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The Effect of Androgens on the Growth and Virulence of Staphylococcus aureus

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THE EFFECT OF ANDROGENS ON THE GROWTH AND VIRULENCE OF STAPHYLOCOCCUS AUREUS

by

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STATEMENT OF THE PROBLEM

The extensive clinical use of sex hormones, such as in fertility control and tumor therapy, without explicit knowledge of the interaction of hormone and patient, represents a paradox in modern biology. The mechanism of hormone action involves a series of steps. First, there must be an interaction between the hormone and pre-existing receptor molecules in the target tissue. Second, this interaction must stimulate a function of the receptor molecule. This is the primary response to the hormone. Third, the primary response is amplified by metabolic action of the target cells, producing a gross physiologic response. At present, virtually nothing is known about the primary action of any hormone.

Interest in the biosynthesis and metabolism of hormones has been greatly stimulated by recent advances and applications of refined isotopic and chromatographic techniques. Among the androgens, there has been a pre-occupation with testosterone, which has been incorrectly termed, "the only natural androgen of biological importance". It is highly improbable that other androgens serve merely as precursors or inactive metabolites of testosterone. In fact, actual proof that testosterone is the active form of the hormone is lacking.

The term androgen usually signifies a C-19 steroid, which acts in man and other animals to produce and maintain the secondary sexual characteristics in the adult male (8).

Androgenic effects on staphylococcal infections represent an especially fertile field of study. Disease caused by these bacteria has caused much concern due to the occasional state of recurrence, problems of treatment, and long duration.

Secretions of the endocrine system have long been implicated in the reactivity of an infectious staphylococcal process. This becomes apparent at infancy, puberty, pregnancy, menopausal and post-menopausal years. In the underlying mechanism of an altered host response, possible hormonal effects on the staphylococci must be considered.

It is the purpose of this thesis to elucidate hormonal-staphylococcal interactions by studying the *in vitro* and *in vivo* effects of androgens on the growth and virulence of *Staphylococcus aureus*. The third step of amplification of the hormonal response will be considered. Mechanisms of androgenic actions will be investigated and an attempt made to correlate *in vitro* bacterial activity and *in vivo* studies of staphylococcal infected animals.

REVIEW OF LITERATURE

An extensive review of the literature revealed a relative lack of information concerning interaction of sex hormones and microorganisms. A few preliminary reports have been published, but caution must be exercised in interpreting these results since high levels of hormones sometimes employed may involve phenomena unrelated to their normal actions. Some reports hypothesize and conclude based on non-therapeutical or unphysiological concentrations. A few possible insights may be obtained in this manner, but extreme care must be taken in applying this data to host defense mechanisms.

Yotis and Stanke (33) reported an *in vitro* bacteriostatic action of progesterone on *Staphylococcus aureus* and other gram positive organisms at levels of 10 to 20 μg/ml. Gram negative bacteria, however, were not subject to the inhibitory action. Pregnenolone, 4-pregnen-20βol-3one, and 5α-pregnane exhibited anti-bacterial properties for gram positive organisms. Two interesting observations were made. Progesterone acted only during the first eight hours of incubation and anaerobic conditions enhanced the hormonal inhibition of bacterial growth.

Nicol (21) found estrogens to be natural stimulants of host defense mechanisms. He treated male white mice with two μg of estrogen for six consecutive days and found increased survival times for infections induced by microbes as *Diplococcus pneumoniae* types 1 and 3, *Pasteurella septica*, *Salmonella typhimurium* and *typhi*, *Escherichia coli*, *Hemophilus pertussis*,

and Pseudomonas aeruginosa. The author postulated a stimulation of the reticuloendothelial system and phagocytic ability as one of the main effects of estrogen therapy.

Kutzsche (17) observed the action of several steroids on Salmonella typhimurium and typhosa, and Diplococcus pneumoniae infections in mice. He stated that the protective action of steroids did not result from detoxification of bacterial endotoxins but rather from diminishing tissue sensitivity to them.

Tokuda (29) described effects of steroid hormones on staphylococcal ocular infections in rabbits. The viable count of bacteria in the aqueous humor was significantly reduced in the hormone treated groups.

Yotis (32) examined norethindrone, a recent progestational contraceptive, for anti-bacterial properties. Bacteriostatic action was found with gram positive but not gram negative microorganisms at 10 to 50 μg/ml under anaerobic conditions. The size of staphylococcal skin lesions and bacterial viable counts were significantly reduced in female rabbits by two injections of norethindrone at 10 to 20 μg/ml. Mestranol alone showed no activity, but it enhanced the inhibitory action of norethindrone. This effectively points out that hormonal interaction does occur and presents a problem pertinent to in vitro hormonal studies and their application to in vivo phenomena.

Ghione (9) found that in vitro 4-chlorotestosterone exerted no bacterial effects, while in vivo it showed inhibitory action in marine staphylococcal infections. This work emphasized two interesting problems. The

bacteriostatic action of this hormone would have been overlooked in the usual in vitro assay procedures. In vitro data indicate that 4-chlorotestosterone is not a direct antibacterial agent but stimulates organic reactive systems of the mice.

Lester (18) reported significant growth inhibition of Neurospora crassa by androgens, including testosterone and androstenedione.

Von Haam and Rosenfield (31) studied the action of various sex hormones on pneumococcal infections in mice. Testosterone propionate, administered at doses of 1 to 10 μg intra-peritoneally, significantly lowered the mortality rate of mice infected three days after a single dose of hormone. They found better protection for the male rather than the female mice.

The aforementioned references have involved only the inhibitory activity of the sex hormones. Rodwell (24), however, noted that specific steroids, especially those with a 3β OH group, could act as growth promoting agents of Mycoplasma mycoides.

