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ESTROGEN-LIKE COMPOUNDS AND PROGESTERONE IN MALE AND FEMALE RATS BEFORE PUBERTY—I.

PATTERN AND ORIGIN

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SUMMARY

In male and female Sprague–Dawley prepubertal rats, plasma levels of progesterone, estrone and estradiol were measured by radioimmunoassay after partial purification on a celite column. Progesterone was determined using a specific antibody. Estrone and estradiol were measured using an anti-estradiol antibody having a 100% cross-reaction with estrone. Progesterone concentration was low during the first 3 weeks of life and increased after 25 days in both sexes. Peaks of immunoreactive estrone ("E_1") and immunoreactive estradiol ("E_2") of 3.8 ± 1.4 and 0.4 ± 0.2 ng/ml of plasma respectively were observed at the age of 13 to 15 days. Measurements by double isotopic dilution, or with highly specific anti-estrone and anti-estradiol antibodies, showed that, in plasma, at least 75% of estrone-like and 90% of estradiol-like substances were neither estrone nor estradiol respectively. However, both substances were able to displace [^{3}H]-estrone from α-fetoprotein and [^{3}H]-estradiol from the cytosol receptor of rat uterus.

These estrogen-like substances totally disappeared from the plasma three days after adrenalectomy plus gonadectomy. The adrenal contents of immunoreactive estrone and estradiol were about 110 times and 90 times higher respectively than the amounts in gonads of 15 day-old rats of both sexes. In adrenals, the highly specific antibodies for estrone and estradiol recognized only 2% of estrone-like and 1% of estradiol-like substances, while in gonads they recognized 60–70% and 70–80% of these substances respectively. These results strongly suggest an adrenal origin for these estrogen-like substances.

INTRODUCTION

The end of the second week of life in the rat seems to be a period of great importance for its sexual maturation. Morphological and histological changes appear in the ovary [1] and the testicle [2], simultaneously with an increase of 3β-hydroxy steroid dehydrogenase in the ovary [1]. Plasma concentrations of LH and FSH show a major peak towards the 12th to 14th day in rats of both sexes [3–5]. The secretion of gonadal steroids before puberty has been suggested by hemi-ovariectomy experiments [6] and the existence of a negative feedback effect of these steroids on the secretions of the gonadotrophins has been claimed [7]. A peak in plasma testosterone has been demonstrated in prepubertal rats [5, 8, 9], the origin of which could be either gonadal [5, 9] or adrenal [8].

Patterns of plasma levels of estrogens [3, 9–12] and progesterone [9–11] have been reported but the age of the rat at which the peak occurs, the concentrations and the origin of estrogens and progesterone are variable according to different authors. These differences could be explained either by the strain of rats employed or by the methods of assays used. The last point is more relevant since it is now well established that plasma estrogens of immature rats as measured with antibodies obtained against estradiol coupled to albumin in the C_{17} position are not true estrone and estradiol [3, 10].

In this article, in addition to the pattern of plasma levels of progesterone and of compounds that have radioimmunological properties of estrogens in rats between 5 and 33 days old, we report adrenal and gonadal contents of those compounds. Moreover we have investigated the origin and the ability of estrogen-like substances to displace labelled estrogens from α-fetoprotein and from uterine cytosol estrogen receptor.

In the accompanying paper [13] we describe the effects of various treatments modifying steroidogenesis on the production of the estrogen-like compounds and progesterone.

MATERIAL AND METHODS

[2,4,6,7-^{3}H]-Estradiol (S.A. = 111 Ci/mmol), [1,2,6,7-^{3}H]-progesterone (S.A. = 105 Ci/mmol), [4-^{14}C]-estrone (S.A. = 58 mCi/mmol) were from NEN Chemicals GmbH, Germany; [2,4,6,7-^{3}H]-estrone (S.A. = 80 Ci/mmol) from the Radiochemical Centre, Amersham, England; [4-^{14}C]-estradiol (S.A. = 40 mCi/mmol) and [^{3}H]-acetic anhydride (S.A. = 100 mCi/mmol) from C.E.A., Saclay, France.

