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Human Fetal Adrenal Definitive and Fetal Zone Metabolism of Pregnenolone and Corticosterone: Alternate Biosynthetic Pathways and Absence of Detectable Aldosterone Synthesis*

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ABSTRACT. In the rhesus monkey and ovine fetus *in utero*, aldosterone concentrations do not rise in response to surgical stress, ACTH, or angiotensin-II, all of which are secretagogues for this mineralocorticoid in the adult. To assess the mechanism of this phenomenon in the human fetus, metabolism of pregnenolone and corticosterone by second trimester human fetal adrenal definitive zone and fetal zone tissue was studied. After incubation of fresh tissue with trace amounts of [^{3}H]pregnenolone or [^{3}H]corticosterone, the products of metabolism were separated using high performance liquid chromatography and quantified. The Δ^5-3β-hydroxysteroids 17-hydroxypregnenolone and dehydroepiandrosterone and their sulfates comprised 85–90% of metabolized pregnenolone. In the fetal zone, cortisol was the predominant secreted Δ^4-3-ketosteroid, accounting for 6–8% of the metabolized pregnenolone. In the definitive zone, progesterone and corticosterone were the predominant secreted Δ^4-3-ketosteroids, each accounting for about 2% of the metabolized pregnenolone. 11-Dehydrocorticosterone and sulfates were the only metabolites detected after incubation of fetal adrenal tissue with corticosterone. 11-Dehydrocorticosterone accounted for more than 80% of the metabolized corticosterone in the definitive zone and 50% in the fetal zone. Incubations with secretagogues or antioxidants (10 nmol/L ACTH, 10 nmol/L angiotensin-II, 21 mmol/L potassium, 100 mmol/L dimethylsulfoxide, 5 μmol/L metyrapone, or 100 μmol/L butylated hydroxyanisole) did not change the pattern or extent of precursor metabolism. No aldosterone, 18-hydroxycorticosterone, or 18-hydroxydeoxycorticosterone was detected in baseline or stimulated incubations of human fetal tissue. In contrast, adult human zona glomerulosa metabolized corticosterone to aldosterone, 18-hydroxycorticosterone, and 11-dehydrocorticosterone under similar conditions. ACTH (10 nmol/L) increased the relative production of aldosterone from 29% to 45% of the metabolized corticosterone, while 18-hydroxycorticosterone decreased from 41% to 26%, and 11-dehydrocorticosterone remained unchanged at 30%. These results indicate that, in the second trimester human fetal adrenal, the non-17-hydroxylated steroid metabolic pathway is localized to the definitive zone, and mineralocorticoid secretion is very low and unresponsive to secretagogues known to be active in the adult. (*J Clin Endocrinol Metab* **70**: 693, 1990)

ALDOSTERONE, the major mineralocorticoid produced by the mature human adrenal cortex, regulates extracellular fluid volume and potassium excretion, primarily by causing sodium reabsorption in exchange for potassium excretion in the distal renal tubule. In the adult, three well defined modulators of aldosterone production are ACTH, angiotensin-II, and potassium. Each acutely stimulates aldosterone secretion *in vivo* and *in vitro* (1).

The initiation, regulation, and physiological significance of mineralocorticoid secretion during fetal development are poorly understood. At birth, the aldosterone concentration in human umbilical cord blood exceeds that in maternal blood and is elevated if the mother has had restricted sodium intake or diuretic treatment, demonstrating responses to some of the same stimuli that regulate aldosterone in the adult (2). While *in vivo* human fetal studies are not feasible, we have shown that circulating aldosterone levels in catheterized rhesus monkey fetuses do not increase after the infusion of ACTH or angiotensin-II (3). In the present study we have examined the extent and regulation of second trimester human fetal mineralocorticoid secretion, measuring steroid metabolism by fresh adrenal tissue in the basal state and in response to ACTH and angiotensin-II. We report here that we have been unable to detect

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fetal adrenal aldosterone secretion in the presence or absence of secretagogues that have been shown to be active in the adult.

