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6-KETOPROGESTERONE AND ITS BIOLOGICAL ACTION

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In 1956 Hogapian, Pincus, Carls and Romanoff found 6-ketoprogesterone in the perfusate of the human placenta; in the following year, 1957, similar evidence was reported by Matsuba in our laboratory. According to his experiments, 6-ketoprogesterone was found in the extract of the placental villus incubated with progesterone. These results suggest that the human placental tissue has the ability of biosynthesis of the compound. Few reports, however, have been published on the biological actions of this compound. This is the reason why we started the present experiments.

In the preliminary experiments, hypertrophy of the preputial gland was observed after the injection of 6-ketoprogesterone. We were interested in this evidence and observed the effects of the compound on the sexual cycles in rats. Since 6-ketoprogesterone has a chemical formula which has only a slight difference in C_6 position with the ketone group from progesterone and there is a possibility of the production of the former in the ovary; and on the supposition that, as Junkmann stated (1954), it may be one of the ovarian androgens which plays the role of controlling the sexual cycles, we inquired into the action of this steroid on the sexual cycles in rats.

Furthermore, in relation to the fact that 6-ketoprogesterone is produced in the placenta and has myotrophic action, the progestin action, myotrophic action and androgenic action were assayed on the steroid extracts of the progesterone-added blood-perfusate of the pregnant dog uterus and on the steroid extracts obtained from placental villus incubated with several steroids.

Synthesis of 6-Ketoprogesterone: 6-Ketoprogesterone was first synthesized from pregnenolone by Ehrenstein *et al.* (1941) by which, as seen below, they synthesized it, irrespective of the metabolism in a living body. This method, however, was abandoned in our experiments because of the poor recovery, only 2% or so. Several methods were then tried and finally, utilizing Fieser's idea (1953) of synthesis

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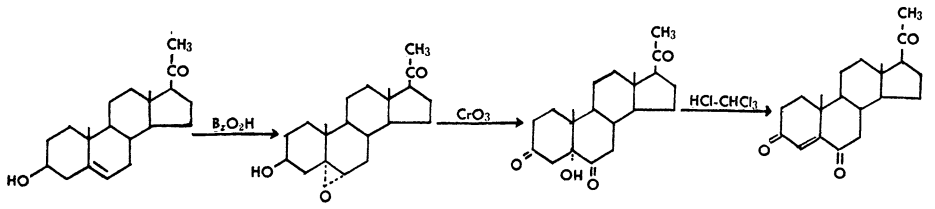
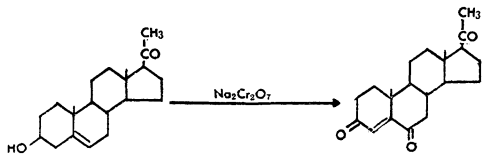
METHOD BY EHRENSTEIN *ET AL.*



Pregnenolone 6-Ketoprogesterone

of $\triangle^4$-cholestene-3,6-diketone from cholesterol by the oxidation procedure with sodium bichromate, we have succeeded in completing a new method of synthesizing it out of pregnenolone in one step with a rich recovery of about 35 %.

NEW METHOD



Pregnenolone 6-Ketoprogesterone

The purification by the rechromatography (alumnina column) and crystalization from the methanol solution yielded the crystal with mp. 194°C. There was no lowering of the melting point when the compound was mixed with the standard substance made by the Ehrenstein's method. The absorption maxima of the substance in methanol and in sulfuric acid were 250 mμ and 348 mμ respectively. These values are the same as those of the standard substance.

MATERIAL AND METHODS

All the steroids, used in this experiment including 6-ketoprogesterone, were dissolved in sesame oil or suspended in 0.04 percent solution of Tween 80. The progestin effect was assayed by Hooker-Forbes' method (1947) on 5-7 week old mice (body weight; 18–22 g) of three strains, DD, ddN and SM: the conclusion was reached from the results in 48 hrs. after the injection of the samples into the uterus on the 18th day after castration done under ether anesthesia.

The androgenic action and myotrophic action were estimated by Eisenberg-Gordan's method (1950): Thirty-day-old rats (average body weight 50 g) of Wistar strain were castrated under ether anesthesia, and from the 21st day after the operation, the samples were given daily for 7 days subcutaneously. On the day after the last injection, seminal vesicle, prostate, preputial gland, adrenal gland and levator ani muscle were weighed.

The effects on the sexual cycles were also observed in the female rats of Wistar strain. The classification of the stages of sexual cycles was done according to Table 1.

Estradiol valerianate (Scherring, A.G. Berlin) was used as the estrogen for prolonged effect suitable to develope the continuous estrus in the castrated rat.

The stage of anesthesia was observed in accordance with the criteria shown in Figure 1 and

Table 1.

