CHAPTER I

TISSUE UPTAKE AND DISAPPEARANCE OF PROGESTATIONAL STEROIDS
INTRODUCTION

After administration the progestational steroids in general, disappear from the blood, get metabolised in the body and are excreted in the urine and faeces. Only a very small amount of the progestational steroid is taken up in the target tissues. It would be of interest to find out whether progestational steroids are incorporated and retained in the target tissues like uterus and vagina as has been found in the case of estradiol (Jensen and Jacobson, 1962). Further, to understand the mechanism of action of a progestational steroid, it is necessary to determine its uptake in the target tissues and also to study as to how it modifies the accumulation of other hormones, particularly estradiol, in the uterus.

This chapter describes a comparative study of the uptake of a 17α-ethynyl 19-nor steroid - norethynodrel and a 17α-acetoxy progesterone
derivative - chlormadinone acetate after a single injection and a constant infusion. The effect of priming with norethynodrel and chlormadinone acetate on the uptake of $^3$H-estradiol in the uterus has been investigated.

**MATERIALS AND METHODS**

**Animals:** Adult female rats, 3-4 months old, from Haffkine Institute, Bombay were used. Immature female mice 22-23 days old were obtained from the animal colony of the AIIMS. All animals were housed in air-conditioned quarters and were maintained on pellet diet supplied by Hindustan Lever Ltd., Bombay.

**Radioactive steroids:** Tritium labelled norethynodrel was prepared by the exposure of inert norethynodrel to tritium gas according to the technique of Wilzbach (1957). Tritiation was carried out at the Bhabha Atomic Research Centre, Trombay, Bombay. The excess of free tritium was removed by equilibration with methanol. The labelled norethynodrel was purified by a slight modification of the technique, of
Arai et al (1962). The radiochemical purity of the purified steroid was checked in the Bush chromatography system, with cyclohexane : benzene (1:1) as the mobile phase and methanol : water (4:1) as the stationary phase. The $^3$H-norethynodrel was spotted along with radioinert norethynodrel and chromatographed in the above system. The radioinert norethynodrel was located by spraying antimony trichloride on the strip. The strip containing radioactive norethynodrel was cut into 1 cm wide pieces, and the paper pieces were put in scintillation vials. The vials were counted in a liquid scintillation counter. A single sharp peak of radioactivity corresponding to the radioinert norethynodrel indicated radiochemical purity of $^3$H-norethynodrel.

In later experiments, specifically labelled norethynodrel as $6\beta\text{-}^3$H-norethynodrel (sp. act. 0.53 mCi/mg) was obtained from C.D. Searle & Co. and used. The results obtained with this preparation were similar to those obtained with randomly labelled preparation of norethynodrel. $1\alpha\text{-}^3$H-chlormadinone acetate (sp. act. 0.222 mCi/mg) was obtained from E. Merck, Darmstadt, Germany. $6\beta\text{-}^3$H-estradiol
(sp. act. 40 Ci/mm) was obtained from the New England Nuclear, Boston, Mass., U.S.A. It was purified by celite column chromatography before use.

Pretreatment of animals with progesterational steroids for uptake of labelled progesterational steroids:
Norethynodrel (Lot No. MA-Q-924) free of estrogenic contamination was a gift from Dr. Victor A. Drill of G.D. Searle & Co. The rats used for $^3$H-norethynodrel uptake study were pretreated with 16.6 μg/100 g body weight of norethynodrel daily for 3-4 weeks while those used for $^3$H-chlormadinone acetate uptake studies were pretreated with chlormadinone acetate 200 μg/100 g body weight for the same period. Both the steroids were administered orally in 0.2 ml olive oil.

Single injection of $^3$H-norethynodrel or $^3$H-chlormadinone acetate: Single intravenous injection of 20.0 μc of the radioactive steroid in 0.5 ml 10% ethanolic saline was given to rats in the femoral vein. Groups of rats, 5-6 in each group, were killed by cervical dislocation at intervals of 1, 2, 5, 10, 20, 40, 60, 80 and 160 minutes after injection. Various tissues like
ovary, uterus, vagina, muscle, intestine, adrenal, liver, thyroid, fat, mammary gland and eye were taken out. The whole brain was removed as rapidly as possible and was freed of adhering blood vessels. The blood vessels surrounding the hypothalamic area on the base of the brain were dissected away and the blood sticking to the hypothalamus was removed by a filter paper. The brain was kept in a clean petri-dish and the various parts of the brain were dissected out. From the ventral side, the entire hypothalamus was taken out as a block limited anteriorly by optic chiasma laterally by hypothalamic fissures and posteriorly by the posterior margin of mammillary bodies. From the dorsal side and the cortical coverings of both sides of cerebral hemispheres were removed exposing the underlying brain areas. The various areas of the brain taken out were the hypothalamus, cingulum, corpus callosum, hippocampus, thalamus, midbrain, pons varolli, cerebellum, medulla oblongata and pineal body. The pituitary gland was removed from the sella turcica. Each tissue, when taken out, was gently blotted, weighed on a torsion balance and dissolved in hyamine hydroxide.
Complete solubilization in hyamine hydroxide was achieved by keeping the vials at 55°C overnight.

