

IN VITRO AND IN VIVO STUDIES OF ACQUIRED
SERUM RESISTANCE OF E. COLI AFTER
INTRACELLULAR RESIDENCE

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INTRODUCTION

The role of the bactericidal action of serum against gram negative organisms in the natural host defense mechanisms is one of probable importance. It has long been recognized as a factor in the total complex of "nonspecific" resistance to infection (Muschel, 1960). As early as 1954, Rowley suggested the importance of serum sensitivity as a determinate of Escherichia coli virulence in the mouse, as tested by intracellular challenge. A proposal by Smith (1960) implicating possible products of the pathogen as agents interfering with the bactericidal action of normal sera and white blood cell extracts lends support to the protective role of the heat labile serum components.

Included among the bacterial genera effected by serum heat labile factors are Escherichia, Salmonella, Proteus, Shigella, Chromobacter, Hemophilis, and Brucella (Osawa and Muschel, 1960). These workers postulated there may not be an absolute distinction between serum susceptibility and resistance, but rather a broad distribution of response. According to Olitzki and Godinger (1963) gram negative organisms belonging to the different genera give rise to similar symptoms upon infection, but there exists striking differences in their pathogenicity and clinical course. This indicates a very complex inter-relationship of the organism and the host, with implications of a correlation of serum sensitivity to virulence.

The initial objectives of the study were to establish the respective roles of humoral and cellular factors in the host defense mechanism, utilizing E. coli and a host cell system of rat peritoneal macrophages. As E. coli is a facultative intracellular parasite, the problem of extensive extracellular multiplication had to be controlled, as it can often mask the intracellular events. Since this organism succumbs to the bactericidal system, an attempt to control extracellular multiplication was made by the addition of unheated rat serum. It was noted that the fresh unheated rat serum failed to exhibit adequate bactericidal effects upon the organisms in the supernatant of the cell system and thus failed to accomplish the intended purpose. It appeared as though some alteration in the serum response had occurred due to intimate cellular association.

In attempting to elucidate and substantiate these initial observations, other techniques were developed. Comparative in vitro studies of "immune" and "non-immune" cells have yielded interesting results, both in regard to the amount of resistance developed and in their interactions with "normal" and rat serum resistant E. coli. Upon extension of the work to the intact animal, the same serum alteration was found to occur. The fate of the organism in vivo, as related to the serum sensitivity, has illustrated some possible correlation of the serum state and persistence in the intact animal. Therefore, the possible relationship of serum sensitivity to virulence and pathogenicity is implicated.

HISTORICAL BACKGROUND

The bactericidal phenomenon was first demonstrated by Nuttal (1888) as the defibrinated blood of a variety of animals was found to be lethal to anthrax bacilli. The first description of bacteriolysis was published by Pfeiffer (1894) after microscopic observation of periodically withdrawn samples from the peritoneal cavity of normal and immune guinea pigs exposed to lethal dosages of Vibrio cholera. The reaction was thought to be specific, as no lysis was observed in non-immune animals, nor did lysis occur with heterologous organisms. However, bactericidal activity of normal sera was observed against Bacillus dysenteriae (Mackie and Finkelstein, 1932, 1931), and fowl cholera organisms (Correll, Miller, and Sherwood, 1940). Von Behring (Myrvik, 1955) also reported bactericidal serum activity against aerobic spore forming bacilli.

From the observations of Pfeiffer (1894) two factors were assumed to be necessary for the bacteriolytic reaction: (1) a heat resistant substance found in immune sera, and (2) a thermolabile component present in both normal and immune sera. The thermolabile substance was first named alexine (Buchner, 1989), but is now commonly known as complement. Although present in the blood of most vertebrate animals, Ehrlich and Morgenroth (1901) noted that complement from a number of animal species differ in their abilities to hemolyze beef erythrocytes sensitized by anti-sera produced in different animals. Guinea pig, rabbit, and human

complements also behave differently in bactericidal tests with Hemophilis influenza (Dingle, Fothergill, and Chandler, 1938). Similar well-marked differences in complement were reported by Bordet and Ehrlich (Boyd, 1956); they also noted that complement was not a product of an immune reaction, but was present in normal sera.

Although there are some marked differences in the bactericidal activity of complement, depending upon the source, Pillemer (1943) reported that the substitution of corresponding parts of human and guinea pig complement increased the bactericidal activity.

Thermolability of complement was characterized by Manwaring (1906) as an inverse relationship between temperature and the time required for complete loss of bactericidal activity. Although such inactivation does occur, the reacquisition of activity resulted if the serum was maintained within a temperature range of 7°C to 37°C; the highest activity reacquired after 24 hr (Gramenityki, 1912).

Further characterization of the role of complement includes increased activity of immune opsonins with the addition of normal sera and complement (Dean, 1907). The activity of normal and immune opsonins are both increased in conjunction with complement; in normal serum the complement action dominates and in immune serum complement enhances the action of immune antibody (Sleeswijk, 1908). Thiele and Embleton (1914, 1915) found the bactericidal action of serum dependent upon the existence of a certain minimum concentration of complement. Too, the speed and completeness of

the reaction increased as the environmental temperature approached 40°C (Zinsser, 1930).

A report of the bactericidal power of complement and a heat stable substance in normal serum (Gordon and Wormal, 1928) initiated further characterization of the bactericidal system. Utilizing adsorption techniques, Mackie and Finkelstein (1931) identified the heat stable substance as specific antibody. Further adsorption studies employing heated washed bacterial suspensions eliminated the bactericidal power of normal serum; however, it was concluded that the heat stable substance was not a classical specific antibody, as there was a loss of activity against a number of organisms (Gordon and Carter, 1932). Due to the antigenic complexity of any given bacterial strain, there are contradictory reports on the specificity of bactericidal antibodies by investigators. Adler (1953) stated that bactericidal action is mediated by specific antibody for surface somatic antigens. The antibodies implicated in the bactericidal action are termed natural or normal antibodies by Muschel (1960) and Osawa and Muschel (1960), respectively.

The importance of antibody in the bactericidal reaction was emphasized by Clawson (1936), in an in vitro study demonstrating increased lysis with immune serum in the cell system, the degree of lysis being directly dependent upon antibody concentration. Furthermore, titration of complement was reportedly least subject to variation in presence of a moderate excess of antibody, indicating not all antibody is actively bactericidal (Muschel and Treffers, 1956). The implicit necessity of antibody in the effectiveness of the bactericidal system can be ascertained from the bactericidal

ineffectiveness of this system in the absence of antibody, even in the presence of properdin, complement, and Mg^{++} (Osawa and Muschel, 1960).

A number of hypotheses have been proposed in an attempt to explain the mode of action of the serum bactericidal system. The more recent work reveals that the bactericidal activity of complement is mediated by antigen-antibody reactions at the cell surface. However, the antigen need not necessarily be a constituent of the cells, but can be artificially absorbed (Adler, 1952). Muschel (1960) suggests the occurrence of a similar process: as antibody and complement combine with the surface antigens or toxic O antigen, a small percentage of the surface is covered with antibody and the cells are uncoated. They persist as protoplasts or are lysed.

Heidelberger (1941) reported the amount of complement fixed on the bacterial surface was of the same magnitude as that of antibody. However, Muschel and Treffers (1956) indicate only a very small portion of the cell wall is covered with antibody (0.03-0.7%), this being approximately equal to 700-860 molecules of antibody to 1.5×10^7 molecules of complement per bacterial cell.

Muschel and Treffers (1956) and Muschel, Carey, and Baron (1959) postulated the reactions of complement might be enzymatic, several or all of the complement components being proteolytic enzymes oriented by specific antibody. Due to the specificity of the reaction, Pillemer (1943) suggested the involvement of a chemical reaction. Too, Ehrlich (Boyd, 1956) thought possible digestion followed sensitization, but Jobling (1915) reported complement

did not appear to be identical to serum protease, and attempts have been unsuccessful to demonstrate digestive action.

As stated by Pillemer (1943), the exact nature and mode of complement action is uncertain, in spite of more than a half century of study. The complexity of the system is illustrated by the diversity of reports on the nature of complement and antibody, the mode of action, and the kinetics of the action. In a most recent report, Michael and Braun (1964) presented results which seem to best characterize and summarize the sequence of the bactericidal serum reaction. The reaction occurs in two stages: (1) a short term reaction involving the factors sufficient in concentration in slightly diluted serum, Ca^{++} and Mg^{++} , and all four complement components, and (2) a long term reaction involving factors sufficient in highly diluted serum. Initial serum exposure does not effect the multiplication potential of the organism, as actual damage occurs during the second stage; furthermore, cell multiplication abolishes the sensitizing effects of the first stage. In addition, the removal of lysozyme did not influence either stage of the reaction.

Properdin, a non-specific protein of normal serum, has been implicated in the bactericidal system. Unlike complement, it is not inactivated or removed by antigen-antibody aggregates and behaves as a mixture of "natural" antibodies (Humphrey and White, 1964). There are conflicting reports in regard to the importance and role of this recently described substance. Wardlaw and Pillemer (1956) and Landy and Shear (1957) related the bactericidal activity

of serum for a variety of micro-organisms to properdin, the properdin acting in conjunction with all complement components and Mg^{++} ; removal of properdin eliminated bactericidal action (Wardlaw and Pillemer, 1956). Osawa and Muschel (1960) reported these three substances destroy gram negative bacteria, protozoa, erythrocytes, and viruses. Although bactericidal activity is reported, Wardlaw (1962) states that there is no involvement of properdin in bacteriolysis due to normal serum.

Nelson (1958) proposed that properdin was involved as a natural mechanism of defense. There have been correlations between properdin levels and host resistance or susceptibility to infection (Osawa and Muschel, 1960). Likewise, a lipopolysaccharide injection induced 2 to 3 fold increases in properdin concentrations; large dosages initiated depressions followed by an increase, and small dosages initiated immediate elevated properdin levels (Landy and Pillemer, 1956; Landy and Shear, 1957). Rowley (1956) suggests that early immune effects are possibly due to changes in available properdin, the action of serum initiated by contact of properdin with the lipopolysaccharide substrate of the bacterial cells. In contrast to these reports, Muschel, Carey, and Baron (1959) and Muschel (1960) describe the bactericidal role of properdin as uncertain; the lethal serum activity may be enhanced by the properdin system, although insufficient evidence is available to correlate properdin with bactericidal action.

The possible synergistic role of lysozyme upon the bactericidal action may also be a part of the natural host defense. Michael and Braun (1959) and Carey, Muschel, and Baron (1960) proposed that

specific antibody, complement, and lysozyme are required for serum action. However, more recent work has revealed the synergistic effects of lysozyme. Muschel, Carey, and Baron (1959) described the conversion of specific strains of E. coli and Salmonella typhosa to protoplasts by fresh normal guinea pig serum and attributed the action to lysozyme and the antibody-complement system. Also noted was the accelerated immune bacteriolysis of two vibrio species due to the presence of lysozyme. Additional observations by Muschel (1960) revealed the persistence of the organism as protoplasts after bactericidal action of serum. However, if lysozyme were employed, the bacteriolytic action of the antibody-complement complex was promoted. Wardlaw (1962) also suggested initial action of the four complement components and antibody with the synergistic lysozyme action, the lysozyme reaction being enzymatic and complement being preparatory.

Wide distribution of lysozyme in fluid and body tissues lends support to the probable role of lysozyme in the natural host defense against gram negative organisms (Muschel, Carey, and Baron, 1959). Ralston and Elberg (1960, 1961) reported that the extracts of parasitized monocytes contained lysozyme, which was effective in the lysis of Brucella in the presence of glycine. Variations in the response of various organisms to the antibody-complement system, whether bacteriolytic or bactericidal, may be attributed to reported variations in lysozyme susceptibility (Muschel, Carey, and Baron, 1959). Muschel (1960) reported smooth organisms, normally refractory to protoplast formation by normal serum, were subject to the action of anti-sera, complement, and lysozyme. Therefore,

it appears that lysozyme might be of major importance in the host defense against such parasites, since this material can accentuate or promote the bactericidal complement-antibody system.

To acquire further understanding of the bactericidal phenomenon, as well as the variations in serum responses of gram negative organisms, the elucidation of the bacterial substrate for antibody-complement action was necessary. This has been accomplished primarily by adsorption studies and serology. As early as 1939, Thebault; (Cundiff and Morgan, 1941) reported that antigenic substance from Shigella dysenteriae specifically inhibited the bactericidal power of fresh rabbit serum. Cundiff and Morgan (1941) reported the production of bactericidal antibody against purified somatic antigen from Eberthella typhosa. Upon addition of somatic antigen to normal and anti-typhoid sera, the union of the antibody and somatic antigen inhibited the bactericidal serum properties. Rowley (1956) also reported purified somatic antigen interfered with the action of normal serum in vitro. Lending support to the described somatic antigenic or endotoxic site of the antibody-complement action, Adler (1952) reported all Salmonella strains were sensitized to complement killing if exposed to immune sera specific for the somatic surface antigens. Landy and Pillemer (1956) and Landy and Shear (1957) stated the lipopolysaccharide injection promoted resistance to the gram negative endotoxin producing organisms. Incubation of bacterial cells with unheated serum initiated removal of endotoxic antigenic properties of the polysaccharides present (Landy et al, 1957; Landy, 1960). Endotoxic antigens elicit previously described characteristic bactericidal

host responses, as properdin levels in the blood increase (Landy and Pillemer, 1956) lysozyme levels increase (Hook et al, 1960), and complement titers decrease (Gilbert and Brande, 1962; Kostka and Sterzl, 1962).

Endotoxin is a characteristic portion of the complex structure of gram negative bacilli; these substances are phospholipid-poly-saccharide complexes identical with the O or somatic antigen (Martin, 1964). The endotoxin is associated with toxic characteristics and is found in the cell walls (Ribi et al, 1959) of gram negative organisms (Thomas, 1954). The chemical composition of endotoxin extracts may vary considerable with the organism employed and with the medium employed for culture (Fukushi et al, 1963; Ribi et al, 1964). Antibodies to these substances can be detected in normal sera, even in sterile environments (Michael et al, 1961, 1962; Landy et al, 1962). The lipopolysaccharide complex has been identified in S. typhosa, as well as other Salmonella species, E. coli, Proteus vulgaris, Serratia marcescens, Pseudomonas aeruginosa, and Brucella melitensis (Johnson et al, 1956).

Differences in serum susceptibility, according to Michael and Landy (1961), reflect qualitative and/or quantitative antigenic differences, either due to the presence or absence of the capsular or somatic antigen, or to the state and amount of endotoxin present. The serum resistant strains are a more potent source of somatic antigen or endotoxin. Adler (1953) observed bactericidal sensitization of smooth Salmonella typhosa 0901 was impeded by antibody specific for the rough antigen, indicating a possible intimate spatial relationship of somatic O antigen and the rough antigen.

The O-inagglutinable phenomenon has been postulated by several investigators as directly responsible for serum resistance (Felix, 1951; Muschel, Chamberlin, and Osawa, 1958; Muschel, 1960; Osawa and Muschel, 1964; Olitzki and Godinger, 1963). Smooth Salmonella typhosa, rich in the Vi capsular antigen, were O-inagglutinable, although containing an abundance of somatic antigen (Felix, 1951). In vivo studies by Olitzki and Godinger (1963) revealed the protectiveness of the Vi antigen against lytic action of serum, this antigen being found in soluble form in the infected host tissue. Osawa and Muschel (1964) states that Vi antigen, like the K antigen, prevents union of the O antibody and the somatic O antigen; however, if the Vi antigen is lost bactericidal activity is regained.

Quantitative somatic antigenic differences have been directly correlated to serum sensitivity responses. Michael and Landy (1961) by attachment of somatic complexes to viable E. coli and Shigella dysenteriae, significantly altered the serum susceptibility of these bacterial cells to normal and immune sera. Adsorption of small amounts enhanced the lethal effects and large uptakes rendered the organism relatively resistant. Reaction of specific antibody with the organism; after adsorption of the antigen, failed to enhance serum sensitivity, the endotoxin uptake eliciting a major change in bactericidal response.

Chemical assay of the cell wall composition of serum susceptible and resistant strains of E. coli have yielded quantitative differences in the amount of endotoxin or somatic antigen. Present in both organisms are 70-80% protein and polypeptide and 14% lipid. Quantitative differences in the lipopolysaccharide content

were observed: 1% in the serum susceptible strains and 9% in the serum resistant strain. Qualitative differences were observed in regard to sugar content, the resistant strain yielding glucose, galactose, rhamnose, and two minor sugars and the sensitive strain only possessing glucose (Wardlaw, 1963).

Correlation of serum sensitivity to the degree of virulence is of major importance in attempts to evaluate the role of the bactericidal system in host defense. According to Muschel (1960) the antibody-complement system undoubtedly operates in vivo, aiding in defense against gram negative organisms. Olitzki and Godinger (1963) report that gram negative organisms cause similar symptoms, but striking differences in pathogenicity and clinical course. Smith (1960) states that pathogenicity is probably due to organismal alterations which interfere with host defense mechanisms; specifically, alterations occur in Brucella which alter the serum action. Differing concentrations of the protective cell wall material of Brucella have been correlated with relative virulence.

Rowley (1954) stated the criteria for virulence of a given organism is the ability to multiply. In vivo observations of multiplication have been correlated to the bactericidal system in vitro. These observations lead to a hypothesized correlation of complement and mouse virulence of Bacterium coli. Also, because of the ability to enhance serum resistance by animal passage and simultaneous mucin injection, further emphasis was placed upon the the serum resistant role in virulence by Rowley (1954). Michael and Landy (1961) also reported that serum resistant strains were more virulent for mice.

Rowley (1956) and Wardlaw (1963) correlated serum sensitivity to the rough and smooth characteristics of E. coli; smooth strains of E. coli having a higher polysaccharide content and being insensitive to bactericidal action, and rough strains being sensitive. The described O-inagglutinability, thought to confer serum resistance, is characteristic of the smooth S. typhosa (Felix, 1951). Muschel, Carey, and Baron (1959) reported specific strains of E. coli and S. typhosa are converted to protoplasts by fresh normal guinea pig serum; however, smooth organisms were refractory to protoplast formation by normal serum, unless specific anti-sera, complement, and lysozyme were added.

Roantree and Pappas (1960) state the bactericidal properties of serum have a minor role in freeing the blood stream of bacteria. However, they feel these properties are of importance due to the presence of serum resistant strains in a bacteremia, a ratio of 10 resistant to 1 susceptible organism observed. The main function of the serum factors is to prevent strains from entering and persisting in the blood (Roantree and Rantz, 1960). Although Muschel (1960) indicates serum resistance does not provide a basis of differentiation of virulent and avirulent bacterial strains of E. coli, the possible necessity of a relatively high serum resistance is stated as a requisite of pathogenicity.

The environmental conditions of culture, as well as those of the bactericidal test, have been shown to influence the serum sensitivity and response of a bacterial strain. The lysis of the bactericidal system is directly dependent upon the metabolism of the bacteria at the time shortly after serum damage to the cell

wall components (Muschel, 1960; Michael and Braun, 1959; Michael and Landy, 1961). If conditions permit elongation or multiplication, S. typhi cultures, when cultured in vitro or in vivo, are affected by immune sera (Olitzki and Kaplan, 1963). Michael and Braun (1959, utilizing S. dysenteriae and E. coli, demonstrated the metabolism of the organism effects the serum sensitivity, in that low concentrations (0.1%) of broth, glucose and amino acids enhanced susceptibility to normal serum; whereas, in contrast metabolic inhibitors decreased susceptibility.

Also, culture in tissue extracts lowers bactericidal activity, due to adsorption, as soluble antigen and organic host material are reported to be adsorbed to the bacterial surface (Olitzki and Kaplan, 1963; Olitzki and Godinger, 1963). The state and amount of endotoxin in the organism represents a factor directly influencing serum sensitivity, and incorporation of soluble endotoxin from the environment occurs most rapidly during the log phase of growth (Michael and Landy, 1961).

Demonstration of marked effects of temperature of culture on serum sensitivity have been reported. E. coli cultured at temperatures of 14°C or 45°C are more sensitive to serum than if cultured at 37°C; the effect of a narrow temperature range readily observed as culture at 42°C has no effect upon serum sensitivity (Muschel, 1960). Paracolobactrum ballerup, an organism insensitive after culture at 40°C, but sensitive to immune and normal sera when cultured at 37°C has been utilized by Osawa and Muschel (1964) to correlate the loss of the Vi antigen to the assumption of the rough state and serum susceptibility.

The actual bacteriolytic reaction is affected by ionic strength of the medium, pH, and temperature (Wardlaw, 1962). Also, the source of serum (Muschel, 1960), as well as the presence and concentration of Mg^{++} (Muschel and Treffers, 1956), influence the bactericidal effects of the system.

MATERIALS AND METHODS

Bacterial Cultures

A serum susceptible strain of E. coli was secured from the Microbiology laboratory of the Department of Biology, Kansas State Teachers College, Emporia, Kansas. The organism was cultured for experimental use at 37°C for 24 hr upon Brain Heart Infusion Agar slants and maintained in stock upon the same medium. By experimentation, it was determined that this strain was sensitive to a 30% unheated rat serum concentration. The viability of the organism, when exposed to heated rat serum of the same concentration, was uneffected.

A rat serum resistant E. coli strain was developed from the serum susceptible strain by means of serial passage through increasing concentrations of unheated rat serum. This strain, because of its stable character of serum resistance, is referred to as a genetically resistant strain. All cultures were grown at 37°C for 24 hr upon Brain Heart Infusion Agar slants.

Experimental Animals

Young adult albino, black, and hooded inbred rats were utilized for all experimental procedures.

Sera

Calf serum was processed from blood collected locally at the Fanestil Packing Co. Inc. The serum fraction was collected, bacteriologically filtered, aliquoted, and immediately frozen. Just prior

to use, it was heat inactivated at 56°C for 30 min in a constant temperature water bath.

Rat blood was drawn aseptically by cardiac puncture and refrigerated. After 30 min, it was centrifuged at 2000 rpm and the serum fraction collected and pooled. A portion was heat inactivated at 56°C for 30 min, the remainder refrigerated until used.

Tissue Culture Medium

The basic medium employed was Scherer's tissue culture medium. For peritoneal macrophage harvesting, the pH was adjusted between 6.8 and 7.0, according to a phenol red indicator in the medium, with a solution of NaHCO₃. To prevent cellular clumping, enough heparin was added to the medium to give a 1:10000 dilution. The tissue culture maintenance medium was composed of Scherer's plus 30% heated calf serum.

Harvest of Peritoneal Macrophages

Using aseptic technique, the skin of the ventral surface of the animal was laid back, and 7 to 10 ml of sterile heparinized Scherer's medium was injected into the peritoneal cavity. The abdomen was massaged to suspend the peritoneal macrophages. An incision was made in the peritoneal wall, and the medium removed with an ungraduated pipette and placed in a flask. The collection from several animals was pooled and a count of the number of cells in suspension was determined with a hemacytometer. The number of cells was adjusted to $3-4 \times 10^6$ cells per ml by means of addition of the appropriate volume of Scherer's medium.

The suspension was distributed to tissue culture bottles and/or flying coverslip tubes in 15 ml and 1 ml volumes, respectively. In most cases the cells were allowed to incubate approximately eight hr for settling and adaptation to the tissue culture environment, after which the medium was removed and replaced with Scherer's plus heated calf serum or maintenance medium.

Infection of Tissue Culture Systems

A 24 hr culture of the appropriate strain of E. coli was suspended in sterile saline, and the suspension adjusted to 20% light transmission on the spectrometer-20 or 160×10^7 organisms per ml. After a series of ten-fold dilutions, the tissue cultures were infected with a 10:1 (organism to cell) ratio. Phagocytosis was allowed to occur for 1 to 2 hr, after which time the infecting medium was removed and replaced with maintenance medium.

