ORAL CONTRACEPTIVES, NORETHYNODREL AND MESTRANOL: EFFECTS ON GLUCOSE METABOLISM

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DAVID, KAI YUI LEI

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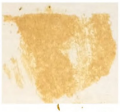

ABSTRACT

ORAL CONTRACEPTIVES, NORETHYNODREL AND MESTRANOL: EFFECTS ON GLUCOSE METABOLISM

By

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Oral contraceptive steroids, norethynodrel and mestranol were fed singly or in combination to 11-week-old female Sprague Dawley rats to provide similar dosages used by women on a body weight basis. Their effects on oral glucose tolerance and in vivo tissue uptake and utilization of glucose-U-^{14}C were determined. Oral glucose tolerance tests were performed on fasted rats after two and four weeks of feeding the steroids. A fasting blood sample was obtained by heart puncture and then the rats were gavaged with glucose. Blood samples were again obtained several times after the glucose load. Blood glucose levels indicated that there was no difference between the steroid-treated and control rats after two weeks of steroid feeding. However, after four weeks there was a tendency for the blood glucose levels to be higher in the treated groups and norethynodrel appeared to the compound responsible for the impairment.

Tissue uptake and utilization of glucose-U-^{14}C in the intact rat were determined in a different set of animals after 6 weeks of steroid feeding. The rats were gavaged with glucose-U-^{14}C and placed in metabolic chambers for collection of expired CO_2. At various time intervals following the oral glucose load, the rats were decapitated and radioactivities in the stomach and intestinal contents, various tissues and expired CO_2 were determined. Although both steroids appeared to be involved, norethynodrel was again found to be the main causative factor in the reduction in the level of radioactivity in parametrial adipose tissue. The radioactivity levels in the diaphragm muscle and liver tissue and the conversion of glucose into respiratory CO_2 were not influenced by the steroids. Mestranol slightly depressed gastric emptying and intestinal absorption but the depression appeared too small to have appreciable effects on the levels of blood glucose and on the rate of glucose metabolism in various tissues.

The effect of oral contraceptive on the level of blood insulin, as measured by radioimmunoassay was determined after a long term feeding of norethynodrel plus mestranol. Impaired glucose tolerance was observed after ten weeks of steroid treatment but the insulin response curve during oral glucose tolerance was found to be unaffected.

The response of peripheral tissues to insulin was studied after 6 weeks of steroid feeding. In the fasted

intact animals, the conversion of blood glucose into adipose tissue fatty acids, after an intravenous injection of insulin and glucose-U-^{14}C to label the blood glucose pool, was found to be lower in the steroid-treated rats than the control. A similar trend was observed in the conversion of blood glucose into diaphragm glycogen but the differences were not significant. In vitro incubation of peripheral tissues in glucose-U-^{14}C medium containing various levels of insulin indicated that less insulin was required to stimulate maximum glycogenesis by diaphragm tissue from control than from steroid-treated rats. Steroid treatment also depressed lipogenesis in parametrial adipose tissue. Results of in vivo and in vitro experiments indicated that the adipose tissues as well as diaphragm muscle of steroid treated rats are slightly resistant to exogenous insulin. The impairment of glucose tolerance following oral contraceptive treatment could be partly the results of peripheral tissue resistance to hypoglycemic action of insulin.

In vitro incubation of peripheral tissues with 2-deoxyglucose-1-^{14}C and various levels of insulin indicated that the uptake of 2-deoxyglucose by the diaphragm and adipose tissue was not affected by the steroids. Since the uptake of 2-deoxyglucose reflects glucose transport across cell membrane, it seems that the resistance to insulin observed occurs not at the point of glucose entry but at subsequent steps in glycogenesis and lipogenesis.

Meal-feeding was imposed on the rats by offering the diet containing the steroids for two hours each day from 5:00 to 7:00 p.m. to the treated rats and during this two hour period, control rats were pair-fed to the treated rats the diet without the steroids. The conversion of glucose into fatty acids by parametrial adipose tissue of meal-fed rats, in an in vitro system was depressed by the steroids, thus the data substantiated the findings of former experiments that oral contraceptive steroids decreased lipogenesis in the adipose tissue.

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David, Kai Yui Lei

A DISSERTATION

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TO MY WIFE AND

HESTIA

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INTRODUCTION

In 1956 Rock, Pincus and Garcia introduced estrogen-progesterone drugs into the field of fertility control. The oral contraceptive pill was licensed for general use in the United States in 1959. Since then, this form of prophylaxis has been widely accepted and applied. It is estimated that more than six million women in the United States are currently taking these compounds. The concern for the safety with which these preparations can be used and the widespread usage of the medication for the control of conception or for various other reasons for long period of time led to clinical studies on large population of women. The FDA declared the contraceptive pill 'not unsafe' for human consumption since no major abnormalities have been related to contraceptive therapy.

Many studies have been performed on the action of oral contraceptive pills on the pituitary, the adrenocortical secretions and the metabolism of various nutrients. More than fifty metabolic changes have been recorded. Despite the large number of studies, the effect of oral contraceptives on carbohydrate metabolism has not been clarified. However, it is well documented that impaired glucose tolerance occurs in women using the combination-type oral

contraceptives. The mechanism(s) by which the oral contraceptives impair glucose tolerance is unknown, but several theories have been proposed. The impairment has been attributed to changes in glucose absorption, in gut insulin-releasing factors and in liver function. The intolerance has also been ascribed to increased insulin resistance of the peripheral tissues and to elevation of plasma glucocorticoids and growth hormone.

The present work was conducted to determine which of the two steroidal agents commonly used in contraceptive preparations is responsible for changes in glucose tolerance. Furthermore, in vitro and in vivo experiments were performed to determine the possible role of liver, depot fat and diaphragm muscle in glucose tolerance. The response of the peripheral tissues to exogenous insulin was also studied in both in vitro and in vivo experiments. In addition, an attempt was made to see if there is any reduction in the rate of glucose transport in the peripheral tissues.

LITERATURE REVIEW

Development of oral contraceptives

As early as 1897, Beard had postulated that the corpus luteum of the ovary, which secretes progesterone, is responsible for the inhibition of ovulation during pregnancy. Makepease et al. reported in 1937 that the administration of progesterone inhibits ovulation in the rabbits. This was confirmed by Pincus and Chang (1953) who also evaluated other steroids for their ability to inhibit ovulation in rabbits. In 1952, norethynodrel (a compound having both the properties of progesterone and high oral activity), the progestin in Encvid, was synthesized by Colton at the Searle Laboratories. The studies performed by Saunders et al. (1957, 1958) and Pincus et al. (1956a and b) established the progestational, estrogenic, pituitary inhibiting and antiovulatory effects of norethynodrel. Rock et al. (1956) and Rice-Wray (1957) later demonstrated in clinical studies the contraceptive effectiveness of norethynodrel in women. An estrogen was subsequently combined with the progestin because it reduces the incidence of bleeding and improves endometrial development. Enovid was finally approved by the FDA for use as an oral contraceptive at the end of 1959.

At present, many oral contraceptives are available under various brand names in different countries. Names of the more common products and their composition are presented below:

Product	Progestin	Estrogen
Enovid	Norethynodrel	Mestranol
Orthonovum	Norethindrone	Mestranol
Anovlar	Norethindrone acetate	Ethynylestradiol
Norlestrin	Norethindrone acetate	Ethynylestradiol
Lyndiol	Lynestranol	Mestranol
Ovulen	Ethynodiol diacetate	Mestranol
Provest	Medroxyprogesterone acetate	Ethynylestradiol
Ovex	Megestrol acetate	Ethynylestradiol
Aconcen	Chlormadinone acetate	Mestranol

Three main types of oral contraceptives are available: combined preparations, sequential preparations, and the 'mini-pills'. The combined preparations, in which each tablet of a 20- or 21- day course contains both an estrogen and a progestin. The sequential or serial preparations provide a course of estrogen-only tablets followed by a short course of tablets containing both estrogen and

progestogen. The 'mini-pills' products, contain only a low dose of a progestogen and the tablets are taken daily continuously without a break.

C-Quens and Oracon were the first sequential oral contraceptives marketed. One of the major problems of sequential therapy is the failure of occurrence of menses following the withdrawal of therapy. In addition, it is not as effective as the combined therapy.

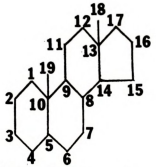
Since most metabolic studies of steroid contraceptives have been conducted with the combined and sequential products, this review will be concerned with only these preparations.

Structural requirement

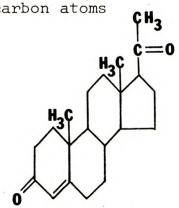
A. For oral contraceptive activity

Norethynodrel, norethindrone, and several other progestins (Fig. 2) have an ethynyl group (-C≡CH) in the 17 position of the steroid molecule and together with the 19 norstructure (19-methyl group replaced by a hydrogen atom) give them their oral activity. Norethynodrel also has a carbon-carbon double bond in the 5(10) position, which is biologically significant, for in addition to being progestational it renders norethynodrel estrogenic and devoid of androgenic effect in both man and animals.

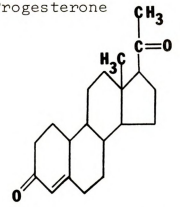
Mestranol and ethynylestradiol (Fig. 3) are estrogens used in combination with progestins for oral contraceptive purposes. Both compounds contain a 17-ethynyl group which imparts high oral potency.



Numbering of the carbon atoms



Progesterone



19-Norprogesterone

Fig. 1. Chemical structure of reference compounds.

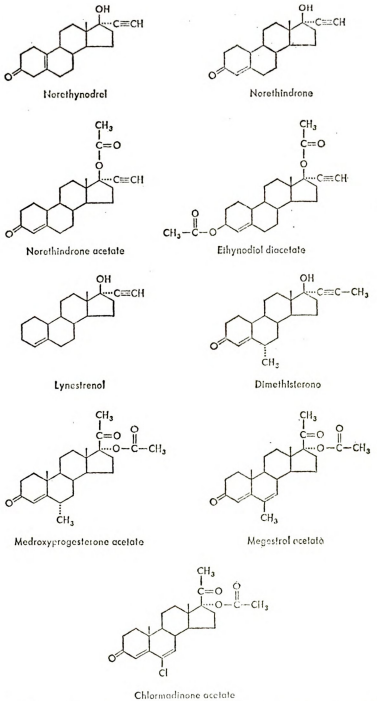
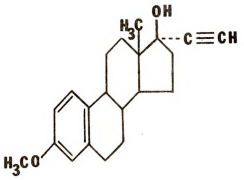
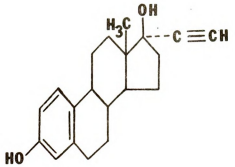


Fig. 2. Chemical structure of the progestins used in oral contraceptive tablets.



Mestranol



Ethynylestradiol

Fig. 3. Chemical structure of the estrogen used in oral contraceptive tablets.

B. Insulinogenic, insulin-antagonistic activities (Table 1)

Mestranol and all of the pregnene and nortestosterone derivatives which are associated with increased insulin production following glucose stimulation possess a partial positive charge at the C_5 position (Fig. 4). Beck (1969a) indicated that steroid compounds with a partial positive charge at C_5 possess insulinogenic and insulin-resistance activities in monkeys which generally neutralize each other when measured in terms of the disposal of intravenously administered glucose. The insulinogenic and insulin-resistance activities may be separated by the introduction of a double bond at C_6 position of the B ring of these steroid compounds.

In addition, mestranol, by virtue of its methoxyl group at the C_3 position, possesses insulinogenic and insulin-antagonistic activities in monkeys and man which are apparently not present in the parent estrogen compound (Beck 1969a).