KOYAMA & NAKAO	LONG & EVANS	Vaginal smear
Diestrus	V	Leucocytes and epitherial cells
Proestrus	I	Epitherial cells
Estrus I	II	Epitherial cells and cornified cells
Estrus II	III, IV	Cornified cells or cornified cells and epitherial cells

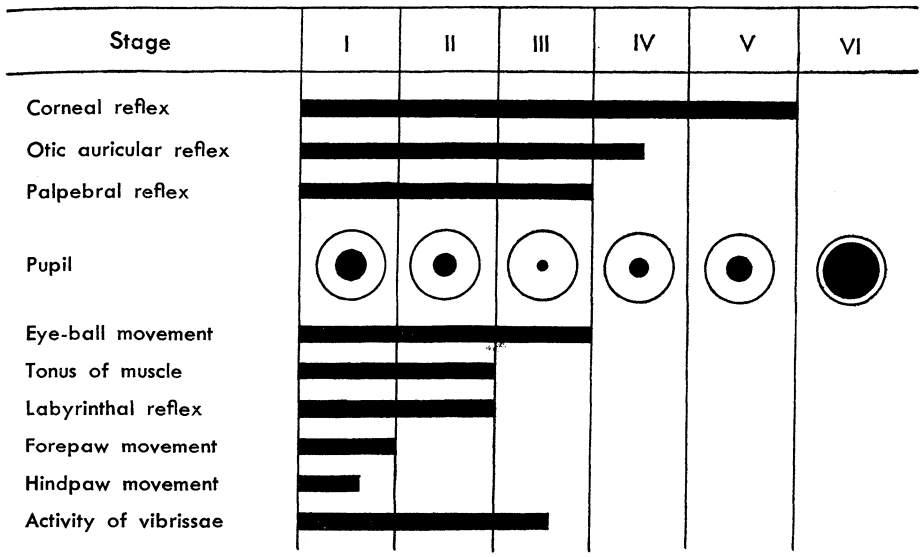


Fig. 1. Stages of anesthesia in rat

the blood pressure was estimated on the non-anesthetized rat by our electric apparatus reported in 1955.

The maximal seizure test was performed by the corneal electrode method, using 13 mA, 0.2 sec. on male DD strain mice which was reported by Swinyard (1949).

For the estimation of the effect on the spontaneous activity, our electronic activity level counter was used in this experiment using DD strain mice weighing 18–20 g. The reading of the activity counter for 2 hrs. after treatment was adopted for the comparison of the activity.

The threshold of the polisynaptic spinal reflex was determined by the contraction of the abdominal muscle using our automatic stepping stimulator with electrode on the tail.

The blood samples for the estimation of myotrophic action, hypertrophic effect on preputial gland etc. were taken from heparinized (10 mg) dog (9.5–12.0 kg) on the last stage of pregnancy under pentobarbital anesthesia. For the perfusion of the pregnant uterus, 680 ml of the blood was collected from the carotic artery of the male dogs and mixed with 15 mg of progesterone in 0.5 ml of propylene glycol. Then, the blood was saturated with oxygen and perfused by our pulsatory perfusing apparatus under the following conditions for one hour: *i.e.*, pressure 95–115 mmHg, pulsation 95 times per minute, 2 ml per each pulse driving. The blood samples were frozen by acetone-dryice soon after systolic perfusion.

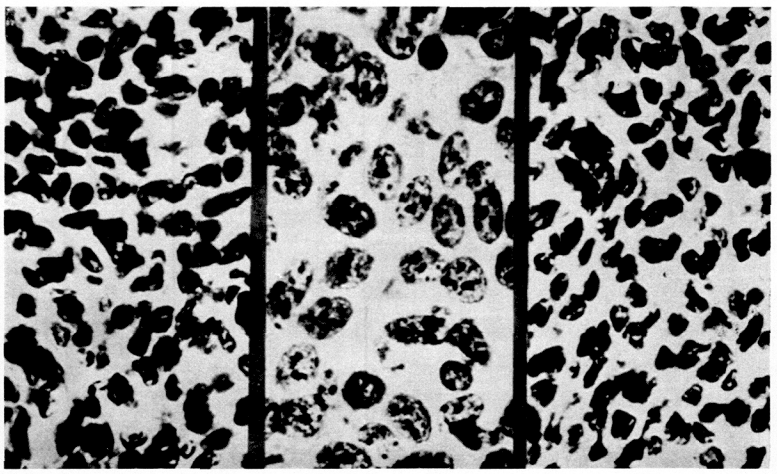
Futhermore, the human placental villus was incubated with several steroids for 3 hrs. at 38°C. Three grams of the tissue (wet weight), 9 mg of the substrate in 0.2 ml of propylene

glycol and 6.0 ml of stock solution were mixed for each incubation. For the estimation of the biological activity of the samples incubated, the crude steroids fraction was extracted, and the extract was dissolved in chloroform. Then, 0.04% solution of Tween 80 was added to cause suspension, and after that chloroform was removed by evaporation.

RESULTS

(1) *Progestin Effect*

At the dose of 0.0005, 0.05, 0.5 and 5.0 μg, progestin action of 6-ketoprogesterone could not be demonstrated (Fig. 2). The results of another 14 compounds are shown in Table 2.



Control Progesterone (+) 6-Ketoprogesterone (−)

Fig. 2. Photomicrographs (×1500) of typical section of endometria of mice ovariectomized 16 days previously. (Hooker-Forbes's)

Control ; No treatment. The stromal nuclei are shrunken and have clumped chromatin.

Progesterone ; After intra-uterine injection of 0.0005 μg progesterone. A characteristic stromal nuclei is oval and has a conspicuous nucleolus and fine, evenly dispersed chromatin particles.

6-Ketoprogesterone ; No reaction.