**Constant infusion with $^3$H-norethynodrel or $^3$H-Chlormadinone acetate:** The radioactive steroid in 4.1 ml volume was infused at a constant rate of 0.0138 ml per minute for 5 hours into the femoral vein of the rat. The infusion was carried out with the help of a microinfusion pump model 600-900 (Harvard Apparatus Co.). After the completion of infusion the animals were killed and the tissues were taken out and dissolved in hyamine hydroxide, as described above. $^3$H-norethynodrel (32.8 μc) was infused in saline containing 10% ethanol and 10% propylene glycol. $^3$H-chlormadinone acetate (44.0 μc) was infused in 10% ethanolic saline.

**Effect of pretreatment of animals with norethynodrel and estradiol on $^3$H-estradiol uptake:** To study the priming effect of norethynodrel on the uptake of 6-7- $^3$H-estradiol, immature female mice were pretreated with different doses of norethynodrel for different periods of time. Norethynodrel was administered subcutaneously in 0.1 ml volume. The injection solution was prepared in 5% ethanolic saline. When large doses
of norethynodrel were administered the proportion of ethanol was increased to achieve solubilization of norethynodrel.

Estradiol pretreatment was given to immature female mice at a dose of 0.01 μg estradiol/0.1 ml 5% ethanolic saline subcutaneously.

$^3$H-estradiol uptake was studied in immature mice after pretreatment with norethynodrel and estradiol. 6:7-$^3$H-estradiol (2 μc) was administered subcutaneously to each animal. The uptake of $^3$H-estradiol was always studied by injecting it one hour before killing. Six to ten immature female mice, 21-23 days old, were used per group. At sacrifice, the uteri were quickly removed, freed of extraneous tissue and uniformly blotted to remove excess fluid. Uteri were weighed on a torsion balance and directly transferred to counting vials. Hyamine hydroxide (0.3 ml) was added to the samples to dissolve the tissues. Complete solubilization in hyamine hydroxide was achieved by keeping the samples at 55°C overnight.

Effect of pretreatment of animals with Chlormadinone acetate (CAP) on $^3$H-estradiol uptake: Adult ovariectomized
rats were pretreated with different doses of CAP. The steroid was dissolved in 10% ethanolic saline and injected intraperitoneally. The animals were sacrificed 4 hours after the injection. One hour before the sacrifice these animals were intravenously injected with $^3$H-estradiol (12.6 μC/0.5 ml 10% ethanolic saline per animal). At sacrifice the uteri were removed and minced and the radioactivity was extracted with acetone and methanol. Each uterus tissue was extracted thrice with acetone and twice with methanol each time using 2.0 ml of the solvent. The extracts from each sample were pooled in a scintillation vial, dried under nitrogen and counted (Pearlman, DeHertogh, Laumas and Pearlman 1969).

To study the long term effect of pretreatment with CAP on $^3$H-estradiol uptake, two groups of rabbits were pretreated with 0.2 mg and 0.5 mg CAP daily. After 6 months of treatment with CAP, the animals were sacrificed and uptake of $^3$H-estradiol was studied in the uterine slices by incubation of the tissue with 0.44 μC (.003 μg) of $^3$H-estradiol for 2 hours in Krebs-Ringer phosphate buffer. At the end of the incubation, the tissue was removed, washed with ice cold buffer, blotted gently and the radioactivity was extracted as described above.

**Determination of radioactivity in the tissues:** Simple scintillation liquid was used as a medium for the counting of radioactivity. It was prepared by dissolving 4.0 gm
of PPO (2,5-Diphenyloxazole) and 0.1 gm of PoPoP (1-4-bis-(2-4-methyl-5-phenyloxazolyl)-benzene) in 1 litre of redistilled toluene. In samples where the water content was a little more diol scintillation liquid was used which contained 3.25 gm of PPO, 0.065 gm of PoPoP and 52 gm of napthalene in 250 ml dioxan, 250 ml toluene and 150 ml methanol.

To each dried extract of the tissue or to the tissue solubilized in hymine hydroxide, 10 ml of simple scintillation liquid was added and the samples were counted for radioactivity in the Liquid Scintillation Spectrometer (Packard, Model 3314). The quenching corrections were carried out with the help of an internal standard.

**RESULTS**

**Uptake of ^3H^-norethynodrel in the uterus, ovary, pituitary and hypothalamus**: The uptake and disappearance of ^3H^-norethynodrel in the uterus, ovary, pituitary and hypothalamus after a single injection of ^3H^-norethynodrel is shown in Fig. 1. It may be seen that within two minutes of the injection, maximum uptake of radioactivity due to norethynodrel and its metabolites is attained in the uterus after which it disappears rapidly up to a period of 20 minutes. Subsequent to that, the radioactivity disappears slowly and at 80 minutes radioactivity became low. The radioactivity which remained at 160 minutes was quite low. The uptake of radioactivity in the ovary showed a pattern similar to that seen in the
Uptake and disappearance of radioactivity in the uterus, ovary, hypothalamus and pituitary after a single injection of 20.0 μc of 3H-norethynodrel. The results are expressed as DPM/mg wet tissue.
case of the uterus except that the maximal uptake in the ovary was higher than that in the uterus and the disappearance after 20 minutes was quite slow. Pituitary also showed an uptake pattern similar to that of ovary in its rapid uptake and disappearance of radioactivity. However, in the case of pituitary, the maximum uptake was attained after 5 minutes of the injection and there was a rapid disappearance up to 40 minutes. The rate of disappearance of radioactivity between 40-160 minutes was quite slow. In the case of hypothalamus, maximum uptake of radioactivity was seen within one minute of the injection and it disappeared rapidly up to a period of 20 minutes. After 20 minutes the rate of disappearance of radioactivity was slow.

