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THE EFFECTS OF NORETHYNODREL AND MESTRANOL ON THE GROWTH AND VIRULENCE OF *STAPHYLOCOCCUS AUREUS*

by

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

1968

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LIFE

James M. Cummings was born on September 30, 1943, in Chicago, Illinois. He was graduated from Harper High School in Chicago in 1961. Enrolling in the pre-medical curriculum, he received a Bachelor of Arts degree in Zoology from the University of Illinois campus at Urbana-Champaign in 1964. He undertook the study of medicine at the Stritch School of Medicine in 1964, and in 1966 began his graduate studies at Loyola University.

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STATEMENT OF THE PROBLEM

Recent interest in the use of oral contraceptive agents by humans has been high. Their widespread popularity as a convenient and efficient means of birth control, and as a therapeutic regime for various gynecological diseases has proven a stimulus sufficient to permeate the literature with documentation of the direct effects of these drugs on the welfare of the human organism. However, extensive research concerning drug effects on the natural course of bacterial disease has only recently begun to appear.

It remains then for the researcher to leave no corner darkened in an attempt to delineate effects of these drugs insofar as they hold practical importance for their human users. The purpose of this work is a clarification, through review and experimentation, of the ability of a specific contraceptive agent to modify the host-parasite relationship as it exists in human bacterial disease.

INTRODUCTION

Oral contraceptives by almost any medical yardstick are a new and largely unknown quantity. They represent the answer of twentieth century scientific know-how and technology to an anguished cry for help from a burgeoning world population. These drugs have brought to a close with door slamming finality a more traditional period of contraception through the promise of a new era hallmarked by efficiency, low cost, simplicity and ease of administration, and safety. Of these factors only the latter has threatened to turn the panacea into a Pandora's box.

Minor side effects accompanying the use of contraceptive drugs are common and include gastrointestinal disturbance, malaise, menstrual irregularities, rash, hirsuitism, alopecia, weight gain and chloasma to mention only a few (40). Possible complications of an inherently more serious nature include potential for the production of thromboembolism, and carcinogenesis.

Despite lingering doubts it is estimated that in Australia and New Zealand 20% of women of reproductive age use oral contraceptives, whereas in the United States 12% are users and in the United Kingdom 3% (18). Projection of such figures calls for a dramatic increase in worldwide utilization of oral contraceptives to the point that between 20 and 40 million of the world population will control their fertility through this method by the year 1980 (18). Such use will no doubt be facilitated by development of new drugs with greater potency and fewer adverse effects.

In addition it should be remembered that oral contraceptive drugs need not be used solely for family planning for they have proven a welcome addition to the therapeutic arsenal of the gynecologist. Therapeutic efficacy has been claimed not only in fertility control but in the treatment of such disease states as amenorrhea, dysmenorrhea, endometriosis and functional uterine bleeding. The value of contraceptives in the treatment of such conditions as menopausal syndrome, acne, chronic vulvar infections and psychiatric disorders remains a subject for further research (19).

Of the oral contraceptive drug combinations in present day use a popular and representative example in Enovid. This drug consists of the synthetic progestogen Norethynodrel (17α ethynyl 17 hydroxy 5(10) estrene 3 one) and the synthetic estrogen Mestranol (17α ethynyl estradiol 3 methyl ether). Although these compounds are characterized biologically through differing effects their basic structure is remarkably similar (53). In fact, structural dissimilarities are limited to ring A of the steroid nucleus and to the chemical grouping substituted on carbon atom 3. Such a close correlation is reflected functionally since it is known that Norethynodrel, while having a high progestational activity possesses as well a low inherent estrogenic activity (6).

In view of chemical details, then, it appears that a consideration of effects of steroid compounds in general is a necessary first step toward understanding the action of Enovid. The high electron affinities of steroids, a property unusual among organic compounds, may be an indication of their

ability to participate in or control biological oxidative processes (13). One of the earliest observed effects of steroid hormones *in vitro* upon enzymatic systems was found to be the general inhibition of respiration effected by a variety of steroids in preparations from diverse sources (48). Specifically, it has been observed that steroid hormones inhibit the reduction of cytochrome C by $NADH_2$ in intact mitochondria from rat liver cells. In addition, mitochondrial particulate preparations are known to exhibit steroid induced permeability changes. It is possible that an increase in permeability in mitochondrial membranes permits a loss of the NAD necessary for respiratory functions. In rat liver mitochondria at least, steroid inhibition of respiration was found to be reversible upon the addition of NAD to the media (48).

In a more immediate sense glucocorticoids are believed to function as passive antagonists of a stress activated intrinsic microcirculatory dilator and thus to oppose relaxation of microvascular sphincters. At the site of irritation dilator synthesis is increased and all small vessels are open. Corticoids by closing sphincters suppress the early hyperemia thereby preventing development of later phases of the inflammatory reaction. In the absence of corticoids mild stress activation of dilator synthesis leads to excessive opening of capillaries while greater activation leads to pooling of blood and circulatory failure (41). More recently such circulatory dilators have been identified and.characterized. Kinins are naturally occurring vasoactive polypeptides thought to be the mediators of the acute

inflammatory response. They are released from a plasma protein substrate by glass activated plasma enzymes (kallikreins) or by isolated intact granulocytes. Cortisol at a concentration of 2.5×10^{-5} M was found to prevent the release of active kinin by granulocytes or contact with glass. Deoxycorticosterone, progesterone and etiocholanolone in comparable concentrations were found significantly less effective in preventing kinin release (41). Cortisol also partially inhibited the release of kinin by purified urinary kallikrein. Thus certain steroids may possess anti-inflammatory effects by virtue of their ability to inhibit the release of plasma kinins. They seem able also to prevent interaction between activated kallikrein and its substrate (11).