(2) *Estrogenic and Myotrophic Actions*

The influences of 15 compounds including 6-ketoprogesterone were observed. Each group of four rats was placed on daily hypodermal injection of one-seventh mg of each compound for one week as shown in Table 2. The treatment of 6-ketoprogesterone did not result in hypertrophy of the seminal vesicles and prostata, that is, the androgenic action could not be demonstrated. However, the hyperplasia of the levator ani muscle was remarkable, similar to the influence of progesterone. 6-Ketoprogesterone had also the effect on the preputial gland enough to be compared with the action of 17(α)-methyl-5-androstene-3β-17β-diol.

Table 2. Promotion of growth and progestin effect

	Progestin effect	Preputial gland	Levator ani	Seminal vesicle	Prostate	Adrenal gland
Control		100	100	100	100	100
6-Ketoprogesterone[1]	−	223	178	118	106	101
Progesterone[1]	+	145	197	100	109	94
Pregnenolone[1]	+	182	173	128	142	92
17(α)-Hydroxyprogesterone[1]	±	240	188	139	121	84
Desoxycorticosterone[1]	+	156	173	127	152	100
Testosterone propionate[1]	+	290	278	1499	1642	88
Dehydroepiandrosterone[1]	+	163	98	122	91	100
Andrenosterone[1]	+	93	120	123	137	109
6-Ketoandrostenedione[1]	+	123	105	122	100	102
17(α)-Methyl-Δ^5-androstene-3(β)-17(β)-diol[2]	−	325	123	139	107	77
Estrone[2]	−	141	130	342	110	110
Estradiol[2]	−	148	117	414	99	112
Estriol[2]	+	129	109	322	88	107
17(α)-Ethynyl-5(10)-estren-17(β)-3-one[2]	−	122	145	231	118	96
17(α)-Ethynyl-testosterone[2]	−	125	121	108	109	97

[1] oil [2] suspension Control value is always equal to 100

(3) *Anesthetic Action and Effect on the Blood Pressure*

The second stage of anesthesia lasting for about 4 hrs. induced in a female rat by the intraperitoneal administration of the 6-ketoprogesterone at the dose level of 30 mg of progesterone per 100 g body weight. All of these results were observed in the experiments on female rats. But, in the case of male rats, both induced much less conditions of anesthesia than those induced in the case of female rats. The change of the blood pressure was scarcely notable.

(4) *Effects on the Maximal Electric Seizure*

Six groups of 20 mice were given intraperitoneally three, four and six mg of 6-ketoprogesterone and 0.5, 0.75, and 1.0 mg of progesterone respectively. The reaction to the maximal electric seizure was estimated 45 mins. after the injection by our above-mentioned method. The determination of ED_{50} and the ratio of potency were carried out as described by Litchfield-Wilcoxon (Table 3).

Table 3.

Compounds	ED_{50}
6-Ketoprogesterone	5.10 (3.92–6.63) mg/mouse
Progesterone	0.80 (0.62–1.04) mg/mouse

(Ratio of potency of 6-ketoprogesterone: progesterone was 1:6.38)

(5) *Effects on the Spontaneous Movements of Mice*

The inhibition of the spontaneous movements of mice were compared.

Table 4. Potential ratio of depressive action

	Correlation coefficient r	Slop b	Regression formulae*	Parallelism F	Ratio of potency (Prog.: 6-Ketop.)
Progesterone	0.9991	−68.3	Y=52.5−68.3 X	15.80	
6-Ketoprogesterone	0.9955	−123-3	Y=43.9−123.3 X	(P>0.05)	6.98:1

