).

**Analytical Procedures**

**Extraction of Steroids from Human Placenta**——As shown in Chart 1, one human term placenta was washed and blood was squeezed out as much as possible. Membrane and blood vessels were removed by peeling. About one-half of this placenta, tissue of 250—280 g in wet weight, was cut into small pieces and homogenized. In order to determine the recovery at each step of the extraction, about $2 \times 10^5$ dpm of each of $^3$H-labeled cortisol, corticosterone, cortisone, 11-deoxycorticosterone, aldosterone, and progesterone was added. In accordance with Menini's method,[10] the placenta was extracted once with 600 ml and 4 times with 100 ml each of 80% EtOH. The extracts were centrifuged at $3000 \times g$ for 10 min in each case, the supernatant (A) was separated, and the residue was warmed at 80° for 20 min in $N_2$ stream. This residue was extracted once with 200 ml and 3 times with 100 ml each of 95% EtOH. The extracts were centrifuged at $3000 \times g$ for 10 min in each case and the supernatant (B) was separated. The combined supernatant (A and B) was evaporated in $N_2$ stream. In order to remove lipids, this residue was dissolved in 50 ml of 70% MeOH, chilled to −15° and kept there for 48 hr, and then centrifuged at $10000 \times g$ for 30 min in a refrigerated centrifuge at the same temperature. Its supernatant was concentrated in $N_2$ stream and the concentrated aqueous solution was extracted with ether to separate into the free steroid fraction and conjugated steroid fraction. The conjugated steroid fraction was submitted to enzymic hydrolysis using 5000 IU of $\beta$-glucuronidase from *Helix pomatia*, which also possesses sulfatase activity, at 37° for 48 hr in acetate buffer (pH 4.7). The hydrolyzed solution was extracted 3 times with 60 ml each of ether and hydrolysate fraction of the conjugates was obtained.

Extraction rate at each step of this extraction was calculated by the measurement of tritium radioactivity in each fraction, and calculated from the recovery of radioactivity to that of $^3$H-labeled corticosteroids initially added to the placental homogenate.

**Group Separation of Steroids by TLC**——Before identification and determination of each of corticosteroids, the fractions of free and hydrolysates of conjugated steroids were examined by TLC on a silica gel plate, developed with solvent systems of $CHCl_3$–EtOH (9: 1, v/v) and benzene–acetone (4: 1, v/v), and the spots of steroids were detected under a short-wave ultraviolet (UV) lamp (256 nm). After development, the plate was scraped off in 1 cm width, filled in a small column, and the column was eluted with 5 ml of $CH_2Cl_2$–MeOH (2: 1, v/v). Each eluate was collected in a counting vial, the solvent was evaporated, and 10 ml of the hydrophobic liquid scintillator was added. Tritium radioactivity of these vials was counted by a liquid scintillation counter to find the fractions with radioactivity.

**Identification of Corticosteroids in Placenta by Reverse Isotope Dilution Analysis**——Corticosteroids in human placenta were extracted by the method shown in Chart 1 and *Rf* values of steroid spots on silica gel TLC were compared with those of unlabeled authentic samples to identify each of the corticosteroids. The radioactive spots on the TLC plate were collected and eluted, 500 $\mu$g of unlabeled corticosteroid presumed from the *Rf* value was added, and the silica gel TLC was repeated 4 times. At each time, silica gel of each spot was scraped off, eluted with methanol, and radioactivity and absorbancy at 240 nm in each fraction were measured. Each of the corticosteroids was identified by the absence of variation in the specific activity to a constant value.

**Determination of Corticosteroids in Human Placenta by the Double Isotope Derivative Dilution Method**——Corticosteroids in the human term placenta were determined by the method described in our preceding paper.[11] To the placental homogenate, about $2 \times 10^5$ dpm of each of $^3$H-labeled cortisol, corticosterone, cortisone, 11-deoxycorticosterone, aldosterone, and progesterone, whose presence was identified as above, was added and corticosteroids were extracted by the method shown in Chart 1. The extract was separated into fractions of free and conjugated steroids and each of these fractions was submitted to silica gel TLC. The corticosteroids were thereby fractionated into 3 groups. Corticosteroids were eluted from each fraction, the solvent was evaporated from the eluates, and 100 $\mu$g of thiosemicarbazide-$^{35}$S (specific activity, 45 mCi/mmol) and 1 ml of 10% AcOH–MeOH solution were added, and the mixture was heated at 65° for 90 min. The corticosteroid thiosemicarbazones-$^{35}$S thereby formed were extracted 3 times with 5 ml each of $CH_2Cl$-$CH_2Cl$. After addition of 20 $\mu$g each of the thiosemicarbazone of unlabeled corticosteroids as a carrier, the

12) A. Kunugi, U. Matsumoto, Y. Aizawa, and Y. Nagase, *Chem. Pharm. Bull.* (Tokyo), **21**, 503 (1973).