Carbohydrate metabolism

A. Glucose and glycogen content of tissues

1. Glucose

A large number of studies of blood glucose levels in women taking oral contraceptives have been published (Table 2). In general, no significant effect on fasting concentrations has been detected, irrespective of type of

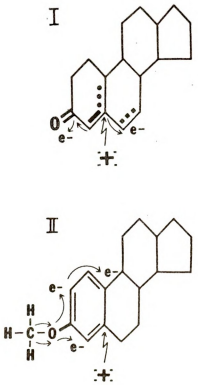


Fig. 4 Structural features of pregnene or nortestosterone (I) and estrogen (II) steroids which are essential for insulinogenic activity. + denotes a partial positive charge; e^-, electrons. Progesterone, and norethindrone are 4-dehydro-, 3-keto-pregnene steroids; norethynodrel is a 5(10) dehydro-, 3-keto-steroid; chlormadinone, 4-dehydro-, 6 dehydro-, 6 chloro-, 3-keto-steroid. Mestranol is a 3-methyl-ether estrogen.

Table 1

SUMMARY OF INSULINOGENIC AND INSULIN-ANTAGONISTIC PROPERTIES OF GONADAL AND CONTRACEPTIVE STEROIDS IN RHESUS MONKEYS.

	Insulin Response	Resistance To Insulin	Δ Glucose Tolerance
Insulinogenic, non-antagonistic			
Pregnene derivative			
Chlormadinone	↑	-	↑
Insulinogenic, insulin-antagonistic			
Pregnene derivatives			
Progesterone	↑	↑	0
Nortestosterone derivatives			
Norethynodrel	↑	↑	0
Norethindrone	↑	-	0
Estrogen derivative			
Mestranol	↑	↑	0
Non-insulinogenic			
Nortestosterone derivative			
Ethynodiol diacetate	0	-	0
Estrogens			
Estradiol	0	-	0
Estriol	0	↑	0
Ethinyl estradiol	0	↑	0

↑ = increased or improved
↓ = decreased or worse
0 = no change
- = not measured

product or duration of treatment, for women with normal values prior to treatment.

2. Glycogen

Relatively few studies have been performed on the effects of contraceptive steroids on glycogen content of various tissues. The glycogen content in biopsies of ectocervical tissues of the uterus in a large population of women, some receiving contraceptives and others not, have been determined by Gregoire and Ledger (1969). No significant differences were observed for glycogen content at different times of the menstrual cycle between the two groups.

In animal studies, estrogens were found to elevate glycogen formation in adult rat liver (Walass 1952). However, estrogen injections did not increase glycogen formation in hypophysectomized or adrenalectomized rats (Fry et al. 1942). Thus, the estrogen effect is probably mediated through the pituitary-adrenal system.

B. Glucose tolerance

Impaired glucose tolerance in women using the combination-type oral contraceptives is well established. It was first reported by Waine et al. (1963) and was confirmed repeatedly since that time (Table 2). Initially, controversy existed regarding which of the two steroidal agents used in contraceptive preparations was responsible for the glucose intolerance, because both agents were

potent chemicals, and because of differences in dosage, duration of use, and other variables. However, the situation is being clarified rapidly.

A decrease in glucose tolerance is often observed as early as the first treatment cycle in some combination-type oral contraceptive users. However, this effect is rapidly reversible on discontinuing treatment, and Halling et al. (1967) have indicated that there is an improvement of this disturbance if patients are switched from an estrogen-progestin combination to mestranol alone. Gershberg et al. (1969) found that medroxyprogesterone acetate, a progestational steroid, also produced decreased glucose tolerance within three months of treatment. The role of the progestational agents was again confirmed by the long term study of Spellacy et al. (1970) which compared patients on sequential agents versus combination agents for periods exceeding 6 years. Abnormal glucose tolerance was observed in 3% of the sequential contraceptive users and 39% in the combination users. In contrast Javier et al. (1968) indicated that mestranol itself is capable of decreasing glucose tolerance. In a recent review, Goldzieher (1970) postulated that both steroidal agents have effects on carbohydrate metabolism but the effect of estrogen seems to be minor.

Table 2. Results of glucose tolerance tests in users of combination-type oral contraceptives

Reference		Months of treatment	Type of tolerance test	% of abnormal results
Wynn and Doar	(1966)	> 3	oral	18
Waine et al.	(1963)	2-12	oral	43
diPaola et al.	(1968)	3- 7	oral	53
Pi-sunyer & Oster	(1968)	1	oral	0
Gershberg et al.	(1964)	3-38	oral	34
Halling et al.	(1967)	3-43	oral	40
Peterson et al.	(1966)	3-84	oral	39
Taylor & Kass	(1968)	12	oral	0
Spellacy et al.	(1968a)	> 100	oral	77
Javier et al.	(1968)	> 12	oral	85
Spellacy et al.	(1971)	6	oral	33
Pyorala et al.	(1967)	1	intravenous	0
Posner et al.	(1967a)	2- 3	intravenous	9
Posner et al.	(1967b)	18	intravenous	13
Wynn & Doar	(1966)	> 3	intravenous	15
Spellacy et al.	(1968b)	12	intravenous	0
Starup et al.	(1968)	12	intravenous	0

1. Oral vs. intravenous glucose tolerance

The results of some of the oral and intravenous glucose tolerance tests are shown in Tables 2 and 3. In general, the percentage of individuals with impaired glucose tolerance (Tables 2 and 3) reported appeared to be lower in the intravenous glucose tolerance tests but the duration of follow-up for the intravenous glucose tolerance was shorter.

A few of the investigators reported no abnormal oral glucose tolerance tests in women who received oral contraceptives but their duration of contraceptive treatment was short and mostly less than one year. The majority of the investigators indicated an increased frequency of impaired glucose tolerance varying from 18 to 77.4 percent. Javier et al. (1968) suggested that there may be an increased incidence of impaired glucose tolerance as the duration of treatment lengthens. However, Wynn and Doar (1966) and Peterson et al. (1966) postulated that there was no relation between the duration of treatment and incidence of abnormality in glucose tolerance.

In studies where both oral and intravenous tests were performed in the same subjects (Buchler and Warren 1966, Wynn and Doar 1966), higher incidences of impaired glucose tolerance were obtained with the oral test. Most investigators agreed that the oral test is more sensitive than the intravenous test, especially when accompanied by a corticoid challenge (diPaola et al. 1968, Javier et al.

Table 3. Results of glucose tolerance test in users of sequential-type oral contraceptives

Reference	Months of treatment	Type of tolerance test	% of abnormal results
Pyorala et al. (1967)	1	intravenous	0
Spellacy et al. (1968c)	6	intravenous	0
Spellacy et al. (1969)	12	intravenous	0
Spellacy et al. (1968a)	80	intravenous	26

1968). The incidence of impaired glucose tolerance appears to be lower in those receiving the sequential-type oral contraceptives than those receiving the combined preparations. But this may be due to fewer sequential-type oral contraceptives studies and shorter duration of sequential-type contraceptives treatment.

2. Plasma insulin and growth hormone (GH) levels before and after glucose administration

Most investigators agreed that the plasma insulin levels were elevated during glucose tolerance tests (Table 4). However, there are some who believe that they are raised at all times in oral contraceptive users. A significant increase in fasting levels of insulin was found after 19 days of treatment with Enovid (Spellacy and Carlson 1966). They published further work with other combined preparations given for longer durations and found elevated plasma insulin levels during fast or after intravenous or oral glucose tolerance tests. Spellacy et al. (1968c, 1969) noted elevated insulin levels in women receiving sequential preparations for 6 months. The elevations disappeared, however, when they received the preparations for another 6 months.

Javier et al. (1968) and Pi-Sunyer and Oster (1968) found in women only a small and non-significant increase in plasma insulin, during oral glucose tolerance tests, after a short duration of treatment. Starup et al. (1968) found no insulin change after women received oral

contraceptives for one year. The progestin in their preparation has no estrogenic properties and this was postulated to be responsible for not finding an increased level of insulin.

Only a few investigators have reported plasma growth hormone levels in oral contraceptive users (Table 4). Yen and Vela (1968) found elevated fasting plasma levels after 3 months of oral contraceptive treatment, but Boshell et al. (1968) could not confirm this. Elevated levels of growth hormone during oral glucose tolerance tests have been reported by Spellacy et al. (1970b). The relevance of these findings is not known, but the elevation of growth hormone may be important in the production of elevated blood glucose and plasma insulin levels.

3. Lactate and pyruvate during glucose tolerance tests

Wynn and Doar (1966) found an elevation of fasting venous blood pyruvate level in oral contraceptive users. The maximum blood pyruvate response during oral and intravenous glucose tolerance tests was also elevated in certain women receiving oral contraceptives. In a recent review, they (Wynn and Doar 1969) extended these observations to a larger group of subjects and found that the blood pyruvate levels were significantly increased in the contraceptive treated group. However, the venous blood lactate/pyruvate ratio during oral and intravenous glucose tolerance tests was found to be similar between the control and contraceptive groups. They also reported preliminary studies of

Table 4. Effect of oral contraceptives on plasma insulin and growth hormone during glucose tolerance test

Reference	Months of treatment	Test	Plasma glucose	Plasma insulin	Plasma growth hormone
Combination contraceptives					
Spellacy et al. (1967a)	6	intravenous	↑	↑	-
(1968a)	12	intravenous	↑	↑	-
(1970b)	72-85	oral	↑	↑	↑
(1971)	6	oral	↑	↑	-
Javier et al. (1968)	12	oral	↑	0	-
	2-17	oral	↑	↑	-
Yen & Vela (1968)	3	oral	0	↑	↑ fasting
Boshell et al. (1968)	12-24	oral	↑	↑	0 fasting
Pi-Sunyer & Oster (1968)	1	oral	↑	0	-
Sequential contraceptives					
Spellacy et al. (1968c)	6	intravenous	↑	↑	-
Spellacy et al. (1969)	12	intravenous	↑	0	-

↑ = elevated
0 = no change
- = not measured

whole body lactate turnover rates and postulated that the elevated blood pyruvate and lactate levels during oral contraceptive treatment resulted from an increased rate of production rather than impaired utilization of pyruvate and lactate.

4. Levels of free fatty acids during glucose tolerance test

In a large cross-sectional study of women taking oral contraceptives, Wynn and Doar (1966) found elevated levels of free fatty acids after fasting as compared to that of a control group. However, in a subsequent study (Wynn and Doar 1969) they failed to show that the mean plasma free fatty acids levels before and after oral or intravenous glucose administration were affected by oral contraceptive treatments.

5. Effect of vitamin B_6 supplementation on oral glucose tolerance test (OGTT)

In a recent review, Theuer (1972) indicated that some oral contraceptives and ovarian-like steroids can alter vitamin as well as carbohydrate metabolism. In an attempt to elucidate the interrelationship between oral contraceptive usage, vitamin B_6 metabolism and carbohydrate metabolic abnormalities, Spellacy et al. (1972) supplemented oral contraceptive users with vitamin B_6 for one month. The subjects were selected on the basis of their progressively deteriorated glucose tolerance

tests during oral contraceptive therapy. A significant improvement in their oral glucose tolerance test was observed after this combined treatment. However, the improvement did not reach the pre-steroid treatment value, thus indicating short term B_6 supplementation is only partially effective.

