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CHARACTERIZATION OF SELECTED MICROORGANISMS
RECOVERED FROM THE HUMAN GENITOURINARY
TRACT.

The Ohio State University, Ph.D., 1971
Microbiology

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CHARACTERIZATION OF SELECTED MICROORGANISMS RECOVERED
FROM THE HUMAN GENITOURINARY TRACT

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy
in the Graduate School of The
Ohio State University

By

Robert Vito Marraro, A.B., B.S., M.S.
Major, USAF, BSC

The Ohio State University
1971

Approved by

Melvin Pfennig
Advisor
Academic Faculty
of
Microbial and Cellular
Biology
Dedicated to

Josephine "Dolly" Marraro

Your son, daughter-in-law, and grandchildren will keep you in their hearts and on their minds and lips always.
“Messieurs, c’est les microbes qui auront le dernier mot”.

--- Louis Pasteur.
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VITA

May 24, 1930 ........ Born - Brooklyn, New York

1951............... A.B., Colby College, Waterville, Maine

1955............... B.S., Columbia University, College of Pharmacy, New York

1955-1957........... Second Lieutenant, United States Air Force
Chief, Department of Clinical Laboratories
78th USAF Hospital
Hamilton AFB, California

1957-1959.......... First Lieutenant, United States Air Force (Reserve)
Associate Director
Plaza Pharmacy
Larchmont, New York

1959-1962.......... Chief, Department of Clinical Laboratories
3535th USAF Hospital
Mather AFB, California

1962-1964.......... Captain, United States Air Force
M.S., Arizona State University
Tempe, Arizona

1964-1965.......... Chief, Department of Clinical Laboratories
7505th USAF Hospital
APO New York 09232

1965-1967.......... Chief, Microbiology Branch
Chief, Phase II Medical Technologist Training Program
Consultant in Microbiology for United States Air Force in Europe
USAF Hospital Wiesbaden
APO New York 09220
1967-1968.......... Major, United States Air Force
Chief, Microbiology Branch
USAF Medical Center
Wright-Patterson AFB, Ohio

1968-1971........... Graduate Student, The Academic Faculty
of Microbial and Cellular Biology, The
Ohio State University, Columbus, Ohio

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FIELDS OF STUDY

Major Field: Pathogenic Microbiology

Studies in Pathogenic Microbiology: Professor M. S. Rheins

Studies in Bacterial Cytology: Professor R. M. Pfister

Studies in Immunology: Professors M. C. Dodd and N. J. Bigley

Studies in Medical Mycology: Professor J. A. Schmitt, Jr.
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Zones of growth inhibition of bacterial variants (RobkWel medium)
INTRODUCTION

The etiology of chronic diseases of the genitourinary tract often is an enigma to the microbiologist. Positive bacteriologic findings, implicating the classic and/or cell wall-defective forms of microorganisms may well explain the syndromes of chronic urethritis in both sexes, chronic prostatitis in the male and chronic trigonitis in the female. However, there remains those disease conditions which have proved to be intractable to appropriate urologic procedures and from which microorganisms frequently are not isolatable by the usual, and in some cases by extensive, microbiologic procedures.

The present investigation was undertaken to develop more sophisticated methodology for examining urine specimens, particularly those from which organisms in the "hydrophilic state" (Fugazzotto and Pinkosz, 1968) frequently are not recovered. The procedures were developed in an attempt to circumvent this problem by affording microorganisms a variety of atmospheric environments for primary isolation and, in doing so, increased the frequency of the recovery of a number of genera of microorganisms.

Further, the role of the cell wall-defective forms of bacteria in human disease also has drawn the attention of many investigators during the past decade (Cuze, 1968; McGee and Wittler, 1969; Sharp, 1970). Recognizing that these variant forms may be implicated in
chronic diseases of the genitourinary tract, the present study included efforts to isolate and identify these forms from urine specimens and to determine their possible role in these specific disease states.

Although the morphology of induced cell wall-defective forms of bacteria as examined by light microscopy has been reported frequently (Hijmans and Dienes, 1955; Crawford et al., 1958; Hadoff and Dienes, 1958; Karston, 1961; Anderson, 1967; Guze, 1968; Berliner et al., 1969), it was felt that a more detailed study concerning the sequential development of these forms over a prolonged period was pertinent to this investigation. Accordingly, included is a study in which the sequential development of induced cell wall-defective forms of *Streptococcus faecalis* on an agar medium is described. The morphologic changes which occurred over a 36 day period are described by light photomicrographs.

The demonstration of morphologic similarity among the L-forms of bacteria studied by light microscopy has prompted a number of workers to investigate the ultrastructure of these forms utilizing the techniques of electron microscopy (Abrams et al., 1964; Weibull, 1965; Dienes and Bullivant, 1967; Corfield and Smith, 1968; Wyrick, 1969). However, no attempt had been made, at the cellular level, to describe the intact, mature classical L-form colony. Therefore, longitudinal ultra-thin sections of such a colony were examined by electron microscopy and the morphology is described.
The hematologic data and the serum protein electrophoresis values gathered during this study were subjected to biometric analyses, and the response patterns of these particular disease states were examined for characteristic aberrations.
LITERATURE REVIEW

The study of urology has evolved over the years into a special branch of medicine specifically dealing with diseases and abnormalities of the genitourinary system of the male and the urinary tract of the female (Leader and Carlton, 1970). However, since the urinary and genital tracts are closely allied, both embryologically and anatomically, the two systems should be considered as a single unit under the name of "genitourinary" or "urogenital" system (Campbell, 1970). So intimate is the development of the two tracts in both sexes that when a malformation of one tract occurs, an anomaly of the other system often is noted. Essentially, the study of this special branch of medicine encompasses the definition and the preservation of the normal and the recognition and correction of the abnormal.

The genitourinary tract may be affected directly by a diversity of inflammatory, vascular, neoplastic and congenital disorders. Regardless of the disease entity, certain discrete groupings and signs are encountered repeatedly in every type of urogenital disease, irrespective of the cause. By far, one of the more important problems, when investigating the etiology of urologic disease presumably attributable to microbial agents, is the consideration of the basic concepts which may play a role in the lowering of host resistance.
These factors, capable of altering host susceptibility, include, but are not limited to, the properties of the microorganisms which enable them to produce disease and the manner in which the infected host responds to the microbial invasion. A general review of host-parasite relations in bacterial diseases (Davis et al., 1967) indicates that a delicate balance exists between host and parasite and that a multitude of factors may be involved in the disruption of the state of health, e.g. age, nutrition, drug therapy, anatomic, physiologic and metabolic imbalances, and the effect of other concomitant disease states.

Primary diseases of the genitourinary tract, such as bacterial infections, require the same types of diagnostic and therapeutic procedures in the two sexes (Everett and Williams, 1970). Certain dissimilarities in anatomic configurations of the tracts between the sexes, however, result in variations in etiologic factors, and even in disease processes themselves. Whether the differences in the rates of acquisition of genitourinary tract disease between the sexes depends on anatomic or physiological differences, or upon a combination of both, as yet remains unclear. However, the fact remains that these disease states are 14 times as common in women as in men (Stamey et al., 1965). It appears likely that the entry of microorganisms in the male urogenital tract is somewhat restricted anatomically and presumably would originate primarily from exogenous sources, from an infected prostate, or from the prepuce in the non-circumcised individual. The role of the male hormones, the
condition of the tissues *in situ* as an optimal environment for the growth and maintenance of the organisms, the influence of antimicrobial prostatic secretions, and the general physiologic status of the individual are to be considered as integral factors in predisposing to bacteria. On the other hand, in the female genitourinary tract, the external one-third of the urethra is often contaminated by the bacteria from the vagina and the rectum. Additionally, the length of the female urethra is shorter than that of the male, and it is highly probable that women do not normally empty their bladder as completely as do men. A final predisposing factor is the displacement of the female urethra during sexual intercourse from the normal external position to an intravaginal one, thus presenting an ideal situation for the massage of bacteria directly into the bladder (Stamey et al., 1965). The role of the female hormones, and, as in the male, the condition of the tissues in the genitourinary tract coupled with the general physiologic status of the individual must be considered as influences effecting the establishment of bacteriuria. The etiology of infections of the urogenital system may well be attributed to additional factors such as developmental anomalies, interference with urinary flow, and abnormal circumstances of urine reflux (Carroll, 1970). As a final premise, it would be remiss not to mention the psychosomatic approach to diseases of the genitourinary tract. Although it seems possible that future investigations may clarify the fact that a psychologic disturbance antedates a functional alteration, for the
present whatever may precede the functional disturbance is still unclear (English, 1970).

Infectious agents, then, may reach the genitourinary tract by three main routes: the lymphogenous, urogenous and the hematogenous. However, other methods such as continuity, contiguity and refluxing of the urine may also account for the bacterial colonization of the urogenital system. The microorganisms incriminated in these disease states may originate from a specific focus of infection, e.g., the oropharynx, the nasopharynx, lungs, and intestines, and may be borne to the genitourinary tract by means of the aforementioned routes of infection. The role that the lymph system plays in the production of infection is not clearly determined (Carroll, 1970) but, based on experimental and clinical evidence, it would appear to be important. It is quite possible that bacteria gain chance entry to the lymphatic system, are conveyed to the thoracic duct, and then into the blood thus constituting a lymphohematogenous route of invasion. In the urogenous route, the most frequent portal of entry of microorganisms is through the exposed meatus, thus causing infection in the urethra and in the bladder. Further, studies have shown that the urine in the prostatic urethra of the male and the proximal two-thirds of the urethra in the female is returned to the bladder during interrupted micturition, and perhaps at the termination of urination as well (Carroll, 1970). This ascending route of invasion from the lower genitourinary tract may account for infections of the upper urinary tract as well. The hematogenous route of invasion is believed to be
the most common by which bacteria may be carried to the urogenital system, and especially to the kidneys. This explanation would appear to be the more plausible since any area of vascularity would be exposed to blood-borne microorganisms. Fortunately, most bacteria that enter the blood are destroyed by natural host defense mechanisms.

Once bacteriuria has been established, the mechanisms for colonization become of interest. Based on the experiments of Cox and Hinnan (1961), it may be postulated that the ability of healthy individuals to rid their urinary tracts of bacteria may depend on either intrinsic bladder defense mechanisms, or upon the mechanisms of the ebb and flow of urine. It would seem likely that persistence of bacteriuria may also depend upon certain other factors, e.g., stasis of the urine; optimal tonicity of the urine; urine pH; and the lack of bacteriostatic/bacteriocidal substances. Additionally, mechanical factors such as the trapping of residual organisms on mucous, debris, or in residual urine, and the aid of irritative factors (node of wiping the vulva, tub baths, and masturbation) produce further change in the voiding pattern and incomplete emptying of the bladder (Kunin, 1969). These factors, arising from anatomic, physiologic, mechanical, and irritative origins account for the development and establishment of chronic bacteriuria.

Undoubtedly, a number of physiologic factors in the body tend to combat the invasion of bacteria. The more common of these and the possibility of a defect in the host defense mechanisms often have been reviewed. Other, more subtle factors include the acidity
of the urine, which discourages the survival of invading organisms in the urinary tract as the pH approaches 5.5 (Carroll, 1970). As a result, the use of ketogenic diets to combat urinary tract infections, as well as the use of methionine, have been successful in acidifying the urine. Further, the efficacy of urea as a urinary tract antiseptic has been studied and it appears to be bacteriocidal in concentrations found in "normal" urine to the more commonly encountered gram-negative pathogenic bacteria.

Regardless of the factors, including antibacterial chemotherapy, which may play a part in opposing the invasion of bacteria, the fact that there are recurrences of infection is well noted. Although the obvious invading organism may be eliminated, it may be assumed that the cause of the original invasion and the anatomic changes brought about by the original infection have not been necessarily corrected. Factors such as slow-healing local lesions with the prolonged presence of viable bacteria, the occult survival of drug-resistant strains of bacteria, the role of the L-forms in disease, the unavailability of protective antibody to the area, general defense mechanisms and the well-being of the host, and the failure to locate and eliminate areas where organisms can lodge and multiply (prostate and para-urethral apparatus) may account for recurrences of infection (Carroll, 1970).

Even though the more common agents causing infections of the genitourinary tract are bacteria, unusual etiologic factors have been noted (Alyea, 1970) and the causative agents involved have
been identified as fungal, notably *Nocardia asteroides* (actinomycosis), *Blastomyces dermatitidis* (blastomycosis) and *Coccidioides immitis* (coccidioidomycosis). Viral agents, e.g. *Herpes simplex*, have been implicated in genitourinary tract disease (Corrissseau et al., 1970) and human parasitoses have been documented (Sanjurjo, 1970), despite a relative immunity noted in the urogenital system directed to a variety of parasites.

Chronic or subacute prostatitis, so-called "non-specific" prostatitis, is probably the most common urologic disease of men. Although its incidence is estimated to vary between thirty-five and fifty percent of the adult male population, only a relatively small proportion of individuals develop symptoms (Austen, 1966). This syndrome frequently follows acute prostatitis or chronic posterior urethritis (Alyea, 1970), but in the absence of any antecedent local prostatitis or infection of the urinary tract, it would appear that this disease is of blood-borne origin, but with the primary focus usually obscure. Irrespective of the focus and the route of invasion, once infection has become established it tends to exist as a low grade, inflammatory reaction which can persist for many years. Factors which promote the tendency to chronicity are related principally to the anatomic structure of the prostate; its honeycomb-like architecture; its poor drainage system, and its relatively inadequate vascular supply (Lich and Howerton, 1970). Another syndrome in the male, termed "non-specific urethritis", has been utilized to describe any urethritis in which the gonococcus
cannot be found to be the etiologic agent. The term thus covers a variety of conditions in which the exact cause is not immediately apparent (Burns and Thompson, 1970). These syndromes are resolved into two major divisions: the conditions in which no specific cause for the symptoms can be found, and those which follow or are a result of a wide variety of bacterial invaders. It is to these clinical conditions that attention has been directed, and the literature offers many reports dealing with chronic prostatitis and urethritis (Chornley et al., 1954; O'Shaughnessy et al., 1956; Stamey et al., 1965; Austen, 1966; Bourne and Frishette, 1967; Lennert et al., 1967; Meares and Stamey, 1968; Burns and Thompson, 1970; Campbell, 1970).

Diseases of the female genitourinary tract, such as chronic, non-specific urethritis and trigonitis, are frequently accompanied by normal or relatively insignificant abnormal findings in the urine. Such conditions usually give rise to urologic symptoms which, at times, may be the result of a gynecologic disorder (Everett and Williams, 1970). However, this should not be assumed until the urinary tract has been proved "normal" by careful study. These diseases of the urethra and trigone, although readily recognized, have not been investigated extensively as such, and therefore the literature contains meager information concerning the clinical and diagnostic aspects of these syndromes. Suffice to say, since anatomically the external meatus is situated in the middle of the vestibule of the vagina, that this field is exposed constantly to bacterial contamination from both the rectum and the vagina.
Chronic, non-specific urethritis and trigonitis may be explained then in terms of probable bacterial etiology, with the symptoms manifested being the sequellae of microbial invasion. There are numerous reports describing microorganisms in the female urinary tract in both health and disease, and further review here would seem unnecessary (Simon et al., 1964; Stamey et al., 1965; Moore et al., 1965; Smart, 1957; Andelman et al., 1968; Leigh et al., 1968; Turck and Petersdorf, 1968; Kunin, 1969; Asscher et al., 1969; Brumfitt and Reeves, 1969; Carroll, 1970).

Undoubtedly, the urine is one of the most frequent and difficult of the body substances from which to culture. Urine is ordinarily an excellent culture medium for the multiplication of the common pathogens of the urinary tract, and often bacteria tend to increase greatly, frequently exceeding $10^6$ per milliliter. It has been established that quantitative cultures of the urine from infected patients will reveal a bacterial count of $10^5$ organisms per milliliter or more (Hoeprich, 1960), whereas specimens from noninfected or normal persons may be sterile or contain up to $10^3$ organisms per milliliter, often due to contamination during collection (Prother and Sears, 1960). It should be pointed out, however, that bacterial counts of less than $10^5$ organisms per milliliter may occur in patients who are receiving specific antibacterial chemotherapy or in patients who are excessively hydrated, with a consequent dilution of urine (Clapp and Grossman, 1964). Therefore, to evaluate the clinical significance of a "positive" urine culture,
some means of estimating the number of organisms present must be employed. There are three methods generally accepted for the routine quantitation of microorganisms encountered in the urine. The first of these, the "calibrated loop-direct streak" method (Hoeprich, 1960), makes use of a calibrated bacteriological loop to inoculate and streak plates of standard or differential culture media. The method is used most frequently as a screening procedure; its confidence level, when compared with actual plate counts, is highest when counts of $10^5$ bacteria per milliliter or more are observed. The second method, termed the "pour plate" method (Pryles, 1960), has been criticized as not entirely reflecting the true bacterial count of the urine specimen. This may be attributed to the fact that clumps of organisms can give rise to single colonies (Bailey and Scott, 1970), and this procedure, although practical, is not used frequently. Finally, the "streak-pour plate" method (Savage et al., 1967), combines both screening and quantitation for detecting significant bacteriuria, particularly in the female. Although no specific shortcomings have as yet been noted for this procedure, it appears that the multiple manipulations involved in processing the specimen have caused its use to decline. Recently attention has been given to chemical tests for the rapid detection of bacteriuria. These include the reduction of triphenyltetrazolium chloride by metabolizing bacteria (Simmons and Williams, 1962), and the Griess nitrate test (Branson, 1966) which is based on the rapid reduction of nitrate by members of the Enterobacteriaceae. Both
tests, however, have been considered unsatisfactory due to substantial numbers of false-negative results.