Chattaway (7) postulated a steroidal effect on the growth of certain dermatophytes. He found that sex hormones enhanced dermatophyte infections, especially in microsporium diseases at puberty.

Alterations in steroid structure are produced by many microorganisms, particularly fungi. Carlstrom (4) noted that several organisms oxidize androstenedione to testololactone. In particular, Aspergillus flavus and Penicillium lilacinum cleave the side chains of progesterone via two separate pathways to produce androstenedione.

Naguchi and Fukushimo (20) studied the numerous hydroxylations of saturated 17-keto steroids by Penicillium molds.

Decomposition of steroids has been reported by Schatz (25). Nocardia erythropolis can metabolize testosterone, progesterone, and dehydroepiandrosterone.

Capek et al. (3) reported steroidal transformation involving progesterone → 11-β-OH-progesterone by Rhizopus nigricans, dehydroepiandrosterone → androstenedione by Actinomyces globisporus, and androstenedione → testosterone by Saccharomyces cerevisiae. In these transformations, the transformable steroid usually inhibited oxygen uptake by the microorganism while the transformed steroid exerted no effect. Therefore, the authors concluded that the transformations probably represented a detoxication procedure by the organisms.

Petersen and Murray (23) gave evidence for the oxygenation of progesterone at the C-11 position by the common mucorales mold upon incubation for 24 to 48 hr. A similar oxygenation occurred with androstenedione.

Jefferson (15) found that asperenone, a major yellow pigment of Aspergillus niger, accumulated under non-growing conditions in the presence of minerals, glycerol, and 10 μg/ml of specific sex hormones, notably estrone, estradiol, and progesterone. Steroid structure appeared to be related to mineral composition for optimal asperenone formation. The author postulated that steroids altered the effective concentration of the specific metallic ions at cell permeability sites.

MATERIALS AND METHODS

Procedure for Measuring In Vitro Effects

Cultures. Staphylococcus aureus serotypes 1 to 13 inclusive (ATCC 12598-12610) were used. Stock cultures were maintained on tryptic soy agar (Difco) slants at 4 C and transferred to new slants every four weeks. The cultures were tested by accepted biological diagnostic methods for glucose and mannitol fermentation according to the modified method of Hugh and Leifson (14), for free and bound coagulase, for growth on staphylococcal media #110 (Difco), for liquifaction of gelatin, for hemolysin production on rabbit blood agar, for reduction of nitrate to nitrite, and for inability to produce indole in peptone water.

Crystalline androstenedione, androsterone, testosterone, epiandrosterone, and dehydroepiandrosterone were obtained from Sigma Chemical Co. Crystalline stanolone; androstanedione, 5α-androstan-3β-17β-diol; 5β-androstan-3αol,-17one; 5βandrostan-3,17-dione; 5β-androstan-3α,17βdiol; 5α-androstan-3α,17β-diol; 5-androsten-3β-ol,17one-3β sodium sulfate; 5α-androstan-3αol, 17 one -3 sodium sulfate were obtained from Mann Research Laboratories, Inc. The purity of these steroids was tested by observations of melting points and maximum absorbance.

Hormone Solutions. A weighed amount of fresh steroid was dissolved in 95% ethanol. A sample (0.5 ml) of this was added to 49.5 ml of sterile 3% tryptic soy broth (Difco) or 3.7% brain heart infusion broth (Difco) in a

300 ml nephelometric flask to obtain final concentrations of 20 to 50 μg/ml in 1% ethanol. Control flasks received 0.5 ml ethanol similarly.

Aerobic Growth Studies. S. aureus was grown for 16 to 20 hr in 5.0 ml of sterile tryptic soy broth (TSB) or brain heart infusion broth (BHI) at 37 C and harvested by centrifugation. A smooth suspension was prepared in sterile saline by mixing for 60 sec with a Vortex Junior Mixer. The cultures were adjusted to 10 Klett units with a No. 42 filter and a Klett-Summerson photoelectric colorimeter. The suspension contained approximately 7.0×10^6 viable cells/ml (33). Each flask was inoculated with a specified amount of the cell suspension and incubated on a rotary shaker at 37 C. Turbidity, as a function of growth, was followed with the Klett-Summerson photoelectric colorimeter equipped with a blue filter.

Anaerobic Growth Studies. Flasks were inoculated with bacteria prepared as in aerobic growth studies. A Precision Thelco anaerobic incubator at 37 C with a CO_2 gas phase was used for static incubation. At initial signs of growth, the flasks were removed and turbidity determined as in aerobic growth studies.

Synthetic Media. Growth studies were undertaken with chemically defined media from Grand Island Chemical Co. (Gibco, Grand Island, N.Y.). The following nutrient mixtures were used: nutrient mixture F-10 with glutamine; GIB medium with glutamine and without serum; L-15 (Leibovitz) medium with glutamine and without antibiotics; Puck's N-16 with glutamine; Scherer's maintenance solution (revised); SRI-14 medium with glutamine, without serum and antibiotics.

Bacteriostatic Studies. Cells were grown 16 to 20 hr, harvested, and a smooth suspension prepared and adjusted to 10 Klett units in saline. Hormone in 95% ethanol was added to a final concentration of 40 μg/ml in 1% ethanol. The control consisted of only the vehicle in 1% ethanol solution. Samples were removed at specific time intervals and serial 10-fold dilutions made in sterile saline. Samples (0.1 ml) of each dilution were spread on tryptic soy agar, incubated at 37 C, and viable counts determined 24-48 hr thereafter.