Compounds that are able to alter the course of inflammation and affect such basic processes as cellular respiration might well be expected to influence the host-parasite relationship as it exists in human infectious disease. Due to the practical implications of such a contingency the effect of glucocorticoids on disease processes has been studied in detail. Both adrenocorticotropic hormone (ACTH) and cortisone have been found to depress the resistance of laboratory animals to infectious agents, activate foci of latent infection and render animals susceptible to intection by ordinarily non-pathogenic flora. Infected animals may succumb to smaller doses of infective agent than do animals not receiving hormones; and, when given in conjunction with antibiotics the steroids generally reduce the effectiveness of a given dose of antibiotic. Factors induced by steroid administration

which may play a role in decreased host resistance include a decrease in the local inflammatory response, increased multiplication of the pathogen and inhibition of phagocytosis and the capacity of the reticuloendothelial system to fix or remove bacteria and their toxins from tissue (24). Steroids reduce the rate of antibody synthesis and it has been found that the bactericidal capacity of leucocytes obtained from the peritoneal exudate of rats pre-treated for 3 days with intramuscular injections of cortisol displayed an impaired capacity to kill phagocytized organisms (3). In addition, wide-spread dissemination characteristic of hormone treated animals may in some instances reflect the inhibitory effect of cortisone on the action of hayluronidase (24). Mineralocorticoids apparently have less drastic effects on the microbial world although it is known that deoxycorticosterone may markedly inhibit the growth of Neurospora crassa in a highly specific manner. In fact, growth of all Gram-positive bacteria, yeasts and molds are significantly affected by this compound (26).

The effects of pregnancy on various disease states may be the result of intrinsic physiologic change related to the economy of endocrine hormones whose concentrations rise dramatically in the gravid female (14). As a result these compounds, notably estrogens and progestogens, have been extensively studied with respect to their effect on pathogenic microorganisms. Casas-Campillo demonstrated that 21-31 dimethoxyprogesterone effectively inhibited the growth of Curvularia lunata and Trichophyton mentagrophytes. Fungistatic effects were found to decrease with increasing complexity of

the modifications on carbon atom 21 (10). Progesterone has been shown to have a bacteriostatic effect on staphylococci in vitro which correlated well with a significant reduction in the oxygen uptake (55). Yotis has carried this work a step farther by demonstrating that Norethindrone, a synthetic progestogen, has in vivo effects serving to protect infected animals against the formation of dermal lesions (54). Some workers feel that the inhibitory capacity of the sex hormones depends upon specific functional groups or chemical properties rather than merely on the general features of steroid structure (46). Information is accumulating to show demonstrable sex differences in respect to immune responsiveness; the female being more responsive than the male. In addition there is evidence that immune responsiveness is lower in the pregnant female of most species including humans (16). In this regard it has been found that progestational agents increase the hazard from anaphylactic shock in rats. Hydroxyprogesterone caproate (Dela lutin) and 6α methyl 17 hydroxyprogesterone acetate have been found to delay the rejection of skin grafts in rabbits (51).

The estrogenic hormone estrone increases the resistance of the castrated rabbit to vaccinia infection (45). Estradiol, however, has been found by other investigators to shorten survival time of animals infected with certain species of Aspergillus. This effect was correlated with leukopenia and relative neutropenia (31). Lurie found that in experimental infections with Mycobacterium tuberculosis, estrogens exerted a retarding effect on the growth of the lesion at the site of inoculation, diminished the extent of

the disease in internal organs and suppressed its dissemination throughout the body (30). Conversely, the gravid state in white mice has been shown to increase their susceptibility to infection with streptococci (51).

The effects of estrogen on the function of the reticuloendothelial system are well documented. It appears that the estrogenic molecule has two unrelated biological functions - modification of sexual organs and stimulation of the reticuloendothelial system (34). Estrogens exert an adjuvant effect on the primary immune response. Their ability to increase phagocytic ability, elevate serum gamma globulin levels and protect experimental animals against severe infection provide strong argument for a generalized arousal of body defenses. Several substances, including endotoxins, lipopolysaccharides, lipid A and zymosan, are also capable of this action; but to date, the strongest reticuloendothelial system stimulants appear to be estrogens both natural and synthetic (35). Cortisone has been shown to act as an antagonist to estrogen in that it depresses phagocytic ability, the total and differential white blood cell count and the serum levels of gamma globulin. In fact, if cortisone and estrogens are administered separately but simultaneously at separate sites, the depressant activity of cortisone is prevented and considerable stimulation of phagocytic activity occurs (17). Other reticuloendothelial depressants include prednisone, 4 hydroxy tetraphenylmethane, cholesterol, ergosterol, stilbene (33) and ethanol (2).

The basic mode of action in currently marketed oral contraceptives is similar in all - the inhibition of ovulation and the initiation of periodic

breakthrough bleeding. However, the potentiality of these compounds to affect human consumers is by no means limited to prevention of pregnancy. In an attempt to completely consider the drug modified human in his context as bacterial host, a brief review of metabolic effects is worthy of mention. It is now well established that the oral contraceptives inhibit ovulation predominantly at the pituitary level where they interfere with the synthesis and release of lutenizing hormone. After cessation of therapy resumption of ovulation is prompt, with return of pituitary function within 6 to 8 weeks.

