STUDIES ON THE INTERACTION OF *NEISSERIA MENINGITIDIS* AND MOUSE PERITONEAL EXUDATE LEUKOCYTES

by

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INTRODUCTION

This thesis is concerned with the problem of host-parasite relationship between Neisseria meningitidis and mammalian phagocytic polymorphonuclear leukocytes (PMN). This introductory section is intended to provide background material for an experimental approach to the following questions of the selected problem:

1) Do certain strains of N. meningitidis survive after phagocytosis by PMN?
2) Do certain strains of N. meningitidis proliferate within PMN after phagocytosis?
3) In an in vitro system, what are some parameters of certain experimental conditions which may provide answers to questions 1 and 2?
4) What experimental evidence from the above studies may be applied to the problem of pathogenicity of N. meningitidis?

A historical review of the literature on N. meningitidis reveals that this diplococcus has been recognized since Weichselbaum isolated and described the bacterium in 1887. In subsequent years, many other workers have conducted studies on the meningococcus. Despite the effort expended, a paucity of knowledge pertinent to pathogenesis of meningococcal disease has been uncovered. This is certainly not to say that the data reported are without value. On the contrary, previous investigators have provided valuable information. These previous reports in conjunction with the current molecular biological trends of research may hopefully lead to a more fundamental understanding of

N. meningitidis involvement in disease processes.

A. Characteristics of the organism

N. meningitidis is a Gram-negative, generally spherical cell approximately 1 micron in diameter. The cells commonly appear in pairs with a slight flattening of contiguous sides to yield a "bean-shaped" morphology. One commonly notes both swollen and poorly stained cells in smears, for autolysis is common and occurs even in growing cultures. No endospores are formed and the cells are nonmotile. The colonial appearance is generally smooth, moist, and elevated with a bluish-grey tint (Smith, Conant, and Overman, 1964). In common with other neisseriae, colonies of N. meningitidis produce a rapid positive cytochrome oxidase reaction when tested with tetra-methyl-p-phenylenediamine. The optimal growth temperature is 37 C with an optimal pH at 7.4 to 7.6. Useful identifying biochemical reactions are fermentations of glucose and maltose with production of acid.

Meningococci are commonly cultivated on blood-agar, chocolate agar, and in meat infusion. For primary isolation from patients, the culture atmosphere is usually supplemented with carbon dioxide (Morton, 1945; Larson and Scherp, 1954). Convenient cultivation of the meningococcus is accomplished by use of Mueller-Hinton Agar (Mueller and Hinton, 1941), or Dextrose Starch Agar or Broth (Marshall, 1947). Both of these latter media are commercially available in desiccated form. Defined media have been devised and used on a restricted basis (Catlin and Schloer, 1962). A good background discussion of various nutritional studies using N. meningitidis is presented by Hoesly (1962).

Metabolic studies upon meningococci have been few relative to those

studies of other bacteria. Sufficient work has been reported, however, to indicate the meningococcus possesses no unusual metabolic pathways. Respiration of the meningococcus is aerobic. Jyssum (1960) reported enzyme activities corresponding to certain enzymes involved in the tricarboxylic acid cycle. In 1961, Jyssum, Borchgrevink, and Jyssum reported that N. meningitidis "adapted" to growth with glucose as sole carbon and energy sources oxidized glucose and several phosphorylated intermediates in the Embden-Meyerhoff pathway of glucose catabolism. Jyssum and Jyssum (1962a) have also presented evidence for the pentose pathway and for the Entner-Doudoroff route. Other enzyme activity has been reported present in N. meningitidis cells. Phosphoenolpyruvic carboxylase activity in extracts of N. meningitidis was reported in 1962b(Jyssum and Jyssum) and polynucleotide phosphorylase in 1963 (Jyssum and Jyssum). Tauber and Russell (1962) demonstrated a reduced diphosphopyridine nucleotide oxidase in N. meningitidis and in other bacteria.

A few workers have reported certain genetic aspects of the meningococcus. Alexander and Redman (1953) showed that N. meningitidis undergoes transformation affecting capsular antigen specificities. Catlin (1960) showed that genetically active DNA may be recovered from lysed N. meningitidis cells and also from the extracellular medium. This same paper reported that streptomycin resistance was a transformable characteristic. An interesting paper by Catlin and Cunningham (1961) concerned the correlation of transformation of streptomycin resistance and base contents of DNA preparations of various neisseriae. The base ratios of N. meningitidis, N. perflava, N. sicca, and N. flavescens were very similar with this similarity expressing itself in interspecies transformants. The base ratios of N. catarrhalis strains were quite different

and the ratios of transformants between N. catarrhalis and the above neisseriae were very low. Jyssum and Lie (1965a; 1965b) have reported a number of recent genetic studies using N. meningitidis. There have not been any reports of transformation of virulence, for a satisfactory test system must first be provided.

The currently accepted classification of the meningococci is that recommended by the Subcommittee on Neisseria of the International Association of Microbiologists (Branham, 1940; Breed, Murray, and Smith, 1957). Four serological Groups A, B, C, and D have been designated on the basis of agglutination reactions with immune serums. Group specificity appears due to a specific capsular polysaccharide for A and C Groups (Branham and Carlin, 1942; Branham and Wormald, 1953). Group B specificity is due to a polypeptide complex (Menzel and Rake, 1942). Group D strains are relatively unstudied for they are difficult to procure. In a very recent paper, a tentative bacteriocin-typing scheme was presented as a means of subgrouping strains of N. meningitidis (Kingsbury, 1966). It may be possible to make a fine distinction between strains, as Kingsbury suggests, and study the epidemiology of bacteriocin types in a closed population where the meningococcus is often a problem.

B. Characteristics of infection

A carrier state exists for N. meningitidis apparently only in the human and not in animals. It is generally held that the nasopharynx of man is the residence site in carriers (Buddingh and Polk, 1937; Buddingh and Polk, 1939; Smith et al., 1964). In epidemic periods, carrier rates as high as 85 per 100 have been reported (Aycock and Mueller, 1950).

During nonepidemic periods, carrier rates may be 10-12 per 100 (Branham, 1940; Branham and Wormald, 1953). Carrier rates may be as low as 5 per 100 in a closed population during nonepidemic periods (Millar et al., 1963). Despite relatively high carrier rates, incidence of meningococcal meningitis is quite low and occurs primarily in humans under 30 years old (Aycock and Mueller, 1950; Smith et al., 1959). It is known that humans possess serum anti-meningococcal antibody (Silverthorne and Fraser, 1935) and also that meningococci appear to be efficiently phagocytized by PMN and monocytic cells (MN) (Leichtenstern, 1885; Councilman , Mallory, and Wright, 1898; Buddingh and Polk, 1939; Sevag, 1956). Yet, cerebrospinal meningitis may unexplicably appear in a person with no nasopharyngeal pathology. The factors which initiate such infections are not understood.

The route of infection appears to be via the nasopharyngeal tissues usually subsequent to local acute inflammation, though inflammation does not invariably precede infection (Smith et al, 1959; Smith et al., 1964; Morehead, 1965). In certain cases, the meningococci invade lymphatics and capillaries of the nasopharyngeal mucosa. A bacteremia may ensue to produce the clinical syndrome, meningococcal bacteremia, a frequently fulminant condition with no prodromal symptoms. This condition is associated with hyperthermia and ecchymosis and/or petechiae of skin and various organs. The lesions appear to be due to direct endotoxic action, for no exotoxins have been reported for N. meningitidis. Meningococci are found within PMN and MN, in lesions such as petechiae, and bacteria may be isolated from hemorrhagic skin lesions. Though not of a quantitative nature, the work of Buddingh and Polk (1937; 1939) presented substantial evidence on the route of infection and associated events.

Cerebrospinal meningitis, as a complication of meningococcal

bacteremia, was described by Councilman, et al., (1898) in a very thorough report. This condition, though ordinarily considered a complication, occurs in individuals with no preceding clinical evidence of nasopharyngitis or bacteremia (Smith et al., 1959). The condition of purulent meningitis develops in less than 3 per cent of exposed persons during epidemics (Smith et al, 1959). Examinations of stained smears of cerebrospinal fluid from such patients are consistent in one major respect, namely, differential counts show the leukocyte population, in septic cerebrospinal fluid and purulent exudates in the brain, to be 100 per cent PMN (Weinberg, 1963; Roy, 1964). Emphasis should also be focused on the observations that PMN frequently contained intracellular meningococci, sometimes in large numbers (Councilman et al., 1898; Flexner, 1907; Wollstein, 1907) and that meningococci have been observed intracellularly only in PMN (Councilman, et al., 1898).

A small number of N. meningitidis infections are complicated by the Waterhouse-Friderichsen Syndrome. This syndrome is primarily observed in association with meningococcal infections (Thompson and Shapiro, 1957), although it is also seen in association with heavy infections with other bacteria such as the streptococci and pneumococci (Smith et al., 1959). The hallmark of this syndrome is bilateral large or massive adrenal hemorrhage (Smith et al., 1959; Morehead, 1965). The Waterhouse-Friderichsen Syndrome appears very similar to the generalized Schwartzman reaction with the skin lesions resembling a local Schwartzman reaction (Schwartzman, 1929). Black-Schaffer, Hiebert, and Kerby,(1947) made a comparative study of strains isolated from patients demonstrating no skin lesions and strains isolated from patients with skin lesions. All strains produced materials eliciting the local Schwartzman reaction, but more

potent filtrates appeared to be from patients exhibiting skin lesions. Indications were that meningococcal purpura may most likely occur through fortuitous infection with a purpurogenic strain. No correlation was shown to exist between antigenic type and ability to produce purpura in rabbits. The report of Good and Thomas (1953) indicated that meningococcal-toxin-induced Schwartzman reaction yielded dermal pathology and bilateral cortical necrosis very smilar to that encountered in the observed cases of Waterhouse-Friderichsen Syndrome. The endotoxin in this study was prepared by using saline to wash N. meningitidis growth off of the surface of agar contained in Kŏllĕ flasks. The saline suspension was then centrifuged at 12,000 g for 30 minutes. The supernatant was considered to contain the endotoxin and was used without further treatment. Heparin prevented occurrence of both local and generalized Schwartzman phenomena in rabbits if given at the time of provocation dose but not when given at the time of the preparatory dose. It would appear that, as stated by the authors, vascular occlusion which is prevented by heparin, does play a role in development of the Schwartzman reaction.

The endotoxin possessed by the meningococci appears to be similar to that found in other Gram negative bacteria. It is commonly held that this endotoxin is a complex cell wall material composed of carboydrate, phospholipid and proteins or polysaccharide-like material (Thomas, 1954; Atkins, 1960). Demonstration of pure cell wall material as the endotoxin has not been done. Neither has the goal of a pure cell wall preparation been achieved, but Vedros and Hill (1966) found that a trypsin-treated meningococcal cell wall preparation was not toxic for mice. It is felt by some workers that neisserial nucleoprotein is responsible for endotoxic properties of the bacteria. This so-called "nucleoprotein" is actually a mixture of

cellular components of the bacterial cell which remain following lipid-solvent extraction and cell lysis. In 1934, Boor and Miller reported that "nucleoprotein" from meningococci was slightly less toxic in mice than intact bacteria. Extraction of the intact meningococci using acetone and ether in the cold did not appear to reduce toxicity. The number of mice used in this study did not permit clear-cut interpretations of the data. In 1944, Boor and Miller reported a similar and more complete study. Meningococci, represented among 78 strains of neisseriae, were extracted with trichloroacetic acid. Meningococcal "nucleoprotein complex" was shown to be responsible for virtually all toxicity in mice. The carbohydrate-lipid portion of the "nucleoprotein complex" was also shown to possess toxicity for mice.

The physiological effects noted in experimental animals appears to be due to endotoxin (Atkins, 1960). Among the effects produced are the following:

1) Profound vasomotor disturbance terminating in shock and characterized by intense, generalized arteriolar constriction.
2) Hyperglycemia followed by hypoglycemia.
3) Pyrexia followed by hypothermia.
4) Polymorphonuclear leucopenia followed by leucocytosis.
5) Rapid appearance of a state of resistance against the same and other endotoxins of Gram negative bacteria.

There does not appear to be a correlation between virulence of a particular strain of meningococcus and endotoxin potency, and involvement of endotoxin in pathogenicity of the meningococcus is still uncertain (Atkins, 1960).