Labeled Testosterone. ^{14}C-testosterone was obtained from the New England Nuclear Corp. with a specific activity of 50.6 mc/ mmole. Bacterial cultures were grown for 24 hr, harvested by centrifugation, and adjusted with sterile broth to 200 Klett units. A homogeneous suspension was prepared by passing the cells through a #27 gauge needle twice. This suspension was divided into two portions. One received labeled testosterone at 10 or 40 μg/ml in 1% ethanol, while the other received only the vehicle. Incubation followed for 30 to 120 min. A similar experiment was performed in which the bacteria were grown in the presence of the labeled testosterone. The cells were harvested by centrifugation and 0.2 ml of the supernatant fluid was placed in a vial of scintillation medium containing: 900 ml p-dioxane, 150 ml anisole, 150 ml 1,2 dimethylethane, 18 g PPO, 60 mg POPOP. The supernatant fluid was discarded and the cells resuspended in sterile 0.9% saline. Similar washings followed, each time removing 0.2 ml of the supernatant fluid. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer, model 314EX. Appropriate blanks were used containing broth or water in place of supernatant.

DNA Determination. Determination of DNA by Ceriotti's (6) indole method confirmed the turbidimetric readings and corresponding plate counts. Calf-thymus DNA was used as the standard.

Manometric Studies. S. aureus was grown in broth for 6 to 8 hr, harvested by centrifugation, washed twice in 0.1 M sodium phosphate buffer, pH 7.0. A smooth suspension was prepared in phosphate buffer and adjusted to 200 or 250 Klett units. This suspension was equally divided. One-half received hormone at 40 μg/ml in 1% alcohol (ethanol or methanol), while the other received only the 1% alcohol. Incubation followed at 37 C for various time intervals. Samples (1.0 ml) of bacterial suspension were placed in the main compartment of a Warburg vessel. The center well contained 0.2 ml 35% KOH with a paper wick and 0.5 ml substrate was added to the sidearm. Total fluid volume was adjusted to 2.2 ml with phosphate buffer. Rates of oxygen uptake in air at 37 C were determined by conventional manometric techniques.

Preparation of Animals and Explanation of Assay Procedures

Mortality in Mice. White, male mice weighing 18 to 21 grams were injected intraperitoneally on 3 consecutive days with hormone at 1 to 20 μg/ml in 1% ethanol-saline solution. Similar control injections consisted of 1% ethanol saline. On the fourth day, the mice were injected in the tail vein with 0.25 ml of a homogeneous suspension of S. aureus previously grown on brain heart infusion agar salnts for 8 to 10 hr, suspended in saline and adjusted to 450 Klett units. Immediately after each intravenous injection,

a fourth intraperitoneal injection of hormone or control solution was administered. Daily observations of mortality rates were made.

Rabbit Skin Lesions. New Zealand, white, male rabbits, 6 months old, weighing 2.5 to 3.5 kg were used. The animals were shaved on both flanks. A smooth suspension of S. aureus in saline adjusted to 200 Klett units was divided into two equal portions. One part received hormone at 40 μg/ml in 1% ethanol saline, while the other received only the vehicle. Appropriate aliquots were injected intradermally on each flank. The size and appearance of subsequent skin lesions were noted. Areas were traced on transparent plastic, transferred to paper, and measured planimetrically.

Growth Studies in Serum. Androgens in 1% ethanol saline were injected subcutaneously in a 1.0 ml volume into New Zealand, white, male rabbits. 5 to 10 min thereafter, blood was removed by cardiac puncture and allowed to coagulate at 22 C for 1 hr, followed by 4 C for 1 hr. Serum was separated by two centrifugations at 3,000 rpm for 15 min. Controls were treated similarly, receiving only the vehicle. Serum was aseptically transferred in 4.0 ml portions to test tubes and inoculated with a smooth suspension of bacteria, prepared as in aerobic growth studies. Incubation followed on a rotary shaker or statically at 37 C. At initial sign of growth, cells were centrifuged, resuspended in sterile saline, and turbidity determined with a Klett-Summerson photoelectric colorimeter. Serial 10-fold dilutions in sterile saline and corresponding plate counts were made to confirm Klett readings. It should be mentioned that a blank of sterile, uninoculated

serum was used for the hormone treated and control sera. This was treated the same as the other inoculated tubes and compensated for turbidity due to protein precipitation. Glucose levels were obtained by the anthrone - H_2SO_4 method (12). Cholesterol levels were determined by the method of Carr-Drekter (1) and Bloor, Pelkin, and Allen (2).

RESULTS

Effect of Androgens on the Growth of S. aureus

Aerobic and Anaerobic Growth Curves. Testosterone inhibits the growth of S. aureus at concentrations of 20-50 μg/ml in tryptic soy broth and brain heart infusion broth. Inhibition is proportional to hormone concentration with slight activity at 20 μg/ml. Table 1 shows rates of growth for 3 serotypes grown in 40 μg/ml testosterone. No effects were observed at 0.1 to 0.003 μg/ml. Anaerobiosis enhanced the inhibitory action of testosterone and of all other hormones tested according to the susceptibility of strains utilized.

Two water-soluble steroids, 5-androsten-3βol-17βone-3-$NaSO_4$ and 5α-androstan-3α-ol-17one-3$NaSO_4$, did not effect growth of serotypes 11 and 12 at 50, 40, 30, and 20 μg/ml. Similarly, anaerobic conditions were ineffectual for serotypes 1 to 13 with these two hormones.

Table 2 indicates the relative potencies of 5 androgens at 40 μg/ml on serotype 3. Maximum inhibition was given by epiandrosterone followed by 5α-androstan-3,17-dione, and dehydroepiandrosterone. These hormones did not inhibit at 2.0 and 0.001 μg/ml.