6. Associated factors

Many investigators have examined factors that might predispose oral contraceptive users to abnormal carbohydrate metabolism during oral contraceptive treatment. The factors included the subject's age, weight, offsprings' infant weight and family history of diabetes. These factors are discussed in the following paragraphs:

a) Age

Spellacy et al. (1968b) demonstrated a positive correlation between the subjects' age and the degree of alterations in insulin or glucose levels during contraceptive therapy. However, Peterson et al. (1966) found no correlation between the subjects' age and the incidence of impaired glucose tolerance.

b) Weight

A significant relationship between the excess weight gained during oral contraceptive treatment and the associated elevation of glucose or insulin level was found by Spellacy et al. (1968b and 1971). Thus, some investigators (Taylor and Kass 1968, Yen and Vela 1968) have excluded

obese subjects from their studies. But several investigators (Wynn and Doar 1966, Posner et al. 1967a, Gershberg et al. 1964, and diPaola et al. 1968) found no relationship between the pre-drug treatment degree of obesity and the incidence of abnormal glucose tolerance observed.

c) Infant weight

Pregnant women with abnormal carbohydrate metabolism frequently give birth to excessively large babies. Javier et al. (1968) and Spellacy et al. (1968a and b) observed significant correlations between the development of abnormal carbohydrate metabolism while taking oral contraceptives and the history of delivering infants whose weight was more than 9 pounds at birth. Whereas, Peterson et al. (1966) and Posner et al. (1967b) found no correlations between the development of abnormal carbohydrate metabolism during contraceptive treatment and the previous infant's weight.

d) Family history of diabetes

Many investigators have examined the relation of a positive family history for diabetes mellitus with the development of abnormal carbohydrate metabolism during oral contraceptive therapy. Some workers (Gershberg et al. 1964, Peterson et al. 1966, Pyorala et al. 1966, Spellacy et al. 1970b) observed significant increases in the incidence of abnormal glucose tolerance in oral contraceptive users with a positive family history of diabetes mellitus but others (Posner et al. 1967b and diPaola et al. 1968)

noted only a significant borderline association or no association at all (Halling et al. 1967, Javier et al. 1968). Thus, several authors excluded from their studies patients with a positive history of diabetes mellitus (Starup et al. 1968, Taylor and Kass 1968).

Mechanism(s) of action

The mechanism(s) by which the oral contraceptives impair glucose tolerance is a matter for speculation but several theories have been proposed:

1. Corticoids

There are many studies on the effects of contraceptive steroids on the levels of blood and urinary corticosteroids and their metabolites (Table 5). Most investigators find a small, but significant, decrease in corticoid excretion in women on oral contraceptive therapy. However, soon after stopping treatment, corticoid excretion returns to normal. Plasma glucocorticoid levels are elevated by most oral contraceptives. This elevation is largely due to the action of estrogens, which raise the level of binding protein, transcortin (Daly et al. 1968). Doe et al. (1960) postulated that there may be a slight increase in the level of free cortisol with contraceptive treatment but this is probably insufficient to account for all the carbohydrate changes observed in oral contraceptive users.

2. Growth hormone

In an attempt to elucidate the mechanism affecting carbohydrate metabolism, several investigators have determined the level of growth hormone in women taking oral contraceptives (Yen and Vela 1968, Garcia et al. 1967, Spellacy and Carlson 1966, Spellacy et al. 1969). In general, growth hormone was increased by oral contraceptives. Estrogen alone has also been found to increase the levels of growth hormone (Frantz and Robkin 1965). But Mintz et al. (1967) indicated that the action of estrogen on growth hormone secretion may be dependent upon the length of treatment and the dosage used. Earlier studies have shown that growth hormone is diabetogenic and may produce elevated levels of blood glucose (Young 1937). However, the elevation of blood glucose levels in subjects receiving oral contraceptives can also lead to a reduction in pituitary growth hormone secretion (Yen and Vela 1968, Spellacy et al. 1968a). The magnitude in which growth hormone affects carbohydrate metabolism in oral contraceptive users is still unknown, but is is possible that persistent elevation of growth hormone may be important in the production of elevated blood glucose and plasma insulin levels.

3. Insulin

a) Insulin secretion

Elevated levels of plasma insulin during glucose tolerance test, in contraceptive users, have been found by many intestigators (Table 4).

Javier et al. (1968) found higher plasma insulin levels in women early in therapy, but with prolonged treatment insulin levels tended to be lower in some subjects as glucose tolerance became further impaired. They postulated that the failure of the pancreas to respond to hyperglycemia may be the cause of lower insulin level with prolonged treatment, and that the estrogen component of these drugs is the major cause of these metabolic changes, though the progestin may exert additional effects by being converted in the body to substances with estrogenic activity.

Gold et al. (1969) found that Ovulen induced hyperglycemia, during fast and glucose tolerance tests, in overt diabetics. The hyperglycemia was not accompanied by increased concentration of endogenous plasma insulin or increased resistance to exogenous insulin. This was interpreted by Gold et al. (1969) as a loss of beta-cell sensitivity or 'indifference' to hyperglycemia while still retaining the capacity to respond to acute increments of blood glucose. It appears that the insulin secretory response of diabetics once initiated, assumes a fixed pattern not related to the magnitude and duration of the hyperglycemic stimulus (Gold et al. 1969)

Spellacy (1969b) postulated that the elevated plasma insulin level may bring the glucose level back to the pre-treatment range after several months of treatment. However, prolonged usage of these agents would again lead to elevation of glucose levels in some women. Spellacy (1969b) suggested that this incidence of decompensation with the resultant abnormal glucose tolerance may be due to an altered insulin secretion mechanism, a delayed insulin release in response to hyperglycemia and perhaps an alteration in the type of insulin (proinsulin) that is released. Perhaps some of the excess insulin measured by immunoassay is biologically inactive and is really proinsulin (Chance 1968).

b) Insulin-xanthurenic acid complex

Alterations in vitamin metabolism in some oral contraceptive users have been observed (Theuer 1972). Metabolic studies of the involvement of pyridoxine (B_6) in tryptophan metabolism have been done in humans by administering an oral tryptophan load (2-5 gms) and measuring the urine metabolites before and after the tryptophan load. The urinary excretion of xanthurenic acid (XA) is increased in subjects receiving oral contraceptives and during pregnancy. Luhby et al. (1971) indicated that the increased XA excretion in oral contraceptive users can be returned towards normal with vitamin B_6 supplementation and estimated one would need approximately 30 mg of B_6 per day to normalize the XA excretion pattern. The average diet contains

only 1-2 mg of vitamin B_6 per day and is insufficient to maintain a normal XA excretion pattern in oral contraceptive users.

Murakami (1968) has shown that 2 moles of XA will bind to one dimer of insulin at the histidine imidazole group through a zinc linkage to form an 'insulin-Zn-XA' complex. A significant reduction in biological activity of the insulin in insulin-complex was observed by means of rat diaphragm and rat epididymal fat pad bioassays (Kotake et al. 1968a). And a 50% reduction in the hypoglycemic effect of the insulin-complex was observed when injected into dogs and rabbits (Kotake et al. 1968a and b). It would appear that insulin bound to XA, drecreases the insulin's biological activity.

In an attempt to investigate the relationship of vitamin B_6 metabolism and glucose tolerance in oral contraceptive users, Spellacy et al. (1972) supplemented oral contraceptive users with vitamin B_6. The subjects were chosen on the basis that they had already demonstrated a deteriorating glucose tolerance while receiving oral contraceptives. A significant improvement in oral glucose tolerance test was observed after one month of vitamin B_6 supplementation. However, the supplementation did not return the glucose level back to the value before oral contraceptive treatment. Thus, Spellacy et al. (1972) postulated that the observed deterioration in carbohydrate

metabolism in oral contraceptive users may be caused by a vitamin B_6 deficiency. They also suggested that the vitamin B_6 deficiency in contraceptive users may increase the blood XA level. The XA then could become bound to insulin, thus decreasing the insulin biologic activity, and finally resulting in elevated blood glucose levels.

c) Peripheral resistance to insulin

Experiments with laboratory animals suggest that steroid hormones may also alter the peripheral tissue response to insulin. A mild peripheral resistance to the hypoglycemic action of exogenous insulin and an enhancement of plasma insulin response to intravenous glucose have been observed when progesterone (Beck 1969b) and mestranol (Beck and Wells 1969) were administered to female rhesus monkeys.

Beck and Wells (1969) also studied two groups of women in the third trimester of pregnancy. Gestational diabetic glucose tolerance tests were observed in one group and normal glucose tolerance tests were observed in the other group. Postpartum glucose tolerance test were normal in both groups. However, 50% of the gestational diabetic subjects and 21% of the normal subjects developed impaired glucose tolerance during oral contraceptive therapy. During pregnancy, the normal subjects have a retarded insulin response in relation to the blood glucose. But on the oral contraceptive therapy the normal subjects showed a substantial increase in plasma insulin levels, which rose

progressively, accompanied by a rise in blood glucose. The oral contraceptive users with abnormal glucose tolerance apparently were unable to effect a compensatory increase in peripheral insulin levels in response to glucose. Beck and Wells (1969a) suggested that the oral contraceptives unmasked diabetes less readily than pregnancy because the initial rate of appearance of insulin in the peripheral plasma was not retarded by the contraceptive treatment. Thus, they concluded that oral contraceptives produce impaired glucose tolerance by causing increased peripheral resistance to the hypoglycemic action of insulin.

4. Intestinal function

In studies where both oral and intravenous glucose tolerance tests were performed in the same oral contraceptive users (Buchler and Warren 1966, Wynn and Doar 1966), more abnormal tests were obtained with the oral route. Most investigators agreed that the oral test is more sensitive than the intravenous test especially when accompanied by a corticoid challenge (diPaola et al. 1968, and Javier et al. 1968). This increased sensitivity may result from a greater release of beta-cell insulin by the orally administered glucose (McIntyre and Holdsworth 1964, and Perley and Kipnis 1967). Spellacy (1969b) suggested that this difference may be caused by gut factors affecting insulin release, whereas Buchler and Warren (1966) postulated that the abnormal oral glucose tolerance tests may be due to

delayed gastrointestinal absorption. The intestinal function may be affected by oral contraceptives, but direct data on this point are not yet available.

5. Liver function

Alterations in many of the laboratory tests for liver function have been observed in pregnant women and oral contraceptive users. In a recent review, Briggs et al. (1970), indicated that these changes usually involve small increases in bromosulphalein retention and thymol turbidity, but occasional increases in serum alkaline phosphatase and transaminases activities have also been reported.

It is well known that the liver is involved in carbohydrate metabolism with glycogen storage and glucose release. Wynn and Doar (1966) indicated that the fasting blood pyruvate levels were elevated in 22% of their subjects on oral contraceptive therapy. This alteration in blood pyruvate level may be caused by abnormal liver metabolism. Another effect, reflective of abnormal liver metabolism, is the elevated blood triglyceride levels in oral contraceptive users (Gershberg et al. 1968, Javier et al. 1968). The additive effects of these alterations in liver function on carbohydrate metabolism in oral contraceptive users are uncertain.

METHODS AND MATERIALS

1. Oral glucose tolerance test

Oral glucose tolerance was performed by forcefeeding glucose (300 mg/100 g body weight), in a 50% solution (W/V), to rats that had been fasted for 18 hours, and obtaining blood samples at specified times for glucose determinations. Sodium pentobarbital (5 mg/100 g body weight) was injected subcutaneously to anesthetize the rat for blood sampling by heart puncture.

2. Assay of serum glucose

A Somogyi (1952) blood filtrate was prepared by mixing 1/19/1/1 (v/v/v/v) of serum/H_2O/1.8% $Ba(OH)_2.8H_2O$/2.0% $ZnSO_4.7H_2O$) solutions. The $Ba(OH)_2$ and $ZnSO_4$ were used to deproteinize and neutralize the serum respectively. The mixture was centrifuged and the supernatant used for glucose determinations. Serum glucose was determined by a semi-micro glucose oxidase method (Washka and Rice 1961) using 'Glucostat' obtained from Worthington Biochemical Corporation.

