EFFECT OF PROLONGED ADMINISTRATION OF NORETHYNODREL AND MESTRANOL ON [U-14C]GLUCOSE METABOLISM IN UTERUS, OVARY AND ADRENAL OF RABBITS

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Summary. The effect of long-term oral administration of norethynodrel and 17α-ethynyl oestadiol-3-methyl ether (mestranol, EO3Me) on biochemical changes in the uterus, ovary and adrenal of rabbits has been studied. It was found that the administration of norethynodrel significantly increased the incorporation of [U-14C]glucose into lipid, RNA and protein of the rabbit uterus. In this respect, the effect of norethynodrel resembled that of an oestrogenic compound besides its known progestational action. EO3Me increased glucose metabolism and its incorporation into lipid and RNA in the uterus. Norethynodrel also increased lipid and protein synthesis in the ovary, but EO3Me enhanced only lipid synthesis in the ovary. Glucose metabolism was markedly depressed in the adrenal gland after the administration of norethynodrel. The mode of action of norethynodrel has been discussed in relation to the disturbance in the biochemical changes it produced in the uterus and the ovary.

INTRODUCTION

It is more than a decade since the introduction of oral contraceptives (Rock, Garcia & Pincus, 1957), which have subsequently become an accepted method for the control of fertility, but there are a number of points associated with their use which still require study. Laumas, Malkani, Bhatnagar & Laumas (1967) have demonstrated the excretion of norethynodrel (17α-ethynyl-17-hydroxyΔ5(10)-estren-3-one) and its metabolites in the milk of lactating women after oral administration of [3H]norethynodrel and have expressed concern at the possible oestrogenic activity of the excreted compounds and their possible harmful effect (Laumas, 1968) on the infant being nursed. Holmes & Mandl (1962) have drawn attention to the possible direct harmful effect of Enovid on the genital apparatus and normal endocrine homeostasis. Little is known of the biochemical changes evoked by the progestational and oestrogenic components of oral contraceptives after long-term administration and their mode of action is also not fully understood.

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In order to study the biochemical changes produced by oral contraceptives, it was considered desirable to investigate the changes produced by the individual components of the contraceptives and experiments were, therefore, undertaken to study the metabolism of [U-14C]glucose and its incorporation in the lipids, ribonucleic acid (RNA) and proteins of the uterus, ovary and adrenals of rabbits after prolonged treatment with norethynodrel and 17α-ethinyl oestradiol-3-methyl ether (EO3Me).

**MATERIAL AND METHODS**

Adult female rabbits weighing about 1.5 kg were used in these experiments. The animals were maintained in separate cages throughout the experimental period and were divided into three groups each containing four to five animals. Group I animals, the controls, were given 0.7 ml olive oil daily by mouth throughout the treatment period. Group II animals were given a daily oral dose of 0.125 mg/kg body weight of norethynodrel in 0.5 ml olive oil for a period of 90 days. After that, the dose was increased to 0.833 mg (0.5 ml olive oil)/kg body weight/day and this was continued for another 200 days. Group III animals were similarly treated with EO3Me (0.41 μg/kg body weight daily for 290 days). After the treatment period, the three groups of animals were killed by a blow to the head. Uterus, ovary and adrenals were removed, freed from adhering fat and weighed. Suitable pieces for incubation were weighed on a Roller-Smith balance. Thin slices of the tissues were separately incubated in 2.0 ml of Robinson's medium (Umbrecht, Burris & Stauffer, 1959) containing 2.5 μCi [U-14C]glucose at 37° C for 2 hr in the presence of carbogen gas (95% oxygen and 5% carbon dioxide). After incubation, the tissues were washed twice with cold Robinson medium and then homogenized in 5 ml of 5% perchloric acid in an all-glass homogenizer.

Acid-soluble nucleotides, lipids and RNA were separated by the method of Ui & Mueller (1963) except that RNA was hydrolysed with 0.2 n-NaOH for 16 hr and estimated spectrophotometrically by reading the maximal absorption at 260 nm. The residue after the separation of RNA was treated with 1.6 n-perchloric acid for 10 min at 70° C. After centrifugation, the supernatant was collected and DNA was determined by the diphenylamine reaction (Burton, 1956). The residue left after the removal of DNA was estimated for protein content by Lowry's method (Lowry, Rosenbrough, Farr & Randall, 1951). Yeast RNA and calf thymus DNA (Sigma Chemical Co.) were used as standards.

