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by

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Certain specific gonadal steroids selectively reduce the growth and virulence of *Staphylococcus aureus*. This work was undertaken to investigate this hormonal mechanism of action at the molecular level.

Under anaerobic conditions, when various hormones, such as progesterone, testosterone, mestranol, or norethindrone, at the pharmacological concentrations of 20 to 40 mcg/ml, were added to *Staphylococcus aureus* that had been previously exposed to ^{14}C-glucose, the rates of leakage of radioactivity from hormone-treated cultures were 10% to 60% greater than the rates of leakage from the control cultures. Similarly, the cellular release of protein was 10% to 50% greater in the presence of progesterone. Penicillin enhanced the steroidal effects of increased cellular leakage.

The uptake of labeled substrates, such as glucose, alanine, lysine, or glutamic acid, was altered 10% to 90% by prior treatment with progesterone, testosterone, or estradiol at 40 mcg/ml, or diethylstilbestrol at 20 mcg/ml. The inhibition of entry of labeled alanine was markedly influenced by metabolic activity, anaerobiosis, temperature, pH, cell concentration, and hormone concentration. Substrate uptake by gram negative organisms, whose growth is not inhibited by the steroids, was also unaffected by the steroids.

Fractionation of staphylococci after exposure to ten ^{14}C-labeled substrates in the presence and absence of hormones, revealed widespread alterations in the entrance of the labels into lipids, proteins, nucleic acids, teichoic acids, and cell wall mucopeptides. No direct relationship of steroid action to macromolecular synthesis emerged. With some substrates, entry into

the cellular fractions was inhibited, while with others, either stimulatory or intermediate effects were detected. Progesterone at 40 mcg/ml, however, altered the entry of eight of the compounds into their respective cellular pools. The hormone inhibited entry 11% to 17% for seven of these eight substrates. For short term incorporation, using ^{14}C-alanine, progesterone initially reduced entrance into the cellular pools 15% within 10 seconds. At this time, no hormonal intervention was apparent in the incorporation into the other cellular fractions. After 15 minutes, however, the steroid reduced the entrance of the ^{14}C-label into the other fractions by 15% to 50%.

Synergistic activity was observed when sub-inhibitory concentrations of penicillin were utilized with sub-inhibitory concentrations of progesterone, testosterone, norethindrone and mestranol, or diethylstilbestrol, to inhibit the entry of ^{14}C-alanine into cell wall mucopeptides. A progesterone concentration above 15 mcg/ml was required to reduce entrance of the labeled alanine into the mucopeptide fraction. If sub-inhibitory concentrations of penicillin were added, progesterone concentrations as low as 5 mcg/ml significantly reduced entry of the label into this fraction.

When ^{14}C-progesterone was added, either to whole cells or to preparations of mucopeptides from the identical quantity of whole cells, the latter preparation contained three times as much radioactivity as the former.

The following hypothesis is presented to explain the mechanism of action of the gonadal hormones for the previously reported inhibition of growth of S. aureus. It is proposed that the steroids, in binding to the cell wall-cell membrane complex, influence bacterial transport mechanisms, producing alterations in the rates of entry of essential nutrients. This in turn, reduces the growth of the staphylococci.

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List of abbreviations

Hormones

PROGEST.	progesterone
TESTOST.	testosterone
DIETHYLSTIL.	diethylstilbestrol
ESTRADIOL	β-estradiol
EPIANDROST.	epiandrosterone
STANOL.	stanolone
17αOHPROGEST.	17α-hydroxyprogesterone
NORETHIN.	norethindrone

Chemicals and medium

TCA	trichloroacetic acid
ETOH	ethanol
TSB	tryptic soy broth

I. INTRODUCTION AND REVIEW OF LITERATURE

The gonadal hormones not only exert diverse regulatory activities on the metabolism of mammalian cells, but also have been implicated in the development of mycotic, parasitic, and bacterial infections. Numerous reports have established the interaction of microorganisms and steroids in an altered host response, which, in turn, may produce increased or decreased susceptibility to infection.

Much has been reported concerning hormonal effects on fungi, molds, and yeasts. Reiss (49) in 1947 did pioneering research on the effects of various steroids on the growth of fungi. He reported inhibitory action of methyl testosterone and α-estradiol. Casas-Campillo et al. (7) likewise reported the fungistatic activity of hormones, especially 21, 21-dimethoxyprogesterone on Curvularia lunata and Trichophyton mentagrophytes. In addition, this steroid inhibited the growth of a variety of other fungi, mycobacteria, and nocardia. Mankowski (39) extended his findings to in vivo effects of hormones. He showed that testosterone exerted a slight protective effect on animals experimentally infected with aspergilli. In sharp contrast, estradiol shortened the survival time of infected animals.

Maxwell et al. (41) found that androstenedione almost completely retarded the growth of Saccharomyces fragilis in nutrient broth. They also isolated several mutants that were highly resistant to the hormone action. In similar work, Lester et al. (37 and 38) reported significant growth reduction of germinating conidia of Neurospora crassa by androstenedione and testosterone. Townsley et al. (60) also worked with Neurospora crassa and found that deoxycorticosterone decreased both the endogenous respiration and the uptake of glucose. They hypothesized that the hormone uncoupled oxidative phosphorylation, thereby interferring with energy yielding processes.

Chattaway's (8) epidemiological studies provided evidence for enhancement of dermatophytic infections by gonadal hormones, especially in Microsporium diseases at puberty. He postulated a direct steroidal effect on the in vivo growth of the specific dermatophytes.

A number of reports describe the action of hormones on various animal systems in combating experimentally-induced infections. Kass and Finland (32) found that adrenocorticotrophic hormone depressed resistance to infection by inhibiting inflamation, producing a negative nitrogen balance, lowering antibody production, and altering the function of the reticuloendothelial system. The hormone diminished the effectiveness

of large macrophages in disposal of their ingested organisms. McDermott (42) emphasized the protective role of steroids in dormant or inapparent infections in animals.

Kutzsche (36) documented the action of several steroids on mice experimentally infected with Salmonella typhimurium, Salmonella typi, and Diplococcus pneumoniae. He suggested that the protective action of hormones resulted from diminishing tissue sensitivity to microbial endotoxins, rather than a direct detoxification of the endotoxins. In related experiments, Von Haan and Rosenfield (62) studied the action of various gonadal hormones on pnemococcal infections in mice. A single intraperitoneal injection of testosterone propionate significantly lowered the mortality of diseased animals. When male and female groups were compared, testosterone provided better protection against the pneumococci in the male group.

Tokuda (59) used rabbits instead of mice and described the effects of sex hormones on staphylococcal ocular infections. The viable count of bacteria isolated from the aqueous humor was statistically lower in the steroid-treated groups. Ghione (20) found that 4- chlorotestosterone inhibited murine staphylococcal infections, but did not affect the in vitro growth of the organisms in broth. The author's analysis illustrated two interesting concepts. First, the bacteriostasis produced by

4-chlorotestosterone would not have been detected if only *in vitro* assay procedures were employed. Secondly, the hormones may act either indirectly against the bacteria by stimulating host-defense mechanisms of the mice, or directly against the organisms after an *in vivo* transformation to an active antibacterial substance.

Other reports detail the *in vitro* action of hormones on specific bacteria. Osborne and Bourdeau (45) investigated the effects of various steroids on the growth of *Vibrio fetus*. If progesterone or testosterone were added to the culture medium, stimulation of *Vibrio* growth was detected. Varricchio et al. (61) tested the antibacterial activity of related azasteroids on *Bacillus subtilis* and *Sarcina lutea*. The amount of growth inhibition was proportional to cell and steroid concentration. In addition, only the active azasteroids were bound to the organisms and this binding appeared to occur at the cell membrane level. Similarly, Smith et al. (55) related the bacteriocidal action of various synthetic steroids to the surfactant properties of the hormones at the membrane level. Smith and Shay (53) investigated the antimicrobial properties of steroids on protoplasts of *Staphylococcus aureus* and suggested a direct hormonal action on the cell membrane.

If the steroids act at the cell membrane level it would

help to explain the differences in hormonal susceptibility of gram positive and gram negative organisms,which possess entirely different cell wall structures surrounding the membranes. Beutow and Levedahl (6) found that natural and synthetic estrogens inhibited the growth of a number of gram-positive bacteria, but did not affect gram-negatives. Smith et al. (54) in testing the antibacterial action of several nitrogen-containing steroids, observed similar results. Most of the compounds used retarded the growth only of gram-positive organisms. Casas-Campillo et al. (7) in like manner, detected inhibitory effects of gonadal steroids only on gram-positive bacteria.

In contrast to the above findings, Nicol, et al. (44) observed hormonal inhibitory effects on gram-negative microorganisms also. They stated that estrogens were stimulants of the host-defense mechanisms of male white mice experimentally infected with Diplococcus pneumoniae, Pasteurella septica, Salmonella typhimurium, Salmonella typhi, Escherichia coli, Hemophilus pertussis, and Pseudomonas aeruginosa. The authors postulated a stimulation of the reticulo-endothelial system with increased phagocytic ability.

Yotis and his co-workers have detailed specific effects of the sex steroids on the growth and virulence of Staphylococcus aureus. Yotis and Stanke (76) reported an in vitro bacteriostatic action of progesterone, pregnenolone, 4-pregnen-

-20 β ol -3one, and 5 α -pregnane on S. aureus, and other gram positive organisms. Gram negative bacteria, however, were not subject to the inhibitory action. Two pertinent observations were made. The steroids retarded growth only during the initial 8 to 10 hours after inoculation, and anaerobiosis enhanced hormonal inhibition of growth.

Yotis (69) examined the antimicrobial properties of norethindrone and mestranol, two synthetic progestational agents used in contraceptive pills. Bacteriostatic action was exerted only on gram positive organisms. The severity and duration of staphylococcal skin lesions and the total viable counts recovered from the lesions were significantly reduced in female rabbits previously treated with norethindrone. Mestranol appeared to enhance the antibacterial activity of norethindrone. This suggested that hormonal interaction does occur and must be considered in evaluating the data obtained from in vitro studies, and in correlating this data with in vivo phenomena.

Yotis and Waner (77) investigated the antimicrobial properties of testosterone and related intermediates on the growth of various microorganisms. Hormonal growth inhibition was evident only with gram positive bacteria, such as S. aureus, S. epidermidis, Streptococcus faecalis, and Listeria monocytogenes. In addition, epiandrosterone and dehydroisoandrosterone significantly reduced oxidation of pyruvate by S. aureus.

Yotis and Baman (70, 71, 72) documented the antistaphylococcal action of diethylstilbestrol, a synthetic estrogen. This agent drastically reduced *in vitro* growth, inhibited oxidation of various substrates, produced significant leakage of intracellular contents, and retarded the progress of induced staphylococcal skin lesions in rabbits.

Yotis and Cummings (74) reported that a combination of norethynodrel and mestranol severely decreased the viable count of microorganisms isolated from the spleens and kidneys of mice previously infected intravenously with *S. aureus*. Furthermore, mortality was significantly lower in hormonally treated animals.

Yotis and Fitzgerald (75) after subcutaneous injection of various androgens into rabbits and subsequent isolation of serum, found that *S. aureus* grew more luxuriantly in the serum of control rabbits than in the serum of androgen treated animals. With tryptic soy broth as a culture medium, a concentration 150 to 300-fold higher than that achieved in the blood of a hormonally-treated animal was required to yield an equivalent effect. Also, the progress of induced furunculosis in rabbits was significantly retarded by their prior treatment with testosterone or 5α -androstan -3, 17-dione.

At this point, a substantial body of information has indicated an interaction of steroids with bacteria, especially

S. aureus. The question arises as to how the hormones inhibit the growth and virulence of staphylococci. What is the mechanism of action of the hormone at the cellular or sub-cellular level?

Theories of hormone action have followed three basic patterns. Initially the hormone-enzyme hypothesis developed when biochemistry was systematically unraveling the mysteries of classic intermediary metabolism. The hormone-permeability thesis followed, and was based on the discoveries that insulin and vasopressin influenced membrane transport. The hormone-gene theory emerged from the advances of molecular biology in elucidating the genetic regulation of protein synthesis.

In considering hormone action, the two essential steps are the association of the hormone with the specific receptor, and the effects resulting from hormone–receptor combination. At present, there are too many uncertainties to point with any clarity to a definite molecular mechanism as the target of action of any hormone. In fact, in the vast majority of cases, there is no guarantee that the responses of enzyme systems, protein synthesis, or permeability functions that have been reported, are not relatively remote secondary consequences of primary actions exerted elsewhere. A basic problem then, is to seperate the initial site of hormonal action in a cell from the

subsequent secondary reactions.

To thoroughly detail hormonal effects at the three levels of enzymes, proteins, or permeation would be a massive undertaking. Therefore, effects on permeability functions, deemed to be of primary importance, were selected for study and are the subject of this dissertation.

The problems associated with the mechanism of action of steroids upon staphylococcal permeability are numerous; important questions are: 1) What is the target or receptor in a bacterial cell that initially interacts with the hormone? 2) Is the affect upon cell permeability, in fact, the primary mechanism of action at the cellular level? 3) What are the secondary effects produced by the steroid-receptor combination? 4) Do all sex hormones regulate permeability processes in staphylococci in a similar fashion? 5) Why are some derivatives more active than others? 6) Why are certain strains of S. aureus more susceptible to hormone action? And, 7) Are the inhibitory effects of these steroids in vitro, as well as in vivo, ultimately the result of hormonal alteration of cell permeability?

The purpose of this dissertation is two-fold in attempting to clarify the above questions. Firstly, a characterization of hormonal effects at the cellular level of bacteria, more specifically of Staphylococcus aureus will be presented; secondly, a

correlation of these cellular effects will be made with the previously mentioned *in vivo* and *in vitro* phenomena of hormone-bacterial interaction, with specific emphasis on the data previously reported (15, 16, 17, 75).

II. MATERIALS AND METHODS

Cultures - S. aureus serotypes 1X, X, X1, X11, and X111 (ATCC 12606-12610), and Rose strain (ATCC 14154) were maintained by periodic transfer on nutrient agar (Difco) slants at 4 C. The bacteria were tested for glucose and mannitol utilization under anaerobic conditions according to Hugh and Leifson (29), and for gelatin liquefaction, pigmentation, hemolysis, and both free and bound coagulase by accepted microbiological techniques (56). Shigella flexneri, Salmonella paratyphi, Proteus mirabilis, and Escherichia coli were procurred from stock culture collections maintained at the Stritch School of Medicine and had been originally obtained from The American Type Culture Collection.

Growth of Bacteria - A loopful of cells was removed from the agar slants and inoculated into 5 ml of sterile 3% tryptic soy (Difco) broth (TSB). After 12 hr, 0.5 ml of this culture was added to 50 or 100 ml of sterile 3% TSB. The cells were grown at 37 C on a rotary shaker to mid logarithmic phase, harvested by centrifugation at 10,000 x g for 8 minutes, and washed twice in 0.89% saline. A smooth suspension in saline was prepared by mixing for two min at high speed with a Vortex Junior Mixer. The cultures were then adjusted to the specified cellular density in saline, 0.1 M sodium phosphate buffer pH 7.0 (21), or the synthetic medium of Hancock and Park (25) which contained

the following; L-lysine, 0.5 μ mole/ml; glycine, 0.5 μ mole/ml; L-glutamic acid, 2.0 μ moles/ml; DL-alanine, 2 μmoles; uracil, 20 mcg/ml; glucose, 4 mg/ml; sodium phosphate buffer pH 7.0, 80 μ moles/ml; magnesium sulphate, 1 μ mole/ml; manganese chloride, 0.1 μ moles/ml; thiamine, 2 mcg/ml; nicotinic acid, 1 mcg/ml. The synthetic medium was slightly modified whenever the incorporation of one of its constituents was examined, by deleting that ^{12}C-constituent. Bacterial suspensions were adjusted to the proper cellular density with a Klett Summerson photoelectric colorimeter containing a number 42 filter. Preliminary work had indicated a direct relationship of turbidity to viable counts of bacteria. For most of the experiments 100 or 150 Klett units were used, corresponding to 1.05×10^8 viable bacteria/ml (126 mcg dry wt/ml) or 2.16×10^8 viable bacteria/ml (222 mcg dry wt/ml).

Chemicals - Crystalline progesterone, testosterone, diethylstilbestrol, β -estradiol, epiandrosterone, stanolone, and 17 α -hydroxyprogesterone were obtained from Sigma Chemical Company, St. Louis, Missouri. Crystalline norethindrone and mestranol were obtained from Syntex Laboratories, Palo Alto, California. The purity of these steroids was confirmed by measurement of the melting points, with a Fisher-Johns melting point apparatus, and maximum absorbancies with a Beckman DK-G spectrophotometer.

The following labeled compounds were secured from Tracerlab, Waltham, Massachusettes: adenine-8-^{14}C, L-alanine -^{14}C (uniformly labeled - u.l.), L-glutamic acid -^{14}C (u.l.), L-phenylalanine -^{14}C (u.l.), sodium acetate -1 -^{14}C, glycerol -^{14}C (u.l.), D-glucose -^{14}C (u.l.), uracil -2 -^{14}C, L-leucine -^{14}C (u.l.), and L-lysine -^{14}C (u.l.). Progesterone -4 -^{14}C and testosterone -4 -^{14}C were obtained from Amersham/Searle Corporation, DesPlaines, Illinois. All other chemicals utilized were of reagent grade.

Crystalline penicillin "G" (sodium), bacitracin, and streptomycin sulphate were obtained from General Biochemicals, Chagrin Falls, Ohio; crystalline chloramphenicol from Sigma Chemical Company, St. Louis, Missouri; crystalline sodium azide and 2, 4-dinitrophenol from Eastman Organic Chemicals, Rochester, New York; and actinomycin D from Nann Research Laboratories, New York, New York. The chemicals for the scintillation fluor, anisole, p-dioxane, and 1, 2-dimethoxyethane were obtained from Eastman Organic Chemicals, Rochester, New York. Crystalline 2, 5-diphenyloxazole (PPO) and 1, 4-bis 2-(5-phenyloxazolyl) -benzene (POPOP) were obtained from Packard Instrument Company, Downers Grove, Illinois.

Buffers - Buffers were made according to the method of Comuri (21) as 0.1 M sodium phosphate buffers at pH 5.7, 6.5,

7.0, 7.5, and 8.0.