Administration of Norethynodrel (1-5 mg per 100 g of body weight) to female white rats was found to produce striking effects in the adrenal glands. Histochemically, lipid was found depleted from the cortical zones while the parenchymal cells of the zona glomerulosa became smaller. Significant lipid depletion occurred in the zona fasiculata and reticularis after only 2 days of treatment (5). Total weight of the glands was found to be increased over that of controls. Corticosterone assays performed on adrenal cortices and plasma from treated animals indicated increased concentrations of hormone (6). In humans oral contraceptives have been observed to produce increased cortisol binding by serum proteins (23). Low levels of urinary 17 hydroxysteroids have been accounted for by Leach and Margulis (25) who observed a variable suppression of pituitary ACTH in response to treatment with progestin.

Studies of thyroid function reveal that the majority of women taking oral contraceptives show increased blood levels of thyroxine binding globulin (TBG) as well as protein bound iodine (PBI) with a simultaneous decrease

in the T_3 RBC uptake. the PBI may rise into the hyperthyroid range on occasion. These thyroid effects are probably secondary to estrogen induced increase of the binding proteins, for in no instance, has clinical evidence of hyperthyroidism been reported.

There is no good evidence that Enovid alters directly the diabetic state. However, in a few cases, some degree of difficulty in the management of diabetic patients has been reported in connection with Enovid therapy. One study has indicated that a high percentage of women taking Enovid for fertility control have impaired glucose tolerance. The incidence of abnormal test results is greater in women with a family history of diabetes than in those without such a history. It seems reasonable to associate this change in glucose tolerance with the "pseudo pregnancy" (15) induced by the drugs since pregnancy has long been known as an efficient screening test for pre-clinical diabetes mellitus. Viewed in this light it is possible that those women exhibiting impaired glucose tolerance during Enovid therapy and having a positive family history should be carefully followed in anticipation of the onset of overt diabestes mellitus. Specifically, this diabetogenic effect seems to be due more to the progestogenic component of the contraceptive combination.

Liver function is yet another parameter subject to alteration by the oral contraceptives. These drugs may produce in certain patients clinical signs of jaundice and modifications in various tests of liver function indicative of biliary stagnation and hepatocellular damage (40). These changes

are apparently mild and reversible on discontinuation of hormonal therapy. Women who have had cholestasis of pregnancy or familial, congenital or acquired defects of biliary secretion are most vulnerable (1). The capacity of certain synthetic steroids to induce abnormalities of liver function has been attributed to the unnatural structural configurations or chemical substitutions that characterize these compounds. For instance, the natural hormone progesterone has not been shown to have this action. The synthetic progestogens, however, have certain functional groups (e.g. the 17 alkyl groups) which are known to confer hepatotoxicity (23). In addition, the natural hormone estradiol and related synthetic estrogens consistently impair the ability of the liver cell to excrete the dye bromsulpthalein -- a sensitive test of hepatocellular function. The possibility of a potentiating effect of estrogenic and progestogenic compounds on liver function has not yet been thoroughly investigated (23). On a subcellular basis Norethynodrel was capable of producing alterations in certain hepatic drug metabolizing systems as measured in vitro. It should be noted that metabolic pathways most affected were those in which hydroxylated or oxidized products were formed. The nature of this inhibition appears to be competitive and may indicate that liver microsomal enzymes which catalyze certain aliphatic or aromatic hydroxylations of drugs and foreign compounds may also catalyze hydroxylation of progestogenic steroids. Evidence that such systems catalyze hydroxylations of androgenic, estrogenic and corticosteroids has already been forthcoming. It is of interest that Norethynodrel seems to stimulate

only certain drug metabolizing enzymes and that progesterone does not have this action (22). Electron microscopic studies of tissue obtained at liver biopsy from women taking oral contraceptives showed consistent mitochondrial changes as evidenced by considerable enlargement and irregularity of shape. The normal inner membrane was partially replaced by systems of laminae oriented parallel to the long axis of the mitochondrion (4).

Possibly a less significant metabolic effect with regard to serum copper levels has been reported by Carruthers (9). He was able to demonstrate a marked increase in the serum copper and ceruloplasmin levels in 25 female subjects taking oral contraceptives. The correlation between copper and ceruloplasmin concentration together with the finding of normal urine copper levels in 10 subjects suggested that the increased serum copper was due to increased ceruloplasmin synthesis.

In a study of 36 patients receiving oral contraceptives, Strauss and Pochi (41) found a significant decrease in sebum production. Separate administration of drug components revealed the effect primarily due to the estrogen. Suppression of sebaceous gland activity was found to be directly correlated with improvement in acne vulgaris. Indeed, recent clinical trials indicate striking effects of contraceptive drugs on dermal abcesses complicating acne (37).

Of all the possible effects attributable to oral contraceptives the tendency to thromboembolic phenomena has remained one of the most enigmatic and potentially lethal. Although there have been many studies by competent

investigators the results have proven conflicting and divergent. It is known that certain of the clotting factors such as fibrinogen and factors VII to X are elevated in women consuming oral agènts on a long term basis (12,32). Unfortunately, even though many of the factors in the blood concerned with clotting can be ascertained, their elevation is not a measurement of hypercoagulability. For example, although fibrinogen and factors VII to X are elevated in pregnancy, thromboembolism occurs most commonly in the puerperium when levels of these factors have returned to normal. At present all that may be said is that available statistics show no clear cut relationship between contraceptive administration and the production of thromboembolism. Indeed the Food and Drug Administration's advisory committee on obstetrics and gynecology has recommended a long term, carefully designed epidemiologic study to shed light on this problem.