Materials and Methods

Tissue Incubations

Fetal adrenal glands were obtained immediately after elective dilatation and evacuation. Incubations were performed using tissue from 10 abortuses (6 females and 4 males). All abortuses were 18–21 weeks gestational age, as assessed by fetal foot length (4). None had congenital anomalies. Fetal and definitive zones were separated as described previously (5). Briefly, the definitive zone was obtained as a layer of cells remaining attached to the adrenal capsule after removal of all fragments of the central fetal zone by careful blunt dissection. Any tissue not clearly identified as adherent to the capsule or as a fragment of the central fetal zone was discarded. The fetal zone and the capsule with the definitive zone were then each processed separately. The tissue from each zone was minced into 0.5-mm cubes. Portions of each tissue, weighing approximately 100–150 mg, were transported to the laboratory on ice in silanized glass tubes containing 1 mL medium 199 with or without added metyrapone,[1] DMSO, and/or BHA.

For comparison, an adult adrenal gland, obtained 1 h after death following head injury and maintained on ice until dissection and incubation 13 h later, was also studied. Zona glomerulosa, consisting of 1–2 mm tissue underlying the capsule, was minced into 0.5-mm cubes and placed in silanized glass tubes containing 1 mL medium 199.

All fetal adrenal incubations were initiated within 1 h of tissue acquisition, as preliminary studies demonstrated that delayed processing of fetal tissue markedly reduced the degree of metabolism of radioactive steroid precursors. Preliminary studies showed that 3-h incubations yielded maximal precursor metabolism. After the addition of 0.6 ng [^{3}H]corticosterone ([1,2,6,7-^{3}H]corticosterone; SA, 85.8 Ci/mmol; New England Nuclear, Boston, MA) or [^{3}H]pregnenolone ([7-^{3}H]pregnenolone; SA, 25 Ci/mmol; Amersham, Arlington Heights, IL), with or without ACTH-(1–24) (Cortrosyn, Organon, West Orange, NJ), angiotensin-II (Sigma Chemical Co., St. Louis, MO), or potassium, tubes were incubated for 3 h at 37 C in a Dubnoff metabolic shaker under 95% O_2-5% CO_2 unless otherwise specified. At the end of the incubation, the tubes were centrifuged for 5 min at 1000 rpm at 4 C. The media were decanted and frozen until prepared for chromatography. The tissue pellets were washed twice with cold phosphate-buffered saline, sonicated into 1 mL distilled water, and frozen until chromatography or protein assay.

Chromatography

Unlabeled steroid standards (aldosterone, 18-hydroxycorticosterone, cortisone, cortisol, 11-dehydrocorticosterone, corticosterone, 11-deoxycortisol, deoxycorticosterone, 17-hydroxyprogesterone, 17-hydroxypregnenolone, progesterone, and pregnenolone) were obtained from Steraloids (Wilton, NH). One microgram of each steroid standard, 1 mL 1 N H_2SO_4, and 0.5 mL acetonitrile were added to 800 μL thawed medium or sonicated tissue. The mixture was extracted for 30 min with 12 mL dichloromethane. The dichloromethane phase was collected, washed twice with distilled water, and dried under nitrogen at 37 C. The residue was reconstituted with 100 μL mobile phase A of the chromatography system and injected onto the column.

HPLC was performed on a Brownlee 5-μm C18 reverse phase column (Brownlee Labs, Santa Clara, CA) using a programmed gradient of mobile phases at a constant flow rate of 1.5 mL/min as previously described (6). Mobile phase A consisted of methanol/1 mM KH_2PO_4 mixed 52:48 (vol/vol). Mobile phase B consisted of methanol/isopropanol mixed 50:50 (vol/vol). Elution was carried out over 36 min using 100% mobile phase A from 0–8 min, then a linear gradient from 0–34% mobile phase B from 8–36 min. Retention times of corticoids (Table 1) were monitored with a variable wavelength absorption detector set at 210 nm. Fractions were collected every 0.25 min, 4 mL scintillation fluid (Scintiverse II, Fisher Scientific, Santa Clara, CA) were added, and the amount of radioactivity was determined at 35% efficiency in a PRIAS scintillation counter (Packard Instruments, Downers Grove, IL).