Data of a related experiment using serotype 12 and the 5 hormones at 40 μg/ml showed similar results, except for androsterone, which exerted no effects. This specificity is also evidenced by the following table:

Serotype 12 Concentration 40 μg/ml

INHIBITORY: 5α-androstan-3,17-dione (greatest)

5β-androstan-3αol,17-one

5β-androstan-3,17-dione (least)

NO EFFECT: 5β-androstan-3α,17β-diol

5α-androstan-3β,17β-diol

5α-androstan-3α,17β-diol

Serotype 3 Concentration 40 μg/ml

INHIBITORY: 5α-androstan-3,17-dione

5β-androstan-3α,17β-diol

NO EFFECT: 5α-androstan-3β,17β-diol

5α-androstan-3α,17β-diol

5β-androstan-3,17-dione

5β-androstan-3αol,17-one

It seems unlikely that a transformation or detoxication mechanism exists since only 5α-androstan-3,17-dione inhibits growth of both serotypes and 3 other hormones produce conflicting activities.

Under anaerobic conditions, the 11 previously discussed hormones and dehydroepiandrosterone, at 20 μg/ml inhibited the growth of serotype 12 (Table 3). Maximum inhibition was attained with epiandrosterone, 5α-androstan-3,17-dione, and dehydroepiandrosterone. The action of 5α-androstan-3α, 17β-diol was questionable, but further work proved it to be inhibitory also.

5α-androstan-3,17-dione, as a potent inhibitor, was chosen for further experimentation. Studies of growth rates (Table 4) indicate the minimum

inhibitory concentration to be 15 to 20 μg/ml. As expected, a progressive decline in hormone concentration resulted in a proportional decrease in growth inhibition. As shown in Table 5, 6 of 7 serotypes did not grow as luxuriantly as comparable controls when grown anaerobically in the presence of this hormone at 20 μg/ml.

Growth of S. aureus in synthetic media was investigated to determine whether 5α-androstan-3,17-dione interferes with essential nutrients. 20 μg/ml produced no effects under anaerobic conditions. Growth, without inhibition, occurred in nutrient mixture F-10, Scherer's maintenance medium, and SR-I 14 medium, while Puck's N-16 medium, L-15 medium, and GIB medium did not support apparent growth after 20 hr incubation. Further investigation of hormonal effects in known synthetic media is in progress.

All steroids exerted a bacteriostatic rather than bactericidal action on serotypes 3, 12, and 13. Cells were suspended in saline and incubated 24 hr in the presence of 40 μg/ml hormone. In a few cases, slight decreases in viability were noted, but these were not significant.

Inhibition was usually overcome 8 to 10 hr after inoculation. To investigate this phenomenon, 2 sets of bacterial cultures were grown to 70 Klett units (early log phase). At this point 5α-androstan-3,17-dione, at 40 μg/ml, was added to one group while the other group received only the hormone vehicle. Growth continued to the stationary phase without any noticeable effect, indicating that inhibition occurs only at low cell concentration and a transformation or detoxication process is probably not involved.

Bacterial selectivity was investigated. Cells were grown to late log phase in the presence of 5α-androstan-3,17-dione at 30 μg/ml. At this stage, hormonal inhibition has been overcome. 2 ml of the culture were aseptically removed, centrifuged, resuspended in saline, and adjusted to 10 Klett units. These cells were inoculated into a second set of flasks containing the hormone at 30 μg/ml and appropriate controls. Inhibition again occurred. This experiment indicates that a selective mechanism probably is not responsible for the loss of inhibition after 8-10 hr of growth.

Labeled Testosterone. ^{14}C-testosterone was used to investigate hormonal entrance into the staphylococcal cell. Cells were grown 24 hr in the presence of the hormone at 40 μg/ml. The cultures were centrifuged at 10,000 rpm for 15 min. A sample (0.2 ml) of the broth supernatant was removed and placed in scintillation fluid. The remaining broth was discarded and the cells were washed four times in saline, each time removing 0.2 ml aliquots of the supernatant. Table 6 shows that 83% of the activity was detected in the broth supernatant. Further washings removed about 6% of the total activity. Similar experiments were performed in which cells were grown 24 hr, hormone was added, and the cells were incubated for 30 to 150 min. Again 80-85% of the activity appeared in the broth supernatant.

Biological assays verified the above experiment. Bacterial cultures were grown 24 hr in the presence of testosterone at 40 μg/ml and in the presence of the hormone vehicle. The cultures were centrifuged and the broth supernatant removed and autoclaved. If 80-85% of the hormone is removed

in the initial centrifugation, enough testosterone should be present to produce inhibition upon reinoculation. Two things should be mentioned here. Previous experiments had shown no loss of hormonal inhibitory ability during autoclaving procedures and the minimal inhibitory concentration of testosterone is 20 μg/ml. Significant inhibition was observed as shown in Table 7. An identical experiment was done with 5α-androstan-3,17-dione at 30 μg/ml and similar results were obtained.

Similar results were also obtained when the cells were grown 24 hr, hormone added to one group of flasks while a second group received only the 1% ethanol and the cells incubated for 30 to 120 min. Upon centrifugation, autoclaving, and reinoculation, inhibition again appeared.

A third type of biological assay was done in which broth was isolated from cultures by using a Seitz millipore filter instead of centrifugation. Inhibition again appeared.

Manometric Studies. Oxygen uptake was studied by conventional Warburg manometric techniques. 5α-androstan-3,17-dione, dehydroepiandrosterone, and epiandrosterone at 40 μg/ml were used in 1% ethanol or 1% methanol. Incubation of the cells with the hormone varied from 15 to 180 min. Serotypes 12 and 13 were tested. The concentration of respiring cells was adjusted to 200 or 250 Klett units. The bacteria were grown in BHI or TSB for six hr, in BHI for 18 hr under static and shaken conditions, and on BHI agar slants. TSB, BHI, sodium pyruvate with niacin and thiamine, rabbit serum, and 1% glucose - all gave inconclusive results as substrates. Thus, no positive

experimental data emerged from the manometric studies at this hormone concentration.