Other long term effects presently being scrutinized include the possible participation in tumorigenesis. In experimental animals there is abundant evidence for such a role. Ovarian granulosa cell tumors have been induced in BALB mice by administration of 19-nor progesterone (21), progesterone (27), Norethindrone and Norethynodrel (28). The neoplastic faculty of the synthetic 19 nor-progestogenic steroids appears superior to that of the natural progesterone. Progesterone and 19 nor-progesterone contraceptives have also been shown capable of producing uterine malignancy, characteristically a sarcoma, in experimental animals. Similar tumors have been found in women receiving 19-nor progestogenic agents (29).

Estrogenic compounds have also been implicated in the production of neoplasia involving cervix, endometrium, ovary, breast, testicle, pituitary, kidney and bone marrow in laboratory animals (1). However, it must be remembered that animal studies in which certain susceptible strains and species are used and excessive dosages are employed, cannot be readily and accurately applied to the situation regarding human beings. Such data does serve to inject a note of caution and emphasizes the need for good, long term statistical studies.

MATERIALS AND METHODS

Cultures. The following microorganisms were used -- *Staphylococcus aureus* serotypes 1-13, Smith Diffuses strain, Rose strain and strain 209. These organisms were kept in stock at 4 C on tryptic soy agar (Difco) slants in screw capped tubes. Transfer to a new slant was made every four weeks. Cultures were checked periodically for morphology, anaerobic utilization of glucose and mannitol, free and bound coagulase, pigmentation and gelatin liquefaction by accepted microbiological procedures (44). In addition, inocula were screened before and after individual experiments for contaminants by Gram staining and the production of coagulase as measured by its ability to clot citrated plasma (Difco).

Hormone. Powdered pure Norethynodrel and Mestranol were obtained from the Searle Drug Company. Fresh solutions were prepared by dissolving 20 mg of Norethynodrel in 10 ml of 95% ethanol and immediately transferring 1 ml of the solution to 49 ml of tryptic soy broth (Difco) in 300 ml nephelometric flasks, yielding a final concentration of 40 μg hormone per ml of growth medium. Control flasks received 1 ml of 95% ethanol. Both sets of flasks were then sterilized by autoclaving. For animal inoculation 10 mg of Norethynodrel and 5 mg of Mestranol were dissolved together in 10 ml of 95% ethanol. Of this solution .1 ml was mixed with 9.9 ml of sterile saline to arrive at a solution containing 10 μg/ml of Norethynodrel and 5 μg per ml of Mestranol. Mice were injected intraperitoneally with .25 ml of the above

solution. Control animals received 0.25 ml of 1% ethanol in saline by the same route and at the same time.

When animals were injected intravenously with bacteria and hormone simultaneously the hormone was dissolved in ethanol and a 1% solution prepared as described previously. S. aureus previously grown at 37 C for 18 hr was then harvested by centrifugation and added to the solution. Adjustment to the desired bacterial density was accomplished by subsequent dilution with hormone solution. At no time were bacteria suspended in hormone solution for periods greater than one hr prior to use.

Growth studies. Cells used in growth studies were grown in 10 ml of 3% tryptic soy broth (Difco) at 37 C for 18 hr and harvested by centrifugation. A smooth suspension was prepared by manual shaking for 1 min and was then adjusted with sterile saline (0.9%) to 10 Klett units by use of a No. 42 filter in a Klett-Summerson photoelectric colorimeter. This bacterial density corresponded to approximately 7×10^6 viable cells per ml. Each nephelometric flask was inoculated with 1 ml of the above suspension and incubated aerobically in a walk-in incubator on a rotary shaker at 37 C, or anaerobically in a stationary carbon dioxide incubator at a similar temperature under 2.5 atmospheres of pressure (CO_2).

Growth was estimated turbidimetrically at varying time intervals using the Klett-Summerson photoelectric colorimeter and previously prepared standard curves. Under these conditions 1 Klett unit was equivalent to 1.5 plus or minus 0.3 μg dry weight of cells per ml.

Animal work. All mice and rabbits were obtained from Abrams Small Stock Breeders, Chicago, Illinois. The mice were maintained on a diet of Rockland Mice Pellets and the rabbits on Dixie Rabbit Pellets. All animals received food and tap water ad libitum.

White Swiss female mice, each weighing between 18 and 22 g, were used in all work. These mice were prepared by intraperitoneal injection of a 0.25 ml volume of solution containing 2% ethanol and varying concentrations of hormone prepared as above. On the fourth day the mice received a bacterial suspension in a solution containing hormone and adjusted to 450 Klett units. This suspension was administered through the dorsal tail vein in a total volume of 0.25 ml which had been adjusted to the desired hormone concentration. Mice were observed for three days after infection and the number of deaths on each day recorded.

New Zealand female rabbits, 6 months old and weighing between 2.7 and 3.2 kg, were used in all experiments. These animals were prepared by shaving bilateral paravertebral areas of skin approximating 9 sq.in. Subsequently they received at this site daily subcutaneous injections of varying quantities of hormone for periods of between 5 and 14 days. They were finally challenged by injecting into the marginal ear vein S. aureus suspended in a total volume of 1 ml of hormone solution. The amount of hormone administered was equivalent to that given on any previous individual subcutaneous injection. Total white blood cell counts were made by the use of a conventional white cell counting chamber, pipette and acetic acid (2.5%) diluent.