* X: Log dose

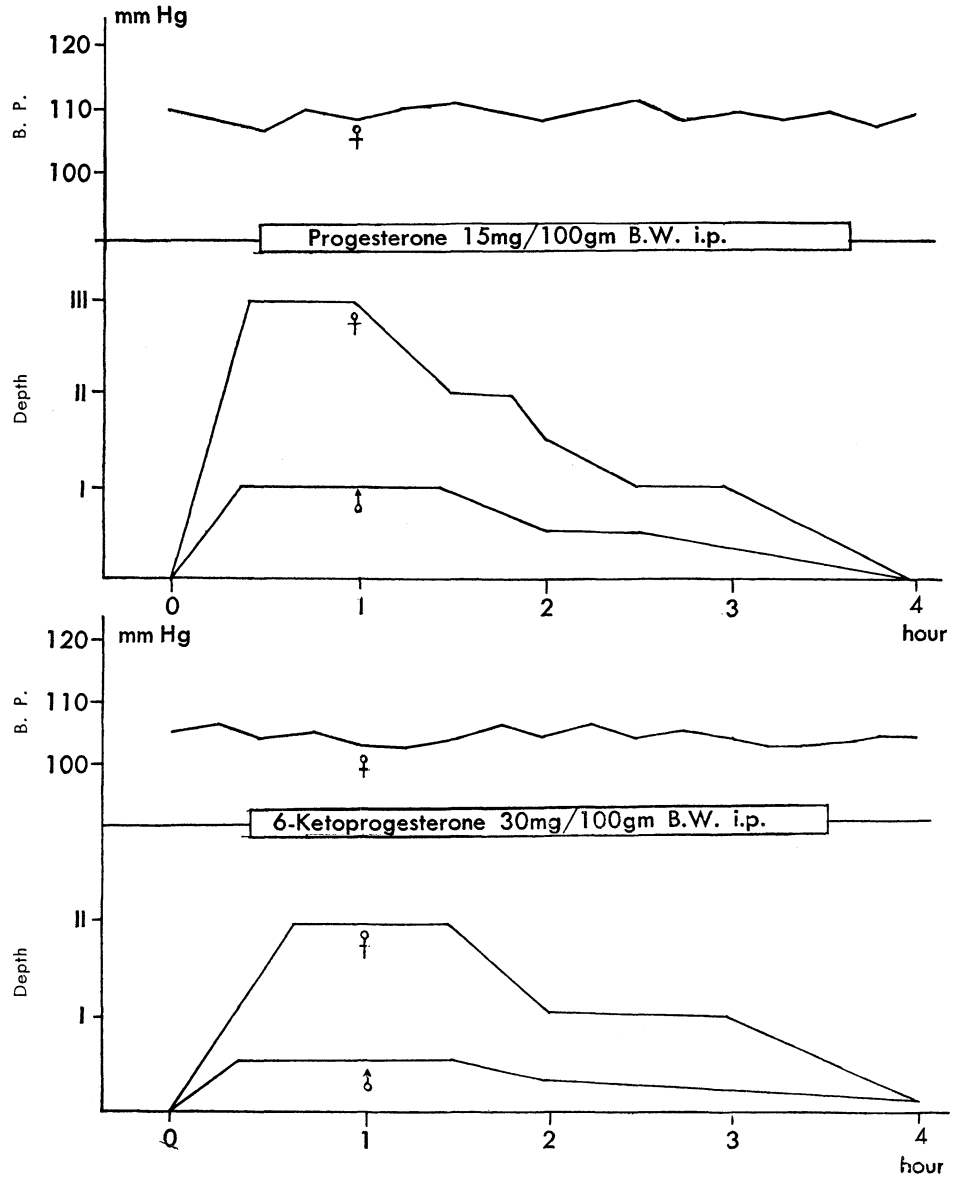


Fig. 3. Anesthetic action and blood pressure

Six groups of 4 mice were injected intraperitoneally one, two and four mg of 6-ketoprogesterone and 0.5, 1.0, 2.0 mg of progesterone per 10 g of body-weight respectively. The log-dose-response curves were straight and parallel to each other. The following potency ratio was deduced; 6-ketoprogesterone : progesterone : 1:6.98 (Table 4).

(6) *Effects upon the Spinal Reflex*

The lowering of the threshold of spinal reflex, that is, acceleration of the reflex induced by the intraperitoneal injection of 6-ketoprogesterone (3 mg per 100 g body weight) was in accordance with anesthesia. Such response as the lowering of the threshold could not be demonstrated in spinal (Th. 5) rat. A similar trend was seen when progesterone was injected.

(7) *Effects on the Sexual Cycles of the Rat*

With the subcutaneous injections of 2 mg of 6-ketoprogesterone per rat, no response was developed on the vaginal smear of the normal, the castrated and the castrated-estradiol-valerianate treated rats. On the contrary, progesterone and testosterone propionate inhibited the appearance of the normal cycle or the continuous estrus of the castrated estradiol-valerianate treated rats even with the injection of the dosage as small as 0.5 mg (Figs. 6, 7).

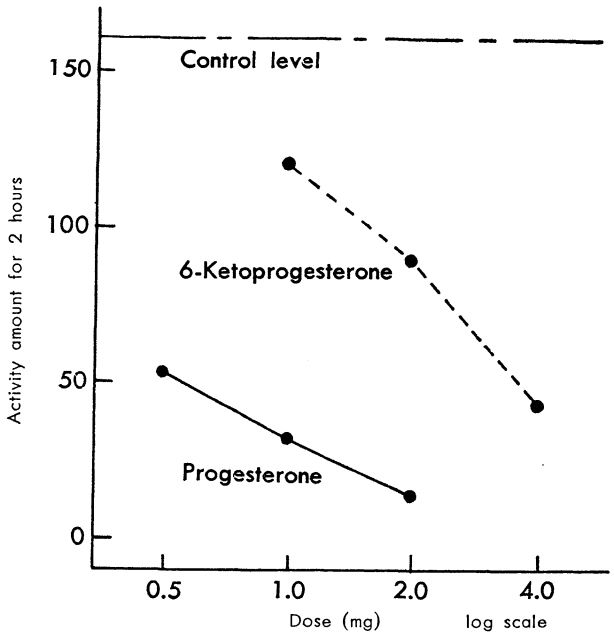


Fig. 4. Activity level of mouse

(8) *Critical Consideration upon Junkmann's Theory of the Sexual Cycle in Rats*

Because of the possibility of the production in the ovary and of the remarkable effects on the preputial gland (Exp. 2), it was expected that 6-ketoprogesterone might be one of such ovarian androgens which control the sexual cycle as reported by Junkmann. However, it was found untrue through Exp. 7, while Junkmann reported that 5–25 mg of progesterone have to be given daily to inhibit the cycle of the rat. Our results in Exp. 7 give evidence that progesterone is able to inhibit the appearance of the normal cycle or of the continuous estrus of the castrated-estradiol treated rats by subcutaneous injection of 0.5 mg (in suspension) per day in the area between scapulae. As shown in Figure 8, the continuous estrus was developed by the treatment with 50 μg of estradiol valerianate in the castrated rat, but not in the normal rat. In the latter cases, the appearance of estrus was rather inhibited, but the subsequent castration caused also the continuous estrus in such animals. As pointed out by Junkmann, these evidences also suggest that the ovary maintains the vaginal cycle even when enough estrogen to develop the continuous estrus remains in the body.