Bactericidal System

The test system utilized to determine the serum sensitivity of the organisms was composed of sterile Scherer's medium supplemented with 30% heated calf serum plus 30% unheated rat serum.

Isolation of E. coli Cultured in vitro

By microscopic examination of May-Grünwald Giemsa stained coverslips, the infection process was closely followed. To control extensive extracellular multiplication, several methods were employed. Unheated rat serum added to the cell system at a specific time during the infection, periodic changing of the medium, and addition of a 10 g/ml concentration of streptomycin for a limited time period were those methods which proved effective.

When extensive intracellular multiplication and cellular damage had occurred, the cells were removed from the culture bottle with a rubber policeman. To remove intact cells present in the suspension three methods were employed: filtration, differential centrifugation, and sonification. The filtration process involved filtering the entire system through Whatman #2 paper, observing microscopically for intact cells, and adding to a specific volume of the filtrate containing organisms enough unheated rat serum to obtain a 30% concentration. To separate the rat cells from the supernatant by centrifugation, the system was spun at 2000 rpm for 15 min. The supernatant was removed with an ungraduated pipette, observed microscopically, and those bacterial cells in suspension were serum tested. To disrupt the cells by sonification, a setting of seven amperes for a two min period proved effective in lysing host cells without effecting the bacterial viability. The organisms in the lysate were then subjected to the bactericidal action of normal unheated rat serum.

Dilution and Plating

All samples were diluted by a ten-fold series, using 4.5 ml saline blanks. One ml or 0.1 ml samples were placed in sterile petri dishes, depending upon the required dilution, and pour plates were made using nutrient agar. The plates were allowed to incubate from 18-24 hr, then the colonies were counted.

Staining

Staining of the coverslips was done using May-Grünwald and Giemsa stains. The coverslips were fixed in absolute methanol

for five min; stained with May-Grünwald thirteen min and Giemsa nine minutes. Acetone, acetone-xylol (1:1), and xylol were utilized for clearing. The coverslips were mounted with permount medium.

Preparation of Glassware

Immediately after use all glassware was placed in a Chlorox-7X soap solution to sterilize as well as prevent particulate protein deposits. After a 24 hr period, the glassware was hand-washed in an Alconox soap solution, rinsed thoroughly with tap water, then rinsed 6-7 times with deionized water. It was drained upon brown wrapping paper until thoroughly dry. The material was sterilized by autoclaving at 15 lb. pressure and 150°C for 20 min.

RESULTS

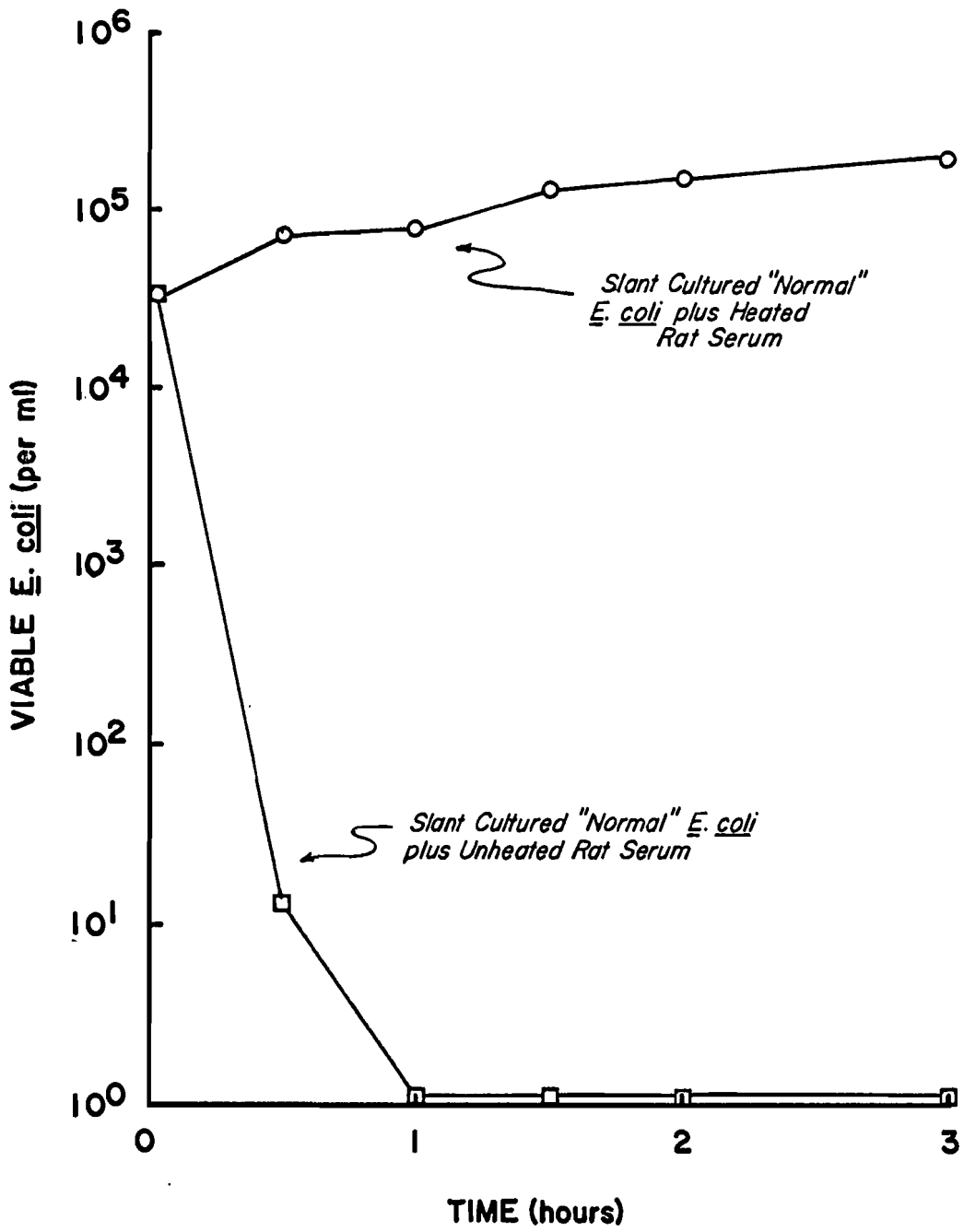
IN VITRO STUDIES

Bactericidal test of "normal" or serum susceptible E. coli.

To determine if unheated rat serum would be effective in controlling extracellular multiplication in the cell system, slant cultured E. coli was exposed to fresh unheated rat serum. This test illustrated the effectiveness of the bactericidal system in reducing the viable number of organisms. The necessity of the heat labile factors for an effective bactericidal system can be seen, as the same organism when exposed to rat serum heat inactivated at 56°C for 30 min did not decrease in viable number but began to increase in number during the three hr testing period (Fig. 1).

Alteration of the serum response of "normal" E. coli present in the cell culture supernatant.

Early work, utilizing a tissue culture system infected with "normal" E. coli was done in an attempt to determine the respective roles of cellular and humoral factors in the host defense mechanisms. To control extracellular multiplication and prevent masking of the intracellular events, 30% unheated rat serum was added to the cell system. When intracellular multiplication became evident by microscopic observation, the supernatant was plated for viable number. A control system of Brain Heart Infusion Agar slant cultured "normal" E. coli plus unheated rat serum was used to determine the effectiveness of the bactericidal system. Those organisms present in the



supernatant of the cell system showed a decrease in count initially, but subsequently the number began to increase. The bactericidal system was effective as illustrated by the drop in viable number of those organisms in the control system during the initial one and one-half hr (Fig. 2). These results provided evidence of a change in the organism after intracellular residence resulting in a serum resistant state as compared to those organisms cultured upon slants.

Effects of culture in the tissue culture medium upon serum sensitivity.

To determine if the acquired resistance was a direct attribute of intracellular residence, the organism was cultured in the defined tissue culture medium in the absence of cells. The medium was inoculated and incubated for a 24 hr period, after which time the organism suspension was adjusted with Scherer's and heated calf serum to 20% light transmission. These organisms were exposed to the bactericidal serum system and the viability curve compared to the slant cultured E. coli in unheated rat serum. There was no significant effect of culture in this complex medium upon the serum sensitivity of the organism (Fig. 3).

Effective methods employed to eliminate experimental artifacts.

Microscopic observation of the supernatant of the cell system revealed a possible artifact in the form of intact macrophages. These floating cells present in the supernatant could protect the organisms from the bactericidal serum action. Thus, the observed resistance could have been due to mere protection of the organisms from the extracellular unheated serum factors by intracellular

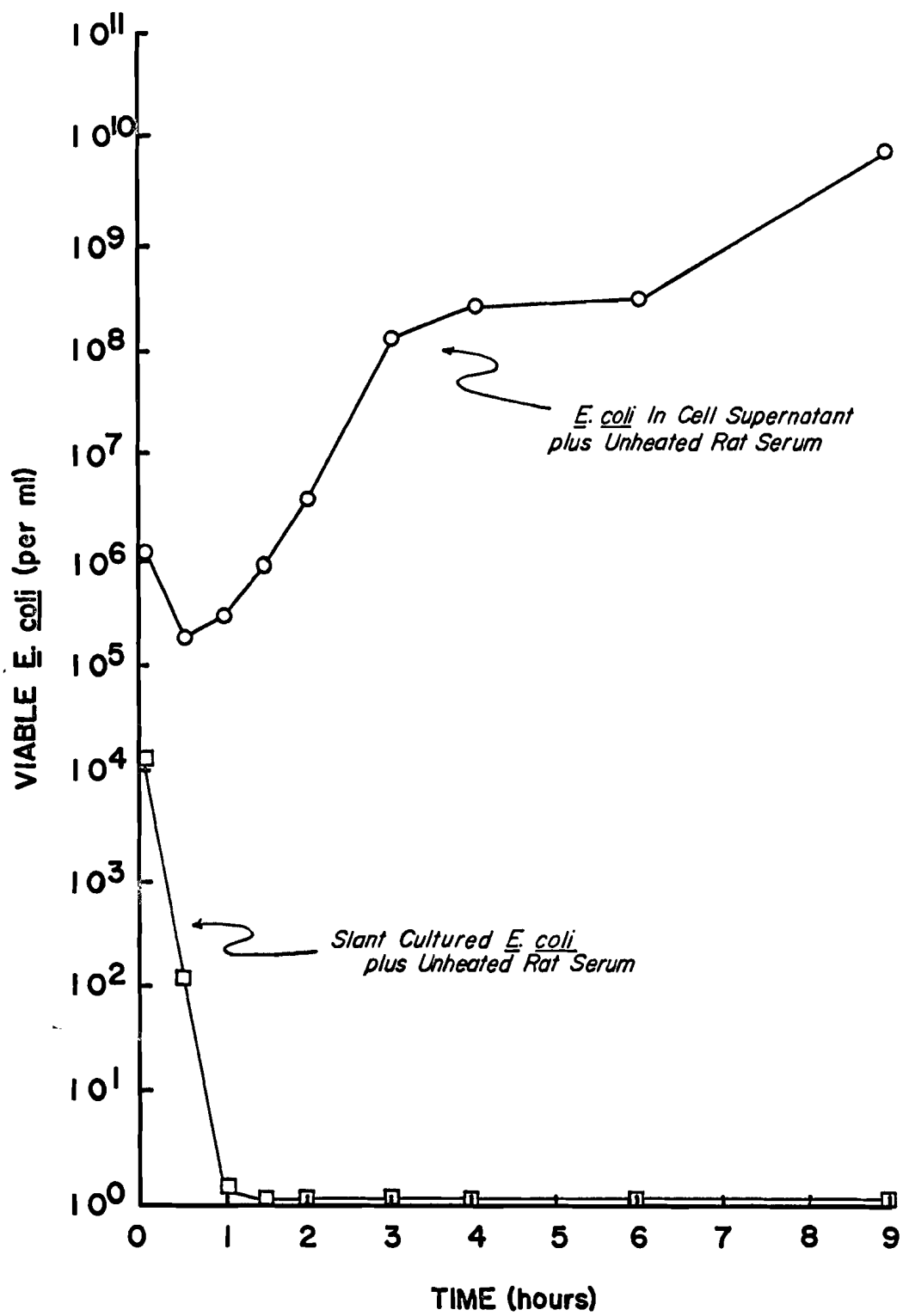
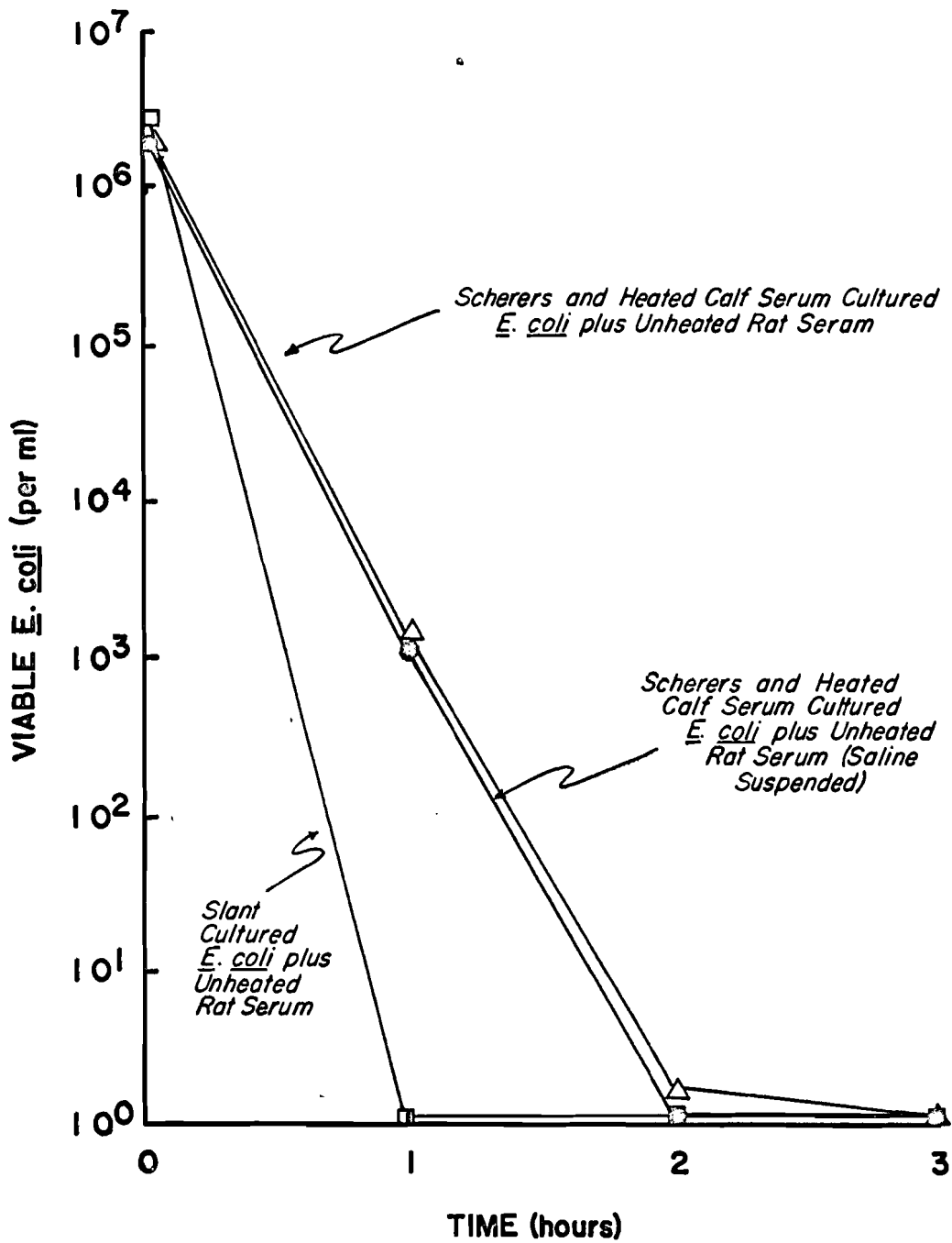


Figure 3: Serum sensitivity of E. coli cultured in tissue culture medium or Scherer's plus 30% heated calf serum.



residence. Therefore a different experimental procedure had to be devised to exclude this possibility.

Initially, sonification was used to lyse those intact cells present in the tissue culture system at the time of bactericidal test. Cells were harvested, the count adjusted to $3-4 \times 10^6$ cells per ml, and a portion of the cell suspension was infected with a 10:1 (organism to cell) ratio. One ml suspensions of infected and uninfected cell suspensions were planted in flying coverslip tubes. The remainder of the infected cell suspension was placed in a sterile sonification apparatus. All systems were incubated for 4 hr at 37°C , and coverslips stained periodically to observe the progression of the infection (Plate 1, 2).

Following the incubation period, the infected host cells were sonified at a setting of 5 amperes for 2 min; this amperage and time were determined effective in lysing intact macrophages (Fig. 4), but did not effect the bacterial viable count (Fig. 5). The lysed macrophage suspension was divided into two equal portions; unheated rat serum was added to one portion and heated to the second in 30% concentrations, and each system plated periodically. A system of "normal" slant cultured E. coli plus 30% unheated rat serum was used as a control system.

This procedure revealed a significant difference in the serum sensitivity of the organism after close cellular contact (Fig. 6). Although there was an initial drop in viable count, this could have been an attribute of the sonification effects, as it occurred in both the heated and unheated systems. Also, bactericidal serum factors in the harvested exudate of the peritoneal cavity

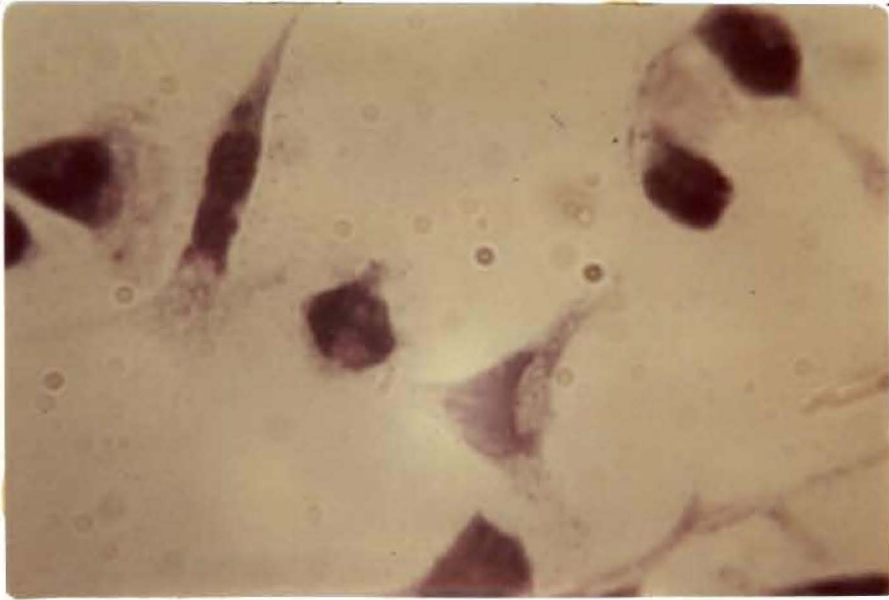


Plate 1: Uninfected "non-immune" rat peritoneal mononuclear cells (1600X).

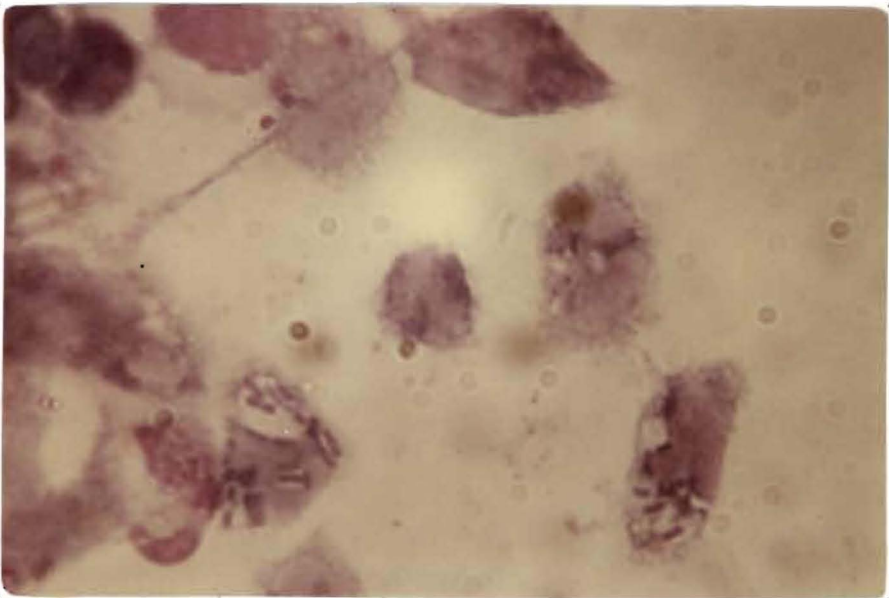


Plate 2: E. coli infected "non-immune" rat peritoneal mononuclear cells (1600X).

Figure 4: Effect of sonification upon intact rat peritoneal mononuclear cells.

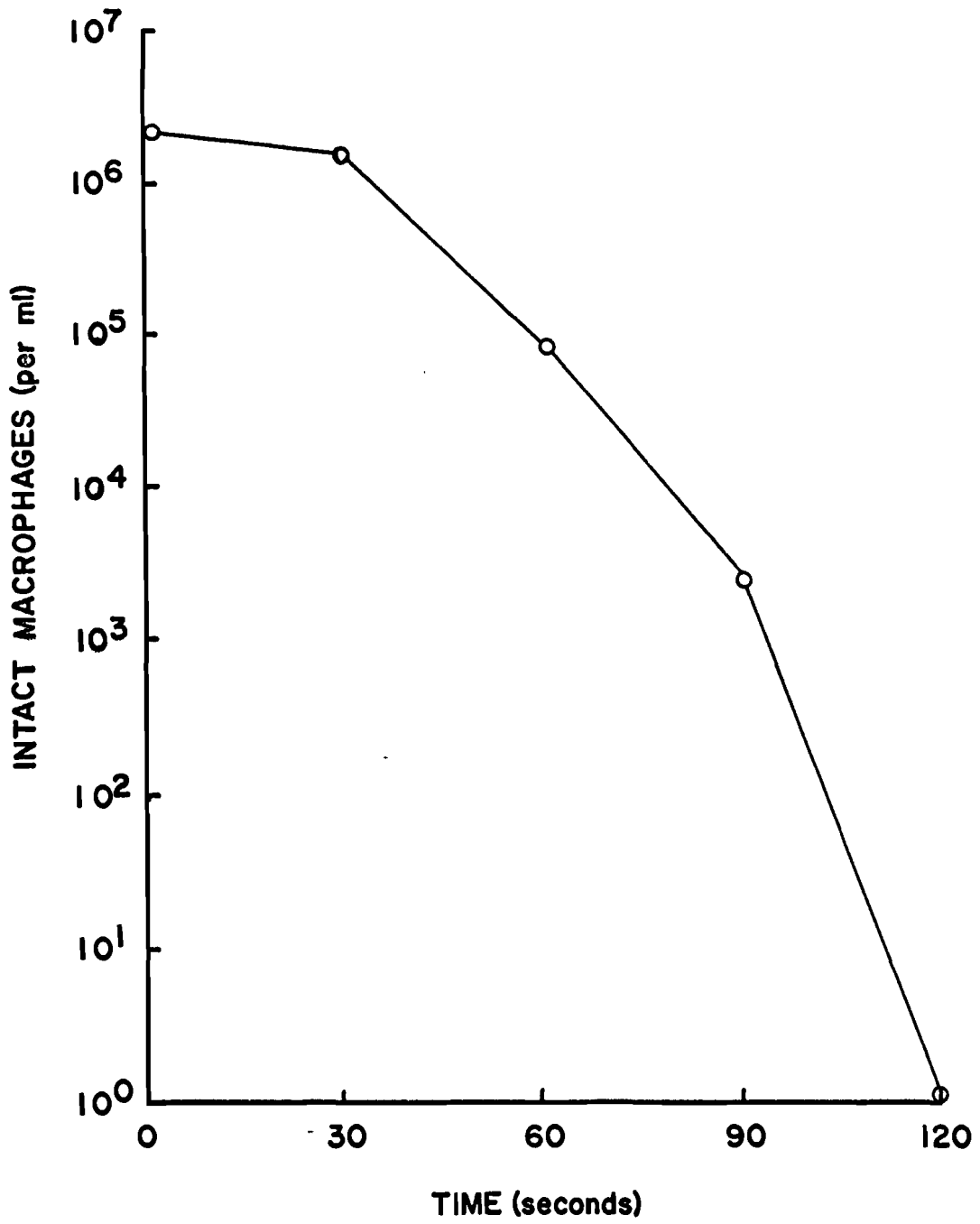


Figure 5: Effect of sonification upon E. coli viability.