extract was submitted to silica gel TLC, repeated 3 times, with solvent system of $CHCl_3$–EtOH (9: 1, v/v), benzene–acetone (1: 1, v/v), and AcOEt, and six kinds of corticosteroid thiosemicarbazone-$^{35}S$ were separated. The silica gel of the spots detected under a short-wave UV lamp were each collected, filled in a small column, and the column was eluted with $CH_2Cl_2$–MeOH (1: 1, v/v). Each eluate was collected in a counting vial, the solvent was evaporated, 10 ml of the hydrophobic liquid scintillator was added, and the radioactivity of $^3H$ and $^{35}S$ was measured with a liquid scintillation counter. The amount of corticosteroids in 250—280 g wet weight of placental gland was calculated from the following equation:

$$C_p = C_s \times \frac{S_p}{H_p} \times \frac{H_s}{S_s} \times \frac{I_p}{I_s}$$

where $C_p$ is the amount (in μg) of corticosteroid in the placenta (250—280 g wet weight), $C_s$ is the amount (in μg) of corticosteroid added to the standard sample, $S_p$ and $H_p$ are the counts (in dpm) of $^{35}S$ and $^3H$, respectively, in the sample after the analytical procedures, $S_s$ and $H_s$ are the counts (in dpm) of $^{35}S$ and $^3H$, respectively, in the standard sample after the analytical procedures, and $I_p$ and $I_s$ are the counts (in dpm) of $^3H$-labeled corticosteroids added to placenta and to standard sample, repectively.

## Results and Discussion

### Extraction of Corticosteroids from Placental Tissue

Extraction of corticosteroids from human term placenta with 80% and 95% ethanol in the procedures shown in Chart 1 and in analytical procedures was examined. As shown in Table I, about 95% of the corticosteroids added to the placental tissue homogenate is extracted by five extractions with 80% ethanol and approximately quantitatively by subsequent extraction with 95% ethanol.

Table I. Extraction of Added Authentic Corticosteroids from Human Placental Tissue[a)]

| Ext. No. | Solvent | Volume (ml) | Extraction rate (%) | Total recovery (%) |
|---|---|---|---|---|
| 1 | 80% EtOH | 600 | 72.6 | 72.6 |
| 2 | 80% EtOH | 100 | 9.2 | 81.8 |
| 3 | 80% EtOH | 100 | 6.8 | 88.5 |
| 4 | 80% EtOH | 100 | 4.6 | 93.2 |
| 5 | 80% EtOH | 100 | 2.2 | 95.4 |
| 6 | 95% EtOH | 200 | 1.8 | 97.2 |
| 7 | 95% EtOH | 100 | 0.7 | 97.9 |
| 8 | 95% EtOH | 100 | 0.3 | 98.2 |
| 9 | 95% EtOH | 100 | 0.2 | 98.4 |

a) Wet weight: 250—280 g.

### Isolation and Identification of Placental Corticosteroids

It is necessary to know which corticosteroids are present prior to the determination. Consequently, repeated TLC and reverse isotope dilution analysis were then carried out to isolate and identify corticosteroids. As shown in Fig. 1, a and b, placental corticosteroids were found to be fractionated into 4—5 groups by the difference in their polarity. Purification of each fraction by repeated TLC showed that the specific radioactivity of each steroid became constant after the second TLC, as shown in Table II. The reverse isotope dilution analysis identified 7 kinds of corticosteroid to be present in each fraction; 11-deoxycorticosterone, 11-dehydrocorticosterone, cortisone, corticosterone, cortisol, aldosterone, and progesterone.

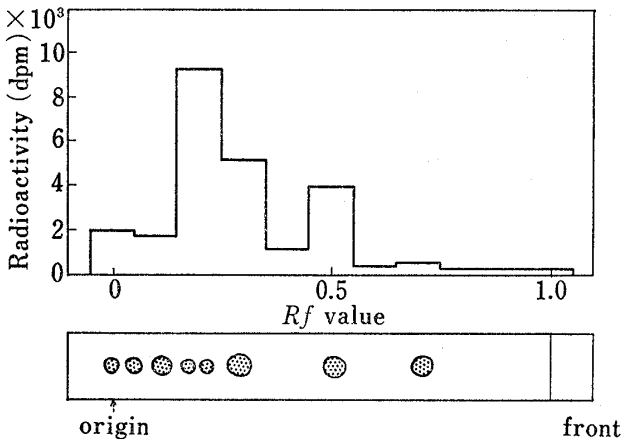


Fig. 1a. Group Separation of Corticosteroids Fraction of Placental Extract by TLC(silica gel)

solvent system: benzene–acetone (4: 1, v/v).
⊛: detected with ultraviolet ray.