From these two evidences on the supposition that, even without taking ovarian androgen into consideration, it is possible to develop the vaginal cycle by estrogen

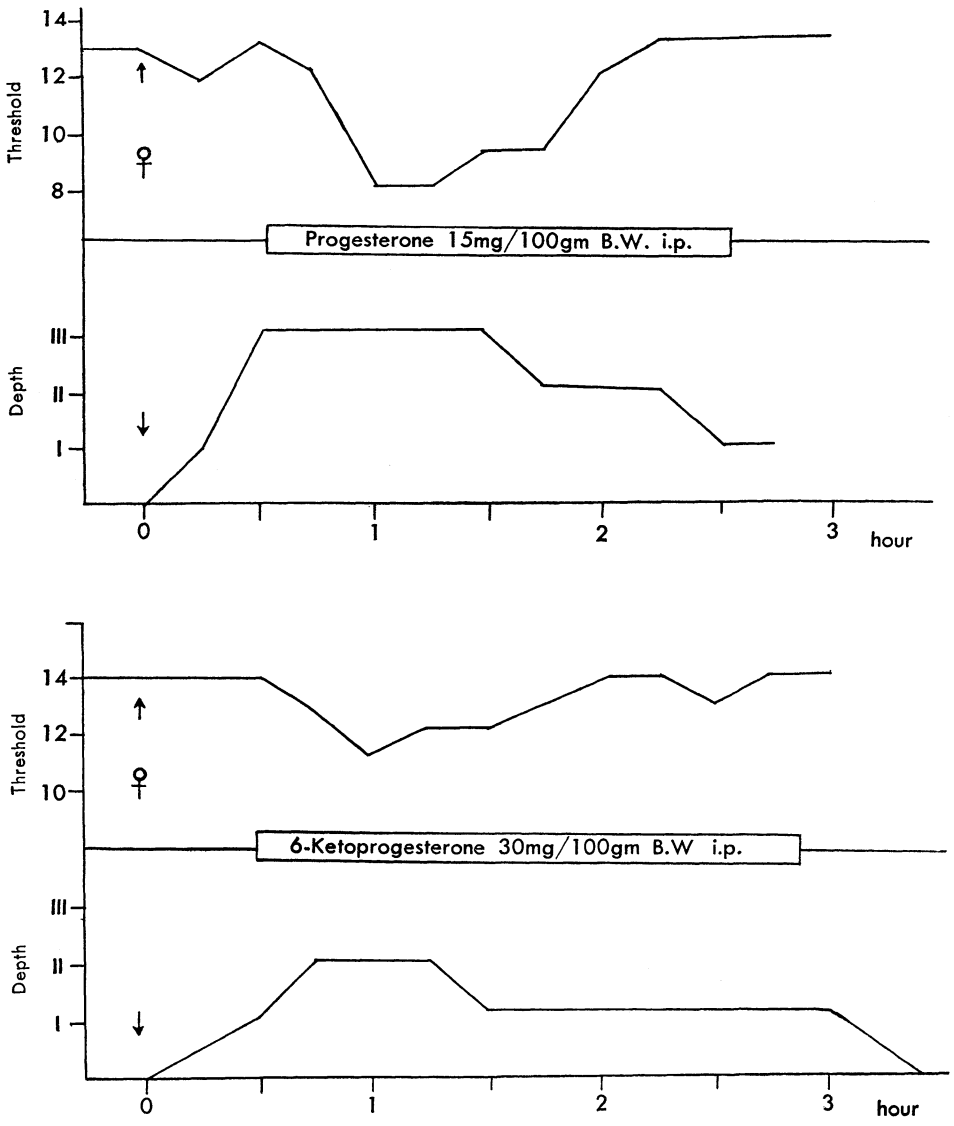


Fig. 5. Spinal reflex and anesthesia

and progesterone, the changes of vaginal smear were observed by the timely administration of 0.5 mg of progesterone to the castrated rats, whose continuous estrus had been developed by the subcutaneous injection of 25 μg of estradiol valerianate (Fig. 8).

In the castrated, estradiol-valerianate treated rats of continuous estrus, when we injected 0.5 mg progesterone, one day after the injection the diestrus