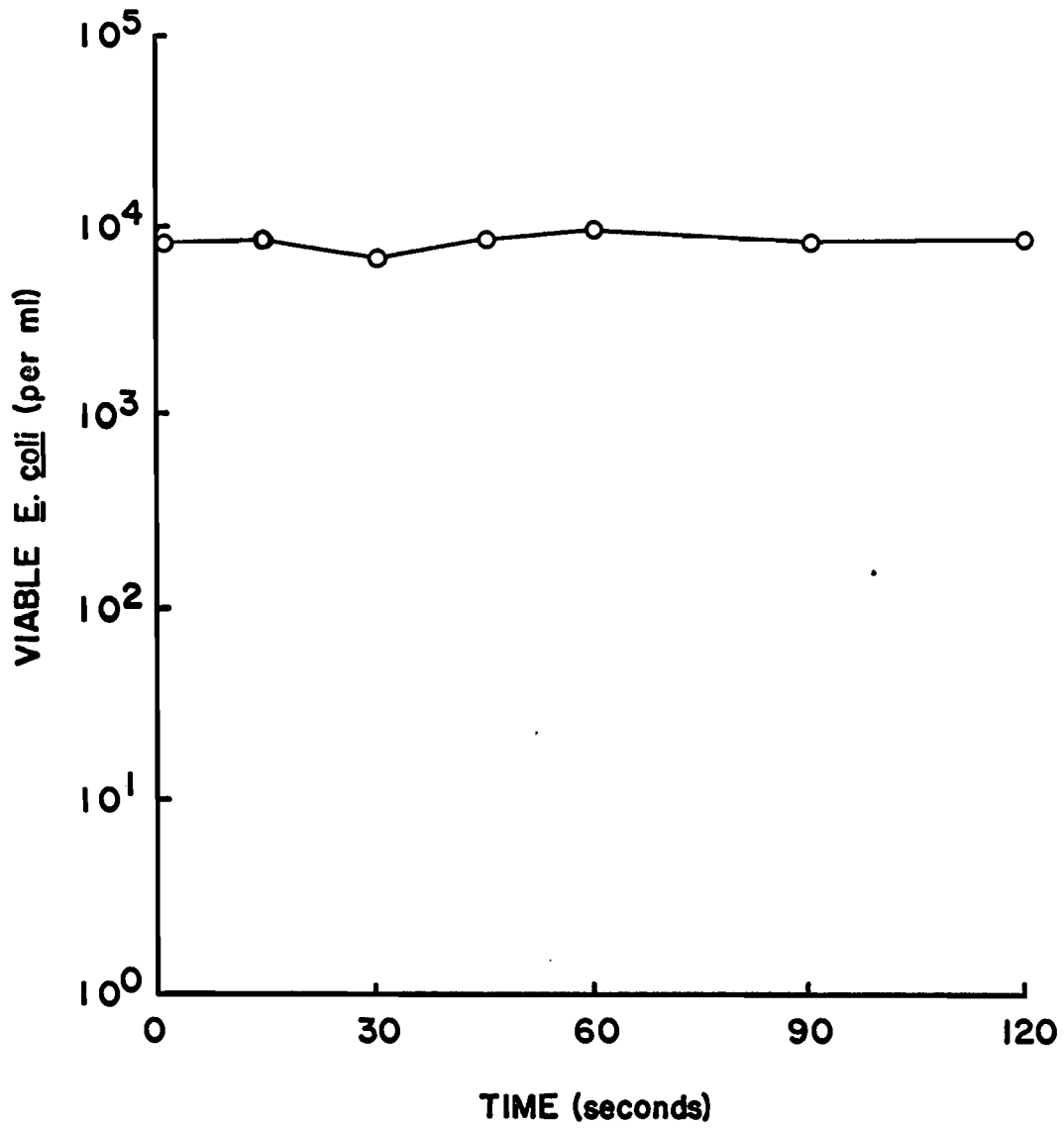
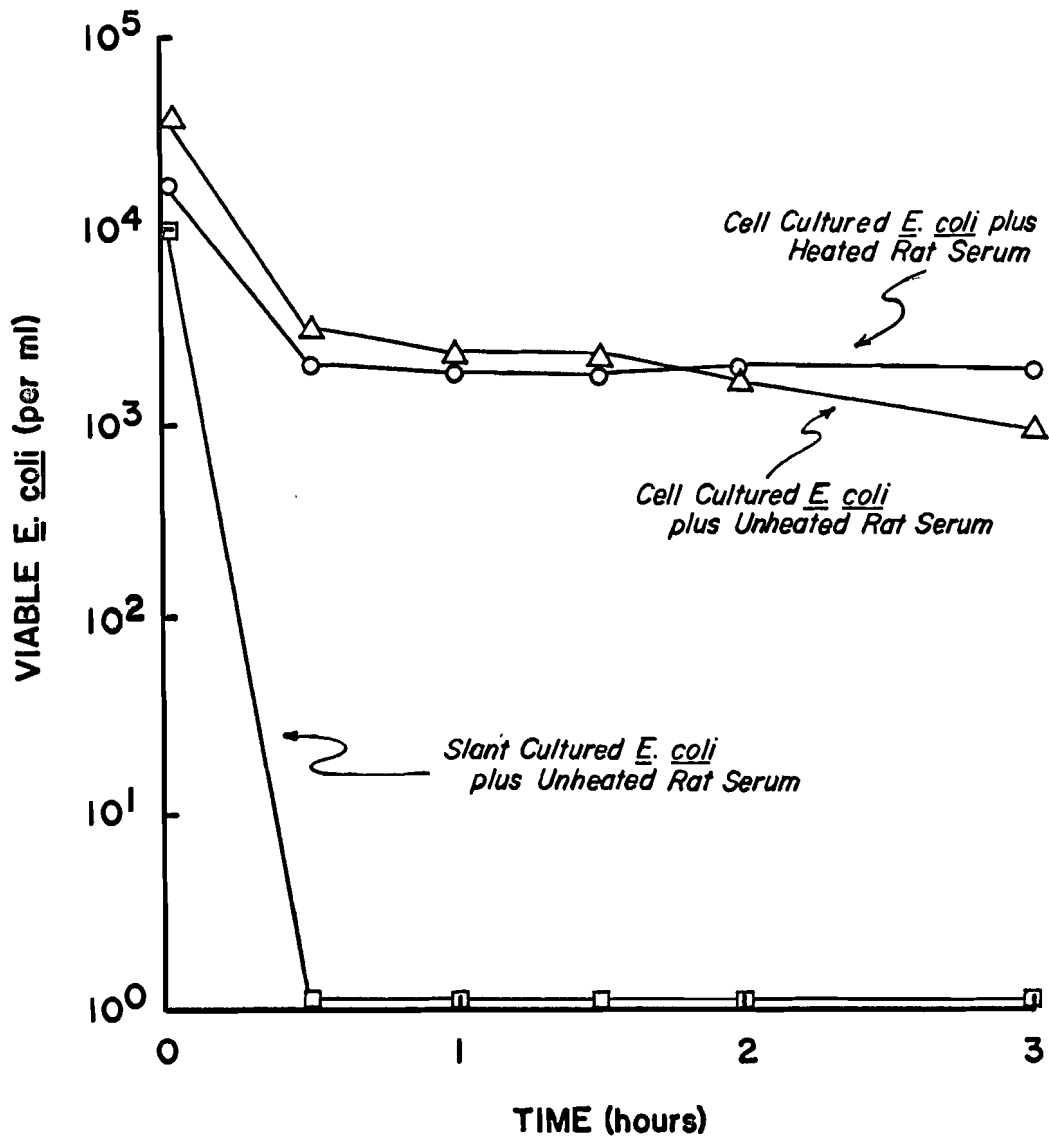


Figure 6: Serum sensitivity of cell cultured E. coli, grown in the presence of exudate factors and isolated from the rat peritoneal mononuclear cells by sonification.



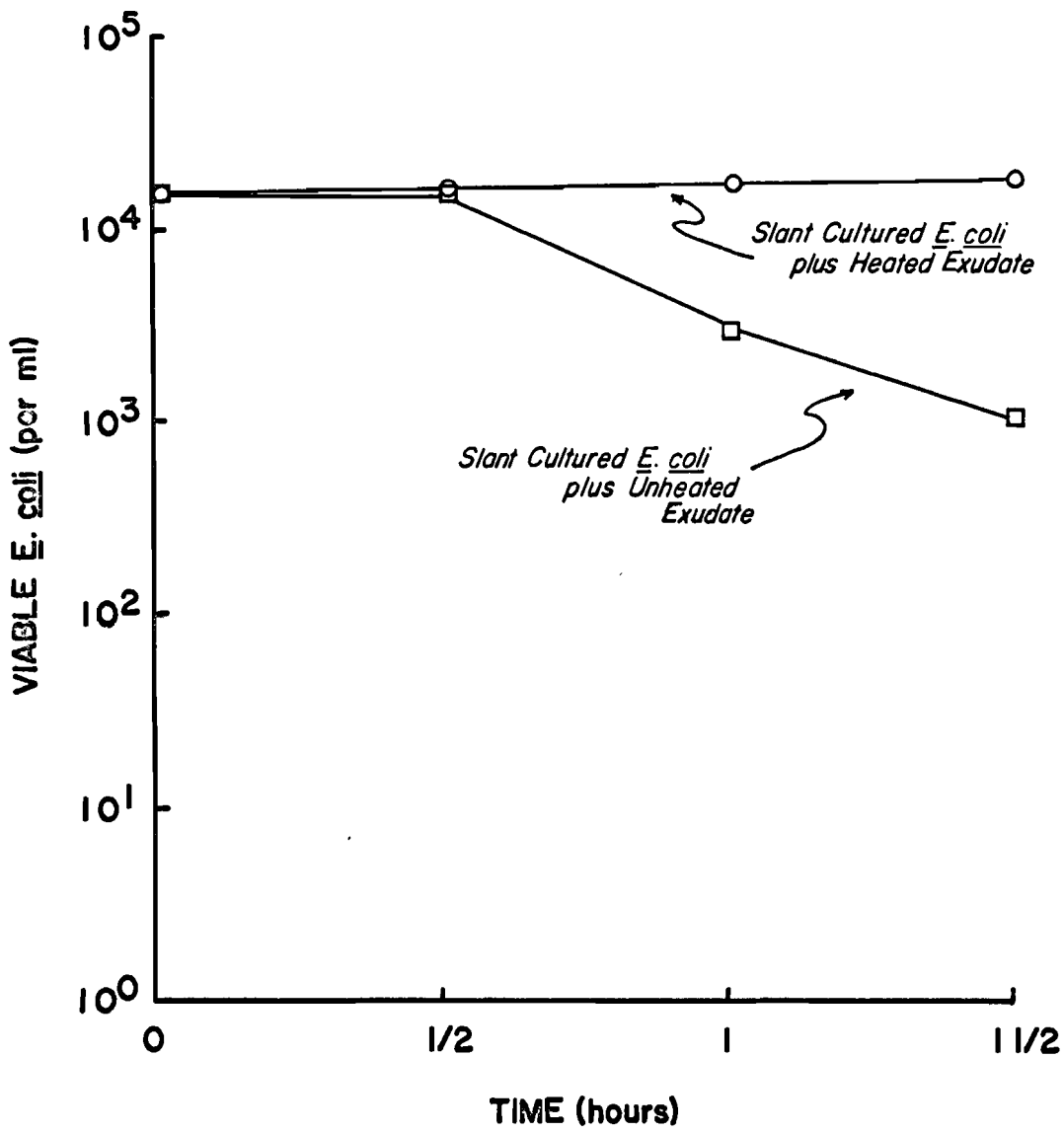
initiate a drop in viable number. The viable count remained relatively stable the remainder of the testing period, while there was a complete loss of viability in the control system after one-half hr.

Due to the harshness of the sonification procedure, another method was sought which would be effective in separating the organisms from the intact cells. If this procedure were traumatic enough to disrupt intact cells, a temporary change in the bacterial cell surface could be effected by this procedure initiating a loss of the resistance obtained upon cell passage.

Also noted in the sonification experiment was the presence of bactericidal serum factors in the harvested peritoneal exudate. Such factors in the medium of the cell culture could have promoted a natural selection phenomena, those organisms possessing the potential genetic character to become serum resistant surviving exposure to low concentrations of unheated factors in the exudate. If the cells served as a place of seclusion from these bactericidal factors and merely temporarily released organisms for serum exposure, the cell culture might promote such a selection process.

To demonstrate the bactericidal action of these factors in the exudate, cells were harvested from the peritoneal cavity, centrifuged out of the medium, and the centrifugate collected. It was divided into two aliquots, and one was heated at 56^oC for 30 min and the other refrigerated. Each aliquot was infected with slant cultured E. coli and plated periodically. The unheated exudate decreased the viable number of organisms due to the heat labile factors present, since the heated fraction had no effect upon the viable number (Fig. 7).

Figure 7: Effect of exudate factors upon the viability of normal
E. coli.



This degree of bactericidal action due to exudate factors in the harvesting medium had been serving to control extracellular multiplication in the supernatant medium. It was noted, that if these factors were removed from the infected cell system, extensive extracellular multiplication masked the intracellular development of serum resistance. Therefore, it became necessary to devise a method to control extracellular multiplication and yet eliminate a possible natural selection phenomenon. A first attempt was made to correct the situation by the addition of unheated rat serum to the cell system at a point in the infection cycle when extracellular multiplication was microscopically observed. As the organisms were free of serum factors from 5 to 8 hr, if resistance were to develop due to intracellular residence, this would be allowed to occur. Such added serum factors would merely eliminate unphagocytized organisms in the medium. Upon complete cellular destruction, the organisms in the supernatant was harvested and serum tested.

To replace the harsh sonification process, differential centrifugation was utilized and the organisms in the centrifugate serum tested. To test the effectiveness of the separation of the host cells from the supernatant, a sample of the centrifugate was examined microscopically. The organisms in an uncentrifuged aliquot were also exposed to unheated rat serum in an attempt to determine if intracellular residence could account for the observed serum resistance. The controls of "normal" slant cultured E. coli in heated and unheated rat serum were utilized. There appeared to be

a higher degree of resistant organisms in the uncentrifuged system as compared to the centrifuged system (Fig. 8). This was probably due to the presence of intracellular organisms which were protected from the bactericidal system. These data indicated a significant difference in the display of resistance by organisms in the unheated control and both test systems.

To unequivocally verify these observations of altered serum response and insure the removal of intact cells from the test system, the method of differential filtration was employed. The process involved the use of filter paper of adequate pore size to remove cells, but not bacteria. To rid the system of extracellular organisms, unheated rat serum was added to the cell system when extensive extracellular multiplication became evident by microscopic observation. Upon complete cell destruction (Plate 3), the medium was removed and filtered, and unheated rat serum was added to an aliquot of the filtrate to serum test those organisms present. The proper control systems were prepared to test the effectiveness of the bactericidal system and each system was plated. As observed in the differential centrifugation procedure, alteration in the serum response of the cellular associated organism was similar (Fig. 9).

Even though added late in the infection cycle, the presence of unheated rat serum in the medium offered the chance of selection for serum resistant organisms should they be present in the extracellular population. Therefore, other methods were sought and utilized in an attempt to control extracellular multiplication and eliminate the possible process of natural selection. Included in these methods were changing of the medium when deemed necessary

Figure 8: Serum sensitivity of E. coli present in centrifuged and uncentrifuged supernatant. Unheated rat serum was added to cell system to control extracellular multiplication.

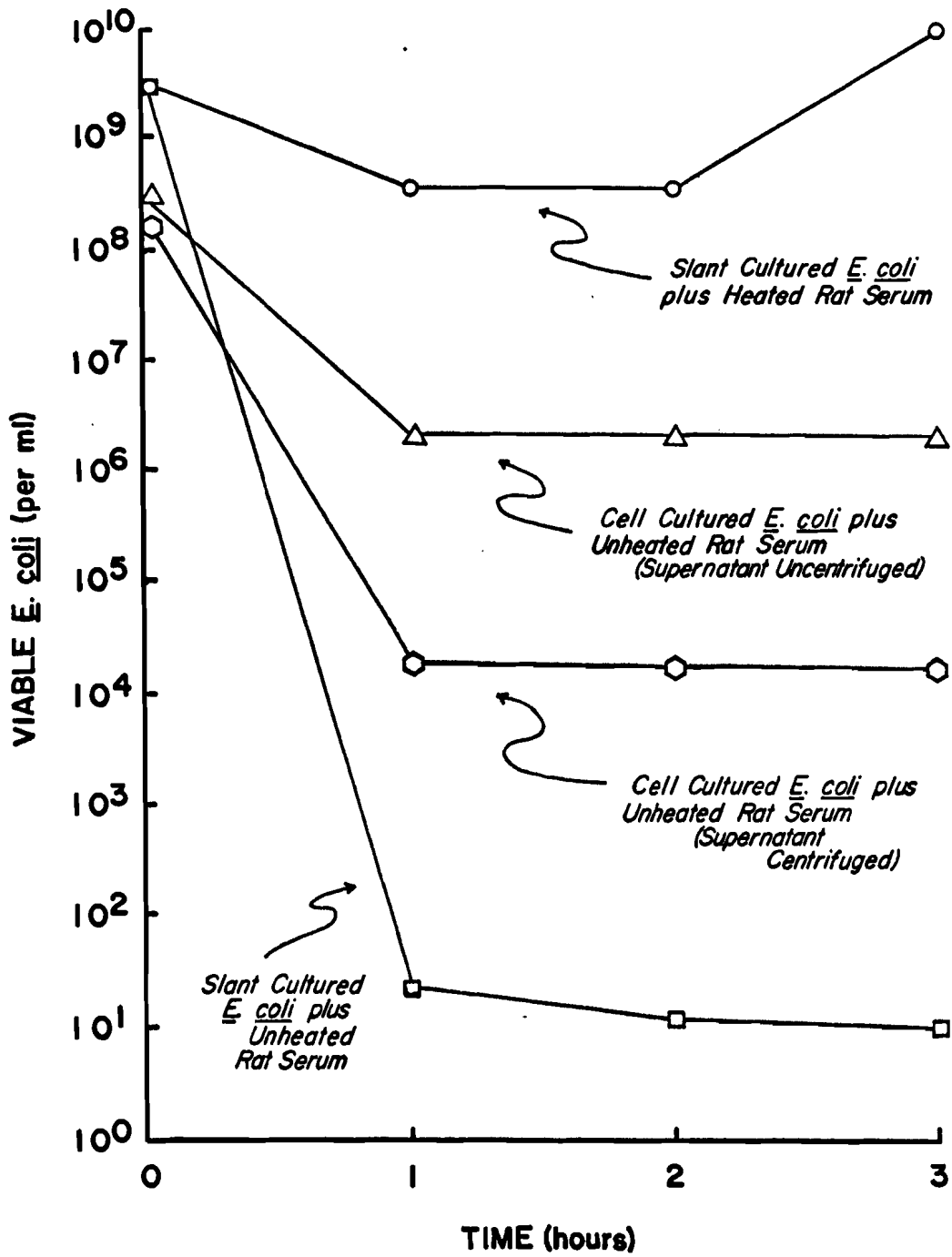


Figure 9: Serum sensitivity of cell cultured E. coli present in the filtered supernatant. Unheated rat serum was added to cell system to control extracellular multiplication.

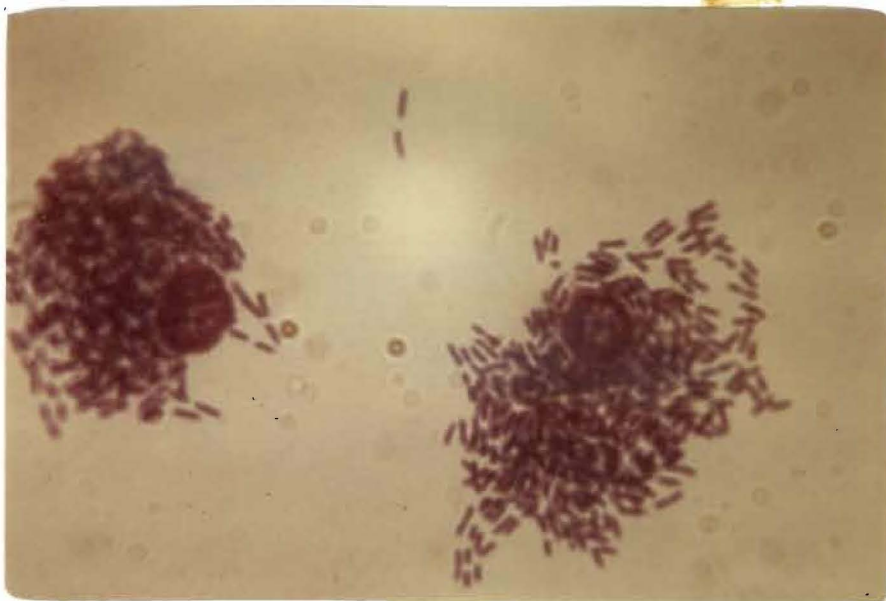
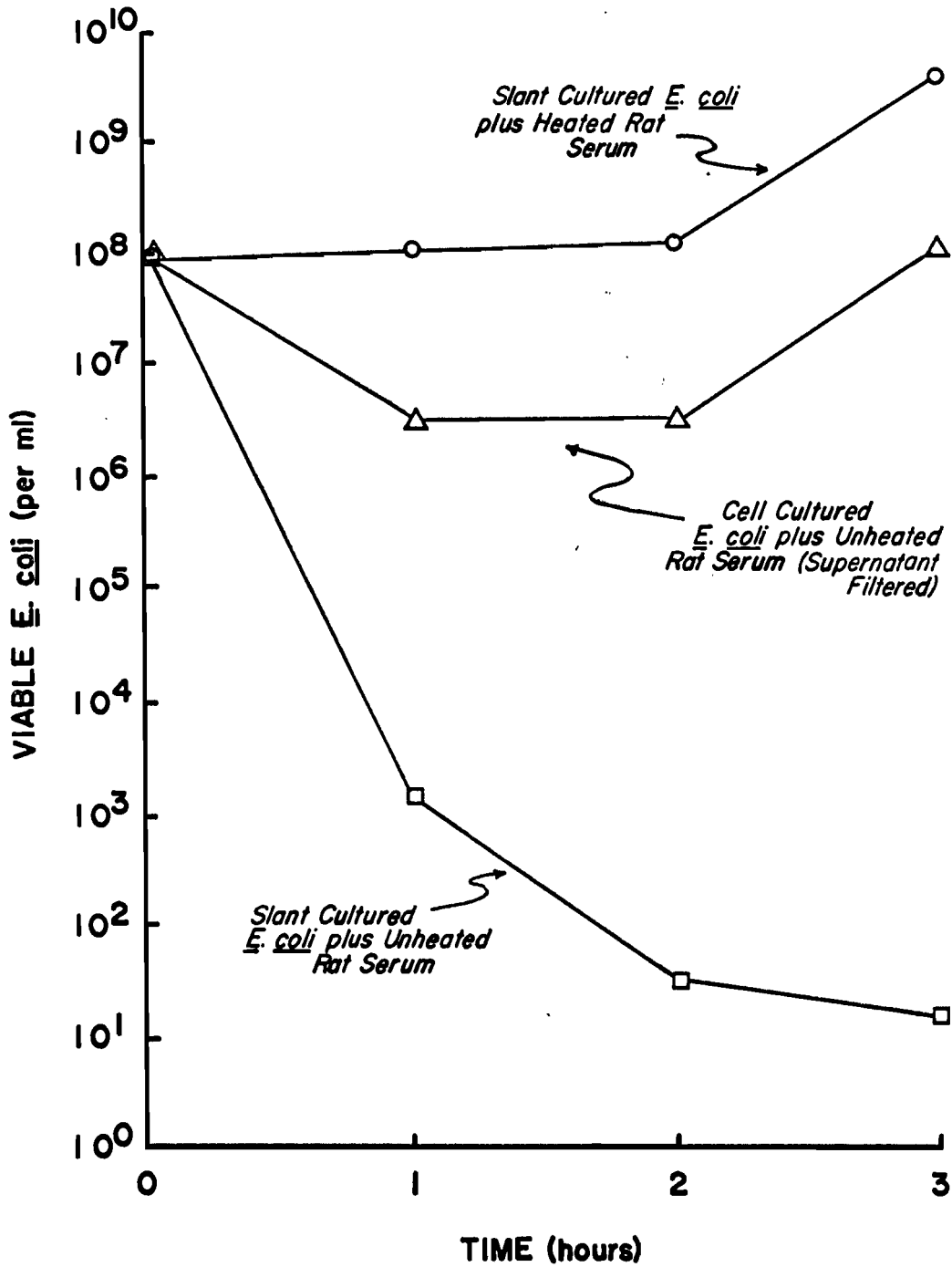


Plate 3: Destruction of "non-immune" rat peritoneal mononuclear cells by gross intracellular multiplication (1600X).