Effect of Androgens on Male, White Mice

Upon daily treatment of mice with 5α-androstan-3,17-dione at 20, 10, and 1 μg/ml for four days and subsequent intravenous injection of S. aureus, no significant protective or destructive action was observed. 100 mice were used in each group.

Effect of Androgens on Male, White Rabbits

Some definitions are in order before presenting the data (Stedman's Medical Dictionary).

Lesion - A more or less circumscribed pathogenic change in the tissues.

Erythema - Redness of the skin, inflammation.

Pus - A fluid product of inflammation consisting of leukocytes and debris of dead cells and tissue elements liquefied by proteolytic and histolytic enzymes elaborated by PMN leukocytes.

Necrosis - Pathogenic death of one or more cells or of a portion of a tissue or organ, resulting from irreversible damage to the cell nucleus.

Lesion Size and Morphology. Four rabbits were used in the hormone treated group and four in the control group. Interpretation of lesion severity was based on areas of pus and erythema. 5α-androstan-3,17-dione was the only hormone investigated. Erythema was observed 24 hr after

intradermal injection of S. aureus and pus 40 hr thereafter. Recession of lesions occurred 60 to 70 hr post-injection. Complete healing required at least four weeks depending on the area of necrosis.

The mean values for all four experiments cannot be totaled because an optimal system was not found. The data in Table 8 represent a typical experiment in which S. aureus produced necrosis. Positive statements should not be made based on four rabbits per group, but it appears as if the hormone does retard lesion production.

Serum Studies. Testosterone and 5α-androstan-3,17-dione were chosen for further experimentation because of their good inhibitory properties as noted in the previous studies. Preliminary work with dehydroepiandrosterone, androstenedione, and epiandrosterone indicated that these also acted as inhibitory agents. Obvious growth usually occurred 10 to 12 hr after inoculation, but occasionally 18 to 20 hr were required. This may be a reflection of the age of the initial bacterial inoculum. Plate counts of the serum grown cultures were made to exclude miscalculations due to any precipitation in the serum. These correlated well with Klett readings.

20 μg of 5α-androstan-3,17-dione produced an inhibitory pattern similar to in vitro growth in broth. The bacteria grew more luxuriantly in the control sera. As shown in Table 9, maximum growth was achieved in eleven hr in the control and in fourteen hr in the hormone treated serum. The following two physiological constants indicate the differences:

	Hormone	Control
Time lag constant, T_L	$= 8.7 \times 10^8$ cells	4.1×10^8 cells
Growth lag constant, L	= 9.2 hr	5.5 hr

The next step was to utilize 13 serotypes of S. aureus with the same hormone at the same concentration. Inhibition of growth was noted in 9 of 13 serotypes (Table 10). Further experimentation to investigate the apparent stimulation of serotypes 1 and 7 was not done due to limitations of time and rabbits. It should be mentioned that the other tables concerning serum studies are also not perfect, but do give a significant indication of inhibition.

At this point, serum glucose levels were determined since any large difference between control and hormone serum might account for the variations in growth rates. Glucose levels varied 0.2 to 0.3 mg % in an inconsistent pattern. This was insignificant.

Reports exist (16,30) that implicate androgens in the production of a hypocholesteremia of the blood. Cholesterol levels were observed in serum isolated by cardiac puncture (growth studies) and in serum obtained by slitting the marginal ear vein. In the latter studies, blood was collected before and at timed intervals after subcutaneous injection of 5α-androstan-3,17-dione. Initial experiments were encouraging, but further work showed a fluctuation too great to be conclusive. The data follow:

	Number Tested	Mean Mg%	Range Mg%
Control	21	57	33-177
Hormone	33	56	35-87

The fluctuation, as seen in the ranges, is a typical characteristic of human serum cholesterol levels. The cholesterol level is highly variable especially after eating.

The effects of temperature on hormonal inhibition were investigated. 20 μg of 5α-androstan-3,17-dione were injected and the serum collected. Before inoculation of the bacteria, the serum was subjected to a variety of temperature conditions. Inhibition was not inactivated by heating for 30 min at 50, 60, or 65 C or by freezing for three days. If heated for 30 min at 70, 80, or 100 C, serum coagulated and could not be used.

The next step was to vary the amount of hormone injected. Testosterone was injected in 20, 10, and 1 μg amounts. Table 11 shows the percent inhibition based on six to eight serotypes per concentration.

Other androgens were injected to determine whether growth inhibition was a general androgenic response. Table 12 shows the relative potencies of other androgens. Dehydroepiandrosterone, epiandrosterone, and androstenedione produced good inhibition.

A few preliminary experiments were done with female rabbits. 20 μg of epiandrosterone were injected. No significant effect was observed. This is in contrast to a similar experiment in which males were used and good inhibition was noted.

Labeled Testosterone. ^{14}C-testosterone was injected into rabbits to determine metabolic clearance rates, as an indication of the optimal time for cardiac puncture. Hormone (29 μg with a specific activity of 0.005 mc/ml)

was injected subcutaneously. Blood was removed from the marginal ear vein before injection and at timed intervals after injection. Serum was isolated and testosterone extracted according to the method of Horton et al. (13). A specific volume of the extract was placed in scintillation fluid and its activity determined. Figure 1 shows the immediate uptake of the hormone. Highest levels of activity were attained five to 10 min after the injection. A slight amount of activity is detectable two hr post-injection. This experiment assumes that the testosterone is not metabolized.

The level of serum ^{14}C-testosterone can be calculated for any certain time based on the amount of hormone injected, its specific activity, and instrument efficiency. Table 13 gives a crude estimation of these levels. The reported values for 5 to 10 min (time of cardiac puncture) are approximately 35 times higher than the normal values for human adult males.