Before use, labelled estrogens and progesterone were purified once a month by paper chromatography in the following systems: hexane–benzene–methanol–water (10:5:12:3, by vol.) and hexane–methanol–water (50:45:5, by vol.) respectively, and purity checked regularly thereafter. Non-radioactive standards were from Mann Research Laboratories, NY, U.S.A., and Steraloids, Wilton, NH, U.S.A.

Anti-progesterone antibodies obtained from rabbits by the injection of progesterone coupled to albumin in the C11 (P-C11) position were kindly given by Roussel-Uclaf.

Three types of anti-estrogen antibodies have been used: anti-estradiol antibodies (SLC-X) obtained with estradiol coupled to albumin in the C17 position (anti-E_2-C17) were given by Dr. B. V. Caldwell (Worcester Foundation for Experimental Biology, U.S.A.). These antibodies exhibit a 100% cross-reaction with estrone. Anti-estradiol (anti-E_2-C_6) and anti-estrone antibodies (anti-E_1-C_6) obtained with antigens E_1 and E_2 coupled to albumin in the C6 position were from Pasteur Institute, Paris. These antibodies are highly specific for E_1 and E_2 respectively [14]*.

Sprague–Dawley rats, 5 to 33 days old, were raised in the laboratory at constant temperature, kept with their mother until the 21st day of life, and subjected to a light–dark rhythm of 12 h out of each 24 h (7 a.m. to 7 p.m.). The animals were sacrificed without anesthesia between 9 a.m. and noon by decapitation. Each determination was performed with the pooled blood of 5 to 15 animals according to their age. Adrenals and ovaries of these animals were immediately excised, weighed, and homogenized two times for 20 s at maximum speed on a Polytron homogenizer in a 0.05 M phosphate buffer, pH 7.4, and kept at −20°C. In each homogenate, proteins were measured by the Lowry method [15]. "Estrone", "estradiol", and progesterone concentrations were determined in the plasma and also in the homogenates. Estrone and estradiol were extracted and purified following the Abraham method [16] as modified by Saez[17]. Progesterone was separated from estrone and estradiol on celite columns (celite–ethylene glycol, 1:0.5, w/v). Progesterone was eluted with 8 ml *iso*octane containing 5% benzene, estrone by 6 ml of *iso*octane with 17% ethyl acetate and estradiol by 6 ml of *iso*octane with 33% ethyl acetate. Dried extracts were redissolved in 1 ml 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% gelatin, 0.9% sodium chloride and 0.1% sodium azide. An aliquot of 0.2 ml was transferred to counting vials for recovery purpose, while three aliquots of 0.1, 0.2 and 0.4 ml were taken for radioimmunoassay.

Progesterone was measured using the anti-P-C11 antibodies described above. The blank of the method was less than 10 pg and the intra and inter-assay coefficients of variation were less than 10% for both.

Estrogens were measured by radioimmunoassay with three different antibodies: anti-E_2-C_{17}, anti-E_1-C_6 and anti-E_2-C_6. With all of them the values for estrone and estradiol in 10 ml plasma from gonadectomized and adrenalectomized rats were below 10 pg/sample. The intra and interassay coefficients of variation were less than 10%. Recoveries during extraction and purification were 80 ± 9% for estrone and 83 ± 8% for estradiol (n = 200). All results were corrected for these losses.

In addition, the estrogen compounds were measured by protein binding assay using either α-fetoprotein purified by the method described by Benassayag *et al.*[18] from plasma of 13–15 day old rats, or cytosol estrogen receptors prepared from adult rat uteri as described by Korenman[19].

Furthermore, on a pool of plasma from 14 day old rats, the estrogen concentration was measured by double isotopic dilution according to the following method:

After addition of [^{14}C]-E_1 (≃4.2 ng) and [^{14}C]-E_2 (≃4.4 ng) steroids are extracted by ether (3 times with 3 vol. each time), subjected to phenolic partition, and paper chromatography in Bush system A hexane–methanol–water (50:45:5, by vol.). Estrone and estradiol are then eluted separately and rechromatographed in Bush system B hexane–benzene–methanol–water (10:5:12:3, by vol.). Estrone and estradiol are then acetylated with [^{3}H]-acetic anhydride at room temperature overnight. The acetylation is stopped by adding 1 ml of water. The acetates are extracted by 9 ml of chloroform and the solvent washed five times by 1 ml of twice distilled water. Estrone acetate and estradiol diacetate are purified four times by t.l.c. on silica gel in a bidimensional system as follows: (1) chloroform (twice); (2) benzene–ethyl acetate (5:2, by vol.), benzene–dichloromethane–methanol (25:25:1, by vol.); (3) benzene–ethanol (9:1, by vol.) (twice); (4) cyclohexane–ethyl acetate (6:4, by vol.) and cyclohexane–ethyl acetate (1:1, by vol.). After the addition of 15 mg of estrone acetate and estradiol diacetate to extracts purified by t.l.c., we proceeded to six recrystallizations in the methanol–water system; the ratio of ^{3}H/^{14}C being constant from the third recrystallization. When a sample of water is analyzed following addition of [^{14}C]-estrogens, the amounts measured after recrystallizations are 4.19 ng for estrone and 4.39 ng for estradiol corresponding to the mass of the tracer added. This blank value is subtracted from the plasma value. The recoveries during extraction and purification were 36.5 ± 5.3% for estrone and 30.4 ± 4.8% for estradiol.

* The following abbreviations are used: "Estrone-like" ("E_1") for estrone measured with anti-estradiol-17β-hemisuccinate bovine serum albumin (SLC-X, gift of Dr. Caldwell). "Estradiol-like" ("E_2") for 17β-estradiol measured with the same antibody. E_1 for estrone measured with antibodies obtained after injection of estrone-6-carboxymethyloxime-bovine serum albumin (Pasteur Institute). E_2 for 17β-estradiol measured with antibodies obtained after injection of 17β-estradiol-6-carboxymethyloxime-bovine serum albumin (Pasteur Institute). Dexamethasone: 9α-fluoro-11β-17α,21-trihydroxy-16-methyl-pregna-1,4-diene-3,20-dione. ACTH-Retard: Long-acting ACTH.