Relative quantities of neutrophils and lymphocytes were estimated under the oil immersion lens of the light microscope using Wright's stain and phosphate buffer (44). The first 100 white cells were counted at random in three widely separated areas of the same slide and standard hematological criteria based on size, shape, cytoplasmic granulation and nuclear morphology were used to identify various cells. Blood counts, both before and after bacterial challenge, were compared with suitable control values. In addition, samples of whole blood were aseptically drawn at various periods after infection from the marginal vein of the ear opposite to that used in inoculation. These samples were then diluted in sterile saline in a ten-fold continuum. Pour plates were made with 0.5 ml of the appropriate dilutions using tryptic soy agar (Difco) and placed in a 37 C stationary incubator for 48 hr. The number of colonies was then counted and the results expressed as colony forming units per ml of blood.

Bacterial counts. Mice were selected at random at 2, 4, 6, 18 and 24 hr after intravenous infection and sacrificed with ether. Utilizing sterile techniques the spleen, kidney, lungs and liver were excised and homogenized for 1 min with a Contorque power unit and 10 ml tissue grinders (Thomas teflon pestle model). The resulting homogenate was then prepared by serial ten-fold dilution in sterile saline (0.9%) and plate counts of viable organisms made as described above. Prior to homogenization organs were immersed in sterile saline (0.9%) and the volume of tissue estimated by observing the displacement of fluid in calibrated vessels. Results were expressed as colony forming units per organ and per ml of tissue.

In all studies outlined above adequate controls were maintained. In animal work the drugs were administered in dosages calculated to approximate as nearly as possible on a per unit weight basis those dosages routinely administered to human beings.

RESULTS

Growth studies. Turbidimetric assessment of the *in vitro* growth of *S. aureus* indicated a strong inhibitory action of Norethynodrel being most noticeable in the early or lag phase of the growth curve and persistent to some extent over a period of 8 hr (Table 1). It should be noted that Norethynodrel in dosage limited only by its solubility (40 μg per ml) evidenced a dose response relationship approximating linearity over the time period studied. After 8 hr of growth the inhibitory effect was found to decrease rapidly until at 12 hr in almost all instances no significant difference was discernible between flasks containing the hormone and the controls.

The effect of Norethynodrel on the *in vitro* growth of *S. aureus* in tryptic soy broth with regard to size of inoculum is emphasized in Table 2. Norethynodrel (40 μg per ml) produced an inhibitory effect on growth at an initial inoculum of 0.1 ml of 10 Klett units, much as in the first table. By varying bacterial density in the initial inoculum in an increasing manner growth curves were obtained which tended to confirm the inhibitory effect of Norethynodrel. Moreover it appeared that a larger inoculum size did not change the bacteriostatic effect while accelerating both its appearance and disappearance in the course of bacterial growth, a simple skewing of the curve to the left.

In an attempt to identify the specific portion of the growth curve at which Norethynodrel exerted its maximal effect the hormone was added to each

TABLE 1

EFFECT OF NORETHYNODREL ON THE GROWTH OF STAPHYLOCOCCUS AUREUS IN TRYPTIC SOY BROTH

	Average Klett readings at varying hormone concentrations		
Hours of growth	0 µg/ml	20 µg/ml	40 µg/ml
3	2.3	0.54	0.46
4	4.9	1.2	0.9
6	44.0	30.0	18.0
8	113.0	94.0	73.0

Inoculum: 0.1 ml of a suspension containing 10 Klett units of *S. aureus* (serotypes I-XIII, strain Rose, strain Smith and strain 209).

Incubation: Rotary shaker at a constant temperature of 37 C.

Results: Average of values obtained with serotypes I-XIII, strain Rose, strain Smith and strain 209.

TABLE 2

EFFECT OF NORETHYNODREL ON THE GROWTH OF *STAPHYLOCOCCUS AUREUS* IN TRYPTIC SOY BROTH WITH REGARD TO THE SIZE OF INOCULUM

Hours of Growth	Size of Inoculum (Klett Units) 10		100		200	
	Control	Hormone	Control	Hormone	Control	Hormone
3	5	0	30	16	50	28
4	20	8	56	26	110	80
6	75	48	128	101	191	170
8	115	95	170	160	246	245

Inoculum: 0.1 ml of a suspension of *S. aureus* (serotypes I, II, III) with a variable bacterial density as indicated above.

Incubation: Rotary shaker at a constant temperature of 37 C.

Hormone: Norethynodrel adjusted to a concentration of 40 μg/ml.

Results: Expressed in Klett units of bacterial density at various time periods after inoculation. Each result is the average value obtained with serotypes I, II, III.

of several flasks at varying time intervals after inoculation with *S. aureus*. It may be seen in Table 3 that each addition was effective in producing a strong inhibition of bacterial growth up to 7 hr after inoculation with *S. aureus*.

In view of the fact that any drug exerting effects on a living organism must do so in a millieu of other chemically active substances, growth studies were conducted utilizing Norethynodrel in combination with another steroid hormone. The presence in the media of such compounds as estradiol, testosterone, deoxycorticosterone, 5α-pregnane, pregnenolone and progesterone in concentrations of 20 μg/ml was not found to modify the action of Norethynodrel. The situation was different, however, with regard to Norethynodrel and hydrocortisone. Table 4 illustrates the fact that a combination of Norethynodrel (20 μg per ml) and hydrocortisone (20 μg per ml) effected bacteriostasis to a greater degree than either compound alone at a similar concentration. Thus, a synergistic effect of these steroidal compounds, one upon the other is noted.