The first successful treatment of meningococcal infection was by means of antiserum near the beginning of the twentieth century. Various aspects of serum treatment for meningococcal infections were reported (Topley and Wilson, 1936). For example, in 1913 Flexner reported on serum treatment of epidemic meningitis and in 1918, Gordon reported on improved methods for production of meningococcus anti-endotoxin. Although serum therapy early in the infection apparently reduced mortality by some 50 per cent, problems attendant upon such therapy have accounted to some extent for lack of acceptance of such treatment (Flexner, 1913). Sulfonamides have been successfully used for treatment of neisserial meningitis for approximately 27 years (Millar et al., 1963). Penicillin has also been well received in treatment (Alexander, 1953). Despite the overall favorable performance of penicillin and sulfonamides, there are incidences of appearance of strains of meningococci resistant to these compounds (Millar et al., 1963; Eickhoff and Finland, 1965). Some advocate use of cortisone in conjunction with sulfonamides or antibiotic therapy (Forbes, 1962; Anglin et al., 1965). The picture is really not clear for efficacy of such combinations and the evidence for use of cortisone is equivocal with some reports indicating no therapeutic advantage (Levin and Cluff, 1965).

Active immunization as a prophylactic measure has never been satisfactorily demonstrated in man. Greenberg and Cooper (1965) point out a number of problems associated with active immunization of humans. The aspect which presents a minor obstacle appears to be the manufacture of a vaccine which will elicit various degrees of protective antibodies in mice and in man. The aspect of the problem which presents a major obstacle is the testing of a vaccine in humans in a large-scale, well-

controlled, field trial. A field trial is requisite, for no other test has been devised to successfully estimate protective immunizing efficacy of a vaccine in man. As emphasized by these authors, at this time there is little point in developing a vaccine composed of purified fraction or fractions of meningococci since each vaccine would have to be tested in a large trial and such testing would be quite impractical.

C. Animal experiments

Early experiments of pathogenicity of N. meningitidis in intact animals were reported by Murray (1923). These studies presented novel ideas but did not greatly expand knowledge of disease caused by the meningococcus. In most early reports, the study of meningococcal infection in small laboratory animals was hampered by lack of a technique permitting infection using relatively small numbers of meningococci. Miller and Castles (1936) pointed out that several hundred million meningococci were usually required to kill a mouse by intraperitoneal injection and that this number was very similar to the lethal dose of killed bacteria. As Miller suggested, "infection" could have been incidental to a primary intoxication. In 1933, Miller reported a technique for infecting experimental animals with low numbers of N. meningitidis organisms. Miller later demonstrated that intraperitoneal injection of hog gastric mucin with meningococci permitted the killing of a mouse with 100 and even fewer bacteria (Miller and Castles, 1936). Miller's technique provided a system particularly useful for immunological studies (Greenberg and Cooper, 1965). Other animals, such as guinea pigs, have been used for infectivity studies but their usefulness has been of minor value (Branham and Lillie, 1933). Attempts have been made to simulate in

monkeys the apparent route of infection in man, which is probably via the nasopharyngeal mucosa, without uniform success, though Flexner (1907) did successfully infect monkeys by intraspinal inoculation.

An experimental animal better suited for study of meningococcal infection was supplied when Buddingh and Polk (1937) published the first work on infection of chick embryos. Infection of 12 day old chick embryos was accomplished by inoculating the chorio-allantoic membrane through a "window" cut in the shell (Polk, Buddingh and Goodpasture, 1938). The meningococcus invaded the blood stream of the embryo at the site of inoculation with subsequent widespread hemorrhage, and capillary and venous thrombosis. Death of the embryo occurred in 24-48 hrs after inoculation. The route of infection in man could be approximated by inoculation into the amnionic cavity, for the embryo was then infected primarily via the nasopharynx and mouth. It was reported that virulence for the chick embryo was not enhanced with passage through embryos (Buddingh and Polk, 1937). This observation is at variance with the unpublished work of Comeau (1963) who found that virulence was enhanced on chick embryo passage.

Hoesly (1962) also found the chick embryo a useful host to evaluate host-parasite response using N. meningitidis strains. In reference to an avirulent control strain of N. catarrhalis, Hoesly was able to demonstrate a difference in virulence among the strains of meningococci tested. It is of interest that a contemporary problem attendant upon the use of chick embryos for meningococcal studies is the difficulty of obtaining eggs free of antibiotics, that is, eggs from flocks not fed on antibiotic-containing rations. This difficulty was non-existent at the time of the work of Buddingh and Polk, (1937) since they experimented in the pre-antibiotic era.

No studies upon N. meningitidis using an in vitro host-parasite system have been reported. One aspect of this study was to devise a technique which may, with modification perhaps, serve as a useful in vitro study system to approach the problem of pathogenicity.

D. Pathogenicity of N. meningitidis

Pathogenicity of N. meningitidis is an unsolved problem. However, as was noted in Part B of this introduction, stains of smears of septic cerebrospinal fluid and purulent brain exudates from patients with meningococcal meningitis provide two important consistent facts:

1) The differential leukocyte counts indicated a population nearly 100 per cent PMN leukocytes.
2) Meningococci appear intracellularly only within PMN leukocytes and PMN leukocytes very often contain numerous meningococci.

These two observations may indicate mechanisms involved in the pathogenicity of the disease, namely, intracellular survival and/or proliferation in PMN.

These considerations have been made by others for indeed, the bacterium was formerly named N. intracellularis. One occasionally reads statements to the effect that intracellular survival is associated with the mechanism of pathogenicity of N. meningitidis (Smith et al., 1964). Scherp (1955) states that "At the risk of oversimplification, one concludes that the pathogenicity of gonococci and meningococci is determined by antiphagocytic and endotoxic

mechanisms." That this statement is an oversimplification is probably correct for it appears to be generally agreed upon that a multiplicity of factors is necessary for initiation of infection (Miles, 1955; Dubos, 1954). As a matter of fact, there is no convincing evidence that intracellular survival of meningococci does obtain in PMN, or that such takes part in the mechanism of pathogenicity. However, the vast literature on the role of phagocytosis in bodily defenses against various other bacteria does suggest that phagocytosis, intracellular survival, and perhaps proliferation are factors involved in the disease mechanism (Hanks, 1957; Burrows, 1955; Hatten and Sulkin, 1966).

It is known that meningococci appear to induce polynucleosis (Olitzki, Avinery, and Bendersky, 1941; Atkins, 1960). It is also apparent that there is general recognition of the fact that meningococci are phagocytized by and closely involved with the PMN leukocytes. Therefore, a number of considerations need be made concerning the intracellular state of the phagocytized meningococcus.

During the phagocytic process by PMN, bacteria are taken into the animal cell by a process termed endocytosis (Novikoff, 1961). The ingested bacteria come to lie within the cell cytoplasm enclosed in a sac composed of cell membrane and cytoplasmic membrane (DeDuve, 1963a; Novikoff, 1961). The bacteria and the enclosing membranes form what is termed a phagosome (DeDuve, 1963a). DeDuve (1963b) postulates that lysosomes or storage granules somehow merge with the phagosome to produce a digestive vacuole. There is some evidence that lysosomes disrupt in the vicinity of phagosomes (DeDuve, 1963a) and there is also evidence that enzymes from the lysosomes are discharged into the phagosome (Hirsch and Cohn, 1960). It is known that a wide spectrum of

various hydrolytic enzymes are associated with PMN leukocyte lysosomes DeDuve, 1963a). Heterogeneity of granule structures seen in electron micrographs seem to indicate that different enzymes are stored in different lysosomal granules (Cohn and Wiener, 1963a). It is felt that degradative functions within PMN cells are at least in part carried out by lysosomal enzymes. Cohn, Hirsch, and Wiener, (1963) present an interesting discussion of cytoplasmic granules of PMN.

At this point in the discussion, the following question should be considered. How is it possible for an ingested meningococcus to survive if the above phagocytic process is correct? One finds that survival of certain bacteria within PMN has been demonstrated (Hirsch and Church, 1960; Cohn et al., 1963) but such a demonstration has not yet been reported for the meningococcus. What hypotheses may one present to explain possible intracellular survival of N. meningitidis within PMN? One may postulate the following:

1) No enzymes are discharged into the phagosome. As previously mentioned (Hirsch and Church,1960) there is some evidence for discharge of enzymes into a phagosome. There is no evidence for such discharge of enzymes under certain conditions in a study using meningococci.

2) All or certain meningococci are resistant to lysosomal enzymes. There is no clear evidence that some or all meningococci are resistant to enzymatic attack. The work of Murray (1923) in which he treated meningococci with extracts of predominantly PMN leukocytes suggests the presence of resistant bacteria. However, Murray found that survival in more dilute extract,

that is, less bactericidal, resulted in bacteria of increased virulence, whereas survival in concentrated extract, that is more bactericidal, did not provide meningococci of increased virulence for the mouse. These results were interpreted to indicate that more factors than merely selection of PMN-extract-resistant forms were involved.

3) No components or only certain components of the meningococcus are enzymatically attacked, for example, the cell wall, but the bacteria remain viable. It is certainly credible that meningococci may survive as L-form bacteria or even as spheroplast forms within phagocytic PMN. In 1964, (Dienes, Bandur, and Madoff) development of L-type growth was demonstrated in Neisseria gonorrheae cultures, although this demonstration in meningococcal cultures has not been reported. Greenberg and Cooper (1965) state that addition of glycine to their semi-defined medium results in a "soft-celled" or "L-form organism" of N. meningitidis. Recent work by Hatten and Sulkin, (1966) indicates that L-form bacteria can be recovered from tissue culture cells infected with Brucella abortus. Perhaps L-form meningococci could similarly survive in the phagosome and proliferate upon death of the host cell or egestion of the bacterium (Wilson, 1954). The possibility of intracellular meningococcal survival as spheroplast forms is not precluded by the fragility and osmotic susceptibility of these bacterial forms. The evidence of Aronson (1963) for bridge formation and cytoplasmic flow between phagocytic cells furnishes a mechanism by which intracellular bacterial forms could be transported from cell to cell

without exposure to extracellular environment. Resistance of ingested meningococci to lysosomal enzymes may not in itself explain intracellular survival and one must make a closer inspection and consider other factors.

It was previously mentioned that the meningococcus is a highly aerobic microorganism. It therefore appears more than likely that an ingested meningococcus must withstand the conditions of anaerobiosis imposed by the restricted confines of the phagosome. Evidence for the presence of the Embden-Meyerhof pathway and the pentose pathway has been previously presented and consequently survival under anaerobic conditions may be a reasonable assumption. One should note that intracellular survival need be for only a relatively brief duration of time. Time durations as short as a few minutes could easily be involved in host-parasite interactions according to the work of some (Evans, Miles, and Niven, 1948; Elberg and Schneider, 1953). These studies indicate that the first few minutes or hours of contact between host and parasite determine the outcome of the infection.

By reason of residence within the phagosome, the intracellular meningococci are further burdened by nutritional exigencies. The work of Hoesly (1962) is the only report of experiments to determine the effect of nutritional dependency or independency on virulence of strains of N. meningitidis. Hoesly divided 7 strains of N. meningitidis into 3 groups on the basis of growth response in Frantz medium, a synthetic medium containing inorganic salts, glucose, L-glutamic acid, and L-cystine. The 7 strains were divided into 3 groups as follows:

1) Group I was composed of 3 strains, M-1027, M-1628, and M-2092,

which were respectively serological Groups A, C, and B. All 3 strains grew in Frantz medium on initial transfer from stock dextrose starch agar.

2) Group II strains were the serologically ungrouped strains M-2 and Bell. These two strains required a single passage through yeast extract-supplemented Frantz medium before growth could be obtained in unsupplemented Frantz medium.

3) Group III was composed of the serologically ungrouped strain M-1 and serological Group D strain M-158. These strains required yeast extract-supplemented Frantz medium both for primary cultivation and subculture.

Hoesly utilized a chick embryo inoculation system to assess the virulence of all 7 strains. A strain of N. catarrhalis, generally considered non-pathogenic for humans, and avirulent for chick embryos, was used as a control strain of neisseria. In reference to use of N. catarrhalis one might recall the work of Catlin and Cunningham (1961). These workers obtained a very low ratio of transformants between N. catarrhalis and several strains of neisseriae. On the basis of this study, one may question whether N. catarrhalis is a suitable choice as a comparative strain of neisseria.

Hoesly found that in comparison to the virulence of N. catarrhalis expressed in the chick embryo, strain M-1027 was virtually as benign as N. catarrhalis. Strain M-1628 proved to be the most virulent of all strains tested. These two strains from the same nutritional group exhibited different orders of virulence. The strains of Group II, M-2 and Bell, were virulent with respect to N. catarrhalis but no more so

than M-1628. Group III strains, M-1 and M-158, were no more virulent than N. catarrhalis. It therefore appears that nutritional independence does not per se confer virulence, a virulence which may be partially a function of intracellular survival and proliferation in PMN leukocytes.