Culture media for routine bacteriology may be selected for either general or for specific purposes. Usually the selection is governed partly by tradition and partly by the experience of the technician. Additionally, the physician's interpretation of the patient's symptoms is helpful, and the subsequent source of the specimen is of great importance. Moreover, the environmental requirements for the cultivation of these microorganisms, e.g., temperature, atmosphere and moisture are of concern. It would appear that the optimal conditions of temperature and moisture are recognized and employed in routine diagnostic practise. However, the importance of varying the atmospheric conditions during the primary cultivation is not apparent. It seems obvious that most specimens are incubated under strictly aerobic conditions. The primary culture of some suspected organisms will require from 2-10% concentration of carbon dioxide. More infrequently, an anaerobic atmosphere is required for the primary isolation of certain microorganisms. However, the majority of microbiologists have been remiss in employing a microaerophilic environment for organisms upon primary cultivation. By failing to do so, organisms in the "hydrophilic state" (Fugazzotto and Pinkosz, 1968), and proven to be obligate microaerophils, therefore cannot possibly be recovered.

Although the classic forms of bacteria have been incriminated as the etiologic agents of infections of the genitourinary tract
(Bailey and Scott, 1970), the vaginal flora in normal females varies considerably with the pH of the secretions and with the amount of glycogen present in the epithelium. These factors, in turn, depend on ovarian function. It would seem, from the foregoing, that certain genera and species of a wide variety of microorganisms are to be encountered in both the normal and the diseased genitourinary tract. Less understood, however, is the role of those microorganisms heretofore considered to be non-pathogenic and, when encountered in laboratory cultures, are thought to be contaminants. The pathogenic potential of Corynebacterium species (diphtheroids) has been documented in chronic prostatitis (Kaged and Khafaga, 1965), in infected urine specimens (Torregrosa, 1968) and in non-specific urethritis by Kaminski et al. (1970). Staphylococcus epidermidis has been implicated in chronic prostatitis (Kaged and Khafaga, 1965), as well as being found in other specimens from infected patients (Person et al., 1969). More recently, Pearson (1970) has reported human infections caused by the Bacillus species. From the evidence available, it would appear that the occurrence of these microorganisms in culture, previously considered to be non-pathogenic, should no longer be disregarded, but must be evaluated on the basis that they are potentially pathogenic.

Investigations of the serum antibody response to bacterial infections of the genitourinary tract were initiated almost four decades ago, but since then, interest has lagged and, as a result, few reports appear. Antibody response to urinary tract infections
of an acute nature have been documented (Griffin, 1934; Needell et al., 1955; Winberg et al., 1963; Reeves and Brumfitt, 1968), but caution is indicated in the interpretation of the results. Not all symptomatic patients have shown rises in serum antibody titer as was demonstrated by Kunin et al. (1964) using hemagglutination, and Kalmanson et al. (1965) using serum bactericidal titers. Further, hemagglutinating titers and whole cell agglutinating titers do not necessarily agree (Reeves and Brumfitt, 1968). Thus the nature of the antibody response to the staphylococci, streptococci and to the coliform bacteria, as well as the species of antibody and the other variables which contribute to the differences in the measurements of antibody, require more detailed investigation. Bacterial infection of the prostate in the male and of the corresponding para-urethral apparatus in the female is considered to be a tissue infection (Brumfitt and Reeves, 1969), and the serum antibody titer to the invading organisms will rise. Studies of the sera and prostatic fluids in patients with chronic prostatitis (Haged and Khafaga, 1965) indicate that a local and general antibody response occurs in infections of the prostate. Although bacteriological cure may be associated with an increase in antibody in the prostatic secretion, a rise in circulating antibody may not necessarily be associated with remission or cure of the disease. The possible significance of antibody, if any, in these syndromes is not clear and further experimentation is indicated.

Although conclusive evidence is not currently available to
establish a role for L-forms -- often referred to as cell wall-defective forms, bacterial variants, spheroplasts, or protoplasts (McGee and Wittler, 1969) -- in human disease, the discovery that numerous bacterial species are able to reproduce after undergoing a major alteration in structure has many implications which are significant. Bacterial L-forms were first described in the laboratory by Klieneberger-Nobel (1935) and, since then, have continued to attract the attention of microbiologists and clinicians alike. The first report on the isolation of an L-form from a human was that of Dienes and Smith (1944). However, since the L-form of Bacteroides species recovered from the clinical specimen could not be maintained in subculture, and upon inoculation onto other media yielded a mixture of L-forms and bacilli, doubts were expressed as to whether the organisms existed in the L-phase in the host. As a result, no further reports appeared until Dolman et al. (1951) described the isolation of the L-forms of Streptobacillus moniliformis from blood cultures of two patients with rat-bite fever. It was not until 1960, that Wittler et al. isolated the L-form of Corynebacterium species from the blood and from the bone marrow cultures of a patient with subacute bacterial endocarditis. During the past decade, the L-forms of bacteria have been investigated extensively and it would seem to be appropriate to consider their production, persistence and potential pathogenicity.

The development of variant forms of microorganisms in vivo
have been investigated, and it would appear that many chemical and physical agents can modify the bacterial cell wall, and thus function as inducing agents for the development of cell wall-defective forms of bacteria. Other factors normally antagonistic to bacterial invasion of the body also seem to injure the cell wall and may be responsible for the induction of variants in vivo. For example, antibody together with complement have been shown to promote production of variants, e.g. *Haemophilus pertussis* and *Salmonella typhosa*, in vitro (Dienes et al., 1950) and in vivo (Wittler, 1952; Carey et al., 1960). Further, it is possible that phagocytized bacteria may be induced to wall-defective variants by such lysosomal enzymes as lysozyme, leucozyme, acid phosphatase and phosphatase. These have been employed in vitro to produce these forms from gram-positive (McQuillen, 1960) and from gram-negative bacteria (Anano et al., 1956). During some infectious processes, the tissue, blood, and urinary lysozyme concentrations may equal or exceed levels shown to induce variants in vitro (McQuillen, 1960), but the relevance of this observation to the possible production of variants by lysozyme in disease states is unknown. In addition, non-specific host tissue factors, such as amino acids, acting alone or synergistically with other enzymes or antibiotics, bacterial enzymes, and bacteriophage have induced variants in vitro (Carey et al., 1957; Stolp and Starr, 1965; Ghosh and Murray, 1967). As yet, however, there is no evidence of their importance in producing variants in naturally-occurring diseases. Of the above inducers, antibiotics are most
likely to be significant in producing L-forms in vivo, considering
the variety of antimicrobial substances capable of producing
variants (Roberts, 1967) and the diversity of bacterial species
that have undergone conversion to these aberrant forms of bacteria
(Dienes and Weinberger, 1951). It seems probable, then, that most
bacteria pathogenic for man can be induced to variants in vivo
by those antibiotics most frequently used in clinical medicine.

Once produced, the persistence of the L-forms in vivo may be
favored by their cell wall-deficiency, their small size, and by
their relative osmotic stability. The seemingly refractory nature
of these aberrant bacterial forms to certain antimicrobials and
host tissue factors may well be a function of their cell
wall-deficiency (McDermott, 1958). This deficiency may also decrease
their chemotactogenicity and, in the presence of specific opsonins
to cell wall or capsule, phagocytic ingestion and intracellular
destruction of these forms may be decreased (Hirsch and Strauss,
1964). Since small particles are cleared by the reticuloendothelial
system more slowly than are larger particles (Kims, 1964), the
smaller size of the L-forms may theoretically give them a selective
advantage for persistence. Reports of the persistence of these
forms of bacteria in the isotonic renal cortex (Kortiner, 1965)
indicate that even variants which might be expected to require a
hypertonic environment may survive in vivo in sites other than those
with an increased osmolality.

The demonstration of the pathogenicity of L-forms has been
most difficult except in the case of variants such as those of
Clostridium tetani in which the pathogenicity depends on the production of tetanospasmin, a neurogenic exotoxin (Scheibel and Assandri, 1959). This toxin has been shown to be elaborated by bacteria devoid of classical cell wall structure. In addition, it is of interest that bacterial variants, in general, can participate in various experimental hypersensitivity phenomena, since some L-forms produce antigens which may be associated with naturally-occurring hypersensitivity diseases (Kagan et al., 1964; Mortimer and Vastine, 1967). However, the pathogenic qualities of these cell wall-defective forms likely to be of greatest importance are their ability to perpetuate the residence of a pathogenic organism in a host under conditions that might destroy organisms in the classic bacterial phase, and their ability to revert to the pathogenic bacterial phase when conditions again become favorable.

Recent studies (Gutman et al., 1965; Gutman et al., 1967; Guze, 1968; Conner et al., 1968; McGee and Wittler, 1969; Sharp, 1970; Domingue and Schlegel, 1970) have demonstrated that the L-forms of bacteria, as well as spheroplasts and protoplasts, may be implicated in persistent, chronic, or recurrent urinary tract infections. These observations on the occurrence of bacterial variants in diseases of the genitourinary system suggest that L-forms may be one cause of persistent or chronic infection of this area. The more important question of the role of the L-forms in sustaining the infection remains unanswered.

As convincing as the implication of the L-forms in these
states may appear to be, to date the frequency of isolation of these variants has been neither frequent nor consistent enough to be accepted completely. Of pertinence is the reliability of the present techniques for the successful isolation and support of any microbial variant possibly present in a clinical specimen. The cell wall-defective form of bacteria are considered fastidious and require complex media and special conditions for cultivation and isolation. Although the requirements are strict (Smith, 1964), these forms can be grown on complex artificial media, the formulae for which vary from laboratory to laboratory. Certain deficiencies are frequently encountered in the attempts for the cultural study of these variants. These include the failure to make use of adequate controls for the detection of contaminants and the failure to recognize that these forms may not have existed \textit{in vivo} but were produced inadvertently \textit{in vitro} by laboratory manipulation. Obviously, what is required for the establishment of a program for the cultivating of L-forms from clinical material is the development and/or standardization of appropriate media and proper methodology for detection of these forms.

The identification of L-forms from clinical material is difficult. The initial procedures customarily employed to determine genus and species of classical bacteria have little, if any, applicability to these aberrant organisms. Gram staining does not sort a cell wall-deficient form into one or the other of the two large categories of gram-positive or gram-negative genera since
L-forms are all gram-negative. Microscopic examination gives no hint of differential morphologic features which would place them among either bacillary or coccal genera, among genera arranged in clusters or in chains, or among those containing or lacking spores, capsules or flagella. As a result, a number of reports have appeared in the literature claiming that peculiarly stained granules found in stained smears of varying clinical material represent bacterial L-forms. On occasion, attempts have been made to attribute illness to infection with L-forms when cultures have been positive only after repeated, blind transfer or unusually prolonged incubation. A number of these reports, although dealing with plausible concepts, have not presented adequate evidence to permit critical judgment regarding the morphologic characteristics of the organisms studied. Much information has been accumulated regarding the conditions which permit the growth of these aberrant forms of bacteria and on the factors which influence their morphology (Dienes, 1953; Sharp, 1954; Hijnans and Dienes, 1955; Kadoff and Dienes, 1958; Crawford et al., 1958; Hijnans and Kastelein, 1960; Morston, 1961 Weibull and Lundin, 1963; Dienes, 1967a; Anderson, 1967; Dienes, 1967b; Anderson, 1968; Burmeister and Hesseltinge, 1968; Guze, 1968; Young and Armstrong, 1969; Berliner et al., 1969). As a result, the morphologic parameters of these forms appear to be well defined, and recognition, by means of light microscopy, should not be difficult. The demonstration of morphological similarity among the L-forms studied by light microscopy, in contrast to the obvious
structural differences existing among their parental bacteria, has prompted a number of workers to investigate the ultrastructure of these forms utilizing the techniques of electron microscopy (Abrams et al., 1964; Weibull and Kohri, 1965; Weibull, 1965; Dienes and Bullivant, 1967; Dienes and Bullivant, 1968; Corfield and Smith, 1968; Guze, 1968; Wyrick, 1969). Studies of ultra-thin sections of these aberrant forms of bacteria have contributed information with regard to their morphology and, when combined with biochemical, biophysical, and genetic investigations, will continue to clarify the structure and function of microorganisms at the submicroscopic and molecular levels.

Another aspect of genitourinary tract diseases which merits mention is the recognition of allergy which has been occasionally reported but, seemingly, is considered a rare occurrence. However, in patients with symptoms of recurrent urinary tract infection, there is a need to consider allergy as a possible etiologic factor. The literature on this subject was reviewed by Powell (1961), but only a few reports have appeared since (Carroll, 1970; Alyea, 1970; Powell et al., 1970). Conditions described as eosinophilic granulomatous disease of the prostate and eosinophilic cystitis, although recognized and reported, have not been linked as yet with any specific causative agent(s). It would seem that allergies of the genitourinary tract, e.g. the urethra, bladder, and prostate, may be caused by factors such as inhalants, drugs, food, bacteria, viruses, and rickettsia (Powell, 1961) with the allergic symptoms
mimicking those of overt bacterial infection. Food allergy has been most often incriminated in these unresolved disease states (Dees and Simmons, 1951; Eisenstaedt, 1951; Walter, 1958; Unger et al., 1959; Powell et al., 1970) and, in some patients, a significant degree of atopic reactivity has been revealed. Since many substances are capable of acting as allergens, bacterial sensitization may occur in the various organs of the urogenital system. This suggests that factors of hypersensitivity to tissue or bacterial products may play a rather decisive role in these syndromes (Burkland, 1951).

Not so apparent is the involvement of the cell wall-defective forms of bacteria as initiators of similar immunologic mechanisms. L-forms can be immunogenic (Lynn and Haller, 1968), and apparently these variants may possess several immunogenic substances which were absent in the parent bacteria. Little further information regarding the immunologic potential of these aberrant forms of bacteria is available at present. Nonetheless, the immunochemical properties of the Group A streptococci, in their variant form, have been explored (Freimer, 1968) and it was concluded that the bacterial membrane contains antigens distinct from those associated with the cell wall and also distinct from those of the cytoplasm. One further report (Bono and Crawford, 1969) concerning animal studies on the antigenicity, serologic activity, protective capacity and toxicity of a stabilized L-form derived from a Group B strain of Neisseria meningitidis demonstrated the immunizing potential of this stabilized L-form.
Investigations regarding the potential of the L-forms of bacteria to enter into autoimmune diseases have not been developed to any extent. Perhaps rheumatic fever may afford an example of how these forms might play a role in autoimmune disease since the possible relationship of streptococcal antigens to the myocardial component of rheumatic fever has been demonstrated (Kaplan and Svec, 1964). If streptococcal cell wall antigens play a part in the pathogenesis of rheumatic fever, then it may seem contradictory to implicate streptococcal L-phase variants, which lack all or part of their cell wall. This has been partly answered by Freimer et al. (1959) and Gooder and Faxed (1961) who found that although K protein was not present on the surface of the streptococcal L-form, it was still produced and elaborated into the milieu. Although these variants have not been shown to produce the specific cell wall antigens which cross-react with human heart valves and muscle, they may yet be shown to do so. Since these aberrant bacterial forms have been reported to form spontaneously in streptococcus-inoculated animals, to persist intracellularly for long periods, and have been isolated from patients who probably had rheumatic fever, their potential ability to serve as an unknown and continuing stimulus for production of cross-reactive antibody in rheumatic fever is not to be overlooked. Related mechanisms possibly may be shown to be involved in diseases of the urogenital tract.
MATERIALS AND METHODS

Anatomic Locale Investigated in these Studies:

The male genitourinary tract, as depicted in Plate I for purposes of anatomic orientation, consists of two ureteral orifices which are the outlets of the fibromuscular tubes (ureters) and which convey the urine from the kidney into the base of the bladder. The bladder trigone, also referred to as the trigonum vesicae (lieutandi), is that triangular space or area circumscribed within the limits of the ureteral orifices and the neck of the bladder. The prostate, a seven-lobed gland surrounding the neck of the bladder and urethra, is made up partly of glandular tissue, the ducts from which empty into the prostatic portion of the urethra, and partly of muscular fibers.

The female genitourinary tract, as seen in Plate II, includes the ureteral orifices and the bladder trigone. Additionally, attention must be drawn to the orifices of the peri-urethral glands (openings into the urethra of the glands occurring around the urethra) which include glands regarded as rudimentary homologues of the prostate gland in the male. The urethra, a membranous canal which conveys urine from the bladder to the surface, and, in the male, additionally transporting the seminal ejaculations, is apparent.
Plate I. Anatomy of the male genitourinary tract indicating the ureteral orifice, the bladder trigone, and the prostate.

Plate II. Anatomy of the female genitourinary tract indicating the ureteral orifice, the bladder trigone, the orifice of the peri-urethral gland, and the urethra.
Categorization of Patients:

Patients were divided into three major groups, each composed of 50 males ranging in age from 23 to 50 and 50 females between the ages of 20 and 45.

Group 1 was composed of 50 males and 50 females designated as Group 1—Male and Group 1—Female and were selected at random from the population at hand. All individuals admitted to this group were asymptomatic and had no history of urologic disease within the previous three years. This group served as the "negative" control.