The radio-activity in lipid residues and RNA aliquots was determined by taking up in 15 ml diol scintillation liquid (PPO, 3.25 g; dimethyl POPOP, 65.0 mg; naphthalene, 52 g; methanol, 150 ml; dioxan, 250 ml; toluene, 250 ml) and counting in a liquid scintillation counter Model No. 3314 (Packard Instrument Co., La Grange, Illinois, U.S.A.). The protein residues were dissolved in 0.5 ml hyamine hydroxide and counted as usual after adding 10 ml simple scintillation liquid (PPO, 4.0 g and POPOP, 100 mg in 1 litre of toluene).

Norethynodrel and EO3Me were obtained from G. D. Searle & Co.,
Effect of norethynodrel and EO3Me on [U-14C]glucose metabolism

Chicago. [U-14C]glucose (specific activity 22 mCi/m-mole) was purchased from Bhabha Atomic Research Centre, Bombay.

RESULTS

Incorporation of [U-14C]glucose into uterine lipids, RNA and proteins

It may be seen from Table 1 that the weight of the uterus increased considerably after prolonged treatment with norethynodrel and EO3Me and was significantly higher \((P<0.001)\) than that of the controls. The incorporation of [U-14C]glucose into the lipid, RNA and proteins of the group of rabbits treated with norethynodrel was significantly higher compared with the control group. In EO3Me-treated group also, there was significantly higher \((P<0.001)\) incorporation of [U-14C]glucose into lipid and RNA but not into protein. The

<table>
<thead>
<tr>
<th>Group</th>
<th>Uterus wt (mg/kg body weight)</th>
<th>Lipid (disintegrations/min/mg DNA)</th>
<th>RNA (disintegrations/min/mg RNA)</th>
<th>Proteins (disintegrations/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>682 ± 136*</td>
<td>9584 ± 819</td>
<td>972 ± 129</td>
<td>43.5 ± 4.6</td>
</tr>
<tr>
<td>II. Norethynodrel</td>
<td>1702 ± 170</td>
<td>29296 ± 729</td>
<td>6027 ± 485</td>
<td>65.0 ± 8.0</td>
</tr>
<tr>
<td>III. EO3Me</td>
<td>2229 ± 348</td>
<td>37439 ± 1096</td>
<td>2563 ± 142</td>
<td>46.0 ± 6.5</td>
</tr>
</tbody>
</table>

Statistical analysis

<table>
<thead>
<tr>
<th>Difference between mean of control and norethynodrel-treated groups</th>
<th>P value</th>
<th>Difference between mean of control and EO3Me-treated groups</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1020</td>
<td>&lt;0.001</td>
<td>1547</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>19712</td>
<td>&lt;0.001</td>
<td>27855</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5055</td>
<td>&lt;0.001</td>
<td>1591</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>21.5</td>
<td></td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

* All values are expressed as mean ± S.E.

The incorporation of [U-14C]glucose into uterine lipids in the norethynodrel-treated group was 29,296 ± 729 disintegrations/min/mg DNA which was lower than the lipid incorporation of 37,439 ± 1096 disintegrations/min/mg DNA of the EO3Me group at the dose levels studied. On the other hand, the incorporation of [U-14C]glucose into RNA in the norethynodrel-treated group was 6027 ± 485 disintegrations/min/mg RNA which was much higher than the RNA incorporation of 2563 ± 142 disintegrations/min/mg RNA in the EO3Me-treated group, and both were significantly higher \((P<0.001)\) than in the control group.

Incorporation of [U-14C]glucose into ovarian lipids, RNA and proteins

The results of these experiments are presented in Table 2. The ovarian weight of the norethynodrel-treated group was 124.0 ± 10.6 (mg/kg body weight) which, although higher than that of the controls, was not statistically significant. The ovarian weight of the EO3Me-treated group was also not
Table 2
INCORPORATION OF [U-14C]GLUCOSE INTO RABBIT OVARIAN LIPID, RNA AND PROTEINS

<table>
<thead>
<tr>
<th>Group</th>
<th>Ovary wt (mg/kg body weight)</th>
<th>Lipid (disintegrations/min/mg DNA)</th>
<th>RNA (disintegrations/min/mg RNA)</th>
<th>Proteins (disintegrations/min/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>82.0 ± 19.4*</td>
<td>8388 ± 1963</td>
<td>1968 ± 646</td>
<td>28.5 ± 3.0</td>
</tr>
<tr>
<td>II. Norethynodrel</td>
<td>124.0 ± 10.6</td>
<td>42235 ± 6308</td>
<td>1890 ± 413</td>
<td>66.3 ± 1.7</td>
</tr>
<tr>
<td>III. EO3Me</td>
<td>84.5 ± 6.5</td>
<td>26875 ± 6375</td>
<td>1052 ± 92</td>
<td>25.2 ± 3.9</td>
</tr>
</tbody>
</table>

