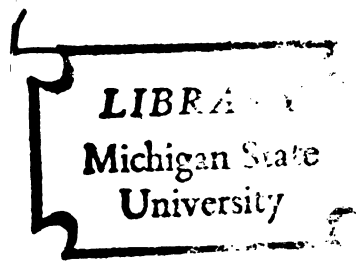


VITAMIN C AND ORAL CONTRACEPTIVES

Thesis for the Degree of M. S.  
MICHIGAN STATE UNIVERSITY  
RAY-SHIANG CHRISTINE SU  
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## ABSTRACT

### VITAMIN C AND ORAL CONTRACEPTIVES

By

Ray-Shiang Christine Su

In order to determine whether norethynodrel or mestranol or a combination of both is responsible for the reported decrease of serum ascorbate concentrations of women receiving oral contraceptives, guinea pigs were fed these compounds and several criteria of ascorbate catabolism measured. For this purpose 40 ovariectomized guinea pigs were divided into four equal groups according to body weight. The guinea pigs were fed either norethynodrel (91  $\mu\text{g/kg}$  body weight), mestranol (1.36  $\mu\text{g/kg}$  body weight) or both or the control diet for 6 weeks. During this time ascorbate was fed to all animals, at 10 mg/kg body weight/day.

At the termination of the treatments, serum ceruloplasmin ascorbic acid oxidase, the enzyme catalyzing the oxidation of reduced ascorbate to dehydroascorbate was measured. No significant changes in the enzyme activity were found. This finding was strengthened by the fact that serum concentrations of ascorbic acid, the substrate for ceruloplasmin, as well as serum dehydroascorbate, the end product of the reaction, were not significantly altered by the oral contraceptive steroids. Since serum



ionized copper [Cu (II)] was also expected to increase the conversion of ascorbate to dehydroascorbate, its non-enzymatic oxidative activity against ascorbate was also determined. This copper oxidative activity was not significantly influenced by mestranol and combination of norethynodrel and mestranol, but a lower ascorbate oxidase activity was found in norethynodrel treated group.

Adrenals, kidneys, livers and spleens were assayed for total ascorbate and dehydroascorbate in order to determine whether the oral steroids might have affected their concentrations and thus provide an explanation for the decreased serum ascorbate concentrations found in human contraceptive users. Except for an increased level of dehydroascorbate in the kidneys of the animals treated with norethynodrel, total ascorbate and dehydroascorbate concentrations and absolute amount in the other organs were not significantly affected by the steroids.

VITAMIN C AND ORAL CONTRACEPTIVES

By

Ray-Shiang Christine Su

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## INTRODUCTION

Several reports indicated that women taking oral contraceptives have decreased plasma ascorbic acid levels (Briggs and Briggs, 1972; Rivers and Devine, 1972; Kalesh et al., 1971). Furthermore, Saroja et al. (1971) showed that guinea pigs fed oral contraceptives had a marked reduction in plasma ascorbic acid concentration and even a greater reduction in the concentration in the blood vessels. Other work done by Kalesh et al. (1971) found that women taking oral contraceptives exhibited significantly lower platelet ascorbic acid levels than controls. Rivers and Devine (1972) reported that total ascorbic acid (reduced plus oxidized) and reduced ascorbic acid concentrations were highest at the time of ovulation and lowest in the late secretory phase and menses, thus suggesting that vitamin C metabolism may be influenced by hormones. However, urinary excretion of ascorbic acid was not consistently related to fasting plasma concentrations during the different phases of the menstrual cycle or ingestion of the oral contraceptives. Ascorbic acid concentrations in both leucocytes and platelets were significantly lower in women taking steroid contraceptives than in untreated controls or in pregnant women. Briggs

and Briggs (1972) suggested that the decrease was likely due to oral contraceptive steroids increasing the breakdown of ascorbic acid perhaps by their stimulant action on liver release of ceruloplasmin. A similar suggestion was made by Clemetson (1968), who presumed that the disturbance of ascorbic acid metabolism caused by high doses of estrogen may well be due to increased ceruloplasmin oxidase activity.

This suggestion came about because Holmberg and Laurell (1951) in their early work on the oxidase activity of ceruloplasmin reported that ascorbic acid was a substrate of ceruloplasmin. The work of Humoller et al. (1960) supported the existence of ascorbic acid oxidase activity of ceruloplasmin. Walter and Frieden (1962) presented evidence for the existence of ascorbate oxidase activity for this enzyme. In 1962, Morell et al. studied the ascorbic acid oxidase activity of ceruloplasmin previously treated with chelex-100 to eliminate non-protein copper ion and concluded that ascorbic acid was not a substrate for ceruloplasmin. But, Osaki et al. (1963) did their work on chelex-treated ceruloplasmin and showed that the reaction was at least many hundred times that predicted from a stoichiometric reaction between ascorbate and Cu (II) of ceruloplasmin.

Therefore, the purposes of this study were to determine the total and dehydroascorbic acid levels in serum, liver, spleen, adrenals and kidneys; and the ceruloplasmin

and copper ascorbic acid oxidase activities in serum of guinea pigs treated with or without mestranol or norethynodrel or both, in order to see whether there is a decrease in ascorbic acid levels and whether the decrease is due to an increased ascorbic acid oxidase activity of ceruloplasmin in the serum.

## LITERATURE REVIEW

### History of Oral Contraceptives

People seeking contraception are as ancient as history. Wide varieties of herbs, plant extracts and mechanical means were employed by various civilizations and societies to prevent pregnancy (Himes, 1936). In modern times, various types of spermatocidal agents, mechanical barriers or the rhythm method were relied on to prevent pregnancy, but a truly effective method was not available until about 1960. With the advent of Enovid in 1962, effectiveness was as high as 100 percent.

Oral contraceptives control fertility by preventing ovulation, in the same way as natural estrogens and progesterone do. It has been known for a long time that ovulation can be prevented by the administration of androgens, estrogens or progesterone. As early as 1897, Beard postulated that corpus luteum was responsible for the inhibition of ovulation during pregnancy (Beard, quoted by Asdell, 1928). Following the isolation of progesterone, the administration of this steroid inhibited ovulation in the rabbit and rat (Makepeace et al., 1937; Astwood and Fenold, 1939).

Since natural steroid hormones are not suitable as oral contraceptives for family planning purposes because of the variable absorption from the gastrointestinal tract (Pincus, 1956), and the occurrence of the severe local irritation at the site of injection, synthesis of oral progestin was therefore advantageous. In 1952, norethynodrel, the progestin in Enovid, was synthesized by Dr. Frank B. Colton at the Searle Laboratories. The progestational effects, pituitary inhibiting activity and other properties of norethynodrel and related structures were studied by Dr. Francis J. Saunders and collaborators. In late 1953, the progestational, estrogenic, pituitary inhibiting and antiovaratory effects of norethynodrel were established by Dr. Gregory Pincus and associates at the Worcester Foundation for Experimental Biology. In 1956, Rock, Garcia and Pincus first demonstrated the contraceptive effectiveness of norethynodrel in women.

In order to decrease the occurrence of spotting and bleeding when a progestin is used alone, an estrogen (mestranol) was incorporated with norethynodrel. It has been found necessary to add an estrogen to other progestins being evaluated as oral contraceptives in order to aid in the maintenance of the endometrium. In 1957, the combination of norethynodrel and mestranol called Enovid was approved for use in the treatment of menstrual problems. Toward the end of 1957, Enovid was approved for use as an oral contraceptive.

A second oral contraceptive, Ortho-Novum, which was approved for use in medical practice in 1962, was a combination of norethindrone and mestranol. Norethindrone was synthesized by Dr. Carl Djerassi and associates at the Syntex Laboratories and was evaluated biologically by Saunders et al. (1957) and by Pincus and coworkers (1956). Its progestational effects led to the development of norethindrone as Norlutin, a new progestin, for the treatment of menstrual disorders. Norlutin was found effective and safe for contraceptive purposes and thus became the second one available in the U. S. market (Goldzieher et al., 1962 and Rice-Wray et al., 1962). Later, several other progestational compounds were prepared and used in oral contraceptive preparations including norethindrone acetate, medroxyprogesterone acetate, ethynodiol diacetate and chlormadinone acetate.

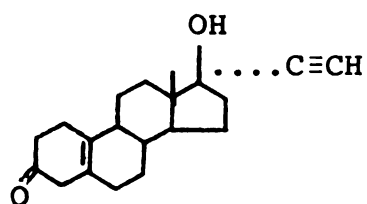
Many oral contraceptive preparations are now available under various brand names in various countries. Common preparations currently available commercially consist of two major types, combined and sequential. The combined oral contraceptives refer to a progestin in combination with an estrogen throughout the treatment period. In the sequential type, estrogen alone is given for 15 days of therapy, followed by the combination of estrogen and progesterone for 5 days. One of the major problems with the use of sequential therapy is the occasional failure to menstruate following the withdrawal of therapy.