The category designated as Group 2—Male included patients who were examined for conditions other than those which would usually involve a possible microbial etiology. In this group were individuals with hydroceles, varicoceles, testicular tumors, hydronephrosis, calculus disease without evidence of positive bacteriologic cultures, and other common noninfectious urologic diseases. Few patients in this category required cystoscopic examination, but those examined exhibited no inflammation of the penile or prostatic urethra. The bladder and especially the trigone showed no evidence of inflammation. Routine quantitative cultures of mid-stream urine specimens failed to reveal the presence of microorganisms.

The category designated as Group 2—Female was comprised of patients who were examined for presumably noninfectious urologic conditions such as cystocele, polycystic kidney disease, calculus
disease without evidence of positive bacteriologic cultures, and other syndromes which were congenital or acquired, but not associated with bacterial infection. Cystoscopic examination did not disclose changes consistent with acute or chronic inflammation of the urethra or trigone. Laboratory findings included sterile quantitative cultures of mid-stream urine specimens.

The patients, both male and female, admitted to the Group 2 category fulfilled the need for the second control group, that is a group which did not present the specific manifestations ascribed to the following patients (Group 3).

The category, Group 3—Male, included patients who were diagnosed as having chronic prostatitis or non-specific urethritis, and manifested one or more of the following symptoms: burning on urination; dysuria (painful or difficult urination); increased frequency of urination both day and night; perineal or low back pain; aching in the inguinal area; suprapubic discomfort or pressure; and urgency. Cystoscopic findings invariably reflected some degree of abnormality in the prostatic urethra. An edematous surface, often with areas of pseudopolypoid formation (inflammatory polyps), was noted, especially lateral to the verrumontanum or colliculus seminalis (a prominent portion of the urethral crest surmounted by the prostatic utricle (vagina masculina; uterus masculinus) or the homologue of a part of the female vagina close to which are the orifices of the ejaculatory ducts). The trigone was occasionally inflamed, but more often was normal as was the remainder of the
bladder. Laboratory findings included negative routine quantitative cultures of mid-stream urine specimens and normal results of urinalysis. If an expressed prostatic secretion was obtained and subsequently subjected to microscopic examination, a moderate number of white blood cells often was noted.

The category designated as Group 3—Female included patients diagnosed as having the so-called "urethral syndrome", or chronic urethritis. The patients in this group manifested one or more of the following symptoms: burning on urination; dysuria; increased frequency of urination both day and night; low back pain; dull, suprapubic pressure discomfort; urgency; and a feeling of incomplete evacuation of the bladder. Associated urethral stenosis (a narrowing or stricture of the urethra) was noted in some cases. These patients were subjected to urethral calibration in order to diagnose and treat the urethral stenosis. Cystoscopy was performed at least once on all patients and invariably revealed chronic granular changes of the mucosa of the trigone. These changes either uniformly involved the trigone, often extending up to the ureteral orifices, or were patchy in appearance. Similar changes were observed at the bladder neck and in the proximal urethra. The remainder of the bladder often showed no abnormality. Occasionally, there was evidence of bullous cystitis (an inflammation of the bladder characterized by blisters or vesicles filled with serous fluid). Laboratory findings included negative routine quantitative cultures of mid-stream urine specimens and urinalysis results were within
normal limits.

Collection of Urine Specimens:

Groups 1 and 2 Male Patients:

These individuals were asked to cleanse the periphery of the urethral meatus three times with benzalkonium chloride (1:750 to 1:5000 dilution), and then were asked to void the first 20 to 30 ml of urine into a standard, sterile disposable urine collection container. These specimens were subjected to a battery of bacteriologic procedures directed toward the isolation and identification of microorganisms which might be present.

Group 3 Male Patients:

These patients cleansed the periphery of the urethral meatus as described above. They were then subjected to prostatic examination with gentle massage. Immediately after the prostatic examination, a similar urine specimen was collected as previously indicated and, within ten minutes, the specimens were cultured bacteriologically.

Group 1 and 2 Female Patients:

These patients were instructed to wipe around the urethral meatus and adjacent labia minora three times with benzalkonium chloride (1:750 to 1:5000 dilution). In addition, the patients were requested to void and discard the initial portion of the urine and to collect the mid-stream urine. The patients were cautioned to keep the labia separated so as not to contaminate the specimen.

Group 3 Female Patients:

Mid-stream urine specimens were obtained from these patients.
in a similar fashion to that just described. Additionally, each patient was placed in the lithotomy position, the perineum cleansed with benzalkonium chloride, and a catheterized sample was recovered either through a female dilator or a cystoscope. All specimens were processed immediately upon collection.

Quantitative Culture of the Urine:

The method of Marraro et al. (1970a) was used throughout this phase of the investigation. Upon receipt of the freshly voided urine sample, the quantitative cultivation of organisms was performed as outlined in Figure 1. The specimen was inoculated with a platinum-rhodium 0.001 ml calibrated wire loop (A. H. Thomas Company, Philadelphia) onto a whole plate of sheep blood agar (5%). In addition, by means of a noncalibrated bacteriologic wire loop, the urine was inoculated to a bi-plate containing eosin-methylene blue and MacConkey agar (B-B-L, Cockeysville, Maryland) respectively. Both petri dishes were placed in the 37°C incubator under 10% carbon dioxide tension and were examined at 18-24 hours and at 48 hours. Upon visualization of growth, the organisms were quantitated from the sheep blood agar plates, identified according to accepted protocol (Bailey and Scott, 1970) and disk antibiogram studies were instituted.

In order to obtain valid quantitation of the organisms, presumably ranging from microaerophilic to anaerobic, a whole plate of sheep blood agar (5%) was inoculated with the 0.001 ml calibrated wire loop (Figure 2). This specimen was placed in an anaerobic GasPak system (B-B-L), incubated at 37°C and observed in 18-24 hours
Figure 1. Technique for the routine quantitative culture of the urine.

Figure 2. Technique for the quantitative culture of the urine for microaerophilic and anaerobic microorganisms.
and again at 48 hours. Further, from the original specimen, 3.0 ml of urine was transferred aseptically to 8.0 ml fluid thioglycollate medium (B-B-L), incubated at 37°C and observed at 18-24 hours and again at 48 hours. The quantitation of the organisms, when possible, was obtained from the sheep blood agar plate under anaerobic conditions, and the fluid thioglycollate medium was utilized for further studies.

The fluid thioglycollate culture was centrifuged at 2500 rpm for 10 minutes (Figure 3). The supernatant fluid was decanted aseptically and the sediment retained for subsequent testing. From the sediment, a smear was prepared and gram stained. Depending upon the tinctorial properties, either a gram-positive or a gram-negative antibiogram was performed in duplicate on Mueller-Hinton medium (B-B-L). The two sensitivity study plates were placed aside until the remainder of the protocol was completed.

Returning to the sediment, and by means of a noncalibrated bacteriologic wire loop, the material was streaked onto a whole plate of sheep blood agar (5%) and to a bi-plate of eosin-methylene blue and MacConkey agars (B-B-L). These plates plus one Mueller-Hinton medium (B-B-L) for antibiogram studies were incubated at 37°C under 10% carbon dioxide tension and observed at 18-24 hours and at 48 hours. Organisms recovered were designated as microaerophilic and identified in accordance with accepted protocol (Bailey and Scott, 1970).

The same procedure was repeated (Figure 3), but in this instance
Figure 3. Technique for the recovery, identification, and antibiogram study of microorganisms recovered from fluid thioglycollate medium.
the cultures were incubated at 37°C in the anaerobic GasPak system (B-B-L) and observed at 18-24 hours and at 48 hours. Organisms recovered were designated as anaerobic and identified accordingly.

Prototype Organisms:

The prototype organisms used throughout these studies were obtained through the courtesy of Dr. Richard R. Facklam, Streptococcal Bacteriology Unit, Center for Disease Control, Atlanta, Georgia 30333. These organisms were: Streptococcus faecalis (SS 441), Streptococcus faecalis var. liquefaciens (SS 275), Streptococcus faecalis var. zymogenes (SS 498), Streptococcus faecium (SS 442), and Streptococcus durans (SS 497).

Specification of the Group D Streptococci (Enterococci):

A schema was devised (Figure 4) for the differentiation of the members of the Group D streptococci utilizing the biochemical and physiological characteristics of the prototype organisms. Once established, this protocol was used throughout the investigation.

The prototype organisms were inoculated to S F medium (B-B-L) as originally described by Hajna and Perry (1943) and incubated at 45.5°C for 18-24 hours under aerobic conditions. Simultaneously, the cultures were inoculated into 0.1% methylene blue milk (Bailey and Scott, 1970) and incubated aerobically for 18-24 hours at 37°C. Fermentation of the S F medium and reduction of the 0.1% methylene blue milk were considered "positive" reactions and evidence that those organisms were Group D streptococci.
Figure 4. Schema for the differentiation of the Group D enterococci. Pos: positive; Neg: negative. Note: Arabinose, mannitol, and melibiose were prepared in 1% concentrations in phenol red broth base and incubated at 37°C for 18-24 hours.
Subculture of these microorganisms to triphenyltetrazolium chloride (TTC) medium as described by Barnes (1956) was accomplished. The medium, as modified by Facklam (1969), contained heart infusion agar (Difco, Detroit, Michigan) 20 g, dextrose (Difco) 2.5 g, and distilled water 500 ml. The pH was adjusted to 6.0 with 1 N hydrochloric acid, and the medium was autoclaved for 15 minutes at 15 psi (121°C). Following removal from the autoclave, the medium was allowed to cool to approximately 50°C, at which time 5.0 ml of 1% TTC solution (B-B-L) was added and the resulting solution thoroughly mixed. The medium was dispensed in the normal fashion into sterile, disposable petri dishes. Cultures were incubated aerobically at 37°C and were examined at 18-24 hours, at 48 hours, and at 72 hours. Tetrazolium reduction was noted when the bacterial colonies developed a deep magenta to red color. Colorless colonies or those with a faint pink hue were considered as having failed to reduce tetrazolium.

The microorganisms incapable of reducing tetrazolium were subcultured in 1% arabinose (Difco), 1% mannitol (Difco), and 1% melibiose (B-B-L), each in phenol red broth base (B-B-L). These cultures were also streaked onto sheep blood agar (5%). Aerobic incubation was carried out for 18-24 hours at 37°C. The media were observed for carbohydrate fermentation, as well as for beta hemolysis (Sharpe et al., 1966; Bailey and Scott, 1970).

Urine Filtration System:

In order to facilitate recovery of cell wall-defective forms of microorganisms, freshly voided urine specimens were collected
in accordance with previously established protocol and were subjected to vacuum, rather than pressure, serial filtration.

Two Swinnex 47 filter holders (Millipore Corporation, Bedford, Massachusetts) were assembled in tandem and attached by means of a nylon connector (Figure 5). Additionally, another nylon connector fitted with approximately 12 inches of autoclavable tubing was attached to the input terminal of the first filter holder. The filter holder itself was fitted with a Millipore filter (LCWP 04700). This mitex (teflon) filter has a mean pore size of 10 microns and is controlled so that absolute particle retention is possible.

The filtrate obtained through the 10 micron filter system was delivered, via a nylon connector, to a second Swinnex 47 filter holder. This unit was fitted with a KF-Millipore filter (HAWP 04700) having a mean pore size of 0.45 microns and is composed of pure and biologically inert esters of cellulose.

A 30 ml sterile, disposable syringe was fitted to the output or terminal end of the serial filtration system. The utilization of this size syringe permitted filtration by creating an adequate vacuum system when the plunger was steadily drawn from the barrel of the syringe. Additionally, it provided a sterile, disposable collection vessel for the filtrate which would be subjected to further bacteriologic analysis.

This assembled unit, but without the disposable syringe, was surgically wrapped and sterilized in the autoclave at 121°C for 30 minutes. Prior to use, the unit was opened, the sterile disposable
Figure 5. Urine filtration system for the recovery of cell wall-defective forms of bacteria. Key: UI: urine input; T: tubing; NC: nylon connector; Sw1: Swinnex 47 filter holder (10 micron filter); Sw2: Swinnex 47 filter holder (0.45 micron filter); S: sterile, disposable 30 ml syringe.
syringe attached, the tubing placed in the urine specimen, and vacuum filtration initiated. Approximately 10 ml of filtrate was collected in this fashion.

Growth Medium for Cell Wall-Defective Forms of Bacteria:

An agar medium, hereafter referred to as Robb medium (patent applied for), was developed specifically for this aspect of the investigation. The formula somewhat resembles that described by Gutman et al. (1965), but was modified in accordance with our experiences in the laboratory. The formula contains, for each liter of medium prepared, sucrose 125 g, phytone peptone (B-B-L) 15 g, sodium chloride 5 g, MgSO$_4$·7H$_2$O 3 g, yeast extract 15 g, cholesterol 0.06 g (dissolve in 10 ml of 95% ethanol), ionagar #2 (Colab, Chicago Heights, Illinois) 8 g, and distilled water to make 1.0 liter. The pH was adjusted to 7.8 with 1 N sodium hydroxide and the medium was autoclaved at 121°C for 15 minutes. The solution was allowed to cool to approximately 50°C. Subsequently, 10 ml of supplement B (Difco) and 200 ml of human serum albumin (Armour Pharmaceutical Company, Kankakee, Illinois) were added. The medium was mixed and 8 ml aliquots were poured into 60 x 15 mm petri dishes. After several hours at room temperature, six petri dishes were randomly selected from each batch of medium prepared and incubated at 37°C for 18-24 hours in order to insure sterility.

Protocol for Culturing Cell Wall-Defective Forms of Bacteria:

Freshly voided urine specimens, collected and cultured as previously described, were processed additionally as illustrated
in Figure 6. A 0.5 ml aliquot of the unfiltered urine sample was aseptically removed by pipette and was transferred to the surface of Rob\textsuperscript{Kel} medium. The urine was streaked uniformly over the surface of the agar in the standard fashion and the plate was placed aside.

Urine was filtered as outlined above, and from the filtrate, 0.5 ml of urine was aseptically transferred to sheep blood agar (5%) and a second 0.5 ml sample was transferred to Rob\textsuperscript{Kel} medium. The urine was streaked uniformly over the surface of the agar as above.

The three petri dishes were placed in a GasPak anaerobic system (B-B-L) and incubated at 37\textdegree C for a period of 14 days. If no growth was observed during this incubation, the petri dishes were discarded and the procedure was terminated.

To minimize the possibility that some constituent of the Rob\textsuperscript{Kel} medium might inadvertently serve as an inducer of bacterial variants, classical parental forms of 18-24 hour old cultures of Streptococcus faecalis harvested from sheep blood agar (5%) were inoculated to Rob\textsuperscript{Kel} medium and incubated simultaneously with the patient specimens as just described.

**Reversion of the Cell Wall-Defective Forms to Parent Bacteria:**

Colonies growing on Rob\textsuperscript{Kel} medium were examined macroscopically and microscopically. Light microscopy was performed utilizing the low power magnification (x100) of a conventional binocular light microscope. Colonies not suggestive of cell wall-defective forms of bacteria were transferred to sheep blood agar (5%) and were subsequently identified by standard techniques.
Figure 6. Schema for the culture of urine for cell wall-defective forms of bacteria. BA<sup>a</sup>: blood agar.
Colonies morphologically suggestive of cell wall-defective forms of bacteria were harvested aseptically as single colonies in agar blocks. Cubes of agar were cut with a scalpel fitted with a Bard-Parker blade, number 11 (American Safety Razor Company, New York). This manipulative technique occasionally was facilitated by the use of a conventional stereoscopic dissecting microscope under low power. These blocks were aseptically transferred to Mycoplasma broth base (B-B-L) enriched with 1% serum fraction (Difco), and the cultures were incubated in a GasPak anaerobic system (B-B-L) at 37°C and examined at 18-24 hours and again at 48 hours for the presence of turbidity (growth). When growth occurred, sub-cultures were made to sheep blood agar (5%) and other standard laboratory media. Identification of the parent bacterium was accomplished in the usual fashion, employing accepted techniques.

**Induction Medium for Cell Wall-Defective Forms of Bacteria:**

An agar medium, hereafter referred to as RobMel induction medium (patent applied for), was developed specifically for this procedure, and the formula and its preparation is identical to that of RobMel medium, as previously described, but with the exception that it contains 1000 units of buffered potassium penicillin G (Eli Lilly, Indianapolis, Indiana) per milliliter of medium.

**Induction and Light Microscopy of Streptococcus faecalis:**

The prototype organism, *Streptococcus faecalis* (SS 441), was arbitrarily selected for this phase of the investigation. The microorganism was reconstituted from the lyophilized state with
brain heart infusion broth (Difco) as the diluent. At 18-24 hour intervals for 4 days, the culture was passed through a series of blood culture bottles with brain heart infusion (Difco) at 37°C to insure viability. The final passage of the organisms was to sheep blood agar (5%) and was accomplished prior to commencing the induction process. A fresh isolate obtained from a patient and identified as Streptococcus faecalis by the schema previously described was treated in a similar fashion.