DISCUSSION

In Vitro Effects

Previous workers have shown a relationship between chemical structure and biological activity of androgens (5,11,22,26). No such correlations were observed regarding antimicrobial activity on S. aureus. Two lines of evidence are indicative of this. First, examination of the chemical structures of the 12 androgens, especially the four most active ones, testosterone, epiandrosterone, dehydroepiandrosterone, and 5α-androstan-3,17-dione, revealed no clear-cut evidence of a specific active group. Unsaturated carbon bonds, keto groups, and the conformational position of hydrogen and hydroxyl groups did not effect inhibitory capacity in any detectable pattern. Secondly, various degrees of inhibition for each hormone were observed for each serotype. Similar degrees did not occur when a different serotype was used as shown by the experiments with five or six hormones and two or three serotypes. Thus, it appears as if the steric configuration of the androgen molecule is responsible for inhibitory activity.

Decomposition of steroids by microorganisms has been reported (3,4,20, 24,25). Capek proposed that transformations represent a detoxication mechanism whereby an inhibitory or deleterious molecule is inactivated. If a transforming mechanism is operating, three things should be evident. First, the time required for overcoming inhibition should vary greatly, depending on the hormone used. This was not the case. Inhibition of growth was overcome eight to ten hr post-inoculation for all inhibitory hormones.

As a second point, all twelve hormones inhibited staphylococcal growth under anaerobic conditions. It is important to note that these twelve androgens represent numerous combinations of chemical groups, and if a simple transformation is involved, some androgens would not effect S. aureus, while others would greatly alter the growth rate.

Thirdly, if a transformation is required in order to overcome inhibition, then hormone added in log phase of growth should inhibit immediately. This did not occur. No effect was observed upon addition of the hormone after initiation of growth. Apparently, inhibition occurs only at low cell concentration. Thus, it seems unlikely that a general detoxication mechanism exists in the system.

The reasons for enhancement of antimicrobial activity by anaerobiosis remain unclear. Further work is in progress using synthetic media to elucidate specific site(s) of hormonal action.

The experiments concerning bacterial selectivity exclude two possibilities of androgen-staphylococcal interaction. A mutation to hormonal resistance to produce a clone of cells uneffected by the androgens has not occurred. Also, no metabolic induction of enzymes to counter-act androgenic inhibition is evident.

Hormonal Entrance Into Cells

The data implicate a relative lack of entrance of testosterone into the bacterial cell. About 83% of the ^{14}C-testosterone (40 μg/ml) remained

outside of the bacteria. The other 17% (equivalent to 7 μg/ml) may be residual on the centrifuge tubes, trapped intercellularly, or taken up or adsorbed by the cells. Even if the entire 7 μg/ml did enter the cell, it would not be enough to inhibit growth.

The biological assays also indicate that the hormone does not enter the bacterial cell. In addition to the reported data using 40 μg/ml, another experiment was done with 5α-androstan-3,17-dione at 30 μg/ml, a more critical concentration. Since the minimal inhibitory concentration of this hormone is 20 μg/ml, inhibition upon reinoculation of the autoclaved broth shows that at least 20 of the 30 μg/ml (67%) did not enter the cell.

Manometric Studies

The data for manometric studies showed no significant inhibition of O_2 uptake. Either 200 or 250 Klett units of bacteria were used. For the *in vitro* growth curves, inhibition had been overcome at this stage. The hormones appear to act only at lower cell concentrations. This may be the reason for lack of positive effects in oxygen uptake.

Mouse Mortality

No significant differences in mortality rates were observed. The system of injections and concentrations may not have been optimal for demonstration of hormonal-staphylococcal interaction. It is also possible that the mouse is not the best species to demonstrate the desired effects.

Rabbit Lesions

Androgenic effects on lesion size and morphology are exerted directly on the bacteria, directly on the rabbit, or on both. In vitro growth data indicate an antimicrobial action directly on the staphylococci. Other papers (19,27,28) have reported a stimulatory effect of androgens on the granulation and connective tissue of animals. The retardation of skin lesions may be due to direct intervention of the hormone on progressive tissue pathogenesis. The hormone may indirectly stimulate or inhibit organic systems of the rabbit which then produce substances against dermal necrotic lesions. More experimentation is needed to determine what is occurring and how it occurs.

Serum Studies

The minimum concentration of hormone in the in vitro experiments required to inhibit S. aureus was 20 μg/ml. In the rabbit studies, injected hormone is immediately diluted at least 100-fold by the total volume of blood and body fluids. Thus, a much lower androgen concentration inhibits growth of bacteria in rabbit serum. The ^{14}C-testosterone experiment provided a crude estimation of hormone levels after subcutaneous injection. It appears as if the hormone is acting synergistically with other serum constituents. Efforts to inactivate the inhibitory agent(s) by heating or freezing were unsuccessful. Cholesterol and glucose levels were not involved. Serum complement played no important role.

It is possible that the hormone is solely responsible for inhibition, and the higher concentration required in broth studies reflects an antagonistic action of broth constituents on the hormone.

All five androgens tested showed good inhibitory powers. Dehydroepiandrosterone and epiandrosterone were especially potent. Detoxication or transformation, which were not important in the *in vitro* broth studies, may play a major role in these rabbit experiments. In the five to ten min that the injected hormone is entering the blood, previous to cardiac puncture and blood removal, numerous changes may be occurring. Conjugation in the liver to glucuronides or sulfates, absorption by albumen fractions, catabolism to related steroids, excretion by the kidney - all are possibilities. Also, serum enzymes may act degradatively on the androgens after separation from the blood.

An interesting phenomenon was observed upon injection of epiandrosterone into female rabbits in a concentration that inhibits bacterial growth in serum from a male rabbit. *S. aureus* grew at similar rates in control and hormone-treated sera. This implicates antagonistic action by substances found exclusively in the female serum, such as female sex hormones.