Hormone Solutions - A specified amount of hormone was dissolved in 95% ethanol (ETOH) and added to saline, buffer, or synthetic medium to obtain a final concentration of 1 to 40 mcg/ml in 1% or 2% ETOH. Controls received only the 1% or 2% ETOH.

Measurement of Radioactivity - Aliquots of 0.1 ml of the filtrates, the supernatants, the soluble fractions, or the dried Millipore filters (Millipore Corporation, New Bedford, Massachusettes, 0.45 micron, 13 or 25 mm diameter) were added to 10 ml of scintillation fluor containing 18 g PPO, 60 mg POPOP, 900 ml p-dioxane, 150 ml anisole, and 150 ml 1, 2-dimethoxyethane (11). ^{14}C-activity was determined in a Packard Tri-Carb liquid scintillation spectrometer, model 3320. In this system carbon-14 was counted with an eighty to eighty-five percent efficiency. A minimum of 10,000 counts were accumulated per vial.

Statistical Analysis - Data were subjected to statistical analysis (3). A "t" test value larger than 2.000 indicated a probability factor of less than 5% and was statistically significant at the 95% level. To obtain a value for the "t" test, three separate formulas were utilized:

standard deviation $$\sigma = \sqrt{\frac{\Sigma(x-\bar{x})^2}{N}}$$

standard error of difference of 2 means $SE_d = \sqrt{\frac{\sigma 1^2}{N_1} + \frac{\sigma 2^2}{N_2}}$

"t" test $"t" = \frac{\text{difference of 2 means}}{SE_d}$

In general, duplicate samples were used and experiments were performed from 2 to 5 times.

Leakage Experiments - The bacteria were grown to mid logarithmic phase, harvested by centrifugation, washed twice in saline, and re-suspended in 0.1M sodium phosphate buffer pH 7.0 at 126 mcg dry wt/ml. To this suspension, 0.1 μ C of ^{14}C-glucose was added. The cells were incubated on a rotary shaker at 37 C for 30 min. Excess ^{14}C-activity not taken up by the cells was removed by centrifugation at 15,000 x g for 10 min, and subsequent washing with saline. The ^{14}C-labelled bacteria were re-suspended in 0.1 M sodium phosphate buffer pH 7.0 or synthetic medium at 126 mcg dry wt/ml. (This was prepared as a smooth suspension as previously indicated to minimize clumping effects). The bacteria were incubated in the presence and absence of the specified hormones at 37 C in a Precision Thelco anaerobic incubator flushed with 95% CO_2 and 5% N_2. At the designated times, duplicate 5 ml portions were removed and centrifuged at 10,000 x g for 10 min. Aliquots of the supernatant fluid were placed

in scintillation fluor and assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer.

An alternative procedure to the above assay of extracellular fluid, was assessment of residual intracellular radioactivity remaining within the bacteria. Aliquots of 0.5 ml of cells were membrane filtered after specific periods of exposure to the hormones. The filters were washed with 4 volumes of 0.1 M sodium phosphate buffer pH 7.0, air dried, and placed into scintillation fluor.

In similar experiments, the leakage of protein and 260 nm absorbing substances from the bacteria into the extracellular fluid was assayed according to Warburg and Christian (63). Protein determinations were made by reading the absorbance of supernatant fluid at 215 and 225 nm.

Uptake and Incorporation - Bacteria were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0, or in synthetic medium, at 126 mcg dry wt/ml. The bacteria were added to 0.01 mC ^{14}C-substrate containing either the specified hormones dissolved in 1% EtOH or only the 1% EtOH at 24C. At various times thereafter, 0.5 ml of the suspension was filtered by membrane filtration. The filters containing the bacteria were washed with 5 volumes of cold buffer, air dried, and placed into scintillation fluor.

The quantity of radioactivity retained on the filter was a direct reflection of the total uptake of ^{14}C-label by the bacteria.

As an alternative procedure to the above, the cell suspensions, after uptake of the ^{14}C-label, were passed through Seitz filters. The filtrates were then assayed for radioactivity as described.

Fractionation of Bacteria - Bacteria were grown to mid logarithmic phase, harvested, washed, and suspended in synthetic medium at 222 mcg dry wt/ml. The cells were pre-incubated 45 min on a rotary shaker at 37 C. Samples of 20 or 30 ml of the suspensions were then exposed to 0.01 mC of ^{14}C-substrate in the presence and absence of the indicated hormones at 24 C for 15 min. The cells were immediately centrifuged at 15,000 x g for 12 min at 0 C, washed twice in cold saline, and extracted according to Park and Hancock (46). The washed cells were suspended in 5% trichloracetic acid (TCA) and maintained at 4 C for 20 min. The preparation was then centrifuged and the supernatant fluid tested for radioactivity (cold TCA soluble fraction). The pellet was suspended and extracted with 75% EtOH for 15 min at 45 C. The extract was centrifuged, decanted, and examined for radioactivity (alcohol soluble fraction). The pellet was trypsinized for 4 hr in a solution containing 0.05M NH_4HCO_3, 0.005N NH_4OH, pH 8.2, and 1% trypsin (E. H. Sargent Company,

Chicago, Illinois). The preparation was passed through a membrane filter to remove the remaining insoluble residue and the filtrate was tested for radioactivity (trypsin-solubilized protein fraction). Filters containing the residue were air-dried and placed directly in a vial of scintillation fluid (residue fraction).

A modification of the above procedure was required for short-term studies utilizing 10, 30, and 60 second exposure of suspensions, as previously described, to ^{14}C-alanine. After exposure to the amino acid with and without the hormone, two volumes of ice water (4C) were immediately added to the suspension. These were placed in a water bath at 4C for 10 min to drastically reduce incorporation of the label, centrifuged, washed twice, and fractionated as described.

Chromatographic Analysis - Experimental procedures for growth, preparation, and exposure to ^{14}C-labels were similar to those described. A large volume of cells, 300 ml, suspended in synthetic medium at 222 mcg dry wt/ml was exposed to 0.01 mC of ^{14}C-alanine or ^{14}C-lysine for 15 min at 24 C in the presence and absence of progesterone at 40 mcg/ml, and subsequently fractionated. Each fraction was concentrated 300-fold by flash evaporation and treated as follows: The cold TCA fractions were washed with 5 volumes of cold ethyl ether to remove residual TCA. The

75% EtOH fractions were subjected to mild alkaline hydrolysis in 0.5N NaOH for 2 hr at 37 C (19) to hydrolyze amino-phosphatidylglycerol. The hot TCA fractions, after washing with ethyl ether, were hydrolyzed in 1 N HCl for 3 hr at 100 C (2) to remove alanyl groups esterified to teichoic acids. The trypsin-solubilized protein fractions were suspended in 6N HCl in sealed ampules at 104 C for 22 hr. The insoluble residue was hydrolyzed in 3 N HCl at 100 C for 16 hr. The excess HCl in the last three fractions was removed by dessication in vacuo over NaOH pellets. All resulting samples, after evaporation to dryness, were dissolved in distilled HOH and applied to a column (1.2 x 11.0 cm) of Dowex 50 (hydrogen ion form). The column was washed with distilled water until no further radioactivity was detected in the eluate. The absorbed compounds were then eluted with 4 N NH_4OH in 5 ml portions, and tested for ^{14}C-activity. Labeled portions were evaporated to dryness and re-dissolved in 1.0 ml water. Eighty microliter portions of each sample were quantitatively applied to Whatmann #4 chromatography paper and developed by conventional descending single dimension chromatography using two separate solvent systems, n-butanol-acetic acid - water (120: 30: 50 v/v) and phenol-water (75: 25 v/v) (52). Amino acids were located by application of ninhydrin reagent with subsequent heating at 105 C for 3 min. Ninhydrin positive spots were identified by comparing R_f values

to the R_f values of amino acid standard solutions and to known published R_f values (52). The specific amino acid spots were cut from the paper chromatograms, placed in scintillation fluor, and assayed for radioactivity. Control sections of an equal size were also cut from the chromatogram and counted similarly.

Exchange Reactions - Staphylococci were grown in mid logarithmic phase, harvested, and re-suspended in buffer or synthetic medium at 222 mcg dry wt/ml. The bacterial suspension was added to various labelled substrates containing hormones in 1% EtOH or only 1% EtOH. After uptake was initiated, unlabelled substrates at 10^{-2}M (300 to 1000-fold higher than ^{14}C-substrate concentration) were added to the suspensions. The high concentration of "cold" substrate acted as an external trap to prevent re-entry of ^{14}C-substrate that had effluxed from the cell. Aliquots of 0.5 ml of the cells were isolated by membrane filtration and assayed for total content of radioactivity at various intervals after exposure to the label.

Inhibitors - The synergistic or antagonistic effect of inhibitors on hormonal alterations in the uptake and incorporation of nutrients was studied by two procedures, the choice of which depended upon the nature of the experiment.

In the first procedure, mid logarithmic phase cells were preincubated in buffer or synthetic medium with and without a

specific inhibitor, such as chloramphenicol, streptomycin, sodium azide, or 2, 4-dinitrophenol for 30 to 60 min. Both groups of cells were exposed to ^{14}C-alanine containing hormones in 1% ETOH or only 1% ETOH. At various times thereafter, total ^{14}C-uptake was determined by membrane filtration and subsequent assay of radioactivity.

In the second procedure, hormonal alterations in the incorporation of substrates into staphylococcal macromolecules, as influenced by cellular inhibitors, were determined by using the fractionation procedure of Park and Hancock (46). Staphylococci were pre-incubated 30 to 60 min in buffer or synthetic medium in the presence or absence of chloramphenicol, streptomycin, or penicillin. The cells were then added to ^{14}C-alanine, ^{14}C-lysine, or ^{14}C-glutamic acid with and without the hormones. After 8 to 10 min incubation, the bacteria were immediately centrifuged, washed, fractionated, and assayed for radioactivity as described.

III. RESULTS

A. Hormonally-induced Leakage of Cytoplasmic Contents

It was apparent from previous work (15, 75) that the gonodal steroids may act in an extracellular fashion without penetrating into the cell proper; thus, the possibility that hormonal inhibition of staphylococcal growth involved changes in cellular permeability was considered. To this end, the effect of various steroids upon the leakage of radioactive substances from cells labelled with ^{14}C-glucose was assayed.

The release of ^{14}C-activity from S. aureus exposed anaerobically at 37C to four steroids is shown in Table 1. Similar rates of leakage were observed for the control and hormonally-treated cells for the initial 12 hours. After 12 hours, however, substantial differences in ^{14}C-leakage were evident. Progesterone promoted maximal loss of radioactivity (44% to 60%); norethindrone, and the combination of norethindrone and mestranol produced slightly lower rates of leakage (35% to 48%); and mestranol alone, and testosterone yielded minimal but significant leakage effects (11% to 29%) relative to the control cultures. The percentage of increased c /min, relative to leakage in control suspensions, induced by each hormone, remained relatively stable for the duration of the experiment. Note the increase in the quantities of radioactivity in the extracellular

Table 1. Effects of various hormones on cellular leakage from ^{14}C-labelled cells

Treatment	Time of exposure					
	12 hrs		17 hrs		23 hrs	
	Activity	% leakage	Activity	% leakage	Activity	% leakage
Control	4200	----	4710	----	5170	----
Progest. 40mcg/ml	6045	44.0±17.0	7540	60.0±13.2	8200	58.6± 2.9
Testost. 40mcg/ml	4805	14.4± 0.7	6075	29.0±5.6	5750	11.2± 3.6
Mestranol 5mcg/ml	4740	12.8± 2.4	5730	22.7±12.6	6350	22.8± 9.2
Norethin. 35mcg/ml	.6050	44.0±3.8	6630	40.8±7.3	6970	34.8±16.1
Mestranol 5mcg/ml and Norethin. 35mcg/ml	5980	42.4±7.8	6960	47.8±13.5	7570	46.4± 8.8

S. aureus serotypes IX and X were grown to mid logarithmic phase in TSB, harvested, washed, and suspended at 126 mcg dry wt/ml in 0.1 M sodium phosphate buffer pH 7.0 containing 1.0 μC ^{14}C-glucose. After assimilation of the label for 30 min, the cellular suspension was washed twice in physiological saline (0.89%) and resuspended in 0.1 M sodium phosphate buffer pH 7.0 at 126 mcg/ml. The bacteria were incubated in the presence and absence of the specified hormones at 37C in a Precision Thelco anaerobic incubator flushed with 95% CO_2 and 5% nitrogen. At the designated times, 5ml portions were removed and centrifuged at 10,000 X g for 10 min. Aliquots of the supernatant fluid were placed in scintillation fluor and assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. Activity is expressed as cpm/ml. The values represent the average of two experiments. The % leakage is defined as $\frac{\text{hormone-treated}-\text{control}}{\text{control}} \times 100$.

fluid of both control and hormonally-treated suspensions with increasing time of incubation.

The quantitative aspects of various steroid concentrations on cellular leakage were presented in Table 2. Staphylococci, pre-loaded with ^{14}C-glucose, were exposed to progesterone or testosterone at 0, 1, 20, and 30 mcg/ml under anaerobic conditions. As in the previous experiment, no differences were apparent during the first 20 hours of incubation. The control and hormonally-treated suspensions exhibited identical rates of leakage (results not shown). After incubation for 20 hours, a significant difference in loss of radioactivity was detected with progesterone and testosterone at or above 20 mcg/ml. At 10 and 1 mcg/ml, neither hormone enhanced the release of ^{14}C-activity. Progesterone was slightly more effective in inducing cellular leakage. After 20 and 26 hours exposure, progesterone at 30 mcg/ml produced 23.2% and 15.9% leakage, while testosterone at 30 mcg/ml produced 16.7% and 12.8% leakage, No hormonal effects were detected after 45 hours of exposure, suggesting that saturation levels had been reached in the efflux of radioactivity from staphylococci.

Previous studies (75, 76, 77) showed that steroids inhibited the <u>in vitro</u> growth of gram positive organisms such as

Table 2. Effect of hormone concentration on the leakage of internal radioactivity

Treatment	Time of exposure			
	20 hrs		26 hrs	
	Activity	% leakage	Activity	% leakage
Control	6120	----	6780	----
Testost. 1mcg/ml	6310	3.1±3.8	6790	0.1±3.5
Testost. 20mcg/ml	6870	12.2±2.0	7240	6.8±0.2
Testost. 30mcg/ml	7140	16.7±6.1	7650	12.8±7.3
Progest. 1mcg/ml	6410	4.7±0.1	6920	2.1±4.1
Progest. 20mcg/ml	6980	14.0±1.0	7780	14.7±1.4
Progest. 30mcg/ml	7540	23.2±4.1	7860	15.9±0.1

Experimental conditions were the same as in Table 1 except that serotypes XII and XIII were used. Activity is expressed as cpm/ml. The data are mean values for two experiments. The % leakage is defined as $\frac{\text{hormone-treated}-\text{control}}{\text{control}} \times 100$.

S. aureus, Streptococcus feacalis, and Bacillus subtilis, but do not affect gram negative growth. Therefore, in postulating cellular leakage as one of the hormonal mechanisms of action, no steroidal effects should be exerted on the leakage of cytoplasmic contents from gram negative microorganisms. Escherichia coli and Proteus mirabilis were pre-loaded with ^{14}C-glucose and exposed to progesterone or testosterone at 40 mcg/ml under identical conditions of pH, temperature, and cell concentration. In sharp contrast to the results obtained with S. aureus, the release of radioactivity in hormone-treated suspensions was quite similar to the control suspensions (Table 3). In fact, in all instances, the extracellular ^{14}C-activity in the cultures containing the hormone was less than the activity in the corresponding controls. Incubation for as long as 45 hours did not significantly increase leakage rates.

S. aureus at 126, 222, and 307 mcg dry wt/ml was exposed anaerobically to progesterone at 40 mcg/ml in 1% ETOH or only 1% ETOH. At specified times, 5 ml of the suspending fluid were isolated by membrane filtration and assayed for protein and 260 nM absorbing substances (Table 4). Again, no differences were observed during the initial stages of incubation. After 22 hours, progesterone treatment resulted in loss of protein at increased rates relative to controls. Maximal differences were

Table 3. The cellular leakage of ^{14}C-activity from gram negative organisms as influenced by hormones

Organism and treatment	Time of exposure					
	20 hrs		26 hrs		36 hrs	
	Activity	% leakage	Activity	% leakage	Activity	% leakage
Escherichia coli						
Control	6060	----	7370	----	8340	----
Progest. 40mcg/ml	5260	Negative	6700	Negative	8330	Negative
Testost. 40mcg/ml	5970	Negative	7220	Negative	8040	Negative
Proteus mirabilis						
Control	13970	----	16710	----	16810	----
Progest. 40mcg/ml	10210	Negative	11140	Negative	11070	Negative
Testost. 40mcg/ml	13370	Negative	15510	Negative	13920	Negative

Experimental conditions were identical to those of Table 1 except that *E. coli* and *P. mirabilis* were utilized. Negative, under % leakage, indicates lower quantities of ^{14}C-activity in the extracellular fluid of the hormone-treated suspensions as compared to the ^{14}C-activity in the extracellular fluid of control suspensions. Activity is expressed as cpm/ml. The data are average values for two experiments. The % leakage is defined as $\frac{\text{hormone-treated} - \text{control}}{\text{control}} \times 100$.

Table 4. Effects of progesterone on the cellular release of protein

Time of exposure	Bacterial concentra. (mcg dry wt/ml)	Mg protein/1000 ml Control	Progest. 40mcg/ml	Relative % difference
22 hrs	126	18.56	20.73	11.7±4.8
	222	35.52	40.60	14.3±2.7
	307	39.73	47.85	20.4±7.2
28 hrs	126	31.32	34.80	11.1±1.6
	222	40.60	49.01	20.7±4.1
	307	52.78	65.10	23.3±2.9

S. aureus serotypes X and X1 were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0 at the specified cellular densities. The cell suspensions were incubated anaerobically at 37C as described in Table 1. After 22 and 28 hrs, 5 ml portions were removed and centrifuged at 10,000 X g for 10 min. The supernatant fluid was assayed for protein according to the method of Warburg and Christian (63). These values represent the average of three experiments. The relative % difference is defined as

$$\frac{\text{hormone-treated} - \text{control}}{\text{control}} \times 100.$$

attained at the highest cell density in which there was a 20.4% and 23.3% difference after 22 and 28 hours, respectively. Note that the percent differences after 22 hours were quite similar to the percent differences after 28 hours at each bacterial concentration. Also, there was a progressive increase in the release of protein not only with time, but also with cell density.