The RobMel induction medium was inoculated by means of a sterile cotton swab heavily seeded with the parent organisms harvested after 20 hours of incubation at 37°C under 2-3% carbon dioxide tension (candle jar technique; Bailey and Scott, 1970). After the initial application of the specimen, the surface of the medium was streaked for isolation using a standard bacteriologic wire loop. Replicate plates were prepared and placed in a GasPak anaerobic system (B-B-L) and incubated at 37°C. The specimens were examined daily for 36 days both macroscopically and microscopically using the Zeiss photomicroscope (Carl Zeiss, Oberkochen/Nuertt, West Germany). Photomicrographs, using 35 mm Kodak Pan X film (Eastman Kodak Company, New York), were prepared of unstained colonies at 36 hours and at 3, 6, 12, 25, and 36 days.

Electronmicroscopy of Induced Cell Wall-Defective Forms

Twelve-day old colonies of induced cell wall-defective forms of Streptococcus faecalis, prepared as described above, were fixed in situ according to the following procedure. Glutaraldehyde
(3% in 0.1 M phosphate buffer, pH 7.2) was carefully layered over
the surface of the agar medium at room temperature. The plates were
placed at 4C for 1 hour, after which the glutaraldehyde solution
was carefully removed and the agar surface was rinsed gently with
distilled water. Agar blocks containing isolated colonies were
excised as previously described, and were fixed in 1% osmium
tetroxide in veronal buffer (Kellenberger et al., 1958). After 4C for
3 hours, the blocks were rinsed with distilled water and placed in
5% uranyl acetate in veronal buffer for 2 hours at room temperature.
Subsequently, the blocks were rinsed with distilled water and dehydrat­
ed through a series of graded ethyl alcohol concentrations (50-100%).
The specimens were imbedded in Epon 812 resin (Shell Chemical Company,
New York) as modified from the method of Luft (1961). The blocks
were sectioned longitudinally through the colony with glass knives
in a Reichert Om U2 ultramicrotome (C. Reichert, Vienna, Austria).
Silver sections were placed on formvar-coated 300 mesh copper grids
(Mason and Norton Limited, Harrow, England) and treated with lead
citrate for 7 to 10 minutes (Reynolds, 1963). The grids were
examined in a Zeiss electron microscope EM 9S.

Serum Electrophoresis:

Serum electrophoresis analyses were performed on 300 fasting
sera utilizing the Beckman microzone electrophoresis system (Beckman
Instruments Incorporated, Fullerton, California). This system
consists of the following: Model R-101 microzone electrophoresis
cell; Model R-110 microzone densitometer; microzone accessory kit;
and expendable materials, e.g., membranes, buffer, Ponceau-S fixative-dye solution. Instruction manuals RH-7H-3, "Model R-101 Microzone Electrophoresis Cell" and RH-7E-005, "Model R-110 Microzone Densitometer" are supplied with the system and describe the technical procedures to be employed throughout.

**Total Eosinophil Count (Whole Blood):**

A sample of blood from each patient was diluted with Pilot's solution (Wintrobe, 1967) which selectively stains the eosinophils, renders the erythrocytes non-refractile and invisible, and lyases all leukocytes other than the base-resistant eosinophils. Following mixing, the specimen is introduced into a Fuchs-Rosenthal or Speirs-Levy hemocytometer and the number of eosinophils in a known volume of blood is counted. The exact technique for the enumeration of eosinophils in whole blood is outlined in detail by Wintrobe (1967). The range of normal values for this procedure is from 150 to 300 eosinophils per cubic millimeter of blood.

**Biometric Analysis of Total Eosinophil Counts and Serum Electrophoreses:**

For each of the seven variables associated with the hematologic and serologic study, the arithmetic means and the standard deviations were calculated through the courtesy of the Biometrics Laboratory, Department of Preventative Medicine, The Ohio State University, employing standard methodology (Sokal and Rohlf, 1969). The results subsequently were tabulated.
RESULTS

Quantitative Culture of the Urine:

Because of the repeated visits and multiple sterile urine cultures of the patients assigned to Group 2 with syndromes of non-bacterial etiology, as well as those in Group 3 whose diseases were of presumed bacterial origin, it was felt that the microbiologic procedures, perhaps, were not offering the clinician sufficient laboratory information. Therefore, the culture methods of Marraro et al. (1970a), developed to improve the frequency of recovery of microorganisms from urine specimens, were employed (Materials and Methods). Accordingly, a possibility of three atmospheric environments was afforded these organisms for primary isolation (Tables 1-4) and it must be emphasized that these environments are essential to this technique. When bacteria were recovered under aerobic conditions, quantitated, and identified, the procedure was considered complete, in a practical sense, and no further laboratory manipulations were carried out unless the patient failed to respond to appropriate urologic management. In all instances in which there was no observable growth under aerobic conditions, the fluid thioglycollate medium was examined closely for the presence of microorganisms. Growth was restricted to the mid- or interface zone of the fluid thioglycollate medium and
appeared as a narrow, hazy band. Organisms recovered in this manner were termed microaerophilic and subculturing presented no difficulty. At this point, further diagnostic procedures were carried out as previously described and the investigation was completed. In instances in which there was no observable growth either in the aerobic or microaerophilic environments on primary isolation, the media incubated in the GasPak anaerobic system were examined and microorganisms, if present, were processed as previously described and were reported as anaerobes.

The urine specimens from 50 male and 50 female patients assigned to Group 1 and serving as the asymptomatic "negative" control group were cultured. The types of organisms recovered are presented in Table 1, where it may be noted that 62 isolates recovered aerobically were distributed among nine genera or groups of microorganisms. The fluid thioglycollate medium supported the growth microaerophilically of an additional 95 isolates divided among nine genera or groups of organisms. There were no anaerobic bacteria recovered on primary isolation from these individuals. It is apparent then, that the availability of a microaerophilic environment for the primary isolation of these microorganisms permitted an increase of approximately 153% in the number of isolates recovered. The genera of bacteria recovered from these urine specimens are those generally associated with specimens obtained from the genitourinary tract and therefore require no further comment.
TABLE 1

Microorganisms recovered from the genitourinary tract of Group 1 patients (50 male and 50 female).

<table>
<thead>
<tr>
<th>Atmospheric Environments (primary isolation)</th>
<th>Number of Times Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td><strong>Aerobic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td>6</td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td>3</td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td>4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
</tr>
<tr>
<td>Klebsiella-Enterobacter</td>
<td>0</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>14</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>1</td>
</tr>
<tr>
<td><strong>Microaerophilic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td>11</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>3</td>
</tr>
<tr>
<td>Klebsiella-Enterobacter</td>
<td>2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>0</td>
</tr>
<tr>
<td>Proteus rettgeri</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus, coagulase positive</td>
<td>3</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>16</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>4</td>
</tr>
<tr>
<td><strong>Anaerobic:</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
The category designated as Group 2 was composed of 50 male and 50 female patients. The urine specimens from these patients were subjected to the culture protocol as described, and the results of these procedures are presented in Table 2. Under aerobic atmospheric conditions, 62 isolates were distributed among eleven genera or groups of microorganisms. Ninety-three isolates were obtained from the fluid thioglycollate medium and were representative of twelve genera or groups of organisms. Eight isolates were obtained from the anaerobic system and were distributed among five genera of microorganisms. The data indicate that the number of isolates recovered was increased by approximately 163% when both the microaerophilic and anaerobic environments were employed. It must be emphasized that although the same type of microorganism was isolated under different atmospheric environments, it is not a different species of organism. It is different only in the sense that, upon primary isolation, it requires a specific atmosphere for cultivation.

Urine specimens from the 50 male and 50 female patients assigned to Group 3 were processed in the same manner and the results (Table 3) indicated that the 58 isolates recovered under aerobic conditions were distributed among eleven genera of microorganisms. The microaerophilic environment permitted the recovery of an additional 107 isolates (thirteen genera or groups of bacteria), while 9 isolates were recovered from the anaerobic system and were distributed among six microbial genera. The
Macroorganisms recovered from the genitourinary tract of Group 2 patients (50 male and 50 female).

<table>
<thead>
<tr>
<th>Atmospheric Environments (primary isolation)</th>
<th>Number of Times Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td><strong>Aerobic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td>0</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>0</td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td>0</td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6</td>
</tr>
<tr>
<td>Klebsiella-Enterobacter</td>
<td>4</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>6</td>
</tr>
<tr>
<td>Staphylococcus aureus, coagulase positive</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>6</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>0</td>
</tr>
<tr>
<td><strong>Microaerophilic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td>5</td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td>0</td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2</td>
</tr>
<tr>
<td>Klebsiella-Enterobacter</td>
<td>1</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>5</td>
</tr>
<tr>
<td>Proteus rettgeri</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>15</td>
</tr>
<tr>
<td>Streptococcus agalactiae (Group B)</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>8</td>
</tr>
<tr>
<td>Streptococcus faecium</td>
<td>0</td>
</tr>
<tr>
<td><strong>Anaerobic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td>1</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>0</td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td>0</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
</tr>
<tr>
<td>Gamma streptococci (not Group D)</td>
<td>2</td>
</tr>
<tr>
<td>Atmospheric Environments (primary isolation)</td>
<td>Number of Times Isolated</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>Aerobic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td></td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus, coagulase positive</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td></td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td></td>
</tr>
<tr>
<td>Streptococcus faecium</td>
<td></td>
</tr>
<tr>
<td><strong>Microaerophilic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td></td>
</tr>
<tr>
<td>Bacillus species</td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td></td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>Klebsiella-Enterobacter</td>
<td></td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus, coagulase positive</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td></td>
</tr>
<tr>
<td>Streptococcus durans</td>
<td></td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td></td>
</tr>
<tr>
<td>Streptococcus faecium</td>
<td></td>
</tr>
<tr>
<td><strong>Anaerobic:</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha hemolytic streptococci (not Group D)</td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td></td>
</tr>
<tr>
<td>Citrobacter group</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus bifidus</td>
<td></td>
</tr>
</tbody>
</table>

Mid: mid-stream urine specimen; Cath: catheterized urine specimen.
number of isolates attributable to the availability of the microaerophilic and anaerobic environments increased by approximately 18.4% the total number recovered. The catheterized urine specimens from the female patients assigned to this group were cultured as described above, but the data were tabulated separately. It may be seen in Table 3, that 7 isolates were recovered from the aerobic atmosphere, 14 from the microaerophilic environment, and 3 isolates from the anaerobic system. These data represent an increase of approximately 24.3% in the number of isolates recovered. These data also indicate a higher frequency of isolation from the female mid-stream urine specimens than was achieved from the catheterized specimens.

The data, as presented in Tables 1, 2 and 3 also reveal the frequency with which each microorganism was recovered upon primary isolation from each of the three atmospheric conditions. Summarized in Table 4 is a comparison of the effectiveness of atmospheric environments upon the recovery of organisms. In the Group 1 Male, it will be noted that 29 isolates, representing 43% of the total number, were recovered in an aerobic environment, 39 isolates (57% of the total number) were recovered from a microaerophilic atmosphere, and no organisms were recovered from the anaerobic system. Although the figures varied for the number of isolates recovered from the Group 2 and Group 3 Male patient population, percentage values designating the efficiency of the various atmospheres were found to be somewhat similar. Additionally, isolates were recovered from the anaerobic
TABLE 4

Comparison of the effectiveness of atmospheric environments upon the number of times microorganisms are recovered on primary isolation

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td>Aerobic</td>
<td>29-43%</td>
<td>24-36%</td>
</tr>
<tr>
<td>Microaero&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39-57%</td>
<td>38-58%</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0</td>
<td>4-6%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Microaero: microaerophilic atmosphere; <sup>b</sup> Mid: mid-stream urine specimen; <sup>c</sup> Cath: catheterized urine specimen.
environment in the latter two groups. In the Group 1 Female, 33 isolates, representing 37% of the total number, were cultivated aerobically, 56 isolates (63% of the total) were retrieved from the microaerophilic environment, and no organisms were recovered from the anaerobic system. It became apparent that although the numbers of isolates recovered from the Group 2 and Group 3 Female patients differed, percentage values showed the same inter-group similarity as was previously described regarding the male patients. The specimens from these latter two groups of female patients did harbor organisms which were recoverable with the anaerobic system and these data are comparable to those obtained with the corresponding groups of male patients. Although fewer organisms were recovered from the Group 3 Female catheterized urine specimens than from the corresponding mid-stream urine specimens, 7 isolates, representing 29% of the total number, were recovered from the aerobic atmospheric environment. Fourteen isolates (59% of the total) were recovered from a microaerophilic environment, and 3 organisms were isolated from the anaerobic system.

Of further interest were the data obtained regarding the frequency with which one or more genera of microorganisms were isolated from each patient in the three study groups (Table 5). Thirty (60%) of the fifty Group 1 Male patient specimens were found to harbor but a single genus of bacteria; 11 (22%) had two genera; urines from 2 (4%) exhibited three genera; and 7 (14%) of the patients were free of organisms, or at least no organisms were
### TABLE 5

Frequency with which multiple genera of microorganisms were isolated from each patient of the three study groups.

<table>
<thead>
<tr>
<th>Frequency of genera</th>
<th>Group 1 (50)</th>
<th>Group 2 (50)</th>
<th>Group 3 (50)</th>
<th>Group 1 (50)</th>
<th>Group 2 (50)</th>
<th>Group 3 Mid Cath (50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>30-60%</td>
<td>28-56%</td>
<td>29-58%</td>
<td>16-32%</td>
<td>17-34%</td>
<td>15-30% 18-36%</td>
</tr>
<tr>
<td>Two</td>
<td>11-22%</td>
<td>13-26%</td>
<td>10-20%</td>
<td>21-42%</td>
<td>22-44%</td>
<td>22-44% 3-6%</td>
</tr>
<tr>
<td>Three</td>
<td>2-4%</td>
<td>0</td>
<td>2-4%</td>
<td>9-18%</td>
<td>8-16%</td>
<td>9-18% 2-4%</td>
</tr>
<tr>
<td>Four</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1-2%</td>
<td>2-4%</td>
<td>1-2% 0</td>
</tr>
<tr>
<td>Sterile</td>
<td>7-14%</td>
<td>9-18%</td>
<td>9-18%</td>
<td>3-6%</td>
<td>1-2%</td>
<td>3-6% 27-54%</td>
</tr>
</tbody>
</table>

(50): number of patients (50) per group; Mid: mid-stream urine specimen; Cath: catheterized urine specimen.
recovered by these procedures. By employing this type of analysis, there exists, regardless of the group to which the patient has been assigned, a rather similar frequency distribution of the number of genera of microorganisms isolated from the male patients. In the Group 1 Female patient category, urines from 16 (32%) of the 50 patients harbored but one genus of bacteria; 21 (42%) had two genera; 9 (18%) had three genera; 1 (2%) had four genera; and the urines of 3 (6%) of the individuals were sterile. Thus, among the female patients, regardless of the clinical condition, the number of genera of microorganisms recovered displayed a quite similar pattern. However, it should be noted that the data, in the instance of the Group 3 catheterized urine specimens, suggest a different pattern of frequency distribution. There were decreases in the number of patients harboring two and three genera of bacteria per urine specimen and an increase in the number of sterile urine specimens.

Induction and Light Microscopy of Cell Wall-Defective Forms of Bacteria:

Because of the recent suggestions by several investigators (Guze, 1968; McGee and Wittler, 1969; Sharp, 1970) that cell wall-defective forms of bacteria may be involved in certain urologic disease states, a study was undertaken to establish the morphologic parameters of these variants to serve as references during the remainder of this investigation.

The prototype organism, *Streptococcus faecalis* (SS 441), as well as a fresh isolate of this organism serving as a control, were
cultivated as previously described (Materials and Methods). Colonies were examined by light microscopy at specified intervals during a 36 day period.

The results obtained by light microscopy clearly established the progress of change in colony morphology which occurred in these bacterial variants during the test period.

It was confirmed that the convertability of the parent organism to a cell wall-defective form was related to the age of the culture since these aberrant bacterial forms could not be obtained from parent bacteria which were still in the early log phase of growth. This observation is in agreement with that of Young and Armstrong (1969) and, as a phenomenon, possibly may be attributed to the accelerated rates of bacterial metabolism and/or the stage of cell wall development during this phase of growth. The parental colonies selected were harvested from 20-hour old cultures and induction of these bacteria to cell wall-defective forms posed no technical difficulties in an anaerobic environment and on an agar medium containing penicillin (RobMel induction medium). It should be noted that the induction of defective forms was accomplished in one passage from the original parental prototype, and also from the fresh isolate. Similarly, reversion of the aberrant forms to the parent organism was readily achieved in a single passage after 18-24 hours incubation in PFLO broth containing 1% serum fraction.

No significant morphologic differences were noted by light microscopy between the prototype organism and the recent isolate.
The colonies shown in the following photomicrographs represent sequential development of the cell wall-defective forms of the prototype organism.

The control colony of *Streptococcus faecalis* (Plate III) was 0.54 mm in diameter and represented a 24 hour growth on brain heart infusion agar (Difco) at 37°C and under anaerobic conditions. This normal, mature colony was smooth, entire, and convex with some surface undulations.

On the plates in which the induction of these forms was carried out, "cartwheel" structures were seen at 36 hours (Plate IV). These early manifestations of "L-form" types of colonies have been previously described by Young and Armstrong (1969) in their study of *Streptococcus liquefaciens*. These young colonies exhibited a circular internal arrangement and an irregular margin attributed to the presence of spherical or globular subunits (S). These subunits (S) and those (S₁) presented in subsequent photomicrographs, and which appeared as structures of different sizes are not necessarily to be construed as identical, nor is it implied that they represent a direct developmental progression of one form to the other. They are identified only as repeating subunits in each instance. The diameter of the colony photographically depicted is approximately 0.1 mm.