Uptake of $^3$H-norethynodrel in the liver, kidney and adrenal- The uptake of radioactivity due to norethy- nodrel and its metabolites in the case of liver, kidney and adrenal is shown in Fig. 2. The uptake of radioactivity in the liver was highest of all the tissues studied and showed a similar pattern of uptake as found in other tissues. The highest uptake of radioactivity was attained within 5 minutes of
Uptake and disappearance of radioactivity in the adrenal, liver and kidney after a single injection of 20.0 μc of $^3$H-norethynodrel. The results are expressed as DPM/mg wet tissue.
Graph showing the comparison of DPM/mg tissue over time for Adrenal, Liver, and Kidney tissues.

- **Adrenal** (O-O)
- **Liver** (△-△)
- **Kidney** (●-●)

**Axes:**
- Y-axis: DPM/mg tissue
- X-axis: Time in minutes

**Legend:**
- Adrenal
- Liver
- Kidney
injection and then there was a rapid disappearance of radioactivity up to a period of 40 minutes. After 40 minutes, the rate of disappearance of radioactivity was very slow. The uptake in the kidney was also quite high. Maximum levels of radioactivity could be found within one minute of the intravenous injection of $^3$H-norethynodrel. However, after 5 minutes the rate of disappearance was very slow and measurable levels of radioactivity could still be found at 160 minutes. It may be noted that in the adrenal the uptake of radioactivity was much higher than that in the other endocrine organs like the ovary and the pituitary. However, the maximum uptake of radioactivity in the adrenal was obtained within two minutes of the injection and after that it disappeared rapidly up to a period of 10 minutes. Subsequent to that the rate of disappearance of radioactivity was rather slow.

**Distribution and uptake after constant infusion of $^3$H-norethynodrel:** The data on uptake and distribution of radioactivity due to norethynodrel and its metabolites in different tissues after constant infusion of $^3$H-norethynodrel into female rats are given in
Fig. 3

Uptake of radioactivity in the genital, endocrine, metabolic, excretory and other tissues after a constant infusion of 32.8 μc of ³H-norethynodrel. The results are expressed as DPM/mg wet tissue.
Fig. 3 and 4. Fig. 3 gives the uptake of radioactivity in the genital, endocrine, metabolic, excretory and other tissues. Fig. 4 shows the uptake in the pituitary and various parts of the brain.

The uptake of radioactivity due to norethynodrel and its metabolites in the uterus and the ovary after a constant infusion of $^3$H-norethynodrel was quite small and of the order of $5.8 \times 10^{-8}$ M and $4.0 \times 10^{-7}$ M respectively. However, as also seen in results with single injection, the uptake in the ovary was higher than that in the uterus.

The adrenal gland showed a very high uptake of the steroids which was next only to liver. Amongst the endocrine tissues studied, the adrenal showed a comparatively very high uptake. However, liver had the highest uptake of $^3$H-norethy- nodrel and its metabolites. Like liver, kidney and intestine had also a high uptake which was significantly higher ($P < 0.001$) than that in the muscle. An almost negligible uptake was seen in the eye.
Fig. 4

Uptake of radioactivity in the pituitary and the various areas of the brain after a constant infusion of 32.8 μc of 3H-norethynodrel. The results are expressed as DPM/mg wet tissue.
The pituitary showed a somewhat higher uptake than the various areas of the brain (Fig. 4). The hypothalamus and other areas of the brain showed a similar uptake which was not significantly different from that found in the muscle. Although the anterior and posterior parts of the hypothalamus had a slightly higher uptake compared with the middle part yet it was not significant.

Uptake of CAP in the uterus, vagina, ovary and the mammary gland: The data on the uptake and disappearance of \( \text{I}\alpha-^{3}\text{H-CAP} \) and its metabolites in the uterus, ovary, vagina and mammary gland are given in Fig. 5. It may be seen that after an i.v. injection of \( \text{I}\alpha-^{3}\text{H-CAP} \) there is a rapid uptake. The maximal uptake in the uterus, ovary and vagina of the steroid is attained within a period of 5 minutes. The level of uptake in the uterus and the vagina are almost the same, but the ovary showed an uptake which was much higher than that of the uterus and vagina. In the uterus, after the maximal uptake at 5 minutes, the levels at 10, 20, 60 and 120 minutes were almost in the same range. In the case of the vagina, there was a decline in the level of radioactivity
Fig. 5.

Uptake of radioactivity due to chlormadinone acetate and its metabolites in the uterus, ovary, vagina and mammary gland of rat after a single injection of 20.0 μc of $1\alpha$-$^3\text{H}$-chlormadinone acetate. The results are expressed as DMP/mg wet tissue weight.
between 5 and 20 minutes and subsequent to that the levels were almost in the same range up to 120 minutes. However, in the case of the ovary, there was a rapid decline in the levels of the steroids between 5 and 60 minutes, followed by a slower disappearance at 120 minutes. The levels of CAP and its metabolites in the ovary at all time intervals were higher than those in the uterus and vagina. The uptake in the mammary gland showed a continued increase at the different time intervals studied. At 120 minutes when the experiment was terminated, it had the maximal uptake. The molar concentration of CAP in the mammary gland at 120 minutes was \(3.0 \times 10^{-6}\) M.