Inconsistent results were obtained for the analysis of 260 nm absorbing compounds. Progesterone has a maximum absorbance at 240 nm, which quite possibly interferred with readings at 260 nm.

At this time, it was of interest to investigate steroidal effects on cellular leakage in the presence of antibiotics. Cultures of staphylococci, pre-loaded with ^{14}C-glucose and suspended in synthetic medium to induce growing conditions, were divided into three portions. To one was added bacitracin at 100 mcg/ml, to another, penicillin at 100 units/ml, and to the third, distilled water.

The interaction of progesterone with the two antibiotics is shown in Table 5. Without antibiotics, after two hours progesterone-treated cells contained 98.8% as much ^{14}C-activity as the corresponding controls, and after 5 hours, 92.2% as much ^{14}C-activity. Note the difference in the presence of penicillin.

Table 5. Interaction of progesterone and antibiotics in enhancing cellular leakage

Time of exposure	Treatment	cpm/ml Control	cpm/ml Progest. 40mcg/ml	% leakage
2 hrs	No antibiotic	9712	9595	1.2±0.1
	Bacitracin 100mcg/ml	9736	9568	1.7±0.7
	Penicillin 100units/ml	9218	8204	11.0±1.0
5 hrs	No antibiotic	7264	6698	7.8±0.3
	Bacitracin 100mcg/ml	8733	8373	4.1±1.2
	Penicillin 100units/ml	7004	4437	36.7±3.6

S. aureus serotypes XI and XII were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0 containing 0.01mC ^{14}C-glucose. After assimilation of the label for 30 min, the cells were washed twice in physiological saline (0.89%) and re-suspended in synthetic medium (25) at 126 mcg dry wt/ml. The bacteria were incubated without antibiotics, with penicillin, or with bacitracin in the presence and absence of progesterone under anaerobiosis as described in Table 1. After 2 and 5 hrs exposure, 0.5 ml aliquots of the suspensions were removed and membrane filtered (13 mm diameter, 0.45 micron pore size). The dried filters containing the bacteria were placed into scintillation fluor and assayed for radioactivity as described in Table 1. The values represent the average of two experiments. The % leakage is defined as $\frac{\text{hormone-treated}-\text{control}}{\text{control}} \times 100$.

Hormonally treated suspensions contain a significantly lower quantity of radioactivity than the corresponding controls. After two hours, progesterone-treated cells contained 89.0% as much activity, and after five hours 63.3% as much activity.

Bacitracin did not alter leakage rates.

B. Hormonal Intervention in the Uptake of Amino Acids

The alteration of entrance of specific substrates could affect the progress of growth by reducing the availability of required nutrients. The initial experiments to test this hypothesis involved exposure of staphylococcal suspensions to ^{14}C-glutamic acid, containing hormones in 1% ETOH or only 1% ETOH.

Table 6 presents the data obtained by Seitz filtration on bacterial uptake of glutamic acid as influenced by progesterone and testosterone at 40 mcg/ml. After 15 min, progesterone retarded entry of the ^{14}C- activity 16%, and testosterone, 15%. After 20 min, progesterone inhibition increased to 32%, while testosterone inhibition remained at the same level of 15%. Progesterone thus appears to be the more potent inhibitor.

Table 6 also shows the results for a similar set of experiments, utilizing synthetic steroids. After 10 min incubation with labeled glutamic acid, norethindrone at 30 mcg/ml reduced

Table 6. Inhibition of glutamic acid uptake by progesterone, testosterone, norethindrone, and mestranol

Time	Control	Progest. 40mcg/ml	Relative % inhibition	Testost. 40mcg/ml	Relative % inhibition
15 min	1458	1692	16.0±4.1	1676	14.9±0.7
20 min	1303	1723	32.2±4.3	1494	14.6±4.6

Time	Control	Norethin. 40mcg/ml	Relative % inhibition	Norethin. 35mcg/ml Mestranol 10mcg/ml	Relative % inhibition
10 min	9080	12160	33.9±0.2	14160	55.9±0.2
15 min	6870	10950	59.4±2.2	9720	41.5±4.5

S. aureus serotypes VI and VII were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0 at 126mcg dry wt/ml. The cell suspension was added to 0.01 mC ^{14}C-glutamic acid containing the specified hormones dissolved in 1% ethanol or the 1% ethanol at 24C. At the indicated times, the suspensions were passed through Seitz filters. The filtrates were assayed for residual ^{14}C-activity as described in Table 1. Activity is expressed as cpm/ml. These values represent the average data for three experiments. Relative % inhibition is defined as $\frac{\text{hormone-treated}-\text{control}}{\text{control}} \times 100$.

entrance of the label 34%, and after 15 min, 59%. The combination of mestranol, at 10 mcg/ml, with norethindrone diminished glutamic acid uptake 56% and 41% after 10 and 15 min exposure. These two steroids exerted better inhibition of uptake than either progesterone or testosterone.

In the remaining experiments of this section, membrane filtration was used instead of Seitz filtration. Staphylococci suspended in buffer were added to ^{14}C-glutamic acid containing progesterone and testosterone at 40 mcg/ml in 1% ETOH or only 1% ETOH. Total uptake was assayed 1, 2, and 3 min after addition of the label (Table 7). Both hormones inhibited uptake. Although the effects were not as pronounced as in the previous experiments (Table 6), progesterone again was slightly more effective in reducing entry of the ^{14}C-activity. This inhibition was transitory, occurring only during the initial 7 min. Thereafter, both control and hormonally treated cells contained equal amounts of radioactivity.

The next group of experiments detailed steroidal effects on the uptake of lysine and glucose. Staphylococci were suspended in buffer and subjected to the labeled substrates in the presence and absence of progesterone at 40 mcg/ml. Table 8 indicates that the hormone significantly stimulated the uptake processes. After 5 min, the entry of ^{14}C-lysine was enhanced 30.2%,

Table 7. Hormonal intervention in the uptake of glutamic acid

Time	Treatment	Activity	Relative % inhibition
1 min	Control	1661	----
	Progest.	1542	7.2±1.2
	Testost.	1553	6.5±1.2
2 min	Control	1842	----
	Progest.	1628	11.6±1.1
	Testost.	1695	8.0±1.6
3 min	Control	2166	----
	Progest.	1986	8.3±0.8
	Testost.	2074	4.3±0.8

S. aureus serotypes XII and XIII were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0 at 126mcg dry wt/ml. The cell suspension was added to 0.01mC ^{14}C-glutamic acid containing progesterone or testosterone at 40mcg/ml, or only the hormone vehicle at 24C. After uptake of the amino acid for the designated time, 0.5ml of the suspensions was filtered by membrane filtration (0.45 micron pore size, 13mm diameter). The filters were washed with 5 volumes of cold buffer, air-dried, and placed directly into scintillation fluor. The radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer. Activity is expressed as cpm/ml. The are the average of three experiments. The relative % inhibition is defined as $\frac{\text{hormone-treated}-\text{control}}{\text{control}} \times 100$.

Table 8. The uptake of lysine and glucose as influenced by progesterone

Time	Treatment	Lysine		Glucose	
		Activity	Relative % stimulation	Activity	Relative % stimulation
5 min	Control	4114	----	1664	----
	Progest.	5356	30.2±3.4	1942	16.7±3.0
9 min	Control	8462	----	2640	----
	Progest.	10890	28.7±3.7	3152	19.4±7.3
16 min	Control	14866	----	3520	----
	Progest.	20010	34.6±3.3	3890	10.5±4.9

The experimental conditions were identical to those of Table 7 except that cells were exposed to 0.01 mC of ^{14}C-lysine or ^{14}C-glucose. The values represent the average data for three experiments. The relative % stimulation is defined as $\frac{\text{hormone-treated} - \text{control}}{\text{control}} \times 100$.

and of ^{14}C-glucose, 16.7%. Stimulation of uptake of both amino acids remained through the 9 and 16 min exposure times.

Hormonal effects on alanine uptake were then investigated. ^{14}C-activity was measured 5, 9, and 16 min post-addition of the label (Table 9). After 5 min, control suspensions contained 2279 cpm and progesterone-treated contained 1821 cpm, a 20.1% inhibition. In like manner, the steroid reduced entrance of the label 18.4% and 14.9% after 9 and 16 minutes, respectively. It is noteworthy, that this inhibition remained at a relatively constant level for as long as 240 min (15.4% inhibition) after addition of the ^{14}C-amino acid, at which time saturation levels of alanine were approached.

These positive results stimulated further examination of hormonal alterations in substrate uptake by staphylococci. The next group of experiments were concerned with designing optimal parameters for detecting these alterations. Hormonal effects were observed on the following experimental parameters: cell concentration, pH, hormone concentration, heat inactivation, age of culture, anaerobiosis, and temperature.

The initial experiment involved an investigation of the bacterial concentration required for optimal hormonal inhibition of alanine uptake. To this end, staphylococci were suspended

Table 9. The uptake of alanine as influenced by progesterone

Time	Treatment	Activity	Relative % inhibition
5 min	Control	2279	----
	Progest.	1821	20.1±4.1
9 min	Control	2833	----
	Progest.	2311	18.4±7.3
16 min	Control	4142	----
	Progest.	3525	14.9±5.5
240 min	Control	23120	----
	Progest.	19559	15.4±3.8

Experimental conditions are described in Table 7. Bac- were exposed to 0.01 mC ^{14}C-alanine. These values represent the average data for four experiments. Activity is expressed as cpm/ml. The relative % inhibition is defined as $\frac{\text{control} - \text{hormone-treated}}{\text{control}} \times 100$.

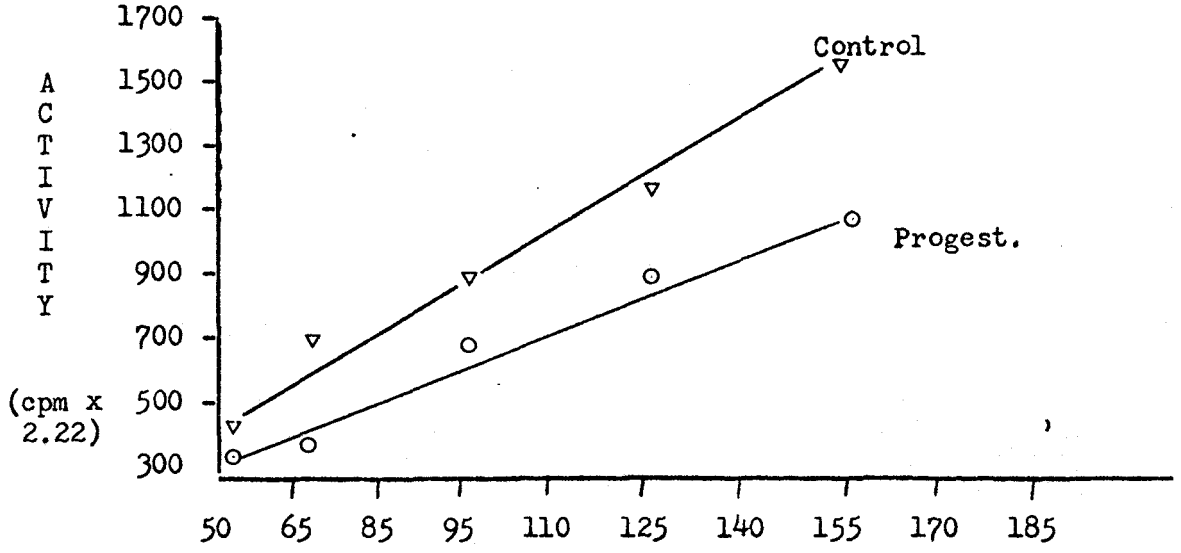
in buffer at various cellular densities. These suspensions were added to the ^{14}C-alanine with and without progesterone at 40 mcg/ml. Incubation was terminated 8 min post-addition of the label. The data (Fig 1) revealed that the hormone significantly reduced the total uptake at all bacterial concentrations. Inhibition ranged from 12% to 27% with maximal effects at the highest concentration.

Because amino acid uptake is mediated by enzyme-like reactions involving permease systems (9, 34), it was of interest to observe hormonal effects at various hydrogen ion concentrations; therefore, phosphate buffers at pH 5.7, 6.5, 7.5, and 8.0 were employed in the incubation. Cells were exposed to the labeled alanine for 8 min and assayed for radioactivity (Fig 2). Maximum inhibition occurred below pH 7.0. At pH 5.7, 1136 cpm were taken up by hormonally-treated bacteria as contrasted to 2209 cpm for the controls (48.6% inhibition); at pH 6.5, 1062 cpm, as contrasted to 2478 cpm (57.2% inhibition). Thereafter, there was a rapid decline in alanine uptake and corresponding inhibitory effects.

The quantitative aspects of various progesterone concentrations on alanine uptake are presented in Figure 3. Staphylococci were exposed to the ^{14}C-amino acid in the presence of the hormone at 30, 20, 10, 1, and 0 mcg/ml for 8 min. A

Figure 1

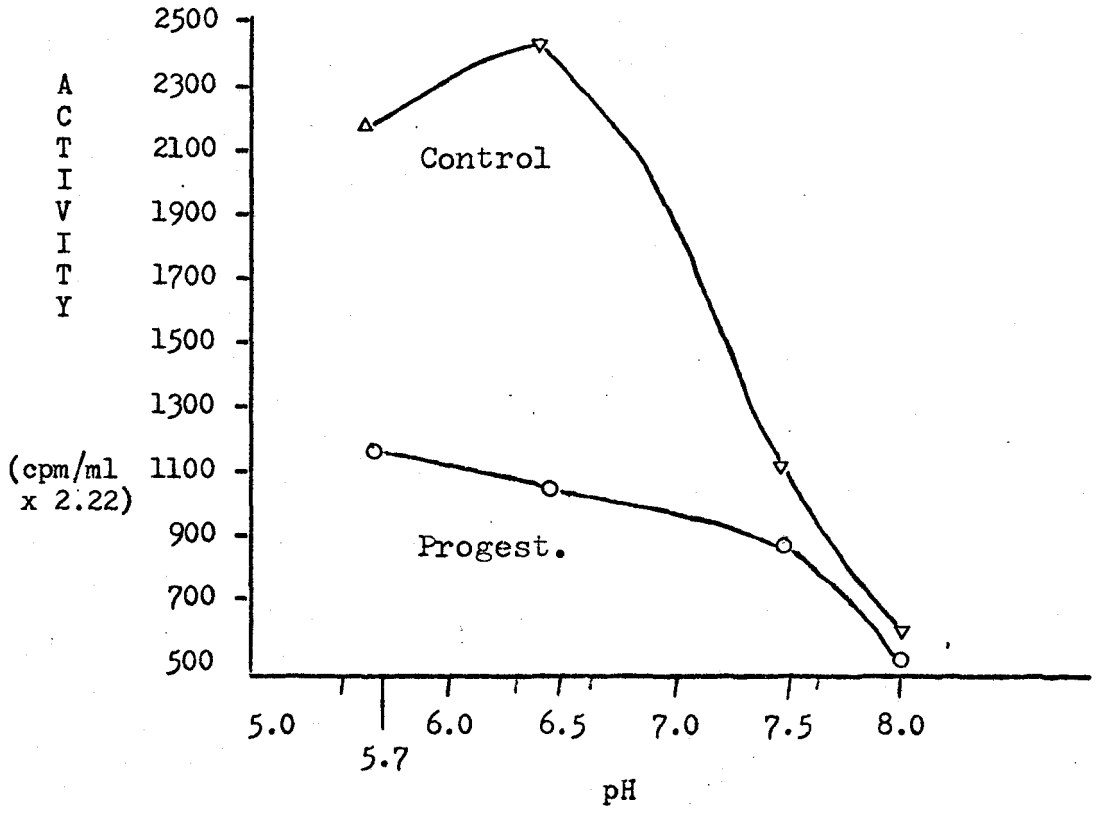
Alanine Uptake as Influenced by
Bacterial Concentration



Experimental conditions were identical to those described in Table 8, except that cellular suspensions were adjusted to 57, 70, 100, 124, and 156 mcg dry wt/ml and exposed to 0.01 mC ^{14}C-alanine. The data represent the average values for three experiments.

Figure 2

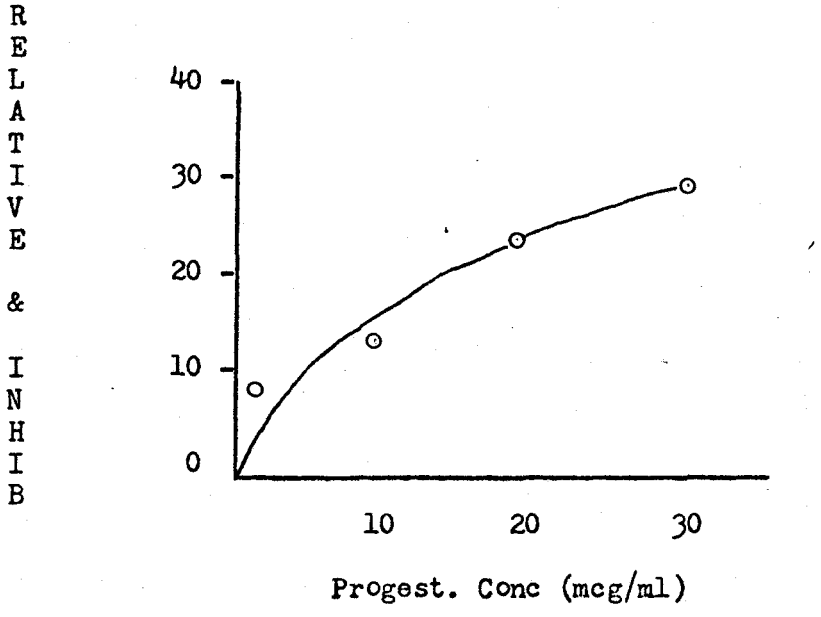
The Effects of Various pH Levels on Alanine Uptake



Experimental conditions were identical to those of Table 8, except that bacteria were suspended at pH 5.7, 6.5, 7.5, and 8.0 and then exposed to 0.01 mC ^{14}C-alanine. The data are average data for four experiments.