In summary, it appears as if androgens inhibit the growth of *S. aureus* without entering the cell. Their mode of action may be similar to that of surface active agents. Hamilton (11) postulates that surface active agents inhibit either production of energy or its coupling to endergonic reactions of the cell's metabolism, with cell leakage as a secondary effect.

The reported regulatory activity of hormones on mammalian cells suggest that a hormonal effect may be of importance in the host-parasite relationship

of staphylococci. The androgen-staphylococcal interaction has been studied with regard to the uptake of androgens by S. aureus, steroid molecular structure, and antimicrobial activity. The data indicate that androgens may play a role in protection against staphylococcal infection.

SUMMARY

1. Various androgens inhibit the *in vitro* growth of *S. aureus*. No clear-cut active (chemical) group or detoxication mechanism is apparent.

2. Anaerobiosis enhances the antimicrobial effects of all twelve hormones tested.

3. The hormones exerted a bacteriostatic effect on the bacteria.

4. Testosterone and 5α-androstan-3,17-dione are unable to enter the bacterial cell in concentrations sufficient to produce inhibition of bacterial growth.

5. Manometric studies of bacterial oxygen uptake and mouse mortality studies showed no significant differences due to the androgens.

6. Staphylococcal skin lesions are significantly retarded by 5α-androstan-3,17-dione.

7. The subcutaneous injection of one to 20 μg of five androgens significantly inhibited the serum growth rates of *S. aureus*.

TABLE 1

EFFECT OF TESTOSTERONE ON VARIOUS SEROTYPES

Time (hrs)	Sero. I		Sero. XII		Sero. XIII	
	Hormone	Control	Hormone	Control	Hormone	Control
6	0	5	0	0	28	50
7	26	43	0	14	80	158
8	78	105	13	55	170	290
9	180	210	70	150	318	370

Inoculum: 1×10^5 colony forming units

Medium: TSB

Incubation: Rotary shaker at 37 C

NOTE: All Klett readings in this table and the following tables represent mean values.

TABLE 2

EFFECT OF VARIOUS ANDROGENS ON THE GROWTH OF S. AUREUS

Hormone 40 µg/ml	Klett Reading at Various Hours 4	5	6	7
Testosterone	0	4	64	206
5α-androstan-3,17-dione	0	8	20	76
Androsterone	1	6	69	211
Epiandrosterone	4	5	12	67
Dehydroepiandrosterone	3	12	58	117
None	8	22	92	230

Inoculum: 1×10^5 colony forming units

Medium: TSB

Incubation: Rotary shaker at 37 C

TABLE 3

ANAEROBIC EFFECTS OF VARIOUS HORMONES

Hormone 20 μg/ml	Klett Reading	% Inhibition
Testosterone	17	78
Stanolone	11	86
Androsterone	16	77
Epiandrosterone	6	92
Androstenedione	44	44
Dehydroepiandrosterone	12	87
5β-androstan-3α,17β-diol	51	34
5α-androstan-3β,17β-diol	52	33
5β-androstan-3,17-dione	56	31
5β-androstan-3αol,17-one	13	85
5α-androstan-3,17-dione	8	91
5α-androstan-3α,17β-diol	70-?	-
Control	78	0

Inoculum: 2×10^5 colony forming units

Medium: TSB

Incubation: Sixteen hr in anaerobic chamber at 37 C

TABLE 4

EFFECT OF VARIOUS CONCENTRATIONS OF
5α-ANDROSTAN-3,17-DIONE

Hormone (μg/ml)	Klett Reading at Various Hours			
	5	6	7	8
40	0	8	45	93
30	0	18	80	126
20	0	35	111	163
10	4	45	124	176
5	0	40	116	170
0	7	52	130	182

Inoculum: 1×10^5 colony forming units

Medium: TSB

Incubation: Rotary shaker at 37 C

TABLE 5

EFFECTS OF 5α-ANDROSTAN-3,17-DIONE ON VARIOUS SEROTYPES

Serotype	Klett Reading		
	Hormone	Control	% Inhibition
1	134	212	37
2	73	93	21
3	105	162	36
4	63	58	--
5	102	130	21
6	50	65	23
12	35	77	45

Inoculum: 20×10^5 colony forming units

Medium: TSB

Incubation: Twenty hr in anaerobic chamber at 37 C

TABLE 6

INABILITY OF TESTOSTERONE TO ENTER THE STAPHYLOCOCCAL CELL

No. of Washing	dps/ml	% of Total
0	5,260	83
1.	309	4
2	94	1
4	11	> 1

^{14}C-testosterone was initially diluted to a final activity of 6,311 dps.

TABLE 7

BIOLOGICAL ASSAY OF SUPERNATANT BROTH

Time (Hr)	Klett Reading	
	Hormone	Control
5	23	28
6	41	63
7	79	110
10	219	231

1×10^5 colony forming units were inoculated in TSB containing either 40 μg hormone/ml or the hormone vehicle. After growth for 24 hr on rotary shaker, cells were removed by centrifugation. The supernatant broth was autoclaved and reinoculated with 1×10^5 colony forming units.

TABLE 8

EFFECT OF 5α-androstan-3,17-dione ON SKIN LESIONS PRODUCTION

Time Post-Injection (Days)	Mean Area (Sq.In.)			
	Control		Hormone	
	Pus	Erythema	Pus	Erythema
2	0.28	1.08	0.07	0.80
3	0.34	0.89	0.05	0.66
4	0.46	0.78	0.01	0.51

Adult male rabbits received intradermally 9.2×10^7 colony forming units suspended in 1% ethanol saline containing 16 μg hormone. Control animals received a similar dose of bacteria suspended in 1% ethanol saline.