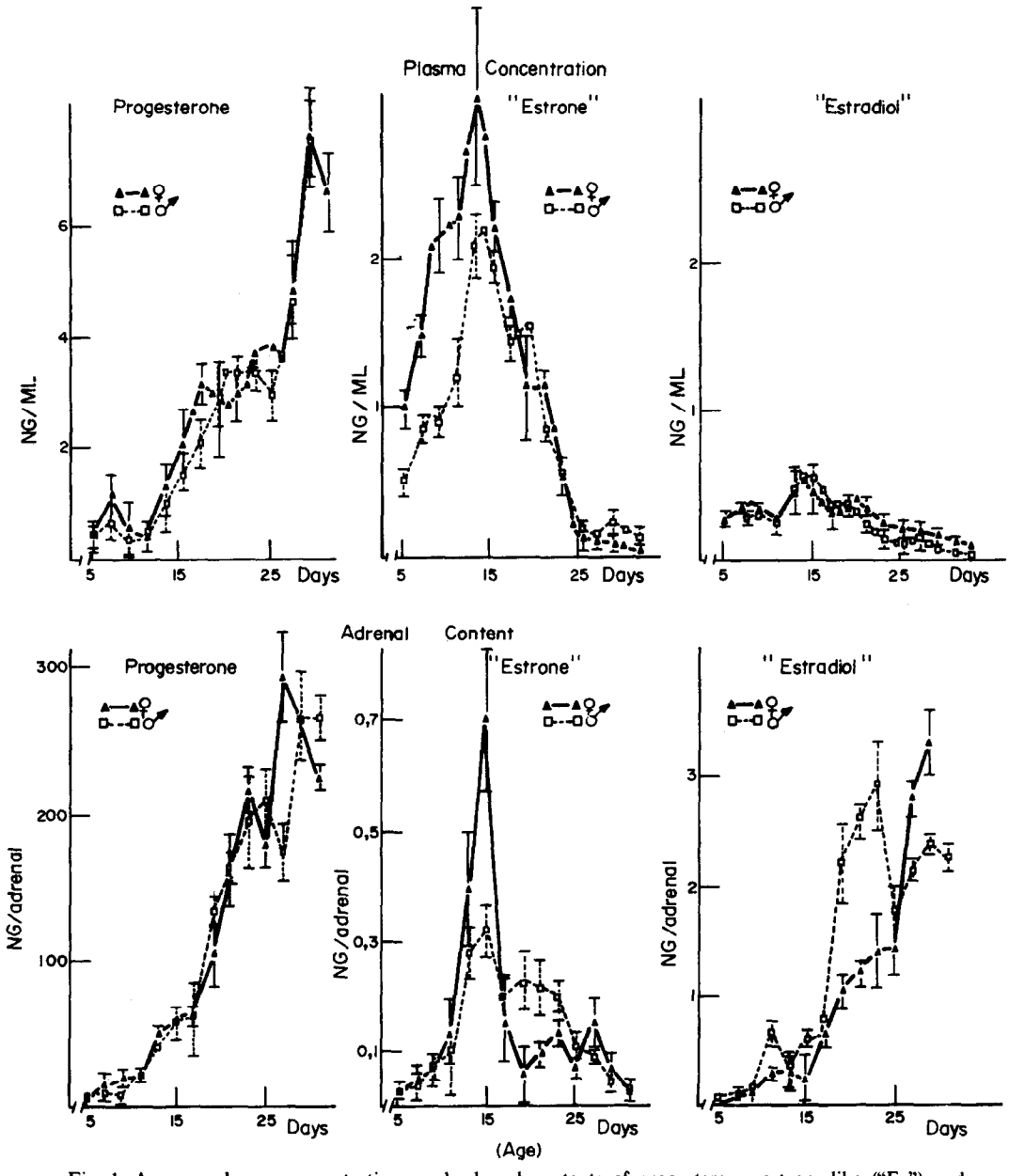

Fig. 1. Average plasma concentrations and adrenal contents of progesterone, estrone-like ("E_1") and estradiol-like ("E_2") in male and female prepubertal rats. The curves represent mean values for two series of experiments. Each measurement corresponds to a plasma pool of 5–15 animals according to age. Each point is the mean ± S.E. of triplicate determinations of two plasma pools.

RESULTS

Plasma, adrenal, and gonadal concentrations of progesterone

In the plasma, there is a rapid increase of progesterone concentrations between 11–17 days, then there is a plateau from the 17 to 25 days, followed by a sharp increase, without sex-linked difference (Fig. 1). In the adrenals, there is a comparable evolution to that of plasma. The progesterone content of the gonads is between 100 and 1000 times lower than the adrenal content at all ages (i.e. in the 15 day old rats the adrenal, ovary and testicle contents were 59 ng, 54 pg and 33 pg for one organ respectively).

Plasma, adrenal, and gonadal concentrations of estrogens (Fig. 1)

The plasma estrogens were measured with anti-E_2-C_{17} antibodies: E_1 and E_2 show a peak in male and female rats towards the 13th to 15th days. These results represent the mean ± S.E. values obtained from two series of experiments; for each series the measurements were made in pooled plasma of 5 to 15 animals according to age. The scattering of plasma levels of E_1 and E_2 is larger at the moment of the peak than when the concentrations are lower. This suggests that the individual estrogen-like substances peak may be shorter. The determination of estrogen

Table 1. Plasma concentrations of estrone and estradiol measured by different methods

Measurement by	Estrone fraction (ng/ml)*	Estradiol fraction (ng/ml)*
Anti-E_2-C_{17}	1.54 ± 0.42	0.487 ± 0.065
Anti-E_1-C_6	0.45 ± 0.21	0†
Anti-E_2-C_6	0†	0.025 ± 0.003
Double isotopic dilution	⩽0.4	⩽0.018
α-Fetoprotein	27.2 ± 3.2	1.75 ± 0.52
Uterine cytosol	0.825 ± 0.197	0.068 ± 0.003

A plasma pool of rats 13–15 days old of both sexes was extracted and purified on the celite column as indicated under Methods. The amounts of estrogens in the estrone and estradiol fractions were measured by the methods indicated. *Mean ± S.E. of triplicate determinations of two plasma pools of 25 rats 13 to 15 days old. †Results similar to the blank of the method (< 10 pg/sample).