Statistical analysis
Difference between mean of control and norethynodrel-treated groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal wt (mg/kg body weight)</th>
<th>Lipid (disintegrations/min/mg DNA)</th>
<th>RNA (disintegrations/min/mg RNA)</th>
<th>Proteins (disintegrations/min/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>86.0 ± 10.8*</td>
<td>12509 ± 2647</td>
<td>2669 ± 139</td>
<td>77.0 ± 19.7</td>
</tr>
<tr>
<td>II. Norethynodrel</td>
<td>127.0 ± 19.5</td>
<td>4409 ± 952</td>
<td>1907 ± 374</td>
<td>33.3 ± 4.0</td>
</tr>
<tr>
<td>III. EO3Me</td>
<td>91.0 ± 4.5</td>
<td>16043 ± 4607</td>
<td>2460 ± 276</td>
<td>77.2 ± 39.0</td>
</tr>
</tbody>
</table>

Statistical analysis
Difference between mean of control and norethynodrel-treated groups

* Mean ± S.E.

Different from that of the controls. Norethynodrel treatment caused an increased rate of [U-14C]glucose incorporation into ovarian lipids and protein which was significantly higher (P<0.001 and P<0.05 respectively) than in the controls. The [U-14C]glucose incorporation into lipids in the EO3Me-treated group was also increased significantly. However, the incorporation of [U-14C]glucose into RNA and proteins in the EO3Me-treated group was not different from that of the controls.

Incorporation of [U-14C]glucose into lipids, RNA and proteins of the adrenal gland
It may be seen from the results shown in Table 3 that [U-14C]glucose incorporation into lipids of the adrenal glands after norethynodrel treatment

Table 3
INCORPORATION OF [U-14C]GLUCOSE INTO RABBIT ADRENAL LIPID, RNA AND PROTEINS

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal wt (mg/kg body weight)</th>
<th>Lipid (disintegrations/min/mg DNA)</th>
<th>RNA (disintegrations/min/mg RNA)</th>
<th>Proteins (disintegrations/min/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>86.0 ± 10.8*</td>
<td>12509 ± 2647</td>
<td>2669 ± 139</td>
<td>77.0 ± 19.7</td>
</tr>
<tr>
<td>II. Norethynodrel</td>
<td>127.0 ± 19.5</td>
<td>4409 ± 952</td>
<td>1907 ± 374</td>
<td>33.3 ± 4.0</td>
</tr>
<tr>
<td>III. EO3Me</td>
<td>91.0 ± 4.5</td>
<td>16043 ± 4607</td>
<td>2460 ± 276</td>
<td>77.2 ± 39.0</td>
</tr>
</tbody>
</table>

Statistical analysis
Difference between mean of control and norethynodrel-treated groups

* Mean ± S.E.
Effect of norethynodrel and EO3Me on [U-14C]glucose metabolism

was 4409±952 disintegrations/min/mg DNA. This was significantly lower (P<0.02) compared to the value of 12,005±2647 disintegrations/min/mg DNA of the control group. The [U-14C]glucose incorporation into RNA was 1907±374 disintegrations/min/mg RNA which appeared to be lower than the control value of 269±139 but the difference was not statistically significant. The incorporation of [U-14C]glucose into proteins in the norethynodrel-treated

| Table 4
RNA CONTENT OF RABBIT UTERUS, OVARY AND ADRENAL GLAND AFTER PROLONGED TREATMENT WITH NORETHYNODREL AND EO3Me |

<table>
<thead>
<tr>
<th>Group</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>1.99±0.32*</td>
<td>4.44±1.31</td>
<td>5.71±1.32</td>
</tr>
<tr>
<td>II. Norethynodrel</td>
<td>3.62±0.50</td>
<td>2.39±0.49</td>
<td>2.09±0.37</td>
</tr>
<tr>
<td>III. EO3Me</td>
<td>2.96±0.48</td>
<td>4.70±2.12</td>
<td>0.90±0.12</td>
</tr>
</tbody>
</table>

Statistical analysis

- Difference between mean of control and norethynodrel-treated groups
  - P value: <0.01

- Difference between mean of control and EO3Me-treated groups
  - P value: <0.05

Results are expressed as µg RNA/µg DNA.
* Mean±S.E.

| Table 5
DNA CONTENTS OF RABBIT UTERUS, OVARY AND ADRENAL GLAND AFTER PROLONGED TREATMENT WITH NORETHYNODREL AND EO3Me |