3. Assay of serum insulin

The insulin content of serum was assayed by using the 'Insulin Immunoassay Kit', obtained from Amersham/Searle

Corporation, which resembles the method described by Hales and Randle (1963). The method uses an antibody specific to insulin. The insulin in the serum competes with added radioactive insulin (iodinated with iodine 125) for reaction with this antibody. The insoluble insulin antibody complex is filtered out, and measured by liquid scintillation for radioactivity. The level of radioactivity is inversely related to the amount of insulin present in the sample.

4. Assay of serum free fatty acids (FFA)

The free fatty acids content in the serum was determined by the extraction-titration procedure of Ko and Royer (1966). The method involved the extraction of FFA with 40/10/1 (v/v/v) isopropyl alcohol/heptane/1.0 N H_2SO_4 mixture. The extract was then titrated against 0.01 N tetrabutylammonium hydroxide using thymophthalein as an indicator. Sodium palmitate was used to prepare the standard curve.

5. Assay for *in vitro* utilization of ^{14}C

The incubation procedure and methods used for isolating ratioactive metabolites were similar to those reported by Leveille (1967).

a) Tissue preparation

Pieces of diaphragm muscle weighing approximately 100 mg were cut free from the rib cage, blotted dry and weighed. Hemidiaphragms were obtained by disecting through

the xyphoid, central tendon and vertebra, leaving intact the attachment of each hemidiaphragm to its half of rib cage. After incubation, the hemidiaphragm was washed three times in saline and the diaphragm muscle disected free, washed three times in saline, blotted dry after each washing and weighed. Parametrial adipose tissues weighing about 100 mg were cut free and weighed before incubation. Slices (approximately 0.5 mm thick) of the left lateral lobe of the liver weighing about 200 mg were prepared by a Stadie-Riggs microtome and weighed.

b) Incubation procedure

The tissue was placed in a 25 ml Erlenmeyer flask containing Krebs-Ringer buffer and substrate. All incubation flasks were precoated with silicone to prevent non-specific loss of insulin by adherence to the vessel wall. The flask was capped with a rubber stopper with a hanging well containing a folded 2 cm square filter paper. The tissue was incubated in a metabolic shaking incubator at 37°C for two hours. At the end of the incubation 0.1 ml of 25% hyamine hydroxide was injected into the hanging well, saturating the filter paper, and 0.5 ml of 0.2 N H_2SO_4 was injected into the buffer. Incubation was continued for another 20 minutes to liberate CO_2 from the buffer. The filter paper was then removed and placed in a scintillation vial. The tissue was rinsed in normal saline three times, blotted dry on filter paper after each rinse and then put

into a saponification tube containing 15 ml 2/1 (v/v) chloroform/methanol mixture.

c) Preparation of fractions for counting radioactivity

i) Filter paper - $^{14}CO_2$

The filter paper in the scintillation vial was flattened on the bottom of the vial and 12 ml of toluene scintillant was then added. For the blank, a clean filter paper and 12 ml of toluene scintillant were used.

ii) Glycogen

The tube containing the tissue was stoppered and placed on a shaking machine for three hours. Excess chloroform/methanol was squeezed out of the tissue by tissue forceps. The tissue was placed into a screw-capped tube containing 3 ml of 30% KOH. The tube was heated in boiling water bath for a few minutes to dissolve the tissue. Aliquots of 0.5 ml Na_2SO_4 (saturated) and 4 ml of 95% ethanol were then added. The tube was warmed but not boiled for one minute and then placed in a refrigerator overnight to precipitate the glycogen. After a 10 minutes centrifugation at 1,000 rpm, the supernatant was discarded and the tube was inverted and allowed to drain for 5 minutes. The precipitated glycogen was dissolved in 2 ml of H_2O and 1 ml of the solution was transferred to a scintillation vial containing 11 ml of "Aquasol'. One ml of water and 11 ml of 'Aquasol' were used for the blank. 'Aquasol' was purchased from New England Nuclear.

iii) Non-saponifiable fraction

Three point eight ml of lipid salty was (0.05% $CaCl_2$) were added to the saponification tubes containing the lipid extract. The two phases were mixed, then allowed to separate by standing and the upper phase was discarded. The lipid extract was then washed with 7.5 ml lipid salty wash upper phase (3/48/47 (v/v/v) chloroform/methanol/0.05% $CaCl_2$ solution) and the upper phase was again discarded. The extract was evaporated to dryness in a hot water bath under a stream of nitrogen. The lipid remaining was saponified by adding 6 ml of 3% KOH in methanol, and heating for 30 minutes for adipose tissue or 3 hours for liver at 80 to 85°C. After cooling to room temperature 6 ml of H_2O were added. The non-saponifiable fraction was obtained by three successive extractions with 5 ml portions of petroleum ether. The petroleum ether fraction was transferred to a scintillation vial, evaporated to dryness, and the residue dissolved in 12 ml of toluene scintillant. The blank consisted of 12 ml of toluene scintillant.

iv) Fatty acids

After the extraction of the non-saponifiable fraction, the content of the saponification tubes was acidified with concentrated HCl. Congo Red Paper was used to check for the effectiveness of the acidification. The fatty acid fraction was obtained by 3 consecutive extractions with 5 ml portions of petroleum ether. The fatty acid extract was transferred to a scintillation vial, evaporated to dryness

and the residue dissolved in 12 ml of toluene scintillant. The blank consisted of 12 ml of toluene scintillant.

v) Glyceride-glycerol

After the extraction of the fatty acid fraction, the aqueous layer in the saponification tube was diluted to a volume of 20 ml in a graduated cylinder. One ml of this solution was transferred to a scintillation vial containing 11 ml of 'Aquasol' (New England Nuclear). The blank consisted of 1 ml of H_2O and 11 ml of 'Aquasol'.

6. Liquid scintillation counting

Three different types of scintillants were used for sample preparation. The toluene scintillant (Tri-Carb Liquid Scintillation Spectrometer Operation Manual, Packard Instrument Company, Inc., La Grange, Illinois, 1959), containing 0.4% PPO and 0.010% POPOP was used for the counting of fatty acids, non-saponifiable fraction or CO_2 and adipose tissue digest in hyamin hydroxide samples. In Experiment 2, a Polytron homogenizer (Brinkmann Instruments) was used to disintegrate intestinal contents, liver and diaphragm. For tissue homogenates and intestinal contents the solvent used was p-dioxane containing 10% naphthalene, 1% PPO, 0.025% POPOP and 4% Cab-O-Sil to hold the samples in a fine suspension (Gordon and Wolfe, 1960). In Experiments 6 and 7, the diaphragm and adipose tissues were solubilized in 'Protosol' (New England Nuclear) and 'Aquasol' was used as the scintillation solution. 'Aquasol' was also used for

glycogen sample preparation. The radioactivity of the samples was determined in a Nuclear-Chicago Unilux I Scintillation system. The counting efficiency of the instument was determined by the channel ratio method (Bush 1963). Samples were each counted to an accuracy of ± 2% according to the statistical procedure of Loevinger and Berman (1951).

7. Animals and diets

Ten-week-old female Sprague Dawley rats (Spartan Research Animals Inc., Williamston, Michigan) weighting between 250 and 270 g were housed individually in suspended wire cages in a temperature (22°C) and light regulated room. The rats were fed ad libitum a nutritionally adequate grain diet (Yang et al. 1969) (Appendix A1) for one week to allow them to acclimatize to the cages and the ration. In the first and second experiments, the rats were divided into groups of fours according to their body weight after the acclimatization period. Mestranol and norethynodrel were fed either singly or in combination by incorporating 0.027 mg mestranol or 1.83 mg norethynodrel or both into each kilogram of the grain diet. Each rat in the three groups received an amount of feed equal to that consumed the previous day by the rat fed ad libitum the diet containing mestranol plus norethynodrel. The diets provided approximately 0.1 mg of norethynodrel or 1.5 Mg of mestranol or both per kilogram of body weight per day. These levels are comparable, on a body weight basis to those used by women

for contraceptive purposes. In subsequent Experiments 3, 4, 5, 6, 7, the rats were divided into two groups, a control and an oral contraceptive fed group, according to their body weight. Mestranol and norethynodrel were fed in combination to the oral contraceptive group. The dosage of the steroids, and the pair-feeding procedure were similar to those of the former experiments. However, in Experiment 8, the concentration of the steroids was increased to compensate for the decrease in food intake because of the meal feeding method employed. In Experiment 8, treated rats were offered the diet ad libitum for two hours from 5 to 7 p.m. During this two-hour period, control rats were pair-fed to the treated rats the diet without steroids. Water was given ad libitum to all groups. Specific treatment procedures and duration for each experiment are shown in the figure legends and table footnotes. Daily feed consumption and weekly weight gain were recorded (Appendix A2, 3, 4).

8. Statistical analysis

All data were analysed statistically by analysis of variance (Steel and Torrie 1960), and the experimental design is indicated in the figure or table legends. Unless stated otherwise, all tests of significance were made at the 5% probability level.

PART I

EFFECT OF ORAL CONTRACEPTIVES, NORETHYNODREL AND MESTRANOL ON ORAL GLUCOSE TOLERANCE, TISSUE UPTAKE OF GLUCOSE-U-^{14}C AND SERUM INSULIN LEVELS

Experiments 1 - 3

OBJECTIVES

The first experiment was conducted to determine which of two common steroidal agents used in contraceptive preparations is responsible for changes in glucose tolerance. In the second experiment, the in vivo tissue uptake and utilization of glucose-U-^{14}C were measured in the liver, diaphragm and parametrial adipose tissue, to determine whether they are responsible for the impairment of glucose tolerance. Gastric emptying and gastrointestinal absorption were also determined in the second experiment since a delay in glucose absorption could result in apparent impairment of glucose tolerance. The $^{14}CO_2$ exhaled by rats gavaged with glucose-U-^{14}C was also measured and used to estimate the rate of glucose oxidation in the intact animal. The insulin response after an oral glucose load was measured during the third experiment to see if the pancreas is involved in the impairment.

EXPERIMENTAL DESIGNS AND MATERIALS

Experiment 1

The experiment involved 4 treatments, a control, a mestranol only, a norethynodrel only and a mestranol plus norethynodrel group. Thirty-two, 11-week-old female Sprague Dawley rats were divided into four groups and were subjected to their respective diets containing the appropriate steroid. The control, mestranol and norethynodrel groups were given the same amount of food as that consumed ad libitum by the mestranol plus norethynodrel group on the previous day. After two, and four weeks of pair-feeding, oral glucose tolerance tests were performed on fasted rats.

Experiment 2

The design of this experiment was the same as that of the first but had a total of 60 rats. Since in Experiment 1, the effect of the steroids did not manifest itself until the fourth week of treatment, the length of time was thus increased to 6 weeks to insure maximal effects. Tissue uptake and utilization of glucose were measured using glucose-U-^{14}C. Glucose (300 mg/100 g body weight) and glucose-U-^{14}C (1.67 μCi/300 mg glucose load) in a 45% glucose solution (w/v) were given by stomach tube to rats fasted for 18 hours. Immediately after gastric intubation the rat was placed in a respiratory chamber and the expired air collected into hyamine hydroxide traps (Yang et al. 1970) to absorb CO_2. The air flow into the chamber was

adjusted to maintain the chamber temperature near 22°C. The hyamine traps were changed every 20 minutes to insure complete absorption of CO_2. The method used for counting $^{14}CO_2$ has been described previously (Yang et al. 1970).