E. Objectives of this thesis

Hoesly studied 7 strains of N. meningitidis and only 4 strains were serologically grouped as A, B, C, and D. These strains were M-1027, M-2092, M-1628 and M-158 respectively. In comparison with N. catarrhalis, M-1027 and M-158 were avirulent for chick embryo whereas M-2092 and M-1628 were virulent. The serological Groups A, B, and C take part most prominently in meningococcal infections (Aycock and Mueller, 1950; Smith et al., 1959). The relative avirulence of strain M-1027 and the relative virulence of strain M-1628 provided meningococcal strains with attributes amenable to study in an in vitro PMN leukocyte-meningococcal host-parasite system. This thesis is concerned with a study of the meningococcal strains, M-1027 and M-1628, in an experimental design which attempts to approach the problem of intracellular survival and proliferation in mammalian PMN leukocytes as part of the mechanism of pathogenicity of N. meningitidis.

MATERIALS AND METHODS

A. Bacterial studies

1. Bacterial strains studied

A history of the two strains of Neisseria meningitidis used in this study is presented in Table 1. Both strains were originally obtained from the American Type Culture Collection, Washington, D.C. Upon reception of these cultures in the laboratory of Dr. E. L. Oginsky, Department of Bacteriology, University of Oregon Medical School, lyophilized stock cultures were prepared and stored under refrigeration. Strain M-1027 was lyophilized in April, 1961, and strain M-1628 was lyophilized in June, 1961.

2. Stock cultures

On initiation of this study in June 1963, both lyophilized strains were suspended in dextrose starch broth (DSB) (Marshall, 1947) which was obtained in desiccated form from Difco Laboratories, Detroit, Michigan. The formulation of this medium is given in Table 2. The suspended bacteria were incubated for 24 hrs at 37C without CO_2 supplementation of the culture tube atmosphere. Petri dishes of Mueller-Hinton agar (M-H) (Mueller and Hinton, 1941) were streaked with growth from the 24 hr growth tubes so as to obtain isolated colonies. The petri dishes were then incubated in candle jars with a slight amount of water at the bottom of the jar to provide a humid atmosphere. In all instances in which candle jar incubation was used, water was placed in the bottom. Incubation was for a period of 24 hrs at 37 C. M-H medium

was obtained from Difco Laboratories, Detroit, Michigan. The formulation of the desiccated product is given in Table 3.

Stock cultures were prepared by stab inoculation of single colony isolates from the above M-H petri dishes into M-H agar deeps in screw-cap tubes. The inoculated tubes were incubated at 37 C both for growth of the bacteria and for storage. Subcultures were carried out at approximately monthly intervals by streaking out agar-growth onto M-H agar petri dishes, incubating for 24 hrs at 37 C, and then reinoculating into M-H agar deeps. In certain instances, cultures stored under this procedure have remained viable for considerably longer than one month; for example, one particular stock of M-1027 yielded viable bacteria after two years of storage.

3. Working cultures

Working cultures were maintained on M-H agar in petri dishes which were streaked so as to obtain isolated colonies. Both strains of meningococci survived over 48 hrs incubation but viability was considerably reduced by 72 hrs. Therefore, subcultures were made at 48 hr intervals by picking single colony isolates and restreaking on M-H agar petri dishes. The inoculated dishes were stored at 37 C in candle jars.

4. Confirmation of strain serological type

Confirmation of the meningococcal strains used in this study as Group A and Group C was carried out by means of an agglutination procedure (Gradwohl, 1956) with minor modifications. Anti-Group A and anti-Group C rabbit serums were provided by the Department of Health, Education, and Welfare, Public Health Service, Bureau of State Services

Communicable Disease Center, Diagnostic Reagents Section, Chamblee, Georgia. The protocol for the agglutination test was as follows:

1. Streak a 5% blood extract-agar plate (sheep blood) and incubate in a candle jar for 24 hr at 37 C.
2. Thoroughly emulsify growth in phosphate-buffered saline (PBS) (0.85% NaCl with 0.01 M K_2HPO_4 : KH_2PO_4 pH 6.8). Mix well to obtain a uniform suspension. Adjust the density of the suspension to 0.8 at 520 mμ using 10 X 100 mm matched tubes.
3. Prepare dilutions of the antiserums in PBS.
4. Place 0.4 ml of diluted antiserum in a 10 X 100 mm agglutination tube and mix with 0.1 ml bacterial suspension. Permit the agglutination reaction to proceed undisturbed for 2 hr at room temperature. Include controls of normal rabbit serum and PBS.
5. Examine the tubes for bacterial agglutination by observing the image of the contents in a concave mirror.

5. Growth of bacteria for experiments

M-H petri dishes were prepared by drying for approximately 20 hrs at 37 C. Medium with dry surface was desirable because the tendency for contaminants to flow in with moisture and spreading of growth on the surface of the plate were both minimized. Single colony picks from working cultures approximately 36 hr old were spread with a bent glass rod on an approximately 50 mm square central portion of the plate, to obviate possible air contamination at the periphery. Cultures prepared

in this manner were termed "spread plates" and were incubated in candle jars for approximately 18 hrs at 37 C.

6. Preparation of bacterial suspensions

Prior to preparation of suspensions, smears were prepared from "spread plates" and Gram stained to verify purity of the cultures. The suspensions were prepared as follows: All suspensions were prepared using matched 10 X 100 mm tubes. The tubes were indexed to permit reproducible placement in the cuvette holder of the Spectronic '20' (Bausch and Lomb Optical Company, Rochester, New York), which was used for all optical density measurements of bacterial suspensions. Optical densities were determined at 520 mμ in cases in which the suspending medium was colorless, and at 600 mμ in case in which the suspending medium was prepared with tissue culture Medium 199 (Microbiological Associates, Bethesda, Maryland). The formulation of Medium 199 is given in Table 4. Approximately 4.0 ml of suspending medium at room temperature was added per tube, which was closed with a Morton Closure (Bellco Glass, Inc., Vineland, New Jersey). Growth from an 18 hr "spread plate" was carefully removed using a sharpened wooden applicator stick. Caution was exercised to prevent collection of agar medium. The bacterial growth on the applicator stick was well emulsified on the side of the tube before suspension in the medium. Emulsification was rapid in the case of strain M-1027. Emulsification of strain M-1628 was more difficult and required longer emulsification time. Relative ease of emulsification is another difference between these strains; others have been reported previously (Hoesly, 1962) or will be described later in this thesis. The tubes with emulsified bacteria were mixed well with a

vortex stirrer (Scientific Industries, Inc., Queens Village, New York) to effect a uniform suspension. Optical density readings at the appropriate wavelength were then made using as a blank a tube with uninoculated suspending medium.

7. Plate counts of bacteria

Plates containing approximately 15 ml of freshly prepared M-H agar were dried for approximately 20 hrs at 37 C in an inverted position. Drying was obligatory for the complete absorption of the 0.1 ml dilution sample, and for the development of well isolated discrete colonies. All operations were carried out at room temperature, with diluting and plating media at room temperature. Dilutions of bacterial suspensions, whatever the composition of their suspending media, were made in DSB by ten-fold dilution of 0.5 ml to 4.5 ml DSB. Mixing was accomplished by use of a vortex stirrer. A 1.0 ml serological pipette was used to transfer 0.1 ml of DSB dilution to the surface of each of duplicate M-H agar plates. The fluid was then uniformly spread over the entire surface of each petri dish until a "rubbery" drag on the triangular glass spreader indicated that spreading was complete. A separate glass spreader was used for each plate to obviate carry-over and to minimize problems of contamination. Plates were incubated in candle jars for 48 hrs at 37 C.

8. Enumeration of colonies

Counts were accomplished using a Spencer Darkfield Quebec Colony Counter (American Optical Company, Buffalo, New York). Questionable colonies were examined for the oxidase reaction, characteristic of neisseriae, by treatment with a 1% aqueous solution of N,N-dimethyl-p-

phenylenediamine. Colony counts in most experiments were within the limits of 30-300 per petri dish. The number of viable bacteria per ml was calculated, as is customary, by multiplying colony count times the dilution plated.

9. Bacterial viability experiments

The effect of a variety of experimental conditions on the survival of both strains of N. meningitidis was determined:

a. Dilution medium

Survival of bacteria suspended in DSB at various dilutions over a three hour period was determined. A bacterial suspension was prepared in 0.85% NaCl buffered with 0.005 M K_2HPO_4: KH_2PO_4 pH 7.0. Serial ten-fold dilutions of the bacterial suspension were made into DSB (0.5 ml to 4.5 ml DSB) from 10^{-1} to 10^{-5}. Viable counts were made at zero time and after three hrs incubation at room temperature.

b. Experimental medium

Viable counts of bacteria suspended in the following preparations of Medium 199 were determined:

1. Medium 199-1X-H_2O - A 1X solution of stock 10X Medium 199 in distilled water containing 1.0 ml 10% $NaHCO_3$. The medium was gassed with 5% CO_2:95% air. Final pH 7.4.

2. Medium 199-1X-PO_4 - A 1X solution of stock 10X Medium 199 in 0.85% NaCl buffered with 0.02 M K_2HPO_4 : KH_2PO_4 pH 7.7. The medium was thoroughly gassed with 5% CO_2:95% air to give a final pH 7.0.

3. Medium 199-1:2-PO_4 - A 1:2 dilution of a 1X dilution

of 10X stock Medium 199 in the same diluent as 2, with gassing. Final pH 7.0.

4. Medium 199-1:4-PO_4 - A 1:4 dilution of a 1X dilution of 10X stock Medium 199 in the same diluent as 2, with gassing. Final pH 7.0.

5. Medium 199-1:8-PO_4 - A 1:8 dilution of a 1X dilution of 10X stock Medium 199 in the same diluent as 2, with gassing. Final pH 7.0.

Bacterial suspensions were prepared in 0.85% NcC1 buffered with 0.02 M K_2HPO_4 : KH_2PO_4 pH 7.0. The various preparations of Medium 199 were contained in 50 ml screw-cap tubes and occasionally in 100 ml screw-cap bottles. A volume of bacterial suspension, 0.1 or 0.01 ml per 25.0 ml of medium, was added at room temperature, the preparation mixed, and a zero time viability count made. The caps were tightened securely and the tubes or bottles incubated in an air incubator at 37 C for the desired duration of time, either 5 or 6 hrs. After incubation, samples were again taken for viability counts.

c. Homogenization

In experiments described in Section E, the bacteria were subjected to homogenization with or without glass slips. It was necessary first to determine whether such homogenization of bacteria affected viability. The homogenizer used for these experiments was a Sorvall Omni-Mixer Homogenizer with Type OM-2000 Micro-Homogenizer attachment (Ivan Sorvall, Inc., Norwalk, Connecticut). Five ml stainless steel chambers were used for this experiment and for all homogenizations reported in this thesis. Bacterial suspensions with and

without 0.25% sucrose, and with and without glass slips (10.5 X 35 mm), were homogenized as follows: Bacteria were suspended in Medium 199-1:8-H_2O. When a glass slip was used in homogenization, one slip was broken in two in a chamber and 4.0 ml of bacterial suspension added. The chamber was quickly covered to prevent escape of CO_2 with subsequent increase of pH. When cover slips were not used, 4.0 ml of bacterial suspension was merely added to a chamber. The filled chamber was cooled in ice-water slush for 1.0 min and then homogenized at a Powerstat setting of 140 for 2 min (The Superior Electric Company, Bristol, Connecticut). The chamber was immersed in ice-water slush during the entire homogenization to prevent overheating of the chamber contents. After homogenization, glass particles were permitted to settle for 1 min and then samples were taken of the supernatants and plate counts performed.

B. Exudate leukocyte preparations

1. Elicitation of polymorphonuclear leukocytes (PMN) of mice

a. Mice

Mice used in this study were of Swiss strain, BP lab-Albino, 6-8 to 12-14 weeks old and were purchased from Berkeley Pacific Laboratories, Berkeley, California. Certain experiments early in this study were conducted using female mice but most experiments were conducted using male mice. Sex and age of mice used in a particular experiment will be noted.

b. Inflammatory agent

Figure 1 is a flow sheet depicting the steps to be followed in the preparation of exudate leukocyte suspension. The inflammatory agent used to elicit exudates in mice was sodium caseinate, C grade (Calbiochem, Los Angeles, California) prepared by dissolving 12 g in 100 ml hot distilled water (Stähelin, Suter, and Karnovsky, 1956). The temperature of the water was maintained near the boiling point as powdered sodium caseinate was added and carefully emulsified to obtain a smooth low-particle preparation. The preparation was filtered through cotton gauze to remove aggregates of sodium caseinate, autoclaved at 15 lbs pressure at 121 C for 10 min, and then quickly cooled to room temperature. Several 50 ml rubber-closed vials were prepared at one time and stored at 4 C until used. A new batch was prepared every 2 to 3 months. Because of the high viscosity of the final preparation, it was warmed in a 37 C water bath for 10 to 15 min prior to use. Heating not only facilitated aspiration and injection but also reduced the trauma associated with injection of cold materials.

c. Injection of mice

A disposible 1.0 ml syringe fitted with a 25 gauge needle was used to inject 0.5 ml of the 12% sodium caseinate preparation into the peritoneal cavity. Prior to injection, the mouse abdomen was scrubbed with 70% ethanol. Injection of mice was carefully done in order to minimize trauma, internal hemorrhage, and subsequent bloody exudate.