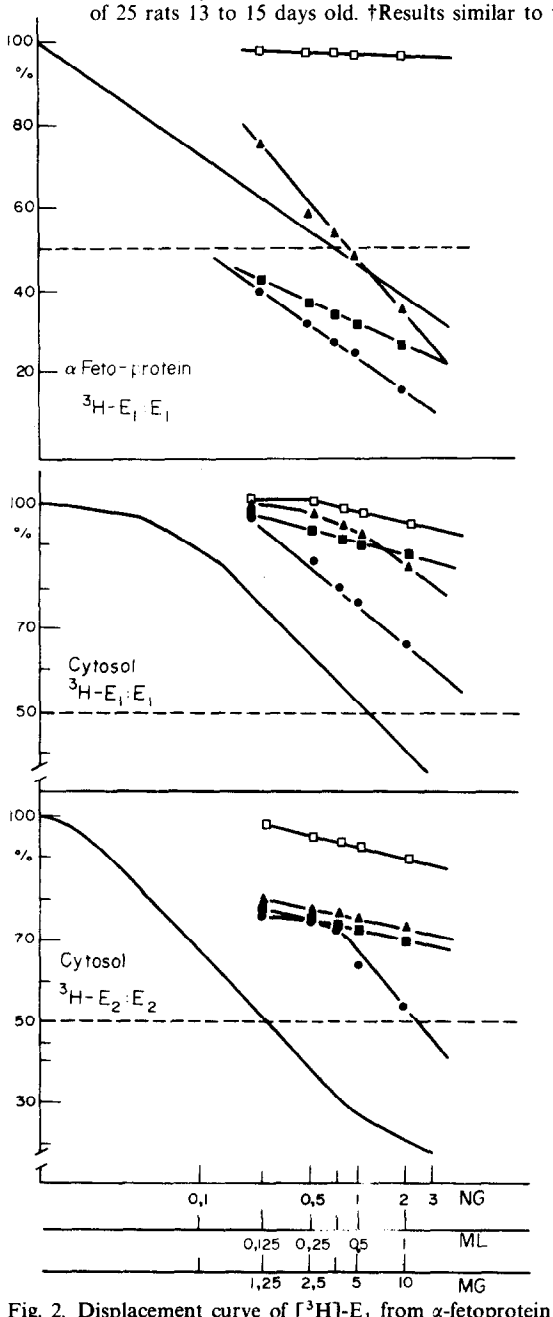

Fig. 2. Displacement curve of [^{3}H]-E_1 from α-fetoprotein (upper panel) and of [^{3}H]-E_1 (middle panel) and [^{3}H]-E_2 (lower panel) from cytosolic proteins from adult rat uteri by increasing of extracts corresponding to 0.125 to 1 ml of plasma ("E_1", ● and "E_2", ▲) and 1.25 to 10 mg of adrenal weights ("E_1", ■ and "E_2", □) and having the same polarity as estrone and estradiol on the celite column.

concentrations in a plasma pool from 14 day old rats as measured by the more specific anti-E_1-C_6 and anti-E_2-C_6 antibodies shows that only a small fraction of the estrogens dosed by the anti-E_2-C_{17} antibodies is recognized by the specific antibodies (Table 1). This would suggest that most of estrogens measured by anti-E_2-C_{17} are neither E_1 nor E_2. This has been confirmed by double isotopic dilution (Table 1). On the other hand, when the compounds were measured using α-fetoprotein as competitive binding protein, the amounts found were several times higher than those measured with antibodies anti-E_2-C_{17} (Table 1). Nevertheless Fig. 2 shows that increasing concentrations of plasma and adrenal extracts having the same polarity as estrone and estradiol on the celite column are able to displace [^{3}H]-E_1 bound to α-fetoprotein, and [^{3}H]-E_1 and [^{3}H]-E_2 bound to cytosol proteins of adult rat uteri. We have used uteri from adult rats in order to avoid the contamination from α-fetoprotein shown in the uterus of immature animals [20].

In the adrenals, there is a parallelism between the plasma concentrations and adrenal contents of "estrone-like"; this parallelism is not evident for "estradiol-like" (Fig. 1). However the more significant finding is that the anti-E_1-C_6 and anti-E_2-C_6 antibodies recognize only about 2% of "estrone-like" adrenal substances and 1% of "estradiol-like" adrenal substances for both sexes. Thus, the adrenals contain essentially estrogen-like substances (Fig. 3).