<table>
<thead>
<tr>
<th>Group</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>2456±683*</td>
<td>97.6±33.2</td>
<td>67±19.92</td>
</tr>
<tr>
<td>II. Norethynodrel</td>
<td>154±35</td>
<td>199.7±4.7</td>
<td>160±31.76</td>
</tr>
<tr>
<td>III. EO3Me</td>
<td>2122±202.4</td>
<td>84±32</td>
<td>248±26.3</td>
</tr>
</tbody>
</table>

Statistical analysis

- Difference between mean of control and norethynodrel-treated groups
  - P value: >0.2

- Difference between mean of control and EO3Me-treated groups
  - P value: >0.05

Results are expressed as µg DNA per uterus, ovary or adrenal.
* Mean±S.E.

group was 33.3±4.0 disintegrations/min/mg protein which was significantly lower (P<0.05) than the corresponding incorporation of 77.0±19.7 disintegrations/min/mg protein in the control group. On the other hand, [U-14C]glucose incorporation into lipid, RNA and proteins in the EO3Me-treated group was not significantly different from that in the control group as seen in Table 3.
The weight of the adrenal glands in the norethynodrel-treated group appeared to be increased but this was not statistically significant. The adrenal glands of the norethynodrel-treated group became sponge-like and dark red in colour while those of control animals remained smaller and unchanged in colour.

**RNA content of uterus, ovary and adrenal**

The data on the RNA content of the three tissues in the different groups are given in Table 4. There was a statistically significant increase in uterine RNA after prolonged treatment with norethynodrel \((P<0.01)\) and EO3Me \((P<0.05)\) which contrasted with a significant decrease in ovarian RNA in the norethynodrel-treated group and no change in that of the EO3Me-treated group. Adrenal RNA was significantly decreased in both the norethynodrel-treated group \((P<0.05)\) and the EO3Me-treated group \((P<0.01)\).

**DNA content of uterus, ovary and adrenal**

As seen in Table 5, there was no change in uterine and ovarian DNA after treatment with norethynodrel of EO3Me but adrenal DNA was significantly increased in both the treated groups.

**DISCUSSION**

The results indicate that prolonged treatment of rabbits with norethynodrel produced marked alteration in glucose metabolism, significantly enhanced incorporation of \([\text{U}-^{14}\text{C}]\)glucose into lipid, RNA and proteins in the uterus and altered its incorporation into ovarian and adrenal lipid, RNA and protein. It is known that the metabolism and incorporation of glucose into lipid, \(\text{CO}_2\), RNA and proteins of rat uterus is markedly increased by oestradiol pretreatment (Nicolette & Gorski, 1964) and that phospholipid metabolism has been shown to constitute an unusually sensitive indicator of oestrogen action (Aziawa & Mueller, 1961; Mueller, Herranen & Jervell, 1958). Norethynodrel, in enhancing the metabolism of glucose and its incorporation into lipid, RNA and protein in the rabbit uterus, appears to behave like an oestrogen. After oral administration of norethynodrel, human urine (Paulsen, Leach, Lanman, Goldston, Maddock & Heller, 1962), rabbit urine (Arai, Golab, Layne & Pincus, 1962; Bialy, Layne & Pincus, 1965) and goat milk (Laumas, 1968) have been found to possess oestrogenic activity. 17\(\alpha\)-Ethynyl-19-norandrost-4-ene-3\(\beta\),10\(\beta\),17\(\beta\)-triol, one of the major urinary metabolites of norethynodrel, has been found to have oestrogenic activity (Bialy et al., 1965). It is likely that the oestrogenic metabolites of norethynodrel are responsible for the oestrogenic changes in the uterus, which, being a target organ for oestrogens, has a capacity to concentrate the hormone from the circulation. This property of oestrogens is shown not only by naturally-occurring oestrogens like oestradiol-17\(\beta\) (Jensen & Jacobson, 1962; Stone & Martin, 1964; Laumas & Farooq, 1966; Laumas, 1967) but also by synthetic oestrogens such as diethylstilboestrol (Laumas, 1969). There remains the possibility that norethynodrel may act directly on the cell membrane promoting increased entry of glucose into the cell with
subsequent increased glucose metabolism. It would be of interest to see whether other actions of oestrogens on the uterus are elicited by pre-treatment with norethynodrel.