Peaks of metabolized radioactive steroids comigrated with unlabeled steroid standards, except for a broad peak eluting

TABLE 1. Retention times of corticoids, androgens, and estrogens

Steroid	Retention time (min)	Fraction (1 fraction/15 s)
Dehydroepiandrosterone sulfate	2.7	11
Aldosterone	8.3	32
18-Hydroxycorticosterone	9.3	37
Cortisone	9.7	38
Cortisol	11.4	45
11-Dehydrocorticosterone	12.5	50
18-Hydroxydeoxycorticosterone	16.8	67
Corticosterone	17.8	71
11-Deoxycortisol	18.8	75
11-Hydroxyprogesterone	20.5	82
Estrone	23	92
Estradiol	23	92
Androstenedione	24.3	97
Deoxycorticosterone	24.5	98
Testosterone	24.7	99
17-Hydroxyprogesterone	26	104
Androstenediol	26	104
17-Hydroxypregnenolone	27	108
Dehydroepiandrosterone	27	108
Progesterone	34.5	138
Pregnenolone	37.5	150

[1] Trivial names and abbreviations used are: BHA, butylated hydroxyanisole, 2- and 3-*tert*-butyl-4-methoxyphenol; 11-dehydrocorticosterone, 21-hydroxypregnen-4-ene-3,11,20-trione; 11-deoxycortisol, 17,21-dihydroxypregnen-4-ene-3,20-dione; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography; 18-hydroxycorticosterone, 11β,18,21-trihydroxypregn-4-ene-3,20-dione; 18-hydroxydeoxycorticosterone 18,21-dihydroxypregn-4-ene-3,20-dione; 17-hydroxypregnenolone, 3β,17-dihydroxypregn-5-en-20-one; 17-hydroxyprogesterone, 17-hydroxypregn-4-ene-3,20-dione; metyrapone 2-methyl-1-2, di-3-pyridyl-1-propanone; pregnenolone, 3β-hydroxypregn-5-en-20-one.

before aldosterone in fractions 9–25. Dehydroepiandrosterone sulfate migrated within the range of this broad peak. Pretreatment of incubation medium overnight at 37 C with sulfatase (Sigma Chemical Co., St. Louis, MO: 50 U in 2 mol/L sodium acetate buffer adjusted to pH 5.0) resulted in at least a 70% reduction of radioactivity in the broad peak and redistribution of that radioactivity among the other steroid peaks present (corticosterone and 11-dehydrocorticosterone when corticosterone was the precursor; primarily pregnenolone and 17-hydroxypregnenolone and/or dehydroepiandrosterone when pregnenolone was the precursor). These findings suggest that sulfates of steroid precursors and products are a major component of the early eluting broad peak, which is referred to as sulfates in this report.

Fractions containing radioactivity that comigrated with aldosterone, corticosterone, or 11-dehydrocorticosterone in the HPLC system were collected, extracted with dichloromethane, and rechromatographed using thin layer chromatography with two different solvent systems. After spotting, the plates were developed once with toluene-acetone-dichloromethane (2:2:1, vol/vol/vol) or twice with dichloromethane-acetone (4:1, vol/vol). Rf values are listed in Table 2. In each case, more than 95% of the radioactivity comigrated with steroid standard. The combination of HPLC and thin layer chromatography data is the basis for the presumptive identification of the steroids found in these experiments.

Relative steroid production is the ratio of radioactivity in the specified steroid product peak to the total radioactivity present in all steroid product peaks. The relative production of steroid metabolites in corresponding medium and sonicated tissue samples differed by less than 5%. Steroid determinations reported are from medium after incubation with tissue. The relative production of steroid metabolites in tissue samples from different specimens treated similarly differed by less than 5%. Incubation of medium with all combinations of additives but without tissue resulted in less than 1% metabolism of precursor. Conversion of at least 0.1% added substrate (0.6 pg [^{3}H]corticosterone or 2 pg [^{3}H]pregnenolone) to a product was required for detection of the product by this system.

Precursor metabolism is the ratio of radioactivity in all steroid product peaks to the total radioactivity eluted (products plus remaining precursor). Precursor metabolism by tissue type and precursor is shown in Table 3. Precursor metabolism did not differ significantly with differing treatment of a given tissue in any experiment or between tissue samples from different specimens treated similarly. Because the amount of tissue placed into each incubation was approximate, the concentration of protein after sonication of the tissue was measured by the method of Bradford (7), using BSA as a standard. The correlation coefficient (r) between the corticosterone metabolism and protein concentration was 0.65 for definitive zone tissue and 0.51 for fetal zone tissue.