Figure 3

Alanine Uptake as Influenced by
Progesterone Concentration



Experimental conditions were identical to those listed in Table 8, except that the concentration of progesterone was 30, 20, 10, 1, and 0 mcg/ml. The data represent mean values for three experiments - Relative % inhibition is defined as $\frac{\text{hormone-treated-control}}{\text{control}} \times 100$.

progressive increase in inhibition was observed with increasing concentrations of progesterone above 10 mcg/ml. Below this concentration, no significant reduction in alanine uptake was detected.

At this point, it was of interest to determine whether the steroidal effects were exerted on the actual transport of the amino acid into the cell, or on the adsorption of the amino acid to the cell prior to its entrance. Heat-inactivated staphylococci (65C for 30 min) were exposed to ^{14}C-alanine with and without progesterone at 40 mcg/ml. Radioactivity was measured after 5, 9, and 16 min. Table 10 compares the data with data from a similar experiment with active cells. The radioactivity within heat-inactivated bacteria was only slightly above background; and thus, progesterone was interferring with an active cellular process rather than physical adsorption.

Alanine uptake as a function of the age of bacteria and hydrogen ion concentration is presented in Table 11. Staphylococci growing, in TSB, were removed at mid-logarithmic phase, harvested, washed, and suspended in phosphate buffers at the indicated pH's. The cells were exposed to ^{14}C-alanine in the presence and absence of progesterone at 40 mcg/ml. Radioactivity was evaluated after 8 min incubation. Twelve hours later, the staphylococci in the TSB had reached stationary phase of growth. They were

Table 10. Hormonal effects on the uptake of alanine by "live" cells and heat-inactivated cells

Time	Treatment	"Live" Activity	"Live" Relative % inhibition	Heat-inactivated Activity	Heat-inactivated Relative % inhibition
5 min	Control	3548	----	98	Counts
	Progest.	2890	18.6±1.3	75	
9 min	Control	5155	----	67	Too
	Progest.	4291	16.8±6.4	62	
16 min	Control	6602	----	53	Low
	Progest.	5685	13.9±1.5	69	

Experimental conditions were identical to those of Table 7 except that sereotypes X and XII were exposed to 0.01 mC ^{14}C-alanine. Staphylococci were inactivated by heating for 30 min at 60C. Activity is expressed as cpm/ml. These values represent the average data for two experiments. The relative % inhibition is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

Table 11. Hormonal effects on alanine uptake as a function of the age of culture

pH	Treatment	Logarithmic cells		Stationary cells	
		Activity	Relative % inhibition	Activity	Relative % inhibition
5.7	Control	6338	----	1558	----
	Progest.	5156	18.7±3.0	1269	21.1±5.9
6.8	Control	5276	----	2049	----
	Progest.	4373	17.1±4.3	1654	19.3±2.8
7.5	Control	4624	----	1743	----
	Progest.	3642	21.2±9.5	1154	33.8±8.0
8.0	Control	3840	----	1461	----
	Progest.	3094	19.4±6.6	1219	16.6±6.1

S. aureus serotypes IX and XIII were grown in TSB to mid logarithmic and stationary phases, harvested, washed, and suspended in 0.1 M sodium phosphate buffers at the specified pH's at a density of 126mcg dry wt/ml. The bacterial cultures were exposed to 0.01 mC ^{14}C-alanine for 8 min in the presence and absence of progesterone at 40mcg/ml and membrane filtered. The filters were washed with 8 volumes of buffer, air-dried, and assayed for ^{14}C-content as described in Table 7. Activity is expressed as cpm/ml. The values represent average data for three experiments. The relative % inhibition is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

harvested, washed, and suspended in the various buffers. The identical experiment was performed. Note that the hormonal inhibition of alanine uptake was fairly similar with bacteria from both phases of growth. The percent inhibition at pH 5.7 was 18.7% in log cells and 21.1% in stationary cells. At pH 6.5, progesterone reduced entry of alanine 17.1% in log cells, and 19.3% in stationary cells. In like manner at pH 8.0, the hormone impeded entrance of the label 19.4% in log cultures, and 16.6% in stationary cultures. The only pH to show a significant difference between the two groups of bacteria was pH 7.5. In log cells there was a 21.2% inhibition which increased to 33.8% inhibition in the older cultures. It should be emphasized that the progesterone-induced inhibition remained relatively constant, in spite of the fact that mid-log phase suspensions had taken up 3 to 4 times as much radioactivity as the slower metabolizing stationary suspensions.

The next parameter investigated was anaerobic versus aerobic uptake. Bacterial suspensions were added to ^{14}C-alanine containing progesterone at 40 mcg/ml in 1% EtOH or only 1% EtOH and placed in a Precision Thelco anaerobic incubator. Samples of 0.5 ml were removed and membrane filtered separately after 5, 9, and 16 min of exposure. The results in Table 12 list uptake effects under anaerobic as well as aerobic conditions. An

Table 12. Alanine uptake under anaerobic conditions

Time	Treatment	Aerobic conditions		Anaerobic conditions	
		Activity	Relative % inhibition	Activity	Relative % inhibition
5 min	Control	1812	----	1676	----
	Progest.	1529	15.6±0.2	1019	39.2±1.0
9 min	Control	2892	----	2779	----
	Progest.	2401	17.0±3.9	1726	37.9±0.1
16 min	Control	4509	----	4234	----
	Progest.	4395	22.5±6.0	2174	48.7±5.2

Experimental conditions were identical to those of Table 7 except that serotypes XII and XIII were exposed to 0.01 mC ^{14}C-alanine. For anaerobiosis, a Precision Thelco Anaerobic Chamber flushed with 95% CO_2 and 5% nitrogen was used. The activity is expressed as cpm/ml. The values represent average values for three experiments. Relative % inhibition is defined as $\frac{\text{control} - \text{hormone-treated}}{\text{control}} \times 100$.

enhancement of steroidal inhibition by anaerobiosis was apparent. For aerobic conditions, the percent inhibition ranged from 15.6% to 22.5%. For anaerobic conditions, the percent inhibition ranged from 37.9% to 48.7%, representing over a two-fold increase in hormonal reduction of alanine uptake.

The effects of various temperatures on the uptake of ^{14}C-alanine is exhibited in Table 13. Staphylococcal suspensions were added to the ^{14}C-amino acid in the presence and absence of progesterone at 40 mcg/ml and incubated at 4, 24, 30, 37, 45, and 55 C for 8 min. Assessment of radioactivity indicated a fluctuating hormonal inhibition. Maximum reduction of alanine uptake occurred at 30 C. Control suspensions contained 7880 cpm, whereas the hormone-treated suspensions contained 5691 cpm (27.8% inhibition). At the other temperatures, progesterone produced statistically significant reductions in alanine uptake ranging from 16.3% to 21.6%.

Previous work (15, 69, 70, 75, 76, 77) showed that steroids inhibited the *in vitro* growth of gram positive organisms but did not affect the growth of gram negative organisms. Thus, it was important to undertake a related series of uptake experiments with gram negative bacteria. *Shigella flexneri*, and *Salmonella paratyphi* were exposed to ^{14}C-alanine under identical conditions of pH, temperature, cell concentration, and progesterone at 40 mcg/ml. The

Table 13. Effects of temperature on hormonal inhibition of alanine uptake

Temperature	Treatment	Activity	Relative % inhibition
4C	Control	831	----
	Progest.	652	21.6±4.9
24C	Control	5204	----
	Progest.	4344	16.5±3.2
30C	Control	7880	----
	Progest.	5691	27.8±5.6
37C	Control	10072	----
	Progest.	8035	20.2±1.7
45C	Control	9697	----
	Progest.	8115	16.3±3.2
55C	Control	4102	----
	Progest.	3257	20.6±2.8

Experimental conditions were similar to those of Table 7. Serotype A was exposed to 0.01 mC ^{14}C-alanine in the presence and absence of progesterone at 40mcg/ml at the indicated temperatures. Activity is expressed as cpm/ml. These values represent the mean values of three experiments. The relative % inhibition is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

suspensions were assayed for radioactivity at 5, 9, and 16 min post-addition of the label. Table 14 summarizes the results and correlates them to the effects on S. aureus. After 5 min, the hormone reduced staphylococcal uptake 20.1%; after 9 min, 18.4%; and after 16 min, 14.9%. In direct contrast, S. flexneri and S. paratyphi, in the presence of the hormone, showed no significant variation in quantity of ^{14}C-activity taken up, compared to the corresponding controls.

The observation that progesterone inhibited the uptake of alanine prompted a comparative study of the effects of other hormones. Bacterial uptake of ^{14}C-alanine was observed in the presence of testosterone and β-estradiol, in addition to progesterone, at 40 mcg/ml, and diethylstilbestrol at 20 mcg/ml. The latter steroid, due to solubility requirements, dictated a 2% ETOH concentration for all four steroids. Controls received only the 2% ETOH. Cells were removed after 5, 9, and 16 min incubation (Table 15). Diethylstilbestrol produced maximum inhibition, retarding uptake 71% to 87%. Testosterone, β-estradiol, and progesterone exerted relatively lower inhibitory effects, retarding uptake 10% to 18%.

In related experiments (results not shown), 17α-hydroxyprogesterone, which did not retard the growth of staphylococci in broth (76), produced no inhibitory effects on alanine uptake.

Table 14. Alanine uptake in gram negatives as influenced by progesterone

Organism	Time	Control	Progest. 40mcg/ml	Relative % inhibition
Staphylococcus aureus	5 min	2279	1821	20.1±4.1
	9 min	2833	2311	18.4±7.3
	16 min	4142	3525	14.9±5.5
Shigella flexneri	5 min	1333	1296	2.8±2.4
	9 min	1733	1794	No inhibition
	16 min	2572	2575	No inhibition
Salmonella paratyphi	5 min	3339	3487	No inhibition
	9 min	4383	5286	No inhibition
	16 min	6721	6972	No inhibition

S. aureus serotypes XI and XII, S. flexneri, and S. paratyphi were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0 at 126mcg dry wt/ml. The cell suspensions were exposed to 0.01 mC ^{14}C-alanine containing either the hormone or the hormone vehicle. At the designated times, bacteria were isolated by membrane filtration, washed with 8 volumes of buffer, and placed into scintillation fluor for assessment of radioactivity as described in Table 7. Activity is expressed as cpm/ml. The values represent average data of two experiments. Relative % inhibition is defined as $\frac{\text{control} - \text{hormone-treated}}{\text{control}} \times 100$.

Table 15. Effects of other hormones on the uptake of alanine

Time	Control	Progest. 40mcg/ml	Relative % inhib.	Testost. 40mcg/ml	Relative % inhib.	Estradiol 40mcg/ml	Relative % inhib.	Diethylstil. 20mcg/ml	Relative % inhib.
5 min	1396	1195	14.4±3.4	1249	10.5±5.4	1217	12.8±3.1	411	70.6±7.6
9 min	2532	2100	17.1±4.1	2281	10.0±4.8	2134	15.8±3.5	402	84.1±3.8
16 min	3517	2994	14.9±4.1	3100	11.9±6.3	3023	14.1±5.7	443	87.4±2.5

Experimental conditions were similar to those described in Table 7. Bacteria were exposed to 0.01 mC ^{14}C-alanine containing the hormones dissolved in 1% ethanol or only the 1% ethanol. Activity is expressed as cpm/ml. The values represent the mean values of three experiments. Relative % inhibition is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

The data presented to this point reflect total radioactivity within the bacterial cells. The next table (16) provides a more detailed analysis of alanine uptake by S. aureus. Bacteria were exposed to the labeled amino acid and progesterone at 40 mcg/ml. At the indicated times, aliquots of the suspension were removed and divided in two. One portion was immediately filtered to determine total uptake. The other was equally mixed with cold 10% TCA and extracted for 15 min at 0C. Residual activity after extraction, filtration, and washing represents total incorporation of the label into cellular macromolecules. The pool of activity, defined as that amount of ^{14}C-activity extractable with cold TCA, can be readily ascertained by appropriate subtraction of total incorporation from total uptake.

Table 16 depicts the nature of progesterone reduction of total uptake. Note after 5, 9, and 16 min exposure, the fairly constant percent inhibition in the rate of total uptake; 19.0%, 18.1%,and 15.5%, respectively. Characterization of this inhibition revealed a two-fold effect: 1) an increasing retardation of cellular incorporation, and 2) a decreasing reduction in pool size. After 5 min, the steroid reduced incorporation 35.0%; after 9 min, 40.7%; and after 16 min, 53.5%. The cellular pools were diminished 15.3% after 5 min, 12.2% after 9 min, and 2.8% after 16 min.

Table 16. Progesterone-induced effects on cellular incorporation and pool sizes

	Total uptake			Total incorporation			Pool sizes		
Time	Control	Progest. 40mcg/ml	Relative % inhib.	Control	Progest. 40mcg/ml	Relative % inhib.	Control	Progest. 40mcg/ml	Relative % inhib.
5 min	3219	2609	19.0±3.8	601	391	35.0±5.1	2618	2218	15.3±4.9
9 min	4825	3954	18.1±3.4	989	587	40.7±12.7	3836	3367	12.2±5.2
16 min	6686	5650	15.5±4.3	1674	779	53.5±8.6	5012	4871	2.9±7.3

S. aureus serotypes XI, XII, and XIII were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in 0.1 M sodium phosphate buffer pH 7.0 at 126mcg dry wt/ml. The cell suspensions were incubated at 24C with 0.01 mC ^{14}C-alanine in the presence and absence of progesterone. At the designated times, 1.0 ml samples were removed and divided in two. One portion (0.5 ml) was immediately membrane filtered and washed with 8 volumes of cold buffer. The other portion (0.5 ml) was diluted with 0.5 ml 10% ice-cold TCA. After extraction for 15 min, the second portion was also membrane filtered and washed with cold buffer. The first portion represents total cellular uptake and the second portion, total incorporation. Pool sizes were readily determined by subtraction of incorporation from total uptake. Activity is expressed as cpm/ml. These values represent the mean values of four experiments. Relative % inhibition is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

At this time, further experiments were undertaken to elucidate the steroidal mechanism of action. The uptake of alanine and hormonal effects thereon, were studied in the presence of various inhibitors, such as sodium azide, 2,4-dinitrophenol, actinomycin D, and chloramphenicol. Any synergistic or antagonistic action of steroidal inhibition would implicate specific cellular processes. Bacteria were incubated with the inhibitors, then exposed to ^{14}C-alanine in the presence and absence of progesterone at 40 mcg/ml. Uptake was analyzed by the standard procedures of membrane filtration.

Sodium azide and 2,4-dinitrophenol restrict energy production in microbial cells (12). Neither of these agents affected the uptake or the inhibition of uptake of labeled alanine under the experimental conditions employed, thereby limiting the possibility of hormonal involvement at the level of energy production via oxidative phosphorylation.

In similar fashion, actinomycin D, an inhibitor of RNA synthesis (12) did not alter the hormonal inhibition of uptake.

When chloramphenicol, an inhibitor of protein synthesis (12), was tested, synergistic results were obtained (Table 17). Bacteria, suspended in buffer and pre-incubated with the inhibitor at 200 mcg/ml for 30 min, contained markedly lower quantities of radioactivity in progesterone-treated cultures. After

Table 17. Effects of chloramphenicol and progesterone on uptake of alanine

Uptake in buffer

Time	Without chloramphenicol			With chloramphenicol		
	Control	Progest. 40mcg/ml	Relative % inhib.	Control	Progest. 40mcg/ml	Relative % inhib.
5 min	3218	2606	19.0±5.1	1065	617	42.1±10.6
9 min	4826	3850	20.2±7.5	1434	752	47.6±8.8
16 min	7490	5524	26.3±5.4	1718	1020	40.6±2.9

Uptake in synthetic medium

Time	Without chloramphenicol			With chloramphenicol		
	Control	Progest. 40mcg/ml	Relative % inhib.	Control	Progest. 40mcg/ml	Relative % inhib.
5 min	2984	2376	20.4±3.1	2620	2182	16.6±3.7
9 min	3120	2528	19.4±3.7	2723	2288	16.0±3.3
16 min	3290	2634	20.0±2.6	2828	2370	16.2±4.2

Experimental conditions were similar to those listed in Table 7. Bacteria were pre-incubated with and without chloramphenicol in 0.1 M sodium phosphate buffer pH 7.0 or synthetic medium (25) for 30 min, then exposed to 0.01 mC ^{14}C-alanine. Activity is expressed as cpm/ml. These values represent the average values. The relative % inhibition is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

5, 9, and 16 min, suspensions without the antibiotic, in the presence of hormone at 40 mcg/ml, contained 19.0% to 26.3% less ^{14}C-activity than the corresponding controls. When suspensions were pre-incubated with the antibiotic, a two-fold increase in hormonal retardation was apparent. Progesterone-treated cultures contained 40.6% to 47.6% less radioactivity.

Similar experiments were performed, with staphylococci suspended in synthetic medium, to test the assumption that chloramphenicol should exert increased effects on actively growing cells, and in turn, produce an enhancement of synergistic action in combination with progesterone; however, the results did not substantiate this, as seen in Table 17. The percent inhibition produced by the hormone was not significantly affected by the antibiotic. Without chloramphenicol, progesterone reduced uptake 19.4% to 20.4%, and with chloramphenicol, 16.0% to 16.6%. The synergistic effect observed in the previous experiment quite possibly was due to the interaction of the inhibitor with cellular processes other than protein synthesis.

C. Hormonal Effects on Entry of Substrates into Various Cellular Fractions

Experiments described in the previous section demonstrated hormonal intervention in the total uptake and total incorporation

of nutrients into S. aureus. A further analysis of the phenomenon was attempted. The fractionation procedure of Park & Hancock (46) was extended to a comparative study of steroidal effects on the entrance of ^{14}C-substrates, and their subsequent incorporation into cellular macromolecules.

The characterization of the five fractions is outlined below. The cold TCA extract contains the pool of amino acids and other acid-soluble low molecular weight compounds. The ETOH soluble fraction is comprised of cellular lipid and very slight amounts of alcohol-soluble protein. Extraction with hot TCA removes the nucleic acids and virtually all of the staphylococcal teichoic acids from the cell wall. Trypsinization converts over 95% of the cell protein to soluble peptides. The residue consists predominantly of the mucopeptides of the wall structure.