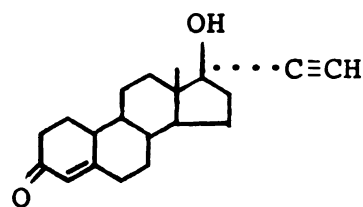
## Structure of Progestins and Estrogens

The chemical structures of both the progestins and estrogens used in oral contraceptives include an ethynyl group ( $-\text{C}\equiv\text{CH}$ ) in the 17 position of the steroid structure (Figure I). This ethynyl group and the nor structure of progestins give the high oral activity of these compounds.

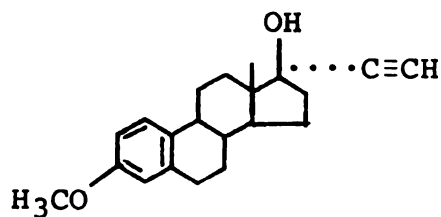
In the basic steroid molecule of progestins, most of the compounds contain a carbon-carbon double bond in 4(5) position, except norethynodrel which is in 5(10) position. The double bond in 5(10) position is biologically significant, since besides being progestational, it makes norethynodrel estrogenic and devoid of androgenic effects in both animals and human (Drill, 1966). However, norethindrone has minimal androgenic effects and is not estrogenic in animals and human.



Norethynodrel



Norethindrone



Mestranol

Figure I. Structure of Progestins and Estrogen

### Mechanism of Action of Oral Contraceptives

The most important effect of oral contraceptives is the prevention of ovulation by inhibiting the secretion of gonadotropins from the pituitary gland. Other mechanisms proposed for the effectiveness of oral contraceptives include the alteration of the cervical mucus so that the sperm penetration of ova is inhibited and the alteration in the endometrium so that it becomes unsuitable for implantation.

#### Pituitary Gonadotropins:

Drill and Saunders (1958) demonstrated that norethynodrel decreases the gonadotropin content of the pituitary gland in the ovariectomized rat. Other studies have found that many progestins are effective in inhibiting the secretion of pituitary gonadotropins in the rat (Saunders, 1964; Kupperman, 1957; Epstein, 1958; Overbeek et al., 1964; Kincl and Dorfman, 1965; Desaulles and Krähenbühl, 1965) and suggest that these steroids depress pituitary gonadotropin function (Pincus and Merrill, 1961; Holmes and Mandl, 1962; Greenwald, 1964).

According to Hayashi (1962), 19-norsteroids inhibit the secretion of luteinizing hormone (LH) rather than follicle stimulating hormone (FSH) from the pituitary of the castrate rat. Mares (1964) reported that the rise in LH occurring in the rat pituitary following ovariectomy was prevented by norethynodrel, norethindrone, norethindrone

acetate and ethynodiol diacetate. Brown et al. (1965) proved that norethynodrel may prevent the release of LH from the pituitary gland of the rat. Besides, Overbeek and Visser (1964) concluded that lynestrenol inhibited FSH secretion, while 6-methyl-lynestrenol inhibited LH.

The estrogens are very effective in inhibiting pituitary gonadotropin secretion in the rat and they are many times more potent than the progestins (Saunders, 1964). Also, several investigators report that estrogen decreases LH content in the pituitary of the ovariectomized rat (Mares, 1964), inhibits pituitary function in the parabiotic rat (Kincl and Dorfman, 1965; Desaulles and Krähenbühl, 1965) and suppresses elevated plasma LH levels in the ovariectomized rat (McCann and Taleisnik, 1961).

The increase in ovarian weight induced by injections of gonadotropin is not inhibited by the administration of norethynodrel (Drill and Saunders, 1957). Thus, it does not affect the stimulating action of pituitary gonadotropins on the ovary. Furthermore, investigators have found that norethynodrel, Enovid, norethindrone and chlormadinone acetate do not block the ovulation induced by pituitary gonadotropin administration (Drill, 1966).

#### Inhibition of Ovulation:

Since oral contraceptives inhibit pituitary gonadotropins, ovulation is not expected to occur. Rock et al. (1956) reported that following cyclic administration of norethynodrel, no functioning corpus luteum was found on

the ovary at laparotomy. After administering norethynodrel and other progestational compounds to women starting from the third to the fourteenth day of the menstrual cycle Matsumoto et al. (1960) examined the ovaries of these women and concluded that ovulation was inhibited when the treatment was started from the sixth day of the cycle. Vasicka and Richter (1959) also found that the final normal maturation of the follicles was prevented in women by taking norethynodrel from day five to day ten of the menstrual cycle.

Besides the absence of functioning corpus luteum in ovaries many investigators have shown that oral contraceptive steroids successfully prevented ovulation either by the absence of the mid cycle peak in estrogen excretion (Brown et al., 1962; Loraine et al., 1963; Bucholz et al., 1962; Brown and Blair, 1960; Shearman, 1964 and Kaiser, 1964), or by the absence of an increase in pregnanediol excretion (Pincus, 1957; Rock et al., 1956; Garcia et al., 1958; Bucholz et al., 1962; Brown et al., 1962; Loraine et al., 1963 and Lin et al., 1964).

#### Effect of Oral Contraceptives on Metabolisms

The metabolism of women receiving oral contraceptive agents is similar in many respects to that of pregnant women (Goldzieher, 1970). Since oral contraceptives contain estrogenic and progestational compounds, they are

likely to produce biological effects ordinarily exerted by natural ovarian hormones.

The effects of oral contraceptives on several aspects of carbohydrate, fat, protein, mineral and vitamin metabolisms have been studied. Various metabolic changes may be associated with the alterations in the secretion and functional rates of endocrine glands.

#### Carbohydrate Metabolism:

An impairment of oral glucose tolerance has been found in women receiving Enovid (Gershberg et al., 1964). A greater incidence of abnormal glucose tolerance was found in women receiving oral contraceptives and having a family history of diabetes than those having no familial diabetes. This finding supported the work of Cochran and Pote (1963) who found that Enovid treatment enhanced the abnormality of glucose tolerance in one half of a group of postmenopausal diabetic women. Wynn and Doar (1966) found impaired oral and intravenous glucose tolerances and increased plasma nonesterified fatty acids and blood pyruvate values in women receiving oral contraceptives. Mean fasting plasma glucose was not significantly changed by oral contraceptive therapy (Wynn and Doar, 1969).

Puchulu et al. (1967) suggested that the estrogenic compounds rather than progestational compounds in the oral contraceptives were responsible for the changes in carbohydrate metabolism, since treatment with progestational

agents alone did not alter the glucose tolerance. The study of Pyörälä et al. (1967) indicated that glucose tolerance decreased during the estrogenic phase of combined estrogen-progesterone treatment. Also, Javier et al. (1968) reported a decreased glucose tolerance in women who were given mestranol. However, Gershberg et al. (1969) found that medroxy-progesterone acetate, a progestational steroid, produced impaired glucose tolerance; Lei and Yang (1972) indicated that norethynodrel impaired oral glucose tolerance and reduced the levels of radioactivity in adipose tissues at various time intervals after an oral glucose-U-C<sup>14</sup> load in rats. Mestranol slightly depressed gastric emptying and intestinal absorption of the radioactive glucose load. This conclusion supported the study of Buchler and Warren (1966) who suggested that the estrogen effects were related to delayed absorption of glucose rather than any diabetogenic effect.

The impairment of glucose tolerance caused by oral contraceptives has not only been attributed to changes in glucose absorption (Buchler and Warren, 1966; Lei and Yang, 1972), but also to gut insulin releasing factors (Jarrett and Cohen, 1967), liver function (Kleiner et al., 1966; Ockner and Davidson, 1967), increased insulin resistance of the peripheral tissues (Beck and Wells, 1969), reduced insulin sensitivity in tissues (Lei and Yang, 1972), elevated plasma glucocorticoids (Metcalf and Beaven, 1963) and growth hormone (Spellacy et al., 1967)..



Lipid Metabolism:

Russ et al. (1955) reported that estrogen in combination with orally active synthetic androgens caused a sharp increase in low density lipoproteins which was followed by a decrease in high density lipoproteins. Aurell et al. (1966) studied the effect of an oral contraceptive, Anovlar, containing 5 µg of 17 ethynylestradiol and 4 mg of norethisterone. They found a significant rise in serum lipids, especially low density lipoproteins, after one year of administration to women. In pre-menopausal women, Pincus (1965) reported that no significant changes were found in either blood cholesterol or  $\beta$ -lipoprotein levels following Enovid therapy.

Wynn et al. (1966) observed elevation of serum triglyceride, cholesterol and low density and very low density lipoprotein levels in women receiving cyclical oral contraceptives.