2. Harvesting and washing of peritoneal exudate leukocytes

Exudate was harvested as outline in Figure 1. Initial cell suspension and early washes in Parts A, B, and C of Figure 1 were in

Medium 199-1X-LB which was a ten-fold dilution in distilled water of stock 10 X Medium 199 tissue culture medium. A sufficient volume of 10% $NaHCO_3$ was added to raise the pH to approximately 7.4. Stock Medium 199 (Microbiological Associates, Inc., Bethesda, Maryland) used in this entire study was a 10X solution from which 1X or higher dilutions were prepared.

Suspension of the thrice-washed cells for the final centrifugation in Step D of Figure 1 was made in Medium 199-1X-HB which was a 1X solution of Medium 199 containing 1.0 ml of 10% $NaHCO_3$ per 100 ml medium. The medium was thoroughly gassed with 5% CO_2:95% air to yield a final pH of approximately 7.4. All gassing operations in this entire study were done with a 5% CO_2:95% air mixture.

Centrifugations were carried out at low g forces during the washing procedures to minimize trauma to the cells and all pipetting procedures were similarly gentle. The final low speed brief centrifugation of cell suspension in Medium 199-1X-HB was done in order to sediment the larger aggregates of cells and to produce a more uniform suspension. A uniform suspension permitted more accurate and reliable hemacytometer counts, and facilitated the uniform distribution of exudate leukocytes settled on glass slips.

3. Hemacytometer counts of exudate leukocytes

The hemacytometer counting chamber used was a Spencer Bright-Line with improved Neubauer ruling (American Optical Company, Buffalo, New York). Dilution fluid was a 1% aqueous solution of glacial acetic acid. Cell counts were done according to the method of Hepler (1952).

C. Attachment of exudate leukocytes to glass slips

1. Culture tubes

The culture tubes used were short style Leighton type, 16 x 85 mm, with a window size of 11 X 55 mm (Bellco Glass, Inc., Vineland, New Jersey).

2. Glass cover slips and preparation

Cover slips were of non-corrosive glass No. 1 thickness, 10.5 X 35 mm (Bellco Glass, Inc., Vineland, New Jersey). The slips were carefully treated according to the following procedure before use.

Each slip was individually dropped into 500 ml of near boiling 1% distilled water solution of 7X (Linbro Chemical Company, Inc., New Haven, Connecticut) in a 600 ml beaker. The slips in 7X solution were autoclaved at 15 lbs pressure at 121 C for 5 min. At the end of the autoclaving period, the pressure was rapidly relieved to permit bubbles to form in the solution presumably to provide more thorough cleaning of the slips. The slips were then individually dropped into 500 ml of near boiling distilled water in a 600 ml beaker. This rinse procedure was repeated 4 more times. Without drying, the slips were individually dropped into 200 ml of a 1% solution of Siliclad (Clay-Adams, Inc., New York City, New York) in distilled water in a 250 ml beaker. The slips remained in contact with the silicone solution for 2-4 hrs at room temperature. A final rinse of the siliconized slips was effected by individually dropping slips into 500 ml room temperature distilled water contained in a 600 ml beaker.

A single washed and siliconized slip was placed in a Leighton tube and the tube closed with a Morton closure. Tubes were autoclaved at

15 lbs pressure at 121 C for 10 min and dried in a hot air oven.

3. Settling of exudate leukocytes onto glass slips

A suspension of 2.0 ml washed exudate leukocytes in gassed Medium 199-1X-HB was added to each Leighton tube containing a slip. The tubes were gassed, stoppered with silicone stoppers, which were used in this entire study, and incubated at 37 C in a horizontal position in a rack specially constructed for this purpose. The "cell side", that is the slip side which is "up" when the exudate cells are settled, should be retained "up" during all procedures.

Most operations were carried out in a hood equipped with a UV lamp which was turned on several hours before each experiment, and turned off when the hood was being used. No antibiotics were used in the medium in this study, and therefore great care had to be taken to minimize contamination.

Leukocytes were settled for 1.5 hrs in all experiments.

4. Fixation and staining of exudate leukocytes attached to slips

Exudate cells attached to glass slips were fixed by immersion in absolute methanol at 37 C for at least 15 min, the slips were then removed and air dried (Aronson and Elberg, 1962). This method of warm fixation provided preparations in which cell morphology was relatively intact. The dried slips were attached cell side up to glass slides using Permount (Fisher Scientific Company, Fair Lawn, New Jersey) and permitted to dry overnight at 37 C.

Staining was carried out by a method modified from Jenkins, Brooke, and Osgood, (1961). The stain was prepared as follows:

1. Twelve ml of Giemsa stain solution (National Aniline Division, Allied Chemical Corporation, New York City, New York).
2. Eighty-eight ml of phosphate buffer pH 6.4 prepared as a 10X stock solution. Two liters were prepared as follows:

a. Na_2HPO_4 51.20 g

b. KH_2PO_4 132.60 g

c. Distilled water to make 2 liters.

d. Dilute 1:10 with distilled water before use.

Staining was done in a 150 ml beaker containing 100 ml of stain solution. The slide was placed on an angle with the slip side down to prevent particles of stain from settling on the slip surface. Staining was for 10 min at room temperature. After the staining period, the slide was placed slip side down in a clean 150 ml beaker and cool tap water slowly run into the beaker and let overflow for at least 30 seconds. The back of the slide was wiped to remove excess stain and the slide air dried.

5. Differential populations of exudate leukocytes attached to slips

Determination of the fraction of PMN and MN exudate leukocytes on slips was accomplished as follows: Exudate was prepared in twenty-four 8 to 10 week old female mice, collected from 4 mice at intervals of 3, 6, 9, 12, and 18 hrs, and treated as in Section B Part 2, and Section C Parts 3 and 4. Differential counts were done by counting a total of 400 cells on the central portion of a stained slip. Cells were classified as PMN and MN with no differentiation of the various MN cell types.

Mast cells were counted as PMN and comprised a very small fraction of the total PMN population.

D. Acid phosphatase activity of exudate leukocytes

1. Acid phosphatase assay

The assay procedure was a modification of a method presented in Bergmeyer (1963).

a. Substrate

Substrate was p-nitrophenyl phosphate, disodium, A grade (Calbiochem, Los Angeles, California). Substrate solution was freshly prepared for one day's experiments by dissolving 1.65 mg per ml p-nitrophenyl phosphate in 0.05 M citric acid-sodium citrate buffer. Buffer at pH 5.8 was used in most experiments but was varied in some experiments, which will be noted. Storage of substrate solution was by refrigeration during the day's use.

b. Saponin

Saponin, Reagent grade (Nutritional Biochemicals Corporation, Cleveland, Ohio) was used in conjunction with substrate solution in certain experiments. In such cases, saponin was dissolved in 0.05 M citric acid-sodium citrate buffer at pH 5.8 and added to substrate solution immediately before use. Concentrations and volumes of substrate and saponin were adjusted to compensate for such additions.

c. Reagent

Sodium hydroxide, Reagent grade, 0.2 N was used to stop

the enzyme reaction, and to intensify the color of p-nitrophenol, the chromagen reaction product.

d. Assay protocols

(A)	Substrate solution	1.0 ml
	Sample	0.2 ml
	NaOH	2.0 ml
(B)	Substrate solution	1.0 ml
	Sample	1.0 ml
	NaOH	1.0 ml

The substrate solution, at pH 5.8, and sample were incubated at 37 C for 120 min, unless otherwise noted, at which time the NaOH was added.

e. Optical density measurements

Optical density was read at 400 mμ with a 1 cm pathlength in a Beckman Model DU Spectrophotometer (Beckman Instruments, Fullerton, California). This value for maximum absorption was determined by measuring the absorption spectrum from 380 mμ to 420 mμ. The OD 400 mμ value represents enzyme activity whenever stated in this thesis in text, table, or figure.

f. Optimum pH

1. Standard acid phosphatase

Boehringer Standard Acid Phosphatase isolated from potatoes (C. Boehringer and Soehne, GmbH, Mannheim, Germany) was

purchased from Calbiochem, Los Angeles, California. The specific activity was stated to be 2.5 enzyme units per mg of protein at pH 4.8 and at 37 C.

Standard acid phosphatase solution was prepared by dissolving the enzyme in 0.001 M citric acid-sodium citrate buffer at pH 6.0. Two ml aliquots, containing 0.002 mg per ml enzyme, were placed in 10 X 100 mm tubes, stoppered, and stored in the deep freeze.

2. Procedure for determining the effect of pH on acid phosphatase activity of leukocyte homogenate and Boehringer's standard enzyme

Homogenate was prepared from pooled and washed leukocytes elicited in three 12 to 14 week old male mice. Three ml of leukocyte suspension in 0.001 M citric acid-sodium citrate buffer at pH 7.0 containing 5.0 X 10^6 cells per ml were placed in each of 3 homogenization chambers. Homogenization was done exactly as described in Section A Part 8 c except that a Powerstat setting of 90 was used for several times of homogenization. The homogenates were pooled and kept in ice.

A series of substrate solutions of varying pH were prepared in 0.05 M citric acid-sodium citrate buffers and Protocol A followed in the assay procedure.

Substrate solution was warmed in 10 X 100 mm tubes in a water bath at 37 C for 5 min. Samples were added, the contents of the tubes mixed, and incubated. After an incubation time of 30 min for tubes containing standard acid phosphatase and 120 min for tubes containing homogenate, sodium hydroxide was added and the tubes mixed on a vortex stirrer. Each preparation of different pH was prepared with a blank by incubating

substrate solution without sample, adding sodium hydroxide after incubation, and then adding sample.

3. Effect of homogenization with or without glass slips on acid phosphatase of exudate leukocytes

Exudate leukocytes were prepared in three 11 to 13 week old male mice. Washed cells were suspended in 0.05 M citric acid-sodium citrate pH 5.8 buffer during the final wash and centrifugation rather than in Medium 199-1X-HB and cell concentration adjusted to 2.5×10^6 per ml. Three ml of cell suspension was placed in each of 4 homogenization chambers without glass slips and 3.0 ml placed in each of 4 homogenization chambers which contained a glass slip broken in two. The contents of each chamber were homogenized for either 15, 30, 45 or 60 sec at a Powerstat setting of 90. After homogenization, 1.0 ml aliqots were removed from all chambers and stored in ice. The remaining chamber contents were then placed in tubes and centrifuged at 3500 g for 5 min at 2 C using a Model PR-2 International Centrifuge with a Multispeed Attachment (International Equipment Company, Boston, Massachusetts). All centrifugations in the acid phosphatase studies were carried out in this manner.

4. Effect of homogenization and sonication on acid phosphatase of exudate leukocyte homogenates

a. Homogenization

Exudates were elicited in three 12 to 14 week old male mice. Washed cells were suspended in 0.05 M citric acid-sodium citrate

buffer at pH 5.8 to a concentration of 3.5 X 10^6 per ml. Three ml of cell suspension was placed into each of 4 homogenization chambers with glass slips and homogenized at Powerstat setting of 90 for 30 seconds.

b. Sonication

The homogenates were pooled and 2.0 ml aliquots delivered into each of six 5 ml snap-top Nalgene vials (The Nalge Company, Rochester, New York). The vials were immersed in ice-water slush in the sonicator chamber (Raytheon Sonic Oscillator, 10 KC, 250 W, Model DF 101; Raytheon Manufacturing Company, Waltham, Massachusetts). Sonication was conducted at 1.25 Amps for periods of 1, 2, 3, 4, 5 and 10 minutes. At each time interval, a vial was removed from the chamber, stored in ice, and the ice in the chamber replenished.

After all sonications were complete, 1.0 ml aliquots were removed from each vial and stored in ice. The remainder of the vial contents were centrifuged at 3500 g for 5 min and Protocol A was used for assay of both the supernatants and the uncentrifuged sonicates.

c. Effect of saponin at 80 mg per ml on acid phosphatase of exudate leukocyte homogenates

Exudate leukocytes were prepared in five 12 to 14 week old male mice. Washed cells were suspended in 0.05 M citric acid-sodium citrate buffer at pH 5.8 to a concentration of 5.0 X 10^6 per ml. Four ml were placed in each of 5 homogenization chambers without glass slips and homogenized at a Powerstat setting of 90 for 30 seconds.