**Uptake of CAP in the fat, liver and adrenal:** These three tissues showed a higher level of uptake (Fig. 6) compared with the reproductive tissues. There was a continued uptake of the steroid and its metabolites in the fat for 60 minutes when the maximal uptake was attained. Subsequent to that there was a slight decline in the level of the steroid at 120 minutes. In the case of the adrenal, the highest
Uptake of radioactivity due to chlormadinone acetate and its metabolites in the fat, liver and adrenal of rat after a single injection of 20.0 μg of 1α-3H-chlormadinone acetate. The results are expressed as DPM/mg wet tissue weight.

**Fig. 6**
uptake was noted at 2 minutes. There was a rapid decline at 5 minutes and also between 10 and 60 minutes. Subsequent to that the levels of the steroids were almost the same between 60 and 120 minutes. The maximal uptake of CAP was attained in the liver at 5 minutes followed by a rapid decline at 20 minutes. The levels of radioactivity between 20 and 120 minutes were almost the same.

Uptake of CAP in the pituitary, cerebral cortex and hypothalamus: These three neural tissues followed an almost similar pattern of uptake and disappearance of $^3$H-CAP and its metabolites (Fig. 7). There was a maximal uptake at 2 minutes, with a rapid decline at 5 minutes followed by a slow disappearance for 120 minutes. In the case of the cerebral cortex and hypothalamus, a slight rise in the levels of the steroids was noted at 10 minutes compared with the levels at 5 minutes. The uptake of $^3$H-CAP and its metabolites in the pituitary was slightly higher than that in the hypothalamus and the cerebral cortex at all the time intervals studied.

Distribution and uptake of CAP after constant infusion: The results presented in Fig. 8 show that after a
Uptake of radioactivity due to chlormadinone acetate and its metabolites in the pituitary, cerebral cortex and hypothalamus after a single injection of 20.0 μc of 1α-3H-chlormadinone acetate. The results are expressed as DMP/mg wet tissue weight.
Fig. 3

Uptake of radioactivity due to chlormadinone acetate and its metabolites in the various tissues of rat after a constant infusion of 44.0 µc of 1α-3H-chlormadinone acetate over a period of 5 hours. The results are expressed as DMP/mg wet tissue weight.
constant infusion of CAP, the ovary showed a much higher uptake compared with the uterus, which was statistically significant (P<0.01). The molar concentration of CAP and its metabolites was $2.4 \times 10^{-7} \text{M}$ in the uterus and $6.6 \times 10^{-7} \text{M}$ in the ovary. Liver showed the highest uptake of all the tissues studied, followed by fat and adrenal. The concentration of CAP and its metabolites in the fat was $1 \times 10^{-6} \text{M}$. These three tissues were noted to have a higher uptake in the single injection studies also.

The pituitary showed a higher uptake compared with the hypothalamus and the cerebral cortex. The difference, however, was not statistically significant.

**Effect of 0.01 µg estradiol or norethynodrel pre-treatment on the uptake of $^{3}$H-estradiol in the mouse uterus:** It may be seen (Fig. 9) that 0.01 µg of estradiol pretreatment to immature female mice produced a statistically significant increase in the uptake of $^{3}$H-estradiol, when pretreatment was given for 2, 4 or 6 hours. When pretreatment with the same dose of estradiol was given for eight hours, it did not produce
Fig. 9

Uptake of $^3$H-estradiol in the mouse uterus, after pre-treatment with 0.01 µg estradiol-17β or 0.01 µg norethynodrel for 2, 4, 6 and 8 hours. The $^3$H-estradiol uptake was studied by injecting 2 µc of $^3$H-estradiol 1 hour before sacrificing the animal. The radioactivity was estimated as DPM/mg wet tissue weight and expressed as percent of the control.
H-ESTRDIOL UPTAKE OF CONTROL.

TIME (HOURS)

3H-ESTRDIOL UPTAKE % OF CONTROL

-0.01uG NORETHYRADOXER PRETREATMENT
-0.01uG ESTRADIOL PRETREATMENT

CONTROL LEVEL
any effect on the uptake of $^3$H-estradiol in the uterus. Pretreatment with 0.01 μg norethynodrel for 2, 6 or 8 hours did not produce any significant increase or decrease in the uptake of $^3$H-estradiol. However, a pretreatment with norethynodrel for 4 hours produced a significant increase in the uptake of $^3$H-estradiol. The pattern of uptake of $^3$H-estradiol after pretreatment with 0.01 μg of estradiol and norethynodrel showed similarities in that the maximal stimulation is attained when pretreatment is given for 4 hours.