The elevated plasma triglyceride level in women taking oral contraceptives has been associated in particular with an increase in the serum pre- $\beta$ -lipoprotein levels (Sachs et al., 1969). Wynn et al. (1969) suggested that the blood lipid increase may be due to the general increase in the levels of serum proteins; however, it has been suggested that this lipemia may be related to the significantly reduced lipoprotein lipase activity in the serum of subjects taking oral contraceptives (Ham and Rose, 1969). The

decreased lipase activity may therefore decrease the hydrolysis of lipoprotein-bound triglyceride to fatty acids.

#### Protein Metabolism:

A marked increase in nitrogen retention was noted by Whitehair et al. (1953), who treated lambs with 24 mg of stilbestrol implanted in the neck region. Landau and Lugibihl (1963) reported that protein catabolism was induced by progesterone which was associated with a decline in plasma amino nitrogen. In their study, fasting concentrations of most of the amino acids were 12-34% lower than control values, but urinary amino acid nitrogen was not affected. The authors suggested that the lower level of amino acids was due to the conversion of the amino acids to urea by the liver. However, Adams (1966) reported that 19-norprogesterin or 17-acetoxy-progesterin caused a positive lean-tissue nitrogen balance and a loss of body fat without changing the body weight in women.

#### Mineral Metabolism:

Ehrlich et al. (1960) reported that progesterone inhibited the salt-retaining effect of aldosterone, thus involving progesterone in the hormonal control of renal sodium excretion during pregnancy. However, Eugenia and Engel (1961) showed that progesterone did not influence electrolyte excretion caused by deoxycorticosterone. Singer et al. (1963) demonstrated that subcutaneous administration of progesterone resulted in a significant

increase in the aldosterone secretion rate and suggested that injected progesterone served as a precursor to aldosterone, or it could have inhibited the effect of endogenous aldosterone on the kidney, which would lead to an increase in aldosterone production. Laidlaw et al. (1962) agreed to this suggestion and explained that the anti-aldosterone action of progesterone was overcome by hypersecretion of aldosterone, thus restoring sodium balance.

Though the mechanism of action of progesterone on sodium balance remains unsolved, incidence of hypertension has been reported by Woods (1967) and Laragh et al. (1967) in patients taking oral contraceptives. Usually, improvement is noted after the treatment is stopped. According to the study of Laragh et al. (1967), most patients receiving oral contraceptives showed very striking and sustained increases in the concentration of renin-substrate, in the serum and a few patients showed increase in renin activity, but none of them suffered sodium depletion, since they had normal ranges of urinary sodium excretion and no clinical edema. Those findings led to the conclusion that the administration of pharmacological doses of estrogen and progesterone required for contraception may be responsible for abnormalities in the renin-angiotensin-aldosterone system and subsequent development of hypertension.

Estrogen inhibits bone resorption (Riggs et al., 1969) which has been confirmed (Anderson et al., 1970). According to Davis et al. (1966) estrogen therapy seems to delay the onset of osteoporosis, but Womack et al. (1950) treated a severely osteoporotic infant with estrogen and found no change in the degree of osteoporosis. The study of Ackermann et al. (1954) showed that estrogen had little effect on calcium balance in subjects initially in positive calcium balance, but increased retention in subjects initially in negative balance. Treatment with progesterone had less effect on calcium balance. Caniggia et al. (1970) compared intestinal absorption of radioactive calcium before and after administration of oral contraceptive agents to post-menopausal women for six months and concluded that estrogen in oral contraceptive agents improved absorption of calcium.

Comparing the menstrual blood loss of normal women and oral contraceptive users, Larsson-Cohn (1966) found that combination oral contraceptives reduced the quantity and duration of bleeding. But Cruickshank (1970) reported that oral contraceptives had no effect on hemoglobin concentration. During oral contraceptive therapy, serum iron levels and serum iron binding capacity were increased to levels normally observed during the second half of pregnancy (Jacobi et al., 1969; Mardell et al., 1969). According to Briggs and Briggs (1970), this is an effect of progestogen rather than of estrogen. The increased

production of apotransferrin and transferrin was considered to be responsible for the increased intestinal iron absorption and for the intensive iron storage observed in the liver of mice treated with synthetic estrogens (Simon et al., 1971).

As early as 1928, the copper content of human maternal blood has been reported to be greatly increased during the later stages of gestation (Krebs, 1928). Administration of oral contraceptives or estrogen also increased serum copper levels significantly (O'Leary and Spellacy, 1968; Russ and Raymunt, 1956); moreover, ceruloplasmin, a copper containing protein is also elevated in the plasma of those receiving oral contraceptives (Tovey and Lathe, 1968). Since a counteracting biological relationship between copper and zinc has been suggested (Hoekstra, 1964), an effect of oral contraceptive agents on zinc metabolism therefore can be expected. McBean et al. studied the effect of an oral contraceptive preparation and its constituent steroids on zinc metabolism of growing females, and reported that estrogenic compounds decreased plasma zinc levels, but increased zinc uptake by liver, spleen, adrenals and uterus.

#### Metabolism of Vitamins:

The blood vitamin A level decreases during the last trimester of pregnancy (Bodansky et al., 1943; Lund and Kimble, 1943); however, the plasma vitamin A levels of

women taking oral contraceptives were reported to be increased significantly over those of non-pregnant controls (Gal et al., 1971).

In women receiving oral contraceptives, their anticoagulant responses to a given dose of bishydroxycoumarin, a vitamin K antagonist, were smaller than subjects not receiving contraceptives (Schrogie et al., 1967). Mellette (1961) studied the interrelationships between vitamin K and estrogenic hormones, and observed that the hemorrhagic phenomenon resulting from hypoprothrombinemia in rats fed a vitamin K-deficient diet could be reversed by treatment with hormone as well as by vitamin K.

Women taking combined progestogen and estrogen preparations excreted considerable amounts of xanthurenic acid following a tryptophan load (Rose, 1966). This disturbance of tryptophan metabolism is an indicator of vitamin B<sub>6</sub> deficiency. According to Rose (1966) and Price et al. (1967), this abnormality could be corrected by the oral administration of pyridoxine hydrochloride. Rose indicated that estrogen was primarily responsible for the abnormal tryptophan metabolism, which was confirmed by Wolf et al. (1970).

Not many studies have been done on the effect of oral contraceptives on niacin metabolism. Rose et al. (1968) reported an increased basal excretion of N-methylnicotinamide, a nicotinic acid metabolite, in women taking



oral contraceptives. This is probably due to an increase in activity of the enzyme which converts tryptophan to niacin (Ross, 1966).

Shojania et al. (1968) studied serum folate level in women taking oral contraceptive agents and found that, in these women, serum folate levels were decreased, but the study of Spray (1968) indicated that there was no general tendency for oral contraceptive agents to decrease folate levels. Additional observation by Shojania et al. (1969) indicated that women receiving oral contraceptives had significantly lower serum and red cell folate levels. They also found a significantly higher urinary excretion of formiminoglutamic acid (FIGLU) following a loading dose of histidine which suggested that a deficient folate status existed in these women. Furthermore, Streiff (1969) and Snyder and Necheles (1969) reported that women taking oral contraceptives for longer than one year had folate-responsive megaloblastic anemia. Another report by Streiff (1970) indicated that despite having a good diet history women were found to have folate deficiency and anemia when they had been taking oral contraceptive drugs for one and a half years or more. The study of Luhby et al. (1971) found abnormal FIGLU excretion after a histidine load, deficient serum folic acid levels in subjects taking oral contraceptives; while none of controls had those abnormalities.

In spite of some investigators claiming that there is no significant difference or even a slight increase in the folic acid levels between women receiving oral contraceptives and control subjects (McLean et al., 1969; Pritchard et al., 1971; Kahn et al., 1970; Castren and Ross, 1970), it is generally accepted that the reduction should it occur, is due to a decrease in absorption of dietary folate. A markedly reduced absorption of a purified preparation of polyglutamic folic acid, which is the main food form of folate, was reported by Streiff (1970) and Snyder and Necheles (1969). The absorption of crystalline folic acid (monoglutamic) was not impaired. A reduction of as much as 50% absorption of polyglutamic folic acid was later found by Streiff (1970).

Streiff (1970) found normal vitamin B<sub>12</sub> levels in the folate deficient women who received oral contraceptives. However, Bianchine et al. (1969) reported that serum vitamin B<sub>12</sub> binding capacity was increased by oral contraceptive hormones. On the other hand, Wertalik et al. (1971) reported that women in the third trimester of pregnancy and women receiving oral contraceptives had significant lower serum vitamin B<sub>12</sub>-binding capacity than controls.

#### Vitamin C and Sex Hormones:

Whether an individual is a male or a female has a direct bearing on blood levels of ascorbic acid. The

effect of sex seems to be important especially after puberty since there is little difference in either intake or serum ascorbic acid between boys and girls aged 4 to 12 years. Above 13 years of age, males, in spite of their higher vitamin C intake, have lower serum ascorbic acid level than females (Dodds, 1959). The same situation was found in subjects over fifty years of age (Morgan et al., 1955). On studies employing equivalent intakes of ascorbic acid, lower plasma values of ascorbic acid were found in male subjects than in females of college age (Fisher et al., 1954; Sabry et al., 1958; Wang et al., 1962).