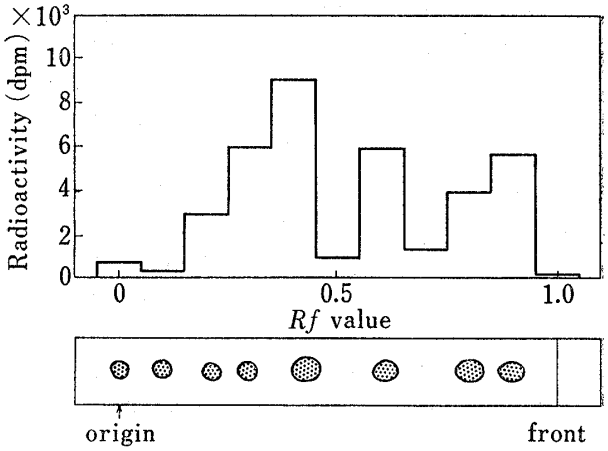


Fig. 1b. Group Separation of Corticosteroids Fraction of Placental Extract by TLC(silica gel)

solvent system: $CHC_3$l–EtOH (9: 1, v/v).
⊛: detected with ultraviolet ray.

TABLE II. Identification of Corticosteroids in Human Term Placenta by Reverse Isotope Dilution Analysis

| Steroid | Specific activity ($dpm/A_{240}$)[a] | | | |
|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th |
| Progesterone | 1610 | 1250 | 1230 | 1250 |
| Deoxycorticosterone | 2205 | 1730 | 1720 | 1735 |
| 11-Dehydrocorticosterone | 4514 | 4035 | 4070 | 4051 |
| Corticosterone | 6117 | 5225 | 5237 | 5240 |
| Cortisone | 7782 | 7280 | 7240 | 7301 |
| Cortisol | 8680 | 7721 | 7690 | 7735 |
| Aldosterone | 8185 | 7830 | 7791 | 7810 |

a) Specific activity was radioactivity per absorbancy at 240 nm in consecutive TLC (silica gel) of corticosteroid extracted from placenta.
solvent system: 1st $CHCl_3$–EtOH (9: 1, v/v), 2nd benzene–acetone (1: 1 v/v), 3rd benzene–acetone (4: 1, v/v), 4 th AcOEt.

## Determination of Corticosteroids in Human Term Placenta

Corticosteroids were determined in the free and conjugated fractions by the double isotope derivative dilution analysis using one-half of three human term placenta, about 250—280 g in wet weight, by the analytical procedures described above. As shown in Table III, the

TABLE III. Corticosteroids Content in Human Term Placenta

| Steroid | Free steroid[a] | | | Conjugated steroid[a] | | |
|---|---|---|---|---|---|---|
| | Placenta[b] | | | Placenta[b] | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| Progesterone | 2.370 | 2.251 | 0.731 | 0.235 | 0.854 | 0.964 |
| Deoxycorticosterone | 1.176 | 0.136 | 0.207 | 0.090 | 0.014 | 0.099 |
| 11-Dehydrocorticosterone | 0.668 | 0.040 | 0.130 | 0.079 | 0.273 | 0.108 |
| Corticosterone | 0.078 | 0.039 | 0.026 | 0.023 | 0.037 | 0.023 |
| Cortisone | 0.050 | 0.016 | 0.023 | 0.014 | 0.031 | 0.019 |
| Cortisol | 0.045 | 0.014 | 0.004 | 0.015 | 0.011 | 0.009 |

a) μg/kg tissue (wet weight).
b) Weight of placental tissue: 1 250 g, 2 260 g, 3 280 g.

amount of non-17-hydroxycorticoids, such as C-21 hydroxylated compound (11-deoxycorticosterone) of progesterone, its 11$\beta$-hydroxylated compound, cortisone (compound E), and its dehydro compound, 11-dehydrocorticosterone (compound A), is relatively high. Especially high content of 11-deoxycorticosterone and 11-dehydrocorticosterone was found. The amount of these steroids was about 10—50 times that of 11-oxo-17$\alpha$-hydroxycorticosteroids, biosynthesized in a minute amount by the route in which 17$\alpha$-hydroxylation of progesterone precedes its 21-hydroxylation, as in cortisone and cortisol, and this was found to be characteristic to the placenta. Presence of 11-deoxycortisterone has been qualitatively pointed out in the past, and the steroid was found in all three samples in a considerable amount by the present method.

Mineral metabolic activity several ten folds stronger than that of 11-deoxycorticosterone had been detected in human placenta, and this substance had been presumed as aldosterone.[6] Sawasaki[8] was unable to detect it by paper chromatography but Berliner, *et al.*[7] indicated the presence of 3 $\mu$g/kg of aldosterone by fractional extraction. Although it is not shown in Table III due to the failure of analysis on two of the placenta samples (No. 1 and 2), because of the unavailability of tritium-labeled steroid, analysis of one placenta sample (No. 3) showed the presence of 0.012 $\mu$g of free aldosterone and 0.009 $\mu$g of conjugated aldosterone per g wet tissue, a larger amount than cortisol.