TABLE 2. R_f values of corticoids in thin layer chromatography with two solvent systems

Steroid	R_f	
	System A	System B
11-Dehydrocorticosterone	0.37	0.39
Cortisone	0.32	0.26
Corticosterone	0.32	0.23
Aldosterone	0.16	0.09
18-Hydroxycorticosterone	0.11	0.04

System A = toluene-acetone-dichloromethane (2:2:1, vol/vol/vol) developed once. System B = dichloromethane-acetone (4:1, vol/vol) developed twice.

TABLE 3. Precursor metabolism by minced definitive zone, fetal zone, and adult human adrenal zona glomerulosa tissue

	% Metabolism of precursor	
	Pregnenolone	Corticosterone
Definitive zone	55 ± 10 (6)	38 ± 16 (12)
Fetal zone	85 ± 5 (9)[a]	44 ± 15 (9)
Adult zona glomerulosa		15 ± 3 (6)

Each value is the mean ± SD of the number of incubations in parentheses.

[a] Differs from definitive zone, $P < 0.05$.

Statistical analysis

The relative steroid production or precursor metabolism of different tissues under various treatment conditions was compared by analysis of variance. A result was considered significant when $P \leq 0.05$.

Results

Table 4 shows the results of incubation of definitive and fetal zone tissue using [^{3}H]pregnenolone as a precursor. The Δ^5-3β-hydroxysteroids 17-hydroxypregnenolone and dehydroepiandrosterone and their sulfates comprised 85–90% of the metabolized tracer. In the fetal zone, cortisol was the predominant secreted Δ^4-3-ketosteroid, accounting for 6–7% of the metabolized pregnenolone. In the definitive zone, progesterone and corticosterone were the predominant secreted Δ^4-3-ketosteroids, each accounting for about 2% of the metabolized pregnenolone. Inclusion of 10 nmol/L ACTH or 10 nmol/L angiotensin-II during incubation did not change either the extent of metabolism or the pattern of metabolites observed. No aldosterone, 18-hydroxycorticosterone, or 18-hydroxydeoxycorticosterone was detected in the incubations using [^{3}H]pregnenolone as precursor.

Table 5 shows the products of [^{3}H]corticosterone metabolism. 11-Dehydrocorticosterone and sulfates were the only metabolites detected after incubation of fetal adrenal tissue. 11-Dehydrocorticosterone accounted for more than 80% of the metabolized corticosterone in the definitive zone and 50% in the fetal zone, a significant difference. Incubations with ACTH, angiotensin-II, or potassium did not change the pattern or extent of metabolism.

Crivello *et al.* (8) have shown that metyrapone and antioxidants are required to maintain aldosterone secretion in cultured adult bovine glomerulosa cells (8). To

TABLE 4. Metabolites of [^{3}H]pregnenolone from minced definitive and fetal zone tissue with various secretagogues

	% of [^{3}H]pregnenolone metabolites recovered as:					
	Progesterone	Corticosterone	17-Hydroxy-progesterone	Cortisol	17-Hydroxy-pregnenolone and Dehydro-epiandrosterone	Sulfates
Definitive zone						
No treatment	2.5 ± 0.4	1.6 ± 0.2	4.8 ± 0.8	0.6 ± 0.2	44 ± 3	46 ± 4
+ 10 nmol/L ACTH	2.1 ± 0.5	1.7 ± 0.2	5.1 ± 0.4	0.7 ± 0.2	43 ± 3	45 ± 4
Fetal zone						
No treatment	—	—	2.8 ± 0.7	5.9 ± 2.7	58 ± 3	27 ± 3
+ 10 nmol/L ACTH	—	—	1.9 ± 0.4	5.7 ± 3.3	60 ± 2	28 ± 1
+ 10 nmol/L angiotensin-II	—	—	2.6 ± 0.4	7.8 ± 2.3	57 ± 3	26 ± 2

Each value is the mean ± SD of three incubations. —, Below limits of detection.