The initial experiments involved quantitative aspects of progesterone intervention in alanine incorporation. Bacterial suspensions were exposed to the labeled amino acid and to the hormone at 40, 30, 15, 1, and 0 mcg/ml, for 15 min (Table 18). In the presence of 1 mcg/ml, no significant inhibition of ^{14}C-entrance was evident. The quantities of radioactivity within hormonally-treated and control fractions were similar. At 15 mcg/ml, progesterone retarded entrance of alanine into

Table 18. Effects of various concentrations of hormone on incorporation of alanine

Fraction	Progesterone concentration (mcg/ml) 0	1	Relative % differ.	15	Relative % differ.	30	Relative % differ.	40	Relative % differ.
Cold TCA	9020	9000	0.3±0.7	8560	5.1±2.1	7600	15.8±3.6	7541	16.4±0.3
75% ETOH	7840	7333	6.5±3.1	7426	5.3±2.4	7346	6.3±1.0	6546	16.5±4.8
Hot TCA	16760	15200	9.3±10.8	11920	28.9±15.6	9700	42.1±16.4	9134	45.5±10.0
Trypsin	67620	64480	4.7±7.5	61800	8.6±6.1	58018	14.2±4.6	55560	17.8±0.5
Residue	4437	4219	4.9±2.6	4623	4.2±3.9	4177	5.9±1.1	3536	20.3±2.2

S.aureus serotypes X and XI were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in synthetic medium at 222mcg dry wt/ml. The bacterial suspensions were exposed to 0.01 mC ^{14}C-alanine in the presence of the indicated hormone concentrations at 24C for 15 min. The cells were immediately centrifuged at 15,000 X g for 12 min at 0C, washed twice incold saline, and extracted according to Park and Hancock (46). Aliquots of the soluble material of the cold and hot TCA, the 75% ETOH, and trypsin fractions were placed in scintillation fluidand assayed for radioactivity as described in Table 1. The insoluble residue was collected on membrane filters, air-dried, placed in fluor, and assayed for ^{14}C-activity. Activity is expressed as cpm/ml. The data are mean values for three experiments. Relative % difference is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

the hot TCA fraction 29%. At 30 mcg/ml, the steroid inhibited entry into the cold TCA fraction 16%, the hot TCA fraction, 42%, and the protein fraction, 14%. At 40 mcg/ml, progesterone reduced entrance of the label into all five cellular fractions. These results correlate with previous findings (76), in that the growth of staphylococci in nutrient broth was significantly retarded by progesterone at a critical concentration of 15 mcg/ml. Below the concentration, no growth inhibitory effects were detected. Above the concentration, there was a progressive increase in retardation of staphylococcal growth.

Earlier work had shown that steroids did not effect the growth of gram negative bacteria in nutrient broth (15, 69, 70, 75, 76, 77). With this in mind, experiments were performed to determine hormonal influences on the cellular incorporation of alanine into Escherichia coli and Shigella flexneri. Table 19 compares this data to results obtained utilizing S. aureus. For the staphylococcal suspensions, progesterone significantly retarded entry of the ^{14}C-label into the five cellular fractions, with inhibition ranging from 16% to 45% (Table 18). In direct contrast, no inhibitory effects were exerted on ^{14}C-entrance into fractions from gram negative bacteria. The quantities of radioactivity within the fractions of progesterone-treated cultures were similar to the corresponding controls.

Table 19. Alanine incorporation into gram negative organisms

Fraction	*Escherichia coli*		*Shigella flexneri*	
	Control	Progest. 40mcg/ml	Control	Progest. 40mcg/ml
Cold TCA	2000	2300	3920	4220
75% ETOH	2620	2840	2040	2300
Hot TCA	2640	2820	2860	3020
Trypsin	21140	22040	20100	22180
Residue	1678	1740	1514	1700

E. coli and *S. flexneri* were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in synthetic medium at 222mcg dry wt/ml. The bacterial suspensions were exposed to 0.01 mC ^{14}C-alanine for 15 min at 24C. Conditions of centrifugation, fractionation, and assay of ^{14}C-quantity were described in Table 18. Activity is expressed as cpm/ml. The data represent mean values of two experiments.

Macromolecular syntheses occurs at a much faster rate in cells in mid-log phase than in cells in stationary phase. If the hormones act specifically on some internal synthetic process, for example, protein synthesis, then increased inhibition of substrate incorporation should be observed in the "younger" cultures. To this end, two suspensions of cells, one from mid-log phase and the other from stationary phase, were exposed to ^{14}C-alanine in the presence and absence of progesterone at 40 mcg/ml (Table 20). Subsequent fractionation revealed no significant deviation in hormonal inhibition of alanine entrance into the five fractions. The percent inhibition in each cellular fraction for both groups of cells was similar: cold TCA, 13% in log cultures, and 13% in stationary cultures; 75% ETOH, 18% and 12%; hot TCA, 43% and 39%; trypsin, 17% and 22%; and residue, 19% and 16%. The smaller quantities of ^{14}C-activity incorporated by stationary cultures was indicative of less metabolically active bacteria. Thus, a slowdown in overall cell metabolism was achieved without a concomitant increase or decrease in hormonal inhibitory effects.

It is known that substrates taken into cells and incorporated into cellular components, must pass through a cellular pool before entering the diverse metabolic pathways (4, 5, 78). In this way, the pool size may directly influence the synthesis of

Table 20. Effects of progesterone on different phases of cultures

Fraction	Logarithmic phase			Stationary phase		
	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.
Cold TCA	15094	13734	13.3±1.0	14920	12976	13.0±4.1
75% ETOH	2680	2200	18.0±3.6	2550	2250	11.7±1.6
Hot TCA	21136	12050	43.0±1.4	18220	11144	38.8±2.4
Trypsin	11736	9760	16.8±1.2	9236	7224	21.8±1.4
Residue	33407	26947	19.3±4.5	24192	20273	16.2±1.7

S. aureus serotypes XII and XIII were grown to mid logarithmic and stationary phases in TSB, harvested, washed, and suspended in synthetic medium at 222mcg dry wt/ml. Experimental conditions were similar to those of Table 18. Activity is expressed as cpm/ml. The data are average values for three experiments. Relative % difference is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

macromolecules. Therefore, it was important to note at what point initial hormonal effects occurred. Staphylococci were exposed for 10, 60, and 900 seconds, to ^{14}C-alanine with and without progesterone at 40 mcg/ml (Table 21). The initial inhibition within 10 seconds (14%) was exerted in the cold TCA fraction. Notice the increasing inhibition in ^{14}C-entry into the hot TCA fraction: 10 seconds, 5% (not statistically significant); 60 seconds, 22%; 900 seconds, 45%. For the protein and residue fractions, hormonal intervention was not apparent until the organisms had been exposed for more than 60 seconds. These results suggested an immediate, initial influence on the transport of alanine into the cellular pool.

Previous investigations (15, 75, 76) relating to the actions of gonodal steroids on the growth of S. aureus revealed that epiandrosterone, at or above 20 mcg/ml, exerted a pronounced growth suppressive action. Stanolone inhibited growth to a lesser extent, whereas 17 α -hydroxyprogesterone did not influence growth of the organisms. With these facts in mind, alanine incorporation was assayed in the presence of the three hormones at 40 mcg/ml, and also, epiandrosterone at the sub-inhibitory concentration of 10 mcg/ml (Table 22). Epiandrosterone at 40 mcg/ml exerted maximum inhibition, retarding entry of the label into all five cellular fractions. Stanolone impeded entrance into the hot TCA (12%) and protein fractions (19%).

Table 21. Time course incorporation of alanine

Fraction	Time of exposure: 10 seconds			60 seconds			900 seconds		
	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.
Cold TCA	3500	3000	14.3±5.5	4070	3500	14.0±4.2	32626	27306	16.3±3.4
Hot TCA	4550	4300	5.5±3.7	5800	4550	21.6±2.0	37466	20453	45.4±1.4
Trypsin	7410	7690	Negative	8890	8980	Negative	39353	33766	14.2±1.2
Residue	2420	2277	5.9±3.1	4000	3780	5.5±3.6	19581	15622	20.2±3.9

S. aureus serotypes X, XII, and XIII were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in synthetic medium at 222mcg/ml. The bacterial suspensions were exposed to 0.01 mC ^{14}C-alanine for 10, 60, and 900 sec in the presence and absence of the hormone. After exposure for the specified times, 2 volumes of ice water were added to the suspensions, which were then placed in an ice bath at 0C for 10 min. The conditios of centrifugation, fractionation, and assay of radioactivity were identical to those of Table 18. Activity is expressed as cpm/ml. The data represent the mean values of four experiments. The relative % difference is defined as $\frac{\text{control} - \text{hormone-treated}}{\text{control}} \times 100$.

Table 22. Effects of other hormones on alanine incorporation

Fraction	Control	Epiand. 40mcg/ml	Relative % differ.	Epiand. 10mcg/ml	Relative % differ.	Stanol. 40mcg/ml	Relative % differ.	17αOH-progest. 40mcg/ml	Relative % differ.
Cold TCA	43040	38060	11.6±3.5	41000	4.7±4.2	46050	Negative	46009	Negative
75% ETOH	4120	3690	10.4±5.8	3910	5.1±3.1	4420	Negative	3869	6.1±5.9
Hot TCA	32280	22870	29.2±2.0	31160	3.5±3.9	28450	11.9±7.0	31989	0.9±2.1
Trypsin	49570	30880	37.7±8.4	44070	11.1±3.6	39980	19.4±5.5	46100	7.0±3.8
Residue	20063	17452	13.0±3.0	20524	Negative	20807	Negative	20524	Negative

Experimental were identical to those listed in Table 18. S. aureus serotypes XII and XIII were exposed to 0.01 mC ^{14}C-alanine in the presence of epiandrosterone at 10 and 40mcg/ml, stanolone at 40mcg/ml, and 17 OHprogesterone at 40mcg/ml. The controls were exposed to the hormone vehicle. Activity is expressed as cpm/ml. The data are average values for three experiments. Negative, under relative % difference, indicates no inhibitory effects. The relative % difference is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

17 α -hydroxyprogesterone did not significantly alter incorporation rates into any of the fractions. Also, epiandrosterone, at the sub-inhibitory concentration of 10 mcg/ml, did not inhibit entrance of the ^{14}C-alanine into four of the five cellular fractions.

Attention was then focused on the incorporation of other substrates. Staphylococcal suspensions were incubated with ^{14}C-glutamic acid or ^{14}C-lysine in the presence and absence of progesterone at 40 mcg/ml (Table 23). The hormone significantly inhibited entry of glutamic acid into the cold TCA (13.1%) and 75% ETOH fractions (16.1%) and stimulated entrance into cell protein (21.0%) and residue (15.7%). After exposure to ^{14}C-lysine, progesterone enhanced entrance of ^{14}C-activity only into the cold TCA fraction and reduced entrance into the other four cellular fractions, 10.5% to 21.3%.

Similar experiments were performed with ^{14}C-phenylalanine and ^{14}C-leucine (Table 24). Progesterone significantly retarded entry of the labeled phenylalanine into the cold TCA fraction (12.2%). Entrance into the other fractions was not altered. The difference in total uptake between hormonally-treated and control cultures was not statistically significant.

Fractionation studies after exposure to leucine disclosed

Table 23. Effect of progesterone on incorporation of glutamic acid and lysine

	Glutamic acid			Lysine		
Fraction	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.
Cold TCA	920	800	13.1±4.7	124240	231640	86.4±9.7
75% ETOH	1413	1186	16.1±5.1	23266	18320	21.3±6.8
Hot TCA	4880	4773	2.2±4.0	6213	5514	11.3±5.8
Trypsin	43226	52293	21.0±6.2	36373	32560	10.5±0.2
Residue	2658	3075	15.7±3.3	36470	31692	13.1±1.2

Experimental conditions were identical to those of Table 18 except that bacteria were exposed to 0.01 mC ^{14}C-glutamic acid or ^{14}C-lysine. Activity is expressed as cpm/ml. These values represent the mean values for four experiments. Relative % difference is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

Table 24. Effect of progesterone on incorporation of phenylalanine and leucine

	Phenylalanine			Leucine		
Fraction	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.
Cold TCA	13800	12120	12.2±1.8	14933	13160	11.9±2.6
75% ETOH	8420	9140	8.5±4.2	3040	2173	28.5±3.1
Hot TCA	4420	4040	8.6±7.3	14853	17720	19.3±10.2
Trypsin	66420	63560	4.3±1.5	49333	46680	5.4±2.7
Residue	20007	18395	8.1±6.5	12863	14891	15.8±4.0

Experimental conditions were identical to those listed in Table 18 except that the bacterial suspensions were exposed to 0.01 mC ^{14}C-leucine or ^{14}C-phenylalanine. The activity is expressed as cpm/ml. The data represent the mean values for three experiments. The relative % difference is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

hormonal alterations in four of the five fractions. Inhibition occurred in the entry of radioactivity into the cold TCA (11.9%) and the 75% ETOH fractions (28.5%). Stimulation was evident in the entrance into the hot TCA fraction (19.3%) and the residue (15.8%).

The previous data involved hormonal alteration in the incorporation of diverse amino acids and dictated further analysis of incorporation, utilizing other nutrients. Bacterial suspensions were incubated with labeled acetate, glycerol, or glucose containing progesterone at 40 mcg/ml in 1% ETOH or only 1% ETOH (Table 25). After acetate exposure, the cold TCA and protein fractions of hormone-treated cultures contained 11.5% and 58.7% less radioactivity than comparable controls. In contrast, progesterone enhanced the entry of the acetate label into the hot TCA fraction 22.2%. After exposure to glycerol, significant hormonal reduction, 11.0%, occurred only in the cold TCA fraction. Entry into the 75% ETOH, the hot TCA, and the residue was increased 17.0%, 11.2%, and 11.1%, respectively. For glucose, progesterone retarded entry of ^{14}C-activity into three of the five fractions. Significantly less radioactivity was detected in the 75% ETOH, trypsin, and residue fractions of hormonally-treated suspensions. No alterations were apparent in the cold TCA and hot TCA extracts.

Table 25. Effects of progesterone on incorporation of acetate, glycerol, and glucose

	Glycerol			Acetate			Glucose		
Fraction	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.
Cold TCA	42270	37640	11.0±4.6	3480	3080	11.5±5.0	48060	46240	3.8±3.1
75% ETOH	110800	129620	17.0±7.3	2040	1880	7.9±5.2	31660	25840	18.4±3.9
Hot TCA	25616	28488	11.2±5.0	1080	1320	22.2±4.2	275760	275740	0.0±2.6
Trypsin	20650	21560	4.4±5.1	1840	760	58.7±9.7	49500	41260	16.7±5.8
Residue	18611	20670	11.1±2.9	1625	1545	4.9±3.6	66077	56249	14.9±3.1

Experimental conditions were identical to those listed in Table 18 except that bacteria were exposed to 0.01 mC ^{14}C-glycerol, ^{14}C-acetate, or ^{14}C-glucose. Activity is expressed as cpm/ml. The values for acetate and glycerol exposure represent average values for three experiments, and for glucose, the values are averages for four experiments. The relative % difference is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

The next group of experiments involved constituents of nucleic acids. ^{14}C-adenine or ^{14}C-uracil were incubated with S. aureus with and without progesterone at 40 mcg/ml (Table 26). Of the five cellular fractions, only two, the cold and hot TCA, contained detectable quantities of radioactivity, after 8 min exposures. For incorporation of adenine, the cold TCA extract of progesterone-treated cells consisted of 37948 cpm, while controls consisted of 45768 cpm, a 17.1% inhibition. In like manner, the entrance of the label into the hot TCA fraction was reduced 21.0% by the hormone. For incorporation of uracil, progesterone retarded entrance solely in the hot TCA fraction. Control cultures contained 93430 cpm and hormone-treated 79889 cpm, representing a 14.5% reduction.

Table 27 summarizes the incorporation data for all ten substrates. Alterations in entrance rates were generally widespread. Inhibition occurred more often than stimulation. With the experimental techniques herein employed, no observable pattern of either hormonal stimulation or hormonal inhibition of specific macromolecular synthesis emerged. Note, however, the steroidal effects on the entry of divers substrates into cellular pools, as reflected by the cold TCA extracts. Progesterone significantly affected eight of the ten substrates tested. Relatively consistent inhibition, 11% to 17%, was observed for seven of those eight compounds.

Table 26. Progesterone-induced effects on the incorporation of adenine and uracil into the cold and hot TCA fractions

	Adenine			Uracil		
Fraction	Control	Progest. 40mcg/ml	Relative % differ.	Control	Progest. 40mcg/ml	Relative % differ.
Cold TCA	45768	37948	17.1±7.7	170730	168190	1.5±2.8
Hot TCA	29718	23466	21.0±6.1	93430	79889	14.5±1.3

Experimental conditions were identical to those of Table 18 except that bacteria were exposed to 0.01 mC ^{14}C-adenine or ^{14}C-uracil. Only background amounts of radioactivity were detected in the 75% ETOH, trypsin, and residue fractions. Activity is expressed as cpm/ml. The values are mean values for three experiments. The relative % difference is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

Table 27. Summary of hormonal effects on cellular incorporation

Fraction	Stimulation	Inhibition	No effect
Cold TCA	LYS-86	acetate-12 ADE-17 ALA-16 GLU-13 glycerol-11 LEU-12 PHE-12	GLC URA
75% ETOH	glycerol-17	ALA-16 GLC-18 GLU-16 LEU-29 LYS-21	acetate PHE
Hot TCA	acetate-22 glycerol-11 LEU-19	ADE-21 ALA-46 LYS-11 URA-14	GLC GLU PHE
Trypsin	GLU-21	acetate-59 ALA-18 GLC-17 LYS-11	glycerol LEU PHE
Residue	GLU-16 glycerol-11 LEU-16	ALA-20 GLC-15 LYS-13	acetate PHE

The numbers after each substrate indicate the % inhibition or the % stimulation produced by progesterone. These values were obtained from Tables 18, 23, 24, 25, and 26.

D. Column and Paper Chromatography

In order to identify the radioactivity associated with the isolated cellular fractions after exposure to labeled substrates, conventional chromatographic techniques were employed. Staphylococci were added to ^{14}C-lysine or ^{14}C-alanine, incubated 15 min, centrifuged, washed, and fractionated. The cellular fractions were concentrated 300 fold by flash evaporation, hydrolyzed, passed through Dowex 50, and spotted on chromatographic paper. The chromatograms were developed in two solvent systems. Ninhydrin-positive spots were assayed for ^{14}C-content and identified by published R_f values and by co-chromatography with authentic standards (52).