The culture observed at 3 days (Plate V) revealed a slightly more advanced type of colony which measured 0.31 mm in diameter. The edge of this colony was not well-defined and coarse granulation
Plate III. A control colony of *Streptococcus faecalis* after 24 hours incubation at 37°C under anaerobic conditions. x 1250.

Plate IV. "Cartwheel" L-form structures seen on induction medium at 36 hours. These colonies exhibit an irregular margin and globular subunits (S). x 1500.
Plate V. Early L-form colony (3 days) showing the presence of spherical or globular subunits (S*) and an inner ring of darker contrast (I). x 1000.

Plate VI. After 6 days of incubation "cartwheel" structures with subunits (S) were still evident and a typical L-form colony showing a distinct inner ring (I) and granular appearance were seen. x 2000.
again was noted. The colony appeared to be constructed of spherical or globular subunits ($S^1$), as were the younger colonies shown in Plate IV. An area (I) was noted and appeared as an inner ring of darker contrast.

After 6 days of incubation, "cartwheel" structures were still discernible (Plate VI) with subunits ($S^1$) as previously described (Plate IV). The diameter of this structure was approximately 0.03 mm. Additionally, a typical "fried egg" colony demonstrating a distinct inner ring (I) and a granular appearance was noted.

The culture observed after 12 days demonstrated many colonies which presented the classical "fried egg" L-form appearance (Plate VII), with distinct granular surfaces in which the inner rings were compact and clearly defined.

At 25 days, the colony (Plate VIII) had a broad and not well-defined margin (M) with large, spherical subunits ($S^1$) scattered throughout. The inner ring (I) was no longer well-defined and the diameter of the colony was 0.47 mm.

A colony, incubated for 36 days, with a highly diffuse vacuolated margin containing large, spherical subunits ($S^1$) was observed and is shown in Plate IX.

Electron Microscopy of Induced Cell Wall-Defective Forms of Streptococcus faecalis:

The observations made during the light microscopy study of the morphogenesis of these forms suggested a further investigation utilizing thin section techniques. Therefore, 12-day old colonies of *Streptococcus faecalis* were selected from RobMel induction
Plate VII. L-form colony after 12 days incubation still shows "fried egg" appearance with a distinct granular surface. x 2200.

Plate VIII. L-form colony after 25 days incubation showed a broad not well-defined margin (M) containing some large spherical or globular subunits (S). The inner ring (I) is less well-defined. x 1050.
Plate IX. A 36 day colony containing a highly diffuse vacuolated margin with large subunits (S1). x 1500.
medium and processed as described in an earlier section. Progressive longitudinal thin sections of the intact epoxy-embedded colony from the deep sub-agar level through the supra-agar surface were examined and photographed. These profiles showed marked differences in the ultrastructure of the bacterial cells at the three levels selected for the study.

The bacterial cell population examined from the deep sub-agar level was composed of cells which were, for the most part, lysed and assumed to be non-viable. Plate X illustrates a typical population of "ghost cells" which were found with regularity throughout this area. The absence of ribosomes, the lack of cellular organization, the dispersion of the nuclear apparatus, and the break-down of the plasmalemma (P) offered evidence to support the concept that these "ghost cells" were non-viable. The cell walls were completely absent in these cells. Within the nucleoid area (N), threads of nuclear material (DNA strands) were visible (D). In Plate XI, higher in the deep sub-agar level, a cell wall-defective cell was observed to be surrounded by "ghost cells". Within the plasmalemma (P) is located a peripheral mass of ribosomes (R) and a centrally located nucleoid region (N) containing DNA strands. Presumably, this cell represents a late stage of a cell wall-defective cell which is in the process of becoming non-viable and will soon become a "ghost cell". Normal cells were not found in this area.

Progressing to the sub-agar surface level, the cell population
Plate X. Electron micrograph of a longitudinal thin section of a 12-day old L-form colony taken from the basal layer of the deep sub-agar level. Within the plasmalemma (P) of the "ghost cell" is the nucleoid area (N) and DNA strands (D). x 22,000.
Plate XI. Electron micrograph of a longitudinal thin section of a 12-day old L-form colony taken from a plane higher in the deep sub-agar level. A cell wall-defective cell is surrounded by a plasmalemma (P) and the ribosomes (R) as well as the nucleoid area (N) are well-defined. x 21,400.
was a mixture of morphologic forms. The "ghost cells" (G), cell wall-defective cells (CWDC), and normal bacterial cells (NC) were observed at this level (Plates XII and XIII). The cell wall-defective cell (CWDC) in Plate XII presents evidence to support the fact that there is a progressive loss of the normal bacterial cell wall (CW) during the process of induction. Internal components of both the "ghost cells" and the cell wall-defective cells have been described above.

At the supra-agar surface level in the area of the elevated dome of the colony (Plate XIV), a mixture of cell types was again apparent, but a higher proportion of normal bacterial cells was noted. Also present were "ghost cells", but in lesser numbers when compared with those observed in the sub-agar and deep sub-agar colony levels. Similarly, fewer cell wall-defective cells were encountered. Of interest in this area of the colony was the frequency with which acellular zones (A and B) were observed and which were presumed to be those areas in the periphery of the colony shown in Plate IX, a concept discussed by Marraro et al. (1971).

A schematic representation of the total 12-day old induced cell wall-defective form of Streptococcus faecalis as revealed by electron microscopy is depicted in Figure 7. The deep sub-agar level (DS) was composed mainly of "ghost cells"; the sub-agar surface level (SUB) was a mixture of cell types, including some "ghost cells" and cells with ribosomes and compacted DNA, cell wall-defective forms, and normal bacterial cells. It will be
Plate XII. Electron micrograph of a longitudinal thin section of a 12-day old L-form colony taken from the sub-agar surface level. A cell wall-defective cell (CWDC) is noted with its receding cell wall (CW). A ghost cell (G) is also observed. x 30,400.
Plate XIII. Electron micrograph of the same colony level as in Plate XII. Normal bacterial cells (NC), cell wall-defective cells (GWDC), and ghost cells (G) are noted. x 22,100,
Plate XIV. Electron micrograph of a longitudinal thin section of a 12-day old L-form colony taken from the supra-agar surface level. A mixture of cell types is again apparent. Acellular zones designated A and B are observed and a linear channel (C) is noted, x 4,800.
Figure 7. A schematic representation of a 12-day old L-form colony. S: supra-agar surface; SUB: sub-agar surface; DS: deep sub-agar; NC: normal cell; AZ: acellular zone; CWDC: cell wall-defective cell; G: ghost cell.
noted that there was a sub-surface compactness of cells, and it was presumed that these cells were less viable and represented an "older" population. The supra-agar surface level (S) contained a mixture of cell types, but a large percentage of "cartwheel" forms and normal bacterial cells were quite apparent.

Culture of Cell Wall-Defective Forms of Bacteria from Urine:

During the past decade, the literature has become replete with accounts of the role of the cell wall-defective forms of bacteria in the diseases of man (Guze, 1968; McGee and Witter, 1969; Sharp, 1970). These data, no longer to be ignored, suggested the need for diagnostic protocols for the direct recovery of these forms from clinical specimens. RobMel medium, as previously described, satisfactorily served for the growth and maintenance of these cell wall-defective forms and procedures for the isolation and identification of the variants recovered were developed.

Having established the reference criteria for the cell wall-defective forms of bacteria, freshly voided urine specimens were collected and cultured as previously described (Figures 1-3). Further, the specimens were processed for the recovery of cell wall-defective variants (Figure 6).

There was some concern that the RobMel medium developed for this study might inadvertently contain some ingredient which would induce bacterial variants. Accordingly, a control culture was prepared as previously described and, after 14 days of incubation, no aberrant bacterial forms were noted either macro- or
microscopically. The colonies harvested from this medium were identified readily by routine laboratory procedures as *Streptococcus faecalis*.

Because of the equivocal information existing in the literature regarding the ability of these aberrant forms to either pass through or to be withheld by filter systems of varying pore sizes (Roberts, 1968; Rose, 1969), all urine specimens were processed in parallel (Figure 6). The culturing of these specimens both with and without subjecting them to vacuum serial filtration established that filtration resulted in a 37% decrease in the recovery rate of these forms. Also, in no instance was a cell wall-defective form of a bacterium recovered from the filtered urine and not from the unfiltered counterpart of the same specimen.

The recovery of cell wall-defective forms of bacteria from urine specimens from the patients in the study groups using both the unfiltered and filtered samples is summarized in Table 6. It will be noted that a total of 24 cell wall-defective forms of bacteria were isolated (one variant from each of 24 patients), representing an 8% recovery rate for the entire patient population. On the other hand, these 24 isolates of bacterial variants represent approximately 3% of the total number of all the bacterial forms recovered. The percentage recovery rate of these aberrant forms of bacteria was almost identical from both the male and female patients assigned to Groups 1 and 2. In terms of actual percentage, it will be seen that the recovery rate of these forms was 2% for the Group
TABLE 6

Recovery of cell wall-defective forms of microorganisms from unfiltered and filtered urine specimens of 300 patients.

<table>
<thead>
<tr>
<th>Group/Sex</th>
<th>Total Number of Patients in the Study Harboring these Isolates</th>
<th>Number of Isolates Recovered</th>
<th>Total</th>
<th>Unfiltered</th>
<th>Filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Group 2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Group 3:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

|          | 24                                                            | 24                          | 24    | 15        |          |
1 Male, the Group 1 Female, and the Group 2 Male, whereas a 4% recovery rate was experienced for the Group 2 Female. In contrast, an 18% recovery rate was accomplished for the Group 3 Male patients and a 20% frequency of recovery was noted for the Group 3 Female category.

The reversion of the isolated cell wall-defective forms of microorganisms to their classical parental forms was accomplished readily when the reversion procedure, as described (Materials and Methods), was enforced. This suggests that no stable cell wall-defective forms were isolated from the patients in the study groups, and that all forms recovered could be reverted to their parental forms and thus identified (Table 7). Of interest, also, was the frequency with which certain aberrant forms were recovered, e.g. Streptococcus faecalis - 10; Staphylococcus epidermidis - 5; Corynebacterium species (diphtheroids) - 2; alpha hemolytic streptococci (not Group D) - 2; Escherichia coli - 1; Klebsiella-Enterobacter - 1; Proteus mirabilis - 1; Staphylococcus aureus, coagulase positive - 1; and Streptococcus faecium - 1.

During this phase of the investigation, a phenomenon was observed which apparently resulted from a parent form of Streptococcus faecalis inhibiting the growth of the cell wall-defective form of the organism. It seemed significant that both the parental form of the streptococcus and the variant were isolated simultaneously and directly from a urine specimen without the purposeful intervention of laboratory manipulations such as the introduction of
TABLE 7

Reversion of the isolated cell wall-defective forms of microorganisms to their classical parental forms.

<table>
<thead>
<tr>
<th>Group/Sex</th>
<th>CWDF No.</th>
<th>Parental Form</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td>Alpha hemolytic streptococci (not Group D)</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>Streptococcus faecalis</td>
</tr>
<tr>
<td><strong>Group 2:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>Corynebacterium species (diphtheroids)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Klebsiella-Enterobacter</td>
</tr>
<tr>
<td><strong>Group 3:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6-8</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td></td>
<td>9-12</td>
<td>Streptococcus faecalis</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Proteus mirabilis</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Alpha hemolytic streptococci (not Group D)</td>
</tr>
<tr>
<td>Female</td>
<td>15-19</td>
<td>Streptococcus faecalis</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Streptococcus faecium</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>Corynebacterium species (diphtheroids)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Staphylococcus aureus, coagulase positive</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

CWDF No.\textsuperscript{a}: cell wall-defective form identification number.
"inducers" into the medium. The urine specimen was obtained from a Group 3 Female patient (E.P.) suffering from chronic urologic disease diagnosed as urethritis. After processing the specimen as described earlier, it was noted that on the fourth day of incubation, the plate exhibited three microbial forms (Plate XV), one of which subsequently proved to be Staphylococcus aureus, coagulase positive. A second white colony of approximately 2 mm in diameter and with a discrete edge was identified as Streptococcus faecalis. The third microbial form grossly appeared as a hazy, lawn-like growth, and microscopically as typical "fried egg" L-form colonies as classically described. These colonies were reverted readily to a characteristic enterococcal parent form upon transfer to other media as previously described, and were identified as Streptococcus faecalis. In an area of the medium immediately surrounding the classical colonial parent form, there were observed areas measuring approximately 6 mm in diameter in which the growth of the L-form colonies was inhibited. It will be observed in Plate XV that the inhibition just described was not characteristic of but a single colony, but involved a number of colonies. The possibility exists that this represented a depletion of nutriments necessary for the cultivation of L-forms, or that the accumulation of toxic metabolic products interfered with colonial development. However, this phenomenon may be attributable to an exocellular substance, bacteriocin-like in nature, which may chemically and biologically have accounted for these observations. Recently, Kalmanson et al. (1970) reported two
Plate XV. RobMel medium showing growth of *Staphylococcus aureus*, coagulase positive (S); parent form of *Streptococcus faecalis* (P); L-form of *Streptococcus faecalis* (L); zone of growth inhibition (Z). x 1.33.
instances in which laboratory strains of a streptococcal L-form were susceptible to bacteriocin produced by its parent bacterium. The phenomenon reported here was not laboratory induced and was presumed to be more directly associated with the in vivo conditions which existed in the patient (Marraro et al., 1970b).

While culturing the urine specimens from 300 patients on RobMel medium for the purpose of recovering cell wall-defective forms of bacteria, it was noted that the medium satisfactorily supported growth of the classical parental forms of bacteria. The genera of microorganisms recovered and the frequency with which each was isolated, using the patients assigned to Group 3 as the model system, are summarized in Table 8. Because of the seemingly increased number of isolates recovered, a survey of the data was initiated to compare the efficiency of the medium employed here with those recommended in the procedure described by Marraro et al. (1970a) for their capacity to support the growth of classical bacterial forms. The results are presented in Tables 9 and 10 using the patients assigned to Group 3 as the experimental design and collating the data obtained here and in Tables 3 and 5 for comparison. Apparently, the use of RobMel medium very significantly increased the frequency with which organisms were recoverable from the urine specimens of these patients (Table 9). Further, this medium also supported the growth of Proteus mirabilis and Streptococcus agalactiae (Group B), two microorganisms which were not recovered utilizing the media employed in the other procedure. Additionally,
TABLE 8

Microorganisms recovered from the genitourinary tract of Group 3 patients (50 male and 50 female).

<table>
<thead>
<tr>
<th>Organism Recovered (RobKel Medium)</th>
<th>Number of Times Isolated</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mid^a</td>
<td>Cath^b</td>
</tr>
<tr>
<td>Alpha hemolytic streptococci</td>
<td></td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>(not Group D)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus species</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Citrobacter group</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corynebacterium species (diphtheroids)</td>
<td>3</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Diplococcus pneumoniae</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>19</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>Klebsiella-Enterobacter</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lactobacillus bifidus</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>5</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Proteus rettgeri</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus, coagulase positive</td>
<td>7</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>35</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td>Streptococcus agalactiae (Group B)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus durans</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>15</td>
<td>32</td>
<td>13</td>
</tr>
<tr>
<td>Streptococcus faecium</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Mid^a: mid-stream urine specimen; Cath^b: catheterized urine specimen.
TABLE 9

Comparison of the efficiency of culture techniques on the recovery of microorganisms.

<table>
<thead>
<tr>
<th>Group/Sex</th>
<th>Culture Technique/Number of Isolates</th>
<th>Percentage Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Harraro et al. (1970a) RobMel Medium</td>
<td></td>
</tr>
<tr>
<td>Group 3-Male</td>
<td>72</td>
<td>103</td>
</tr>
<tr>
<td>Group 3-Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid(^a)</td>
<td>102</td>
<td>134</td>
</tr>
<tr>
<td>Cath(^b)</td>
<td>24</td>
<td>74</td>
</tr>
</tbody>
</table>

Mid\(^a\): mid-stream urine specimen; Cath\(^b\): catheterized urine specimen.
TABLE 10

Comparison of the effectiveness of culture techniques on the reduction of sterile urines.

<table>
<thead>
<tr>
<th>Group/Sex</th>
<th>Culture Technique/Number of Sterile Urine Specimens</th>
<th>Percentage Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marraro et al. (1970a) RobMel Medium</td>
<td></td>
</tr>
<tr>
<td>Group 3-Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Group 3-Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid(^a)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cath(^b)</td>
<td>27</td>
<td>10</td>
</tr>
</tbody>
</table>

Mid\(^a\): mid-stream urine specimen; Cath\(^b\): catheterized urine specimen.
the use of RobMell medium very significantly increased the opportunity
to recover and report microorganisms from a larger number of urine
specimens which previously had been reported as being sterile
(Table 10).

Speciation of the Group D Streptococci (Enterococci):

The results obtained from the cultivation of microorganisms
recovered from the human genitourinary tract revealed that certain
members of the Lancefield Group D streptococci (Lancefield, 1933;
Lancefield, 1938) often were isolated. In view of this, the need
for a simple, rapid, and yet reliable schema for the differentiation
of the members of the enterococci became apparent. Within the last
decade, a more definitive speciation within the enterococcus
division as previously defined by Sherman (1937) has been suggested
(Deibel et al., 1963). With this in mind, the literature was re-
viewed for existing diagnostic protocols, but no one specific
schema appeared to be entirely satisfactory for the differentiation
of the enterococci. Therefore, a protocol was developed from the
literature for the differentiation of the Group D streptococci
based on the biochemical and physiological characteristics of the
prototype organisms. This schema was used throughout this investi-
gation and has been previously described (Figure 4).