Effect of pretreatment with different doses of norethynodrel on the uptake of $^3$H-estradiol in the mouse uterus: The effect of pretreatment with 0.1, 1.0 and 5.0 μg of norethynodrel for 4, 8 and 18 hours on the uptake of $^3$H-estradiol is given in Fig. 10. Pretreatment for 4 hours with any of the above doses of norethynodrel resulted in a statistically significant increase in the uptake of $^3$H-estradiol in the uterus. However, by 8 and 18 hours a decline in the uptake of $^3$H-estradiol took place. The $^3$H-estradiol uptake at 8 and 18 hours after pretreatment with 0.1 and 5.0 μg of norethynodrel is almost in the range of the control.
Uptake of $^3$H-estradiol in the mouse uterus after pre-treatment with 0.1, 1.0 and 5.0 
μg of norethynodrel for 4, 8 and 18 hours.
The estradiol uptake was studied by injecting
2.0 μc of $^3$H-estradiol 1 hour before sacri-
ficing the animal. The radioactivity was
estimated as DPM/mg wet weight and expressed
as percent of the control.
However, pretreatment with 1.0 µg of norethynodrel for 8 and 18 hours still showed a stimulation of uptake of $^3$H-estradiol which was significantly higher than the control level.

The pattern of stimulation of $^3$H-estradiol uptake after priming with 0.1, 1.0 and 5.0 µg of norethynodrel was similar to that presented for the pretreatment with 0.01 µg estradiol and 0.01 µg norethynodrel. The only noteworthy difference is that the pretreatment with 1.0 µg norethynodrel had lasting stimulating effect on $^3$H-estradiol uptake and this could be observed even up to 18 hours.

**Stimulation of $^3$H-estradiol uptake in the mouse uterus after pretreatment with different doses of norethynodrel for 4 hours**—The data for the effect of 4 hours pretreatment with different doses of norethynodrel on the uptake of $^3$H-estradiol are given in Fig. 11. It showed that the maximal stimulation of $^3$H-estradiol uptake was obtained with 1.0 µg norethynodrel and doses higher than 1.0 µg produced lesser stimulation in the uptake of estradiol. Pretreatment with 10 µg norethynodrel did not show any increase or decrease of $^3$H-estradiol uptake. However,
Uptake of $^3$H-estradiol in the mouse uterus after pre-treatment with 0.01, 0.1, 1.0, 5.0, 10.0 and 50.0 µg of norethynodrel for 4 hours. The $^3$H-estradiol uptake was studied by injecting 2.0 µg of $^3$H-estradiol 1 hour before sacrificing the animal. The radioactivity was estimated as DPM/mg wet weight and expressed as percent of the control.
$^{3}\text{H}-\text{ESTRADIOL UPTAKE} - \% \text{ OF CONTROL}$

\[ \mu \text{g NORETHYNDREL} \]

CONTROL LEVEL

100  200  300
50 μg of norethynodrel produced a statistically significant (P<0.01) decrease in the uptake of $^3$H-estradiol compared with the control.

**Priming effect of different doses of CAP on the uptake of $^3$H-estradiol**: Different doses of CAP 1, 10, 25, 50 and 100 μg were intraperitoneally administered to groups of ovariectomized rats. Three hours after the administration of CAP 12.2 μc of $^6$H,$^7$H-estradiol was administered to study the priming effect of CAP on the estradiol uptake. The animals were killed one hour after the administration of $^3$H-estradiol. The results given in Table 1 showed that pretreatment with 1 μg of CAP for 4 hours significantly increased the uptake of $^3$H-estradiol in the uterus and the pituitary. Pretreatment with higher doses of CAP, i.e. 10, 25 and 50 μg caused a decrease in the uptake of $^3$H-estradiol in the uterus which was statistically significant only at the 100 μg level. In the case of the pituitary also there was a decrease in $^3$H-estradiol uptake after 4 hours pretreatment with 10, 25, 50 and 100 μg of CAP and this was significant only at the 50 and 100 μg levels. The decrease in the uptake of $^3$H-estradiol was not significant in the hypothalamus and vagina.
The means of two groups are compared using Student's t test. The means of the two groups are not significantly different.

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Hypothalamus

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Vaguna

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Uterus

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Tissue

In the ovariecctomized rat tissues, an increase in uptake of 67-G-H-estradiol was observed in vitro with 12.6 µl of 67-G-H-estradiol. The four hour primaic effect of different doses of opharmazine...
Effect of long term treatment of CAP on the in vitro uptake of $^3$H-estradiol in the various tissues of the rabbit: The results on the long term pretreatment with 0.2 mg and 0.5 mg of CAP on the uptake of $^3$H-estradiol are given in Table 2. These showed that the uptake was significantly decreased in the uterus at both the dose levels. In the mammary gland the decrease was statistically significant in the 0.2 mg pretreatment group while it was not significant in the 0.5 mg pretreatment group. There was no significant difference in the $^3$H-estradiol uptake in the vagina, diaphragm and pituitary at the dose levels of treatment studied.