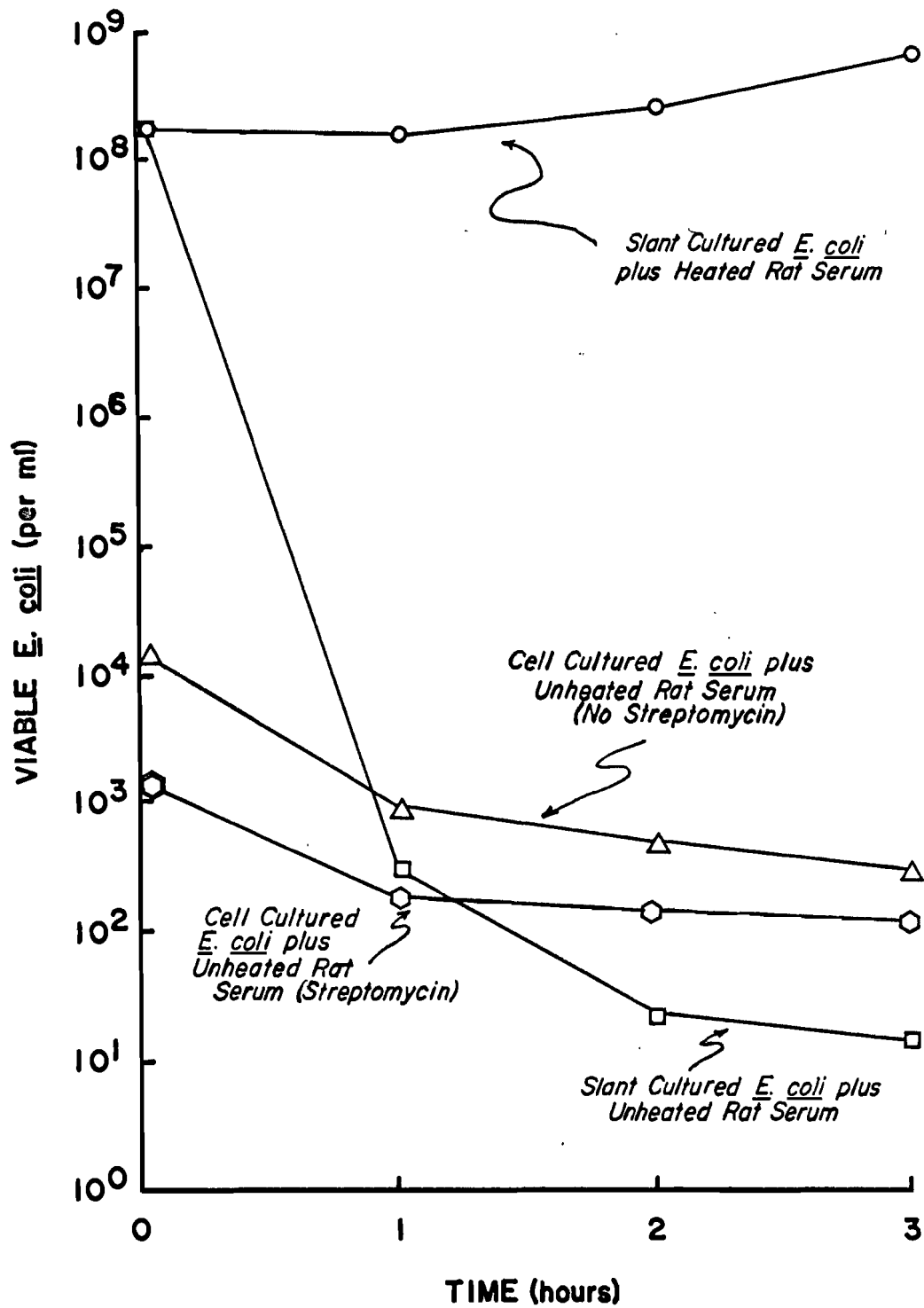
by microscopic examination and addition of antibiotics to the medium for a short time period at a point when extracellular multiplication became evident. Also, when harvesting the organism for the serum test, the cells were removed from the glass with a rubber policeman in an attempt to obtain larger numbers of intracellular organisms.

Each method utilized provided results supportive of those results obtained with other procedures (Fig. 10). Upon comparison with the unheated rat serum control, those organisms from the cell system displayed a significant amount of resistance. Antibiotics in the medium had a slight effect upon the number of organisms recovered from the system, but the amount of resistance in both systems was comparable.

Time of appearance and persistence of serum resistance of cell cultured E. coli.

In an attempt to ascertain the time period of intracellular residence necessary for the appearance of the observed phenomenon, as well as to determine the necessity of organismal multiplication, intracellular organisms were harvested periodically after the initial infection of the cell system. The cell culture was infected with a 5:1 (organism to cell) ratio for one hr, after which time the infecting medium was replaced with Scherer's and heated calf serum. After an additional one and one-half hr infecting period, a selected number of tubes were removed, the coverslips ground to disrupt the infected macrophages, and the supernatant medium pooled. The medium was divided into two portions; heated rat serum was added to one and unheated rat serum to the other. The heated and unheated serum

Figure 10: Serum sensitivity of cell cultured E. coli present in the filtered supernatant. The extra-cellular multiplication was controlled by medium change or by addition of streptomycin.



controls were prepared, and all systems were plated periodically. The same procedure was repeated at 19 and 24 hr to determine if further development and persistence of the serum resistant state occurred.

The effects of serum exposure of the organisms from the cell system exhibited a change in serum sensitivity as early as two and one-half hr after the initial infection (Fig. 11). Bactericidal testing at 19 and 24 hr after the initial infection, also revealed a significant degree of resistance (Fig. 12, 13). Some initial drop in viable number occurred, but this was probably a direct attribute of extracellular multiplication, as no measures were employed to control the number of organisms present in the supernatant medium.

Therefore, it would appear that a relatively short time period in the cell was necessary for an alteration in the serum response to develop. Maintenance of this resistance was displayed throughout the entire 24 hr infection period. The importance of the intracellular multiplication of the organism can not be determined from these data, as several generation times could have elapsed from the initial infection time to the first testing period.

Transient nature of the cell cultured resistance.

If cellular association resulted in a natural selection of organisms with the potential genetic character to become serum resistant, this type of resistance would not be lost if the organisms were allowed to multiply extensively. In those experimental procedures in which unheated rat serum was utilized to control

Figure 11: Serum sensitivity of cell cultured E. coli
isolated from the cell system two and one-half
hr after the initial infection.

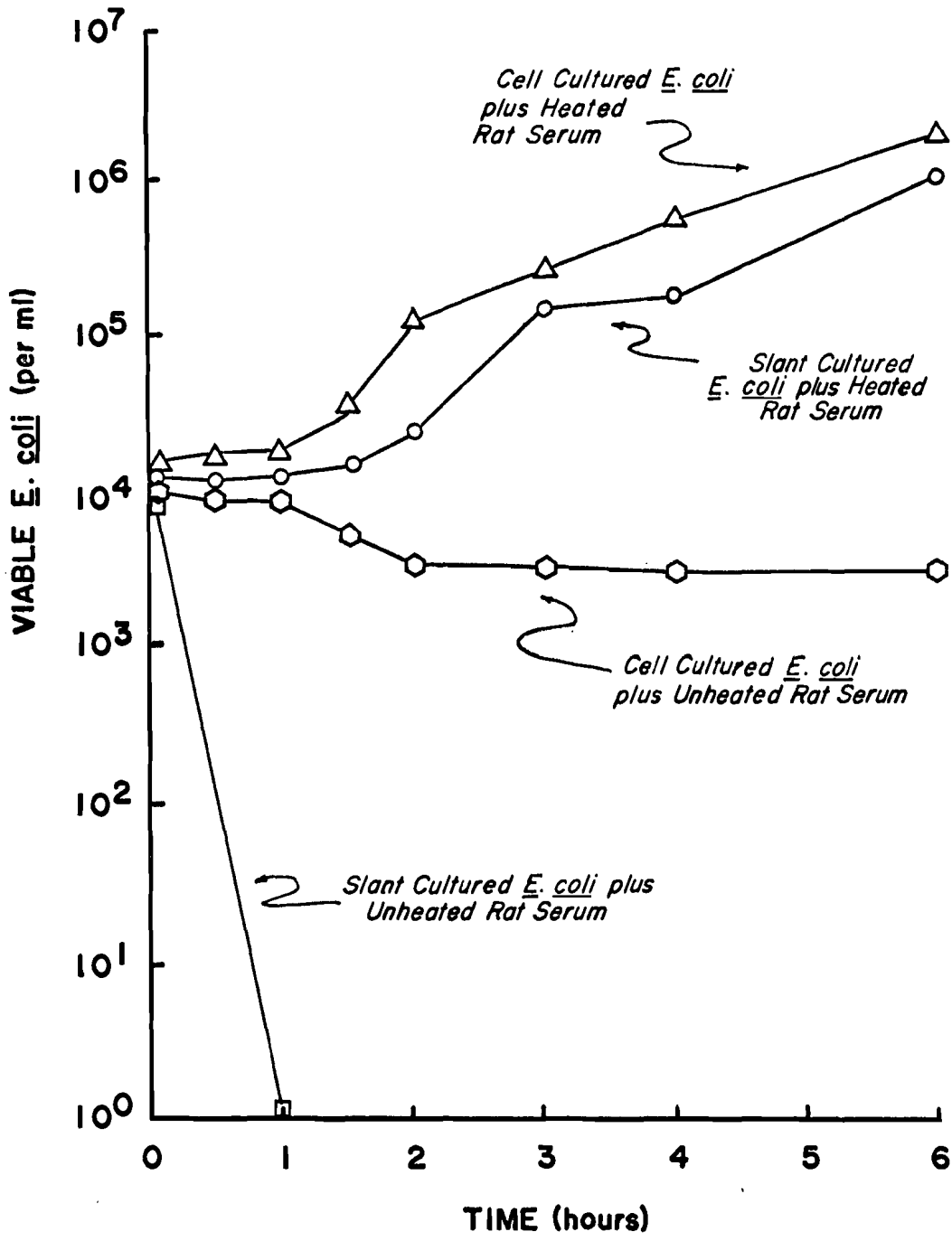


Figure 12: Serum sensitivity of cell cultured E. coli isolated from the cell system 19 hr after the initial infection.

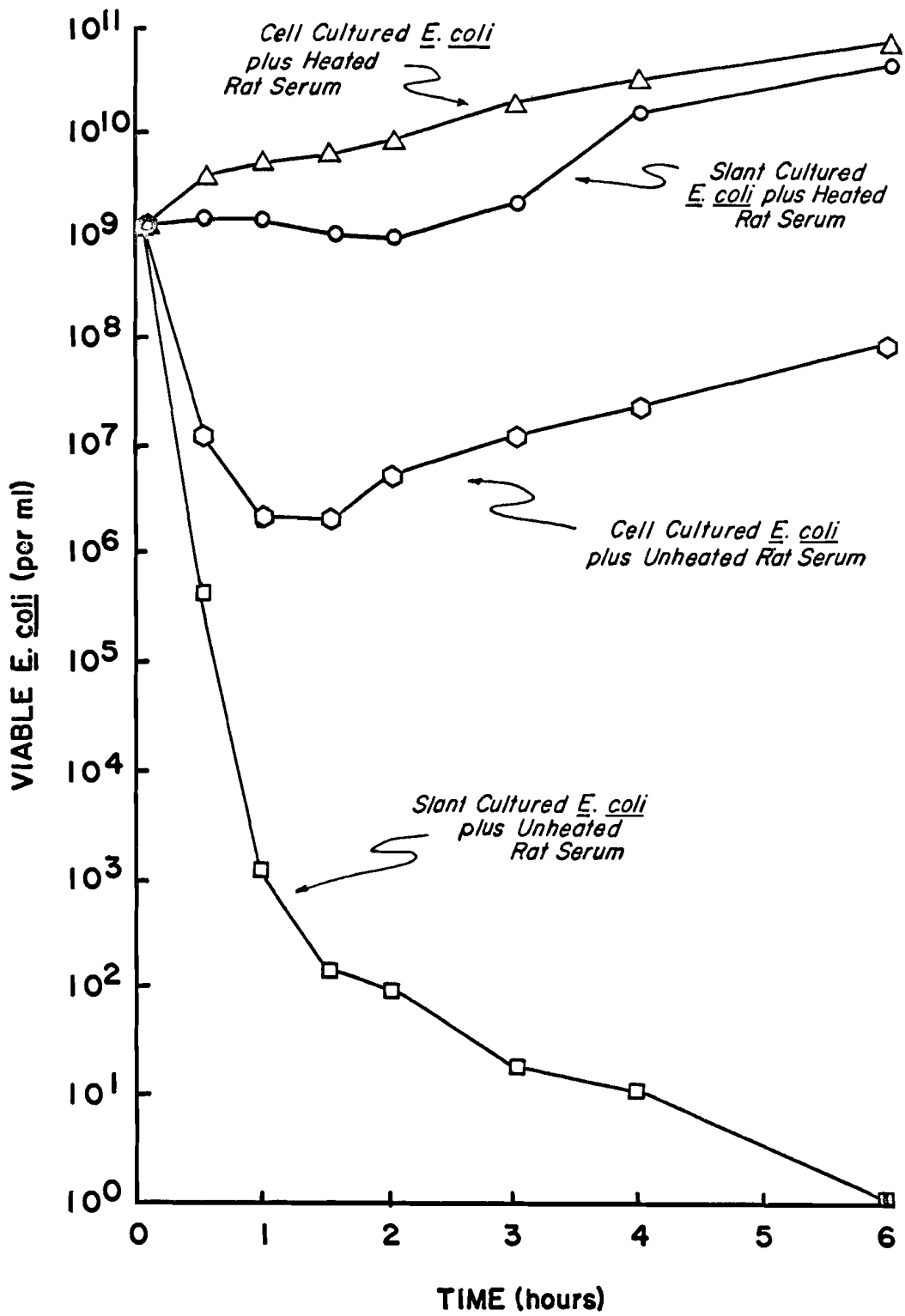
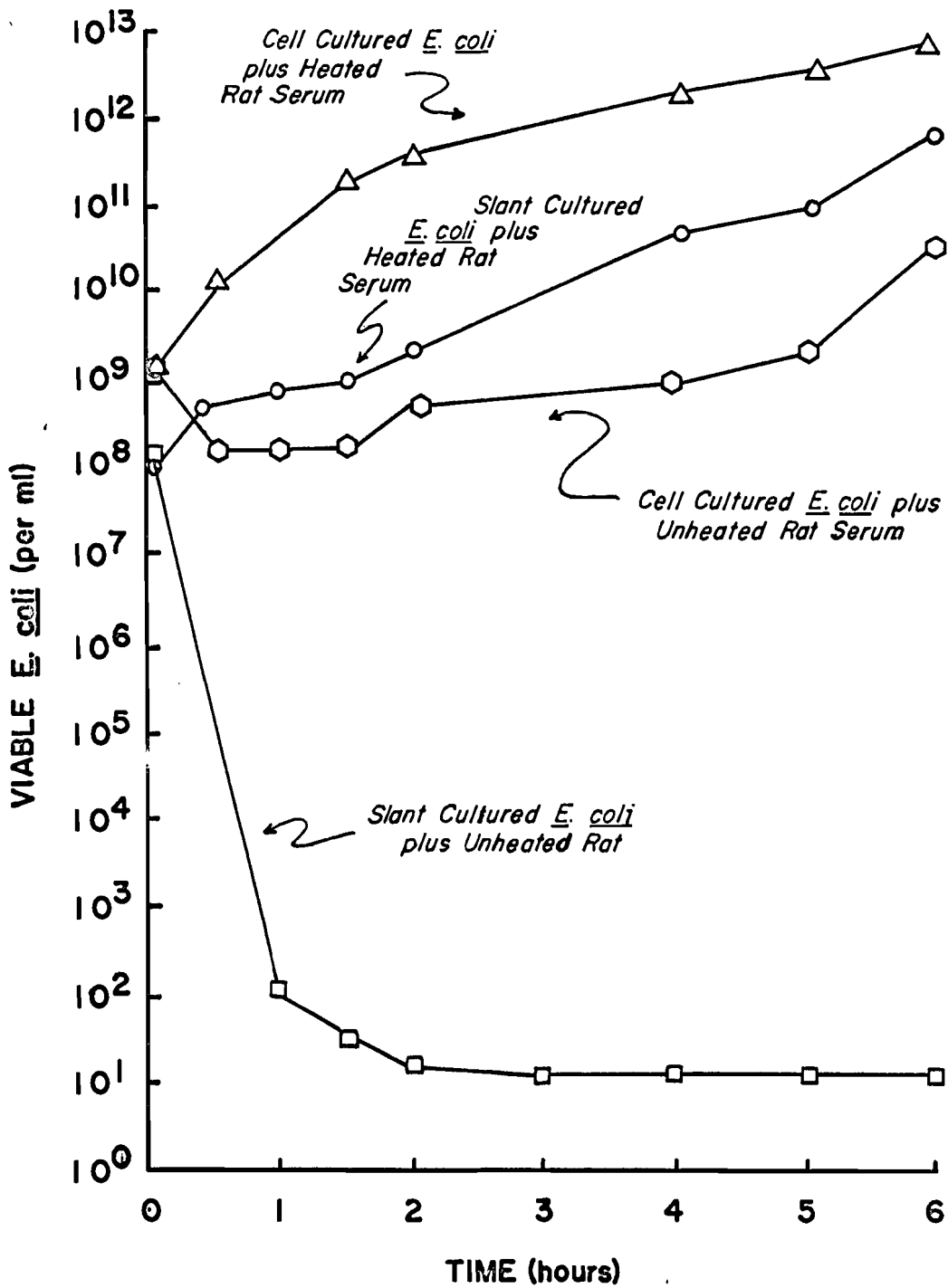


Figure 13: Serum sensitivity of cell cultured E. coli
isolated from the cell system 24 hr after the
initial infection.



extracellular multiplication, subculture methods were of utmost importance in determining the nature of the resistance. For if the cells were allowed short time exposures to the serum factors and natural selection occurred as a result, this would be apparent upon subculture as the resistance would persist.

Organisms exhibiting serum resistance were subcultured upon Brain Heart Infusion Agar slants or in a medium of Scherer's plus 30% heated calf serum. Twenty-four hr culture suspensions were prepared, adjusted to 20% light transmission, and tested for serum sensitivity. With (Fig. 14) or without (Fig. 15) serum exposure during the cellular infection period, those organisms which displayed resistance when isolated, no longer retained this characteristic after subculture upon artificial media. This was indicative of a transient nature of the newly acquired resistance, and if allowed to multiply several generation times, the organism did not possess the genetic character necessary to retain the resistance.

Effect of washing upon the newly acquired resistance.

As subculture procedures revealed the transient nature of the resistance of the organism, temporary alterations in surface characteristics of the bacterial cell altering the antigenic structure of the cell might be responsible for these observations. Surface alteration due to cellular deposition, bacterial adsorption, or altered metabolism of the organism thus inhibiting the bactericidal action of serum.

Figure 14: Effect of subculture upon cell cultured serum resistant E. coli. Unheated rat serum was added to cell system to control extracellular multiplication.