It is recognized that there are five microorganisms classified
as members of the Group D streptococci: Streptococcus faecalis;
Streptococcus faecalis var. liquefaciens; Streptococcus faecalis
var. zymogenes; Streptococcus faecium; and Streptococcus durans.
Since differentiation, in some instances, is based solely upon a specific hemolytic pattern or upon the ability of the organism to liquefy gelatin, there purposefully has been no attempt to distinguish among *Streptococcus faecalis*, *Streptococcus faecalis var. liquefaciens*, and *Streptococcus faecalis var. zymogenes*. These organisms have been grouped and reported, for clinical purposes, simply as *Streptococcus faecalis*.

The first consideration in the speciation of these microorganisms was the hemolytic pattern observed after 18-24 hours of aerobic incubation on 5% sheep blood agar at 37°C. The terms alpha, beta or gamma used to report the hemolytic patterns, or absence thereof, of the microorganisms were as described originally by Brown (1919). *Streptococcus faecalis var. zymogenes* and *Streptococcus faecium* were alpha hemolytic and the typical "greening" effect was noted. *Streptococcus durans* was the sole prototype culture which demonstrated beta hemolysis with a characteristic clear, cell-free zone of hemolysis surrounding the colony. *Streptococcus faecalis* and *Streptococcus faecalis var. liquefaciens* produced no change in the blood agar—neither hemolysis nor "greening" -- and therefore were classified as gamma streptococci.

Of interest also were the morphological and tinctorial properties displayed by this group of microorganisms. The organisms were morphologically compatible with their classical description and exhibited gram-positive staining characteristics. It was noted that the individual cocci frequently appeared in a characteristic lanceolate shape.
Without exception, the prototype organisms inoculated into S F medium, a selective broth for the enterococci, fermented the dextrose in the medium and effected a change in the indicator (bromthymol blue) from a purple to a yellowish color. Similarly, these organisms, when inoculated to 0.1% methylene blue milk (Sherman, 1937; Bailey and Scott, 1970) fermented lactose and reduced methylene blue. A positive reaction is evidenced by a change in the color of the skim milk medium from a purple or blue to a white, indicating that the methylene blue has served as an electron acceptor during the fermentation of lactose and has itself been reduced.

Having established the microorganisms as members of the Group D enterococci, the schema further employed triphenyltetrazolium chloride medium (Barnes, 1956; Whittenbury, 1965; Facklam, 1969). Upon inoculation and aerobic incubation for 18-24 hours, 48 hours, or 72 hours at 37°C, some colonies develop a deep magenta to red color, or, contrarily, remain colorless or at most develop a faint pink hue. Strains of fecal streptococci vary in their ability to reduce tetrazolium to formazan in a glucose-containing medium at an initial pH of 6.0. Therefore, the procedure takes advantage of the difference in the reducing properties of these species, and, as a result, 2,3,5 triphenyltetrazolium chloride has been used as a redox indicator which is colorless in its oxidized form and red in its insoluble reduced state (triphenylformazan). Colonies of Streptococcus faecalis, Streptococcus faecalis var. liquefaciens,
and *Streptococcus faecalis* var. *zynogenes* will develop a deep magenta to red color indicating that reduction has taken place. Colonies of *Streptococcus faecium* and *Streptococcus durans* remain colorless or faint pink, attesting to the fact that the indicator has remained in its oxidized form.

*Streptococcus faecium* and *Streptococcus durans* are quite readily distinguished on the basis of carbohydrate fermentation (Sharpe et al., 1966). *Streptococcus faecium* utilizes arabinose, mannitol, and melibiose, whereas *Streptococcus durans* does not. In order to insure against reversion of the reaction due to the depletion of the carbohydrate, 1% of the sugar concentrations were prepared in phenol red broth base and "positive" fermentation reactions were readily discernible by the change in the color of the phenol red indicator from pink or red to yellow.

This schema for the speciation of the members of the Group D streptococci (enterococci) employed throughout this investigation has proven to be simple, rapid and reliable, with the great majority of microorganisms identified within 18-24 hours, and the remainder requiring not more than 48 hours. All enterococci were harvested from RobMol medium and the speciation of these microorganisms is presented in Table 11. Of some interest was the observation that no single patient specimen harbored more than one species of enterococcal microorganism at the time of the culture. Generally speaking, it is evident that 32 of 150 male patients (approximately 21%) carried a recoverable enterococcal organism,
**TABLE 11**

Speciation of Group D streptococci (enterococci) recovered from 300 patients.

<table>
<thead>
<tr>
<th>Organisms Recovered from RobMel Medium</th>
<th>Group 1</th>
<th></th>
<th>Group 2</th>
<th></th>
<th>Group 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>6</td>
<td>21</td>
<td>8</td>
<td>20</td>
<td>15</td>
<td>32</td>
</tr>
<tr>
<td>Streptococcus faecium</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Streptococcus durans</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total Number</td>
<td>6</td>
<td>21</td>
<td>8</td>
<td>22</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Percentage/50^a</td>
<td>12%</td>
<td>42%</td>
<td>16%</td>
<td>44%</td>
<td>36%</td>
<td>72%</td>
</tr>
</tbody>
</table>

Percentage/50^a: the percentage is based on the fact that a major group is composed of 50 males and 50 females.
whereas 79 of 150 female patients (approximately 53%) harbored these microorganisms. This represents approximately a two and one-half fold increase in the recovery rate of the Group D streptococci from the female patients as compared with the percentage recovery from the males. Additionally, of the 111 enterococcal organisms recovered, 102 (approximately 92%) were identified as *Streptococcus faecalis*; 8 (approximately 7%) were *Streptococcus faecium*; and 1 (less than 1%) was reported as *Streptococcus durans*.

**Biometric Analysis of Total Eosinophil Counts and Serum Electrophoreses:**

While conducting the physical examinations on the patients selected for this investigation, certain findings appeared with regularity during cystoscopy. The examining urologist (L.B.) was able to establish that cystoscopic examinations, when performed on the patient population assigned to Groups 1 and 2, failed to reveal changes consistent with acute or chronic inflammation of the urethra, trigone, or bladder. In contrast, the cystoscopic findings of the Group 3 Male patient category invariably reflected some degree of abnormality in the prostatic urethra, specifically an edematous surface, and occasionally the trigone was inflamed. Cystoscopic findings of the Group 3 Female patients regularly revealed chronic granular changes of the mucosa of the trigone, bladder neck, and proximal urethra. Because of the presumed microbial etiology of these disease states and evidence offered by the cystoscopy procedure, a clinical impression developed on the part of the urologist (L.B.) based on previous experience. It was felt that there may exist
a possible inflammatory or allergic phenomenon in these patients
due to the microorganisms involved.

Accordingly, the values obtained from the serum electrophoresis
patterns for total protein, albumin, alpha-1-globulin, alpha-2-
globulin, beta globulin, and gamma globulin along with the data
obtained from the total eosinophil counts were analyzed as previously
described (Materials and Methods). Table 12 summarizes the arith-
metic means and standard deviations of these factors with comparisons
as to disease group and sex. Table 13 summarizes identical data,
but offers the comparisons as to sex and disease group for the
convenience of the reader.

In reviewing the values obtained for the concentration of
total protein, it became apparent that all males in the study, as
a group, had higher concentrations in the serum than did all
females. Further, the Group 2 Males revealed the lowest mean
value as compared to all other males. The Group 1 Females displayed
the lowest mean as contrasted to all other females and also as
compared to all other patients included in this investigation.
On the other hand, the Group 1 Males possessed the highest mean
value of all individuals examined in the study.

The mean values obtained for the serum albumin levels pointed
to the fact that all males, as a group, had higher concentrations
than did all females. Further, the Group 2 Males exhibited the
lowest mean value as compared to all other males, and also as
contrasted to all other patients in this study. The Group 1
TABLE 12

Means and standard deviations of serum electrophoresis and total eosinophil values by disease group and sex.

<table>
<thead>
<tr>
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<tbody>
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<td><strong>Group 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>( \bar{x} = 7.33 )</td>
<td>( \bar{x} = 61.26 )</td>
<td>( \bar{x} = 2.97 )</td>
<td>( \bar{x} = 10.83 )</td>
<td>( \bar{x} = 8.72 )</td>
<td>( \bar{x} = 16.20 )</td>
</tr>
<tr>
<td>s</td>
<td>0.35</td>
<td>4.78</td>
<td>0.97</td>
<td>1.86</td>
<td>1.36</td>
<td>3.51</td>
</tr>
<tr>
<td>Female</td>
<td>( \bar{x} = 7.02 )</td>
<td>( \bar{x} = 58.93 )</td>
<td>( \bar{x} = 3.28 )</td>
<td>( \bar{x} = 11.38 )</td>
<td>( \bar{x} = 9.23 )</td>
<td>( \bar{x} = 17.14 )</td>
</tr>
<tr>
<td>s</td>
<td>0.42</td>
<td>5.22</td>
<td>1.32</td>
<td>2.28</td>
<td>1.43</td>
<td>3.73</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>( \bar{x} = 7.23 )</td>
<td>( \bar{x} = 57.73 )</td>
<td>( \bar{x} = 3.91 )</td>
<td>( \bar{x} = 12.21 )</td>
<td>( \bar{x} = 9.31 )</td>
<td>( \bar{x} = 16.81 )</td>
</tr>
<tr>
<td>s</td>
<td>0.49</td>
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<td>1.68</td>
<td>2.49</td>
<td>1.80</td>
<td>3.51</td>
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<tr>
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<td>( \bar{x} = 58.99 )</td>
<td>( \bar{x} = 3.24 )</td>
<td>( \bar{x} = 10.74 )</td>
<td>( \bar{x} = 10.42 )</td>
<td>( \bar{x} = 16.58 )</td>
</tr>
<tr>
<td>s</td>
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<td>6.12</td>
<td>1.36</td>
<td>2.33</td>
<td>2.13</td>
<td>3.35</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>( \bar{x} = 7.30 )</td>
<td>( \bar{x} = 61.20 )</td>
<td>( \bar{x} = 2.87 )</td>
<td>( \bar{x} = 9.27 )</td>
<td>( \bar{x} = 9.26 )</td>
<td>( \bar{x} = 16.66 )</td>
</tr>
<tr>
<td>s</td>
<td>0.45</td>
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<td>1.30</td>
<td>2.37</td>
<td>2.11</td>
<td>3.98</td>
</tr>
<tr>
<td>Female</td>
<td>( \bar{x} = 7.18 )</td>
<td>( \bar{x} = 59.82 )</td>
<td>( \bar{x} = 3.19 )</td>
<td>( \bar{x} = 9.99 )</td>
<td>( \bar{x} = 10.44 )</td>
<td>( \bar{x} = 16.35 )</td>
</tr>
<tr>
<td>s</td>
<td>0.40</td>
<td>5.39</td>
<td>1.32</td>
<td>2.07</td>
<td>2.05</td>
<td>3.74</td>
</tr>
</tbody>
</table>

T.P.: Total protein (gm%)  
Alb.: albumin (%)  
A-1: alpha-1-globulin (%)  
A-2: alpha-2-globulin (%)  
Beta: beta globulin (%)  
Gamma: gamma globulin (%)  
Eosin.: eosinophils/cmm  
\( \bar{x} \): means  
s: standard deviation.
TABLE 13

Means and standard deviations of serum electrophoresis and total eosinophil values by sex and disease group.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>x:7.33</td>
<td>x:61.26</td>
<td>x:2.97</td>
<td>x:10.83</td>
<td>x:8.72</td>
<td>x:16.20</td>
<td>x:217.76</td>
</tr>
<tr>
<td></td>
<td>s:0.35</td>
<td>s:4.78</td>
<td>s:0.97</td>
<td>s:1.86</td>
<td>s:1.36</td>
<td>s:3.51</td>
<td>s:101.96</td>
</tr>
<tr>
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<td>x:7.23</td>
<td>x:57.73</td>
<td>x:3.91</td>
<td>x:12.21</td>
<td>x:9.31</td>
<td>x:16.81</td>
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</tr>
<tr>
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<td>s:1.68</td>
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<td>s:188.56</td>
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<td>x:9.27</td>
<td>x:9.26</td>
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</tr>
<tr>
<td></td>
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<td>s:2.37</td>
<td>s:2.11</td>
<td>s:3.98</td>
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<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>x:7.02</td>
<td>x:58.93</td>
<td>x:3.28</td>
<td>x:11.38</td>
<td>x:9.23</td>
<td>x:17.14</td>
<td>x:199.22</td>
</tr>
<tr>
<td></td>
<td>s:0.42</td>
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<td>s:1.32</td>
<td>s:2.28</td>
<td>s:1.43</td>
<td>s:3.73</td>
<td>s:157.24</td>
</tr>
<tr>
<td>Group 2</td>
<td>x:7.24</td>
<td>x:58.99</td>
<td>x:3.24</td>
<td>x:10.74</td>
<td>x:10.42</td>
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<td></td>
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<td>s:1.36</td>
<td>s:2.33</td>
<td>s:2.13</td>
<td>s:3.35</td>
<td>s:201.94</td>
</tr>
<tr>
<td>Group 3</td>
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<td>x:59.88</td>
<td>x:3.19</td>
<td>x:9.99</td>
<td>x:10.44</td>
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<tr>
<td></td>
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<td>s:2.07</td>
<td>s:2.05</td>
<td>s:3.74</td>
<td>s:101.82</td>
</tr>
</tbody>
</table>

T.P.: Total protein (gm%) ; Alb: albumin (%) ; A-1: alpha-1-globulin (%) ; A-2: alpha-2-globulin (%) ; Beta: beta globulin (%) ; Gamma: gamma globulin (%) ; Eosin: eosinophils/cm³ ; x: means ; s: standard deviation.
Females displayed the lowest concentration of all of the females in this investigation, whereas the Group 1 Males revealed the highest value of all individuals examined.

Regarding the alpha-1-globulin fraction of the serum, all females, as a group, possessed a higher concentration than did all males. The Group 3 Females displayed the lowest mean as contrasted to all other females. On the other hand, the Group 3 Males revealed not only the lowest concentration for the entire male population but also for all other patients included in this study. The Group 2 Males exhibited the highest mean value for the entire study group.

The alpha-2-globulin fraction of the sera was greater in all males, as a group, than in all females. The Group 3 Males had the lowest concentration as compared to all other males, and also as compared to all other patients included in this investigation. The Group 3 Females displayed the lowest level as contrasted to all other females. The Group 2 Males revealed the highest mean value of all individuals examined.

All females, as a group, exhibited higher concentrations of beta-globulin than did all males. The Group 1 Females had the lowest concentration among the three female groups. The Group 1 Males displayed the lowest mean value for the entire male population, and also as compared to all other individuals incorporated into this study. The Group 3 Females possessed the highest concentration of beta-globulin.
The data concerning the gamma-globulin and the total eosinophil counts were not considered further since the standard deviations (Tables 12 and 13) were considered to be too great and the analysis not reliable.
DISCUSSION

Often the clinical microbiologist is unable to report positive bacteriologic findings after attempting to culture urine by means of the more frequently employed methods, e.g. "Calibrated loop-direct streak method" (Hoeprich, 1960); "Pour plate method" (Pryles, 1960); and the "Streak-pour plate method" (Savage et al., 1967). Additionally, attention also has been given to a series of chemical tests for the rapid detection of bacteriuria based on the metabolic and physiologic characteristics of the microorganisms (Simmons and Williams, 1962; Branson, 1966; Parker et al., 1966). However, these procedures have been generally unsatisfactory as evidenced by a substantial number of false negative tests (Bailey and Scott, 1970).

The results of this investigation, employing the culture methods of Marraro et al. (1970a), clearly established that microorganisms were present in the urine specimens of "normal" individuals (Table 1). In view of the fact that the urines of 86% of "normal" males (Group 1) and 94% of the "normal" females (Group 1) harbored one or more genera of recoverable bacteria, the question arose as to what was the origin of these bacteria. Although it is often assumed that the "normal" urinary tract is sterile from the bladder neck to the glomerulus (Stamey et al., 1965), there exists the
problem of urethral contamination during the collection of the voided urine specimen. Therefore, within recent years, urologists tend to employ the technique of suprapubic needle aspiration of the bladder in order to circumvent the shortcomings in the collection procedures. Nonetheless, the localization of infections in the lower urinary tract in the male may be demonstrated satisfactorily by means of voided urines (Stamey et al., 1965). While there is no satisfactory substitute for suprapubic bladder aspiration in the female, urethral contamination can be minimized during the collection of a mid-stream aliquot of urine (Stamey et al., 1965). With the above comments in mind, the manner in which organisms may reach the kidney became controversial. Three possible routes of invasion have been described by Heptinstall (1969): (a) by the lymphatics, (b) by the bloodstream, and (c) by an ascending pathway from the bladder along the ureter (vesico-ureteric reflux). It would appear, then, that microorganisms recovered from the bladder urine by suprapubic aspiration, or by catheterization, may have originated either from the lower urinary tract or may have descended from the colonized kidneys themselves. Further, it would seem acceptable that other areas of the body may act as foci for the continued supply of microorganisms to the bloodstream and the lymphatics. Sites such as the oropharynx, respiratory tract, and intestines, under certain conditions of ecosystem imbalance, may be involved in the release of bacteria to the circulatory and lymphatic systems. It would not be difficult to postulate the manner by which colonization may take place in the lower urinary tract of the male, e.g., from exogenous
sources, from an infected prostate, or from the prepuce in the non-circumcised individual. On the other hand, in the lower urinary tract of the female, the external one-third of the urethra is continuously contaminated by the bacteria from the vagina and rectum, and this may explain colonization of the urethra on an anatomic basis. This may account for the fact that urinary tract infections are 14 times as common in women as in men. It should be considered also that in the female: 1) the urethra is shorter than in the male, 2) it is highly probable that women do not empty their bladder, under normal conditions, as completely as do men and, 3) during sexual intercourse, the urethra may be displaced from the normal external position to an intravaginal one and thereby presents an ideal situation for massage of bacteria directly into the bladder (Stamey et al., 1965). More recently, Kunin (1969) has associated the differences in the rates of acquisition of infection between males and females with the differences in the length of the urethra, the influence of antimicrobial prostatic secretions, and the role of instrumentation. The postulates, as described above, are confirmed by the data obtained during this investigation (Table 5). It is apparent that the total male population under investigation had a greater number of sterile urine specimens or, at least, urine specimens with no recoverable microorganisms, and also had fewer genera of recoverable bacteria per individual urine than did the entire female patient population.