DISCUSSION

Uptake and disappearance of Nor ethynodrel and CAP

The study of the uptake of $^3$H-norethynodrel and $^3$H-chlormadinone acetate in the various tissues of the rat after an intravenous injection showed that these steroids are taken up rapidly in the genital, neural and other tissues of the rat. The steroids
Table II

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<th>Treatment</th>
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<th>Mean ± S.E.</th>
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<td>Plasma</td>
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In the rabbit tissues, on the in vitro uptake of 67-3H-estradiol, the long-term pretreatment effect of CAP (0.5 mg) was not significant.
initially disappear rapidly followed by a slow disappearance from these tissues. It thus became obvious that a 17α-ethynyl-19-nor steroid like norethynodrel showed similarities in its profile of uptake with a 17α-acetocyc progesterone derivative like chlormadinone acetate, and in this respect both norethynodrel and chlormadinone acetate showed similarities to progesterone (Laumas and Farooq, 1966b) which has also been found to be rapidly taken up by these tissues and then disappears initially rapidly followed by a slow disappearance. The slow disappearance part of the curve indicated some retention of the progestational steroid and/or its metabolites. In contrast to the neural and genital tissues, mammary gland showed a continued uptake of CAP upto 120 minutes when the experiments were terminated. Fat also showed a continued uptake upto 60 minutes and this was followed by a slow decline upto 120 minutes. Thus in the action of CAP its storage in the fat and retention in the mammary gland may have an important bearing on its mechanism of action.

The uptake of CAP or norethynodrel and their metabolites in the ovary was higher than that
in the uterus and vagina. The molar concentration of CAP and its metabolites in the ovary was \(6.6 \times 10^{-7}\) M which was higher than the molar concentration of \(2.4 \times 10^{-7}\) M in the uterus after constant infusion of \(1\alpha-^3\text{H}-\text{CAP}\). In fact both single injection and constant infusion studies showed retention of small amounts of CAP and its metabolites in the ovary. A similar pattern of retention was observed after the administration of norethynodrel. The uptake of norethynodrel and its metabolites in the uterus and ovary was \(5.8 \times 10^{-8}\) M and \(4.0 \times 10^{-7}\) M respectively. In women also, a higher uptake of \(^3\text{H}-\text{CAP}\) and its metabolites in the ovary has been found (Dugwekar, Narula and Laumas, 1973b). These results are suggestive of a direct action of norethynodrel and CAP on the ovary. A direct action of contraceptive steroids on the ovary has been debated (Diczfalusy, 1968). Norethynodrel and other contraceptive steroids have been reported to reduce ovarian sensitivity to gonadotropins (France and Pincus, 1964, Purshottam, Mason and Pincus, 1961). It has been found that prolonged treatment of rabbits with norethynodrel
causes increased incorporation of $\text{U-C}^{14}$-glucose into lipids and proteins of the ovary (Yadava and Laumas, 1969), thereby indicating that norethynodrel causes a disturbance in the usual biochemical changes in the ovary. After administration of CAP to women, increased urinary excretion of estrogens has been found in some cases suggesting an altered biosynthesis of estrogens in the ovary (Larsson-Cohn et al., 1970). Whether CAP, like norethynodrel (Yadava and Laumas, 1969), causes disturbed biochemical changes in the ovary is not known. Although the evidence so far accumulated in the literature is indicative of a direct action of the contraceptive steroids on the ovary, conclusive evidence for the same needs to be provided.

A high uptake of radioactivity was observed in the liver and the kidney after the administration of $^3\text{H}$-norethynodrel or $^3\text{H}$-CAP. This high uptake in these tissues may be attributed to their metabolic and clearance role. Similar high uptake of radioactivity in these tissues has been found after a single injection or constant infusion of radioactive
estradiol (Jensen and Jacobsen, 1962, Laumas, 1967), progesterone (Laumas and Farooq, 1966b, Falk and Bardin, 1970), testosterone (Roy Jr. and Laumas, 1969) and diethylstilbestrol (Laumas, 1969). A high uptake of radioactivity in the adrenal after $^3$H-CAP or $^3$H-norethynodrel administration was interesting. It has been found that norethynodrel suppresses the rate of lipid, RNA and protein synthesis in the adrenal (Yadava and Laumas, 1969). Besides norethynodrel and CAP, a high uptake of estradiol (Laumas, 1967, Laumas, 1969), progesterone (Laumas and Farooq, 1966b) and diethylstilbestrol (Laumas, 1969) has also been found in the adrenal.

No analysis of radioactivity was made after the uptake in the various tissues. Therefore it represents the total radioactivity due to norethy­

nodrel or CAP and their metabolites. It is not known whether norethynodrel or CAP as such would also show the same pattern of disappearance.

The demonstration of an almost insignificant uptake of norethynodrel and its metabolites in the eye indicated that the progestational compound may not have any direct action on the eye. The possibility of an indirect action needs consideration.
An interesting feature of the results with the uptake studies of $^3$H-CAP was a high uptake of CAP and its metabolites in the mammary gland and the fat. In the mammary gland, a continued uptake of CAP and its metabolites was noted up to 120 minutes, suggestive of incorporation and retention of the steroid in the mammary gland. CAP has been found to cause the development of nodules in the breast of beagle bitches (Goldenthal, 1968, 1969). However, it is not yet known how CAP causes the formation of nodules in the breast. The biochemical mechanisms leading to the participation of the steroid in this process is not fully understood. It is possible that there are species variations in the effect of CAP on the mammary gland, as in beagle bitches CAP is reported to be 45 times more potent than in women (Hill, Averkin, Brown, Gagne and Segre, 1970). On the other hand, prolonged treatment of monkeys with CAP for nearly two years did not produce any apparent nodules in the breast (Laumas, unpublished observation). CAP showed an initial rapid disappearance with a half-life of 2.25 hours.
followed by a much slower disappearance with a long half life of the order of 81 hours in the plasma of women (Dugwekar, Narula and Laumas, 1973a). The prolonged half-life in the plasma explains the continued excretion of CAP and its metabolites in the milk of lactating women for 7 days or longer after the oral administration of $^1\alpha-^3\text{H}\text{-CAP}$. The present observation of a prolonged uptake of CAP in the mammary gland indicated that this tissue has a specific affinity for concentrating CAP from the blood and consequent excretion in the milk. It is possible that CAP might have some receptor sites in the mammary gland, which need to be investigated.