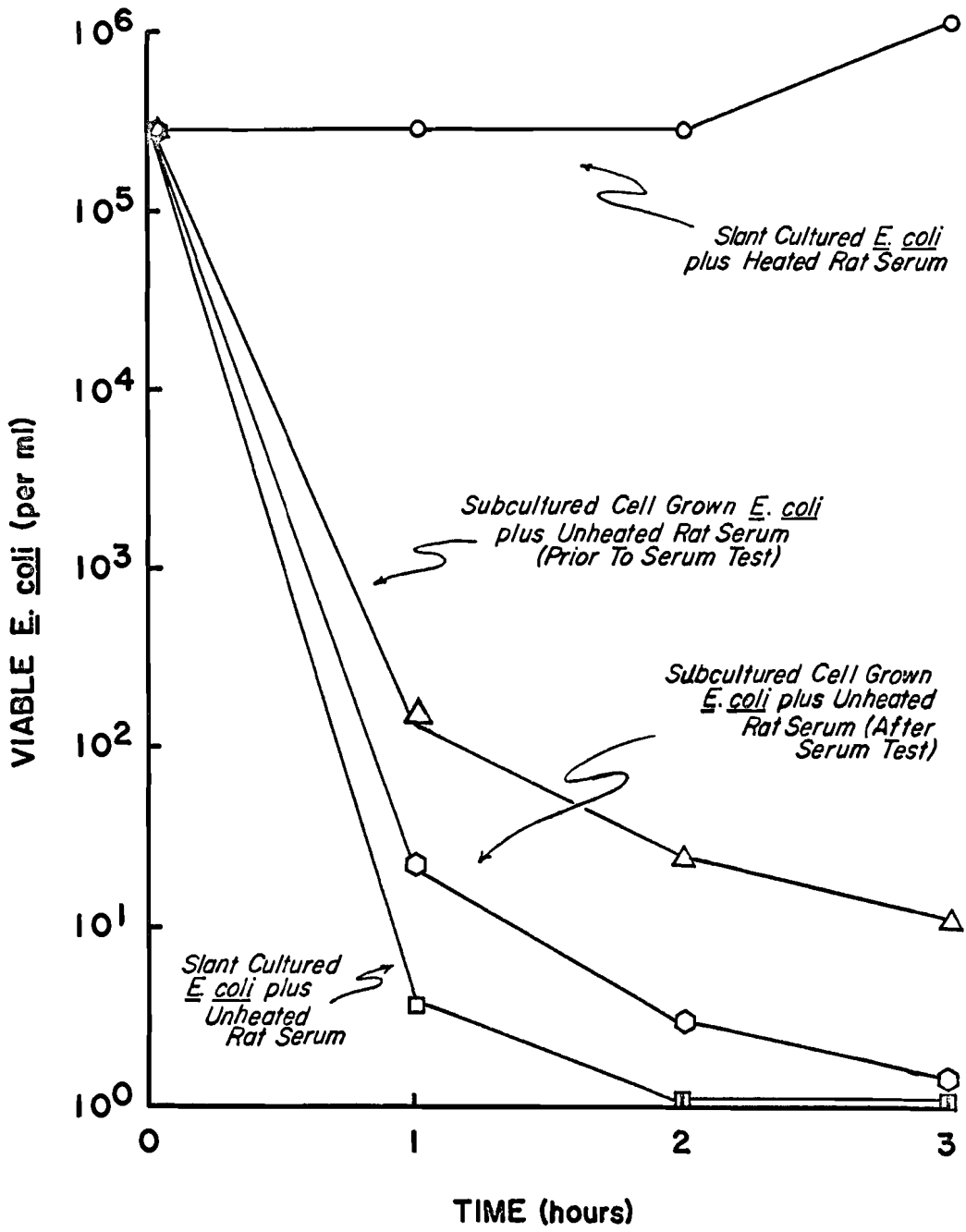
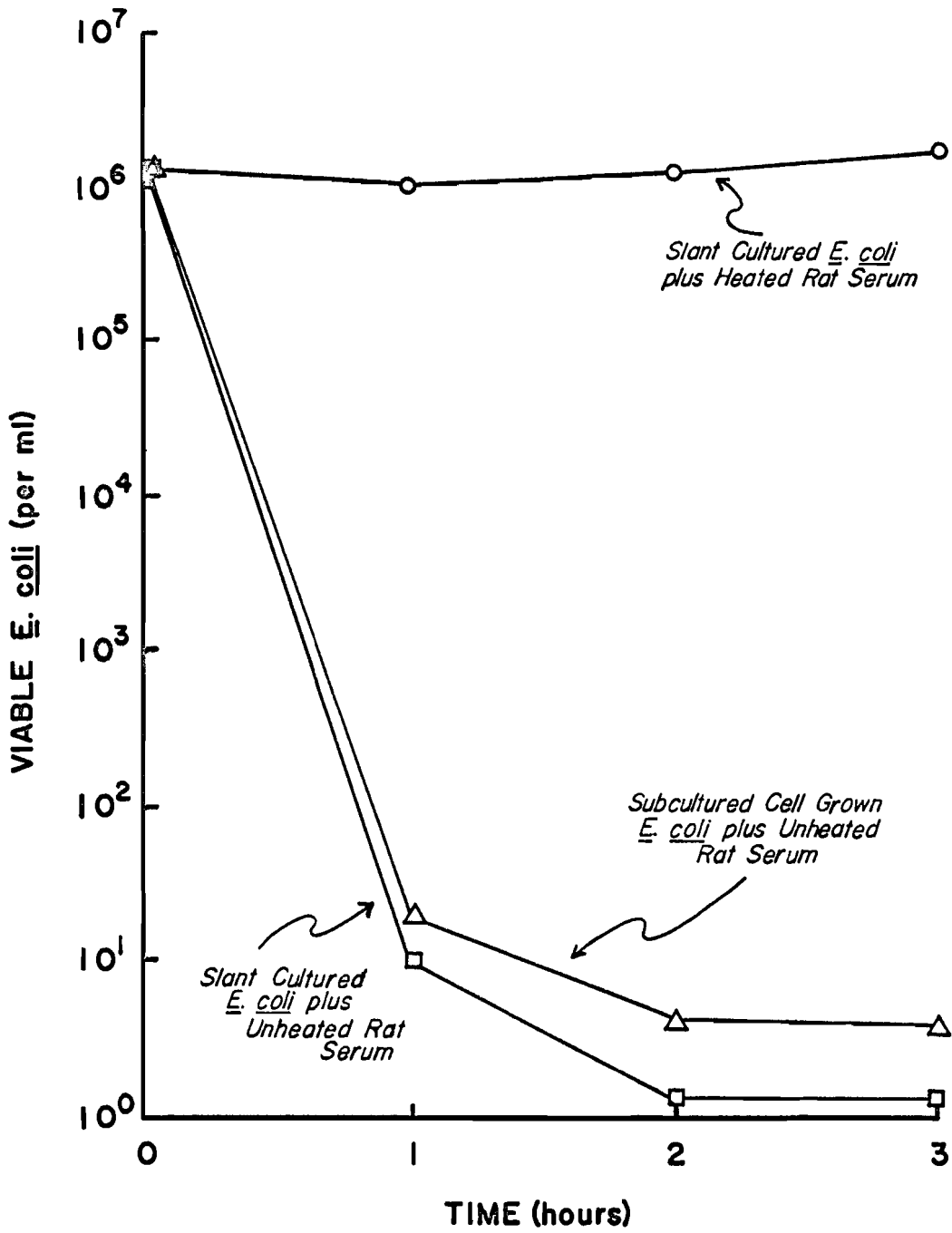


Figure 15: Effect of subculture upon cell cultured serum resistant E. coli. Cell system free of bactericidal heat labile factors.



To further establish the nature and relative stability of the acquisition, washing effects were observed. This was accomplished by centrifugation and resuspension of the organisms in the appropriate medium. Two suspending media were utilized, each yielding results indicative of the nature of the resistance obtained. The resistance of the organism was not effected by washing with Scherer's and heated calf serum or cell supernatant (Fig. 16), whereas it was lost if physiological saline were utilized (Fig. 17). This may be indicative of a temporary change in the surface characteristics of the bacterial cell due to the presence of some type of water soluble material. If such were the case, the material would go into solution when the organism was exposed to an aqueous medium, but would be protected in the complex Scherer's and serum or supernatant medium due to the organic material present.

Lysate culture.

To ascertain the necessity of the cellular state for resistance to develop, attempts were made to culture the organism in cellular lysate. Such a procedure had a two fold purpose: (1) to determine the necessity of an intact cell for resistance to develop, and (2) to determine if the resistance was due to deposition of cellular material upon the bacterial surface. Initial attempts to culture in lysate were hindered due to the presence of exudate factors in the prepared lysate. As previously described, the exudate from the animal at harvest has unheated serum factors present in a degree sufficient to inhibit bacterial growth. Thus, it became necessary to wash the cell suspension before lysate preparation.

Figure 16: Effect of washing the cell cultured serum resistant E. coli with complex tissue culture medium or cell system supernatant.

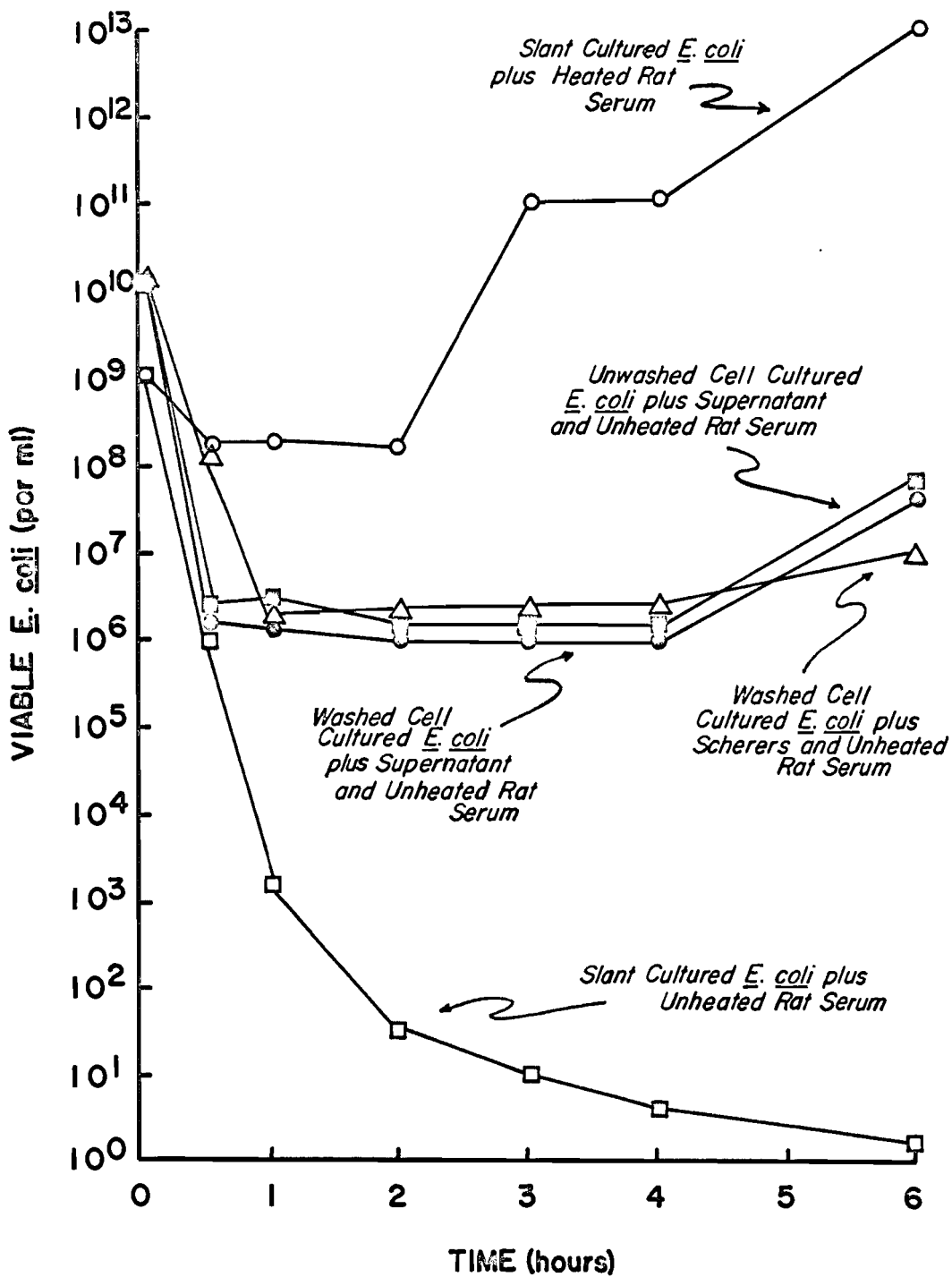
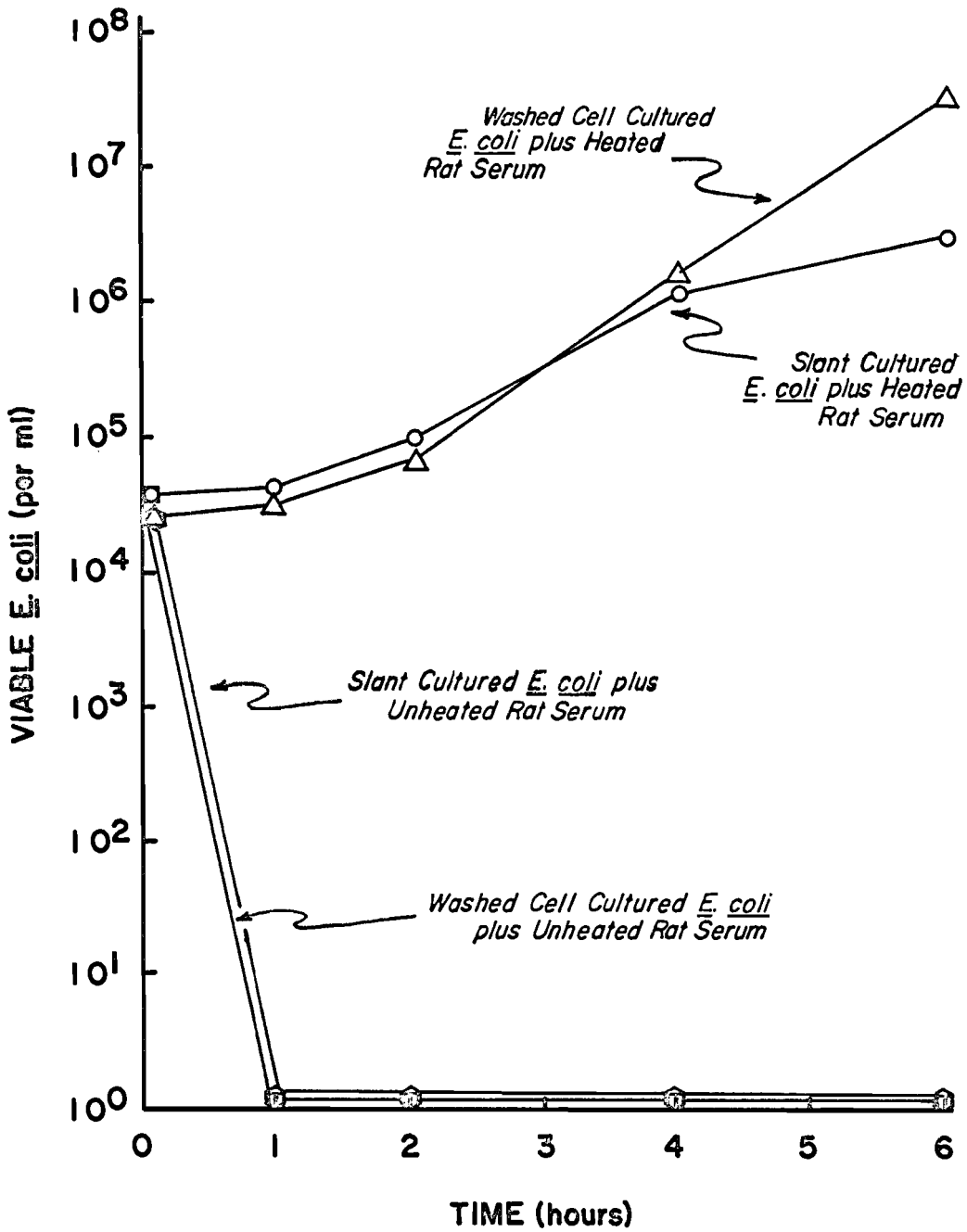


Figure 17: Effect of washing the cell cultured serum
resistant E. coli with physiological saline.

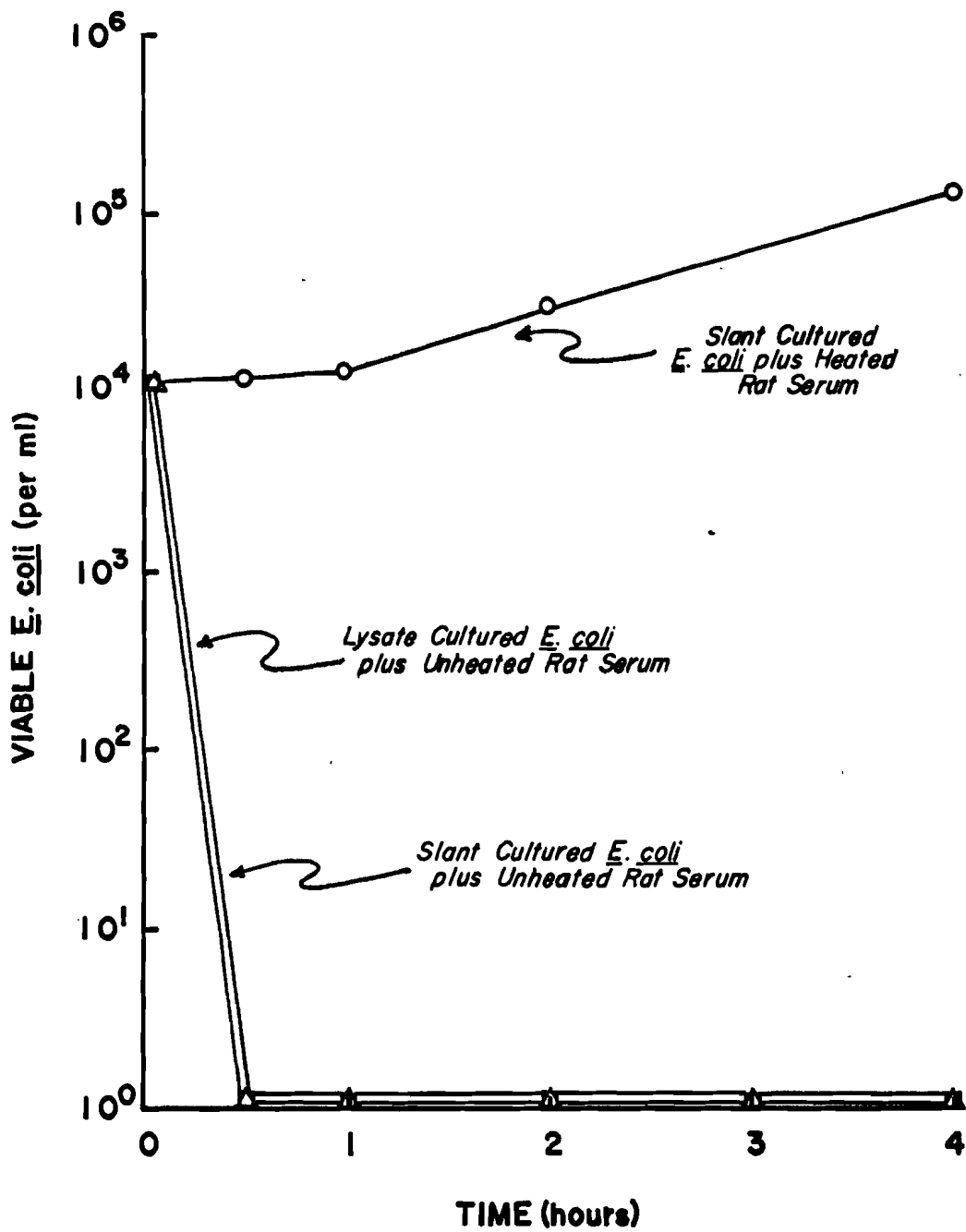


Cells were harvested in sterile heparinized Scherer's, spun out of the medium, and resuspended in Scherer's plus 30% heated calf serum; this procedure concentrated the cell suspension to $40-50 \times 10^6$ cells per ml as well as removed peritoneal exudate factors present in the medium. The cell suspension was sonified for three min at a setting of 4-5 amperes. The prepared lysate was inoculated with one ml of 160×10^7 organisms per ml. After allowing multiplication to occur until a suspension of 20% light transmission was obtained, the lysate cultured organisms were exposed to unheated rat serum and plated. These organisms failed to exhibit resistance, and behaved identically to the slant cultured organisms in unheated serum (Fig. 18). Thus, the necessity of the intact cell in the alteration of the serum response of the bacterial cell was evident. Also, it appears that mere masking of active cell sites for bactericidal serum action by cellular debris is not likely to be responsible for serum resistance after growth in the cell culture.

Double cellular passage

The observation of an alteration in the serum response of "normal" E. coli after a single cellular passage, raised the question of the effect of a second cellular passage upon this organism. After harvesting organisms from a cellular system, and demonstrating a degree of serum resistance, a second cell system was infected with these organisms. The organism suspension was standardized by the use of the spectrophotometer-20, the proper blank of Scherer's and heated calf serum utilized. A separate cell system was infected

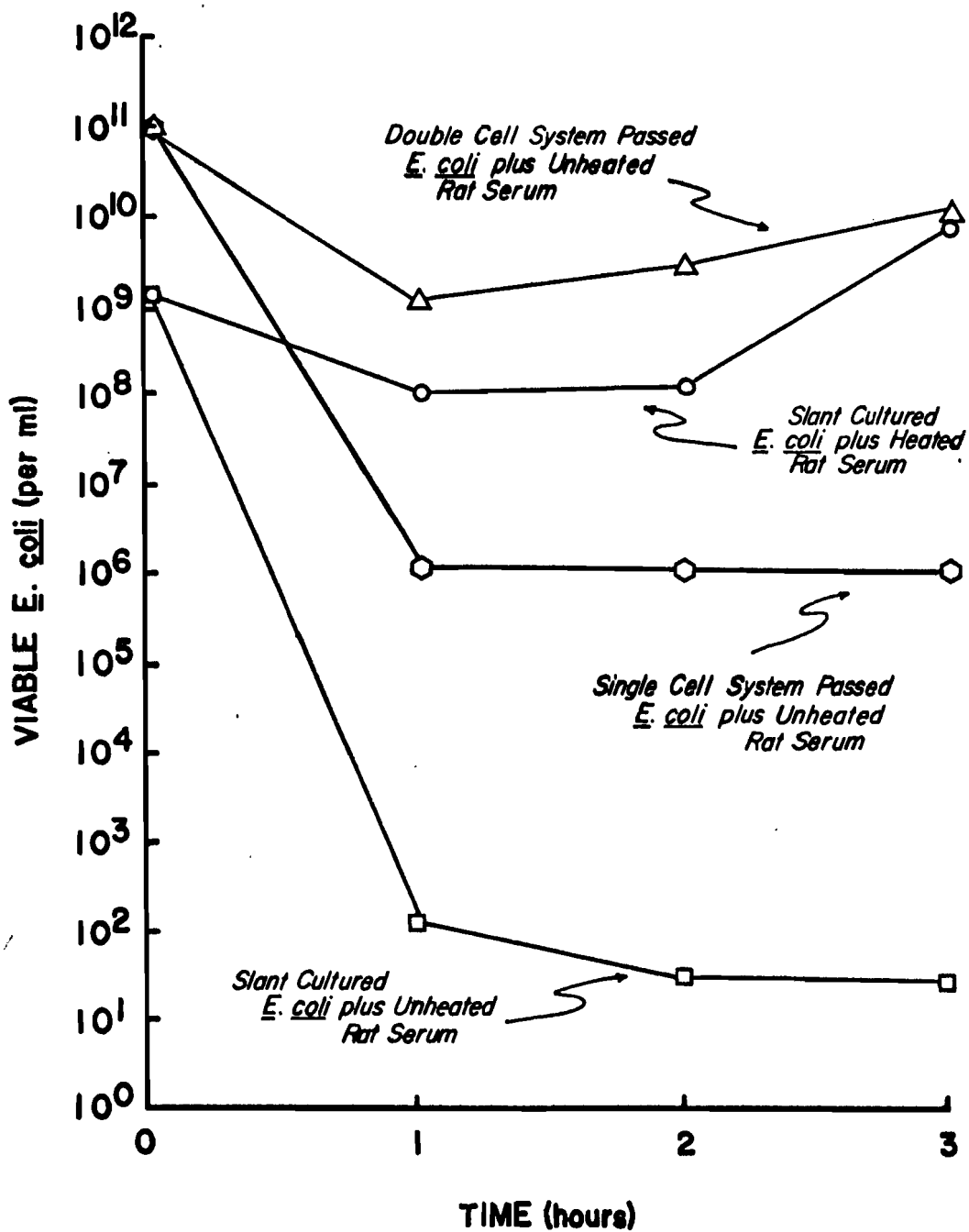
Figure 18: Effect of lysate culture upon the serum
sensitivity of "normal" E. coli.



with slant cultured "normal" E. coli, this system acting as a control. Multiplication was allowed to occur until extracellular multiplication became extensive, then unheated rat serum was added to the system to rid the supernatant of extracellular organisms. After complete cell destruction had occurred, the exudate from both systems was collected, filtered, and an aliquot mixed with unheated rat serum. Usual E. coli control systems were employed, each system being plated periodically.