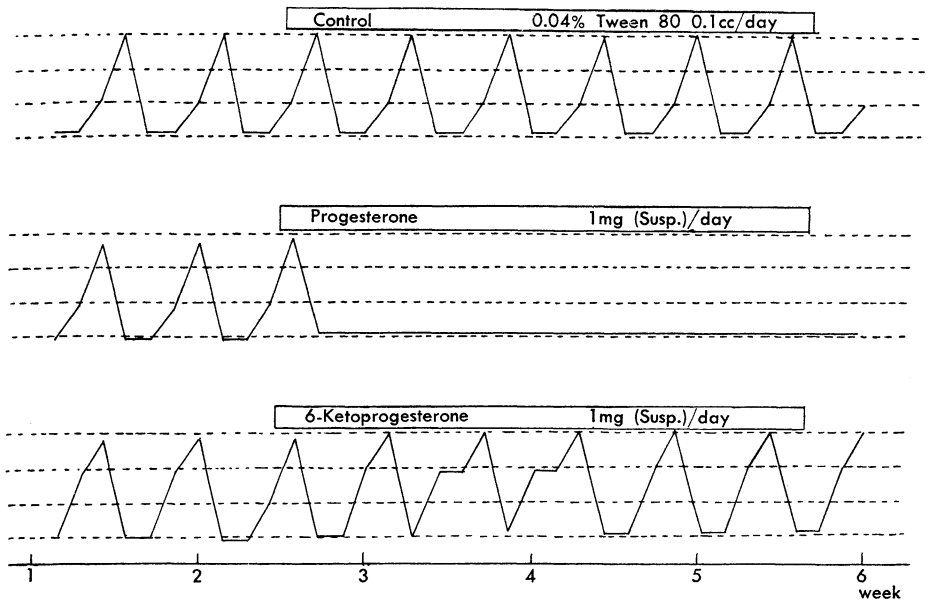


Fig. 6.

was developed. But after that they returned to the estrus through the stages of proestrus and estrus I (cf. Table 1) owing to the long acting estrogen. When progesterone was given in these several stages, the changes of the vaginal smear similar to the typical normal cycle was observed only when progesterone was given each rat at proestrus or estrus I (Fig. 9). So we administered again 0.5 mg of progesterone in the course of these returning stages to estrus. The changes of the vaginal smear similar to the typical normal cycle was observed only when progesterone was given at proestrus and estrus I at these returning stages.

(9) *Progestin, Androgenic and Myotrophic Actions of the Crude Extracts of the Blood of the Pregnant Dogs and Perfusate of their Uteri*

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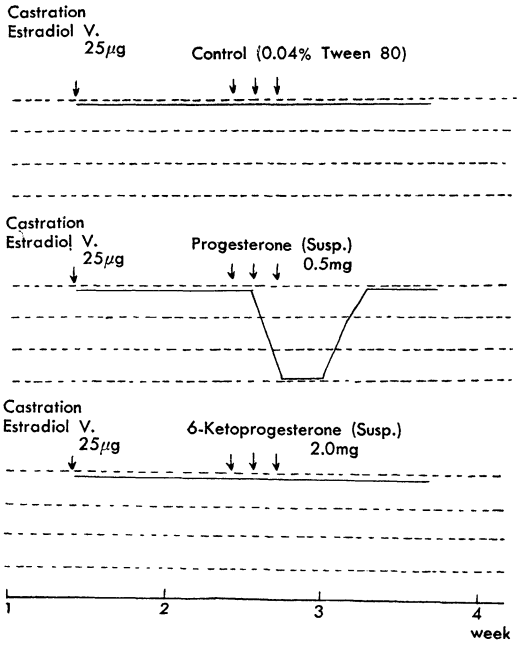
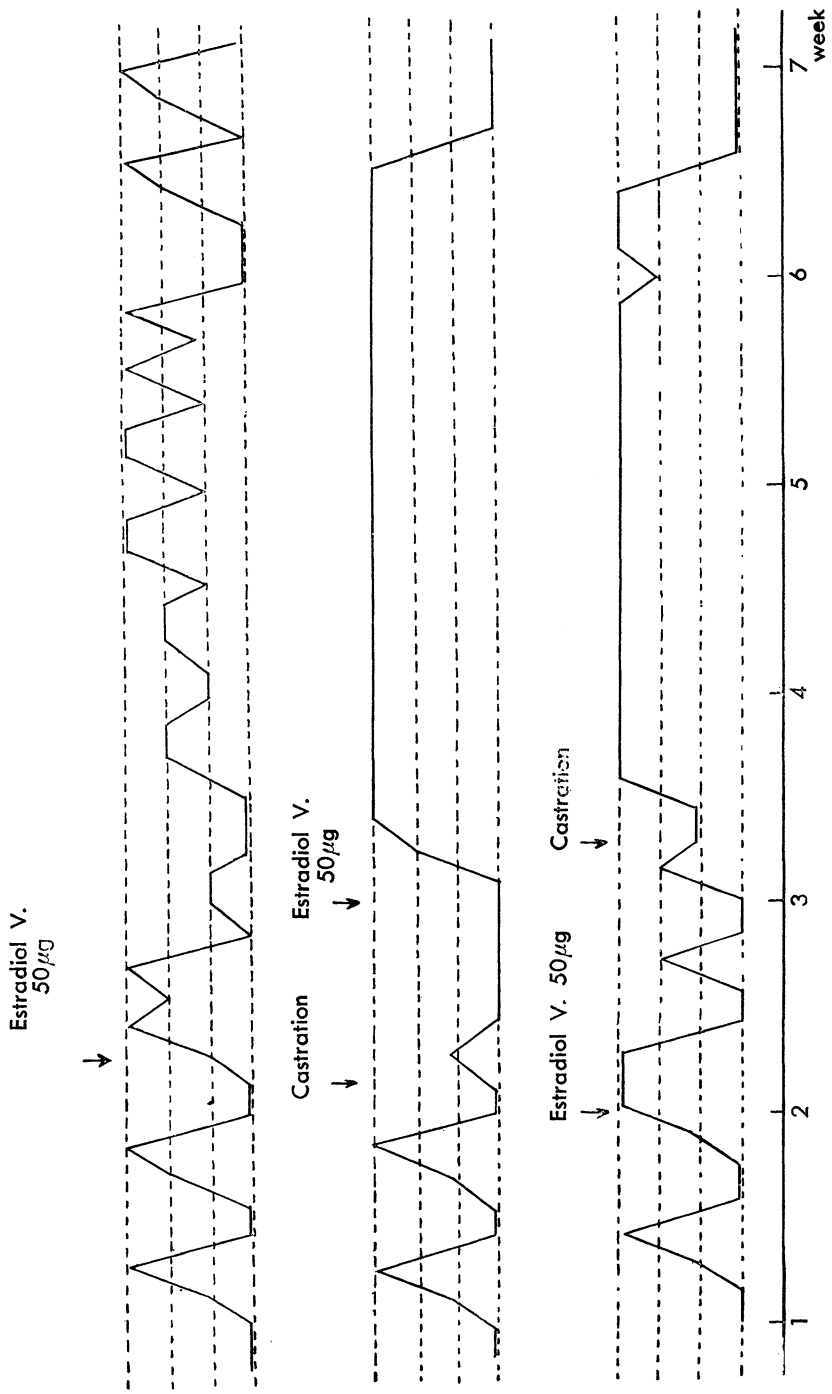