In reviewing the microorganisms recovered from the genitourinary
tract of 300 male and female patients in this study, it was noted that the genera of organisms isolated from all groups were quite similar and occurred throughout each group. Variations were noted, but the most significant fact became the differences in the frequency with which these organisms were isolated under varying conditions of nutrition and atmospheric environments. It was considered that the genitourinary tract offered a suitable environment for the selective growth of these microorganisms. The problem then became one of duplicating, in vitro, similar environmental surroundings in order to permit the recovery of these isolates. The in vitro nutritional requirements of the various microorganisms encountered have been described in detail and, therefore, require no further elaboration.

The need to supply differing atmospheric environments in vitro for the primary isolation of microorganisms has been neglected in clinical practice and, as a result, existing methods for evaluating the bacteriologic status of urine specimens were considered to be highly inadequate. Often overlooked is a group of bacteria classified as the microaerophils which can but tolerate, or even prefer oxygen at low tension, but not at the concentration normally found in the atmosphere. It is to this group that attention was directed while, at the same time, recognizing that aerobes as well as anaerobes also constitute a part of the bacterial flora often associated with the mucous membranes (Bailey and Scott, 1970; Finegold, 1970). In 1968, Fugazzotto and Pinkosz reported, on
primary isolation, organisms which were in the "hydrophilic state". These bacteria, recovered from trypticase soy broth as well as from fluid thioglycollate, were mostly obligate microaerophils. In the present study, approximately 60% of the total isolates recovered from the urine specimens required a microaerophilic environment for primary isolation. These data emphasize that 146 patients, representing 49% of the total population assigned to this study, under other circumstances, would have had "negative" cultures of the urine. Additionally, 36% of the isolates were recovered aerobically and 4% required an anaerobic system for primary cultivation. These data confirm the observations of Fugazzotto and Pinkosz and emphasize the need for providing varying atmospheric environments for the primary isolation of microorganisms from urine specimens. It would appear that the primary isolation of organisms is not influenced by, nor related to, the clinical state of the patient, but rather by the atmospheric environment available for the growth of the organisms. The data (Table 4) indicate that the bacteria isolated from all patients in this investigation, regardless of groupings, displayed somewhat similar patterns during recovery dependent solely upon the atmospheric environments employed.

The isolation of bacteria from the urine specimens of the patients assigned to Group 1 may indicate that "normal" populations, both male and female, often are colonized with microorganisms although clinically they are considered to be asymptomatic or free
of urologic disease. An increase in the frequency of isolation of organisms from the patients assigned to Group 2 (diseases of presumed non-bacterial etiology) was apparent (Table 2) and perhaps the disease itself, in these cases, has modified the genitourinary tract so as to lower the defense mechanisms and thereby permit increased colonization. This premise is based on the fact that several of the patients, originally examined and placed in Group 2 were, at a later date, transferred to Group 3. Although the frequency of isolation of organisms is quite similar, certain patients may progress from Group 2 to Group 3 (diseases of presumed bacterial etiology) based on the fulfillment of predetermined clinical criteria, as previously discussed.

It is generally accepted that, in quantitating cultures of the urine, the recovery of 100,000 or more bacteria per milliliter of urine indicates urinary tract infection (Kass, 1956). Recent studies (Asscher et al., 1969), using these criteria, have shown that 3% of 3,578 asymptomatic, nonpregnant females exhibited bacteriuria. Previously, Freedman et al. (1965) found the overall prevalence of bacteriuria in young and middle aged women to range from 1% to 3%. Bacteriuria is distinctly unusual in young males (Kunin, 1969), ranging from 0.03% in schoolboys to only 0.5% in the general population. However, the data obtained during this study, although based on a comparatively smaller number of samples, indicate that 4% of the "normal" male population (Group 1) and 10% of the "normal" female population (Group 1) demonstrated
asymptomatic bacteriuria. This increase in the incidence may be attributed to the development of more sensitive procedures employed throughout the microbiological analyses. Another aspect to be considered, as postulated by Stamey et al. (1965) in their studies, is that the urine of 33% of the patients with urinary tract infection had bacterial counts of less than 100,000 bacteria per milliliter of urine. The present investigation has shown that of all patients having microorganisms capable of being quantitated—and therefore not in the "hydrophilic state"—7% had urine specimens with greater than 100,000 bacteria per milliliter, 22% had 10,000 to 100,000 bacteria per milliliter, and 25% of the patients had less than 10,000 bacteria per milliliter of urine. Consequently, it would appear that the clinician should now re-determine the significance of the numbers of microorganisms recovered per milliliter of urine and re-evaluate his concepts as to which organisms are capable of causing, or at least of being incriminated, in human infections. The pathogenic potential of Corynebacterium species (diphtheroids) has been documented in chronic prostatitis (Kaged and Khafaga, 1965), in infected urine specimens (Torregrosa, 1968), and in non-specific urethritis by Kaminski et al. (1970). Staphylococcus epidermidis has been implicated in chronic prostatitis (Kaged and Khafaga, 1965), as well as in other specimens from infected patients (Person et al., 1969). More recently, Pearson (1970) has reported human infections caused by species of the Bacillus genus.
The quantitation of microorganisms recovered from the urine specimens of the patients in this study became significant since bacteria were found so frequently in all groups of patients. The role of the postulated defense mechanisms of the bladder (Anonymous, 1968), and the antibacterial properties of prostatic fluid (Gupta et al., 1967; Stamey et al., 1968) merit consideration. Although the efficiency of these substances in vivo is, as yet, undetermined, human prostatic fluid is bacteriocidal in vitro for some gram-positive and gram-negative bacteria. Additionally, similar substances capable of antibacterial activity may be present in the secretion of the para-urethral glands of the "female prostate". Therefore, it must not be overlooked that the data, in the case of the Group 3 female mid-stream urines as compared with the catheterized specimens, clearly indicate that 54% of the catheterized urine specimens were sterile (Table 5) and, in general, fewer isolates and consequently fewer genera of microorganisms were recovered. Whether these data can be explained strictly on an anatomic basis, on the basis of bladder defense mechanisms, or by a combination of both remains unanswered. Further, the recovery of five isolates from catheterized urine specimens, but not from the parallel mid-stream urines from the same patients, are of interest. Perhaps these organisms descended from a colonized kidney, or they may have been introduced by direct urethral catheterization. Still another possibility is that these bacteria may have represented a residual of organisms colonizing the bladder from prior bacterial infection and their presence may be attributed to an appropriate environment resulting
from imbalances in hydrostatic mechanisms and loss of normal physiologic functions.

As a result of the laboratory identification of the microorganisms recovered in this study, the recognition and differentiation of the enterococci assumed a significant role since the urine of 21% of all male and 53% of all female patients harbored an organism belonging to the enterococcus group. Among the reports in the literature concerned with the significance of the enterococci in a variety of human diseases is the work of Evans and Chinn (1947). More recently, Medrek and Kunz (1970) have reported that 75% of all streptococci isolated from urine cultures were Group D streptococci, and the acceptance of these organisms as pathogenic flora in urine has been reported (Bailey and Scott, 1970). The significant increase in the isolation of these bacteria from the patients assigned to Group 3, both male and female, as compared to the frequency of isolating these organisms from the urines of the Group 1 and 2 patients, was of some interest. It was felt that since the syndromes of this group were of presumed bacterial origin, these findings were attributed to a general ecosystem imbalance with an accompanying displacement of microorganisms from their "normal" locale. No attempt was made to differentiate between Streptococcus faecalis, Streptococcus faecalis var. liquefaciens, and Streptococcus faecalis var. zymogenes. Since differentiation is based solely upon differences in gelatin liquefaction and hemolytic patterns on blood agar, it was felt
that these criteria were too variable to be reliable. It is suggested also that there is very little difference among these three species and perhaps the variations do not merit distinct speciation (Deibel et al., 1963; Deibel, 1964; Facklam, 1970). Therefore, these streptococci were reported solely as \textit{Streptococcus faecalis}. Further, it is noted that the literature describes a Group Q streptococcus (Nowlan and Deibel, 1967) which is reported to be a fecal streptococcus resembling the enterococci in most reactions, but differing in one or more commonly accepted critical characteristics used in classification. However, two different prototype species of Group Q streptococci obtained through the courtesy of the Center for Disease Control (Facklam, 1969) did reduce 0.15% methylene blue milk, a finding not in keeping with the reactions as described by Nowlan and Deibel (1967). As a result and in light of similar findings at the Center for Disease Control, the Group Q streptococci were dismissed from consideration and classified as an aberrant biotype, or as a type-specific variety of the Group D streptococci.

In order to insure that the role of the cell wall-defective forms in diseases of the genitourinary tract was taken into account during this investigation, an effort was made to isolate and identify these forms from the urine specimens examined.

The first report on the isolation of an L-form from human infection was that of Dienes and Smith (1944), and since then, and especially within the past several years, the literature has offered
an increased number of reports concerned with the L-forms of bacteria in human diseases (Guze, 1963; McGee and Wittler, 1969; Sharp, 1970). Although conclusive evidence is not available to establish definitely a role for the L-forms in disease, their presence in a variety of clinical materials, including those of the genitourinary tract, e.g., urinary tract infections, recurrent pyelonephritis, and chronic bacteriuria, has been documented. Whether L-forms persist in the genitourinary tract and account for progressive infection, either through reversion to a classical parent organism or through persistence in the form of stable L-forms, is a fundamental and as yet unresolved issue (Domingue and Schlegel, 1970).

One of the most arbitrary areas among investigators is the selection and composition of an artificial medium for the growth of these aberrant forms of bacteria from clinical material. Because of this, the physiologic requirements for the growth of these cell wall-defective forms was reviewed (Smith, 1964) and an agar medium was developed (Robbiel medium) which would presumably offer these organisms a more satisfactory medium for growth, devoid of substances which inadvertently might induce the formation of these cells. The function of certain key substances included in this formulation reflected the complexity of the metabolic requirements of these aberrant forms. Since these variants are cell wall-defective by definition and thereby possess only a cell membrane, sucrose was incorporated for purposes of osmotic stabilization and to preserve the osmolarity of the environment offered these cells
upon transfer from an in vivo environment. Phytone, a peptone substance high in vitamin content, served as the main source of thiamine essential to the growth of these forms. Additionally, yeast extract was incorporated as the source of B complex vitamins of low molecular weight, and supplement B offered both the thermolabile and thermostable growth accessory factors of fresh yeast, especially the "v" factor or coenzyme and the "X" factor of hematin. Cholesterol was employed to provide lipid substances necessary in the formation and support of the fragile cell membrane. Magnesium sulfate and sodium chloride supplied essential inorganic magnesium and sodium ions and ionagar #2 was selected because of its purity and associated lack of toxic factors. Since the literature indicated that L-forms in media with a pH of less than 7.0 and greater than 9.0 generally lost viability and lysed (Smith, 1964), a pH of 7.8 was arbitrarily selected and the medium was so adjusted. The major concern in the development of this medium became the selection of a source of protein. The literature abounds with references to the use of bovine serum albumin (Montgomerie et al., 1967), heat inactivated human serum (Kagan, 1968), human ascitic fluid (Young and Armstrong, 1969), inactivated horse serum (Kagan et al., 1969), and a variety of other substances as the protein source. The selection of human serum albumin resulted from reviewing the findings of others in which it was established that the protein component of mammalian serum (Smith and Morton, 1951) could be replaced by larger quantities of
bovine albumin (Smith, 1960). From this and rather than to cross species boundaries and perhaps introduce into the experimentation undefined forces which might act as inducers, human serum albumin was selected rather than an albumin of non-human origin. Additionally, this protein growth factor served as a detoxifying agent as well as to regulate sterol uptake of the cell wall-defective forms of bacteria (Smith, 1964).

The reports regarding the gaseous requirements for the growth of cell wall-defective forms of bacteria are confusing. The importance of these requirements may be clouded by the use of many diversified formulae in the preparation of media employed for the growth of these organisms (Morton et al., 1951). Although it has been reported that the gaseous requirements for the L-forms appear to mimic those of the parent bacteria (Dienes and Weinberger, 1951), studies were instituted to establish, for the system under examination, the atmospheric environment most suitable for the recovery of these forms. After performing multiple parallel studies utilizing aerobic environments, atmospheres of 4% and 10% carbon dioxide, and an anaerobic system during the forced induction of prototype organisms (to be discussed later), it was established that an anaerobic system at 37°C was the most satisfactory for the recovery of the greatest number of these aberrant forms.

The use of RobMol medium, under the conditions described above, has proven to be satisfactory for the primary isolation of cell wall-defective forms from clinical specimens. It will be recalled
(Table 7) that aberrant forms of bacteria were recovered from the urine specimens of 24 patients, representing each of the groups and both sexes. The recovery of cell wall-defective forms from the male and female patients assigned to Groups 1 and 2, although few in number, established the fact that these organisms could be isolated from "normal" and from non-bacterially involved patient specimens if the clinical material was manipulated properly in the laboratory. Since a minimal number of these forms are found scattered throughout the patient population, then of interest was the observation that there was a significant increase in the number of cell wall-defective forms isolated from the Group 3 Males and from the Group 3 Female patients (Table 7). It would seem reasonable that these forms may develop as a result of an ecosystem imbalance, e.g., general debility, exposure to sudden or unaccustomed psychic and environmental changes, or development of an infectious process in some other part of the body (Austen, 1966). The concomitant physiologic alterations which occur in response to these "stresses" in the host may be responsible for modifying, to some extent, the "normal" concentration of lysozyme in the kidney and in the urine enabling the induction of cell wall-defective forms in vivo (McGee and Wittler, 1969). However, the very real possibility exists that these forms simply may have been a result of the effect of antibiotic medication administered to these patients at an earlier date. Antimicrobial agents are capable of interfering with the synthesis of cell wall peptidoglycan, resulting in the formation
of microbial variants (Feingold, 1969) which are capable of survival in the osmotic gradients afforded by the kidney (Kalman son and Guze, 1964).

It will be recalled that selected urine specimens were processed in parallel, both filtered and non-filtered, in order to evaluate the efficiency of the two procedures on the recovery rate of the organisms present in the specimens, particularly the cell wall-defective forms of bacteria. The concept of pre-filtering the urine was not new and reports in the literature indicated that most investigators utilized a system employing a single filter with a pore size of 0.45 microns (Klieneberger-Nobel, 1949; Gutman et al., 1965; Roberts, 1968). However, it was felt that this technique might be improved upon by using serial filtration (Figure 5) employing a 10 micron filter to remove first the proteinaceous material and other debris generally associated with urine specimens. The function of the second filter with a pore size of 0.45 microns, in theory, was to withhold the classical, parental forms of bacteria and yet permit the passage of cell wall-defective forms into the filtrate which was to be used for culture purposes. Along with these considerations, it was felt that the use of pressure filtration might be too harsh and the cell wall-defective forms, if present, might be inadvertently destroyed. Therefore, vacuum serial filtration was employed throughout this study in order to limit the system to only one atmosphere of differential pressure and to maintain a lower flow rate of filtration (Rose, 1969). It is recognized that
the efficiency of filtration systems may be somewhat limited by electrostatic charges in the system, variations in pore size, differences in flow rates, retention efficiency, absorption/adsorption characteristics, and fluctuations in the thickness of the filter. To eliminate these effects from consideration, urine specimens were cultured for the cell wall-defective forms of bacteria in parallel (see Figure 6). The data (Table 6) established that the use of vacuum serial filtration resulted in a significant decrease in the number of these forms recovered from the urine.

The observations described as the inhibition of the growth of bacterial variants (Plate XV) noted during the culture of the cell wall-defective forms of bacteria represents a phenomenon which may be attributable to substances which are bacteriocin-like in nature (Davis et al., 1967). Recently, Ambrozaitis and Deibel (1970) reported that Streptococcus faecalis was capable of producing an inhibitory agent(s) in vitro which possessed an unusually wide spectrum of activity on enterococci. Additionally, Kalmanson et al. (1970) reported the in vitro effect of bacteriocin from Streptococcus faecalis on microbial L-forms of the same and other genera of microorganisms. Further studies are in progress to elucidate this phenomenon.