The contents of all chambers were pooled, a portion reserved at 0 C, and the remainder mixed with saponin in the ratio of

1.9 ml of homogenate to 0.1 ml of 1600 mg per ml saponin solution. Mixtures were allowed to stand at room temperature for 30 to 60 min at which time 1.0 ml aliquots were removed and stored in ice and the remainder of the contents of the chambers centrifuged. Assays were conducted by using Protocol A with the samples before and after centrifugation, as well as the homogenate without saponin treatment.

d. Relationship of acid phosphatase activity to number of exudate leukocytes treated and untreated with saponin at a final concentration of 0.0125 mg per ml

1. Exudate leukocyte preparations

Exudate leukocytes were prepared in five 9 to 11 week old male mice. Washed leukocytes at a concentration of 1.0×10^6 per ml were suspended in Medium 199-1X-Lb at a pH of approximately 6.5. A series of samples of leukocyte suspension, from 0.05 ml to 0.60 ml, was delivered individually to the chambers of Leighton tubes without glass slips. A volume of Medium 199-1X-LB at pH 6.5 was then added to bring the total volume in each chamber to 1.0 ml. These additions were done in random order. The tubes were gently rocked to distribute the cells, the tubes closed with Morton closures, and incubated at 37 C for 40 min to permit the cells to settle.

2. Enzyme assay

Protocol B was used for the assay procedure. Tubes containing the leukocytes were cooled to room temperature for 10 to 15 minutes. Warm substrate at 37 C without or with saponin (at a

concentration of 0.025 mg per ml) was added to the tubes in randon order. After the contents were gently mixed by rocking, the tubes were closed with Morton closures, and placed in a 37 C air incubator. Care was taken to maintain tubes in a horizontal position to prevent loss of leukocytes and fluid on glass surfaces not in contact with substrate solution. At the end of the incubation period, NaOH was added in random order and the tubes mixed with a vortex stirrer.

Each tube of leukocytes was matched by a blank prepared by incubating substrate without or with saponin, adding NaOH after incubation, and then adding cell suspension in Medium 199-1X-HB at pH 6.5 in the proper volume. A standard acid phosphatase control was included to determine the effect of saponin on enzyme activity.

e. Acid phosphatase activity of homogenate supernatants incubated for 5 hrs at 37 C

Exudate was prepared in four 7 to 9 week old female mice. Washed cells were suspended in Medium 199-1X-HB to a concentration of 1.0×10^6 per ml. A glass slip was broken in half in each of 4 homogenization chambers, 5.0 ml of cell suspension placed in each chamber, and homogenization carried out at a Powerstat setting of 90 for 30 seconds. The homogenates were pooled, centrifuged, and the supernatant used for the remainder of the experiment as follows: Two ml of supernatant was delivered into the chambers of Leighton tubes, the tubes gassed, stoppered, and incubated at 37 C. At intervals of 0, 2, and 5 hrs, the supernatant was removed from a tube and assayed by Protocol B. The tube was inverted to drain. Warm 37 C substrate was placed in the tube and washed over the inner surface, the tube closed with a Morton

closure, and incubated. At 2 hrs. NaOH was added to the tube and OD 400 mμ values determined.

E. Exudate leukocyte-meningococcus interaction

1. Infection of exudate leukocytes

a. Infection procedure

For a single experiment, 11 exudate cell cultures were prepared as follows: Two ml of washed exudate cell suspension containing 1.0 X 10^6 or 2.0 X 10^6 cells per ml in Medium 199-1X-HB were delivered into each of 11 Leighton tubes containing glass slips. The tubes were gassed for 10 sec, stoppered, and incubated at 37 C for 1.5 hrs to permit attachment of cells to the slips.

After incubation, 5 randomly selected slips were individually removed, rapidly placed into clean Leighton tubes, and infected as described below. The remaining 5 tubes were then similarly treated. Rapid manipulation of the tubes was necessary because of the fairly rapid rise in pH when the slips were removed from the 5% CO_2 atmosphere.

Multiplicity of infection was calculated on the basis of the number of leukocytes in the original suspension and not on the basis of the number of leukocytes actually attached to the slips. In different experiments, multiplicities of infection were varied over a range of approximately 5 to 40 bacteria per exudate leukocyte.

An 18 hr old "spread plate" culture was used to prepare a bacterial suspension in Medium 199-1X-HB using 10 X 100 mm matched tubes. Preparation of the suspension was done as described in Section

A Part 6 and only one meningococcal strain was used in a single experiment. On the basis of previous experience, the optical density of the suspension was adjusted using Medium 199-1X-HB to contain an estimated number of bacteria which was verified by plate count after the exudate leukocytes were infected. Two ml of such a bacterial suspension were delivered in random order into first 6 tube unit, and then the 5 tube unit. The tubes were gassed for 10 sec, stoppered, and incubated at 37 C for 1.0 hr.

b. Washing of infected exudate leukocytes

After incubation, the infected leukocytes were treated as follows: In random order, each of the 11 slips was removed individually from its tube and rinsed by gently "swishing" to and fro 25 times in 100 ml of 0.85% NaCl buffered with 0.002 M K_2HPO_4:KH_2PO_4 at pH 7.0. Each slip was rinsed in a separate 100 ml aliquot of buffered saline in a 150 ml beaker. This washing step was effected to wash off bacteria which adhered to the surfaces of the slips and leukocytes. After each slip was rinsed, it was placed into a clean Leighton tube. When all 10 slips had been rinsed and placed into Leighton tubes, 2.0 ml of Medium 199-1:8-PO_4 were delivered into random order into each tube, the tubes gassed for 10 sec, and stoppered.

c. Experimental controls

In order to ascertain the number of meningococci which remained attached to glass slips even after a washing of the slips, controls were prepared in the following manner: The same bacterial suspensions used to infect a set of exudate cells were delivered in

2.0 ml aliquots into each of a set of 5 Leighton tubes containing glass slips without attached leukocytes. The tubes were gassed, stoppered, and incubated for 1.0 hr at 37 C.

After incubation, the slips were individually rinsed in 0.002 M K_2HPO_4:KH_2PO_4 buffered 0.85% NaCl at pH 7.0 Each slip was washed in a separate 100 ml aliquot by "swishing" to and for 25 times After washing, each slip was placed into a clean Leighton tube, and after all 5 slips had been washed and transferred to 2.0 ml of Medium 199:1:8-PO_4 were delivered into each tube, the tubes gassed for 10 sec, and stoppered.

2. Procedures for monitoring viable bacteria during exudate leukocyte-meningococcus interaction experiments

Several procedures were followed in order to study, in several ways, the numbers of viable meningococci interacting with infected exudate leukocytes.

Three procedures which were followed will be briefly described to point out differences between these procedures. A more detailed description of the procedures will follow.

a. Procedure 1

A homogenization of infected exudate leukocytes was carried out. Plate counts were conducted at zero time, and the homogenate incubated. At intervals of 1, 2, 3.5, and 5 hrs, samples were removed and plate counts conducted.

b. Procedure 2

Slips with infected exudate leukocytes were incubated and at 0, 1, 2, 3.5, and 5 hrs following infection, a single slip was sacrificed and homogenized and a plate count conducted.

c. Procedure 3

Control slips without exudate leukocytes were incubated and at 0, 1, 2, 3.5, and 5 hrs, a single slip was sacrificed and homogenized. The procedure order of 1, 2, and 3 was followed throughout the experiment, that is, the timing of samplings was so arranged that at the time intervals of 0, 1, 2, 3.5, and 5 hrs, Procedure 1 was followed first, Procedure 2 second, and Procedure 3 was always last. Incubation for Procedures 1 and 2 was begun after both 0 hr samples had been plated for viable bacterial count; then, the 0 hr sample for Procedure 3 was plated, and the remaining control slips incubated. In more detail, these Procedures were as follows:

1. Procedure 1

Two tubes containing infected exudate leukocytes were selected at random from the set of 11. Each tube was treated as follows: The slip was removed and carefully broken in half in a homogenization chamber. One and one-half ml of medium from the Leighton tube from which the slip was removed was added to the chamber. Three ml of thoroughly gassed fresh Medium 199-1:8-PO_4 was added to the chamber, the chamber closed, and placed in crushed ice to cool for 1 min. Homogenization was done at a Powerstat setting of 140 for 2.0 min. The homogenate in the chamber was then held at room temperature while the entire procedure was followed for the other tube.

After the contents of both tubes had been homogenized, the homogenates were pooled and centrifuged at 100 g for 1 min at room temperature to sediment glass particles. The supernatant was carefully aspirated and transferred to a 50 ml screw-cap tube. One ml was removed, diluted, and plated for a viable count. The remainder of tube contents of approximately 8 ml were gassed for 1.0 min, the screw-cap tightly closed, and the tube incubated at 37 C in an air incubator.

At the time intervals of 1, 2, 3.5, and 5 hrs, 1.0 ml samples were removed from the tube, diluted, and plate counts conducted. After the removal of each sample, the contents of the tube were gassed for 1.0 min before the tube was returned to the incubator. The sampling operations were quickly done to minimize cooling of the tube contents.

2. Procedure 2

At each incubation interval, 0, 1, 2, 3.5, and 5 hrs, one tube containing infected leukocytes was selected at random and the tube treated as follows: The slip was removed and carefully broken in half in an homogenization chamber. One and one-half ml of medium from the Leighton tube from which the slip was removed was added to the chamber. Three ml of thoroughly gassed fresh Medium 199-1:8-PO_4 was added to the chamber, the chamber closed, and cooled in crushed ice for 1 min. Homogenization was done at a Powerstat setting of 140 for 2.0 min.

After homogenization, the homogenate was centrifuged at 100 g for 1 min at room temperature to sediment glass particles. The supernatant was carefully aspirated and a plate count conducted.

A second tube of infected exudate cells was selected, the slip removed, and dropped into 37 C absolute methanol for fixation.

A slip was fixed only at the time intervals of 0, 1, 3.5, and 5 hr.

The remaining tubes of washed infected exudate cells were incubated at 37 C in an air incubator.

3. Procedure 3

One experimental control tube was selected at random at each time interval (0, 1, 2, 3.5, and 5 hrs) and treated as follows: The slip was removed and carefully broken in half in a homogenization chamber. One and one-half ml of medium from the Leighton tube from which the slip was removed was added to the chamber. Three ml of fresh Medium 199-1:8-PO_4 thoroughly gassed was added to the chamber, the chamber closed, and cooled in crushed ice for 1 min. Homogenization was done at a Powerstat setting of 140 for 2.0 min.

After homogenization, the homogenate was centrifuged at 100 g for 1 min at room temperature to sediment glass particles. The supernatant was carefully aspirated, diluted, and a plate count conducted.

3. Estimation of differential population shifts and numbers of infected exudate leukocytes after incubation.

The slips utilized were those fixed as described in Section E Part 2, 2. The slips were stained as previously described.

By methodically examining the stained slips, it was possible in a single experiment to compare the numbers of infected leukocytes attached at zero time to the numbers attached at 1, 3.5, and 5 hrs. Concomittantly, differential counts on the same slips yielded information concerning changes in cell population ratios following infection

and incubation. The methods for cell enumeration and for differential counts were as follows:

a. Method for enumerating infected exudate leukocytes on slips

An area approximately 10 PMN cells wide, as viewed using the oil immersion objective lens, was outlined across the ocular lens with Time tape (Professional Tape Company, Inc., Riverside, Illinois). Twenty separate fields, not immediately adjacent to each other, were examined in a direction parallel with the long axis of the glass slip. The 20 fields were approximately equally spaced so as to cover a 10 cell wide strip extending approximately 4 mm from one edge to 4 mm from the other edge of the slip. All cells in a field were counted and the counts summed. A total of 60 fields was examined and counted on a single slip as follows: Twenty fields were examined along a strip about 4 mm from the top edge of the slip, 20 fields were examined along a strip about 4 mm from the bottom edge of the slip, and 20 fields were examined across the center portion of the slips.

b. Differential counts of infected exudate leukocytes on slips

The slips utilized were those fixed as described in Section E Part 2, 2, and used in the method for enumerating infected exudate leukocytes on slips.

Differential counts were carried out by counting a total of 400 cells over the central portion of a stained slip. The cells were classified as PMN and MN. Mast cells, present in very small numbers, were included in the counts as PMN cells.