Table 28 correlates the results obtained from five separate experiments in each of the two solvent systems. After exposure to ^{14}C-alanine and subsequent fractionation, the cold TCA fraction contained three distinctly labeled compounds. Of the total radioactivity applied to the chromatogram, 16% was recovered in the spot corresponding to ethanolamine, 12% corresponding to arginine, and 66% as unchanged alanine. Five labeled compounds were identified in the cold TCA fraction of cells exposed to ^{14}C-lysine: ethanolamine accounted for 15%; alanine, 10%; proline, 12%; arginine, 8%; and unchanged lysine, 50%.

Table 28. Chromatographic analysis of the cellular fractions after exposure to ^{14}C-alanine and ^{14}C-lysine

	15 min exposure to:			
Fraction	Alanine		Lysine	
Cold TCA	Alanine βalanine	66%	Lysine	50%
			Ethanolamine	15%
	Arginine	12%		
			Alanine	10%
	Ethanolamine	16%		
			Proline	12%
			Arginine	8%
75% ETOH	Omitted		Lysine	98%
Hot TCA	Alanine	98%	Omitted	
Trypsin	Reflects cold TCA		Reflects cold TCA	
Residue	Alanine	95%	Lysine	99%

The per cents indicate the quantity of ^{14}C-activity for each identified compound in relation to the total amount of radioactivity found in that fraction. Experimental procedures for growth, exposure to 0.01 mC ^{14}C-alanine or ^{14}C-lysine were identical to those listed in Table 21. Each fraction was concentrated 300-fold by flash evaporation, acid or alkaline hydrolyzed, passed through a Dowex 50 +H column, spotted on Whatman #1 chromatography paper, and developed in either phenol: water (4;1 v/v) or in butanol: acetic acid: water (12:3:5 v/v). Amino acids were located by application of ninhydrin spray with subsequent heating at 105 C for 3 min. Ninhydrin-positive spots were

identified by co-chromatography with authentic standards and by comparison of R_f values with known published R_f values. The spots were cut out and placed in scintillation fluor. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. The data represent the mean values for five experiments in each solvent system.

The trypsin solubilized protein fraction of suspensions added to the alanine and lysine reflected their respective cold TCA fractions. Slightly less radioactivity was detected, but again, the same compounds, with the exception of ethanolamine, were observed.

In the 75% ETOH fraction, after exposure to labeled lysine, 98% of the ^{14}C-activity applied to the paper was recovered as unchanged lysine. This probably represented the alkaline hydrolysis product of the cellular lipid lysylphosphatidylglycerol as reported by other investigators (18, 19, 28).

In the hot TCA fraction, after exposure to ^{14}C-alanine, 98% of the radioactivity was isolated as unaltered alanine. Other reports (2, 46) have identified an alanyl group esterified to the cell wall teichoic acids of S. aureus. This alanyl group, liberated by acid hydrolysis, could account for the radioactivity in the hot TCA fraction.

In the residue, 95% of the radioactivity was identified as unchanged alanine and 99% as unchanged lysine, after exposure to ^{14}C-alanine and ^{14}C-lysine. These amino acids are constituents of staphylococcal mucopeptides in the cell wall structure (46).

The results of the chromatographic analysis of the cellular

fractions suggested that metabolism of ^{14}C-substrates did occur within the 15 min exposure time, and dictate caution in the interpretation and application of the data.

The suggestion was made that the hormones were binding directly to the labeled substrates, making them unavailable for cellular uptake and incorporation, and, in effect, producing the reported alterations. To examine this possibility, ^{14}C-alanine and ^{14}C-lysine were incubated with and without progesterone at 40 mcg/ml in buffer for 15 min. Each of the solutions was then spotted on chromatography paper and developed separately in solvent A(n-butanol - acetic acid - HOH) and solvent B (phenol-HOH).

If the steroid binds the labeled amino acid, its R_f value would be altered, thereby reducing the quantity of radioactivity at the appropriate alanine or lysine position corresponding to the controls. Assessment of the ^{14}C-content of ninhydrin-positive spots revealed no significant binding by the hormone (Table 29). The quantities of radioactivity in spots corresponding to control and hormone containing solutions for both alanine and lysine were quite similar in each of the solvent systems.

An important sidelight corroborating the above investigation is that hormonal binding to ^{14}C-substrates, with

Table 29. Analysis of hormone binding capacity to ^{14}C-alanine and ^{14}C-lysine

Solvent system	Control	Progest. 40mcg/ml	Relative % differ.
n-butanol:acetic acid:water			
Alanine	2820	2780	1.4±2.2
Lysine	9120	9038	0.9±1.7
Phenol:water			
Alanine	2490	2570	3.1±2.0
Lysine	8880	8630	2.8±0.8

Labelled alanine or labelled lysine, 0.01 mC, was added to 0.1 M sodium phosphate buffer pH 7.0 with and without progesterone at 40mcg/ml. After incubation at 24C for 15 min, unlabelled alanine or unlabelled lysine at 10^{-3} M was added to the appropriate sample, and 80 microliters of each sample was spotted on Whatman #1 chromatography paper. The chromatograms were developed in the two indicated solvent systems separately. Alanine and lysine were identified by spraying with ninhydrin reagent and heating at 105C for 3 min. These spots were cut out and assayed for ^{14}C-quantity as described in Table 28. Activity is expressed as cpm/ml. The data represent average values for five experiments. The relative % difference is defined as

$$\frac{\text{control} - \text{hormone-treated}}{\text{control}} \times 100.$$

consequent unavailability for cellular uptake, could not explain the stimulatory alterations produced by the steroids.

E. Exchange Reactions Involving Labeled and Unlabeled Substrates

The ability of unlabeled alanine to exchange with radioactive alanine present within cellular pools, at a faster rate in the presence of progesterone at 40 mcg/ml is demonstrated in Table 30. After 4.0 and 4.5 min, there was an 11% and 12% hormonal inhibition in the uptake of labeled alanine. An exchange reaction was immediately apparent upon addition of a 300-fold excess of "cold" alanine at 4.5 min. Control cultures effluxed 5.3% of the total radioactivity within 90 seconds, while progesterone-treated cultures effluxed 16.9% of the total radioactivity. The total exit of labeled alanine between 4.5 and 10.0 min was similar for both groups of cells. The activity in the controls dropped from 9900 to 7142 cpm (27.9%) and in hormone-treated from 8702 to 6235 (28.4%). The percent inhibition of uptake produced by the steroid initially was 12%, increased to 23% after 6.0 min, and returned to 12% after 10.0 min. The result, then, was an immediate three-fold enhancement of efflux of the label from cells exposed to progesterone.

To investigate the possibility that the hormonally-induced increased efflux was an indirect effect of the smaller pool

Table 30. Exchange of ^{12}C and ^{14}C-alanine as influenced by progesterone

Time (min)	Control	Progest. 40mcg/ml	Relative % inhib.
4.0	9421	8427	10.6±3.2
4.5	9900	8702	12.0±4.2
Add ^{12}C-ala at 4.5			
6.0	9374	7231	22.9±1.9
8.0	7892	6475	18.0±0.8
10.0	7142	6235	12.7±1.5

S. aureus serotypes XII and XIII were grown to mid logarithmic phase in TSB, harvested, washed, and suspended in synthetic medium at 222mcg dry wt/ml. The cell suspension was added to 0.01 mC ^{14}C-alanine in the presence and absence of progesterone at 40mcg/ml and incubated at 24C. At the designated times, 0.5 ml of each suspension was filtered by membrane filtration (0.45 micron pore size, 13 mm diameter). The filters were washed with 5 volumes of cold 0.1 M sodium phosphate buffer pH 7.0, air-dried, and placed into scintillation fluid for radioactivity determination. After incubation for 4.5 min, 10^{-3}M ^{12}C-alanine was added to each suspension. Activity is expressed as cpm/ml. The data represent the mean values for four experiments. Relative % inhibition is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

size in progesterone-treated staphylococci, at the time of addition of unlabeled alanine, a similar series of experiments were performed. The steroid, however, was added to the suspension at the same time as the "cold" amino acid, after uptake of the ^{14}C-alanine had been initiated. As in the previous experiments, an immediate hormonal effect was manifested (Table 31). The activity within the control suspensions dropped from 9910 to 7335 cpm, a 26% efflux, as opposed to the hormone-containing suspensions, which dropped from 9900 to 6380 cpm, a 36% efflux. Due to the increased exit or exchange of radioactivity an inhibitory effect was apparent after 6.0 min. Controls contained 7335 cpm, whereas hormone-treated contained 6380 cpm, a 13% inhibition. More importantly, these experiments revealed that the phenomenon of progesterone enhancement of staphylococcal exchange was not an indirect result of smaller cellular pool sizes.

Further experiments were undertaken to determine whether this efflux effect could account for the previously reported alterations in the uptake and incorporation of nutrients. Exchange reactions were performed with ^{14}C-lysine or ^{14}C-glutamic acid with and without progesterone at 40 mcg/ml.

Repeated experiments with glutamic acid disclosed that an

Table 31. Effects of progesterone on the exchange mechanisms of ^{12}C and ^{14}C-alanine in cultures containing equal internal pools of labelled alanine

Time (min)	Control	Progest. 40mcg/ml	Relative % inhib.
4.0	9233	9285	----
4.5	9910	9900	----
Add ^{12}C-alanine and progest.			
6.0	7335	6380	13.0±1.5
8.0	6668	5730	14.1±1.6
10.0	6209	5482	11.7±1.5

Experimental procedures were identical to those listed in Table 30 except that progesterone was added at 4.5 min, at the same time as the ^{12}C-alanine. Activity is expressed as cpm/ml. The data represent mean values for three experiments. Relative % inhibition is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

internal-external exchange mechanism did not occur under the experimental conditions employed. There was no detectable decrease in cellular ^{14}C-content upon addition of excess unlabeled glutamic acid.

The initial experiments with lysine presented similar problems which were overcome by using a high concentration of "cold" lysine (1000-fold excess), by suspending the cells in buffer instead of synthetic medium, and by pre-incubation of staphylococci for 60 min at 37 C. The data in Table 32 indicate a different hormonal effect on the exchange mechanism. Upon addition of unlabeled lysine, the ^{14}C-content of control cultures decreased sharply from 3453 to 2320 cpm, a 33% efflux, whereas progesterone-treated cultures dropped only slightly from 4242 to 4214 cpm. In this case, the steroid retarded the efflux of radioactivity, which is in direct contrast to the enhancement of alanine efflux presented in the previous tables.

F. Effects of Various Cellular Inhibitors

In elucidating the hormonal mechanism of action on alterations of staphylococcal incorporation rates, attention was directed to the effects of various metabolic inhibitors. Synergistic or antagonistic interaction of hormones with inhibitors, would implicate involvement of specific processes of

Table 32. The effects of progesterone on exchange of ^{12}C and ^{14}C-lysine

Time (min)	Control	Progest. 40mcg/ml	Relative % differ.
4.0	3202	3797	18.5±1.9
4.9	3453	4242	22.8±0.1
Add ^{12}C-lysine at 4.9			
8.0	2320	4214	81.6±10.9
12.0	2260	4026	78.1±8.0

Experimental conditions were identical to those listed in Table 30 except that cells were suspended in 0.1 M sodium phosphate buffer pH 7.0; cells were exposed to 0.01 mC ^{14}C-lysine; and the labelled lysine was diluted with 10^{-2} M ^{12}C-lysine. Activity is expressed as cpm/ml. The data represent mean values for five experiments. Relative % difference is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

cellular function(s). No interaction was detected with streptomycin. A very slight antagonistic effect, no larger than 5%, was produced by chloramphenicol. This effect, however, was too insignificant to warrant further investigation. Meaningful results were obtained with the combination of penicillin and progesterone, which exhibited a synergistic action on inhibition of cell wall synthesis. This was expected, based on earlier findings that revealed a steroidal enhancement of penicillin inhibition of staphylococcal growth in nutrient broth (73) and a steroidal enhancement of penicillin-induced cellular leakage.

The mode of action of penicillin has been thoroughly documented by Strominger, Park, Wise, and Tipper (57, 58, 67). They offered convincing evidence that the target enzyme, through which penicillin reduces cell wall synthesis, is the transpeptidase which cross-links the mucopeptide polymers through a penta-glycine bridge. Part of the structure of the mucopeptide is composed of D & L-alanine, L-glutamic acid, and D-lysine.

The residue from the fractionation of staphylococci (46) contains this mucopeptide from the cell wall structure. The quantities of radioactivity incorporated into the residue after exposure to labeled alanine, lysine, or glutamic acid are indicative of the cellular metabolic activity. The reduction in incorporation of these amino acids into the mucopeptides,

induced by penicillin treatment, is directly related to the cellular inhibitory effects of this antibiotic. Thus, the tools are available for a detailed investigation of penicillin-hormone interaction.

Penicillin inhibition requires active cellular growth, so all experiments were performed with bacteria suspended in synthetic medium (25). S. aureus, strain Rose, a penicillinase-producing strain, was used.

The retardation of alanine entry into cell wall mucopeptides under two different conditions of cell metabolism is shown in Table 33. Staphylococci were suspended in synthetic medium in the presence and absence of penicillin, preincubated on a rotary shaker at 37 C for 10 min to 120 min, and then exposed to ^{14}C-alanine with and without progesterone at 40 mcg/ml for 8 min. By this procedure, bacteria were either in a static period of adjustment to the suspending medium or were actively growing. The comparison is made in Table 33. After 10 min preincubation, progesterone impeded the rate of entry of ^{14}C-activity into the residue fraction, only in the presence of penicillin at 330 units/ml (11.4% inhibition). The combination of progesterone and penicillin reduced entry 37.2%. The steroid did not significantly affect the staphylococcal suspension not containing the antibiotic or that containing 3 units/ml. After

Table 33. Incorporation of alanine into residue after 10 and 120 min pre-incubation

Treatment	Control	Progest. 40mcg/ml	Relative % inhib. by progest.	t test	Relative % inhib. by progest. & penicillin
Pre-incubate 10 min					
Without penicillin	1873	1721	8.1±4.7	0.678	----
3units/ml penicillin	2133	2003	6.1±4.2	1.245	Negative
300units/ml penicillin	1327	1176	11.4±5.9	2.392	37.2±10.5
Pre-incubate 120 min					
Without penicillin	2650	2363	11.1±4.7	2.576	----
3units/ml penicillin	2279	1946	15.1±2.2	2.777	26.6±7.2
300units/ml penicillin	1755	1373	22.2±3.9	2.073	48.2±4.0

S. aureus strain Rose was grown to mid logarithmic phase in TSB, harvested, washed, and suspended in synthetic medium at 222mcg dry wt/ml. The suspensions were pre-incubated with and without penicillin on a rotary shaker at 37C for 10 and 120 min. Cells were then exposed to 0.01 mC ^{14}C-alanine in the presence and absence of progesterone for 8 min. The bacteria were immediately centrifuged and fractionated as indicated in Table 18. Radioactivity of the residue fraction was assayed. Activity is expressed as cpm/ml. The data represent the average values for three experiments. Relative % inhibition by progesterone is defined as $\frac{\text{control--hormone-treated}}{\text{control}} \times 100$. Relative % inhibition by progesterone and penicillin is defined as $\frac{\text{control without penicillin--hormone-treated with penicillin}}{\text{control without penicillin}} \times 100$.

120 min pre-incubation, however, the hormone significantly reduced the rate of alanine entry into the cell wall mucopeptides for cells not exposed to penicillin (10.8% inhibition), for cells exposed to 3 units/ml (14.6% inhibition), and for cells exposed to 330 units/ml (21.8% inhibition). The combination of penicillin and progesterone inhibited entry 26.6% and 48.2%, respectively.

The activity of the antibiotic in blocking cell wall synthesis is evidenced by the quantities of radioactivity in the control cells. No significant reduction in radioactivity was apparent at the lower penicillin concentration after 10 min of pre-incubation. Control fractions without the antibiotic contained 1873 cpm and controls with penicillin at 3 units/ml contained 2133 cpm. In contrast, control cells in the presence of 330 units/ml contained 1327 cpm, representing a reduction of 29%. Note that this was the only fraction of the 10 min pre-incubation group that exhibited hormonal reduction in ^{14}C-activity into the residue. After 120 min, without the antibiotic, controls contained 2650 cpm; at 3 units/ml, controls contained 2279 cpm, a 14% reduction; and at 300 units/ml, controls contained 1755 cpm, a 34% reduction. Thus, pronounced synergistic effects required conditions of actively growing bacteria and conditions in which penicillin was actively

inhibiting cell wall mucopeptide synthesis.

In the remaining experiments cells were pre-incubated with and without penicillin for 60 min.

It was of interest to determine hormonal effects at or below the critical progesterone concentration of 15 mcg/ml. Staphylococci were exposed to ^{14}C-alanine in the presence of progesterone at 0, 5, and 15 mcg/ml. 1650 units/ml of penicillin were used. Fractionation (Table 34) revealed that progesterone alone at 5 or 15 mcg/ml did not inhibit entrance of radioactivity into the cell residue. In the presence of penicillin and the hormone at 5 mcg/ml, bacterial suspensions contained 10.1% less ^{14}C-activity than the corresponding controls. At 15 mcg/ml, bacterial suspensions contained 19.3% less activity than the corresponding controls. Table 34 also lists the percent inhibition by the antibiotic. The controls without penicillin contained 10970 cpm and with penicillin 6340 cpm. This corresponds to a 42.3% reduction. Similarly, with progesterone at 5 and 15 mcg/ml, penicillin reduced entry of the label 52.8% and 60.3%, respectively. The combination of progesterone at 5 mcg/ml and penicillin inhibited entry 48.1%; when the progesterone concentration was increased to 15 mcg/ml, entry was inhibited 53.3%.