In the gonads, the concentrations of estrogens are very low, even when measured with anti-E_2-C_{17} antibodies. At day 15, the gonadal contents of "E_1" and "E_2" were ≃ 6 pg and ≃ 2 pg for the ovary and ≃ 5 pg and ≃ 7 pg for the testicle respectively, far less than the adrenal contents (Fig. 1). There does not seem to be any correlation between gonadal and plasma concentrations. The results obtained with anti-E_1-C_6 and anti-E_2-C_6 antibodies (Fig. 3) show that these antibodies recognize respectively 60–70% of E_1-like substances and 70–80% of E_2-like substances in the ovaries. It consequently seems that the gonads, and

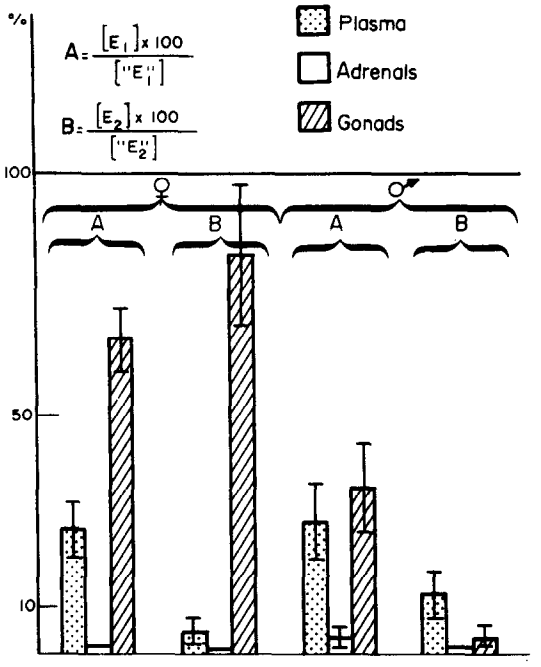

Fig. 3. Percentage of estrone (E_1) and estradiol (E_2) (measured by anti-E_1-C_6 and anti-E_2-C_6 antibodies) compared with estrogen-like substances ("E_1" and "E_2" measured by anti-E_2-C_{17}) in the plasma, adrenals and gonads of 14 day-old male and female rats (mean ± S.E.).

especially the ovaries, produce estrogens essentially recognizable by the antibodies anti-E_2-C_6 and anti-E_1-C_6.

Origin of progesterone and estrogenic compounds

The adrenal and/or gonadal origin of these estrogen-like compounds in the plasma is demonstrated by their complete disappearance from the plasma by the third day after adrenalectomy plus gonadectomy (Table 2). After gonadectomy, the majority of plasma estrogens (≃99%) measurable only by the antibodies anti-E_1-C_6 and anti-E_2-C_6, have practically disappeared. This suggests that these estrogen-like substances are essentially of adrenal origin. There is however a minor but not insignificant gonadal estrogen production, these estrogens being recognized by the more specific antibodies.

The adrenal content of progesterone is higher than the gonadal content. However, if one considers the plasma concentrations after only adrenalectomy or only gonadectomy, the gonads appear to play an important role in progesterone production, especially in the female (Table 2). It must be considered in the interpretation of these results that ovariectomy might provoke a stress and consequently cause an increase of ACTH which could in turn augment progesterone and decrease the concentration of adrenal "estrogen-like" substances [13]. On the other hand, adrenalectomy could raise the plasma levels of LH [7] and therefore increase ovarian progesterone also.

DISCUSSION

The peak of immunoreactive estrogens measured by anti-E_2-C_{17} (SLC-X) that we have described in both male and female 10 to 15 day old rats is not constituted by estrone and estradiol, for the majority: this was evidenced by the measurement with more specific antibodies and double isotopic dilution.

Weisz *et al.*[12] have reported the existence of estrogen-like compounds in the plasma of prepubertal rats using a different anti-E_2-C_{17} antibody (SLC-6 of Caldwell). A difference in the specificity of antibodies could explain the discrepancy in the plasma levels reported by these authors and by us. However this would not explain the difference of age at which the peaks of both compounds occur, i.e. 10 days for "E_1" and 10 and 18 days for "E_2", rather than 13–15 days as we have found. Weisz *et al.*[12] suggested that plasma "E_1" as measured by SLC-6 antibodies is not different from true estrone, since they were able to obtain the same plasma concentrations by various methods. Our results (Table 1 and Fig. 3) clearly show that the estrone fraction from the celite column contains other substances than E_1, that are measured by SLC-10 antibodies.