Fig. 7.


Estradiol V.
50μg
Castration
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Castration
1
2
3
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Fig. 8.

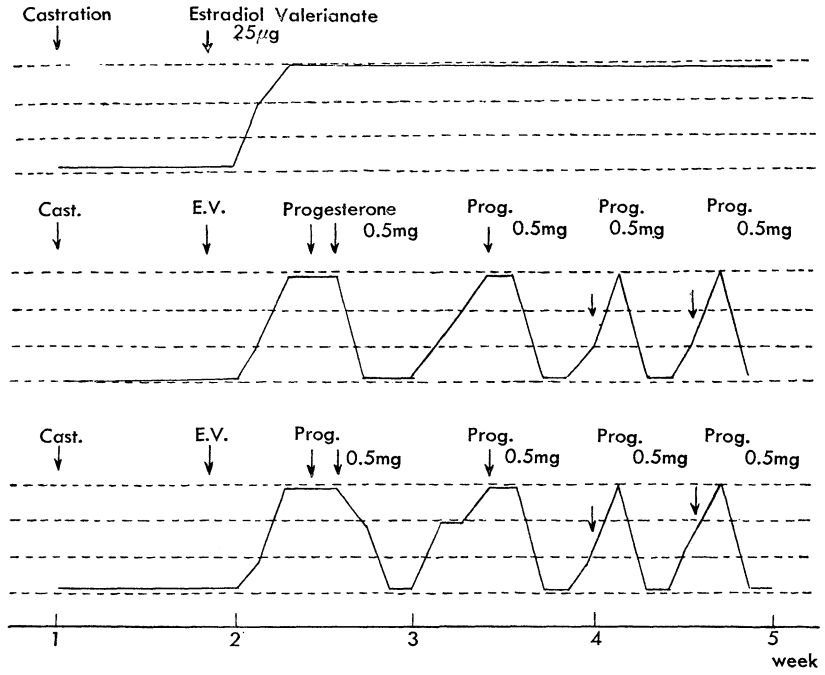

Fig. 9.

nique of testing and scoring is given elsewhere. The definite volume of the steroid extracts equivalent to the volume of the original blood samples used in these experiments was as follow:

Progestin action: Equivalent to 0.00025 ml of the original blood samples.

Androgenic and myotrophic action: Blood samples drawn from carotic artery and uterine vein—5 ml of original blood; umbilical artery and vein—40 ml of original blood; perfusate of the uterus—equivalent to 0.8 mg progesterone added.

As shown in Table 5, it is noteworthy that, while the blood samples drawn from the umbilical vein and the blood perfusate of the pregnant uterus have strong action on the preputial gland and levator ani muscle, their androgenic

Table 5. Promotion of growth and progestin effect (Blood)

		Preputial gland	Levator ani	Seminal vesicle	Prostate	Adrenal	Progestin effect
Pregnant dog blood	Control	100	100	100	100	100	
(Total blood 5 ml/rat Progestin effect 0.00025ml)	Calotic A.	108	105	114	106	88	+
	Uterus V.	111	132	117	100	100	+
Dog blood of navel cord	Control	100	190	100	100	100	
(Total blood 40 ml/rat Progestin effect 0.00025ml)	Umbilical V.	219	163	83	100	125	+
	Umbilical A.	138	121	88	100	95	±
Pregnant dog uterine perfusion blood	Control	100	100	100	100	100	
	Preperfusion	112	123	92	190	119	
	Postperfusion	195	139	108	140	124	

actions are rather weaker than those of the umbilical arterial blood and samples before perfusion.

(10) *Progestin Action, Androgenic Action and Myotrophic Action of the Crude Steroid Extracts of the Human Placental villus which were Incubated with Several Steroids*

Progesterone, pregnenolone, dehydroepiandrosterone and 17(α)-hydroxyprogesterone were used as the substrates. For the progestin action 0.05 μg, and for the observation of androgenic and myotrophic action, 1.0 mg of the crude extracts were injected.

The results are summarized in Table 6. The extracts of the samples, in which progesterone and dehydroepiandrosterone were used as substrate resulted in the strongest hypertrophy of the preputial gland and had the strongest myotrophic action of all. Both of the actions of the products which come from the incubation of the villus with 17(α)-hydroxyprogesterone were much weaker.