The fact that the growth inhibition induced by Norethynodrel is not a lasting one, but is dependent upon the continued presence of the compound in the media, is illustrated in Table 5. Bacteria were incubated in broth containing hormone at a concentration of 40 μg per ml for a period of 18 hr. The organisms were then harvested, washed and used as inocula for hormone and control flasks prepared as in previous studies. Results reveal no difference in the subsequent growth or growth inhibition using as inocula

TABLE 3

EFFECT OF NORETHYNODREL ON THE GROWTH OF
STAPHYLOCOCCUS AUREUS IN TRYPTIC SOY BROTH
WITH REGARD TO THE TIME OF ADDITION OF HORMONE TO MEDIA

Hour of growth	Control	Hormone added at 2 hr	Hormone added at 4 hr	Hormone added at 6 hr	Hormone added at 7 hr
2	0	0	0	0	0
4	20	0	18	20	20
6	70	40	30	70	70
8	280	242	204	241	275

Inoculum: 0.1 ml of a suspension containing 10 Klett units of *S. aureus* (serotype III).

Incubation: Rotary shaker at a constant temperature of 37 C.

Results: Expressed in Klett units of bacterial density at various time periods after inoculation.

TABLE 4

POTENTIATION BY HYDROCORTISONE OF THE EFFECT OF NORETHYNODREL ON THE GROWTH OF STAPHYLOCOCCUS AUREUS IN TRYPTIC SOY BROTH

Hormone	Klett readings at various times (hr) 2	6	9
Control (Ethanol - 2%)	3	29	80
Norethynodrel - 20 μg/ml	0	3	54
Hydrocortisone - 20 μg/ml	0	15	75
Norethynodrel - 20 μg/ml Hydrocortisone - 20 μg/ml	0	0	25
Norethynodrel - 40 μg/ml	0	0	40

Inoculum: 0.1 ml of a solution containing 10 Klett units of *S. aureus* (serotype III).

Incubation: Rotary shaker at a constant temperature of 37 C.

TABLE 5

EFFECT OF NORETHYNODREL ON THE GROWTH OF *STAPHYLOCOCCUS AUREUS* GROWN PREVIOUSLY IN TRYPTIC SOY BROTH WITH NORETHYNODREL

Hours of growth	Klett reading with inoculum grown in the absence of Norethynodrel		Klett reading with inoculum grown in the presence of Norethynodrel	
	Control	Hormone	Control	Hormone
2	0	0	0	0
4	7	4	5	4
6	57	33	60	31
8	115	75	115	76

Inoculum: 0.1 ml of a solution containing 10 Klett units of *S. aureus* (serotypes IV, V, VI, VIII, IX, XI, XII) which had been maintained for 18 hr in broth containing either hormone or 2% ethanol.

Incubation: Rotary shaker at a constant temperature of 37 C.

Hormone: Norethynodrel at a concentration of 40 μg/ml.

organisms previously grown in the presence of Norethynodrel as compared to those grown in the presence of the hormone vehicle alone.

In Table 6 the effect of Norethynodrel on anaerobic staphylococcal growth can be seen. Single determinations of bacterial density at 24 hr after inoculation indicate a striking growth inhibition of *S. aureus* serotypes II and III. In this instance a maximal effect was approximated with a smaller concentration of Norethynodrel (20 μg per ml) than was found to be the case under aerobic conditions.

Clearance studies. The *in vitro* growth prompted exploration of the possible *in vivo* effects of contraceptive agents. Table 7 represents the effect of Enovid administration on the recovery of *S. aureus* (serotypes III, IX and XIII) from selected murine viscera at varying periods after intravenous bacterial challenge. In both spleen and kidney recovery of viable organisms was quantitatively reduced in those animals pretreated with Enovid, for a period encompassing the 6 hr after infection. At 18 hr after infection this effect was still noticeable. Similar clearance studies in mice revealed no such effect in liver and lung, nor were Norethynodrel or Mestranol individually capable of suppressing bacterial recovery.

Since it was felt that a decreased ability to recover viable organisms from viscera of hormone treated animals might be due to a decreased avidity of the reticuloendothelial system, a study of experimentally induced bacteremia in the rabbit was undertaken. In Table 8 it may be seen that with the exception of the 4 hr reading quantities of viable organisms recoverable

TABLE 6

EFFECT OF NORETHYNODREL ON THE ANAEROBIC GROWTH OF STAPHYLOCOCCUS AUREUS

Serotype	Klett readings with varying hormone concentrations (µg/ml) 0	20	40
I	15.0	12.5	10.0
II	51.5	19.0	6.0
III	66.5	15.0	16.5

Inoculum: 1 ml of a solution containing 10 Klett units of *S. aureus* (serotypes I, II, III).

Incubation: In a CO_2 stationary incubator at 2.5 atmospheres of pressure and 37 C.

Results: Average Klett readings with serotypes I, II, and III.