The role of the cell wall-defective forms of bacteria probably should be considered in urologic diseases. Accordingly, the morphologic parameters of these organisms were established to serve as references during the culture of the urine specimens. From the
literature, it was noted that the induction of cell wall-defective forms \textit{in vitro} was not a new technique, and that there were a variety of light photomicrographs available (Sharp, 1954; Hijmans and Dienes, 1955; Crawford \textit{et al.}, 1958; Madoff and Dienes, 1958; Hijmans and Kastelein, 1960; Marston, 1961; Anderson, 1967; Burmeister and Hesseltine, 1968; Berliner \textit{et al.}, 1969). However, it was felt that a more detailed study concerning the sequential development of these forms of \textit{Streptococcus faecalis} during a prescribed test period was indicated.

The choice of an induction medium was difficult since the literature was again replete with a variety of formulae. It appeared that these formulae, although capable of achieving the induction process, were different in composition from the media employed for growth or maintenance of these organisms from the clinical material. Therefore, rather than to select or formulate a different medium for the \textit{in vitro} induction of these forms, and since RobMel medium had served adequately to support the growth of cell wall-defective forms from clinical material, RobMel induction medium was prepared and used throughout this portion of the study. This medium was identical to RobMel medium with the exception that 1000 units of buffered potassium penicillin G were added per milliliter of medium. This medium has proved to be quite satisfactory for the \textit{in vitro} induction of these aberrant forms of bacteria.

The earliest cell wall-defective form observed in the induction
process was the "cartwheel" (Plate IV). The appearance of these immature colonies in 24 to 48 hours has been reported by Karston (1968) and Young and Armstrong (1969) and presumably, these young colonies evolve into the more classical cell wall-defective variants.

Granulation initially appeared within 3 days (Plate V), increased throughout the twelfth day (Plates VI and VII), began to disappear by the twenty-fifth day (Plate VIII), and was gone by the thirty-sixth day (Plate IX). Spherical or globular subunits appeared within 36 hours (Plate IV), and were present in the periphery of the variant form throughout the study. These subunits, although remaining peripherally located, seemed to increase in size and became more vacuolated during the course of observation. A type of granulation also appeared within these subunits (Plate VI) which was subsequently lost (Plates VIII and IX). Dienes and Weinberger (1951) noted the presence of granules within these large bodies, and more recently, Dienes and Bullivant (1967) postulated that these granules multiply rapidly to form large bodies and that the granules are viable.

A radically altered colonial morphology was observed after 25 days (Plate VIII) and represented a point in the death phase of the cell population, as demonstrated by these changes.

After 36 days (Plate IX), further changes in colonial morphology might be attributed to such factors as depletion of nutrients, accumulation of waste metabolic products, elaboration of "autotoxic" substances, drying of the agar, and/or environmental stress.
The induction process of cell wall-defective forms could not be initiated with other than a heavy inoculum which suggested that the growth of these variants did not originate necessarily from a single or from a few parental bacteria. These observations agree with those of Dienes and Weinberger (1951) and may relate to undefined factors which play a role in the initiation of growth, such as influences which the organisms exert on one another.

An agar medium was selected since studies have shown that broth menstrua fail to support the growth of cell wall-defective forms. This suggests that the gel structure of the agar serves as a substitute for the functions of the rigid cell wall (Dienes, 1967b), and that multiplication of the organism usually occurs only if the "cartwheel" structure can penetrate into the medium (Dienes and Bullivant, 1967).

The greatest and most striking morphologic changes occur during the early stages of induction (Plates IV-VI) and may be related to the composition of the induction agar medium. The modified bacteria resulting from this treatment, under appropriate conditions, are capable of multiplication, significant enlargement, and formation of a characteristic colony resulting from the surface and agar penetrating growth (Young and Armstrong, 1969). Marston (1968) stated that the dense center of the colony is an area which represents growth of the variant forms into the agar medium, while peripheral growth is surface growth.

Moreover, Anderson (1967) has described these colonies as
consisting of a dense central area embedded in the agar with less
dense peripherally oriented granular large bodies. The results of
this study confirm these observations. Zones of varying density,
observed as margins and inner rings, appeared during the maturation
and the aging process and suggest that there is a variation among
the various cells which form the individual colonies. Marston (1968)
observved large bodies (transitional stages in the development of
these forms from parent bacteria) at the edge of the developing
colony and felt that this phenomenon was indicative of a phase of
intense activity.

After comparing Plates IV-VI, a theory of development and
maturation of these forms would be more closely akin to an
evolutionary rather than to a transitional concept. Maturation —
the events involved in the evolution of the variant from the
"cartwheel" to the more classical "fried egg" form — was observed
within 3 days at the earliest, with the average time being between
the third and sixth day of incubation. Similar findings (Marston,
1968) demonstrated mature colonies of staphylococcal L-forms
within 3 to 5 days. This phenomenon may depend on the physical
properties of the medium and their influence on the growth, size,
and shape of the organism (Dienes, 1968).

Because of the morphologic changes observed during the light
microscopy of the induced cell wall-defective forms of bacteria,
studies of ultra-thin sections as examined by electron microscopy
were employed in an attempt to verify the concept that cells of
varying morphology and assumed viability occur throughout the typical "fried egg" colony of *Streptococcus faecalis*. A variety of cell types observed as thin sections from the deep sub-agar through the supra-agar dome of the colony were analyzed. The deep sub-agar portion of the colony revealed principally "ghost cells", which contained little identifiable cellular structure, other than a few strands of DNA and a distinct plasmalemma. In the area above this, all other types of cells were observed ranging between normal, cell wall-defective forms, and "ghost cells". In the supra-agar region, normal cells seem to predominate, but occasionally areas (Plate XIV- A and B) appeared devoid of structured cells, and seemed to contain some membranous residues.

Not only the more circular acellular zones, but also linear channels (C) equally free of structural material were observed (Plate XIV). These areas may be associated with differences in internal colony pressure resulting from the spatial attitudes of the varying morphologic types. Perhaps these voids may simply represent areas in which no cells developed. This seems unlikely since this was not noted in studies of a normal colony.

In order to take advantage of the hematologic data and the serum electrophoresis patterns accumulated during this investigation, all data were subjected to statistical analyses. The possibility exists, as a result of certain disease states, that alterations in the serum protein fractions may occur. Further, the fact that dysproteinemia does occur in patients suffering from certain
urologic disorders, e.g. acute infections of the urinary tract, ureteric calculi, chronic renal failure, and the nephrotic syndrome, is well accepted (Jencks et al., 1956; Wall, 1958; Ogryzlo et al., 1959; Brackenridge and Csillag, 1962; Bernstein et al., 1968) and these disease states will produce characteristic aberrations of the protein fractions. In a similar fashion, the implication of the circulating eosinophils and their increase in numbers in allergy and parasitic infections is well documented (Davis et al., 1967; Samter, 1965). With these facts in mind and because of the clinical impression of the urologist (L. B.), it was felt that characteristic response patterns might be produced in these patients and subsequently used as an adjunct in the diagnoses of these chronic syndromes.

In order to validate the data insofar as was possible, all patients in this study were managed by only one physician (L. A.), and therefore physician-associated error could be judged to be distributed randomly across the groups. Further, any variability in laboratory methodology over the extended period of the study could be judged to be distributed randomly across the groups since all of the protocols and personnel involved remained unchanged. Additionally, it was felt that the establishment of "normal" values, derived from the data obtained from the Group 1 patients, would be an unquestionably valuable adjunct to quality control procedures (O'Halloran et al., 1970) required for the study of these specific disease states.
The results of the biometric analyses for the total protein, albumin, beta-globulin, and gamma-globulin fractions of the sera, as well as for the total eosinophils, were disappointing since no apparent trend was revealed which might be used as an aid in the diagnoses of these syndromes. However, the alpha-1-globulin fraction as well as the alpha-2-globulin fraction of both the Group 3 Males and Females showed a decrease from the other mean values and seemed to suggest a trend. These findings are directly opposed to those of Jencks et al. (1956) in which diseases of the genitourinary tract, in some cases similar to those in this study group, did demonstrate increases in the alpha-1- and alpha-2-globulin fractions and were associated with inflammation and tissue destruction. Other studies (Wall, 1958; Brackenridge and Csillag, 1962; Jenson, 1967; Bernstein et al., 1968) indicated that a variety of urologic diseases, both acute and chronic, often are associated with increased levels of alpha-globulin. The contradictory findings of the present investigation are not clearly understood and, at this time, no further discussion is practicable.

Prior to terminating this discussion, it seems pertinent to consider briefly the possible nature of the etiologic agents which may be responsible for the symptoms of chronic, recurrent infections of the genitourinary tract as noted for the patients in test Group 3. Bacterial infections of the genitourinary tract affect patients of all age groups and both sexes, and vary in severity from an unsuspected infection to a condition consistent
with severe systemic disease. The most obvious etiologic agent responsible would seem to be the classic, parental forms of a variety of microorganisms. However, in recent years, the concept that bacteria in the diseased host may assume, or evolve to, a remarkably altered morphologic form which is insusceptible to many antibiotics, undetectable by routine culture procedures, but capable of reverting later to the parent bacterium has intrigued microbiologists and clinicians alike. The role of these cell wall-defective forms of bacteria in disease has become the focus of a number of investigations. To date, conclusive evidence is not available to establish the role of these forms in disease, but speculation as to the potential of these aberrant forms for causing disease has been offered. In man, there is good evidence (McGee and Wittler, 1969) that variants may be produced in vivo by many of the same mechanisms, e.g. tissue concentrations of antibiotics and/or anaerobic conditions, employed for the production of these forms in vitro. Under conditions preventing growth and reproduction of these aberrant forms, their survival would be brief and their importance therefore limited unless circumstances permitted rapid reversion to bacteria. Yet, if these forms were able to reproduce in the host tissues, they might play a major role in the pathogenesis of disease by causing tissue injury directly or by providing a mechanism for perpetuity by which these microorganisms can revert to the classic bacterial form to cause exacerbations following periods of dormancy. Among the mechanisms for provoking disease
which have been considered are competition of the microbe with the host for essential nutrients, synthesis of enzymes by the invading organisms which act on host structures, formation of toxic substances from damaged host tissue, derangement of essential metabolic activities of the host, induction of an inflammatory reaction, and occurrence of hypersensitivity reactions (Guze, 1968; McGee and Wittler, 1969; Sharp, 1970).

At this point, if the data regarding the bacteriologic status of all urine specimens processed were reviewed critically, several pertinent comments would appear to be warranted. This investigation showed that by devising more refined bacteriologic techniques and by developing more sophisticated growth media, the number of isolates recovered from these urine specimens was greatly increased. Additionally, the frequency of isolation of a variety of genera of microorganisms was improved to a very considerable degree. However, by doing so, microorganisms are now able to be detected from those cases with diseases of suspected bacterial etiology (Group 3) as well as from those not generally associated with microbial agents (Group 2), as well as from the urine specimens of "normal" control individuals (Group 1). As a result, the genera of bacteria isolated from all groups and both sexes were quite similar and occurred throughout each group. Variations were noted, but the most significant facts were the differences in the frequency with which these organisms were isolated. Of specific interest, in this regard, were the increased numbers of enterococci recovered from
both the male and female patients assigned to Group 3. In addition to the classic, parental forms of microorganisms, the data revealed that the L-forms of bacteria were isolated throughout each group of patients and from both sexes with the greatest number of isolates recovered from the individuals assigned to Group 3. From the foregoing, it would appear that further investigation in this area should concern itself with the significance, if any, of the enterococci as pathogenic flora in the urine and the potential of the L-forms to act as etiologic agents in producing diseases of the genitourinary tract.

Regardless of the accepted role of the classic forms of microorganisms in diseases of the genitourinary tract and the possible, but as yet unproven, role of the L-forms in a variety of disease states, the fact remains that the patients assigned to Group 3 have continued to return to the urologist with chronic, recurrent symptoms of genitourinary tract disease. As previously reported (Results), the cystoscopic findings in these patients invariably reflected some degree of abnormality in the urethra, bladder neck, and/or trigone suggestive of an inflammatory or allergic phenomenon occurring in this locale. It is well known that allergic reactions may be manifested in many areas of the body and there is no apparent immunophysiological reason why the mediators of such reactions should spare the tissue and cells of the genitourinary tract. The infrequency of reports of allergy of this area indicates that the recognition of allergy as a causative factor of genitourinary
tract disease has been slow to evolve. It would appear reasonable, when primary symptomatology proved intractable to appropriate urologic procedures and management, that there develops a need to consider allergy as a possible etiologic factor. Allergy of the genitourinary tract has been shown to involve both the urethra and bladder in both sexes and the prostate in the male (Powell, 1961). Interestingly, the symptoms and findings were much like those of common urologic disease and, in all cases, mimicked those of overt genitourinary tract infection (Burkland, 1951; Powell et al., 1970). Recently, a significant degree of atopic reactivity has been revealed in a group of patients with urologic symptoms resembling those of the individuals assigned to Group 3 in the present study (Powell et al., 1970). In light of these findings with patients who had previously remained unresolved after standard treatment, and because of their noticeable improvement under appropriate management, an investigation would seem merited for possible atopic allergy, a form of human allergy which is familial and probably heritable, in the patient population assigned to Group 3 in this investigation.

The role of the delayed-type of allergic response, often called tuberculin-type allergy, hypersensitivity, or the allergy of infection, is not to be overlooked. If, indeed, the patients assigned to Group 3 demonstrate clinical symptomatology which may be directly attributable to bacterial origin, then the delayed-type responses may well be involved. Delayed hypersensitivity is known to occur during or after infections, including bacterial, fungal and viral
infections -- especially those of a more chronic nature (Kabat, 1968), and it would appear that this is descriptive of the syndromes diagnosed here (Group 3) as chronic prostatitis or chronic urethritis. Since polysaccharides seem relatively inactive as inducers of the delayed-type of hypersensitivity (Davis et al., 1967), the inciting antigens in diseases of bacterial origin are the protein substances which constitute an essential part of the bacterial cell wall or fragments thereof. However, in the absence of a cell wall, the immunogenic stimulus may be derived from the cytoplasmic membrane of the bacteria. Further, protein elements within the cytoplasm or within the nucleoid area of the bacteria may act in a similar capacity. Whatever the origin of the protein material essential for the initiation of this phenomenon may be, there does exist a need to investigate the possibility that such immune reactions may be incriminated as the etiologic agents of these chronic, recurrent diseases of the genitourinary tract. Whether the units which might determine the specificity of the delayed-type of allergy and mediate the response are "sensitized" lymphocytes and macrophages, or a special class of high-affinity antibodies remains speculative at this time.

As with the various allergic reactions, other immunological phenomena involving cell or tissue damage may occur. These syndromes, generally termed "autoimmune diseases", as yet have not been investigated extensively in regard to urologic disease. However, one report does appear in the literature (Miller et al., 1970), but
this study in experimental pyelonephritis did not support the hypothesis that an autoimmune process contributes to the progression of the pathologic process in "sterile" chronic pyelonephritis. Further investigation in this area is warranted, and perhaps pathological mechanisms similar to those occurring in glomerulonephritis and/or rheumatic fever may be involved.
SUMMARY

1. Procedures are described for the more sophisticated processing of urine specimens for quantitative bacteriologic analysis. The more frequently employed methods did not offer sufficient information for evaluating the microbiologic status of the clinical material. Comparative data indicate the proposed procedure was more effective in recovering a variety of genera of microorganisms, as well as increasing the frequency of recovery of the isolates. The advantages offered by this procedure are based upon the availability of aerobic, microaerophilic, and anaerobic atmospheric environments for the primary isolation of the organisms involved; 2 The results obtained from the cultivation of organisms from these specimens indicated that the enterococci often were isolated. Since no existing schema was satisfactory for the speciation of these microorganisms, a protocol was developed which has proven to be simple, rapid, and reliable for the differentiation of the enterococci; 3. In order to recover the cell wall-defective forms of bacteria which may play a role in diseases of the genitourinary tract, RobinEL medium was developed to offer these variants a suitable medium for growth and/or maintenance upon direct cultivation from the urine specimens. Aberrant forms of bacteria were
isolated throughout the investigation from both filtered and non-filtered urine samples and efforts were made to identify these forms by the process of reverting them to their classical, parental forms. 4. To enable a detailed study of the morphology of the cell wall-defective forms of bacteria, RobMel induction medium was formulated so that induced bacterial variants could be examined utilizing light microscopy. The observations of the sequential development of these forms during a specified time interval served as the morphologic parameters employed in studying the isolates from the clinical material; 5. Because of the morphologic changes observed during the light microscopy of these induced forms, ultra-thin sections were examined by electron microscopy. Attempts were made, at the cellular level, to verify the concept that cells of varying morphology and viability occurred throughout the typical "fried egg" colony. Based on these findings, a schematic representation of the classical colony is offered; 6. Since certain associated disease states will produce characteristic dysproteinemias, the hematologic data and serum protein electrophoresis values gathered during this study were subjected to biometric analyses. Although no characteristic aberrations of the response patterns were noted in these particular chronic disease states, the alpha-globulin moiety of the diseased group (Group 3) was decreased.
LITERATURE CITED


Wyrick, P. B. 1969. Ultrastructure of Streptococcus faecalis F-24 L-forms in broth grown culture. Presented at the 69th Annual Meeting of the American Society for Microbiology, May 4-9, Miami Beach.