Careful observation of the cell systems during infection yielded some definite differences in the progression of the infection process. Upon comparison of the pH at 14 hr after infection, considerable difference could be seen in regard to the changes which had occurred in each system. The pH of the system infected with the cell associated organism was acid (yellow) and the "normal" control system neutral (red); a pH indicator paper revealed the pH values as 5.5 and 7.0, respectively. Furthermore, a difference could be seen by microscopic observation of the cell condition at various times during the infection period. It was noted that the system infected with the cellular cultured organisms was destroyed 8-12 hr sooner than the control system. Also, after isolation and exposure to unheated serum, there was a higher percentage of serum resistant organisms in the double cell passed system (Fig. 19). Thus, subsequent cellular passage appeared to enhance cell damage, and a greater number of resistant organisms were recovered upon exposure to the bactericidal system. Subsequent passage might initiate the development of numbers of resistant bacterial cells and promote the development of a stable resistant strain.

Figure 19: Effect of double cellular passage upon the serum
sensitivity of cell cultured E. coli.



Bactericidal test of genetically rat serum resistant E. coli.

In order to better study the true role of the bactericidal system in natural host resistance, it was necessary to attempt to develop a strain of E. coli which would not succumb to the lethal action of the bactericidal system. Such an organism was successfully developed by serial serum passage, and was shown to be fully resistant upon exposure to unheated rat serum when compared to the "normal" organism in the same medium. The growth curve of the resistant organism in the unheated rat serum approaches that of the "normal" organism in heated serum (Fig. 20).

Infection of "non-immune" cell system with "normal" and serum resistant E. coli.

The rapidity with which the cell system was destroyed by the cell cultured serum resistant organism strongly indicated a direct correlation of pathogenicity and serum sensitivity. To provide additional evidence of this relationship, the stable rat serum resistant strain and the "normal" sensitive strain were employed to infect a cell culture system harvested from non-immune animals. Unheated rat serum was added to each system after an initial infection period of 2 hr, the coverslip ground, and the organism viable number determined (Fig. 21). The viable number of "normal" E. coli declined during the first 2 hr period and then began to increase. In the case of the cell system infected with rat serum resistant E. coli, no initial decrease in viable number was observed after addition of unheated rat serum. Comparison of these data to the rate of destruction of a cell system infected with cell cultured

Figure 20: Serum sensitivity of rat serum resistant E. coli.

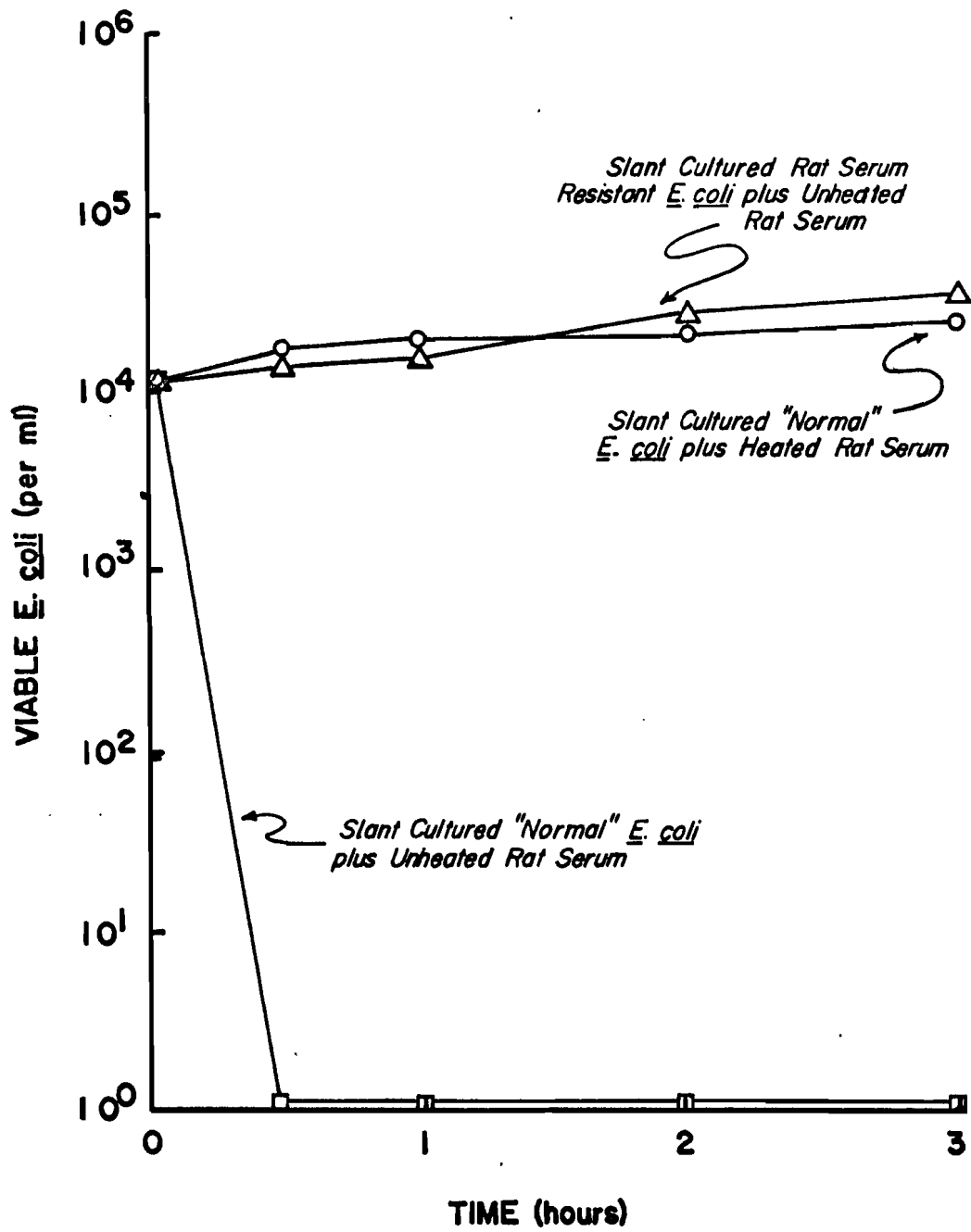
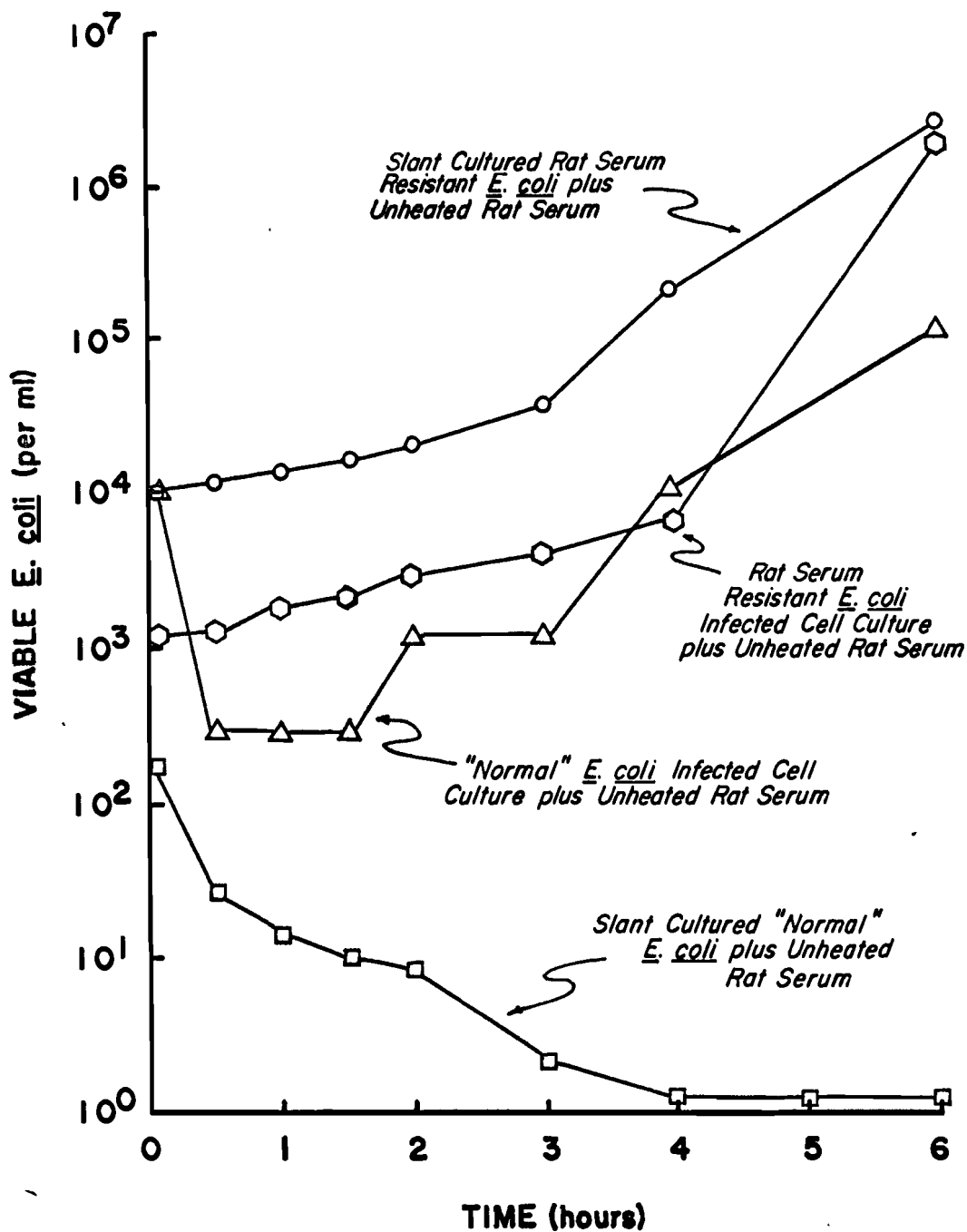


Figure 21: . Fate of "normal" and rat serum resistant E. coli
in an in vitro rat peritoneal mononuclear cell
system.



serum resistant E. coli (Fig. 19), revealed a direct relationship of serum resistance, and the amount and rapidity of cell destruction.

Comparative Amount of Resistance Developed in "Immune" and "Non-immune" Cell Cultures.

Much experimental work has been done in an attempt to reveal the respective cellular roles and/or humoral factors in the mechanism or mechanisms of immunity. As the serum resistance obtained after intracellular residence may serve either as a host protective mechanism or a factor increasing organismal pathogenicity, comparative studies of cells from immune and non-immune animals were done in an attempt to elucidate the above question (Plates 4, 5).

For immunization, an initial injection of a one ml suspension of 20% light transmission was administered intraperitoneally. After two days a two ml 1:1 preparation of Freund's adjuvant and a viable E. coli saline suspension of 20% light transmission was injected intraperitoneally. A two ml challenge dosage was injected two weeks prior to use.

Observations of the serum response of organisms isolated from "non-immune" and "immune" cell systems revealed a difference in the extent and persistence of the resistance developed. The organisms from the "non-immune" system acquired a resistant state significantly higher than those harvested from the "immune" cell system (Fig. 22). Although resistance developed in the "immune" system when compared to the unheated serum control, it was less than that in the "non-immune" system.

Observations of the test systems beyond the three hr bactericidal viability testing period, revealed pH changes indicative of

Figure 22: Comparative study of the serum response of cell cultured E. coli harvested from "non-immune" and "immune" cell cultures.

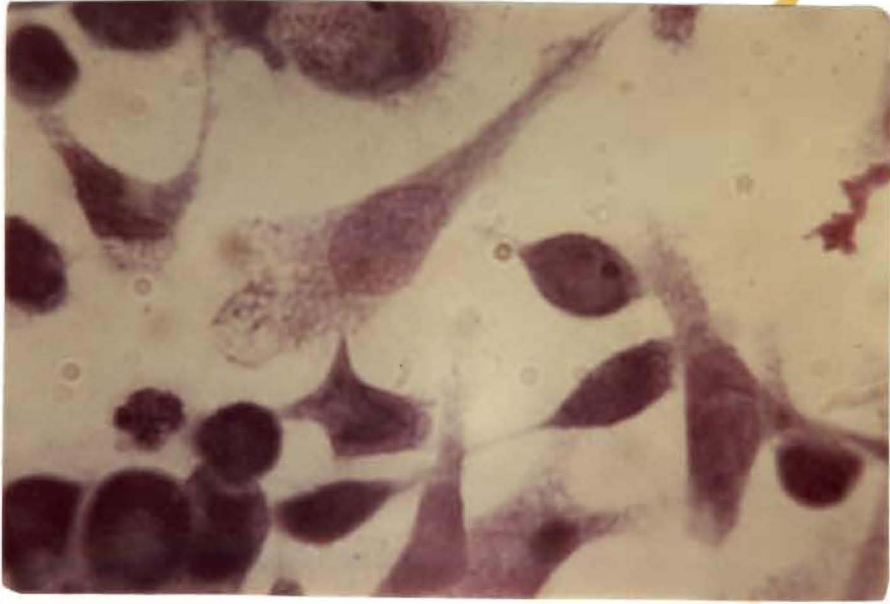


Plate 4: Uninfected "immune" rat peritoneal mononuclear cells (1600X).

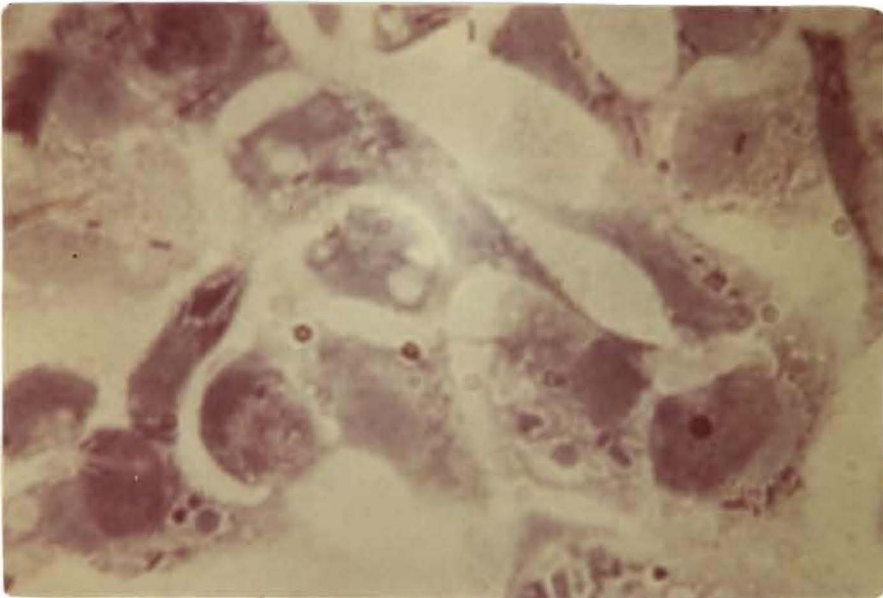
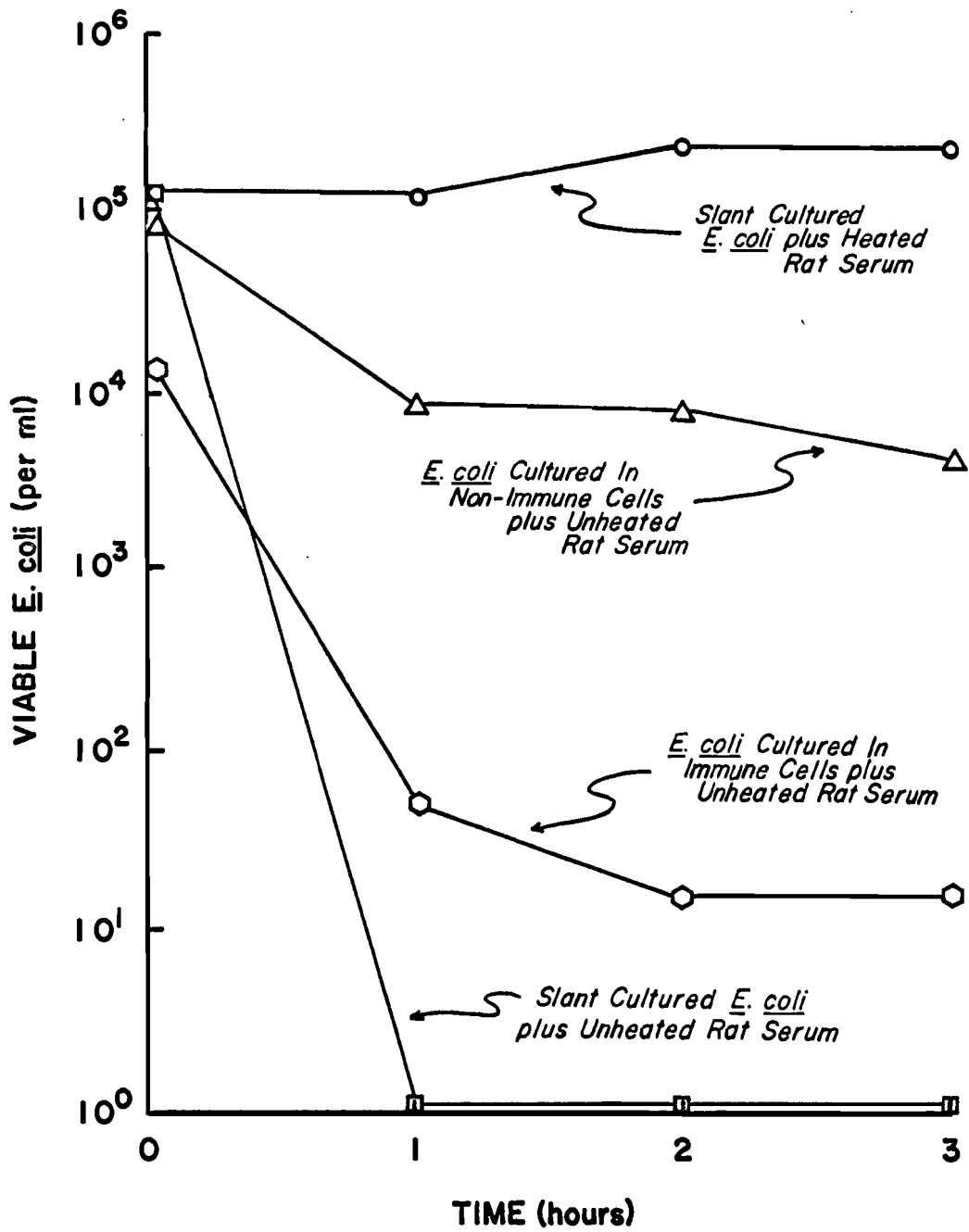


Plate 5: E. coli infected "immune" rat peritoneal mononuclear cells (1600X).



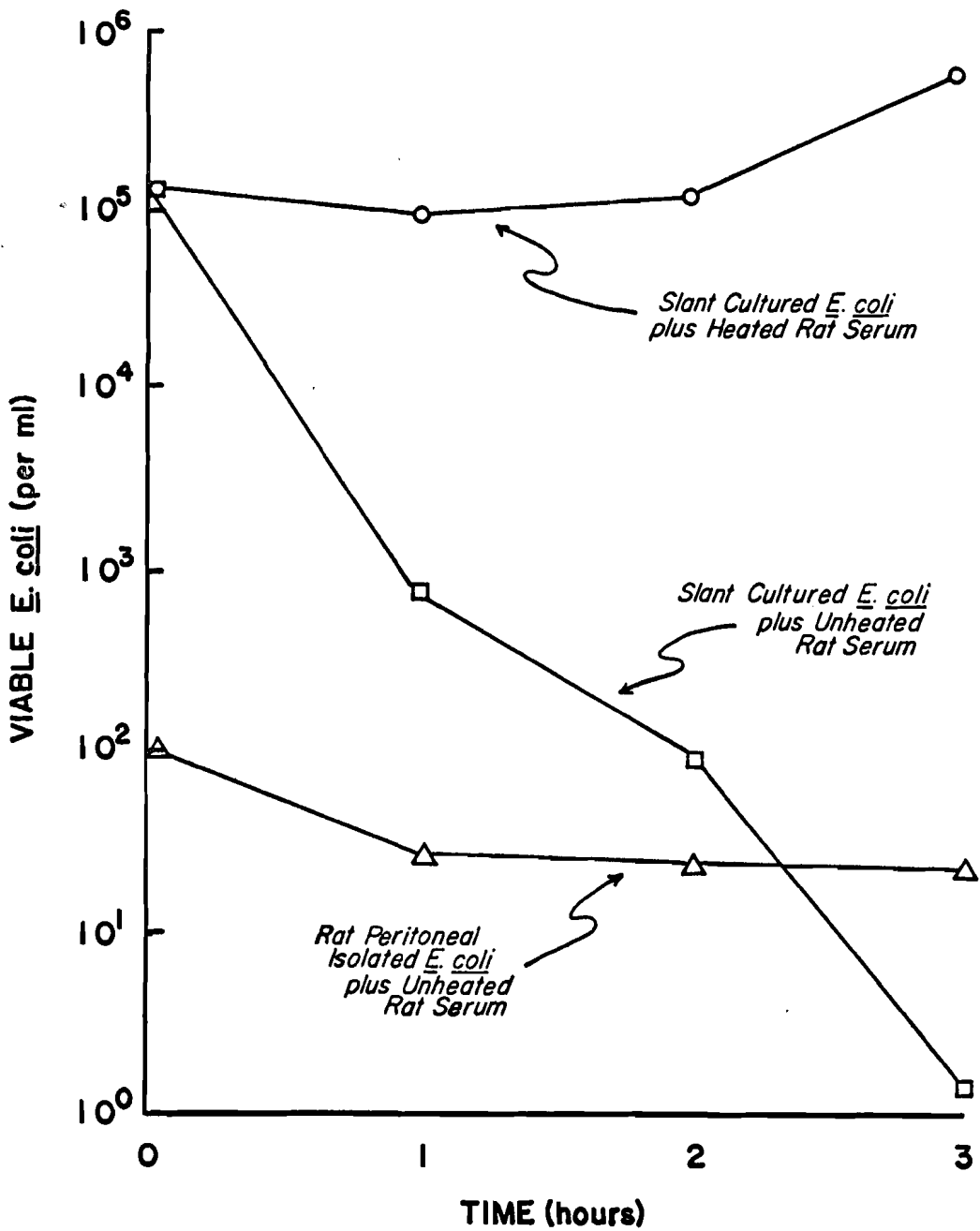
the degree of resistance observed. After 24 hr incubation of the test systems, the pH of the "non-immune" system became acid, which revealed metabolism and/or possible multiplication, whereas the "immune" system showed no pH change. This coupled with the viable counts revealed substantial evidence as to a basic difference in the nature and extent of resistance developed in "immune" and "non-immune" cell systems.

IN VIVO STUDIES

Demonstration of Serum Resistance of *E. coli* Isolated after In Vivo Culture.

To correlate the described in vitro observations to the living system, an attempt was made to isolate *E. coli* after residence in the rat peritoneal cavity. Rats were injected intraperitoneally with 160×10^6 organisms and after 24 hr the cells were harvested in sterile heparinized Scherer's. In order to release intracellular organisms, it was necessary to immediately disrupt the peritoneal mononuclear cells after harvest, remove the exudate factors present, and test the serum sensitivity of those organisms in the lysate suspension. Sonification proved to be an effective method. The cell suspension was sonified at 5 amperes for two min and the lysate examined microscopically for intact cells. Those bacterial cells present in the lysate were spun out of the medium at 3500 rpm for 20 min and subjected to the bactericidal system. When tested the organisms isolated from the peritoneal cavity (Fig. 23), displayed a degree of resistance comparable to that of organisms tested from the in vitro system (Fig. 10). A greater

Figure 23: Serum sensitivity of in vivo cultured E. coli.