TABLE 7

FATE OF *STAPHYLOCOCCUS AUREUS* IN THE SPLEEN AND KIDNEY OF MICE TREATED WITH ENOVID

Colony forming units per ml of tissue homogenate

Hours after infection	Spleen		Kidney	
	Control	Hormone	Control	Hormone
2	2.9×10^6	1.6×10^6	3.6×10^4	2.4×10^4
4	3.4×10^6	1.5×10^6	4.9×10^4	2.3×10^4
6	3.8×10^6	1.9×10^6	1.9×10^5	5.1×10^4
18	7.4×10^5	4.1×10^5	3.7×10^6	2.9×10^6

Inoculum: 450 Klett units of *S. aureus* (serotypes III, IX, XIII).

Hormone: 5 µg Norethynodrel intraperitoneally for 5 days and 2.5 µg Mestranol in the same manner. On the day of bacterial challenge the drugs were administered intravenously in the same dosage.

Results: Average of trials utilizing serotypes III, IX, XIII.

TABLE 8

EFFECT OF ENOVID ON EXPERIMENTALLY INDUCED BACTEREMIA IN THE RABBIT

Group	Colony forming units per ml of blood at varying intervals (hr) after intravenous infection		
	2	4	6
Control	157	1670	673
Hormone	246	146	755

Inoculum: 1 ml of a suspension containing 400 Klett units of S. aureus, serotype III injected intravenously.

Hormone: Each rabbit received a total of 4000 μg Norethynodrel and 150 μg Mestranol subcutaneously over a period of 10 days.

from the circulating blood were remarkably similar in both control and hormone treated animals.

Mortality studies. These results would be of limited value if they did not correlate with the clinical response of the host to staphylococcal challenge. Consequently, hormone treated mice were observed for three days after infection and the time of their death noted. The results in Table 9 show that animals receiving a combination of Norethynodrel and Mestranol suffered fewer lethal infections on a percentage basis over the period of observation than did the control group. This protective action was most noticeable during the 24 hr immediately following infection when only 10% of hormone treated animals died as opposed to 33% of controls.

Blood studies. Norethynodrel was administered to white female rabbits in the hope of determining its capacity to modify the total white blood cell count and differential count, either before or after bacterial challenge. The results expressed in Table 10 suggest that there was no consistent difference between control and hormone treated animals in this respect.

TABLE 9

EFFECT OF NORETHYNODREL AND MESTRANOL IN COMBINATION ON THE MORTALITY OF MICE INFECTED WITH STAPHYLOCOCCUS AUREUS

Time (Days)	Per cent cumulative mortality: Hormone	Control
1	10.0	32.8
2	30.0	44.7
3	55.7	62.8

Inoculum: 450 Klett units of *S. aureus*, serotype III.

Hormone: 20 μg Norethynodrel and 10 μg Mestranol over a four day period.

Results: Expressed in percentage of total animals dying at various periods after intravenous infection.

TABLE 10

EFFECT OF NORETHYNODREL ON THE WHITE BLOOD CELL COUNT IN THE RABBIT

Group	Hormone			Control		
	Total	N	L	Total	N	L
Blood count before drug administration	10,025	32	68	12,640	43	57
Count after Norethynodrel 200 μg/5 days	9,675	27	73	13,500	56	44
Count 24 hr after giving 220 Klett units	9,200	64	36	9,200	68	32
	Total	N	L	Total	N	L
Blood count before drug administration	7,275	30	70	7,200	52	48
Count after Norethynodrel 4000 μg/10 days	8,000	46	54	8,000	41	59
Count 24 hr after giving 300 Klett units	28,850	76	24	16,700	62	38

Method: All hormone was given subcutaneously. Bacteria were given intravenously.

Results: Expressed in total white blood cells per cubic ml of blood. N indicates percentage neutrophils and L percentage lymphocytes.

DISCUSSION

The inhibitory action of Norethynodrel on the growth of staphylococci is a phenomenon not without experimental precedent. Similar effects have been attributed to progesterone (55) and the synthetic progestogen Norethindrone (54). The mechanism of such effects remains open to speculation. There is some evidence to indicate the possibility that progestogens, both natural and synthetic, may interfere with membrane permeability and active transport (48), and in so doing deprive cells of needed nutrients. Alternatively, it has been shown that progesterone may slow oxidative processes by impeding the flow of electrons in the cytochrome system resulting in a decrease of available cellular energy for support of growth and anabolic processes (24,48,50). Militating against such conclusions is the fact that anaerobic growth of *S. aureus* only served to accentuate the inhibitory effect of Norethynodrel (Table 5).

An interesting result consistently observed in the present studies was the manifest tendency of Norethynodrel to quell bacterial proliferation most efficiently early in the growth cycle, with a rapid subsidence of action after 8 hr of growth (Table 1). It must be remembered that at the onset of growth the concentration per organism of the hormone initially added is a maximum quantity. However, variation in the number of organisms in the initial inoculum did not appear to affect the magnitude of growth inhibition (Table 2), but only served to produce a simultaneous skewing of the zones

of growth inhibition and the growth curve itself without affecting their temporal interrelationships.

Cells in the log phase are multiplying at a rapid pace and consequently are characterized by accelerated patterns of metabolism -- a critical period for exposure to metabolic inhibitors and antagonists. By adding Norethynodrel to varying portions of the growth curve (Table 3) inhibitory effectiveness was found prominent throughout the first 7 hr of growth. This data shifts emphasis from the quantity of organisms present to their metabolic state and activity which at this time is beginning its entrance into the phase of deceleration of growth.

In addition it is quite possible that such actively metabolizing and proliferating cells achieve the quantitative ability to modify the hormones in some way so as to neutralize its antibacterial action.