Equally significant was the observation of a high uptake of CAP in the fat. Investigations on women have also shown that CAP is retained in the fat (Gallegos, Gonzalez-Diddi, Merino and Martinez-Manautou, 1970, Dugwekar, Narula and Laumas, 1973b). In fact storage of CAP in the fat and its slow release from there has been suggested to account for the prolonged half-life in plasma and continued excretion of the steroid in the urine of women and monkeys (Dugwekar, Laumas and Narula, 1973a, and see Chapter III).
The uptake and disappearance of CAP or norethy nodrel in the pituitary and the hypothalamus followed a pattern almost similar to that observed in the case of the genital tissues. It is well-known that oral progestins bring about suppression of pituitary gonadotropins by a negative feed back mechanism on the hormone releasing centres in the anterior hypothalamus (Harris and Naftolin, 1970). The increase of eosinophil cells and PAS reacting substances and decrease of pituitary weight after long term use of contraceptive steroids in animals has been interpreted as an indirect or direct effect of the steroids on the pituitary (Toth, 1964). There are reports in the literature which show that administration of CAP to women resulted in the suppression of mid cycle LH peak (Taymor and Levesque, 1971). It is possible that small amounts of CAP retained in the hypothalamus produce this effect. On the other hand there is abundant evidence to show that norethynodrel in combination with mestranol causes suppression in the pituitary LH levels in women (Ryan, Goss and Reid, 1966, Bell, Herbst, Krishnamurti, Loraine, Mears, Jackson and Garcia, 1967). No direct evidence of
Retention of CAP or norethynodrel in the hypothalamus is available except that the later part of the disappearance curve of these steroids in the hypothalamus indicated a very slow disappearance which might be interpreted as their retention in the hypothalamus, and this may participate in the action of the progestational steroid in the CNS.

**Pricing Effect of Norethynodrel and Chlormadinone Acetate (CAP) on the Uptake of $^3$H-Estradiol in the Uterus.**

These studies showed that priming with norethynodrel or CAP stimulated or depressed the $^3$H-estradiol uptake depending upon the dose of the steroid. The studies with norethynodrel further showed that the time of pretreatment with the steroid also modified the estradiol uptake. Pretreatment for 4 hours with 1.0 μg of norethynodrel caused the maximum stimulatory effect on the $^3$H-estradiol uptake while prolongation of time of pretreatment with norethynodrel to 8 or 18 hours did not stimulate the
uptake of $^3$H-estradiol. A higher dose of 50.0 µg of norethynodrel produced an inhibition of the uptake of $^3$H-estradiol in the mouse uterus.

Similarly, 4 hours pretreatment with 1 µg of CAP produced stimulatory effect on the uptake of $^3$H-estradiol in the rat uterus and pituitary, while a dose of 100 µg of CAP inhibited the uptake in both. In the pituitary, the uptake of $^3$H-estradiol was also inhibited at a still lower dose of 50 µg CAP pretreatment. It has been reported that 1 hour pretreatment with CAP in doses of 1 - 100 µg intravenously produced a competitive inhibition of $^3$H-estradiol uptake (Rosner, Macome, Dinari and De Carli, 1973a). When the CAP pretreatment was extended to 4 hours as in the present study, 1.0 µg CAP pretreatment produced a stimulation of $^3$H-estradiol uptake instead of the inhibition observed by Rosner et al. (1972a). However, Rosner, Greta, De Perez, Guerra (1972b) observed that prolonged treatment with CAP in organ culture increased $^3$H-estradiol uptake in the rat uterus. Of all doses only a high dose of 100.1 µg of CAP produced a competitive inhibition of $^3$H-estradiol in the uterus and a dose of 50 and 100 µg
CAP in the pituitary. Thus like norethynodrel, short term CAP priming under different conditions inhibited or stimulated $^3$H-estradiol uptake while on the other hand long term treatment with CAP inhibited the uptake of $^3$H-estradiol in the uterus at both the dose levels studied. At a dose level of 0.2 mg, CAP was also effective in inhibiting the uptake of $^3$H-estradiol in the mammary gland.