4. Addition of M-1027 and M-1628 to homogenates of infected exudate leukocytes

Each meningococcal strain was dealt with in separate experiments. Multiplicity of infection using M-1027 was 5 and for M-1628 was 12. The number of added bacteria per ml in Medium 199-1:8-PO_4 was determined by viable plate counts on the control (Preparation 3 to be given). The volume of fresh Medium 199-1:8-PO_4 added to the homogenization chamber prior to homogenization was adjusted to compensate for the volume of bacterial suspension added so that the total volume per chamber would be 4.5 ml. The exudate leukocytes used were settled from suspensions containing 1.0 X 10^6 cells per ml. The preparation of homogenates was as described in Section E, Part 2, 1, Procedure 1. Viable plate counts were conducted on samples removed from the homogenates at zero time, before incubation, and at 1, 2, 3.5, and 5 hrs of incubation at 37 C. The following 3 preparations were studied in these experiments:

II. Meningococci in suspension were added to a homogenization chamber containing a broken-in-half slip with infected exudate leukocytes.

III. The same volume of bacterial suspension as was used in Procedure 2 was added to a homogenization chamber containing a broken-in-half glass slip without attached leukocytes and homogenization immediately effected.

IV. Homogenates of infected exudate leukocytes without added bacteria.

5. Addition of M-1027 and M-1628 to homogenates of uninfected exudate leukocytes and infected exudate leukocytes

These experiments were carried out exactly as those described in 4 immediately above. The following Preparations(II and III were made as in 4 above) were used in these experiments:

I. Homogenates of uninfected exudate leukocytes with added bacteria.

II. Homogenates of infected exudate leukocytes with added bacteria.

III. The same volume of bacterial suspension as was used in Preparation 2 was added to a homogenization chamber containing a broken-in-half glass slip without attached leukocytes and homogenization immediately effected.

6. Infection of exudate cells with meningococci recovered from incubated infected exudate leukocytes

These experiments consisted essentially in the sequential linking of two separate experiments on exudate leukocyte-meningococcus interaction. By judicious timing of various operations, meningococci recovered from incubated infected exudate cells were used to infect a second set of exudate cells according to the following two-part plan:

a. Infection of exudate leukocytes and recovery of meningococci leukocytes

Exudate leukocytes were prepared in five 8 to 10 week old male mice. A washed suspension of exudate leukocytes containing 2.0×10^6 per ml was delivered into each of 4 Leighton tubes containing glass slips. The tubes were gassed for 10 sec, stoppered, and incubated at 37 C for 1.5 hrs to permit the cells to settle.

After incubation, each tube was individually treated by removing the glass slip and transferring it to a clean Leighton tube. Only a single meningococcal strain was used in an experiment. Into each Leighton tube was delivered 2.0 ml of bacterial suspension in Medium 199-1X-HB. The multiplicity of infection of approximately 7 was verified by plate counts of the number of bacteria after infection of exudate cells had been completed. The tubes were gassed for 10 sec, stoppered, and incubated at 37 C for 1.0 hr. After incubation, each tube was treated as follows: The slip was removed and rinsed in 100 ml of 0.85% NaCl buffered with 0.002 M K_2HPO_4:KH_2PO_4 at pH 7.0. Each slip was washed in a separate 100 ml aliquot of buffered saline by gently "swishing" to and fro 25 times. After each slip was washed, it was transferred to a clean Leighton tube and when slips from all 5 tubes had been removed, washed and transferred to clean Leighton tubes, they were treated as follows: Two slips were removed from their Leighton tubes and placed in a single homogenization chamber. To the chamber was added 4.5 ml of thoroughly gassed Medium 199-1X-HB and the chamber closed. A second set of two slips was prepared in an identical manner. Both chambers were cooled for 1 min in crushed ice and then homogenized at a Powerstat setting of 140 for 2 minutes. The homogenates were pooled and centrifuged at 100 g for 1 min at room temperature to sediment the glass particles. The supernatant was carefully

aspirated and diluted to approximately 32 ml. One ml was removed, diluted, and a plate count conducted. The supernatant obtained was immediately used as the infecting bacterial suspension.

b. Exudate leukocyte-meningococcus interaction in exudate leukocytes infected with meningococci recovered from incubated infected exudate leukocytes

Ten tubes containing exudate leukocytes on slips were prepared as follows: Two ml of washed exudate cell suspension, prepared in five 8 to 10 week old male mice, in Medium 199-1X-HB, were delivered into each of 11 Leighton tubes containing glass slips. The tubes were gassed for 10 sec, stoppered, and incubated at 37 C for 1.5 hrs to permit the cells to settle. After incubation, the slips were individually removed, rapidly placed into clean Leighton tubes, and infected with the supernatant described above in Part a.

The multiplicity of infection in this part of the experiment was not immediately known, because the number of meningococci recovered from incubated washed infected exudate cells was determined only after plate counts had been incubated and colonies counted. Two ml of supernatant prepared in Part a were delivered in random order into each of the 11 tubes containing exudate cells. A randomly selected 6 tube unit was prepared first, and then the second 5 tube unit next. The tubes were gassed for 10 sec, stoppered, and incubated at 37 C for 1.0 hr. An experimental control set of tubes was also prepared as described in Section E Part 1 c. After incubation, the procedures described in Section E Part 2 were followed precisely.

RESULTS

A. Bacterial studies

1. Confirmation of strain serological type

The meningococcal strains used in this study were confirmed as serological Groups A (M-1027) and C (M-1628) using the previously described agglutination protocol. The agglutination reactions were clear and uncomplicated by cross-reactions or non-specific agglutinations (Table 5). Positive results were easily observed through dilutions of each anti-serum to 1:16, the highest dilution used.

2. Bacterial viability studies

In various experiments, meningococcal strains were subjected to conditions and treatments which could have affected viability. Bacteria were suspended in various media, subjected to incubation at 37 C with 5% CO_2 :95% air for different periods of time, and homogenized with or without glass slips. It was therefore necessary to determine the effect of these conditions and treatments on viability of meningococci quantitated by plate count.

a. DSB diluting medium

In order to determine the numbers of viable meningococci in a diluted experimental sample, it was first essential to know that the diluting fluid was not inimical to the meningococci. Incubation periods of 3 hrs were used in these experiments, in the event that it was necessary to retain bacterial dilutions for more protracted periods

at room temperature than the usual few minutes.

The collated data displayed in Figure 2 indicate that DSB was a suitable choice as diluting medium. The plate counts for both strains showed that these strains held in DSB at room temperature for as long as 3 hrs in dilutions varying from 10^{-1} to 10^{-5} did not result in loss of colony forming units. The 3 hr counts rather indicated apparent growth of both strains. Such growth was actually inconsequential in this study because dilutions and plate-counting procedures were always completed in a matter of minutes.

b. Medium 199 preparations

Medium 199 is a defined tissue culture medium widely used in the cultivation of animal cell lines. Table 4 lists the components of this complex medium. The choice of this medium was based in part on its complexity. In preliminary experiments, methyl cellulose at several different concentrations, was used in Medium 199 to increase viscosity and to presumably better protect animal or bacterial cells suspended in it. Results of these experiments indicated that the difficulties of manipulating the viscous medium, such as entrapping of air bubbles when mixed, or slow aspiration and draining when pipetted, outweighed the advantages in using it and incorporation of methyl cellulose in Medium 199 was discontinued.

As described later (Section B Part 2), this medium maintained exudate leukocytes in good morphological condition for several hours incubation. It was therefore selected as the menstruum for infecting bacterial suspensions, and for preparing dilutions for incubation of infected leukocytes. Another advantage of using Medium 199 was

that no animal serums were required to supplement the medium. Use of animal serums was not desirable primarily because of the problems associated with their use, such as antibody activity and non-specific effects on phagocytosis of bacteria.

1. Medium 199-1X-HB for preparation of infecting bacterial suspensions

Bacterial suspensions for infecting exudate leukocytes were prepared in Medium 199-1X-H_2O which was identical with Medium 199-1X-HB. The infection was carried out for 1.0 hr at 37 C. It was therefore essential that meningococci in suspension retain viability for at least this period of time.

The data presented in Table 6 and Figure 3 indicate that in experiments 1 and 2, Medium 199-1X-H_2O did not decrease viability of the meningococci incubated at 37 C over a 6 hr period. Growth occurred for both strains, although this medium may be more suitable for growth of M-1628 than for M-1027. The increment over a 6 hr period indicates that growth over 1 hr, the period of infection, was probably negligible.

2. Medium 199 dilutions for incubation of infected leukocytes

The medium used for incubation of infected exudate leukocytes had to satisfy two criteria: 1) The medium had to maintain exudate leukocytes in acceptable morphology over a period of 5 or 6 hrs. This aspect will be discussed in Section B. 2) The medium had to

permit no growth or only minimal growth of both meningococcal strains.

The second criterion had to be satisfied because no antibiotics were used to control growth of extracellular bacteria. It was not possible to wash all of the bacteria in the infecting suspensions from the surfaces of the leukocytes and glass slips. It therefore was considered most sensible to choose that dilution of Medium 199 in phosphate-buffered saline which would best satisfy criterion 2. Phosphate-buffered saline was used as diluent rather than distilled water, in order to circumvent the difficulty of maintaining pH in aqueous dilutions of Medium 199 using various CO_2:air concentrations. Table 6 and Figure 3 present data of experiments testing for growth of both strains in dilutions of Medium 199 in phosphate-buffered saline.

Experiments 1 and 2 were conducted following identical protocols. In these experiments, bacteria were carefully removed from "spread plates" using a sharpened wooden applicator stick and suspended in phosphate-buffered saline. Appropriate volumes of suspension were then added to dilutions of Medium 199. In experiment 3, bacteria were collected as in experiments 1 and 2, but were suspended at low numbers per ml in the dilutions of Medium 199. In experiment 4, bacteria were washed two times in the phosphate-buffered saline before suspension in phosphate-buffered saline and addition to the dilutions of Medium 199. The useful data in Table 6 and Figure 3 are those from experiments 3 and 4, which were conducted using relatively low numbers of meningococci. The low numbers correspond well to the low numbers usually present in homogenates of infected leukocytes. The data from experiments 3 and 4 are similar; however, the conditions of experiment 4 probably best approximate the treatments given infected

exudate leukocytes on slips, and are perhaps most applicable to choice of an incubation medium. However, on the basis of the data from experiment 4, one is hard put to select either Medium 199-1X-PO_4 or Medium 199-1:8-PO_4. An overall view of the data of all 4 experiments indicated that when viable counts increased, M-1628 always showed a greater increase than did M-1027 in the same dilution of Medium 199. In certain experiments and dilutions, there was also considerable disparity between response in growth of the two strains. For example, in some experiments in certain dilutions, M-1027 appeared to decrease in numbers and M-1628 appeared to increase in numbers. Such an incubation medium would be unsuitable for one could not compare the effect of leukocytes on viable counts using samples of bacteria suspended in this medium. Final selection of the dilution medium was made with a cognizance of the apparent differences between the two strains. The selection of Medium 199-1:8-PO_4 as incubation medium for washed infected exudate leukocytes was based on the overall view of all 4 experiments, which indicated less difference between growth responses of the two strains and minimal growth of these strains over a 5 or 6 hr incubation period in this medium.

c. Effect of homogenization on viability of M-1027 and M-1628

In certain experiments, it was necessary to homogenize infected exudate leukocytes in an attempt to release phagocytized meningococci. Control slips in these experiments, consisting of glass slips with attached bacteria, had also to be homogenized. It was necessary to determine whether homogenization with glass slips affected viability of the two strains as reflected in plate counts.

Preliminary experiments at a Powerstat setting of 90 and homogenization for 15 sec, indicated that M-1027 suspended in Medium 199-1X-HB was not killed by this treatment. Plate counts were 3.0×10^7 before homogenization and 3.2×10^7 after homogenization. The data in Figure 4 indicate that the viability of both strains was essentially the same, whether or not glass slips were used in the homogenization at a Powerstat setting of 140 for 2.0 min. The number of bacteria per ml of strain M-1027 and M-1628 in this experiment was within the range of the number of bacteria recovered from infected exudate leukocytes.

Certain reports, such as that of Hatten and Sulkin (1966), have indicated that bacteria may exist as L-form cells after phagocytosis. In the introduction to this thesis the possibility of spheroplast formation of phagocytized meningococci was also considered. L-forms apparently withstand fairly severe treatment such as trypsinization, freeze-thawing techniques, and brief ultrasonic treatment. On the other hand, spheroplasts are considerably more fragile and have been protected from osmotic disruption by use of 0.25% sucrose (Razin and Argaman, 1963). Although intact meningococci, not either L-forms or spheroplasts, were used in this study, it was considered that 0.25% sucrose might confer some degree of osmotic protection to the bacteria forms released from leukocytes on homogenization, and it was necessary to determine whether this concentration of sucrose would affect viability of intact meningococcal cells. Accordingly, meningococci were homogenized with 0.25 % sucrose with and without glass slips. The data in Figure 4 indicate that strains M-1027 and M-1628 show a differing response to homogenization with sucrose. Homogenization of M-1027 with sucrose did not appear to decrease viability and actually slightly

increased the colony forming units. Homogenization of M-1628 with sucrose appeared to produce a substantial loss of viability. The homogenization of M-1628 with glass slips and sucrose may possibly have reduced viability to a greater degree than homogenization of M-1628 with sucrose alone. It was therefore decided to omit sucrose in later experiments on homogenization of infected leukocytes.