TABLE 5. Metabolites of [^{3}H]Corticosterone from minced definitive zone, fetal zone, and adult human adrenal zona glomerulosa tissue with various secretagogues

	% of [^{3}H]corticosterone metabolites recovered as:			
	Aldosterone	18-Hydroxycorticosterone	11-Dehydrocorticosterone	Sulfates
Definitive zone				
No treatment	—	—	87 ± 6	13 ± 6
+ 10 nmol/L ACTH	—	—	95 ± 4	5 ± 4
+ 10 nmol/L angiotensin-II	—	—	86 ± 7	14 ± 7
+ 21 mmol/L potassium	—	—	80 ± 8	20 ± 8
Fetal zone				
No treatment	—	—	38 ± 11	62 ± 11
+ 10 nmol/L ACTH	—	—	54 ± 5	46 ± 5
+ 10 nmol/L angiotensin-II	—	—	50 ± 9	50 ± 9
Adult zona glomerulosa				
No treatment	29 ± 4	41 ± 6	30 ± 2	—
+ 10 nmol/L ACTH	45 ± 2[a]	26 ± 3[a]	30 ± 1	—

Each value is the mean ± SD of three incubations. —, Below limits of detection.
[a] Differs from no treatment, $P < 0.01$.

test whether oxidative inactivation of aldosterone synthetase might be causing the failure to detect aldosterone, incubations were performed under reduced oxygen concentrations in the presence of metyrapone, DMSO, and BHA. The addition of antioxidants either alone or in combination did not significantly alter the pattern of corticosterone metabolism, as shown in Fig. 1. No aldosterone, 18-hydroxycorticosterone, or 18-hydroxydeoxycorticosterone was detected in baseline or stimulated incubations of human fetal tissue.

In contrast to the results obtained with fetal tissue, adult zona glomerulosa metabolized [^{3}H]corticosterone to aldosterone, 18-hydroxycorticosterone, and 11-dehydrocorticosterone (Table 5). ACTH (10 nmol/L) significantly increased the relative production of aldosterone from 29% to 45% of the metabolized corticosterone, while 18-hydroxycorticosterone decreased from 41% to 26%. 11-Dehydrocorticosterone remained unchanged at 30%. 18-Hydroxydeoxycorticosterone was not detected.

Discussion

The factors that regulate aldosterone secretion during various stages of fetal development are not known. Our *in vivo* and *in vitro* studies of rhesus monkey fetuses during the last month of gestation have demonstrated that basal levels of aldosterone are detectable but do not increase in response to surgical stress, ACTH, or angiotensin-II (3). Similar observations have been made in the surgically catheterized ovine fetus (9–11). Since the adult pattern of response of aldosterone secretion to stimulation is present in primate and ovine neonates, it is likely that the regulatory mechanisms of the adult do not become operative until late in gestation or immediately after birth.

Synthesis of small amounts of aldosterone by second trimester human adrenal tissue has been detected after fetal perfusion with high concentrations of corticosterone (12) or incubation of homogenized tissue with excess substrate (13). In this study we were unable to detect

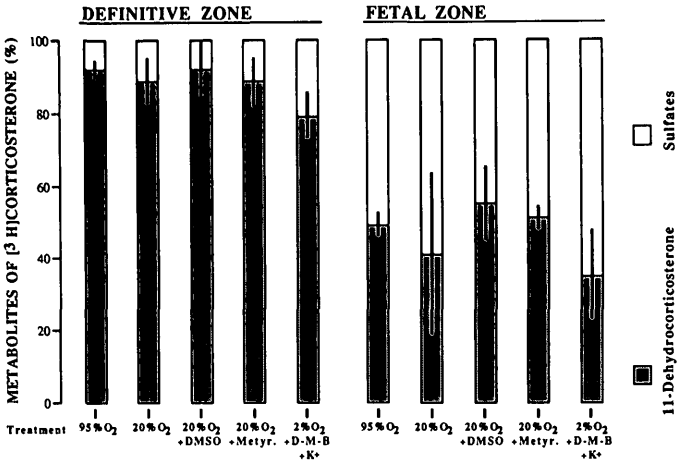

FIG. 1. Metabolites of [^{3}H]corticosterone from minced definitive and fetal zone tissue with various oxygen concentrations and antioxidants. Each *bar* indicates the mean ± SD of four definitive zone or three fetal zone incubations. DMSO, 100 mmol/L DMSO; Metyr., 5 μmol/L metyrapone; D-M-B, 100 mmol/L DMSO, 5 μmol/L metyrapone, plus 100 μmol/L BHA; K^+ 21 mmol/L potassium.