Table 6. Promotion of growth and progestin effect (Biological Materials)

Materials	Preputial gland	Levator Ani	Seminal vesicle	Prostate	Adrenal	Progestin effect
Control	100	100	100	100	100	
Incubation control	123	99	94	107	89	+
Progesterone control	132	121	93	140	95	+
Progesterone incubation	181	132	114	132	95	+
Pregnenolone control	140	107	106	148	107	+
Pregnenolone incubation	156	118	114	170	102	+
Dehydroepiandrosterone control	139	105	114	115	88	+
Dehydroepiandrosterone incubation	237	124	123	123	85	+
17(α)-Hydroxyprogesterone control	191	146	107	119	90	+
17(α)-Hydroxyprogesterone incubation	137	134	112	132	90	+

COMMENT

6-Ketoprogesterone, which only slightly differs from progesterone in ketone group at C_6 position, does not reveal the progestin action at a large dosage level of 5 μg by Hooker-Forbes' method. Androgenic action is as difficult demonstrate by 6-ketoprogesterone as by progesterone. The myotrophic (anabolic) action has about two-thirds the potency of testosterone propionate, but is not significantly different from progesterone. The hypertrophic action of 6-ketoprogesterone for the preputial gland was strong enough to be compared with the action of 17(α)-hydroxyprogesterone, testosterone propionate and 17(α)-methyl-Δ^5-androstene-3(β)-7(β)-diol and is much stronger than that of progesterone. The function of the preputial gland has not been known in detail, but it is believed generally that the organ is one of the target organs of androgen. From the fact of the atrophy of the preputial gland after the ovariectomy, Junkmann concluded that some kind of androgen taking part in the sexual cycle might be secreted in the ovary. Since the ovary has the possibility of secretion of 6-ketoprogesterone, it may be reasonable to suppose that this compound might be the ovarian androgen as mentioned by Junkmann or any kind of similar compound. The compound, however, had no influence on the estrus cycle. The inhibitory action of pro-

gesterone on the sexual cycle has been found to be developed with a smaller dose (subcutaneous injection of 0.5 mg of the suspension in the area between scapulae) than the dose reported by Junkmann (5–25 mg daily).

On the other hand, it has been found that the sexual cycle of the normal rat is under the control of the ovary in spite of the presence of estrogen which is able to develops the continuous estrus as reported by Junkmann. Therefore, the following experiment has been successfully done. To the castrated rat, whose continuous estrus had been developed by administrating 25 μg of estradiol valerianate, 0.5 mg of progesterone was appropriately given : and when it was given in the stage of proestrus or of estrus I, the change of vaginal smears corresponding to the typical vaginal cycle of the normal rat was obtained. Although progesterone given (0.5 mg) may be too large a dose, it may possibly be concluded that the normal vaginal cycle will be developed by estrogen and progesterone, if we consider the difference of the action of endogenous progesterone to that of exogenous.

The lowering of the threshold of the spinal reflex, that is, the acceleration of the reflex, was observed by 6-ketoprogesterone similar to that by barbiturate. But the depressant effect on the central nervous system and its region seems not to fall into the same category as the latter, because of the following three evidences : *i.e.*, 1) no excitation seen at the induction of anesthesia, 2) almost no effect on the blood pressure, 3) the effect varies with sex.

Among the blood samples taken from the carotic artery, umbilical vessels, uterine vein and the blood (with progesterone) before and after the perfusion of pregnant uterus, those from the uterine artery and the umbilical vein and the perfusate had the strongest myotrophic action and hypertrophic effect on the preputial gland. But the androgenic action was in reverse order. When the hypertrophic effect on the preputial gland and myotrophic action of the steroid extracts of human placental villus, incubated with progesterone, pregnenolone, dehydroepiandrosterone, and 17(α)-hydroxyprogesterone, was assayed, the samples incubated with progesterone and dehydroepiandrosterone were strongest. Both actions were weaker when 17(α)-hydroxyprogesterone was incubated as a substrate.

From these evidences it is suggested that the precursor of the steroid, which has myotrophic action, hypertrophic effects on preputial gland and weak androgenic action and which is produced in placenta, might be progesterone or dehydroepiandrosterone. This new steroid is possibly 6-ketoprogesterone or its related compound.

SUMMARY

1) 6-Ketoprogesterone (6 kp) can not demonstrate the progestin effects by Hooker-Forbes' method.

2) 6Kp has little androgenic and estrogenic action.

3) 6Kp has myotrophic (anabolic) action almost as strong as progesterone.

4) 6Kp has the hypertrophic action on the preputial gland which is almost equivalent to that of 17(α)-hydroxyprogesterone, 17(α)-methyl-Δ^5-androstene-3(β)-17(β)-diol.

5) 6Kp (large dose), has depressant effect on the central nervous system,

resembling progesterone in variation with sex and lack of excitation at the induction of anesthesia, displays one-sixth to one-seventh of its potency.

6) The spinal reflex is accelerated by 6kp similar to progesterone.

7) 6kp has little influence on blood pressure.

8) The steroid, which is produced in the placenta and which has myotrophic, anabolic action and hypertrophic effect on the preputial gland without androgenic action, is possibly 6kp or its related compound.

9) The vaginal smear cycle in the rat is under the control of estrogen and progesterone.

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