The rates of gastric emptying and of glucose absorption from the gastrointestinal tract were determined by methods similar to those described by Cori (1925). For this purpose, rats were removed from the respiratory chamber at different time intervals and decapitated. The alimentary tract was exposed and ligatures were placed at the cardiac, pyloric and ileo-cecal sphincters. The stomach and intestine were removed separately, blotted dried and their contents washed out and made up to 100 ml with isotonic saline. Portions of liver (150-200 mg) and diaphragm muscle (150-200 mg) were rapidly excised and homogenized in saline. Radioactivities in the gastrointestinal contents, liver and diaphragm homogenates were determined using a method similar to that described by Yang et al. (1972) and described in the methods and materials section. Samples of parametrial adipose tissue (100-150 mg) were digested in 2 ml of hyamine hydoxide. The radioactivity of the digest was counted as described in the methods and materials section.

Experiment 3

The third study consisted of two treatments, a control and a combination contraceptive (mestranol plus norethynodrel) group. Ten, 11-week-old female Sprague Dawley rats were

involved in each treatment. After 10 weeks of steroid treatment, the rats were fasted for 18 hours and oral glucose tolerance tests were performed on them. The serum collected by heart puncture at various time intervals during the oral glucose tolerance test was used for glucose and insulin determinations.

RESULTS

Experiment 1

After two weeks of steroid treatment, there were no significant differences in the glucose tolerance curves among the four groups of rats (Fig. 5A). However, an additional two weeks of treatment resulted in a higher serum glucose level for the steroid-treated than for control rats (Fig. 5B). Analysis of variance revealed that norethynodrel was responsible for the overall increase in serum glucose levels in steroid-treated rats. These elevations of serum glucose in the norethynodrel-treated rats were most prominent at 40, 80 and 160 minutes. Thus, these data revealed that norethynodrel was the major determinant of the elevated serum glucose and the impaired glucose tolerance. Mestranol alone did not modify the glucose tolerance curve significantly, in fact, the addition of this estrogenic compound seemed to partially revert the effect of norethynodrel.

Experiment 2

Gastric emptying and gastrointestinal absorption of an oral glucose load in the combination and in the mestranol

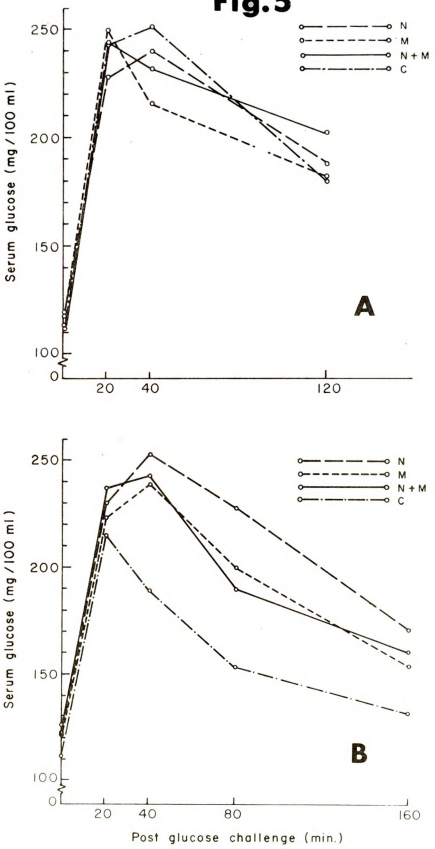
WATCH

FOR

L.H.P.

Figure 5. Serum glucose levels during oral glucose tolerance test: (A) two weeks, (B) four weeks of norethynodrel (N) and/or mestranol (M) treatment (8 animals/group), (C) represents the control group. Analysis of variance by split plot design.

Fig.5


250
200
150
100
0
Serum glucose (mg/100 ml)
N
M
N + M
C
A
20
40
120
250
200
150
100
0
Serum glucose (mg/100 ml)
N
M
N + M
C
B
20
40
80
160
Post glucose challenge (min.)

group were significantly lower than in the norethynodrel and control group (Figs. 6A and B). Mestranol appeared to be the main factor in decreasing gastric emptying and gastrointestinal absorption of glucose.

There were no significant differences in the uptake of radioactivity from the glucose load by liver (Fig. 7A) and diaphragm muscle (Fig. 7B), between rats fed the control diet or the diet containing one or both steroids. However, the ability of adipose tissue (Fig. 7C), to take up radioactivity was significantly lower in the combination and in the norethynodrel group than in the control and mestranol group. Norethynodrel was again the major causative agent in reducing uptake of radioactivity from glucose-U-^{14}C. There were no significant differences in the conversion of glucose into CO_2 (Fig. 8) among rats fed the control diet or diets containing the two hormones or the combination of hormones. In all cases, as expected, the uptake of radioactivity by tissues and the conversion of glucose into CO_2 increased with time after oral loading (Figs. 7 and 8).

Experiment 3

No significant differences in the levels of serum insulin (Fig. 9) during the oral glucose tolerance test were observed between the control and the steroid-treated groups. However, the steroid treatment resulted in the impairment of glucose tolerance. The glucose tolerance curve (Fig. 9) of the treated group was significantly

Figure 6. Gastric emptying and gastrointestinal absorption of a glucose-U-^{14}C load after 6 weeks of norethynodrel (N) and/or mestranol (M) treatment (5 animals/group/time interval). (C) represents the control group. Analysis of variance by a completely randomized design.

Fig. 6

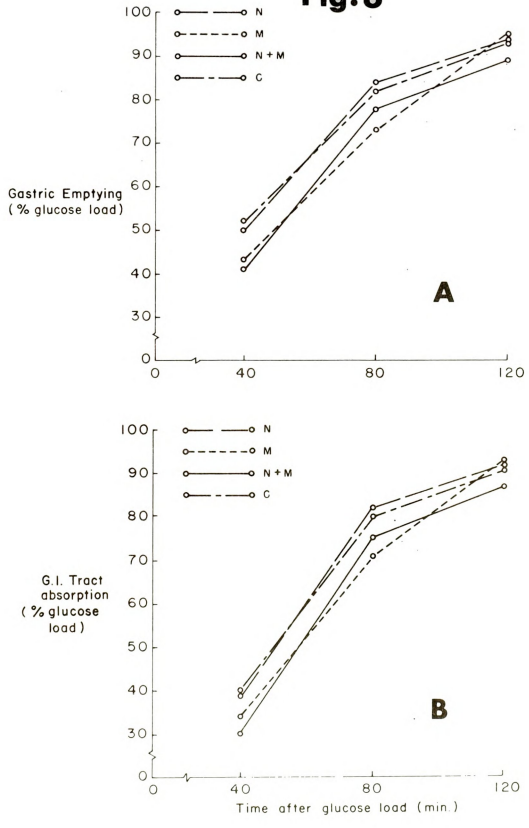

N
M
N + M
C
Gastric Emptying
(% glucose load)
100
90
80
70
60
50
40
30
0
40
80
120
A
G.I. Tract
absorption
(% glucose
load)
B
Time after glucose load (min.)

Figure 7. Radioactivities in liver, diaphragm and adipose tissues at various time intervals after a glucose-U-^{14}C load. Six weeks of norethynodrel (N) and/or mestranol (M) treatments were employed (5 rats/group/time interval). (C) represents the control group. Analysis of variance by a completely randomized design.

Fig.7

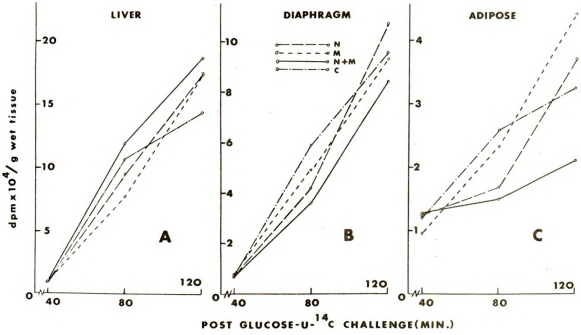
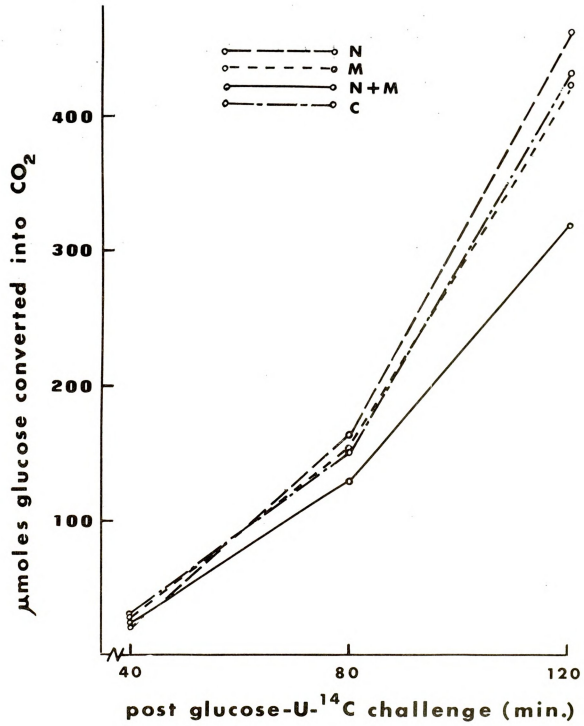

LIVER
DIAPHRAGM
ADIPOSE
N
M
N+M
C
A
B
C
dpm x 10⁴/g wet tissue
POST GLUCOSE-U-¹⁴C CHALLENGE(MIN.)
0
40
80
120
5
10
15
20
2
4
6
8
1
3

Figure 8. Glucose-U-^{14}C converted into expired $^{14}CO_2$ after 6 weeks of norethynodrel (N) and/or mestranol (M) treatment (5 animals/group/time interval). (C) represents the control group. Analysis of variance by a completely randomized design.

Fig.8


N
M
N+M
C
400
300
200
100
μmoles glucose converted into CO_2
40
80
120
post glucose-U-^{14}C challenge (min.)

different from that of the control. The elevations of serum glucose in the steroid-treated rats were most prominent at 40, 80 and 160 minutes.

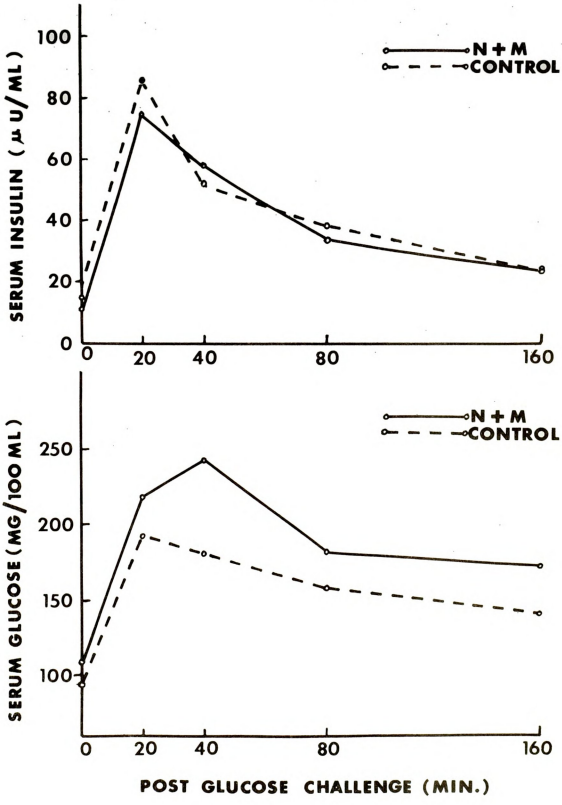
DISCUSSION

In the present experiments, norethynodrel appeared to be responsible for the decrease in oral glucose tolerance (Fig. 5) and in tissue utilization of glucose (Fig. 7C). This conclusion is strengthened by the finding that norethynodrel decreased also the rate of glucose uptake by adipose tissue in the *in vivo* experiment (Fig. 7). This effect required at least four weeks of steroid treatment. A similar glucose intolerance has also been observed in some, but not in all rats when norgestrel was given alone or with ethynyl estradiol for two weeks (Fenichel *et al.* 1969). Thus, about four weeks of treatment appears to be necessary for the development of glucose intolerance. This is suggested also by the experiments of Young and Yang (1971). Steroid treatment for 10 weeks also resulted in impairment of glucose tolerance (Fig. 9) and thus it seems that no recovery occurred and the influence of the steroid was similar whether the treatment was for four or ten weeks (Figs. 5 and 9).