In summarizing this subject, Dodds (1969) reported that there is little difference between males and females in blood responses to intake of ascorbic acid for youngsters before the age of 12. At thirteen to twenty years of age males begin to show lower levels of ascorbic acid in the blood than females on equivalent intakes. This difference persists in the older age group twenty years and over. These findings suggest a hormonal influence on ascorbic acid metabolism.

#### Vitamin C Levels During the Menstrual Cycle:

The results of studies on blood levels and urinary excretion of ascorbic acid during the menstrual cycle are contraversial. Mickelsen et al. (1943) found a sharp increase of plasma vitamin C in mid-cycle. This was confirmed by Kofoed et al. (1965) who reported that

dehydroascorbic acid was highest and reduced ascorbic acid low in the middle of the cycle. Fujino et al. (1965) on the contrary, found a lower mean blood ascorbic acid level during menses, and Hauck (1947) found no evidence of unusual variability in fasting plasma ascorbic acid values associated with the menstrual cycle. In recent studies, Rivers and Devine (1972) found that total ascorbic acid and reduced ascorbic acid concentrations of subjects with a normal diphasic menstrual cycle were highest at the time of ovulation, lowest in the late secretory phase and menses, and in between these extremes in the proliferative and early secretory phases.

Different results were also found in ascorbic acid excretion during the menstrual cycle. Slight fluctuation in the daily vitamin C excretion was noticed by Mickelsen et al. (1943), but Hauck (1947) indicated that no variation in urinary ascorbic acid levels exist in the menstrual cycle. In the work done by Loh and Wilson (1971), three days before the rise in basal body temperature, ascorbic acid excretion rose to a peak, then diminished reaching a minimum value on the day before the temperature rise. The excretion rose dramatically at the same time as the elevation in basal body temperature on the 14th day, followed by a slight fluctuation and fall in excretion on and after the 16th day.

According to Kofoed et al. (1965) the dehydroascorbic acid concentration of cervical mucus was greatest in mid

cycle and minimal at the beginning and at the end. The reduced ascorbic acid concentration of cervical mucus was lowest in the middle part of the cycle and greatest at the beginning or the end of the cycle.

#### Steroid Administration and Vitamin C Levels:

Exogenous estrogens and certain oral contraceptives may cause significant decreases in ascorbic acid concentrations in blood plasma, leucocytes, platelets, blood vessels, adrenals, uteri, liver and adenohypophysis in one or more of the following species: guinea pigs, rats, mice, and humans (Mosonyi, 1936; di Martini et al., 1950; Leathem, 1959; Clemetson, 1968).

The study of Clemetson (1968) showed that plasma ascorbic acid concentrations could be reduced as much as 56% in guinea pigs following the injection of estradiol valerate as compared to controls. Liver and adrenal ascorbic acid concentrations were also significantly decreased by the estrogen treatment. Intramuscular injections of mestranol in virgin female guinea pigs significantly reduced the ascorbic acid by 23% in plasma and 38% in blood vessels. The adrenal ascorbic acid concentration was also lower in the mestranol treated group. Schreiber et al. (1971) demonstrated that the ascorbic acid concentration of the adenohypophysis of rats fell very slightly after castration but very distinctly

after daily intramuscular administration of estradiol dipropionate.

According to Hall et al. (1971) and Fahim et al. (1970), female rats treated intraperitoneally with a natural progesterone increased their urinary ascorbic acid concentration, but in male rats progesterone treatment decreased the concentration.

In human studies, Briggs and Briggs (1972) reported that ascorbic acid in both leucocytes and platelets was significantly lower in women taking steroid contraceptives than in untreated controls or in pregnant women. A depletion study by Kalesh et al. (1971) showed that the decline in platelet ascorbic acid was 6.1% in the control group after two weeks on a vitamin C poor diet. The corresponding fall in the contraceptive groups was 20%. This difference was statistically significant. However, the plasma ascorbic acid levels fell at comparable rates in both groups. Other work done by Rivers and Devine (1972) indicated that plasma reduced ascorbic acid and total ascorbic acid concentrations for subjects taking oral contraceptives were higher during menses, when they were not ingesting the drugs, than at any other time during the cycle. The dehydroascorbic acid values for control subjects, without contraceptives averaged 0.18 mg/100 ml over the entire cycle with no differences among the different stages of the cycle. Oral contraceptive users had dehydroascorbic acid concentrations equivalent to

control subjects on two phases, i.e., (1) from the termination of menses (or day six) to day 11, and (2) 9 days following the ovulation to menses; but lower than control subjects in other phases. In the same study, urinary and plasma ascorbic acid levels following load doses were also investigated. The results showed that excretion of ascorbic acid was not consistently related to fasting plasma concentrations, phase of cycle or ingestion of drugs; subjects taking oral contraceptives did not excrete more ascorbic acid than controls. Ascorbic acid excretion following the load doses was related to the rise in reduced ascorbic acid and the total ascorbic acid of plasma.

Possible Reasons for Depression of Vitamin C Levels in Various Tissues by Oral Contraceptive Administration:

Although no study has been performed to discover the reason why there is such a significant reduction in the concentration of ascorbic acid in various tissues, suggestions have been made that increased serum ceruloplasmin and copper in women using oral contraceptives may be responsible for the change (Briggs and Briggs, 1972; Theuer, 1972; Clemetson, 1968; Saroja et al., 1971). It is also likely that oral contraceptive steroids increase the breakdown of ascorbic acid perhaps by their stimulant action on liver release of ceruloplasmin, a copper containing protein which has been reported to possess ascorbic acid oxidase activity (Osaki et al., 1964;

Humoller et al., 1960). The increased blood concentrations of ceruloplasmin and copper in women using oral contraceptives have been well documented (Briggs et al., 1970). However, the observation of a significant drop in plasma and tissue ascorbic acid level in the absence of any appreciable increase in plasma copper after administration of contraceptive steroids by Saroja et al. (1971) suggests the possibility of other mechanisms for the ascorbic acid depletion.

### Properties of Ceruloplasmin

In the early study of Holmberg and Laurell (1948), a blue protein was isolated from pig serum and named ceruloplasmin. It was described as a copper containing protein and consisted of 8 atoms of copper per molecule. On further study, it was shown that the protein has the ability to enzymatically degrade ascorbic acid and other substrates (Holmberg and Laurell, 1951). The substrates of ceruloplasmin, all of which are reducing agents, fall into three different classes: (a) aromatic polyamines and polyphenols or a combination of these, e.g. epinephrine, serotonin, p-phenylenediamine; (b) enediols, e.g., ascorbic acid; (c) a miscellaneous group of selected reducing agents, e.g.,  $\text{Fe}^{++}$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NH}_2\text{OH}$ , etc., (Osaki et al., 1966). Many monovalent anions, as for instance, the rhodanide, the nitrate, the chloride, the bromide,



the formate and the acetate ions, have an accelerating effect on this enzymatic process, but when these ions are present in higher concentrations they may actually have an inhibitory effect in the degradation of the classes of compounds mentioned above. Both of these effects increase with decreasing pH. For this reason, it was assumed that two types of cationic groups exist on the enzyme surface. One of these has a high affinity for different anions. When these groups were blocked by monovalent anions, the result was generally increased enzymatic activity. The other type of cationic group has a lower affinity for anions. Blocking of these groups leads to inhibition (Holmberg and Laurell, 1951).

The enzymatic activity of ceruloplasmin is also sensitive to the ionic strength of the milieu even at a constant pH. At lower ionic strength the enzymatic activity was greater (Humoller et al., 1960).

The addition of EDTA to plasma containing ceruloplasmin not only destroyed 50% of the amine oxidase activity but also removed 50% of the copper from the enzyme. As the further addition of EDTA seemed to have no added effect on ceruloplasmin, it was then concluded that the 8 copper atoms are held by forces of unequal strength - 4 of the copper atoms are easily removed by EDTA at room temperature, and the other 4 are resistant to such treatment. Furthermore, since removal of the 4 loosely held copper atoms reduces the oxidase activity by 50% of the

amine substrates, it was assumed that enzymatically all copper atoms are equally effective in this reaction. Considering the fact that EDTA destroyed 98% of the oxidase activity of ceruloplasmin on ascorbic acid, Humoller et al. (1960) postulated that ceruloplasmin has two distinct sites for enzymatic activity, one of these is capable of catalyzing the oxidation of both N,N-dimethyl-p-phenylenediamine (DPP) and ascorbic acid and the other site can only mediate the oxidation of substances like DPP and is inhibited progressively by increasing amounts of ascorbic acid.