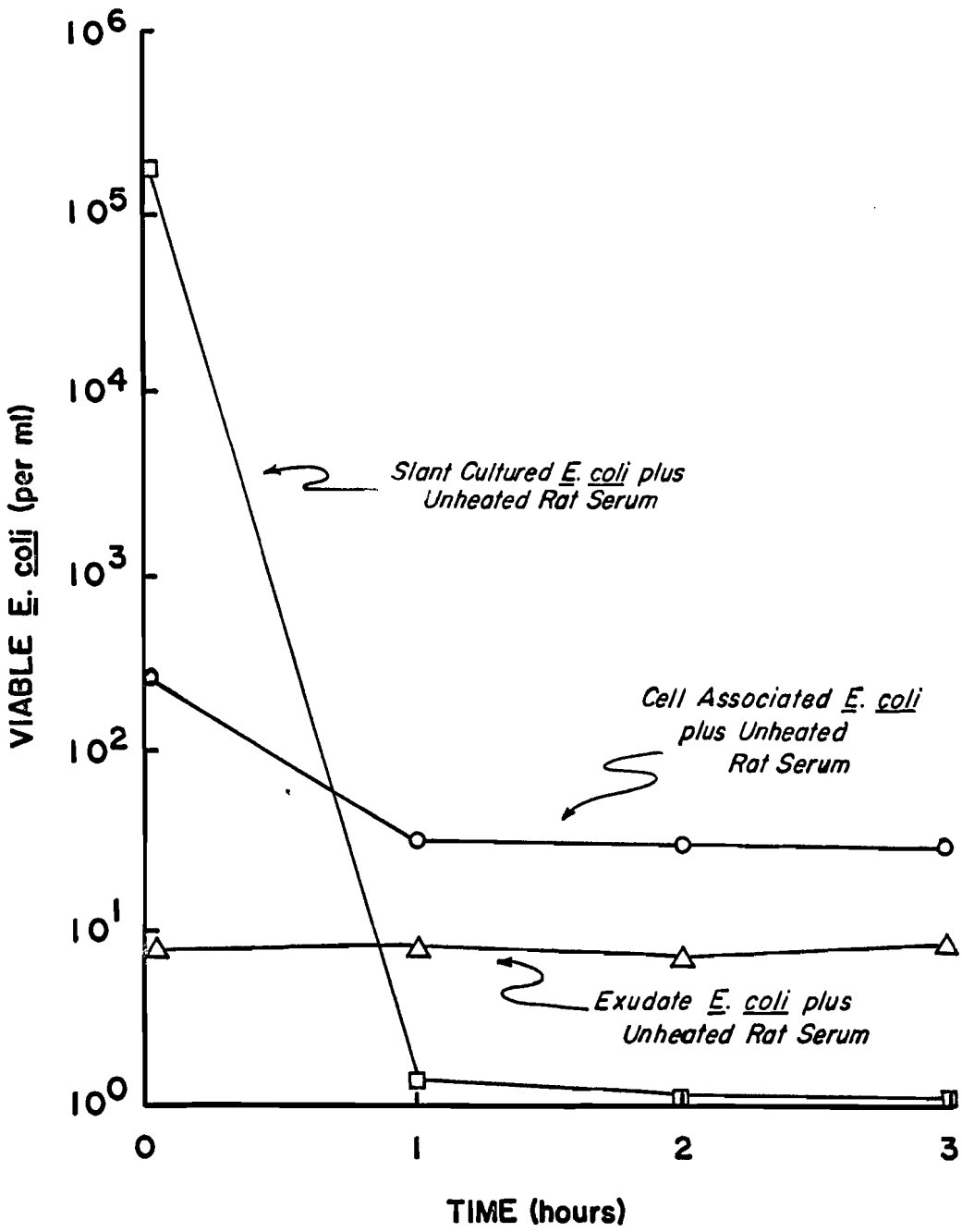


decrease in viable count occurred in the control system when compared to the decrease of the in vivo isolated E. coli.

As the extent of cellular association of those organisms isolated from the cell suspension was not known, a method was sought to determine if these organisms were intracellular or closely associated with the cells. In such an attempt, the cells were harvested and spun out of the medium at 1500-2000 rpm for 15 min. The exudate fraction was removed and placed in thick-walled centrifuge tubes, while the pellet of cells was resuspended in Scherer's and heated calf serum. This suspension was sonified at 5 amperes for two min and observed microscopically for intact cells. The exudate fraction was spun at 3500 rpm for 20 min, the exudate removed and the organisms resuspended in the Scherer's and calf serum medium. The organisms in the cell lysate fraction and exudate fraction were each serum tested by the addition of enough unheated rat serum to obtain a final 30% concentration, then plated for viability. A slant cultured unheated serum control was also set up and plated.

Upon comparison of the viable organisms in the cellular and exudate fractions (Fig. 24), the majority of the organisms appeared to be associated with cellular material. Actual counts revealed an approximate 5:1 ratio of organisms in the cellular fraction as compared to the exudate fraction. Therefore, the majority of the organisms tested for serum sensitivity seem to be either intracellular or intimately associated with the cells. In explanation of the organismal resistance displayed by the exudate fraction, several hypotheses could be made. These organisms may have been recently released from the cells and were temporarily present extracellularly.

Figure 24: Serum sensitivity of in vivo cultured E. coli present in the exudate and cellular fractions.



Also, these organisms may have been intracellular, but present in a few cells remaining in suspension in the exudate fraction. The exudate portion revealed no initial drop in viable number, whereas there was a decrease in the organisms in the cellular fraction. This decrease in the cellular fraction may be attributed to effects of the sonification process. Temporary resistance could be effected drastically by traumatic sonification processes, if the resistance was due to a temporary change in surface characteristics. Both systems, however, displayed a significant level of resistance upon comparison with the control system of slant cultured E. coli and unheated rat serum.

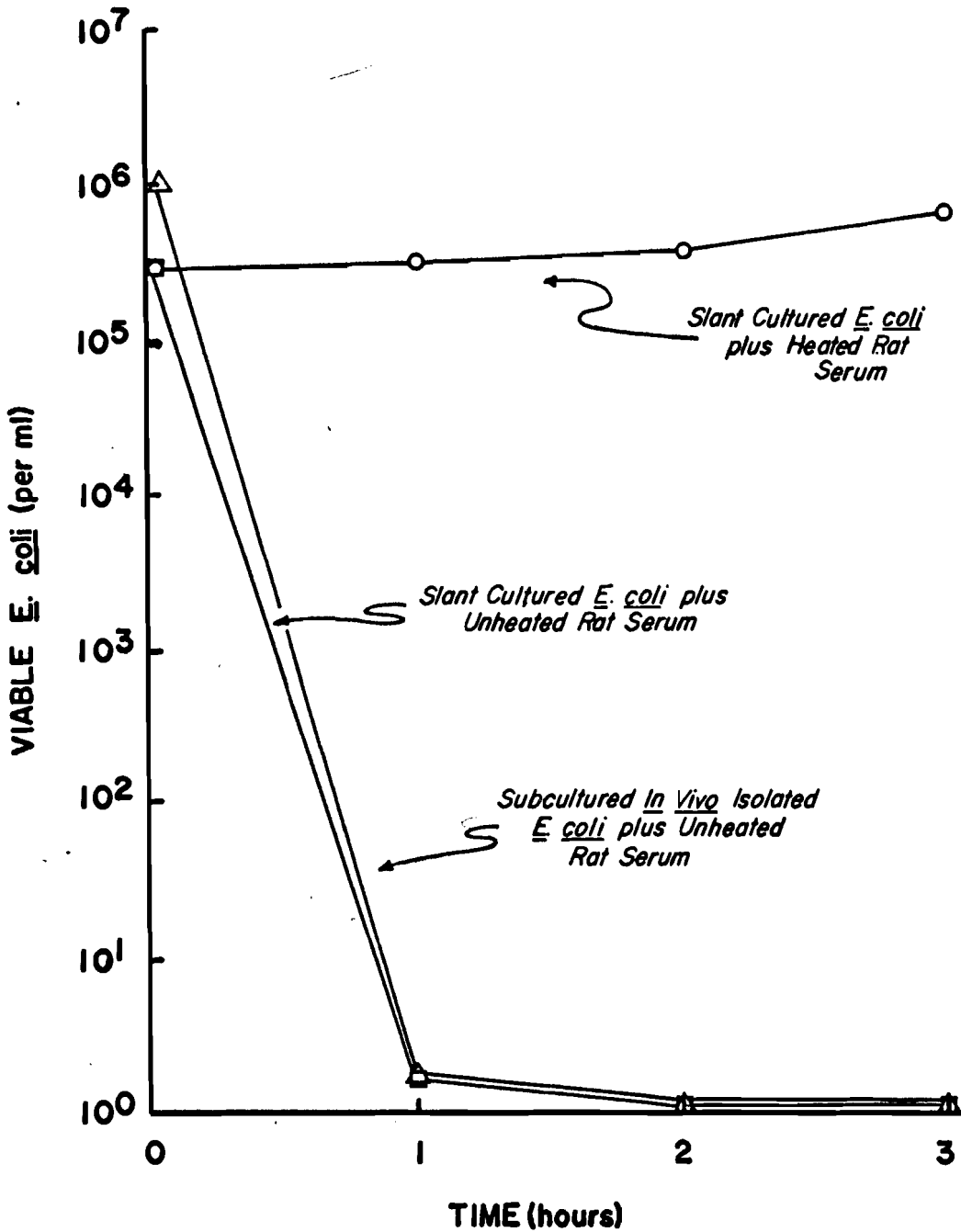
Subculture effects upon in vivo developed resistance.

Further correlations of the in vivo and in vitro work were made by subsequent subculture of those organisms isolated from the rat peritoneal cavity. Again to substantiate if a natural selection phenomenon occurred, those organisms isolated were allowed to multiply extensively upon Brain Heart Infusion Agar slants, then serum tested. As was observed with the in vitro cultured organism, these organisms isolated from the peritoneal cavity no longer exhibited a resistance upon exposure to unheated rat serum after subculture (Fig. 25). The decrease in viable count was comparable to the slant cultured organism in unheated rat serum.

The fate of E. coli in the rat peritoneal cavity.

In order to investigate the relationship of serum resistance to the pathogenicity of E. coli, the fate of this organism was tested after injection into the peritoneal cavity of the animal.

Figure 25: Effect of subculture of in vivo cultured serum
resistant E. coli.



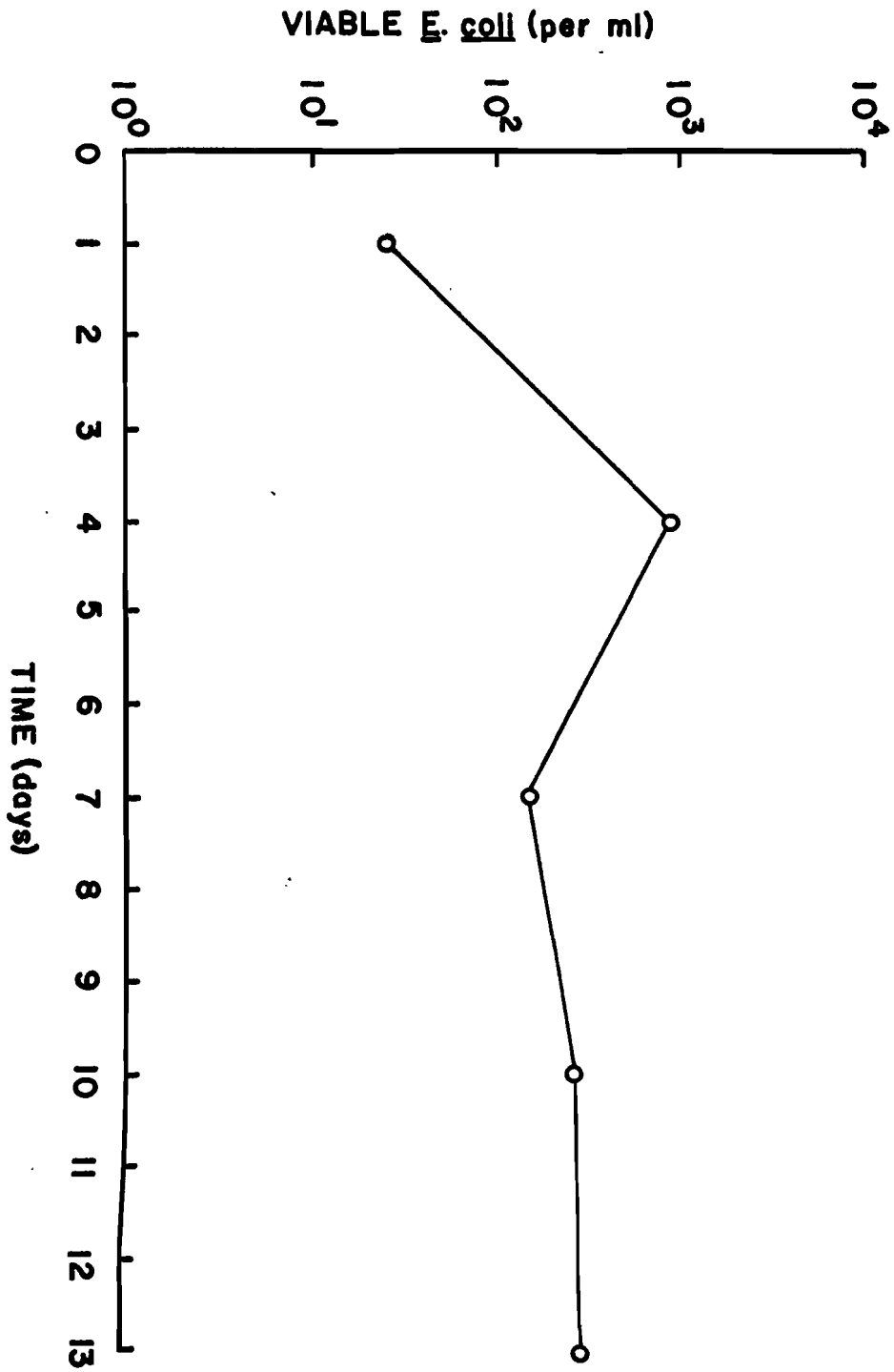
Such resistance, when developed in vivo after cellular association could influence the pathogenesis of a gram negative infectious agent in the host. A two ml suspension of 160×10^7 organisms per ml were injected intraperitoneally into the animal, and the viable count followed over a two week period by periodically harvesting exudate from the peritoneal cavity. One ml aliquots were planted in coverslip tubes and after one-half hr settling period, the coverslips were ground, the supernatants pooled, and a known volume plated for viable number. As the animals were injected with 2 ml of 160×10^7 organisms per ml, there was significant decrease in viable count during the first 24 hr period after inoculation. However, after this point the viable count of the organism leveled and became constant, showing no significant change during the next 12 days (Fig. 26).

As this viability level was comparable to the level of serum resistance of those organisms isolated from the rat peritoneal cavity, it would appear that the viable number might be a function of the resistant state. Maintenance of viable organisms may either contribute to the pathogenicity of the organism or may aid the host defense mechanisms by initiating the antibody response.

Bactericidal test of "pathogenic" E. coli.

Correlation of serum sensitivity and persistence in the animal with pathogenicity was attempted by utilization of a strain of E. coli known to be pathogenic. This strain had been isolated from a small child, and diagnosed as the cause of gastrointestinal disturbances in the child. To ascertain the role of serum responses

Figure 26: Persistence of "normal" E. coli in the rat peritoneal cavity.



in respect to pathogenicity, this organism was serum tested. It was found that this organism did display resistance, the viability level remaining constant over the testing period (Fig. 27). Thus, it would appear that serum resistance may be directly correlated to the pathogenicity of this particular E. coli strain.

Correlation of persistence in vivo and serum sensitivity.

As the viable "normal" E. coli persisted at a stable level for a 12 day period, it can be postulated that this may be a function of an alteration in serum sensitivity. To demonstrate the influence of the serum response upon persistence, the rat serum resistant strain of E. coli and the "pathogenic" strain, shown experimentally to be serum resistant, were injected into the rat peritoneal cavity. The viability of serum resistant strains and "normal" serum susceptible E. coli was determined every 24 hr for a five day period (Fig. 28).

An initial viability drop was observed in each of the three groups of animals, as 160×10^7 organisms were injected per animal. The "pathogenic" strain exhibited the highest level of viability, stabilizing during the testing period. The rat serum resistant strain stabilized at a higher level than the "normal". These observations indicate an influence of serum sensitivity upon the level of organismal persistence. The persistence indicated possible acquisition of resistance by the "normal" E. coli strain, because the viability curve resembles the two resistant curves, even though a lower level of viability existed.

Figure 27: Serum sensitivity of "pathogenic" E. coli.

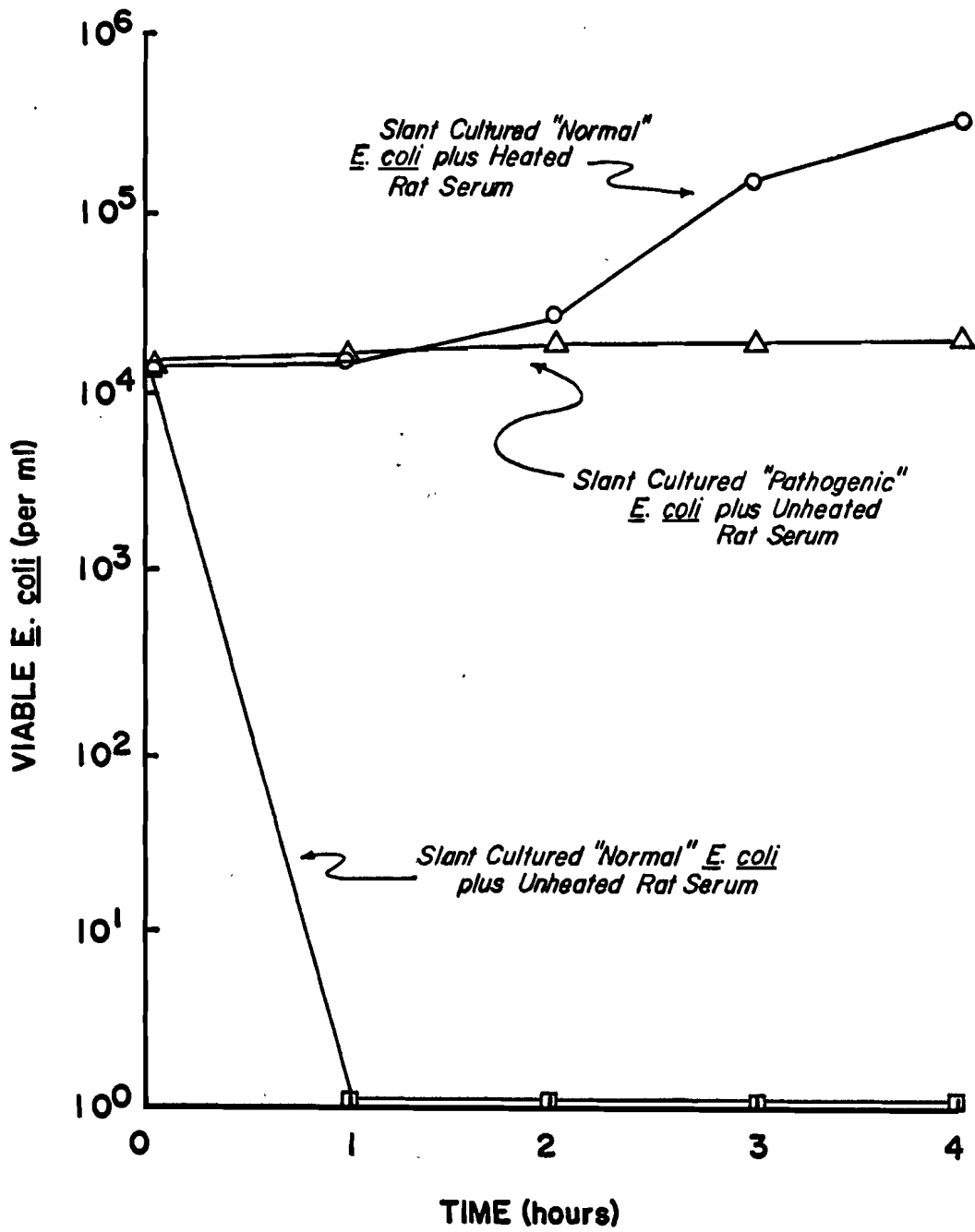
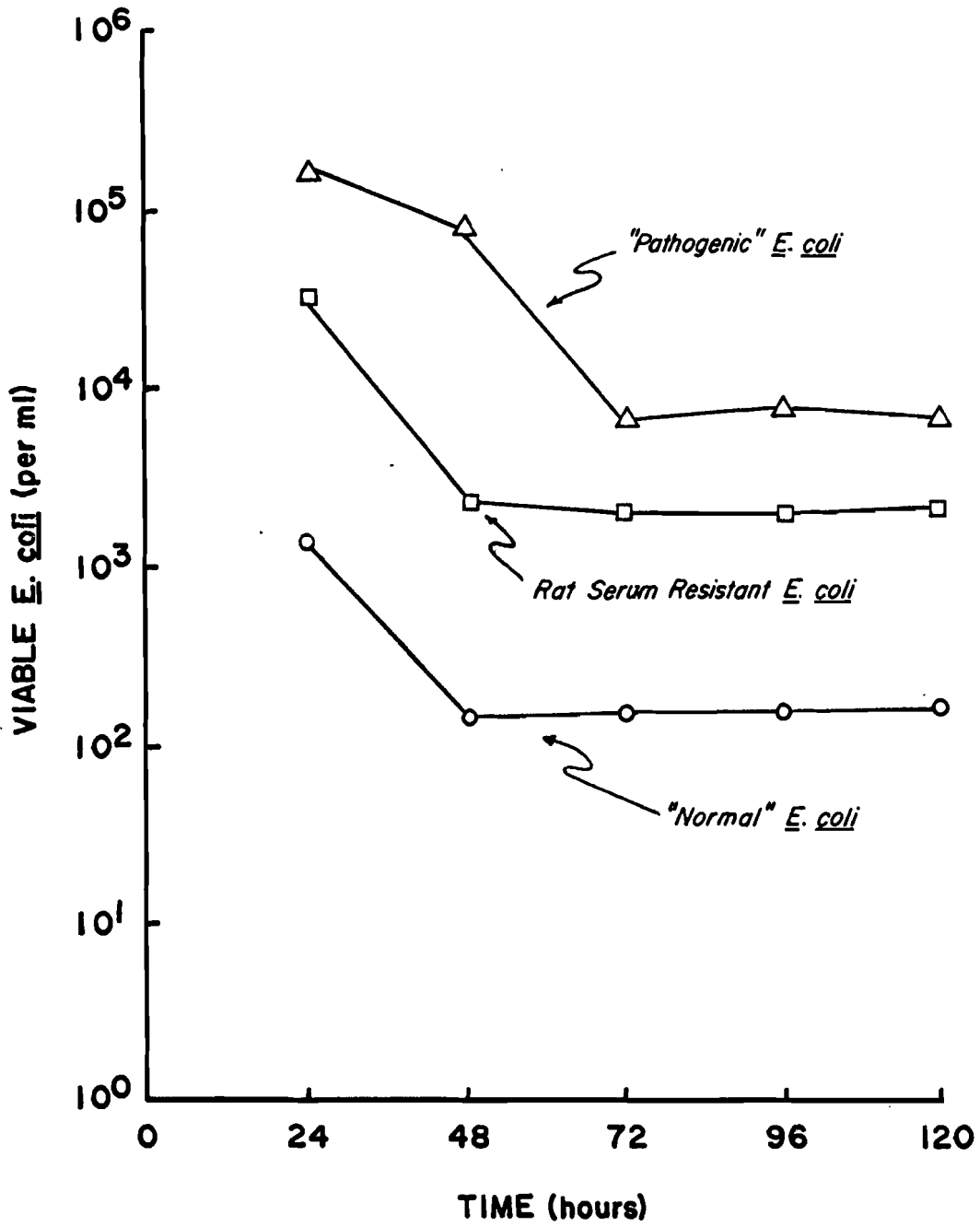


Figure 28: Comparative study of the persistence of "normal" E. coli, rat serum resistant E. coli, and "pathogenic" E. coli in the rat peritoneal cavity.