The slower rate of gastric emptying and gastrointestinal absorption observed in rats treated with mestranol and with mestranol plus norethynodrel, although statistically significant, appears to be too small to have appreciable

Figure 9. Serum insulin and glucose levels during oral glucose tolerance test after 10 weeks of norethynodrel plus mestranol (N + M) treatment. Broken line represents control group. Ten rats/ treatment. Analysis of variance by split plot design.

Fig.9


N + M
CONTROL
SERUM INSULIN (μU/ML)
100
80
60
40
20
0
0
20
40
80
160
N + M
CONTROL
SERUM GLUCOSE (MG/100 ML)
250
200
150
100
0
20
40
80
160
POST GLUCOSE CHALLENGE (MIN.)

effects on the level of blood glucose and on the rate of glucose metabolism in various tissues (Fig. 6). Buchler and Warren (1966) found that the oral administration of diethylstilbestrol or norethynodrel plus mestranol in women decreased their tolerance for oral glucose but not that for intravenous glucose. Thus, they postulated that estrogen might impair the gastrointestinal absorption of glucose rather than exerting a diabetogenic effect.

No statistically significant differences between the rate of conversion of glucose into respiratory CO_2 by the four groups of rats in the second experiment were noted (Fig. 8). This finding is not in accord with the results of Young and Yang (1971). In the latter experiments, however, the rats were treated for a much longer period of time and the radioactive glucose was injected intravenously rather than given orally. Norethynodrel and mestranol had little adverse effect on the oxidation of glucose to CO_2 in short-term feeding trials, confirming the importance of the length of treatment.

Although oral glucose tolerance was impaired after ten weeks of steroid treatment, the serum insulin level during the glucose tolerance test was unaffected (Fig. 9). This is contrary to the elevated plasma insulin levels (Table 4) during glucose tolerance tests observed in women on oral contraceptive treatment. In humans, it would appear that the insulin levels become higher in response to the elevated blood glucose level during glucose

tolerance test. However, the present findings would indicate that the response of the β cells of the rat pancreas to a glucose load was not affected by the steroid treatment despite the impaired glucose tolerance.

In a long term study with women receiving the combination drug, Spellacy (1970) found significant elevations in plasma insulin and glucose values after one year of therapy. Subsequent tests at 2 and 3 years have demonstrated that the average insulin and glucose curves return toward normal but some individuals still developed abnormal glucose tolerance curves. It is possible that the oral contraceptive treated rats may have developed elevated plasma insulin levels during the impaired glucose tolerance tests after short term steroid treatment, but the insulin levels reverted towards normal after ten weeks of steroid treatment.

PART II

EFFECT OF ORAL CONTRACEPTIVES, NORETHYNODREL AND MESTRANOL ON THE RESPONSE OF PERIPHERAL TISSUES TO INSULIN

Experiments 4 - 7

OBJECTIVES

The purpose of Experiment 4 was to determine the effect of oral contraceptives on the influence of exogenous insulin on the conversion of blood glucose into fatty acids and glyceride-glycerol in the adipose tissue and glycogen in the diaphragm and adipose tissue of rats. Furthermore, the influence of exogenous insulin on the levels of serum glucose and free fatty acids was also examined in these rats. The 5th experiment was conducted to elucidate the effect of oral contraceptive steroids on insulin sensitivity of peripheral tissues, in terms of glucose conversion into glycogen by the diaphragm muscle and into non-saponifiable lipids, fatty acids and glyceride-glycerol by the parametrial adipose tissue, in an in vitro system. The CO_2 evolved during the incubation of these tissues was also measured. The effect of oral contraceptive steroids on the conversion of glucose into CO_2, glycogen, non-saponifiable lipids, fatty acids and glyceride-glycerol by liver slices was also determined. Since glucose is freely

permeable in the liver, insulin was not added to the incubation medium.

EXPERIMENTAL DESIGNS AND MATERIALS

Experiment 4

The study consisted of a control and a combination contraceptive group. Sixteen, 11-week-old female Sprague Dawley rats were involved in each group. After 6 weeks of treatment, the rats were fasted for 12 hours. Eight pairs of rats were injected intravenously with a tracer dose of glucose-U-^{14}C and 0.2 U insulin/kg body weight. Another eight pairs were injected with glucose-U-^{14}C but not insulin to use as controls for the insulin-treated rats. Fifteen minutes after the injection, the animals were decapitated. The diaphragm and parametrial adipose tissues were rapidly excised and the radioactivity of various metabolites fractionated and processed as described in the method section. Blood was also collected for serum glucose and free fatty acids determinations.

Experiment 5

The experiment consisted of two groups of animals, a control and a norethynodrel plus mestranol fed group. Ten, 11-week-old female Sprague Dawley rats were used in each group. After 6 weeks of steroid treatment the rats were fasted for 12 hours, decapitated and various tissues rapidly excised. Pieces of parametrial adipose tissue weighing about 100 mg, slices of the left lateral lobe of the liver weighing about 200 mg and pieces of diaphragm

muscle weighing approximately 100 mg were used to determine the incorporation of ^{14}C from glucose-U-^{14}C into various metabolic products. Various levels of insulin were added to the incubation medium to determine the insulin sensitivity of the tissue. The concentrations of substrate and of insulin in the Krebs-Ringer bicarbonate buffer (pH 7.4) are indicated in the legends of figures and table. The incubation procedure and methods used for isolating radioactive metabolites have been described in the method section and were similar to those reported by Leveille (1967). However, hyamine hydroxide was used instead of NaOH for trapping $^{14}CO_2$ evolved during incubation.

Experiments 6 and 7

Each study consisted of 2 treatments, a control and a norethynodrel plus mestranol group. Ten, 11-week-old female Sprague Dawley rats were involved in each group. The care of animals, length of experiment, tissue preparation and incubation procedure were similar to Experiment 5. However, 2-deoxyglucose-1-^{14}C was used instead of glucose-U-^{14}C. The concentrations of 2-deoxyglucose and of insulin in the Krebs-Ringer bicarbonate buffer (pH 7.4) are indicated in the table footnotes.

RESULTS

Experiment 4

Blood glucose conversion into adipose fatty acids, glyceride-glycerol and muscle glycogen was found to be significantly higher in the insulin-injected than the

saline-injected rats (Table 6). The only exception in this respect was adipose tissue glycogen. However, the levels of serum free fatty acids, serum glucose, and the serum radioactive materials were observed to be significantly lower in the insulin injected than in saline-injected groups (Table 6).

The effect of exogenous insulin on the conversion of blood glucose into fatty acids by the adipose tissue was significantly lower in the steroid-treated than that of the control rats (Table 6). No significant differences were observed between the insulin or saline injected steroid-treated and control rats in levels of serum glucose, free fatty acids and serum radioactive materials, and also in the conversion of blood glucose into adipose tissue glyceride-glycerol, adipose tissue and diaphragm glycogen. However, the effect of insulin on the conversion of blood glucose into adipose tissue glyceride-glycerol appeared to be lower in the oral contraceptive group than the control. In addition the levels of serum fatty acids and serum radioactive materials appeared to be higher in the insulin injected oral contraceptive group than that of the control.

Experiment 5

The conversion of glucose into CO_2, non-saponifiable lipid, fatty acids and glyceride-glycerol of the control adipose tissue was increased significantly with each level of insulin added into the incubation medium (Fig. 10). On the other hand, the conversion in the tissue from the

Table 6. Effect of insulin on in vivo utilization of glucose by oral contraceptive-fed and control rats[a]

Tissue	Product	Injection: Saline — Control	Injection: Saline — Steroids	Injection: Insulin — Control	Injection: Insulin — Steroids
			dpm/100 mg tissue		
Adipose	Fatty acids	8.2±1.0[b]	7.7±0.9	186.4±73.9	35.4±12.4
	Glyceride-glycerol	166±18	178±25	1159±232	837±72
	Glycogen	22.9±2.6	25.6±3.7	24.6±4.0	24.2±2.2
			dpm/100 mg tissue		
Diaphram muscle	Glycogen	346±81	226±44	1146±121	1044±169
			mg/100 ml		
Serum	Glucose	79.3±4.9	74.6±2.3	48.3±5.1	47.1±2.2
			dpm/ μ mole glucose		
	Radioactive materials	14144 ±837	14864 ±1561	10514 ±1241	13333 ±1060
			μ eq/L		
	Free fatty acids	598±48	600±61	266±24	337±28

[a] Contraceptive-fed and control rats maintained without food for 12 hours were injected intravenously 15 mins. prior to killing with 24 μCi glucose-U-^{14}C/kg body weight and, where indicated, 0.2 U insulin/kg body weight.

[b] Mean for 8 observations ± standard error of mean.

steroid-treated rat plateaued near the lowest level of insulin used in the medium. Therefore, the conversion into non-saponifiable lipid and fatty acids in the control tissue was significantly higher than in the treated tissue, when either 100 or 200 μU of insulin were added to 1 ml of incubation medium ($P < 0.1$). These two levels of insulin caused a progressive response in the control but had only a slight effect on the steroid-treated tissue (Figs. 10B and C). Fatty acids ($P < 0.06$) and non-saponifiable lipid, but not CO_2 and glyceride-glycerol, synthesis from glucose were significantly lower in the steroid-treated than in the control tissue.

The conversion of glucose to CO_2 and glycogen (Fig. 11) by the control and steroid-treated diaphragm muscle was significantly increased by insulin. Maximum effect in the control and in the treated group was obtained with 300 and with 500 μU of insulin per ml of incubation medium, respectively. Thus the control tissue was more sensitive to insulin than the steroid-treated tissue.

No significant differences were observed between the conversion of glucose to CO_2, glycogen, non-saponifiable lipid, fatty acids or glyceride-glycerol, by liver slices of control and treated rats (Table 7).

Experiment 6

The uptake of 2-deoxyglucose by the hemidiaphragm was found to be significantly higher at 5,000 μU of insulin than the 500 μU of insulin for both groups of rats.

Figure 10. *In vitro* glucose-U-^{14}C conversion into (A) CO_2, (B) non-saponifiable fraction, (C) fatty acids, (D) glyceride-glycerol by adipose tissue of control (broken line) and contraceptive (solid line) treated rats. Six weeks of steroid treatment were involved. All buffers contained (per ml): glucose, 10 μ moles; glucose-U-^{14}C, 0.2 μCi; and insulin 0, 10, 100, 200 μU. Analysis of variance by split plot design.