#### Ascorbic Acid Oxidase Activity of Ceruloplasmin:

Humoller et al. (1960) investigated the enzymatic activity of purified ceruloplasmin with various substances as substrates. The results showed that ascorbic acid was one of the more effective substrates for this enzyme. Further studies showed that EDTA reduced the oxygen uptake to 50% of normal when DPP was used as substrate, but with ascorbic acid as substrate the reduction of oxygen uptake amounted to 80 to 90%. When EDTA systems contained both DPP and ascorbic acid, the oxygen uptake was greatly depressed. These results suggested that although ascorbic acid could not be oxidized by the four tightly bound copper atoms, it would inhibit the oxidation of other substrates. However, this inhibiting effect became significant only with concentrations of ascorbic acid outside physiologic ranges.

The fact that ascorbic acid oxidase activity is solely concentrated in the more labile atoms naturally raises the question that perhaps these dissociate sufficiently so as to catalyze the oxidation of ascorbic acid as inorganic ions rather than as a copper protein. The work of Humoller et al. (1960) supported the theory that ascorbic acid was one of the substrates of ceruloplasmin. Morell et al. (1962) doubted that ceruloplasmin had activity as an oxidase toward ascorbic acid, and treated the ceruloplasmin solution with chelex-100 to eliminate non-protein copper ion. They found that the rate of oxidation of ascorbic acid in the presence of ceruloplasmin copper was not appreciably greater than the rate in the absence of ceruloplasmin; also, in the presence of both 1  $\mu\text{g}$  per ml of ceruloplasmin copper and 1  $\mu\text{g}$  per ml of inorganic copper, the rate of oxidation of ascorbic acid, although appreciable, was only as great as that produced by 0.06  $\mu\text{g}$  per ml of inorganic copper alone. Therefore, it was concluded that ceruloplasmin, freed of non-ceruloplasmin copper, was not an ascorbic acid oxidase. Osaki et al. (1963) investigated the ascorbic acid oxidase activity of chelex treated ceruloplasmin by measuring the disappearance of ascorbate spectrophotometrically and oxygen uptake manometrically. In some of their experiments, neocuproine or serum albumin was added to the reaction mixture as Cu (II) inhibitor. The results of both methods showed that the rate of the reaction catalyzed was at least many

hundred times that predicted from a stoichiometric reaction between ascorbic acid and the Cu (II) of ceruloplasmin. Since it has been observed that ascorbic acid catalysis by ceruloplasmin proceeds, despite the addition of an excess of neocuproine, bovine albumin or thyroxine which is known to block all the Cu (II) catalyzed reactions, the ceruloplasmin catalysis cannot be due to Cu (II) contamination.

In order to substantiate the existence of ascorbic acid oxidase activity of ceruloplasmin, several factors were reported by Osaki et al. (1964): (1) Difference between susceptibility of ceruloplasmin and Cu (II) toward various inhibitors. Those inhibitors that affect ceruloplasmin at specific concentrations do not affect Cu (II), e.g., citric acid; those inhibitors that inhibit Cu (II) do not affect ceruloplasmin activity, e.g., neocuproine and serum albumin. (2) Difference in product formed. The oxidation of ascorbic acid catalyzed by Cu (II) results in hydrogen peroxide formation, whereas the ceruloplasmin catalyzed reaction does not produce hydrogen peroxide. (3) Numerous kinetic differences exist between the two reactions. They are different in the dependence of activity on pH and ascorbic acid concentration. Cu (II) possesses greater activity at the higher pH within a range of 5.0 to 7.5. Ceruloplasmin gives maximum activity around pH 6.0, the activity gradually declines on both sides of this optimum pH, yielding a typical enzymic pH

dependent curve. At low ascorbic acid concentrations, the ceruloplasmin shows a typical enzymic velocity vs. substrate curve. In this same range of ascorbic acid concentration, the Cu (II) reaction is not a true first order with respect to ascorbic acid concentration.

Factors that Influence the Ascorbic Acid Oxidase Activity of Serum Ceruloplasmin:

Some organic acids such as citric acid, D,L-isocitric acid, oxalic acid and oxaloacetic acid are inhibitory to ceruloplasmin activity. However, only citric acid is in high enough concentration in the serum to exert an effect on the oxidation of ascorbic acid by ceruloplasmin; the other organic acids are not present in sufficient concentrations to contribute significantly to ceruloplasmin inhibition in normal human serum (Osaki et al., 1964).

Both apotransferrin and transferrin have been found to be strong inhibitors of ceruloplasmin activity. The inhibition appears to be competitive and reversible. The evidence for a direct interaction between apotransferrin and ceruloplasmin has not been found. The inhibitory effect of apotransferrin and transferrin is extremely pH sensitive. The inhibition of ceruloplasmin by apotransferrin is virtually complete above pH 6.0 and declines rapidly until no inhibition at pH 5.0. The behavior of transferrin is similar to apotransferrin from pH 7.5 to 6.3 but consistently more transferrin is required for



equivalent inhibition than apotransferrin. At lower pH, the ceruloplasmin inhibition is markedly reduced until an activation begins below pH 5.8. This activation is believed to be associated with the release of  $\text{Fe}^{++}$  from transferrin (Figure II) (Osaki et al., 1966).

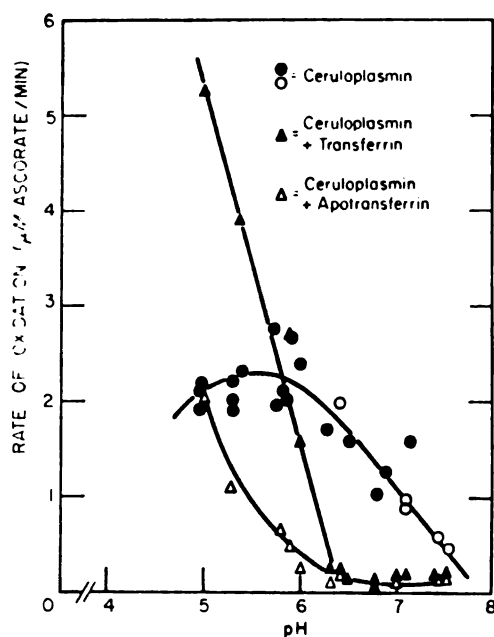


Figure II. The effect of pH on the rate of ascorbic acid oxidation by ceruloplasmin with and without apotransferrin and transferrin. The rate with  $0.17 \mu\text{M}$  ceruloplasmin at  $30^\circ\text{C}$  and  $100 \mu\text{M}$  ascorbic acid in  $0.2 \text{ M}$  acetate buffer is indicated by  $\bullet$  and in  $0.02 \text{ M}$  imidazole- $0.20 \text{ M}$  acetate buffer by  $\circ$ ; with  $0.076 \mu\text{M}$  transferrin,  $\blacktriangle$ ; and with  $0.073 \mu\text{M}$  apotransferrin,  $\Delta$ . The same buffers were used at the corresponding pH's in all experiments.



## MATERIALS AND METHODS

### Animals

Forty ovariectomized guinea pigs of the Hartley strain from Camm Research Institute Inc., weighing 300 to 350 grams, were divided into four groups. The guinea pigs were ovariectomized in order to determine the effect of the separated and combined oral estrogen and progesterone treatment, uncomplicated by endogenous ovarian hormones. It was hoped that effects of the oral steroids would be more uniform, especially for control animals, since estrous rhythmicity may influence ascorbic acid concentrations in blood plasma and perhaps other tissues of the control animals.

### Experimental Design:

A two by two factorial design was used, thus involving four subgroups. Each group consisted of ten guinea pigs. The four groups of guinea pigs were composed of: Group I--control, Group II--fed norethynodrel, Group III--fed mestranol and Group IV--fed norethynodrel plus mestranol.

### Diet:

Reid and Briggs (1953) purified guinea pig diet was modified by using cellulose instead of cellophane

(Appendix I). The diet was devoid of ascorbic acid but nutritionally adequate in all other aspects.

#### Dose of Ascorbic Acid and Contraceptive Steroids

A. Ascorbic acid: 10 mg ascorbic acid were provided per kg body weight per day for each animal in each group during acclimatization and treatment periods.

B. Contraceptive steroids: The doses of oral steroids was the "normal" amount, based on body weight and normal steroid levels used by women. Thus, the dose was 91  $\mu$ g norethynodrel, or 1.36  $\mu$ g mestranol, or both, per kg body weight. The dose was equivalent to a 55 kg woman receiving 5 mg of Enovid.

Steroids or ascorbic acid or both were given by incorporation into a 20% sucrose solution as a carrier, so that the guinea pigs would consume the allotted steroids or ascorbate readily. Solutions were made so that 1 ml contained: (I) 10 mg ascorbic acid, (II) 10 mg ascorbic acid plus 91  $\mu$ g norethynodrel, (III) 10 mg ascorbic acid plus 1.36  $\mu$ g mestranol, or (IV) 10 mg ascorbic acid plus 91  $\mu$ g norethynodrel plus 1.36  $\mu$ g mestranol.