These results showed for the first time that a progestational steroid like norethynodrel, not only inhibited the uptake of $^3$H-estradiol in the uterus and acted as its competitive inhibitor but also stimulated its uptake. Eisenfeld and Axelrod (1966) using a high dose of norethynodrel (220 µg/100 gm body weight) for the priming of animals found an inhibition of $^3$H-estradiol uptake. They concluded that norethynodrel acted as a competitive inhibitor of estradiol uptake. Sauzier, Banerjee, Bazeau and Husain (1970) showed that administration of 100 µg norethynodrel per 100 gm body weight to rats 15 minutes before and 15 minutes after the administration of $^3$H-estradiol produced reduction in the uptake of $^3$H-estradiol. These authors also concluded that norethynodrel produced a
competitive inhibition of the uptake of $^3$H-estradiol. The present results are in agreement with the above studies, when a relatively high dose (50.0 µg) of norethynodrel is used for the pretreatment. The findings of stimulation of $^3$H-estradiol uptake by relatively small doses of norethynodrel have a similarity to some of the earlier reports (Terenius, 1965, Laumas, 1967, 1969, Kraay and Black, 1970) in which pretreatment with small dose of estradiol were shown to cause stimulation in the uptake of $^3$H-estradiol by the uterus. It was seen that 0.03 µg estradiol pretreatment for 3-6 hours produced a maximal stimulation of $^3$H-estradiol uptake.

In the present study, the stimulation of $^3$H-estradiol uptake with norethynodrel priming may be due to two possibilities. Firstly, it may be due to the inherent estrogenic activity of norethynodrel. Norethynodrel has been reported to possess 3-5 per cent (Drill, 1966) or 7 per cent (Edgren, Jones and Peterson, 1967a) of the estrogenic potency of estrone. The second possibility may be due to estrogenic metabolites of norethynodrel which may be exerting a
mild estrogenic effect on the uterus as also noted with very small doses of estradiol. Layne, Golab, Arai and Pincus (1963) isolated 17α-ethynyl, 19-nor androst-4-ene-3β, 10β, 17β triol as one of the major urinary metabolite of norethynodrel from women, which has been found to possess estrogenic activity (Bialy, Layne and Pincus, 1965). It is possible that the mild estrogenic activity of this metabolite of norethynodrel stimulates the uptake of 3H-estradiol in the uterus. Yadava and Laumas (1969) studied the metabolism of U-C14-glucose in the rabbit uterus after prolonged administration of norethynodrel and found an increased rate of synthesis of lipid, RNA and proteins. This observation further, confirmed the estrogenic properties of norethynodrel and its metabolites since estrogens are known to increase lipid, RNA and protein synthesis in the uterus (Ui and Mueller, 1963). However, the mechanism by which mild estrogenic activity of norethynodrel per se or that of its metabolites can stimulate the uptake of estradiol is not completely understood. The effect on cell permeability, transport, protein synthesis and possible increase in estradiol binding sites are worth considering.
The results further brought out that priming with 0.01 µg of estradiol for 4 hours produced 364 percent uptake of $^3$H-estradiol when the uptake in the control uterus was taken as 100%. A similar magnitude of uptake of $^3$H-estradiol was observed with the priming dose of 1.0 µg of norethynodrel for the same period of time. It thus becomes obvious that a priming dose of norethynodrel which is 100 times more than that of estradiol produced an effect similar to estradiol pretreatment. According to this criterion, norethynodrel may be 1.0 percent estrogenic activity compared with estradiol, an estimate which is comparable with the earlier reports using bioassay procedures (Drill, 1966, Edgren, Jones and Peterson, 1967).

CAP is a progestational steroid with no estrogenic activity (Drill, 1966). Although urinary and other metabolites of CAP have not yet been isolated these may not be possessing any estrogenic activity because the parent steroid is not estrogenic. Therefore the possibilities enumerated above to explain stimulatory effect of norethynodrel on the uptake of $^3$H-estradiol may not be valid in the case of CAP. It
is possible that CAP may increase the cell permeability and thus contribute to its stimulatory effect of $^3$H-estradiol uptake in the uterus and pituitary.

These investigations on the priming effect of norethynodrel and CAP on $^3$H-estradiol uptake showing stimulation and inhibition of $^3$H-estradiol uptake, point to the fact that these contraceptive steroids may be disturbing the normal make up of the uterus. A very delicate ratio of estrogen and progesterone is necessary for implantation. It is possible that in the presence of contraceptive steroids the uterus may become non-receptive and prevent implantation. It has been postulated by Maqueo-Topete et al. (1963) that CAP produces morphological modifications in the human endometrium which could be held responsible for the failure of implantation. There are several reports about the mode of action of CAP which indicate that its primary site of action is not at hypothalamo-pituitary axis. The LH peaks after continuous daily administration of 0.5 mg of CAP are not abolished (Larsson-Cohn et al., 1970), instead abortive LH peaks are formed which may or
may not be accompanied by ovulatory pregnanediol excretion in the urine (Diczfalusy, Coebelsman, Johannisson, Tillinger and Wide, 1969). This clearly indicated that the effect of low dose of CAP may or may not be ovulation inhibition. Martinez-Manautou et al. (1966) indicated that contraceptive effect of CAP (0.5 mg daily) is due to its anti-estrogenic effect on cervical mucus while Maqueo-Topete et al. (1963) ascribed it to the abnormal endometrial changes which prevent implantation of the blastocyst. The present work supports the view that the antifertility action of CAP may be at the endometrial level. On the other hand norethynodrel alone (Saunders, 1964) or in combination with mestranol is known to act by suppression of ovulation (Ryan, Goss and Reid, 1966, Bell, Herbst, Krishnamurti, Loraine, Mears, Jackson and Garcia, 1967). However in those cases where "break-through" ovulation may take place norethynodrel like CAP could still produce anti-fertility action at the endometrial level.