B. Exudate cell studies

1. Exudate cell populations

In order to study interaction of meningococci and PMN, it was essential to elicit an exudate population predominating in this cell type. As Table 7 indicates, the use of 12% sodium caseinate as the inflammatory agent produced a population which at 6 hrs was approximately 85-90% PMN. Preliminary experiments were carried out to determine if an exudate higher than 90% PMN could be collected by thoroughly massaging the abdomens of mice before collection. The results of these experiments indicated that massaging neither increased the PMN to MN ratio in exudates, nor increased the number of exudate leukocytes collected per ml. Massaging did not appear detrimental in any way, however, and it was routinely used prior to collection to presumably insure a better intraperitoneal wash. The sodium caseinate preparation used was slightly modified from that of Stähelin et al., (1956). The modification consisted merely of not neutralizing the preparation to pH 7. The pH of such unneutralized 12% sodium caseinate was about pH 6. Stähelin et al. used guinea pigs in their study, and were able to achieve populations of peritoneal exudate leukocytes approximately 80-90% PMN. However, though the per cent PMN population achieved in

this study correlates well with that achieved by Stähelin et al., the highest PMN population in mice in this study occurred at approximately 6 hrs as opposed to 18 hrs in guinea pigs. These differences may merely reflect species differences.

The data displayed in Table 7 were gathered using relatively young mice, 8 to 10 weeks old. A single preliminary experiment indicated that exudates prepared in older mice (circa 16 weeks old) elicited by the same procedure yielded a PMN population approximately 70 per cent. In order to circumvent the possible effect of age of the mice on exudate elicited, the subsequent experiments on interaction of exudate leukocytes and meningococci were done using mice approximately 10 weeks old.

The data from these experiments further indicate that the ratio of PMN to MN decreased with time following injection of the inflammatory agent. It was possible to prepare an exudate at least 90% MN by delaying the harvesting of exudate for several days after injection of sodium caseinate.

It is recognized that the cell populations, though classified as PMN or MN, are in reality a complex population of intergraded cell types both in PMN and MN morphology. It should also be pointed out that the ratios of PMN to MN were determined by counting populations of attached leukocytes. It was not determined whether a high PMN to MN ratio existed in the original leukocyte suspension prior to washing, centrifugation procedures, and settling. Preliminary experiments on settling times indicated that at 30 min, there were many more MN lymphocyte-like cells attached to the slips than at 90 min. It appeared that the MN lymphocyte-like cells did not firmly attach for the most part whereas the PMN cells did attach firmly. The PMN to MN ratios at 90 min thus

were greater than at 30 min. Only the population of cells attached to the slips was considered pertinent to the purpose of this study and therefore a 90 min settling time for exudate leukocytes was used in all interaction experiments.

2. Incubation medium for infected exudate leukocytes

The incubation medium selected for infected exudate leukocytes had to satisfy the criteria set forth previously. These criteria were as follows: 1) The medium had to maintain exudate leukocytes in acceptable morphology over a period of 5 or 6 hrs. 2) The medium had to permit no growth or only minimal growth of both meningococcal strains. This criterion has already been discussed.

As regards criterion 1, if the judgment of "suitable morphology" is to be made, certain guidelines must be established. These guidelines were formulated by examining stained exudate leukocytes settled from suspension in Medium 199-1X-HB (Jackson, 1954). In reference to this "standard" preparation, the following morphological features were noted:

1. Uniformity and depth of staining of nuclei and cytoplasm.
2. Cell integrity, diameter, and whether cells were in a contracted state.
3. Estimated per cent of population with pseudopodia.

Stained cells were examined, compared to the "standard" preparation, and morphologically classified as excellent, good, or poor.

Various dilutions of Medium 199 in phosphate-buffered saline and in water were used as incubation medium. These dilutions were as

indicated in Figure 3. Cells were also incubated in phosphate-buffered saline without addition of Medium 199. Exduate leukocytes on glass slips were incubated for 6 hrs in the particular medium and examination made of the stained cells.

The results of these observations indicated that cell morphology following incubation in phosphate-buffered saline was very poor. All of the other incubation media were classified as good, and none were classified as excellent. Cells incubated in Medium 199-1X-HB for 6 hrs contained many more cells in "motile posture", that is more cells had pseudopodia than cells incubated in various dilutions of Medium 199 in phosphate-buffered saline. In fact, cells incubated in dilutions of Medium 199 in buffer were virtually devoid of pseudopodia. In all other respects, the cells incubated in Medium 199-1X-HB and dilutions of Medium 199 in buffer were comparable. Therefore, Medium 199-1X-HB was used for the infecting bacterial suspension and provided conditions for apparently good phagocytosis of the meningococci. Medium 199-1:8-PO_4 was used to maintain good morphology of the infected leukocytes and to minimize growth of extracellular meningococci. Medium 199-1:8-PO_4 also provided apparently poor phagocytic conditions which may have reduced continued phagocytosis of extracellular bacteria during the 5 hr incubation period.

C. Acid phosphatase studies

The problem of enumerating the total population of attached exudate leukocytes was encountered early in this study. Preliminary attempts were made to quantitate exudate leukocytes on slips by counting random microscopic fields and, by knowing the area of the fields counted,

extrapolating to the entire population on the slip. However, cell aggregation was particularly troublesome and impossible to overcome completely. Various methods were used in experiments attempting to minimize aggregation. Heparin at final concentrations up to 10 U.S.P units per ml were used in the exudate harvesting medium to prevent fibrin formation and aggregation of exudate leukocytes. Heparin has been commonly used for this purpose (Sbarra and Karnovsky, 1959).

Experiments were conducted testing the effects of several different exudate leukocyte harvesting media on aggregation of PMN. Some of the media tested were as follows: 1) Ungassed Medium 199-1X-HB at pH about 7.6. 2) 0.1 M Tris buffered 0.85% NaCl at pH 7.4. 3) Phosphate-buffered saline pH 6.5 and 8.0 0.1 M. 4) Normal horse serum diluted with Tris buffer above, 1:2 or undiluted.

None of the media or combinations of the media with various concentrations of heparin resulted in preparations free of PMN aggregates. Centrifugation was finally selected as the method for removing cell aggregates. This was accomplished during the exudate leukocyte washing procedures, for the differential nature of the centrifugations removed many cell aggregates. A preliminary attempt to approach the problem of counting in a statistical mode was unsuccessful, for distribution of cells on slips did not appear to occur in a random manner. Undoubtedly, clumping primarily accounted for nonrandom attachment.

A method permitting enumeration of total exudate leukoctyes attached to a slip was highly desirable. Total enumeration would minimize the undesirable influence of cell aggregates on the final count, since the distribution of total exudate leukocytes per slip was more uniform than distribution of aggregates in random fields of a

single slip. However, direct counts of all the exudate leukocytes on a slip was impractical, since each slip might have several hundred thousand attached cells. It was therefore desirable that the method of total cell enumeration be less laborious and time-consuming than direct counts.

It was considered that possibly the attached exudate leukocytes could be enumerated by measurement of one of the leukocyte enzymatic activities. If a linear relationship obtained between attached exudate leukocytes and enzyme activity, one might then more easily quantitate the total number of attached cells.

The enzyme selected for study was acid phosphatase because it has been well-studied and assay systems are readily available, simple, and precise. It was also recognized that the lysosomal granules of PMN possess considerable acid phosphatase activity (DeDuve, 1963a). Acid phosphatase could be used not only to quantitate the number of exudate leukocytes, but could also serve as a tool in the status of a barometer to reflect changes in infected cells as compared to uninfected cells. For example, a marked increase in acid phosphatase activity in the medium could indicate lysis of leukocytes and release of lysosomal enzymes.

The results of the attempts at quantitation of exudate leukocytes by acid phosphatase assays are described below.

1. Effect of pH on acid phosphatase activity

The data in Figure 5 indicate that the greatest pH for acid phosphatase activity both for Boehringer standard enzyme and exudate leukocyte homogenate was approximately 5.8. It may be recalled that

Boehringer standard enzyme was stated to have a specific activity of 2.5 enzyme units per mg of protein at pH 4.8. This value was experimentally determined to be correct at pH 4.8. It should be noted, however, that the activity of Boehringer standard is greater at pH 5.8 than at pH 4.8. The assay for specific activity of the commercial product might have been done at pH 4.8 rather than at pH 5.8 because most acid phosphatase assay protocols given in the literature call for a pH 4.8 assay.

Assay for acid phosphatase in exudate leukocyte homogenates in the presence of meningococci was considered valid because no acid phosphatase was detected using intact meningococci. Assays were conducted at pH 5.8 using Protocol A to test for acid phosphatase in meningococci. Six million meningococci of strain M-1027 did not possess acid phosphatase detectable by the method used, although these bacteria might have had phosphatase activity expressed at a different pH or under different assay conditions.

The presence of mouse erythrocytes (Rbc) in the exudate leukocyte suspensions could have complicated assay. In order to determine the effect of Rbc in exudate leukocyte suspensions, experiments were carried out assaying Rbc for acid phosphatase. The Rbc were collected and treated as follows: The cardiac blood from two etherized mice was separately collected and heparinized at a final concentration of 5 units per ml in Medium 199-1X-HB. The Rbc were washed 2X in non-heparinized Medium 199-1X-HB with centrifugations done at 500 g for 5 min. The washed Rbc were suspended in Medium 199-1X-HB at a concentration of 1.3×10^9 per ml for one mouse and 9.9×10^8 per ml for the other mouse, and homogenized in 2.0 ml volumes at a Powerstat setting

of 90 for 15 sec. The uncentrifuged homogenates were diluted 1:10, 1:100, and 1:200 in Medium 199-1X-HB, and assays for acid phosphatase performed using Protocol A. The assay values at a 1:200 dilution of Rbc at $OD_{400\ m\mu}$ were for mouse one 0.624 and for mouse two 0.479. Preliminary experiments had indicated that a macroscopically-non-bloody exudate prior to washing was found to contain approximately 1.0×10^6 Rbc per ml. After washing, the number of Rbc was decreased to approximately 2×10^5 per ml. By calculation, a concentration of mouse Rbc in Medium 199-1X-HB at 2×10^5 per ml would give an $OD_{400\ m\mu}$ of approximately 0.020. Following the procedures of attachment to glass slips, infection, washing, addition of fresh medium, and incubation for some 5 hrs, the number of Rbc remaining attached to the slips would be few indeed. That such was indeed correct was confirmed by microscopic examination of incubated slips for attached Rbc. It was considered that the presence of the very few Rbc in the final preparation would not interfere with acid phosphatase assay.

2. Acid phosphatase activity of homogenized exudate leukocytes

In order to make available acid phosphatase activity within exudate leukocytes attached to glass slips, homogenization was carried out to rupture the cells and release lysosomal granules. The lysosomal membrane appears to be quite fragile (Tappel, Sawant, and Shibko, 1963) and exposure to acid pH appears to rupture the lysosomal membrane and release enzyme contents (Cohn et al., 1963). It was considered that placing homogenates with released lysosomal granules in the acid pH substrate preparation might serve to rupture the lysosomal membranes and release acid phosphatase activity.

It was necessary to determine what times of homogenization were necessary to rupture exudate leukocytes attached to glass slips. Preliminary experiments had determined that a Powerstat setting of 90 and homogenization for 15 sec shattered slips into uniformly small pieces. Hence, a Powerstat setting of 90 and homogenization for at least 15 sec were used to accomplish homogenization of the slips in the acid phosphatase experiments.

Figure 6 displays data on acid phosphatase assays of preparations homogenized for 15, 30, 45 and 60 sec. The degree of breakage of exudate leukocytes by these homogenizations, though not quantitatively determined, was excellent with only one or so vagrant cells every few oil immersion fields. A 15 sec homogenization of exudate leukocytes with or without glass slips yielded exudate leukocyte breakage as thorough as at 60 sec homogenization. As shown in Figure 6, acid phosphatase activity appeared to vary inversely with the duration of homogenization, that is, the longer homogenization with or without slips, the lower the acid phosphatase assay values. Unfortunately, the assay values of preparations homogenized with glass slips were lower than for preparations homogenized without glass slips. These data were unexpected, since it was considered that homogenization of cells with glass slips would effect increased cell breakage and release of lysosomal granules. Furthermore, the supernatants of centrifuged homogenates prepared with or without glass slips were less active than the uncentrifuged preparations.