All of the solutions for supplementation were stored in small vials, wrapped with aluminum foil and frozen at  $-40^{\circ}\text{C}$  and stored for not more than one week in order to minimize oxidation of ascorbic acid.

### Method of Treatment

When the animals arrived from the supplier, they were placed two in a cage and given the modified Reid and Briggs purified guinea pig diet ad libitum. Ascorbic acid in the sucrose solution was given daily according to weight by means of a 1 ml syringe with a wide bore-blunted needle, so that the guinea pigs could lick the solution from the needle. This adjustment period continued for 18 days, in order that all animals would be acclimatized to their environment, diet, and supplementation method. They were then divided into four equal groups, so that all groups had similar average body weight. Starting from the 19th day, each group was supplemented according to their predetermined treatment as described above. The treatment lasted for six weeks. Water was offered ad libitum.

### Animal Sacrifice and Sample Collection

After six weeks of treatment, four animals were killed each day (one from each group) by a sharp blow on their heads in order to avoid the stresses associated with etherization. The process was repeated until all animals from all groups were sacrificed. After stunning, the abdominal and chest cavity were opened and blood obtained with a needle inserted into the heart. Adrenals, kidneys, spleen and liver were quickly removed and weighed (Mettler

P162), then kept in plastic vials and stored in dry ice for future determinations of ascorbic acid.

Blood was centrifuged (Sorval RC2-B) for 10 minutes at 482 x g at 5°C, and serum collected. Part of the serum was used immediately for determinations of ceruloplasmin oxidase activity, and part of it was quickly frozen in dry ice and stored in a freezer for determination of ascorbic acid.

#### Homogenization and Extraction of Serum and Tissues

All the frozen tissue samples were partially thawed and chopped into small pieces with a pair of scissors, whenever necessary. All procedures were performed in an ice bath, so that loss of ascorbic acid would be minimized.

##### Serum:

To 1.5 ml of serum, 1.5 ml of 5% metaphosphoric acid in 10% acetic acid were added, mixed and left standing for 5 minutes, centrifuged at 1086 x g for 20 minutes and the supernatant collected.

##### Adrenals, Kidneys and Spleen:

The chopped tissue was placed in a 50 ml centrifuge tube and 5 ml of 5% metaphosphoric acid (or 7% metaphosphoric acid for kidneys) in 10% acetic acid were added. The mixture was then homogenized with a Polytron (Brinkmann

Instrument). The homogenate was centrifuged in the refrigerated centrifuge (Sorvall RC2-B) for 20 minutes at 5°C at 1086 x g, the supernatant collected and the sediment reextracted with another 5 ml of 5% (or 7% for kidneys) metaphosphoric acid in 10% acetic acid and the reextractant was combined with the previous extractant. The extractant was then passed through a büchner funnel to remove traces of contaminating particles.

#### Liver:

Fifty ml of 7% metaphosphoric acid in 10% acetic acid were added to a portion of the chopped tissue and homogenized in a Polytron (Brinkmann Instrument). Ten gm of the homogenate were placed in a 50 ml centrifuge tube, and centrifuged at 27140 x g for 10 minutes at 5°C. The precipitate was reextracted with 5 ml of 7% metaphosphoric acid in 10% acetic acid. The reextractant was then combined with the previous extractant.

### Chemical Methods

#### Ascorbic Acid:

The amount of dehydroascorbic acid and total ascorbic acid were determined by a modified Schwartz and Williams' (1955) method, in which two drops of 2,6 dichlorophenolindophenol were used instead of one; and samples were incubated for four hours instead of three (Appendix II). Preliminary

work indicated that this modification produced more repeatable and precise results.

Reagents:

Ascorbic acid - 3 mg/ml in a mixture of 5% (or 7% for liver and kidneys) metaphosphoric acid in 10% acetic acid, was stored at -5°C.

5% (or 7% for liver and kidneys) metaphosphoric acid in 10% acetic acid - was used for extraction and dilution of samples.

2,6 dichlorophenolindophenol - 100 mg of dye were dissolved in 50 ml of water and the solution filtered using Whatman 12.5 cm filter paper (2v). It was stored at -5°C.

Thiourea - 1% thiourea in 5% (or 7% for liver and kidneys) metaphosphoric acid.

2,4 dinitrophenylhydrazine - a 2% solution was prepared in a solution of 1 part of concentrated sulfuric acid and 3 parts of water. This reagent was stored at -5°C and was filtered through Whatman 12.5 cm filter paper (2v) before use.

A solution of 3 parts of concentrated hydrochloric acid and 2 parts of 85% phosphoric acid was prepared just before use.

After preparing the samples with the reagents, absorbance of the solutions was determined with a Beckman Spectrophotometer (Acta C III). Concentrations of unknowns were then calculated from standard curve, which was derived from known amounts of ascorbic acid.

During the sample preparation, all procedures were performed in an ice bath so that oxidation of ascorbic acid would be minimized.

#### The Ascorbic Acid Oxidase Activities in Serum:

For these determinations, all glassware was acid washed, and all water chelex-100 (Bio·Rad) treated.

##### Reagents:

Buffer - 0.2 M acetate buffer pH 5.2 was passed through a chelex-100 column.

Ascorbic acid - 0.9247 gm ascorbic acid (Sigma Chem. Co.) were dissolved in 50 ml chelex treated water, so that the concentration was  $10.5 \times 10^{-4}$  M. It was prepared just before use.

Albumin - 3 gm bovine albumin (Sigma Chem. Co.) were dissolved in 50 ml of chelex treated water and stored at 5°C.

##### A. Ultrafiltration:

To a centriflo membrane cone (Amicon 224-CF-50) which was fitted into a support, 0.9 ml serum and 3.6 ml acetate buffer were added. It was then placed in a centrifuge tube and the weight of the whole set recorded. After centrifuging for 15 minutes at  $482 \times g$  at 5°C, the final weight was taken. The decrease in weight was considered to be due to vaporization and compensated for by adding buffer solution to the ultrafiltrate. After the vaporization had

been corrected, the ultrafiltrate was placed in acid washed plastic vials, and kept in a  $-40^{\circ}\text{C}$  freezer for later determination of ascorbic acid oxidase activity catalyzed by copper. The residue remaining in the membrane cone was used immediately for the determination of ceruloplasmin ascorbate oxidase activity.

#### B. Ascorbate Oxidase Activities of Ceruloplasmin and Copper:

The following test system was set by adding the reagents in the order given:

	Ceruloplasmin		Copper	
	Sample	Reference	Sample	Reference
Buffer	2.7 ml	2.7 ml	.95 ml	1.0 ml
Ultrafiltrate	-	-	.50 ml	.50 ml
Residue	.1 ml	.1 ml	-	-
Albumin	.1 ml	.1 ml	-	-
Ascorbic Acid	.1 ml	-	.05 ml	-

Excluding ascorbic acid, these reaction mixtures were prepared in the ice bath and then transferred to 1 cm quartz cuvettes, and placed in the cell holders of the spectrophotometer, which was equipped with a recorder and a water bath around the cell chamber. The temperature of the water was regulated so that the reaction mixture inside the cells was maintained at  $30 \pm 1^{\circ}\text{C}$ . After both sample and reference cells were placed in the cell holders, the spectrophotometer was zeroed at 265  $\text{m}\mu$ , then ascorbic acid solution



was stirred into the sample cell, and recording of change of absorbance continued for 40 minutes. The ascorbic acid regression line was thus traced on the chart by the recorder.

#### C. Protein:

Protein concentration in the reaction mixture which was saved from the ceruloplasmin ascorbate oxidase activity determination was determined by Lowry's method modified by Hartree (Hartree, 1972) (Appendix III).

#### Reagents:

Solution A - 2 gm potassium sodium tartrate and 100 gm  $\text{Na}_2\text{CO}_3$  were dissolved in 500 ml 1 N NaOH and diluted with water to 1 liter.

Solution B - 2 gm potassium sodium tartrate and 1 gm  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were dissolved in 90 ml water plus 10 ml 1 N NaOH.

Solution C - 1 ml Folin-Ciocalteu reagent was diluted with 15 ml water.

After the sample was prepared with the reagents, absorbance of the solutions was determined with a Beckman Spectrophotometer (Acta III).

Concentrations of unknown were calculated from standard curves using bovine serum albumin as standard.

#### Calculation

All data were calculated by a two by two factorial analysis of variance with a computer.