The previous table indicated that progesterone at or

Table 34. Incorporation of alanine into residue at various hormone concentrations

Treatment	Hormone concen. 0 mcg/ml	5	Relative % inhibition by hormone	t test	Relative % inhibition by hormone & penicillin	t test
Without penicillin	10970	12070	Negative	----	----	----
1650units/ml penicillin	6340	5700	10.1±1.5	1.000	48.1±5.5	6.478
% inhib. by penicillin	42.3	52.8				

	0	15				
Without penicillin	10970	12890	Negative	----	----	----
1650units/ml penicillin	6340	5120	19.3±4.8	2.302	53.3±7.9	9.750
% inhib. by penicillin	42.3	60.3				

Experimental conditions were identical to those listed in Table 33 except that cells were pre-incubated with and without penicillin for 60 min. Activity is expressed as cpm/ml. The data are

mean values for three experiments. A t test value larger than 2.000 indicates a value that is significant at a 5% level or better. Relative % inhibition by hormone is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}}$ X 100. Relative % inhibition by hormone and penicillin is defined as $\frac{\text{control without penicillin}-\text{hormone-treated with penicillin}}{\text{control without penicillin}}$ X 100. The % inhibition by penicillin is defined as $\frac{\text{with penicillin}-\text{without penicillin}}{\text{with penicillin}}$ X 100.

below 15 mcg/ml did not retard entry of ^{14}C-alanine into the residue fraction. In order to establish distinct synergistic action, the critical concentration of penicillin should also be determined. To this end, staphylococci were exposed to ^{14}C-alanine and various concentrations of penicillin for 8 min and subsequently fractionated (Table 35). The critical concentration of the antibiotic was between 1.65 and 3.30 units/ml. Above this level, penicillin increasingly retarded entrance of ^{14}C-activity into the cell residue and below this level, no inhibitory effects were detected.

Based on these facts, Table 36 presents the data for incorporation of labeled alanine into cell wall mucopeptides with progesterone at 40 and 15 mcg/ml and penicillin at 0.82 and 1.62 units/ml. Without the antibiotic, the steroid at 40 mcg/ml, significantly reduced entry into the residue 13.6%. At 15 mcg/ml, hormonally-treated suspensions contained quantities of radioactivity similar to the corresponding controls. With penicillin at 0.82 units/ml, progesterone at 40 and 15 mcg/ml significantly impeded entry of the label 20.2% and 15.6%, respectively. With penicillin at 1.62 units/ml, the inhibition increased to 23.9% and 18.5%. Most importantly, the hormone at the sub-inhibitory concentration of 15 mcg/ml and penicillin at the sub-inhibitory concentrations of 0.82 and 1.62 units/ml

Table 35. Effect of penicillin concentration on the incorporation of alanine into residue

Penicillin concen. units/ml	Activity	Relative % inhibition
0.00	4255	----
0.82	4868	Negative
1.65	4443	Negative
3.30	3887	8.7±0.4
4.95	3373	20.7±2.1
6.60	2988	29.8±4.2

Experimental conditions were identical to those in Table 33 except that no progesterone was used. The activity is expressed as cpm/ml. The data represent average values for three experiments. The relative % inhibition is defined as

$$\frac{\text{with penicillin} - \text{without penicillin}}{\text{without penicillin}} \times 100.$$

Table 36. Effect of inhibitory and sub-inhibitory concentrations of progesterone and sub-inhibitory concentrations of penicillin on incorporation of alanine into the residue fraction

Treatment	Hormone concen. mcg/ml 0	40	Relative % inhib. by hormone	t test
Without penicillin	2556	2209	13.6±1.3	2.283
0.82units/ml penicillin	3245	2569	20.2±3.9	2.224
1.65units/ml penicillin	3107	2366	23.9±6.0	2.259
	0	15		
Without penicillin	2556	2553	0.1±2.6	0.012
0.82units/ml penicillin	3245	2739	15.6±5.4	2.279
1.65units/ml penicillin	3107	2532	18.5±3.8	2.447

Experimental conditions were identical to those of Table 33 except that the suspensions were pre-incubated for 60 min. Activity is expressed as cpm/ml. These data are mean values for four experiments. A t test value larger than 2.000 represents avalue that is significant at a 5% level or better. Relative % inhibition by hormone is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

acted synergistically to reduce entrance of ^{14}C-alanine into cell wall mucopeptides.

Similar experiments were performed with penicillin at 1.62 units/ml and decreasing concentrations of progesterone. Cells were exposed to the ^{14}C-alanine and to the hormone at 15, 10, 5, 1, and 0 mcg/ml (Table 37). No significant inhibition was detected at 1 mcg/ml; but at 5, 10, and 15 mcg/ml, progesterone significantly retarded entrance of the label 11.8%, 14.0%, and 18.1%. Note that hormone concentrations as low as 5 mcg/ml produced inhibition in the presence of sub-inhibitory amounts of penicillin.

The data to this point reflect the inhibitory effects of progesterone on the incorporation of alanine into cell wall mucopeptides. Table 38 presents the effects of other hormones at sub-inhibitory concentrations in the presence of penicillin at 1.62 units/ml. Diethylstilbestrol at 2 mcg/ml produced maximum inhibition. Control fractions contained 12140 cpm and hormone-treated 7010 cpm, corresponding to a 42.3% reduction. The combination of norethindrone at 13 mcg/ml and mestranol at 2 mcg/ml exerted 26.9% inhibition; testosterone at 20 mcg/ml, an 18.1% inhibition; and progesterone at 15 mcg/ml, a 17.7% inhibition. This again correlates with previous work in which diethylstilbestrol affected maximal

Table 37. Effects of various progesterone concentrations on alanine incorporation into residue in the presence of sub-inhibitory concentrations of penicillin

Hormone concen. mcg/ml	Activity	Relative % inhibition	t test
0	12760	----	----
1	12260	3.9±0.2	0.735
5	11254	11.8±0.2	2.124
10	10980	14.0±2.6	2.163
15	10450	18.1±2.0	2.924

Experimental procedures were identical to those of Table 33 except that the suspensions were pre-incubated for 60 min. Activity is expressed as cpm/ml. These data are mean values for three experiments. A t test value larger than 2.000 indicates a value that is significant at a 5% level or better. Relative % inhibition is defined as $\frac{\text{control—hormone-treated}}{\text{control}} \times 100$.

Table 38. Effect of various hormones at sub-inhibitory concentrations on incorporation of alanine into residue

Treatment	Activity	Relative % inhibition
Control	12140	----
Progest. 15mcg/ml	9990	17.7±3.3
Testost. 20mcg/ml	9940	18.1±4.3
Diethylstil. 2mcg/ml	7010	42.3±6.0
Norethin. 13mcg/ml and Mestranol 2mcg/ml	8870	26.9±2.8

Experimental conditions were identical to those listed in Table 33 except that cells were pre-incubated for 60 min. Activity is expressed as cpm/ml. The values are averages for three experiments. Relative % inhibition is defined as $\frac{\text{control}-\text{hormone-treated}}{\text{control}} \times 100$.

retardation of growth in broth (70), and maximal reduction in incorporation of nutrients (17).

The cell wall mucopeptides of S. aureus also contain lysine and glutamic acid (46). The next group of experiments assessed progesterone-penicillin interaction in the incorporation of these two amino acids into the residue. A slightly lower critical concentration of penicillin, 0.82 units/ml, was observed for the incorporation of lysine. When bacteria were exposed to the labeled lysine and progesterone at 15 mcg/ml, stimulatory effects were detected (Table 39). Controls contained 51503 cpm, and hormone-treated contained 118533 cpm, representing a 130.1% stimulation. In the presence of penicillin, less stimulation was apparent (94.3% stimulation). Another way of stating this, is that, in the presence of progesterone, penicillin inhibited residue incorporation 12.9%.

The results for hormone-antibiotic interaction in the incorporation of glutamic acid were not as clearly defined. No synergistic effects were noted under the experimental conditions employed.

The synergistic action of hormones with penicillin provided speculation that the steroids act somewhere at the level of cell wall mucopeptides. This led to a re-examination of previous work

Table 39. Effect of progesterone and penicillin on incorporation of lysine into residue

Treatment	Hormone concen. mcg/ml 0	15	Relative % difference
Without penicillin	51503	118533	130.1±17.8
0.82units/ml penicillin	53167	103283	94.3±15.4
% inhib. by penicillin	Negative		12.9±1.5

Experimental conditions were identical to those of Table 33 except that the cells were pre-incubated for 60 min and then exposed to 0.01 mC ^{14}C-lysine. Activity is expressed as cpm/ml. These data represent the mean values for three experiments. Relative % difference is defined as $\frac{\text{control--hormone-treated}}{\text{control}} \times 100$. The % inhibition by penicillin is defined as $\frac{\text{without penicillin--with penicillin}}{\text{with penicillin}} \times 100$.

(15, 76), which had shown approximately 2% or 3% of ^{14}C-testosterone or ^{14}C-progesterone bound to whole cells of S. aureus. The residual 98% or 97% remained free in the extracellular fluid. A thick suspension of staphylococci was exposed to ^{14}C-progesterone for 5 min in synthetic medium. The cells were then fractionated, according to Park and Hancock (46). The residue was washed twice, re-suspended in buffer and assayed for ^{14}C-content. (Millipore filtration was not used because of adsorption of the label to the filters). In the same experiment, whole cells were extracted with cold TCA, then exposed to ^{14}C-progesterone, and fractionated. In similar fashion, after each extraction procedure, the insoluble matter was exposed to the labeled hormone and fractionation was continued to the residue. The data in Table 40 show that 7.3% of the total progesterone added remained bound to the mucopeptides if the label was added directly to whole cells. If the hormone was added after cold TCA extraction, 7.8% remained attached to the residue. Similarly, 11.7% was bound after hot TCA extraction and 10.6% after trypsinization. The largest increase in binding ability was observed when ^{14}C-progesterone was added directly to the mucopeptides. 23.0% was attached to the preparation. If a much denser suspension of staphylococci was utilized, as much as 55% of the added label was firmly bound to the residue fraction.

Table 40. Binding of ^{14}C-progesterone to bacterial mucopeptides after various treatments

Added ^{14}C-progest. to treated cell suspensions	Activity	% ^{14}C-activity bound
Whole cells	8040	7.3±0.1
Cold TCA	8610	7.8±0.1
Hot TCA	12930	11.7±0.2
Trypsin	11670	10.6±0.5
Residue	25260	23.0±0.7

S. aureus strain Rose was grown to mid logarithmic phase in TSB, harvested, washed, and suspended in synthetic medium at 1350mcg dry wt/ml. One-fifth of the cell suspension was exposed to 0.22 mC (110,000 cpm) of ^{14}C-progesterone. This portion, plus the other four portions were extracted with cold TCA. Again, one-fifth of the suspension was exposed to 110,000 cpm of the labelled hormone. All five portions were extracted with hot TCA. Again, a portion of the original suspension was exposed to the labelled hormone. All five portions were trypsinized. A portion of the original suspension was added to ^{14}C-progesterone. The remaining residue in the last portion, was exposed to the labelled hormone. All five portions were washed twice with two volumes of 0.1 M sodium phosphate buffer pH 7.0. The residue fractions of the five portions were assayed for radioactivity by re-suspension in buffer and placing 0.1ml into scintillation fluid. Activity is expressed as cpm/ml. The data are mean values of two experiments. The % ^{14}C-activity bound is defined as $\frac{\text{observed activity}}{\text{total activity added}} \times 100$.

IV. DISCUSSION

It has been previously demonstrated (15, 16, 17, 69, 70, 71, 72, 73, 74, 75, 76, and 77) that steroids effectively diminish the staphylococcal processes of growth in nutrient broth, in the skin of rabbits, and in the spleens and kidneys of mice. In attempting to explain these findings at the molecular level, consideration has been given to hormonal intervention in cellular permeability.

The growth of a microorganism is the result of the synthesis of all of its cellular components. The entry of most of the organic nutrilites required for growth is mediated by specific and nonspecific permeation systems (31), usually involving active transport. This active transport can be defined as the transfer of a specific nutrient, with expenditure of energy, from a given external concentration to a higher level within the cell. Numerous models for active transport have been proposed, one of which follows (14). When energy is applied to bring about active transport, the specific carrier protein, T, is converted on the inner side of the membrane to a relatively inactivated form T_i, with a much lower affinity for the substrate, S. Hence, TS by conversion to T_iS can unload S within the cell, even when the internal concentration is high; and T_i will return unloaded until that concentration becomes high enough to satisfy

the high Michaelis constant of T_i (or to block its formation from TS). During this cycle, T_i, in a spontaneous exergonic reaction, becomes I on the outer aspect of the membrane, and can again pick up external S at a low concentration.

Numerous reports have been published on microbial transport systems, for example, the amino acid permeases of E. coli (64) and Ps. aeruginosa (33), the tryptophan permease of N. crassa (65), the carbohydrate permease of Cl. perfringens (23), and the histindine and aromatic amino acid permeases of S. typhimurium (1). Thorough investigations have also been made on the carbohydrate transport mechanisms of S. aureus (14, 26; 27, and 51). A phosphoenolypyruvate transferase system has been implicated that is very similar to that described in E. coli (35). It consists of enzyme I and enzyme II, a heat stable protein, and a factor III. Hengstenberg et al. (26) postulated a model in which the carbohydrate molecule diffuses through the membrane and is acted upon by the phosphotransferase system at the inner surface of the membrane. The phosphorylated derivative then passes into the cell cytoplasm.

Thus, permeability, as the regulator of the entry of cellular substances, is a most important function. By controlling the rates of entry of specific substrates, transport systems control not only the activities of intracellular enzymes, but also their induced synthesis (9). Any damage, or alteration of

cell integrity, may produce consequent alterations in permeability.

Previous results (15, 70, and 75) indicated an apparent extracellular action of the gonadal hormones on staphylococci. With this in mind, and based upon the reported observations in this paper, the following hypothesis is presented. Binding of the hormones may occur on the cell wall mucopeptide, and at the same time, the close proximity of the cell membrane to the mucopeptide allows for binding and/or interaction of the steroids with the cell membrane regions. Willmer (66) proposed models in which steroid hormones became incorporated into phospholipid monolayers with hydrophilic groups extending outward. In our system, the hydrophobic hydrocarbon backbone of the steroid could bind to the nonpolar membrane with the hydrophilic groups attached to cell wall mucopeptides. Varricchio et al. (61), in their study of the structural features of azasteroids required for antibacterial activity, concluded that the steroids must have both a hydrophobic group and hydrophilic group in order to inhibit growth of the organisms.

Thus, a means has been provided whereby steroids might influence structures at a phase boundary (cell membrane) with consequent alterations in the flux of metabolites across the phase boundary. These alterations, due to the binding of steroids,

could conceivably produce the following two events, affecting bacterial permeability: 1) the prevention of access of some other molecule to a region near, or at the point of attachment of the steroid to the receptor; and 2) the production of a conformational change and, hence, a change in function of the receptor molecule itself. The local disturbance produced by the change in conformation could directly, or indirectly, influence permeability by affecting spatially separate transport systems contained within the membrane proper.

Since the transfer of a substrate into a cell is an obligatory prelude to subsequent metabolism, steroids, via regulation of substrate entry, should exert many secondary consequences on diverse aspects of cell metabolism. Many enzymes located in the membrane profoundly affect the integrated functioning of the cell. Any disturbance or alteration in the activities of these enzymes could also produce numerous secondary consequences.

In support of this hypothesis, the present study provided four lines of evidence for hormonal effects on cell permeability. 1) Bacteria exposed to the steroids showed increased rates of cellular leakage of ^{14}C-labeled materials and protein, 10% to 60% higher than the corresponding controls. This suggested action at the level of the cell membrane. 2) The uptake of

specific nutrients, such as alanine, lysine, or glutamic acid, was altered 10% to 90% by prior treatment with various hormones. Fractionation of the bacteria after exposure to ^{14}C-alanine indicated an initial hormonal intervention in the entrance of the label into the cellular pool. This immediate effect was followed subsequently by effects on macromolecular syntheses. 3) The exchange of labeled alanine or lysine with unlabeled alanine or lysine was altered by progesterone. For alanine, ^{14}C-activity effluxed three times as fast in the presence of the hormone. The opposite results were obtained for lysine. The control cultures exchanged 30% of the internal ^{14}C-activity, while the activity within the hormone-treated cultures remained constant. Again, this implicated steroidal effects on specific permeability mechanisms. 4) Lastly, synergistic activity was detected when penicillin was used in combination with steroids to reduce the entry of ^{14}C-alanine into cell wall mucopeptides. This implied a similar site of action of hormones at the cell wall, cell membrane level.

The data concerning hormonal inducement of cellular leakage provided evidence that the antibacterial action of steroids entails alterations in permeability processes. The overall effectiveness of the hormones may well be the result of several parameters which cause detrimental effects to staphylococci. The inhibition of growth, then, may not be due entirely to leakage

of cellular components; the fact remains, that involvement of the cell membrane was demonstrated.

The leakage of substances from staphylococci exposed to steroids was clearly shown by assessing rates of loss of internal radioactivity and cellular protein. Prolonged exposure for 8 to 12 hr was required before hormone-treated suspensions showed increased rates of leakage over and above the leakage in the control suspensions (Tables 1, 2 and 4).

There were a number of reasons to support the view that leakage effects were in some way related to the hormonal inhibition of staphylococcal growth. Cellular leakage was enhanced at the identical critical concentration, 20 mcg/ml (75, 76), of progesterone and testosterone that was required for growth inhibition (Table 2). In comparing testosterone and progesterone, the latter steroid was slightly more active in inducing leakage, and slightly more active in retarding growth (75, 77). Progesterone, in conjunction with penicillin, produced increased rates of growth reduction (73) and increased rates of cellular leakage (Table 5).

No effects were discernible in the leakage of useful nutrients from gram negative organisms (Table 3), which were also not susceptible to growth inhibition (75, 77). Explanations of insensitivity of gram negative bacteria to hormonally-induced

leakage may involve the chemical differences in the cell envelopes with resulting inability of the organisms to bind the hormones. The cell envelopes of gram negative bacteria are chemically and physically more complex than those of gram positive species, containing large amounts of lipoproteins and lipopolysaccarides, in addition to the rigid mucopeptides. Varricchio et al. (61) suggested that the resistance of gram negative organisms to azasteroids involved the cell walls, which are 20% lipid, as contrasted to the cell walls of gram positive organisms, which contain only 2% lipid.

The results of the uptake experiments revealed specific hormonal inhibition in the permeation of glutamic acid (Table 7) and alanine (Table 9), and stimulation in the permeation of lysine and glucose (Table 8). The dual effect of stimulation and inhibition of uptake of the different substrates was puzzling. It is possible that the binding of the hormone to the specific cell receptors could inhibit permeability by directly hindering some aspect of the adsorption-transport reaction of alanine and glutamic acid. At the same time, the conformational change due to the hormone-receptor interaction, might produce stimulation of uptake in an indirect manner, by affecting spatially separate, but proximate, transport systems of glucose and lysine.