Fig. 10

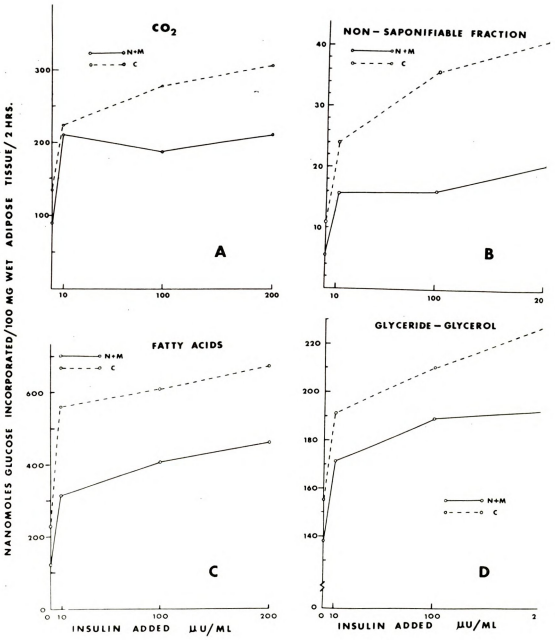

CO2
N+M
C
A
NON – SAPONIFIABLE FRACTION
B
FATTY ACIDS
C
GLYCERIDE – GLYCEROL
D
NANOMOLES GLUCOSE INCORPORATED/100 MG WET ADIPOSE TISSUE/2 HRS.
INSULIN ADDED μU/ML
INSULIN ADDED μU/ML

Figure 11. *In vitro* glucose-U-^{14}C conversion into carbon dioxide and glycogen by diaphragm muscle of control (broken line) and contraceptive - (solid line) treated rats. Six weeks of steroid treatment was involved. All buffers contained (per ml): glucose, 10 μ moles; glucose-U-^{14}C, 0.2 μCi; and insulin 0, 300, 500, 700 μU. Analysis of variance by split plot design.

Fig. II

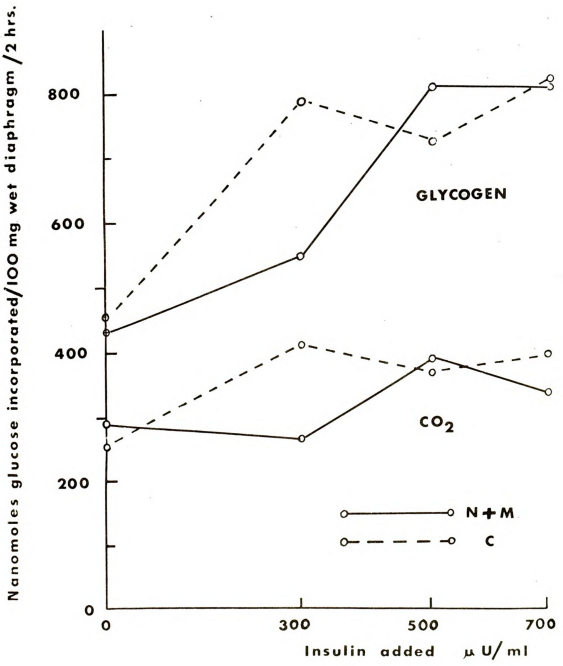

Nanomoles glucose incorporated/100 mg wet diaphragm /2 hrs.
800
600
400
200
0
GLYCOGEN
CO_2
N+M
C
0
300
500
700
Insulin added μU/ml

Table 7. In vitro glucose-U-^{14}C utilization by liver slices of control and oral contraceptive-fed rats[a]

Treatment[b]	Incorporation of labeled substrate into				
	Carbon dioxide	Glycogen	Non-saponifiable lipids	Fatty acids	Glyceride-glycerol
	Substrate incorporated Nanomoles/100 mg tissue/2 hrs				
Control	545[c]±41	752±121	14.0±3.4	239±33	177±14
N + M	565± 45	727±152	12.3±2.7	193±33	181±10

[a]The buffer contained (per ml): glucose 100 μ moles, glucose-U-^{14}C, 1 μCi.

[b]10 animals per treatment. N + M represents norethynodrel plus mestranol. Six weeks of steroid treatment was involved.

[c]Mean for 10 observations ± S.E.M.

However, the uptake of 2-deoxyglucose was not significantly higher in the control as compared with the steroid-treated rats at both levels of insulin (Table 8).

No significant differences were observed in the uptake of 2-deoxyglucose by the adipose tissue between the various insulin levels and among the two groups of rats (Table 8).

Experiment 7

The uptake of 2-deoxyglucose by hemidiaphragm (Table 9) appeared to be higher in the control as compared to the steroid-treated rats at both levels of insulin. However, the differences were not statistically significant. The uptake of 2-deoxyglucose by the hemidiaphragm was higher at 1,000 μU of insulin than the 300 μU of insulin for both groups of rats. But the differences were not significant.

No significant differences were observed in the uptake of 2-deoxyglucose by the adipose tissue between the various insulin levels and among the two groups of rats.

DISCUSSION

The effect of exogenous insulin on the conversion of blood glucose into fatty acids by the adipose tissue was significantly lower in the steroid-treated than that of the control rats (Table 6). A similar trend was observed for the conversion of blood glucose into adipose glyceride-glycerol even though the results were nonsignificant. Because of the reduced incorporation of blood glucose into adipose tissue fatty acids by the insulin injected, steroid-

Table 8. *In vitro* uptake of 2-deoxyglucose-1-^{14}C by hemidiaphragm and parametrial adipose tissue of control and oral contraceptive-fed rats[a]

	Control		Oral Contraceptive	
	Level of insulin μU/ml buffer[b]			
	500	5000	500	5000
Tissue	Uptake of substrate (nanomoles/100 mg tissue/ 90 mins.)			
Hemidiaphragm	692[c]	914	584	847
	±35	±55	±17	±59
Parametrial adipose	134	139	123	118
	± 9	±11	±13	±22

[a] After 6 weeks of steroid treatment, the rats were decapitated following an 18 hour fast.

[b] All buffer contained (per ml): 2-deoxyglucose, 10 μ mole; 2-deoxyglucose-1-^{14}C, 0.1 μCi; and various levels of insulin as indicated. Each incubation flask contained 4 ml of buffer for hemidiaphragm and 3 ml of buffer for adipose tissue.

[c] Mean for 10 rats ± S.E.M.

Table 9. In vitro uptake of 2-deoxyglucose-1-^{14}C by hemidiaphragm and parametrial adipose tissues of control and oral contraceptive-fed rats[a]

Tissue	Control		Oral contraceptive	
	Level of insulin μ U/ml buffer[b]			
	300	1000	300	1000
Hemidiaphragm	Uptake of substrate (nanomoles/100 mg tissue/ 90 mins.)			
	518±55[c]	565±57	458±60	520±67
	Level of insulin μ U/ml buffer[b]			
	100	1000	100	1000
Parametrial adipose	Uptake of substrate (nanomoles/100 mg tissue/ 90 mins.)			
	124±4	135±5	128±8	134±8

[a] After 6 weeks of steroid treatment, the rats were decapitated following an 18 four fast.

[b] All buffers contained (per ml); 2-deoxygluce, 10 μ moles; 2-deoxygluce-1-^{14}C, 0.1 μCi; and various levels of insulin as indicated. Each incubation flask contained 4 ml of buffer for the hemidiaphragm and 3 ml of buffer for the adipose tissue.

[c] Mean for 10 rats ± S.E.M.

treated rats (Table 6), the lipogenic effect of exogenous insulin appeared to have diminished. This is strengthened by the findings of a similar reduction in the conversion of glucose into fatty acids, at various insulin levels, by the adipose tissues of steroid-treated rats observed in the in vitro experiment (Fig. 10C). It is of interest to observe that the steroid-treated adipose tissue is resistant to the lipogenic effect of exogenous insulin in both in vivo and in vitro system.

The results of the in vitro experiments (Figs. 10 and 11) indicate that less insulin was required to stimulate maximum glycogensis by diaphragm tissue from control than from steroid-treated animals and thus suggest that muscle and adipose tissue may play a significant role in the impairment of glucose tolerance in steroid-treated rats.

Steroid treatment also depressed lipogenesis and conversion of glucose into non-saponifiable lipid of adipose tissue (Fig. 10). There was a two-fold increase in lipogenesis in the control tissue when 10 μU of insulin were added to the medium and further addition of insulin produced only a slight increment of lipogenesis. A similar type of dose-response relationship was observed by Crofford (1968) using fat cells isolated from rat epididymal tissues. Thus, our results suggest that the impairment of glucose tolerance following oral treatment with contraceptive steroids could be partly the result of a mild resistance to insulin in the adipose and muscle

tissues. A mild peripheral resistance to the hypoglycemic action of exogenous insulin and an enhancement of plasma insulin response to intravenous glucose have been observed also in progesterone - (Beck 1969b) and mestranol - (Beck and Wells 1969b) treated female rhesus monkeys. Furthermore, elevated fasting serum glucose concentrations, decreased glucose tolerance and increased serum insulin concentrations have been reported in women receiving oral contraceptives (Spellacy et al. 1971). In these women, peripheral insulin resistance may have caused persistent hyperglycemia and hyperinsulinism. Since insulin may increase the synthesis of glycogen (Moody and Felber 1966) and of fatty acids from glucose independently of its effect on glucose transport, Experiments 6 and 7 were designed to determine whether the resistance to insulin occurs at the point of glucose entry into the cells or at subsequent steps in glycogenesis or lipogenesis.

The 2-deoxyglucose taken up by the tissue is not metabolized any further after its initial phosphorylation to 2-deoxyglucose-6-phosphate (Kipnis and Cori 1959) and it was used to measure the effect of the steroids on glucose transport. In both Experiments 8 and 9, the uptake of 2-deoxyglucose by the hemidiaphragms appeared to be slightly lower in the steroid-treated than the control rats. But the differences were small and not statistically significant. Since no significant differences were observed in the uptake of 2-deoxyglucose by the diaphragm and adipose

tissue of the control and steroid-treated rats, the glucose transport across the cell membrane appeared not to be affected by the steroid treatment. So it seems that the resistance to insulin observed in Experiments 4 and 5 occurs not at the point of glucose entry or at its immediate phosphorylation but at subsequent steps in glycogenesis and lipogenesis.

PART III

THE EFFECT OF ORAL CONTRACEPTIVES ON LIPOGENESIS AND GLYCOGENESIS IN MEAL-EATING RATS

Experiment 8

OBJECTIVES

Since oral contraceptives depress feed intake slightly in rats (Manoharan et al. 1970), the pair-feeding procedure was utilized in the former experiments to equalize the feed intake between the oral contraceptive fed and control rats. Questions were raised as to whether there is a tendency for the pair-fed rats to develop into meal-eaters if the restriction of feed intake was too severe and the daily feed comsumption highly variable (Florence and Quarterman 1972). Among the functional changes associated with meal-feeding include increased sensitivity of adipose tissue to insulin (Vrana et al. 1969) and of the animal to diabetogenic agents such as growth hormone (Owen and Engel 1957) and alloxan (Cohn and Joseph 1960). Since some oral contraceptives are diabetogenic (Gershberg et al. 1964) and no studies have been done to determine the effects of oral contraceptives on meal-eating animals, Experiment 8 was designed to investigate the effect of norethynodrel plus mestranol on the influence of insulin on in vitro conversion of glucose-U-^{14}C into various metabolic products by hemi-diaphragm and adipose tissue of meal-eating rats.

EXPERIMENTAL DESIGN AND MATERIALS

Twenty, 11-week-old female Sprague Dawley rats were randomly divided into two groups, a control and a steroid group. The steroids were incorporated into the grain diet (Appendix 1) to provide similar quantities of norethynodrel and mestranol/kg of body weight as in previous experiments. However, the concentrations of the steroids were increased to compensate for the decrease in food intake because of meal-feeding. The treated rats were offered the diet ad libitum for two hours from 5 to 7 p.m. During this two hour period, control rats were pair-fed the diet without the steroids. Both groups of rats had access to water at all times. As expected, both groups of rats lost weight in the first two weeks of meal-feeding but regained most of the weight lost so that at the end of six weeks of experimental period body weights were 225 g for treated and 217 g for control rats. The 8 g difference in body weight was not statistically significant. At this time, the rats were fasted for 18 hours, decapitated and hemidiaphragm and parametrial adipose tissue were used in in vitro studies to determine the conversion of glucose-U-^{14}C into glycogen, fatty acids, glyceride-glycerol and non-saponifiable materials. The methods used were an exact duplication of the method used in Experiment 5.