## RESULTS AND DISCUSSION

The body weight of animals at the beginning and the end of the experiment were as follows (g  $\pm$  S.D.):

	Initial	Final	Weight gain
Control	359.9 $\pm$ 46.55	500.00 $\pm$ 43.60	140.10
Norethynodrel	359.6 $\pm$ 43.02	578.78 $\pm$ 75.08	219.18
Mestranol	357.0 $\pm$ 40.75	535.00 $\pm$ 70.55	178.00
Norethynodrel + Mestranol	357.4 $\pm$ 38.76	535.80 $\pm$ 83.48	178.40

No significant difference among these four groups in body weight was found at the end of the treatment.

The catabolism of ascorbic acid has been studied by various investigators using both humans (Hodges, 1971; Baker et al., 1971; Tolbert et al., 1967; Baker et al., 1969; Atkins et al., 1964; Baker et al., 1962; Baker et al., 1966; Hellman and Burns, 1958; Hughes and Kilpatrick, 1964) and guinea pigs (Grimble and Hughes, 1968; Hughes, 1964; Dayton et al., 1959; Salomon, 1958; Chan et al., 1958; Burns et al., 1956; Burns et al., 1951; Penney and Zilva, 1946; Penney and Zilva, 1943; Schultz et al., 1938; Shimazono and Mano, 1961; Ashwell et al., 1961; Salomon and Stubbs, 1961; Abt and Von Schuching, 1961). Although

we know very little about many of the enzymes involved in the catabolism of this vitamin, several have been well studied. One of these that is directly related to the present study is ceruloplasmin which catalyzed the oxidation of ascorbic acid to dehydroascorbic acid. This key step in the catabolism of ascorbic acid is considered of primary importance since dehydroascorbic acid is more unstable than ascorbic acid and could contribute to lower ascorbic acid level if this side of the reaction is favored. In the present study, ceruloplasmin ascorbic acid oxidase activities of the residue from ultrafiltration were statistically similar among the four groups of guinea pigs (Table I). The averages and their standard deviations of the oxidase activities expressed as a change in absorption/300 mg protein/minute were  $1.81 \pm .46$ ,  $1.83 \pm .43$ ,  $1.76 \pm .36$  and  $1.55 \pm .32$  respectively for control, norethynodrel, mestranol and norethynodrel plus mestranol group. This finding is in accord with the serum concentrations of dehydroascorbate and total ascorbate found in the four groups of guinea pigs (Table I). Both dehydroascorbate and total ascorbate concentrations were not significantly affected by the oral contraceptive steroids. However, Rivers and Devine (1972) reported that women taking oral contraceptive pills for one year or more showed significant depression of reduced and total ascorbic acid concentration in plasma. Saroja et al. (1971) found that daily intramuscular injection of 50  $\mu$ g mestranol to guinea pigs for

two weeks dropped plasma reduced ascorbic acid by 23%. This difference could relate to whether functioning ovaries are present in the experimental subjects. In the present study, the guinea pigs were ovariectomized whereas those of the other investigators' had intact ovaries. How the ovary interacts with oral contraceptive steroids is not clear. Other works however had found that plasma dehydroascorbate concentrations were not depressed by oral contraceptive steroids (Rivers and Devine, 1972).

Several investigators have found that women receiving oral contraceptives had increased serum concentrations of ceruloplasmin (Mendenhall, 1970; Briggs et al., 1970; Russ and Raymunt, 1956). However, this increase was based on the determination of the degradation of N,N-dimethyl-p-phenylenediamine (DPP) as the substrate. For this reason, there are questions as to whether the increased ceruloplasmin found in contraceptive users is able to degrade ascorbate at a rate rapid enough to show a statistical difference among the four groups of guinea pigs in the present study. A partial answer to this question would have been provided if ceruloplasmin DPP oxidase activities had also been measured in the present study. On the other hand, ceruloplasmin determined with DPP as the substrate may actually have no correlation with ceruloplasmin as determined with ascorbate as the substrate. This however has still to be proven with research even though various investigators had previously suggested that

the decrease in serum ascorbate in oral contraceptive users was mediated by ceruloplasmin as determined by DPP as the substrate.

Since serum ionized copper [Cu (II)] may non-enzymatically oxidize ascorbate to dehydroascorbate, this activity was measured in the serum ultrafiltrate (Osaki et al., 1963). The serum ultrafiltrate that was used contained most likely only about 20% of the serum copper, for according to several investigators, 80% of the copper is bound to ceruloplasmin in normal rats, dogs, pigs, sheep and humans (Butler, 1963; Cartwright, 1950; Milne and Weswig, 1968; Starcher and Hill, 1965; Wintrobe et al., 1953). Attempts to determine the Cu(II) in the ultrafiltrate was not successful because of the low concentration as well as the small volume of ultrafiltrate available for this purpose. However, the determination of the Cu(II) ascorbic acid oxidase activities of the ultrafiltrate revealed that norethynodrel decreased this reaction in the guinea pigs (Table II). There is also the possibility that the decreased ascorbate concentrations found in contraceptive users was caused by mechanisms other than by oxidation with ceruloplasmin and Cu(II). Evidence to support this came from Saroja et al. (1971) who found that serum total copper was not increased appreciably in guinea pigs which had lowered serum ascorbate concentrations because of treatment with mestranol.

Still another enzyme which affects ascorbic acid breakdown, is dehydroascorbic acid reductase. Under physiological conditions of pH and temperature, ascorbic acid undergoes conversion to unstable dehydroascorbic acid and continuously undergoes breakdown to biologically inactive substances, unless a reducing mechanism can rapidly reduce the dehydroascorbic acid back to ascorbic acid. Dehydroascorbic acid reductase catalyzes the reduction of dehydroascorbic acid to ascorbic acid (Grimble and Hughes, 1968). Whether oral contraceptives inactivate the activity of dehydroascorbic acid reductase was not measured in this study and needs more investigation.

Another consideration which would be important in determining factors responsible for the reported decrease in serum ascorbic acid concentrations is the redistribution of the vitamin or its metabolites in organs and tissues as a consequence of oral contraceptive treatments. In previous work by other investigators, oral contraceptive treatments resulted in either a decrease in reduced ascorbic acid or total ascorbic acid in blood platelets (Kalesh et al., 1971), leucocytes (Briggs and Briggs, 1972) and blood vessels (Saroja et al., 1971). Furthermore, Clemetson (1968) injected guinea pigs intramuscularly with estradiol and found a significant decrease in reduced ascorbic acid in the adrenals as compared to controls. There were no statistically significant changes however, in the concentration of the reduced ascorbic acid in either spleen or liver.

In the present study, total ascorbate and dehydro-ascorbate concentrations as well as absolute amounts in the spleen, adrenals, kidneys and liver of the guinea pigs were measured (Table III-VI). From these data, reduced ascorbate (total ascorbate minus dehydroascorbate) can also be calculated.

In comparison of the reduced ascorbic acid levels in liver, kidneys, spleen and adrenals with previous studies, Clemetson (1968) showed that liver, adrenals and spleen of their control group contained 19.7, 139.5 and 37.4 mg/100 gm tissue, respectively, which were much higher than those determined in present study. However, the ascorbic acid intake greatly influenced the tissue content of ascorbic acid (Penney and Zilva, 1964). Probably these results were assayed from guinea pigs fed a diet with a high ascorbic acid level. According to the study of Penney and Zilva (1946), level of reduced ascorbic acid in liver, kidneys, adrenals and spleen determined from guinea pigs with 12 mg/day ascorbic acid intake were close to the levels determined in this study.

Except for the significant increase in the proportion of dehydroascorbic acid in the kidneys of guinea pigs treated with norethynodrel, no other parameters were significantly affected by the oral contraceptive steroids used in the experiment. Even in the case of the kidneys, the biological significance of this increase is probably of no consequence since the absolute quantity of

dehydroascorbate per pair of kidneys constitutes only a small fraction of the metabolic pool. Whether this increase in the proportion of dehydroascorbate in the kidneys as a consequence of norethynodrel treatments was mediated through an increase in ceruloplasmin or Cu (II) or both or through a reduction in dehydroascorbate reductase is not known.



Table I. Concentrations of serum dehydroascorbic acid, total ascorbic acid and ceruloplasmin ascorbic acid oxidase activity of residue from ultrafiltration. (Mean  $\pm$  S.D.)

	Dehydroascorbic Acid		Total Ascorbic Acid	Ceruloplasmin Ascorbic Acid Oxidase Activity*
	$\mu\text{g}/100\text{ ml}$	% of total ascorbic acid	$\mu\text{g}/100\text{ ml}$	change in absorption/300 mg protein per minute
Control	277.40 $\pm$ 45.50	68.50 $\pm$ 15.63	429.00 $\pm$ 99.49	1.81 $\pm$ .46
Norethynodrel	301.93 $\pm$ 75.63	74.50 $\pm$ 10.86	417.94 $\pm$ 117.43	1.83 $\pm$ .43
Mestranol	252.00 $\pm$ 76.59	63.62 $\pm$ 17.37	409.02 $\pm$ 123.91	1.76 $\pm$ .36
Norethynodrel + Mestranol	248.90 $\pm$ 109.85	69.99 $\pm$ 20.60	355.03 $\pm$ 89.92	1.55 $\pm$ .32

\*Determined at pH 5.2, 30 $\pm$ 1°C with added bovine albumin as an inhibitor of Cu (II) oxidase activity.