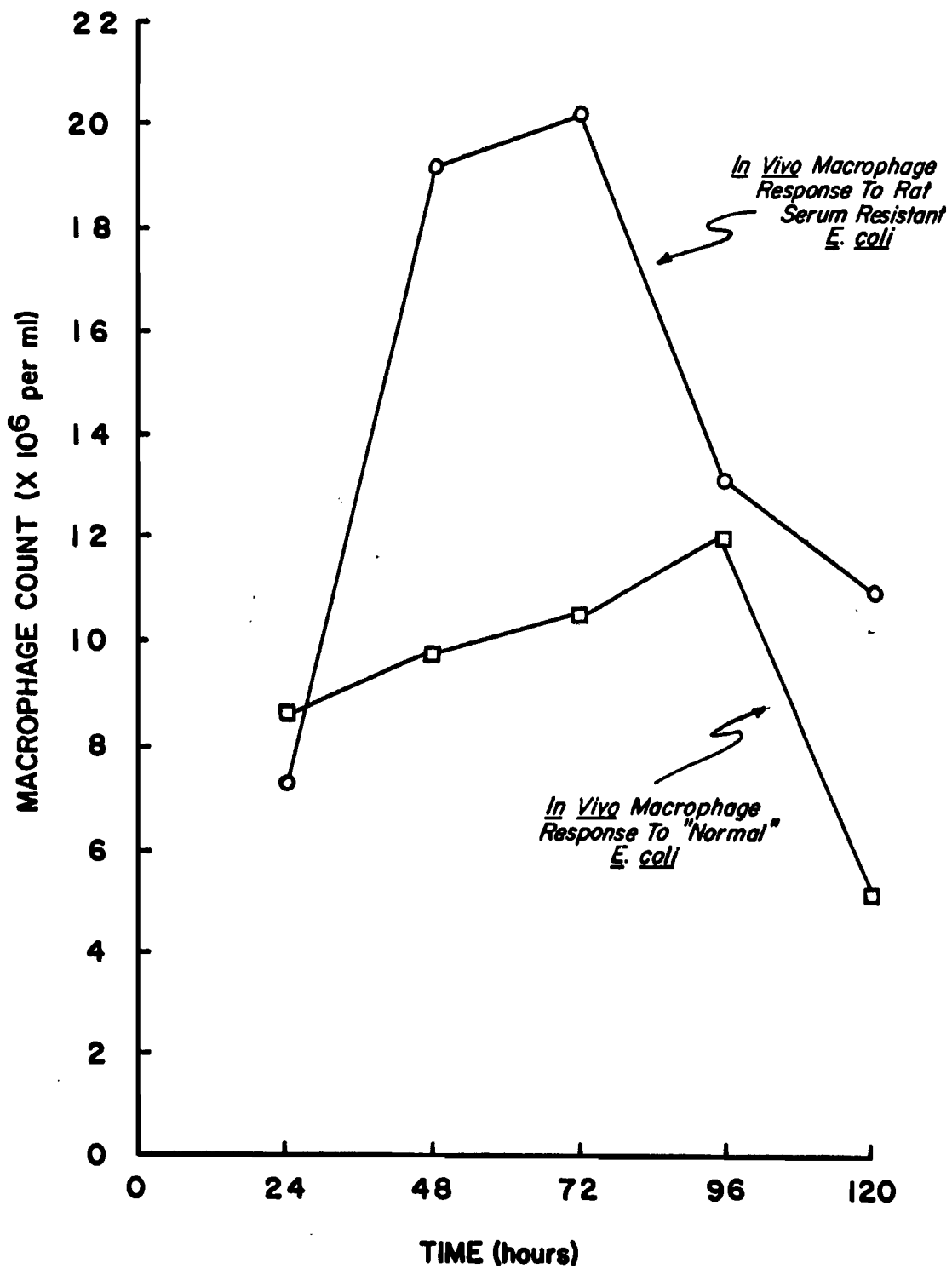


In Vivo Studies Revealing Antigenic Differences Responsible for the Serum Response.

The endotoxin or somatic lipopolysaccharide of gram negative organisms is the antigenic complex thought to be the active site of bactericidal serum action. The responses of the host to the invading organism is probably dependent upon the antigenic structure of these organisms. Utilizing the serum sensitive strain of E. coli and the rat serum resistant strain, in vivo studies were done to determine whether antigenic differences were directly responsible for the response of the organism to serum or whether the response was dependent upon some more complex mechanism. As the bactericidal system and the macrophage system are thought to be two of the most important mechanisms of defense against invading organisms, there is probably a very complex inter-relationship between these two systems. Therefore, comparative macrophage responses of the host to living strains and prepared vaccines of "normal" and rat serum resistant E. coli were done to ascertain if there was a difference in response which could be attributed to antigenic differences or a more complex mechanism involving viability.

Upon the assumption that the host macrophage response to these two strains of E. coli might be different, a suspension of the living organisms of each strain was injected into two groups of animals. Periodically the animals were sacrificed and a macrophage count was made, as well as the organismal viable count. Observations of the macrophage responses indicated a significant difference in the stimulation of the animal by the two organism strains (Fig. 29). Although larger numbers of rat serum resistant E. coli are

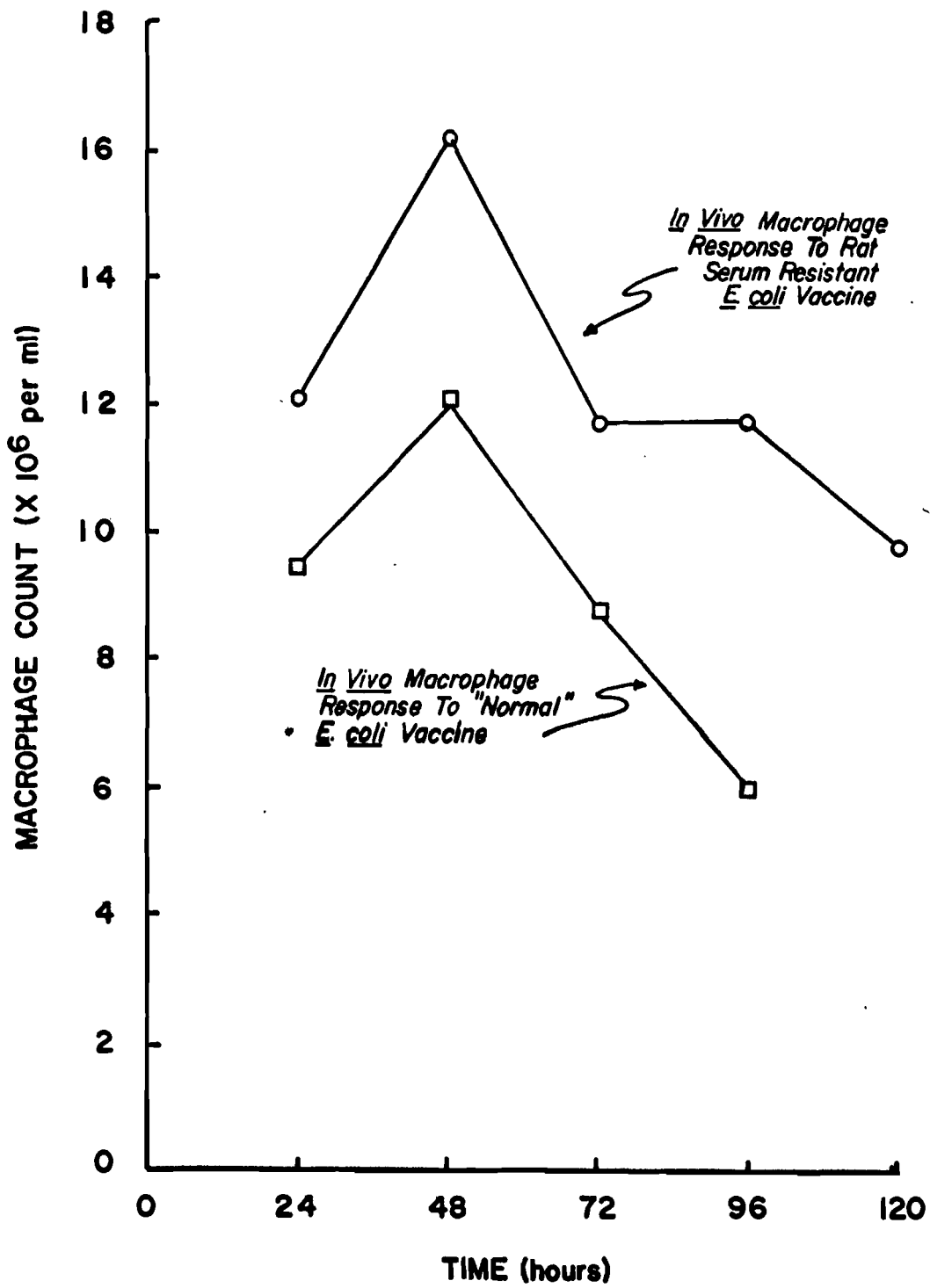
Figure 29: In vivo rat macrophage responses to viable "normal" and rat serum resistant E. coli.



recovered from the cavity, the viability curves of this organism resembles that of "normal" E. coli (Fig. 28). Therefore, the macrophage response did not appear to be a function of variations in organism number, but of the structure or metabolism of the individual organisms.

To substantiate the theory of antigenic differences influencing the macrophage response, a vaccine of each strain was prepared and injected. For vaccine preparation, a 24 hr culture of each organism was harvested in saline and adjusted to 20% light transmission. Enough formaldehyde was added to the suspension to obtain a 1% solution, the suspension was allowed to refrigerate 24 hr, washed with saline, and plated for viability. The vaccine suspension was injected intraperitoneally and the macrophage response determined periodically. If antigenic composition induced differences in the macrophage response, the vaccine induced macrophage curves should correlate closely to the responses to the living organisms. However, if the response was a function of the living state of the organism, there would be no similarity of the macrophage responses. Direct correlation of the viable E. coli and vaccine induced macrophage responses were observed (Fig. 30). Thus, the variations in the host response to "normal" and rat serum resistant strains of E. coli appear to be directly correlated to variations in the antigenic composition.

Figure 30: In vivo rat macrophage response to "normal"
E. coli and rat serum resistant E. coli pre-
pared vaccines.



DISCUSSION

These observations of an acquisition of serum resistance of E. coli after intracellular residence in rat peritoneal mononuclear cells are in agreement with the observations of Stinebring (1960), who reported an acquired resistant state of Brucella abortus after intracellular residence in guinea pig monocytes. Because serum sensitivity has been linked to the pathogenicity of gram negative organisms (Michael and Landy, 1961; Rowley, 1954, 1956), the acquisition of serum resistance would seem to be a contributing factor to the pathogenicity of these organisms. Rowley (1954) reported increased virulence of Bacterium coli following animal passage although there was no apparent change in the state of dissociation or antigenic properties of the organism. This increased virulence was directly correlated to the serum resistance of the bacterial cell. Olitzki and Godinger (1963) observed enhanced lethal effects of S. typhi after in vivo passage which was lost upon subculture. Smith's (1960) discussion of pathogenicity from the standpoint of organism alteration which interferes with host defense mechanisms, specifically, the alteration of the serum response of Brucella to the bactericidal system is supportive of these data. These reports implicate alterations in the organism after host association which lead to changes in the serum response and enhancement of virulence and pathogenicity of these parasites.

Since the bactericidal action of normal serum has been equated with the action of at least two substances, complement and

antibodies, upon some antigenic complexes of the cell wall (Muschel, 1960), it would seem most likely that intracellular residence leading to increased resistance involves an antigenic change in the bacterial cell. Specifically, the toxic O antigen or lipopolysaccharide portion of the cell wall has been designated as the site of action of the heat labile bactericidal system (Muschel, 1960; Muschel, Carey, and Baron, 1959; Landy and Pillemer, 1956; Landy et al, 1957). Michael and Landy (1961) proposed the state and/or the amount of endotoxin represents one of the several factors which determine the serum susceptibility, the serum resistant organism being a more potent source of somatic antigen. Chemical analysis of a serum sensitive rough strain of E. coli and the serum resistant smooth strain revealed significant lipopolysaccharide-polypeptide differences, a 1% composition in the rough and 9% in the smooth strain (Wardlaw, 1963).

The presence of other antigens upon or within the bacterial cell structure may mask the endotoxin or somatic antigen, altering the serum sensitivity of the organism. Adler (1953) reported the specific bactericidal sensitization of a smooth strain of S. typhosa 0901 cells was inhibited by a second specific antibody for the R-antigen. From these observations, it was assumed that the smooth strain of Salmonella possessed an intimate spatial relationship of the characteristic O antigen and R antigens, as antibody specific for the R antigen masked the reactive site of the somatic antibody. More recent workers report masking of the O antigen by the Vi or capsular antigen and termed the phenomena as O-inagglutinability

(Osawa and Muschel, 1964; Muschel, 1960). Osawa and Muschel (1964) state that the Vi antigen of S. typhosa, like other K antigens, masks the O antigen, thereby preventing union with the O antibody. Also, the same investigators demonstrated the loss of the Vi antigen, assumption of the rough state, and alteration of the serum response to P. ballerup when cultured in certain environmental temperatures. Supportive of immunological alteration of an organism cultured in the intricate host system are results reported by Segal (1965). A comparative study of the immunogenic properties of in vivo and in vitro grown Mycobacterium tuberculosis revealed different antigenic properties in the bacterial cells isolated from each system.

If the accepted active site of serum action is the bacterial somatic O or lipopolysaccharide antigen, it is possible the amount or availability of this antigen can be effected by its intracellular residence. Due to the intimacy of the host-parasite relationship, organisms in direct contact with cellular constituents and compounds could adsorb some host material upon the bacterial surface. Olitzki, as early as 1935, suggested the possible alteration of an organism by host tissues. Later work (Olitzki and Godinger, 1963) strongly supported the adsorption of organic host substance upon the cell surface of S. typhi, this termed autoantigenic adsorption. Due to the inability to obtain significant serum resistance of E. coli cultured in lysate, it seems less likely that organic host material is adsorbed by the organism or host deposition occurs after phagocytosis. However, a fallacy exists in assuring deposition does not occur, as the intracellular milieu is more concentrated in organic materials than the lysate medium.

Another mode of antigenic alteration could involve adsorption of endotoxin or somatic O antigen to the surface of the bacterial cell. In 1952, Adler reported the bactericidal action occurred at the cell surface, and the antigenic site could be material not integrally a part of the cell, but an artificially adsorbed material. Reduction of the bactericidal serum action occurred upon addition of an infected organ extract to a bacterial suspension (Olitzki and Kaplan, 1963). This is attributed to soluble antigen, the Vi or surface antigen, present in the infected tissues, the concentration of antigen increasing with time duration of the infection (Olitzki and Godinger, 1963). A study by Cohn (1964) concerning the fate of E. coli agglutinin in peritoneal macrophages, revealed the antigenicity of this substance was not destroyed after intracellular residence. Since such is the case, one could hypothesize some organismal degradation could occur in the infected cell system, the antigen remaining intact and being adsorbed by the viable organisms. Michael and Landy (1961) demonstrated the attachment of somatic endotoxic complexes to viable E. coli and Shigella dysenteriae resulted in significant alterations in the sensitivity to normal and immune serum. Incorporation of small amounts of endotoxin enhanced the lethal effect, whereas large uptakes rendered the bacteria relatively resistant. Therefore, antigen in the environment could be adsorbed, resulting in an altered serum response.

A report by Muschel, Carey, and Baron (1959) suggests a third hypothesis in regard to the intracellular alteration of E. coli which occurs. Utilizing specific strains of E. coli and Salmonella

typhosa, the conversion of these cells to protoplasts by fresh, normal guinea pig serum was noted. These investigators suggest that protoplast formation may initiate the persistence of certain organisms in the body. Diena, Wallace, and Greenbern (1954) reported the reversion of spheroplasts to complete bacterial cells if the cell wall components are synthesized and incorporated to form the bacterial cell. If protoplasts were formed, the active site for the bactericidal system could be absent and the organism could survive serum exposure, after which time multiplication and reappearance of the complete, functional cell could occur.

The three proposed hypotheses of serum alteration intracellularly are attributed to the complex interactions of the organism and the host cell. However, alteration in the serum response could result due to organismal metabolic differences, the micro-environment of the cell influencing the metabolic machinery of the organism. The physiological state of the organism or age of the organism reportedly effects the bactericidal action of serum (Michael and Braun, 1959; Michael and Landy, 1961). Olitzki and Kaplan (1963) reported different nutrients may enhance bactericidal serum action. Furthermore, studies of the serum responses of Shigella dysenteriae and E. coli strains revealed enhancement of bacterial susceptibility to normal human serum in vitro in the presence of low broth concentrations, glucose, or amino acids (Michael and Braun, 1959). They also observed metabolic inhibitors decreased the serum sensitivity of organisms. If the host cell and organisms were metabolically active, organic waste materials and metabolites might be

produced which could inhibit the metabolism of the bacterial cell. Possibly degradation products could metabolically inhibit the organism with the resistant state temporarily appearing upon isolation. Such effects upon bacterial metabolism may be induced within the peritoneal macrophage, thereby accounting for serum alteration.

According to Martin and Marcus (1964), the endotoxin or somatic O antigen characteristic of the gram negative bacilli reportedly accounts for pyrexia, or the fever inducing capacity of these organisms. As these organisms could induce host changes in temperature due to the endotoxin present, these temperature changes could in turn induce the phenotypic expression of factors altering the response of the organism to the heat labile bactericidal system. The effects of temperature upon the serum sensitivity of E. coli (Muschel, 1960) and P. ballerup (Osawa and Muschel, 1964) have been reported and associated with antigenic changes directly affecting the serum response. Osawa (1960) also suggested temperature influences upon the host defense mechanisms.

Besides effects upon the bacterial physiology resulting in serum response differences, the environment may directly effect the bactericidal reaction. Wardlaw (1962) reported that the ionic strength, pH, and temperature effected this reaction. The complex cell system might induce significant changes in the medium to allow for the observed altered serum sensitivity.

Expanding the initial observations of the alteration of serum sensitivity of E. coli in vitro to the intact animal expounds the significance of such organismal change. Extension of test tube

observations to the living host is difficult because of the complexity of the intact animal. Duplication of the in vivo system with an in vitro system is impossible, therefore this must be considered in interpreting the data. The in vitro results can be artifacts, not actually occurring in vivo due to the complex interactions of the parasite and intact animal. Extension of the observations made in the test tube to the in vivo system however, revealed those organisms isolated from the peritoneal cavity were serum resistant as were those isolated from the test tube cell environment. Furthermore, separation of the cellular fraction of the infected peritoneal exudate by centrifugation, revealed the majority of the organisms exhibiting serum resistance were associated with cells present in the harvest.

The correlation of these observed changes in serum response in vivo to the virulence is of probable importance. Data illustrating the persistence of the "normal" E. coli in the peritoneal cavity supports this hypothesis, as the number of organisms recovered is comparable to the amount of serum resistance displayed. Although demonstration of the action of the bactericidal system in vivo has not been possible because of technical difficulties, Muschel (1960) suggested it undoubtedly operates in the host defense against invading gram negative organisms.

As the number of "normal" E. coli persisting in the rat peritoneal cavity is comparable to the display of resistance of the in vivo cultured organism, there may be a direct correlation of persistence and serum resistance. Olitzki and Godinger (1963) reported

the persistence of viable Salmonella typhi for one month in a non-lethal infection. Alteration of such a dynamic state by environmental factors might result in pathogenic effects of the organism upon the host, leading to possible lethality.

Correlation of the viability of "normal", serum resistant, and "pathogenic" E. coli in the rat peritoneal cavity, indicate possible effects of serum resistance upon the relative levels of viability. The two serum resistant strains, one being "pathogenic", persist at a level significantly higher than the "normal" organism. However, the level of persistence of the "normal" organisms seems to be due to an acquired resistant state, as the number remaining in the cavity is comparable to the amount of resistance displayed after in vivo culture.

Determination of the rat peritoneal mononuclear cell response to viable strains of "normal" and rat serum resistant E. coli as compared to vaccine preparations, suggests a difference in the cell response to the organisms, which is not due to the living state. Evidence of an antigenic difference in the two strains is provided by the response differences which occur to both the viable and vaccine injection. Furthermore, the similarity of the viability curves indicate some quantitative or qualitative difference in factors in the E. coli strains which initiate a different macrophage response. This may be due to differences in endotoxin or lipopolysaccharide content, as chemical analysis of sensitive and resistant E. coli strains (Wardlaw, 1963) yielded quantitative differences of this material.

In vitro studies of infected cell cultures with "normal" and rat serum resistant E. coli, also yielded evidence in regard to the relative virulence and pathogenicity of these strains. To project these observations further, the more rapid organismal proliferation and the increased detrimental effect of the cell cultured resistant E. coli upon the in vitro system when compared to "normal" E. coli, indicate this acquired resistance may be related to virulence. Rowley (1954) and Olitzki and Godinger (1963), reported that increased virulence or lethality after in vivo passage, supporting these observations.

Initial comparative studies of "immune" and "non-immune" cell systems, indicate a difference in the degree of developed resistance. Early recovery of organisms from the two systems revealed a greater display of resistance by those organisms from the "non-immune" cells. However, as the infection progresses, there is a greater similarity of the degree of resistance developed in the two systems. Although the differences have not been well-defined due to technical difficulties, if true differences do occur, a new explained host immune mechanism would be revealed. This would implicate some basic cellular difference of macrophages harvested from immune and non-immune animals, as the degree of resistance developed would be an attribute of previous exposure to the organism.

However, infection of "immune" and "non-immune" cell systems with "normal" E. coli results in an initial drop in viability in both cell systems, although some quantitative differences may occur. Sorkin and Boyden (1959) described a greater amount of antigenic breakdown in cells in presence of specific anti-sera. If antibody

is present at the cell surface (Rowley, 1963) in the immune system, antigenic degradation could occur to a greater extent than in the "non-immune" cell system. If adsorption of endotoxin or O antigen occurs, due to bacterial degradation, less antigen would be present in the "immune" cell system due to the action of specific anti-sera. The amount of developed resistance, thus would be less in this system. However, at the present time the observed differences are inconclusive and further work needs to be done in regard to these observations.

SUMMARY

The role of the bactericidal serum factors against gram negative organisms is one of probable importance in the natural host defense mechanisms. The primary components of the bactericidal system are the complement components and antibody. Utilization of a rat peritoneal mononuclear cell system infected with E. coli revealed an alteration in the serum response after intracellular residence. Further attempts to characterize and elucidate the phenomenon indicated a temporary change in the surface characteristics.

Demonstration of the same alteration in the organismal serum response in the intact animal substantiates the implications of the in vitro observations. The persistence of the serum sensitive organism in the animal at a level comparable to the level of serum resistant organisms implicated the serum susceptibility of the organisms as a determinant of its in vivo fate. Comparative studies of serum resistant and susceptible E. coli strains in vitro and in vivo support the role of the serum response in the pathogenesis of gram negative organisms.

Immune studies indicated a basic difference in the amount of organismal serum resistance developed in "immune" and "non-immune" cells. Further study might disclose a new mechanism of immunity.

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