RESULTS

The incorporation of glucose into glycogen by the diaphragm was found to be significantly higher at 1,000 μU/

ml buffer than at 300 μU/ml buffer for both groups of rats (Table 10). Although the conversion of glucose into glycogen appeared to be lower in the steroid-treated than the control group at 300 μU/ml buffer, the differences between the two groups were not significant.

For the parametrial adipose tissue, the conversion of glucose into several products was significantly lower in the treated than in the control rats at either level of insulin in the incubation medium (Table 10).

DISCUSSION

The incorporation of glucose by the adipose tissue into products measured was significantly lower in the treated than in the control rats at either level of insulin in the incubation medium (Table 10). These data thus substantiate previous findings that six weeks of Enovid treatment decreased lipogenesis in adipose tissue (Fig. 10). However, meal-feeding produced two effects distinctly different when compared to those produced in non-meal-eating rats (Fig. 12). First, the incorporation of glucose into fatty acids in the adipose tissue was enhanced by meal-eating in both control and oral contraceptive-treated rats (Fig. 12). Second, the effect of the oral contraceptive was more pronounced on the meal-eating rats since the difference in the conversion of glucose to fatty acids between contraceptive treated and controls was more than two-fold compared to less than one-fold in the non-meal-eating rats. Thus, meal-eating potentiated the effect of the oral contraceptive.

Table 10. Effect of oral contraceptives on in vitro conversion of glucose-U-^{14}C into various metabolic products by hemidiaphragm and adipose tissue of meal-eating rats[a]

Tissue	Product	Oral contraceptive fed rats		Control rats	
		Levels of insulin μU/ml buffer[b]			
		300	1000	300	1000
Hemi-diaphragm	Glycogen	773±103[c]	1355±150	908±61	1281±113
		Levels of insulin μU/ml buffer[b]			
		100	1000	100	1000
Adipose	Glycogen	49.9±12.6	66.8±14.9	139.2±30.3	168.3±26.3
	Non-saponifiable fraction	3.23±1.18	3.59±0.97	6.06±2.54	7.07±1.20
	Fatty acids	636±106	928±116	1413±176	1758±181
	Glyceride-glycerol	193±15	231±14	264±26	304±25

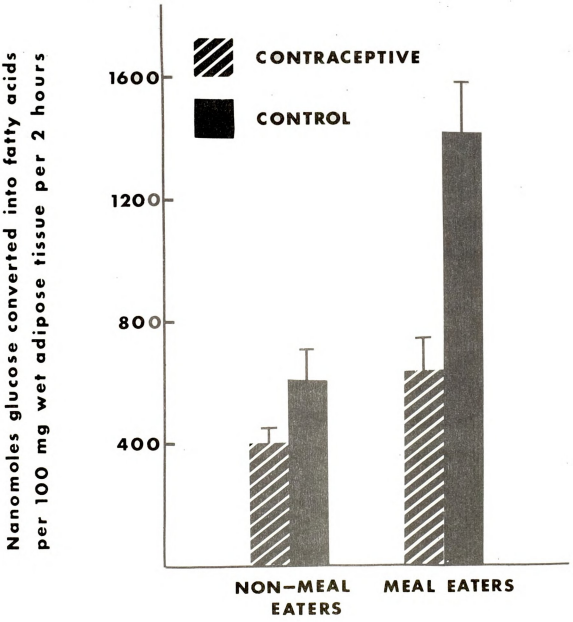
[a]After 6 weeks of steroid treatment, the rats were decapitated following an 18 hour fast.

[b]All buffers contained (per ml); glucose 10 μ moles; glucose-U-^{14}C, 0.2 μCi; and various levels of insulin as indicated. Each incubation flask contained 4 ml of buffer for hemidiaphragm or 3 ml of buffer for adipose tissue.

[c]Mean for 10 rats ± S.E.M.

Figure 12. Comparison of glucose conversion into fatty acids by parametrial adipose tissues of meal-eating vs. non-meal-eating rats. After 6 weeks of contraceptive treatment, tissues from the rats were incubated at 37°C in buffer containing (per ml): glucose, 10 μ moles; glucose-U-^{14}C, 0.2 μCi and 100 μU of insulin. Values for non-meal-eating rats obtained from Fig. 10C. Bars represent averages of 10 rats ± S.E.M.

Fig. 12


Nanomoles glucose converted into fatty acids per 100 mg wet adipose tissue per 2 hours
CONTRACEPTIVE
CONTROL
1600
1200
800
400
NON-MEAL EATERS
MEAL EATERS

Because meal-eating in women is not unusual, the compounding effects of oral contraceptives on lipogenesis in these individuals and other species merits further investigation.

SUMMARY AND CONCLUSIONS

Oral contraceptive steroids, norethynodrel and mestranol were fed singly or in combination to 11-week-old female Sprague Dawley rats to determine their effects on oral glucose tolerance, in vivo tissue uptake and utilization of glucose-U-^{14}C. Although both steroids appeared to be involved, norethynodrel was found to be the major causative factor in the impairment of oral glucose tolerance and the reduction in the level of radioactivity in adipose tissue at various time intervals after an oral glucose-U-^{14}C load. The radioactivity levels in the diaphragm muscle and liver tissue and the conversion of glucose into respiratory CO_2 after the glucose-U-^{14}C load, were not influenced by the steroids. Mestranol slightly suppressed gastric emptying and gastrointestinal absorption of the radioactive glucose load. But the depression appeared to be too small to have appreciable effects on the level of blood glucose and on the rate of glucose metabolism in various tissues. Despite an impairment of oral glucose tolerance, the steroids did not exert any effect on the serum insulin response curve during oral glucose tolerance test after a long term steroid feeding trial (10 weeks). It appeared that the pancreatic beta-cells

were less sensitive or indifferent to hyperglycemia but they still retained the capacity to respond to acute increments of blood glucose.

The conversion of blood glucose into adipose tissue fatty acids in the intact animal, after an intravenous injection of insulin, was found to be lower in the steroid-treated rat than the control. A similar trend was observed in the conversion of blood glucose into diaphragm glycogen but the differences were not significant. The levels of serum free fatty acids and radioactivity in the serum appeared to be lower in the insulin injected control as compared to the steroid-treated rats but the differences were not significant.

Results of the *in vitro* experiment indicate that less insulin was required to stimulate maximum glycogenesis by diaphragm tissue from control than from steroid-treated animals. Steroid treatment also depressed lipogenesis and conversion of glucose into non-saponifiable lipid of adipose tissue. Thus the results of the *in vivo* and *in vitro* experiments indicate that the steroid-treated adipose tissue as well as the diaphragm muscle were slightly resistant to exogenous insulin. The impairment of glucose tolerance following oral contraceptive treatment could be partly the result of peripheral tissue resistance to the hypoglycemic action of insulin.

Since no significant differences were observed in the *in vitro* uptake of 2-deoxyglucose-1-^{14}C, by the isolated

diaphragm and adipose tissue of the control and steroid-treated rats, glucose transport across the cell membrane appeared not to be affected by the steroid treatment. So it seems that the resistance to insulin observed occurs not at the point of glucose entry but at subsequent steps in glycogenesis and lipogenesis.

The data of the meal-feeding experiment substantiated the findings that oral contraceptive steroids decreased lipogenesis in adipose tissue. When compared to a non-meal-eating regime, meal-eating was found to enhance the incorporation of glucose into adipose fatty acids in both control and oral contraceptive-treated rats. And the effect of oral contraceptive in depressing lipogenesis in adipose tissue was more pronounced. Thus, meal-eating potentiated the effect of the oral contraceptives or increased the tissue sensitivity to diabetogenic effect of the steroids.

The alteration of carbohydrate metabolism by oral contraceptives appeared to be small but nevertheless significant in rats. It is hoped that by using animals and human experiments, several steroids will be discovered which when given either together or separately will adequately control fertility and yet produce only minimum adverse metabolic effects. Until this goal is attained, it would seem reasonable to urge physicians to periodically monitor the carbohydrate metabolism of women taking oral contraceptives for long durations.

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APPENDICES

APPENDIX A1

COMPOSITION OF BASAL GRAIN DIET (in %)

Ground corn 60.7; soybean meal (50% protein), 28.0; alfalfa meal (17% protein), 2.0; fish meal (60% protein), 2.5; dried whey (67% lactose), 2.5; limestone (38% Ca), 1.6; dicalcium phosphate (18.5% P, 22-25% Ca), 1.75; iodized salt, 0.5. Supplementary minerals and vitamins were added to provide per kg. of diet: (in mg) Mn, 121; Fe, 95; Cu, 7; Zn, 4; I_2, 4; Co, 2; Choline chloride, 400; Ca pantothenate, 6; riboflavin, 3; niacin, 33; menadione, 2; DL-methionine, 500; (in microgram) vitamin B_{12}, 7; (in I.U.) vitamin A, 8010; vitamin D_2, 750; vitamin E, 5.

APPENDIX A2

Average body weight (± S.E.M.) of experimental animals (g/rat)

Expt.	Treatment	Weeks of steroid treatment: Start	2	4	6
1	control	245±4	255±2	255±4	
	norethynodrel	250±3	254±4	256±4	
	mestranol	246±4	250±4	253±4	
	combined	243±4	251±5	256±4	
	N = 8				
2	control	245±3	249±2	264±3	273±4
	norethynodrel	248±3	252±3	268±4	274±4
	mestranol	246±3	244±2	256±3	266±4
	combined	249±3	253±5	268±5	269±4
	N = 15				
		Start	5	10	
3	control	244±3	237±5	284±6	
	combined	251±5	275±6	286±6	
	N = 10				

APPENDIX A3

Average body weight (± S.E.M.) of experimental animals (g/rat)

Expt.	Treatment	Weeks of steroid treatment: Start	2	4	6
4	control	246±2	247±2	253±2	262±3
	combined	248±3	249±3	256±4	266±4
	N = 16				
5	control	233±3	234±4	250±5	266±6
	combined	236±2	237±3	250±5	259±5
	N = 10				
6	control	246±3	251±4	268±5	279±5
	combined	247±4	251±4	266±4	277±4
	N = 10				
7	control	239±5	244±6	261±6	270±5
	combined	243±6	252±7	266±7	270±6
	N = 10				
8	control	238±3	199±3	207±3	217±3
	combined	239±3	203±2	214±3	226±3
	N = 10				

APPENDIX A4

Average weekly feed consumption (± S.E.M.) of steroid-treated rats (g/ rat/week).

Expt.	Weeks of steroid treatment 1	2	3	4	5	6	7	8	9	10
1	99 ± 3	101 ± 2	95 ± 2	100 ± 3						
N = 8										
2	91 ± 2	99 ± 2	100 ± 3	99 ± 2	95 ± 3	94 ± 3				
N = 15										
3	105 ± 3	110 ± 4	105 ± 4	106 ± 3	107 ± 4	102 ± 5	108 ± 3	107 ± 5	103 ± 3	102 ± 3
N = 10										
4	87 ± 1	93 ± 1	100 ± 3	105 ± 4	102 ± 5	103 ± 2				
N = 16										
5	94 ± 5	97 ± 4	97 ± 2	102 ± 2	100 ± 3	100 ± 1				
N = 10										
6	102 ± 2	113 ± 2	115 ± 3	118 ± 2	113 ± 2	113 ± 2				
N = 10										
7	101 ± 3	109 ± 3	113 ± 3	116 ± 2	109 ± 2	106 ± 3				
N = 10										
8	48 ± 2	67 ± 3	76 ± 2	83 ± 2	91 ± 2	100 ± 2				
N = 10										