Table II. Cu (II) ascorbic acid oxidase activities determined at pH 5.2 and  $30 \pm 1^\circ\text{C}$ . (Change in absorption/min/ml ultra-filtrate)

Animal no.	Control	Norethynodrel	Mestranol	Norethy. + Mestranol
1	.076	.039	.076	.040
2	.128	.050	.053	.023
3	.070	.031	.076	.088
4	.031	--	.060	.020
5	.034	.031	.079	.067
6	.095	.033	.050	.084
7	.063	.030	.027	.047
8	.066	.043	.032	.036
9	.026	.023	.041	.026
10	.062	.096	--	.022
mean	.065	.042*	.055	.045
$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
S.D.	.031	.022	.019	.026

\*p < .05

Table III. Dehydroascorbic acid and total ascorbic acid levels in spleen. (Mean  $\pm$  S.D.)

	Dehydroascorbic Acid			Total Ascorbic Acid	
	mg/100 g tissue	mg/organ	% of total ascorbic acid	mg/100 g tissue	mg/organ
Control	10.948 $\pm$ 1.492	.102 $\pm$ .030	46.51 $\pm$ 9.08	23.862 $\pm$ 2.592	.221 $\pm$ .052
Norethynodrel	11.419 $\pm$ 1.590	.116 $\pm$ .033	46.37 $\pm$ 6.65	24.670 $\pm$ 1.682	.248 $\pm$ .046
Mestranol	12.403 $\pm$ 3.009	.125 $\pm$ .021	52.72 $\pm$ 12.38	23.673 $\pm$ 2.640	.245 $\pm$ .054
Norethynodrel + Mestranol	10.386 $\pm$ 2.989	.110 $\pm$ .034	49.68 $\pm$ 16.20	21.323 $\pm$ 4.900	.226 $\pm$ .059

Table IV. Dehydroascorbic acid and total ascorbic acid levels in adrenals. (Mean  $\pm$  S.D.)

	Dehydroascorbic Acid			Total Ascorbic Acid		
	mg/100 g tissue	mg/pair of organ	% of total ascorbic acid	mg/100 g tissue	mg/pair of organ	
Control	14.502 $\pm$ 5.041	.048 $\pm$ .018	23.97 $\pm$ 7.39	60.108 $\pm$ 10.613	.197 $\pm$ .030	53
Norethynodrel	17.873 $\pm$ 8.971	.056 $\pm$ .025	26.64 $\pm$ 8.50	64.272 $\pm$ 18.779	.208 $\pm$ .079	
Mestranol	14.208 $\pm$ 9.355	.042 $\pm$ .027	20.70 $\pm$ 11.44	65.191 $\pm$ 17.690	.204 $\pm$ .057	
Norethynodrel + Mestranol	13.052 $\pm$ 5.187	.041 $\pm$ .015	21.90 $\pm$ 6.35	58.661 $\pm$ 9.607	.185 $\pm$ .031	

Table V. Dehydroascorbic acid and total ascorbic acid levels in kidneys. (Mean  $\pm$  S.D.)

	Dehydroascorbic Acid			% of total ascorbic acid	Total Ascorbic Acid	
	mg/100 g tissue	mg/pair of organ	mg/100 g tissue		mg/100 g tissue	mg/pair of organ
Control	2.134 $\pm$ .466	.091 $\pm$ .020	56.72 $\pm$ 8.01	3.744 $\pm$ .513	.160 $\pm$ .029	
Norethynodrel	2.456 $\pm$ .657	.106 $\pm$ .034	65.42 $\pm$ 12.53*	3.737 $\pm$ .556	.160 $\pm$ .030	
Mestranol	2.158 $\pm$ .448	.095 $\pm$ .016	56.44 $\pm$ 7.49	3.836 $\pm$ .649	.172 $\pm$ .036	
Norethynodrel + Mestranol	2.233 $\pm$ .427	.094 $\pm$ .021	62.71 $\pm$ 8.15	3.577 $\pm$ .575	.150 $\pm$ .029	

\*p < .016

Table VI. Dehydroascorbic acid and total ascorbic acid levels in liver. (Mean  $\pm$  S.D.)

	Dehydroascorbic Acid			Total Ascorbic Acid	
	mg/100 g tissue	mg/organ	% of total ascorbic acid	mg/100 g tissue	mg/organ
Control	4.295 $\pm$ 1.000	.866 $\pm$ .199	54.18 $\pm$ 8.92	7.909 $\pm$ 1.052	1.596 $\pm$ .361
Norethynodrel	4.639 $\pm$ .808	1.112 $\pm$ .329	61.00 $\pm$ 10.49	7.776 $\pm$ 1.688	1.815 $\pm$ .419
Mestranol	4.855 $\pm$ .777	1.104 $\pm$ .363	60.80 $\pm$ 12.21	8.158 $\pm$ 1.455	1.856 $\pm$ .622
Norethynodrel + Mestranol	4.465 $\pm$ 1.006	.971 $\pm$ .442	59.34 $\pm$ 11.06	7.587 $\pm$ 1.300	1.596 $\pm$ .521

## CONCLUSION

The increase in ceruloplasmin as determined by the use of N,N-dimethyl-p-phenylenediamine in the serum of women using oral contraceptives has been suggested as an explanation for the decreased serum ascorbate concentrations of these women. In order to verify this suggestion, ovariectomized guinea pigs were fed norethynodrel or mestranol or both and ceruloplasmin ascorbic acid oxidase activities determined. Results from these determinations indicated that the steroids did not significantly affect the enzyme activity. The serum ionized copper [Cu (II)] was also determined to establish whether it might have increased the non-enzymatic oxidation of ascorbate to dehydroascorbate. However, serum ionized copper [Cu(II)] oxidative activity was significantly decreased by norethynodrel. Furthermore, concentrations and proportions of serum total ascorbate and dehydroascorbate were not altered by the level of oral steroids used in this study. The latter findings thus substantiated the results obtained from measurements of the enzymatic and non-enzymatic conversion of ascorbate to dehydroascorbate.

Adrenals, livers, kidneys and spleens from guinea pigs were also assayed for ascorbate and dehydroascorbate. Only

in the kidneys was there a significantly increased level of dehydroascorbate due to treatment with norethynodrel. This study therefore did not confirm the observation that oral contraceptives decrease serum ascorbic acid. Furthermore, ceruloplasmin and Cu(II) ascorbic acid oxidase activities were not increased by the levels of oral contraceptive steroids used in this study.



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## **APPENDICES**

## APPENDIX I

### REID AND BRIGGS'S DIET (Reid and Briggs, 1953)

Ingredient	gm/100 gm diet
Casein	30
Corn starch	20
Sucrose	10.3
Glucose	7.8
Cellulose	15
Corn oil	7.3
Briggs' salt mix	6
Potassium acetate	2.5
Magnesium oxide	0.5
Choline	0.2
Inositol	0.2
	mg/100 gm diet
Thiamine HCl	1.6
Riboflavin	1.6
Pyridoxine HCl	1.6
D-Calcium or sodium pantothenate	4
Niacin	20
Biotin	0.6
Folic acid	1
Vitamin B <sub>12</sub>	0.004
Vitamin A acetate	0.06
D,L- $\alpha$ -Tocopheryl acetate	2
Menadione	0.2
Calciferol	0.004

## APPENDIX II

### ASCORBIC ACID DETERMINATION (Schwartz and Williams, 1955)

1. 2 drops of 2,6 dichlorophenolindophenol, 0.5 ml thio-urea and 2,4 dinitrophenylhydrazine were added in order to 0.3 ml of sample solution.
2. Mixed with vortex, and incubated at 37°C for 4 hours.
3. 2.0 ml of freshly prepared acid mixture, as mentioned previously in methods and materials, was added, and again mixed by vortex.
4. Optical density was read at 540 mμ.
5. The dehydroascorbic acid was analysed by the same procedures except 2 drops of 2,6 dichlorophenolindophenol and 0.5 ml thiourea were mixed in each tube before adding 0.3 ml sample.

### APPENDIX III

#### PROTEIN DETERMINATION (Hartree, 1972)

1. To clean dry test tubes add 1 ml of water (blank), standard protein solution or unknown.
2. Add 0.9 ml of solution A. Mix and place in a water bath at 50°C for 10 minutes.
3. Cool to room temperature (21-25°C) and treat with 0.1 ml of solution B. Then leave the solution at room temperature for at least 10 minutes.
4. Rapidly force in 3 ml of solution C to ensure mixing within 1 second. Then heat at 50°C for 10 minutes.
5. Cool to room temperature and read in 1 cm cuvettes at 650 nm.

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