TABLE 9

GROWTH OF S. AUREUS IN RABBIT SERUM

Time (hr)	Klett Reading		
	Control	Hormone	% Inhibition
7	37	10	63
8	137	20	86
11	252	51	80
13	252	202	20
14	250	256	--

Male adult rabbits received subcutaneously a single dose of 20 μg 5α-androstan-3,17-dione. Controls received the hormone vehicle only. Serum was removed 5 min after injection and 4.0 samples inoculated with 1×10^5 colony forming units.

TABLE 10

EFFECT OF 5α-androstan-3,17-dione ON SERUM GROWTH OF VARIOUS SEROTYPES

Serotype	Hormone (20 μg/ml)	Control	% Inhibition
1	112	70	--
2	37	147	75
3	35	47	26
4	20	85	77
5	51	78	35
6	16	21	24
7	139	53	--
8	19	77	76
9	12	25	52
10	35	32	--
11	57	114	50
13	20	85	77

Inoculum: 1×10^5 colony forming units

Medium: Rabbit serum

Incubation: Eleven hr at 37 C

TABLE 11

EFFECT OF TESTOSTERONE CONCENTRATION ON THE GROWTH OF S. AUREUS

µg Injected	Mean Klett Reading at 8-10 hr	% Inhibition
20	46	48
10	57	35
1	68	23
0	89	0

Inoculum: 1×10^5 colony forming units

Medium: Rabbit serum

Incubation: 37 C

TABLE 12

EFFECT OF VARIOUS ANDROGENS ON THE GROWTH OF S. AUREUS IN RABBIT SERUM

Hormone	Mean Klett Reading at 8-10 Hr 20 µg	0	% Inhibition
Dehydroepiandrosterone	1	49	98
Epiandrosterone	2	73	97
Androstenedione	10	49	80
Testosterone	73	113	35
5α-androstan-3,17-dione	46	70	34

Inoculum: 1×10^5 colony forming units

Medium: Rabbit serum

Incubation: 37 C

TABLE 13

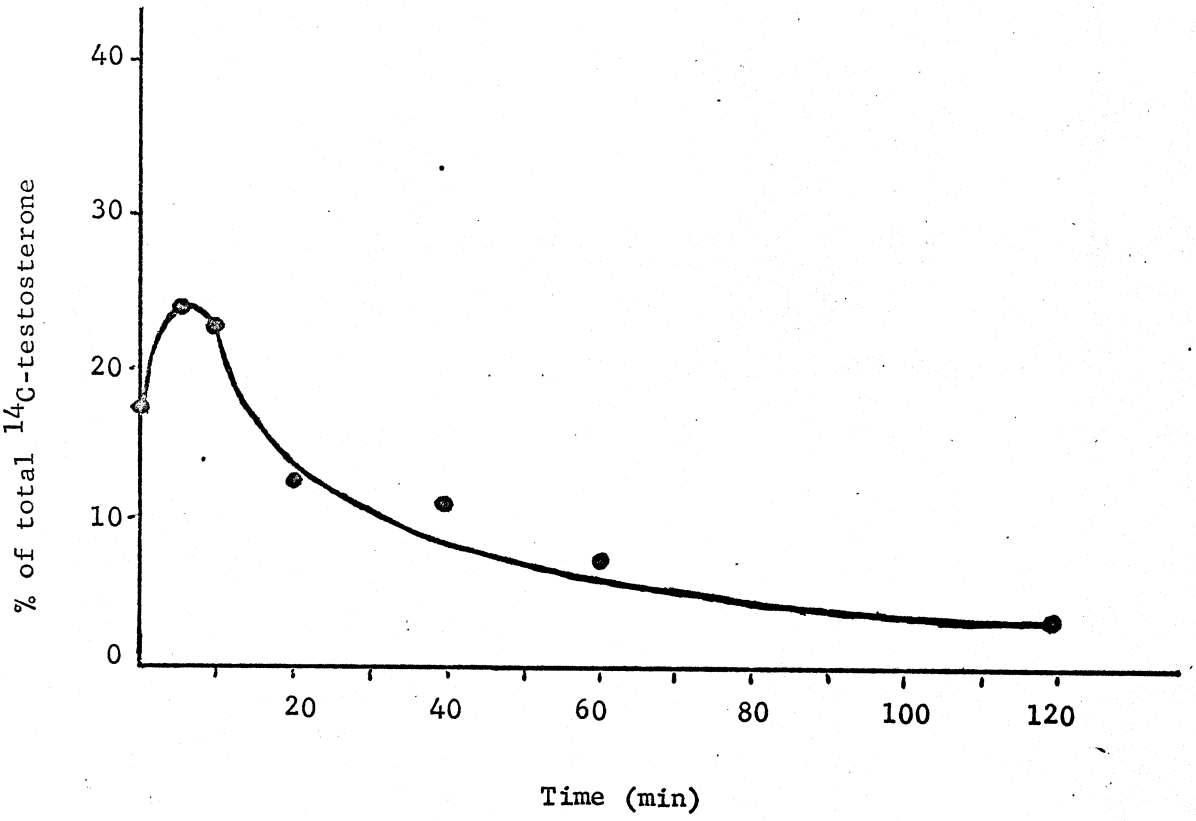
RATE OF HORMONAL UPTAKE BY THE BLOOD AFTER SUBCUTANEOUS INJECTION

Time (Min)	Hormone (μg/ml)
0	0.24
5	0.35
10	0.32
20	0.21
40	0.15
60	0.10
125	0.04

Injection: 29 μg labeled testosterone containing 1.71×10^5 dps.

FIGURE 1

^{14}C UPTAKE BY THE BLOOD



^{14}C-testosterone (29 μg in 1.0 ml containing 0.005 mc) was injected subcutaneously at 0 time. These are the mean values for the amount of ^{14}C in two separate experiments.

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