Matsuba[13] had suggested that 11-oxo-17$\alpha$-hydroxycorticosteroids of cortisol group would not be produced in the placenta because it lacked 17$\alpha$-hydroxylase. Detection of cortisol and cortisone, 11-oxo-17$\alpha$-hydroxycorticosteroids, in the placenta was suggested to be contamination of blood.[14] In recent years, biosynthetic system for steroidal hormones is being clarified for feto-placental unit constituted by the fetus and the placenta.[15] In humans, corticosteroids produced by the fetal cortex transit to the placenta through the cord plasma, and thought to be metabolized there to be excreted from the maternal body. Among $C_{21}$-steroidal hormones, progesterone is known to be produced in a large amount and secreted from the placenta. Placental progesterone is not converted into $C_{19}$-steroids, and transits *per se* into the fetus and the mother. This progesterone is considered[15] to be converted into 11-deoxy- and 11-oxo-corticosteroids, *via* 17-hydroxyprogesterone, in the fetal cortex where the 11,17,21-hydroxylase activity is high. 11-Deoxycortisol (17$\alpha$-hydroxy-11-deoxycorticosterone, compound S), whose presence in the placenta was qualitatively pointed out by Staemmler,[5] was not detected by Berliner, *et al.*[7] The fact that 11-deoxycortisol was not also detected by our experiment is thought to be due to the absence of 17$\alpha$-hydroxylase in the placenta. Based on the lack of this enzyme in the placenta, biosynthesis of cortisol and cortisone *via* 11-deoxycortisol after 17$\alpha$-hydroxylation of progesterone in the fetal cortex may be presumed.

As shown in Table III, relative amount of cortisone is larger than that of cortisol in the placenta, different from the patern in adult plasma. This is considered to reflect the markedly accelerated conversion of cortisol to cortisone in the fetus, with consequent inactivation of cortisol.[16]

Amount of free is larger by one order than the conjugated form in progesterone and 11-deoxycorticosterone while the quantitative ratio of the free to conjugate form of 11-dehydrocorticosterone is reversed in placenta No. 1 and No. 2. In other corticosteroids, a larger amount is isolated as the conjugated fraction. This fact seems to indicate that corticosteroids are metabolized as sulfates in the fetus,[17] while still a large amount of the sulfate

13) M. Matsuba, *Saishin-Igaku*, **12**, 1170 (1957).
14) H.A. Salhanick, L.M. Neal, and J.P. Mahoney, *J. Clin. Endocrinol. Met.*, **16**, 1120 (1956).
15) E. Diczfalusy, *Fed. Proc.*, **23**, 791 (1964); *idem*, Proceedings of the 2nd International Congress of Endocrinology, London, August, 1964 (Excerpta Med. Int. Congr. Ser., n83, Amsterdam, 1965), Part 2, p. 732.
16) D.H. Hillman and C.J.P. Giround, *J. Clin. Endocrinol. Met.*, **25**, 243 (1965).
17) G. Pérez-Palacios, A.E. Pérez, and R.B. Jaffe, *J. Clin. Endocrinol. Met.*, **28**, 19 (1968).

transits from the fetus to the placenta as a conjugate. Since the activities of sulfatase and glucuronase, whose activity is absent or quite low in the fetal adrenal cortex, are markedly high in the placenta, hydrolysis of the conjugate originating in the fetus in the placenta and its metabolism seem to be similar to the metabolic route of estrogen.

Determination of a trace amount of corticosteroids is possible by our double isotope derivative dilution method, and this method is considered to be valuable in the elucidation of the conversion of $C_{21}$-steroids in the feto-placental unit, especially the biosynthesis mechanism of corticosteroids.

Majority of past reports on the chemical detection of steroidal hormones by fractional extraction from the placenta used a large number (20—27) of placenta, and this made it impossible to find the distribution of steroids in the placenta or their individual difference. Berliner, *et al.*[7] expressed their analytical results by micrograms of the steroid per kilogram of placental tissue, using 9 kg of placenta, and they did not make fractional determination of free and conjugated steroids. Correction of the recovery at each step of extraction and isolation was not considered. In the present series of work, about one-half of each placenta was used as the sample, and loss at each step of isolation was corrected in the determination of corticosteroids. It was thereby found that there is a considerable individual variation in the distribution of corticosteroids in the placenta. Consequently, distribution of corticosteroids in the placenta would indicate the individual difference in the biosynthetic functions of corticosteroids in the feto-placental unit and indirectly in the fetal cortex. In order to make this knowledge more useful, it would be necessary to make further examinations with a large number of samples for the determination.