In defining optimal parameters for the reduction of alanine

uptake, a few notable relationships to *in vitro* growth reduction were evident. Progesterone-induced inhibition of growth was detected up to a cell concentration of approximately 600 mcg/ml dry wt (77). Likewise, alanine uptake was significantly affected in the same range of cell densities (Figure 1). Amino acid uptake by gram negative microorganisms was unaffected by the hormones (Table 14). A variety of hormones displayed various degrees of growth inhibition (75, 76, and 77), and similar degrees in reduction of alanine uptake (Table 15). Progesterone significantly impeded the uptake of alanine only at, or above, the critical concentration of 15 mcg/ml required to inhibit growth (Fig. 3).

Anaerobiosis, besides enhancing the antimicrobial activity of steroids on growing cultures of *S. aureus* (75), also enhanced the reduction of alanine uptake (Table 12). It has been reported (18, 30) that shifts from aerobic to anaerobic conditions bring about substantial alterations in membranes of staphylococci, such as in the levels of phosphatidyl glycerol, cardiolipin, glucolipid, vitamine K_2, and protohemes. These changes in membrane structure may increase the binding affinities of hormones, producing increased inhibition of growth, or increased permeability effects.

That hormonal alteration of staphylococcal uptake does not

depend solely upon the rates of internal cellular biosynthesis, as indicated by the following pertinent observations. Firstly, the overall metabolic activity of cells suspended in buffer for long periods of time, such as 4 hr, should be greatly reduced. As noted in Table 9, however, the percent inhibition of alanine uptake after 240 min, 15%, remained comparable to the percent inhibition during the initial stages of exposure, 15% to 20%. Secondly, progesterone intervention in the uptake of alanine was relatively consistent for both the metabolically active logarithmic, and the less active stationary phase cultures (Table 11). Thirdly, cellular metabolism is highly dependent on temperature, being least active at temperature extremes (4C or 55C), and most active at intermediate temperatures. The hormonal inhibition of the uptake of alanine, however, was relatively constant in the range of temperatures tested (Table 13). Fourthly, anaerobiosis severly limits energy production with consequent slowdown in cell metabolism. In direct contrast, hormonal inhibition of amino acid uptake was enhanced two-fold under anaerobic conditions (Table 12).

The pH dependence of hormonal inhibition (Fig. 2) may reflect steroidal interaction with the enzyme-like permease systems. An explanation for the increase in inhibition as pH was decreased, is the ionization of carboxyl or acidic groups

making available greater numbers of charged groups within the cell surface. This, in turn, may result in greater attraction and binding of hormones. Furthermore, organic acids are more permeable as unionized acids than as ionized anions.

Fractionation of bacteria after exposure to diverse ^{14}C-substrates clearly indicated steroidal intervention in the entry of labeled nutrients into cellular pools, lipids, lipid-soluble proteins, nucleic acids, teichoic acids, proteins, and cell wall mucopeptides (Tables 18 to 26). Specific relationships with previous data were recognized. The incorporation of alanine into the cellular fractions was impeded by progesterone (15 mcg/ml), and epiandrosterone (20 mcg/ml) only at, or above, the critical concentrations required to retard growth (Table 18, and 22). Entry rates into the cell fractions of gram negative organisms, on the other hand, were not significantly altered by the steroids (Table 19). Progesterone-induced reductions of incorporation of amino acids were similar for cultures in the logarithmic and stationary phases (Table 20). No significant changes in the percent inhibition of ^{14}C-entry into the five fractions were detected in the less metabolically active stationary cells. Epiandrosterone exerted a pronounced growth supressive action (75), and also exerted maximal inhibition of incorporation of alanine, relative to the actions of stanolone

and 17 α -hydroxyprogesterone. The latter steroid did not affect staphylococcal growth (77), and also did not influence the incorporation of amino acids into the bacteria. Stanolone exerted slight growth inhibitory effects (75), and slight inhibitory effects on incorporation rates (Table 22).

Substrates taken into cells and incorporated into cellular components must pass through cellular pools before entering the various metabolic pathways (4, 5, 78). In this way, the pool sizes may directly influence the synthesis of macromolecules. The experiment involving the time course of ^{14}C-uptake into cells following short term exposure to ^{14}C-alanine indicated that the initial inhibitory effects of progesterone, occurring within 10 seconds, a 14% inhibition, were exerted on the entry of the label into the cellular pools of staphylococci. At this time, the quantities of radioactivity in control and hormonally-treated fractions of the hot TCA, trypsin, and residue were identical. The steroid inhibited entry of ^{14}C-activity into the latter three fractions only after a minimum of 60 sec exposure (Table 21). These results suggested an initial, immediate hormonal influence on the transport of alanine into the cellular pool.

For incorporation of other substrates, no obvious relationship of steroid action to macromolecular syntheses emerged.

With some substrates, entry into the cell fractions was inhibited, while with others, either stimulatory or a combination of inhibitory and stimulatory effects were detected. Of the ten substrates tested, eight showed significant alterations in pool sizes (Table 27). The only two substances not effected were glucose and uracil, both of which are extremely active metabolically. As a possible explanation, the activity of the transport mechanisms for these two compounds may be so great, that any inhibition of permeability function may have been overridden by the high entrance rates of glucose and uracil.

Analysis of the incorporation of phenylalanine (Table 24) and glycerol (Table 25) revealed hormonal inhibition solely in the entry of ^{14}C-activity into the cold TCA fraction, 12% and 11%, respectively. Longer incubation periods may have resulted in steroidal alterations in ^{14}C-quantity in the other four fractions.

The chromatographic assessment and identification of the radioactivity associated with the five cellular fractions after exposure to alanine and lysine (Table 28) dictates extreme caution in interpretation of the data. A thorough quantitative and qualitative investigation is necessary to distinguish between ^{14}C-activity and the original ^{14}C-substrate. Bacterial suspensions, in an active metabolic state, will catabolize

substrates at a rapid rate with resultant entry of the ^{14}carbon into various biosynthetic pathways. For example, after addition of labeled phenylalanine, or leucine to staphylococci, the small, but significant, amount of radioactivity detected in the residue fraction, probably represented breakdown and re-utilization of the original ^{14}C-labels. The recent work of Gupta and Framer (24) itemized the metabolic fate of absorbed valine in a filamentous fungus. Catabolism of the amino acid was observed as early as 4 min post-addition of the label. After 15 min incubation, the label from the valine appeared no less than 12 other compounds.

Chromatographic analysis of the radioactivity within the cold TCA and protein extracts of S. aureus after 15 min exposures to ^{14}C-alanine or ^{14}C-lysine indicated that 40% to 50% of the activity represented compounds other than the original alanine or lysine (Table 28). The radioactivity within the other fractions was unaltered. This provided a useful biological tool for further experimentation. In the study of the synergism between progesterone and penicillin, it was assumed that after exposure to alanine, or lysine, and subsequent fractionation, the ^{14}C-activity in the residue reflected 95% to 99% of the unchanged original substrates. Similar assumptions can be made for incorporation into cell wall teichoic acids (hot TCA) and cellular lipids

(75% EtOH). These results for alanine and lysine correlate well with other reports concerning the identification of amino acids incorporated into various cellular fractions (2, 18, 19, 22, 24, 28, 46).

Not too many conclusions can be drawn from the exchange reactions, in which labeled alanine or lysine exchanged with unlabeled alanine or lysine. Progesterone produced an immediate 3-fold increase in the efflux of alanine, relative to the control (Table 30). In contrast, the hormone reduced the rate of efflux of lysine. It should be recalled, that the entry of alanine into the cold TCA fraction was inhibited (Table 18), while the entry of lysine was stimulated (Table 23). This hormonal influence on exchange reaction was not observed for glutamic acid. Thus, a generalized cellular exchange mechanism, due to steroid exposure, does not appear to be responsible for differences in the quantities of substrates taken into cells. However, it is not necessary for the hormones to influence all components in order to effect cell growth. In any case, these data further implicate a hormone-permeability interaction.

The effects of chloramphenicol provided interesting results for speculation. In buffer, the antibiotic interacted with the hormone to enhance inhibition of substrate uptake, while in synthetic medium this effect did not occur (Table 17). Bacteria suspended in synthetic medium were actively growing, and in this

state should be more susceptible to the inhibition of protein synthesis by chloramphenicol. Resting cells in buffer should be less susceptible because of lowered rates of protein synthesis. Thus, the synergistic effects of chloramphenicol and hormones upon substrate uptake were possibly due to an interaction of the inhibitor with cellular processes other than protein synthesis. In fact, it has been reported that chloramphenicol at 200 mcg/ml, the concentration used in these experiments, influences permeability processes (25). If this conclusion is correct, and if hormones also influence permeability, synergistic effects would be expected.

Synergistic action was observed when penicillin was used in combination with specific steroids. The antibiotic enhanced hormonally-induced leakage of internal constituents from staphylococcal cells (Table 5). In addition, in the presence of penicillin, sub-inhibitory concentrations of progesterone, testosterone, diethylstilbestrol, and norethindrone plus mestranol, reduced the rate of entry of alanine and lysine into cell wall mucopeptides (Table 38 and 39). The synergistic action was entirely feasible in light of the fact that penicillin acts at the cell wall level (12).

The above findings for penicillin were consistent with the attachment of labeled progesterone to the cell wall mucopeptides.

The detection of radioactivity within the residue specifically implicated attachment thereon. When ^{14}C-progesterone was added either to whole cells or to preparations of mucopeptides from the identical quantity of whole cells, the latter preparations contained three times as much radioactivity as the whole cells. It is possible that cell fractionation removed cellular components, and thus, exposed additional areas for hormone attachment.

Based on the hypothetical model presented and the reported observations, some pertinent questions can be answered. What was the target or receptor in a bacterial cell that initially interacted with the hormone? According to the model presented, the receptor was at the cell wall-cell membrane interface and may be a combination of polar mucopeptides of the cell wall and non-polar lipids of the membrane.

Was the affect on permeability the primary mechanism of action at the cellular level? Although this is a most difficult question to answer with assurance, the results pointed to permeability as the primary target for steroidal mechanism of action. The summation of hormonal effects on incorporation of the ten substrates indicated a relatively uniform affect only upon cellular pools of staphylococci. The short term incorporation experiment showed that the initial intervention by the hormones was in

the cold TCA fractions. The lack of dependence on overall cell metabolism apparently excluded primary affects on incorporation into macromolecules. The results of glycerol and phenylalanine incorporation showed steroidal intervention of entry into the cold TCA fraction, without concomitant alterations of incorporation rates into the other four fractions. The progesterone-induced alterations of cellular incorporation were similar for cultures in the logarithmic and stationary phases, suggesting hormonal intervention in the activity of a cellular function (s) equally shared by both groups of cells. Such a function could be permeability as implicated by the experimental data.

What were the secondary effects produced by the hormone receptor combination? Transport processes determine the nature and quantity of the solutes that enter the cell. Peters (47) proposed the cell membrane as the coordinating factor in cellular physiology. If a hormone alters a cell membrane, it would be expected to modify several enzyme reactions, producing many varied, rather than simple, changes. In animal cells, Wool (68) hypothesized that insulin acts on fat and protein metabolism as secondary, or derived phenomena; that is, derived from the action of the hormone in making glucose available to intracellular enzymatic processes. The glucose, in turn, provides, energy for the synthesis of fat or protein. For the reported incorporation

studies in the present dissertation, progesterone intervention was demonstrated for most of the substrates utilized. The incorporation of these substrates reflected a wide range of bacterial metabolic syntheses - proteins, cell wall structures, lipids, sugars, amino acids, and nucleic acids. That the hormone would directly influence all of the enzymatic machinery herein involved, is most unlikely. Probably, the steroidal effects on cellular incorporation represented secondary reactions subservient to primary reactions at permeability levels.

Do all the sex hormones regulate permeability in staphylococci in similar fashion? Most of the accumulated data in this and previous studies were the result of analysis of progesterone action. Other steroids, including androgens, progestens, synthetic progestens, estrogens, and synthetic estrogens, appeared to affect the various staphylococcal processes in related ways (15, 16, 17, 69, 70, 71, 72, 73, 74, 75, 76, and 77).

Why were some derivatives more active than others? The derivatives of hormones utilized in these studies were closely related, differing only in the nature and/or sterochemistry of such groups as hydrogen atoms, hydroxyl groups, methyl groups, or keto groups. Such chemical differences could result in changes in binding capacities of the hormone to the receptor(s). This

could produce increased, or decreased effects relative to the parent compound.

Why were certain strains of S. aureus more susceptible to hormone action than other strains? Strain differentiation often reflects subtle changes in bacterial cells. Redai and Rethy (48) reported that various strains of S. aureus differed in the fatty acid composition of their membranous structures with resultant changes in antibiotic sensitivity. Serotypes 1X to X111 are very likely, closely related metabolically and physiologically. Differences may exist in cell wall-cell membrane composition. If this is true, then fluctuations in binding of hormones to their respective receptor sites could produce more, or less, susceptibility to steroid action.

Could the alterations of permeability observed in the present study account for the previously reported in vivo antimicrobial activities of the hormones? Although further research will be required to answer this question, the results of this study suggested a direct hormone-staphylococcal interaction. It is probable that permeability changes with subsequent secondary reactions of cell metabolism, may account for some, but not all, of the in vivo antimicrobial effects of the steroids. Many other physiological factors must also be considered in the

interplay of host-defense mechanisms with the gonadal hormones.

Hormonal intervention in cell permeability is not an altogether new hypothesis. For example, Edelman et al. (13) isolated skeletal muscle membranes and found that they bound ^{131}I-insulin by both electrovalent and covalent (disulfide) linkages. This, they thought, was evidence that the permeability changes caused by insulin were initiated by a thiodisulfide interchange reaction between the disulfide bond of the hormone and the thiol groups of the membrane. Also, Crabbe and deWeer (10) found that aldosterone stimulated active sodium transport in the toad bladder by increasing the sodium permeability of the mucosal border.

There are still other similar examples. Thus, adrenocorticotropic hormone inhibited the active incorporation of ascorbic acid in adrenal cortical slices. As several cortical steroids also inhibited the uptake, Sharma et al. (50) proposed that adrenocorticotropic hormone acted through steroids produced in its presence. Also, Noall and Allen (43) observed that estradiol stimulated the uptake of α-aminoisobutyric acid by the uterus. This occurred only if the hormone was offered *in vivo*, but not *in vitro*.

As a final example from a microbe, Lester et al. (38)

observed that deoxycorticosterone inhibited the uptake of sugars, amino acids, and rubidium by Neurospora crassa. The results suggested that the action of the hormone was directed against specific permeability processes of the fungal cell.

V. SUMMARY

Certain specific gonadal steroids selectively reduce the growth and virulence of Staphylococcus aureus. This work was undertaken to investigate this hormonal mechanism of action at the molecular level.

Under anaerobic conditions, when various hormones such as progesterone, testosterone, mestranol, or norethindrone, at the pharmacological concentrations of 20 to 40 mcg/ml, were added to Staphylococcus aureus that had been previously exposed to ^{14}C-glucose, the rates of leakage of radioactivity from hormone-treated cultures were 10% to 60% greater than the rates of leakage from the control cultures. Similarly, the cellular release of protein was 10% to 50% greater in the presence of progesterone. Penicillin enhanced the steroidal effects of increased cellular leakage.

The uptake of label ed substrates, such as glucose, alanine, lysine, or glutamic acid, was altered 10% to 90% by prior treatment with progesterone, testosterone, or estradiol at 40 mcg/ml, or diethylstilbestrol at 20 mcg/ml. The inhibition of entry of label ed alanine was markedly influenced by metabolic activity, anaerobiosis, temperature, pH, cell concentration, and hormone concentration. Substrate uptake by gram negative organisms, whose growth is not inhibited by the

steroids, was also unaffected by the steroids.

Fractionation of staphylococci after exposure to ten ^{14}C-label ed substrates in the presence and absence of hormones, revealed wide-spread alterations in the entrance of the labels into lipids, proteins, nucleic acids, teichoic acids, and cell wall mucopeptides. No direct relationship of steroid action to macromolecular syntheses emerged. With some substrates, entry into the cellular fractions was inhibited, while with others, either stimulatory or intermediate effects were detected. Progesterone at 40 mcg/ml, however, altered the entry of eight of the compounds into their respective cellular pools. The hormone inhibited entry 11% to 17% for seven of these eight substrates. For short-term incorporation studies using ^{14}C-alanine, progesterone initially reduced entrance into the cellular pools 15% within 10 seconds. At this time, no hormonal intervention was apparent in the incorporation into the other cellular fractions. After 15 minutes, however, the steroid reduced the entrance of the ^{14}C-label into the other fractions by 15% to 50%.

Synergistic activity was observed when sub-inhibitory concentrations of penicillin were utilized with sub-inhibitory concentrations of progesterone, testosterone, norethindrone and mestranol, or diethylstilbestrol, to inhibit the entry of ^{14}C-alanine into cell wall mucopeptides. A progesterone concentration above 15 mcg/ml was required to reduce entrance of the

labeled alanine into the mucopeptide fraction. If sub-inhibitory concentrations of penicillin were added, progesterone concentrations as low as 5 mcg/ml significantly reduced entry of the label into this fraction.

When ^{14}C-progesterone was added, either to whole cells, or to preparations of mucopeptides from the identical quantity of whole cells, the latter preparation contained three times as much radioactivity as the former. In fact, with concentrated suspensions of mucopeptides, as much as 23% to 55% of the added hormone remained firmly bound.

The following hypothesis is presented to explain the mechanism of action of the gonadal hormones, in the previously reported inhibition of growth of S. aureus. It is proposed that steroids bind to the cell wall-cell membrane complex, and in so doing, produce two molecular events, affecting bacterial permeability: 1) the prevention of access of some other molecule to a region near, or at the point of attachment of the hormone to the receptor; and 2) the production of a conformational change, and hence, a change in function of the receptor molecule itself. The local disturbance produced by the change in conformation could affect spatially separate transport systems contained within the membrane proper.

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APPROVAL SHEET

The dissertation submitted by Thomas Fitzgerald has been read and approved by the members of the advisory committee listed below.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 12, 1971
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