Finally, it may be adduced that Norethynodrel exerts its effect throughout the growth curve. The dwindling difference between hormone treated and control flasks thus would be attributable not to progressive failure of hormonal inhibition late in the cycle, but to progressive inhibition of the growth of control organisms in the presence of accumulating toxic metabolites and depleted stores of nutrient. Such a phenomena probably does occur to an extent. However, the possibility of growth inhibition after 7 hr has been effectively ruled out by the results illustrated in Table 3,

Characteristically exposure of staphylococci to Norethynodrel <u>in vitro</u> induces no lasting change since upon reisolation the organisms do not exhibit any alteration in growth patterns or heightened susceptibility to

subsequent hormonal exposure (Table 5). The significance of such data is twofold. Firstly, the *in vitro* growth inhibitory effect is readily reversible Secondly, the ability of the staphylococcus to overcome this inhibitory effect may not be attributed to the emergence of, or the selection of, organisms possessing a metabolic constitution resistant to the effects of Norethynodrel.

In vivo experimentation utilizing white female mice revealed a quantitatible reduction in viable organisms recoverable from the spleen and kidney in those animals pretreated with Enovid (Norethynodrel and Mestranol). Since the dose, period of administration and time interval following injection have been shown to be of outstanding importance for the effect of hormones on the course of infection (49,52), these factors were carefully regulated and dosages chosen to approximate on a weight basis those given to human beings. Methods of bacterial challenge are equally important, however, and the course of experimentally induced bacteremia in laboratory animals has been carefully reviewed by Rogers (38). Contrary to popular belief most microbes are less capable of provoking disease when injected intravenously than when they are administered by any other route. Rogers divides the bacteremia which ensues into 3 phases. The first phase, between 10 min and 5 hr after infection, is characterized by the disappearance of 90 to 99.9% of the circulating bacteria. The second phase may last from 4 hr to several days; and depending on the animal and the pathogen, microorganisms may persist in the circulation at lower concentrations or be

removed at slower rates. During the third phase bacteria producing fatal infections reappear in the peripheral blood in large quantities and persist until the death of the animal. In the event of survival there may be a temporary rise in blood levels of bacteria with subsequent complete clearance. The virulence of the organisms in question largely governs the late blood findings.

Certain cells of the reticuloendothelial system, the Kupffer cells lining liver capillaries and phagocytic cells in splenic sinuses, are the most important sites of initial clearance of bacteria (7). Microbes are removed at a constant rate characteristic for the bacteria and the uptake by organs is related in a definite manner to their disappearance from the bloodstream. It is during this first phase of bacteremia that Norethynodrel was found effective in reducing the quantity of recoverable organisms in spleen and kidney (Table 8). In view of what has been said above such a phenomenon could be due to an increased avidity of the blood for the organisms, decreased uptake by the fixed phagocytes of the reticuloendothelial system or a more rapid killing of ingested organisms by the phagocytizing cell.

Several workers have found that pathogenic staphylococci possess the capability to remain viable within phagocytes for several hours after ingestion (39,42,43). A differential effect due to intracellular killing within the first 2 hr of infection would appear unlikely. Studies on levels of bacteremia in rabbits as well as total white blood cell counts and

differential counts have consistently revealed no difference between rabbits treated with hormone and control animals (Tables 9, 10). Thus it would appear that phagocytic ability of the white blood cell has not been altered either quantitatively (4) or qualitatively.

Decreased phagocytic ability of the fixed phagocytes of the reticulo-endothelial system is a possible explanation. However, the mechanism of such an action or why it should selectively affect only cells of the spleen and kidney is obscure and could be dependent on local humoral or anatomic factors. Although during phagocytosis by leucocytes there is a large increase in the oxygen uptake, most of this does not yield energy for the process of ingestion (36). In fact, Brogan has shown that the serum independent mechanism of phagocytosis relies almost completely on glycolytic energy (8). Thus demonstrated effects of progesterone on electron transport may not be invoked as an explanation here (50,53).

Whatever the mechanism, the end result is apparently a decreased mortality in mice protected by hormone (Table 8). Experimental evidence suggests that bacteremia persists or resurges into the "third phase" only when there is active seeding of the bloodstream (38). There is some indication that certain bacteria may reseed the circulation from the reticulo-endothelial tissues in which they are originally sequestered. If indeed such sequestration is prohibited, or if sequestered organisms are rendered non-viable then it might reasonably be expected that reseeding and mortal dissemination could be delayed or prevented. Decreased deposition and

proliferation of pathogenic organisms in vital organs such as the kidney may prolong life by decreasing the probability of resurgent bacteremia and the likelihood of destruction of renal tissue necessary for the maintenance of life.

SUMMARY

The effect of Norethynodrel on the growth of *S. aureus* was studied *in vitro*; and the effect of the combination of drugs, Norethynodrel and Mestranol (Enovid) on the course of *in vivo* bacteremia was determined. The results may be summarized in the following manner:

1. Norethynodrel inhibits the growth of a variety of serotypes of *S. aureus in vitro*.

2. This effect is most marked in the lag phase of growth but persists well into the exponential portion of the growth curve.

3. Hydrocortisone and Norethynodrel in combination were able to produce a greater degree of growth suppression than either drug alone.

4. Growth in a CO_2 atmosphere potentiates the inhibitory effect of Norethynodrel.

5. The combination of Norethynodrel and Mestranol reduces the viable organisms recoverable from the spleen and kidney of hematogenously infected mice.

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