Norethynodrel caused an increased incorporation of \([U-^{14}C]\)glucose into ovarian lipids and proteins but no increase in RNA synthesis. These effects could be explained on the basis of the action of oestrogenic metabolites of norethynodrel, but it is also possible that DNA-dependent RNA synthesis may not be necessary for the stimulation of protein synthesis in the ovary by norethynodrel. The stimulation might be effected by the action of norethynodrel on translation of messenger RNA rather than on transcription, as has been found in the case of insulin (Wool & Cavicchi, 1966). It is also possible that the pool size of RNA is quite small and subject to a rapid turn-over. There may also be inadequate formation of RNA precursors since the route involved from glucose breakdown to RNA synthesis is quite long. Ovarian lipid synthesis was also enhanced by EO3Me but it had no effect on RNA and protein synthesis at the dose levels used in the present investigation.

The decrease in the incorporation of \([U-^{14}C]\)glucose into lipid and protein in the adrenal gland after prolonged treatment with norethynodrel appears to be an oestrogenic type of effect. Kitay (1963) observed an increase in adrenal weight in male rats which had been given oestradiol and this resulted in an increase in RNA but no change in DNA, i.e. hypertrophy. No change in the weight of the adrenal glands was observed in female animals but a fall both in RNA and DNA was noted. At the dose level used in the present investigation, no significant increase in the weight of the adrenal glands of norethynodrel- or EO3Me-treated animals was observed, though a fall in adrenal RNA and an increase in DNA content of both the treated groups occurred. Long-term treatment with EO3Me did not produce a decrease in lipid and protein synthesis in the adrenals. From the present experiment, it is not possible to understand the stage at which norethynodrel inhibits lipid and protein synthesis. McKern & Bell (1960) have found that glucose-6-phosphate dehydrogenase is inhibited by oestrogen, thus blocking the pentose monophosphate shunt. Norethynodrel and its metabolites may also act by blocking the same shunt.

Baker, Kahan & Zanotti (1965) found that a daily subcutaneous dose of 1.5 mg norethynodrel/100 g body weight given for 27 to 34 days to female rats caused lipid depletion from all adrenal cortical zones and diminution in size of the parenchymal cells of zona glomerulosa. In women, the secretion rate of cortisol (Layne, Mayer, Vaishwanar & Pincus, 1962) and the excretion of 17-hydroxy corticosteroids are reduced (Layne et al., 1962; Wallach, Garcia, Kistiner & Pincus, 1963). It appears that the action of oestrogens on the adrenal may be biphasic in that larger doses inhibit, and smaller doses stimulate corticosteroid production (Kitay, 1963; Cushman, Pulido & Hilton, 1965) but the literature is full of conflicting reports.

Caution will be needed in extrapolation to women from the results presented in this paper, particularly as it is difficult to compare the dosages and life spans for different species. Similarly it is not possible to generalize for all progestational contraceptives from the results obtained with prolonged treatment using norethynodrel. 17α-Acetoxy compounds like chloromadinone acetate are
not converted into metabolic products with oestrogenic activity and it is not known whether they will also produce biochemical changes similar to those caused by norethynodrel.

In spite of the extensive work on oral contraceptives, the exact mechanism of their action is not completely understood (Diczfalusy, 1965). The present results show that a progestational compound like norethynodrel is capable of evoking oestrogenic as well as progestational changes in the endometrium. This disturbance may inhibit implantation and norethynodrel may act as an anti-implantation agent, this action being compared to the post-coital effect of oestrogens (Morris, van Wagenen, McCann & Jacob, 1967). Another suggested action of oral contraceptives is their inhibition of the ovarian response to gonadotrophins (Purshottam, Masson & Pincus, 1961; France & Pincus, 1964; Taymor & Rizkallah, 1965; Bettendorf, 1962). In the present experiments, norethynodrel was found to enhance lipid and protein synthesis in the ovary producing a disturbance in the synthesis of these constituents which, in turn, may alter the sensitivity of the ovary to gonadotrophins.

Chang (1967) concluded from his studies that the extraordinary effectiveness of oral contraceptives may be due to disturbances of many reproductive processes besides the inhibition of ovulation suggested by Pincus (1966). According to Chang (1967), sperm transport, sperm capacitation, rapid egg transport leading to failure of fertilization, subsequent degeneration in the uterus and expulsion from the uterus may all contribute to their effectiveness. To these factors may be added the altered biochemical changes in the uterus and ovary, evidence of which has been presented in this paper.

ACKNOWLEDGMENT

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REFERENCES


Chang, M. C. (1967) Effect of progesterone and related compounds on fertilization, transportation and development of rabbit eggs. Endocrinology, 81, 1251.


Effect of norethynodrel and EO3Me on [U-14C]glucose metabolism

Lancet, ii, 1174.


Stone, G. M. & Martin, L. (1964) The uptake of tritiated estradiol and estrone by the uterus of the ovariectomised mouse following local application. Steroids, 3, 699.