The fall in plasma estrogens like levels observed after adrenalectomy by Weisz *et al.*[12] is confirmed in this study. This suggests that adrenals play a more significant role than gonads in the production of these substances. This hypothesis is strengthened by the fact that adrenal content of "E_1" and "E_2" is clearly higher than that of the ovary or testicle at 15 days. An adrenal origin of estrogen-like compounds has also been described in adult female rats [21, 22]. The adrenal content of "E_1" is parallel to the pattern of its plasma levels. On the contrary, the adrenal content of "E_2" increases at times when its plasma level decreases. It is difficult to give a fully satisfactory interpretation for this discrepancy but it could be due to an increase of metabolic clearance rate of this compound due to the well known decline of the plasma levels of α-fetoprotein during the first 3 weeks of life [23].

Our work has also shown an ovarian production of estrogens in the prepubertal rats. The specific anti-E_1-C_6 and anti-E_2-C_6 antibodies recognize the ovarian estrogens but they do not recognize those of adrenal origin. This suggests that the ovaries produce true estrone and estradiol while the adrenal produces essentially estrogen-like substances. These results support those of Meijs-Roelofs *et al.*[3] who have demonstrated, with the use of anti-E_2-C_6 antibodies, a plasma peak of estradiol of ovarian origin in 13 to 15 day old rats. However, it must be pointed out that the maximal levels that they observed (≃50–60 pg/ml) are ten times lower than those obtained with anti-E_2-C_{17} antibodies, but similar to those measured by anti-E_2-C_6 and by uterine cytosol as protein binding (Table 1).

The pattern of plasma levels of progesterone in both males and females is similar to that reported

Table 2. Plasma concentrations of progesterone (P), estrone ("E_1"), and estradiol ("E_2") in female and male rats. 14 days old, 96 hours after removal of adrenals and/or gonads

	Plasma ng/ml		
♀ 14 days	P ng/ml	"E_1" ng/ml	"E_2" ng/ml
Controls (a)	1.52 ± 0.75	4.03 ± 1.80	0.41 ± 0.21
Adrenalectomized (b)	0.90 ± 0.11	0.14 ± 0.02	0.057 ± 0.02
Ovariectomized (b)	0.80 ± 0.15	0.84 ± 0.17	0.26 ± 0.013
Adrenalectomized and ovariectomized (c)	0	0	0
♂ 14 days			
Controls (a)	1.37 ± 0.50	3.58 ± 1.60	0.50 ± 0.25
Adrenalectomized (b)	0.35 ± 0.06	0.10 ± 0.01	0.031 ± 0.003
Castrated (b)	0.83 ± 0.06	1.91 ± 0.20	0.37 ± 0.03
Adrenalectomized and castrated (c)	0	0	0

a—Mean ± S.E. of three plasma pools of 25 rats.
b—Mean of triplicate determinations of a plasma pool of 25 rats.
c—Results not different from the blank of the method (< 10 pg/sample).

by Döhler *et al.*[9] in the same strain of rats, but different from that described by Meijs-Roelofs *et al.*[11] in prepubertal Wistar female rats. Data obtained after isolated adrenalectomy or gonadectomy (Table 1), as well as the measurement of the progesterone content in both adrenals and gonads, suggest that the main source of this steroid in 14-day old rats are the adrenals. On the contrary, the results of Meijs-Roelofs *et al.*[11] suggest that in 18-day old females most progesterone comes from the ovaries.

The role of the adrenal in sexual maturation has been suggested by various authors. For instance, Gorski *et al.*[24] have shown that adrenalectomy before the 24th day retards puberty. Meisenheimer *et al.*[25] have reported that adrenal steroids are involved in regulating somatotrophin activity in the anterior pituitary of neonatal rats and Döhler[7] has described an increase of plasma LH in the rat after adrenalectomy. However, in these studies no one determined which compound(s) of adrenal origin was responsible for the effects observed.

The possible physiological role of the estrogen-like substances is unknown. Since they recognize the α-fetoprotein and the uterus intracellular receptors of estrogens and since adrenal steroids are involved in regulating somatotrophic activity in the anterior pituitary of the neonatal rats[25], it is tempting to speculate on the estrogenic or anti-estrogenic role of these compounds. The role and the nature of these compounds still remains to be determined.

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