either basal or stimulated aldosterone production after the addition of trace amounts of [^{3}H]pregnenolone or [^{3}H]corticosterone to fresh fetal tissue minces. Under identical conditions, the adult human adrenal was capable of such metabolism. On the other hand, cultured second trimester human fetal adrenal cells both secrete aldosterone and respond to added ACTH (14). With increasing duration of culture, the products of *in vitro* fetal adrenal steroidogenesis differ markedly from those observed *in vivo* and represent a pattern more similar to that of the adult. These studies taken together suggest that although it has the potential to synthesize aldosterone, the midtrimester human fetal adrenal does not normally synthesize and secrete significant amounts of aldosterone.

Both the fetal and definitive zones converted pregnenolone primarily to other free Δ^5-3β-hydroxysteroids and steroid sulfates. The Δ^4-3-ketosteroid products of pregnenolone metabolism, however, differed significantly between the two zones. Cortisol was the predominant Δ^4-3-ketosteroid product of the fetal zone, while progesterone and corticosterone accounted for most of the definitive zone's Δ^4-3-ketosteroid production. The non-17-hydroxylated steroid pathway leading to progesterone and corticosterone is relatively more active in the definitive zone of the fetal adrenal, a situation analagous to that in the zona glomerulosa of the adult. This demonstrates that this steroid biosynthetic pathway is already localized to the adrenal subcapsular region before midgestation.

Although it is generally accepted that corticosterone is the major intermediate precursor of aldosterone in most species, including man, there is evidence in the rat of an alternative biosynthetic pathway involving conversion of deoxycorticosterone to 18-hydroxydeoxycorticosterone and then to 18-hydroxycorticosterone (15). Although they have been found in the monkey fetus near term (3), neither of the 18-hydroxylated derivatives of deoxycorticosterone was detected as a product of human fetal pregnenolone or corticosterone metabolism in this study.

A single mitochondrial enzyme, P450c11, mediates 11β-hydroxylase, 18-hydroxylase, and aldehyde synthetase activities (16). However, findings in both rat and bovine cell cultures indicate that 11β-hydroxylase activity is less susceptible to inactivation by lipid peroxides than are 18-hydroxylase and aldehyde synthetase activities (17). Similarly, our findings of conversion of precursors to corticosterone, but not to 18-hydroxycorticosterone or aldosterone, suggested possible oxidative inactivation of 18-hydroxylase and aldehyde synthetase activities. Metyrapone is a reversible inhibitor of cytochrome P-450 enzymes that also stabilizes them against oxidation. DMSO scavenges hydroxyl radicals, and BHA blocks free radical propagation in lipid (8). Despite the presence of metyrapone, DMSO, and BHA as well as reduced oxygen concentration neither 18-hydroxylase nor aldehyde synthetase activity was observed in the incubations of human fetal adrenal tissue. These results suggest that the failure to detect these activities was not the result of oxidation.

Although 11-dehydrocorticosterone is a minor circulating metabolite of corticosterone in most species, it was the predominant metabolic product of corticosterone in these studies of human fetal adrenal tissue. An increase in 11β-hydroxysteroid dehydrogenase activity has been previously noted in rat adrenal cells damaged by physical trauma, enzymatic dispersion, or extremes of pH (18). The 11β-hydroxysteroid dehydrogenase activity observed in this study may have been caused by tissue preparation. In addition, it could conceivably affect detection of 18-hydroxylase activity by depleting the common substrate, corticosterone. Substrate depletion is an unlikely cause of failure to detect aldosterone in our incubations of fetal tissue, because substantial amounts of corticosterone remained unmetabolized. Furthermore, adult zona glomerulosa produced detectable aldosterone and 18-hydroxycorticosterone as well as 11-dehydrocorticosterone under identical experimental conditions.

The regulation of fetal fluid and electrolyte balance probably differs considerably from that in adults. At midgestation, the non-17-hydroxylated steroid metabolic pathway is localized to the subcapsular region of the human adrenal, but it is likely that the adult pattern of

mineralocorticoid formation is not functional until about the time of transition to extrauterine life.

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