The results of these experiments presented in Figure 6 indicated that homogenization did not solubilize all enzyme activity, since even the relatively light centrifugation at 3500 g for 5 min sedimented a

considerable portion of the enzymatic activity.

3. Sonication of homogenized exudate leukocytes

Consideration of the fact that centrifugation of homogenates removed nearly 50% of acid phosphatase activity indicated that possibly: 1) The homogenization, while sufficient to rupture cells did not permit release of all lysosome granules or either 2) Lysosomal granules or adsorbed enzyme activity, or both, were associated with the grosser sedimentable particulate matter following homogenization, for the g forces and times of centrifugation used were lower than those reported in the literature to sediment lysosomal granules alone (Novikoff,1961). Any expected increase of enzyme activity with increased homogenization time may have been masked by denaturation of the acid phosphatase enzyme.

Sonication has been shown to be an effective means for treating tissues and lysosomal granules to release enzymatic activity (Novikoff, 1961). Therefore, sonication of exudate leukocyte homogenate was attempted in order to release additional acid phosphatase activity. The data described in Figure 7 indicated that sonication of homogenate resulted in a decrease of acid phosphatase activity. The longer the duration of sonication, the more pronounced the decrease in assay values. Centrifugation of sonicated homogenate still resulted in lowering assay values. Apparently these conditions of sonication did not effectively solubilize acid phosphatase activity and indeed decreased the total measurable activity.

4. Saponin treatment of exudate leukocyte homogenates

It was apparant at this point that neither homogenization, nor homogenization and subsequent sonication of exudate leukocytes released acid phosphatase activity not sedimentable by centrifugation. That this sedimentable activity was not merely intact cells was shown in microscopic examinations which revealed absolutely no intact exudate leukocytes.

The detergent, Triton X-100, has been used to release enzymatic activity of lysosomes, and deoxycholate has also been used for this purpose (Novikoff, 1963). These reagents disrupt membrane integrity and it was considered that the detergent sodium lauryl sulfate, a surface active agent, might also serve to solubilize acid phosphatase activity from exudate leukocyte homogenates. Experiments were first conducted to determine the effect of this agent on Boehringer standard acid phosphatase. It was discovered that sodium lauryl sulfate drastically reduced acid phosphatase activity levels using Boehringer standard enzyme. In one experiment in which control standard enzyme gave an $OD_{400m\mu}$ = 0.8 at pH 5.8 for 30 min incubation using Protocol A, assay of the same amount of enzyme with sodium lauryl sulfate present at the final concentrations of 7, 14, and 20 mg per ml, gave an $OD_{400m\mu}$ = 0.15 under the same conditions as above. In another experiment, sodium lauryl sulfate at the final concentrations of 7, 14, and 20 mg per ml, reduced activity levels from 0.3 to zero at $OD_{400\ m\mu}$.

In view of the above results, saponin, another membrane disrupting agent, was selected and tested by experiment. In Figure 8, the data from these experiments indicate that saponin at a final concentration of 80 mg per ml appeared to exert some increase in supernatant

acid phosphatase activity after 30 min contact of saponin and homogenate. Increased contact time of 60 min did not result in additional acid phosphatase activity. It was noted that Boehringer standard enzyme was not noticeably affected by this concentration of saponin.

5. Relationship of acid phosphatase activity to exudate leukocyte number

Because of the unsuccessful attempts at solubilizing acid phosphatase from homogenized exudate leukocytes, the assay procedure was modified to use intact cells in suspension. The exudate leukocytes were permitted to settle onto the surface of the window in Leighton tubes before assay for acid phosphatase was conducted. Several experimental attempts were made in an effort to achieve a linear relationship between acid phosphatase values and exudate leukocyte number. In these early experiments, using 10 or 5 mg per ml saponin in the substrate preparation, there was an apparent non-linear trend in a line fitted to points plotted from assay data. This non-linear trend appeared to be in the assay values determined on exudate leukocyte numbers approximately 1 to 3 X 10^5 per ml. When the saponin concentration in the substrate preparation was reduced to 0.025 or 0.0125 mg per ml, the data yielded a straight line (Figure 9). The data in Figure 9 also indicate the large increase in activity achieved by use of 0.0125 mg per ml saponin in the substrate preparation in this experimental design, as opposed to the activity achieved using substrate preparation without saponin.

6. Effect of incubation of exudate leukocyte homogenate in Medium 199-1X-HB on acid phosphatase activity

As previously mentioned, assay of activity in the medium bathing infected exudate cells could signal changes taking place in the infected cell population. For such an assay to be of value, the enzyme must be stable in the incubation medium over the duration of the experiment, so that enzyme released early in the incubation period is assayable at the end. Data from Table 8 indicate that acid phosphatase activity of exudate leukocyte homogenates in Medium 199-1X-HB rapidly declined over an incubation period of 5 hrs at 37 C. Assay of the total contents indicated that activity was not merely settling of suspended material or adsorption of particulate matter of glass surfaces. Boehringer standard enzyme in Medium 199-1X-HB subjected to a similar experimental protocol also underwent a rapid decline in activity. In an experiment, the zero time value for a standard acid phosphatase preparation was at OD 400 mμ approximately 1.7 and at 5 hrs, the value had declined to approximately 0.4.

The results of the experimental studies on acid phosphatase indicated that, despite the many attempts to improve and quantitate the procedure, assay of this enzyme could not profitably be used as an index of numbers of leukocytes settled on glass slips, or of amount of destruction of infected exudate leukocytes during incubation.

D. Exudate leukocyte - meningococcus interaction

The preceding experiments were designed so as to provide information sufficient for a rational approach to the main thesis of this study: the relationship of leukocyte-meningococcus interaction to pathogenesis of N. meningitidis.

It is recognized that the data on interactions in this study deal primarily with the gross events transpiring after meningococcal infection of exudate leukocytes. The data derived from this study do not permit direct assessment of events occurring within individual cells, or events occurring within populations of cells within leukocyte types. The data to be subsequently presented do, however, permit a panorama of events following infection, in a manner analogous to the observance of a battlefield from a vista: the direction of the battle towards victory or defeat may be indicated by a sweeping view of the conflict without knowing success of individuals or groups of combatants.

The interaction experiments were all carried out using a similar protocol. This protocol was essentially as follows: Washed exudate leukocytes were settled and attached to glass slips. The leukocytes were then infected at various multiplicities of infection (m) either with strain M-1027 or strain M-1628. The infected leukocytes were washed to remove most extracellular bacteria, and were incubated with fresh medium. At approximate time intervals of 0, 1, 2, 3.5, and 5 hrs, viable bacterial counts were made of samples from the following preparations:

1. An incubated homogenate of infected leukocytes.
2. The homogenate of an incubated infected leukocyte slip.
3. The homogenate of a control slip with no attached

exudate leukocytes.

All of the zero time samplings were carried out on preparations before incubation at 37 C. All subsequent samplings were carried out after incubation.

Preliminary experiments had been conducted to determine whether exudate leukocytes would indeed phagocytize meningococci in serum-free medium. In these experiments, exudate leukocytes were attached to slips and were infected with meningococcal strain M-1027 at various m. The leukocytes were then fixed and stained. Determination of the number of intracellular bacteria was accomplished by counting 200 cells in random oil-immersion fields on a single slip. The bacterial counts were divided into three classifications as follows:

1. Intracellular bacteria - The meningococci appeared to be located within the confines of the leukocyte membrane.
2. Extracellular bacteria - The meningococci appeared to be located outside the confines of the leukocyte membrane.
3. Doubtful - The meningococci appeared to be located on the periphery of the leukocyte membrane and it could not be ascertained whether the bacteria were within or outside of the membrane.

Single meningococci and diplococci were both counted as single cells. The results of these experiments indicated that at m's of 3 or 5, approximately 20 per cent of the leukocytes contained intracellular meningococci. At m = 19 and m = 32, approximately 26 and 77

per cent of the leukocytes respectively contained intracellular bacteria. And at m's= 52 and 100, approximately 78 and 87 per cent of the leukocytes respectively contained intracellular meningococci.

It has been previously shown that the exudate leukocyte population on the slips in this study was approximately 85-90% PMN and 10% MN. Although the infection of PMN was of principal concern, it was also necessary to ascertain approximately to what extent the 10% MN population phagocytized meningococci. Table 9 presents data of experiments carried out as the experiments described in the preceding paragraph. However, the data in Table 9 differ in that only 100 total MN as opposed to 200 total PMN cells were counted. The data indicate that at an m = 50, there are intracellular meningococci in about 67% of the PMN and intracellular meningococci in about 49% of the MN population. Obviously, the MN population, though only 10% of the total, did take part in the infection and must be considered to play a role; not necessarily a minor role because of the low number of MN involved.

It was of interest to determine if "normal" mouse serum (NMS) in Medium 199-1X-HB would provide enhanced phagocytosis as compared to Medium 199-1X-HB without serum. Table 10 presents data from experiments with M-1027 carried out as described in the above two paragraphs. The data were collected from experiments using Medium 199-1X-HB without or with 10% "normal" mouse serum. The m's used were 3 and 100. The serum used was not assayed for anti-A or anti-C meningococcal antibody. Determinations were made of the number of intracellular meningococci per exudate leukocyte and categories of zero, doubtful, 1 to 5, 6 to 10, and greater than 10 meningococci per exudate leukocyte were defined. As the data indicate in Table 10, at an m = 100, the number of intracellular

meningococci with 10% NMS in the medium was 89%, and 86% without NMS. At an $\underline{m}$ = 3, the number of intracellular meningococci was 29% with NMS in the medium, and 22% without NMS in the medium. It thus appeared that 10% NMS did not increase phagocytosis under these conditions. Of course, had phagocytosis been increased in the presence of NMS, the serum would have been assayed for antibody to Group A and Group C meningococci. Infection at $\underline{m}$ = 100, resulted in a much higher per cent of leukocytes with intracellular bacteria than infection at $\underline{m}$ = 3, either without or with NMS in the medium. A further study of the data in the categories of 1 to 5, 6 to 10, and greater than 10 meningococci per exudate leukocyte, indicated that the use of NMS did not shift the values for number of meningococci per exudate leukocyte from those values obtained using medium without serum. All interaction experiments hereafter were conducted using Medium 199-1X-HB without serum for bacterial infecting suspensions.

In order to determine viable counts of bacteria which had been phagocytized by exudate leukocytes, it was necessary to rupture the leukocytes in some manner which would leave the meningococci in an extracellular state and unharmed, at least as far as viability was concerned. As has been previously mentioned, saponin (Novikoff, 1961) has served as a membrane disrupting agent. It was at first considered that saponin would perhaps permit leukocyte rupture and release of meningococci. Preliminary experiments were done testing the effect of 0.5 mg per ml saponin in Medium 199-1X-LB on the viability of M-1027 and M-1628. It was found that the viability of the meningococci declined over a three hr period. The plate counts for M-1027 decreased from approximately 7.0×10^3 per ml to 1.3×10^3 per ml and the plate

counts for M-1628 decreased from 2.0 X 10^3 per ml to 1.0 X 10^3 per ml. These data indicated, that even at very low concentrations of saponin, viability of meningococci, at relatively low numbers per ml, was decreased. In view of these results and in an effort to avoid any membrane-disrupting agents which could have been inimical to the viability of the meningococci, high-speed homogenization of glass slips was tested and finally selected to shatter glass slips and to rupture the attached exudate leukocytes. The very violence of homogenization served to quickly free and disperse intracellular bacteria. Experiments already reported have shown that leukocyte breakage was excellent using short period of homogenization, and that viability of the meningococci was apparently not affected by the procedure. Therefore, homogenization at a Powerstat setting of 140 for a time period of 2.0 min was used in all of the interaction experiments to prepare homogenates of infected leukocytes and control slips.

The exudate leukocyte-meningococcus interaction experiments were designed to furnish the following information:

1. Whether or not, and to what degree, the homogenates of infected leukocytes "killed" the infecting strain of meningococcus.
2. Whether the numbers of phagocytized meningococci increased or decreased with time in exudate leukocytes.
3. To what degree meningococci in the extracellular environment contributed to the determination of 2.

These experiments were monitored after infecting a constant number

of exudate leukocytes at various m. Several experiments were also conducted using higher numbers of leukocytes and infecting them at low m.

A question attendant on these experiments was whether or not the infection of exudate leukocytes caused them to detach from the slips. It was essential to know if such a phenomenon occurred, for a decrease in numbers of infected cells on slips would be reflected in the viable counts. The data pertaining to this problem and results of experiments are considered below.

1. Estimation of population shifts and number of exudate leukocytes attached to glass slips after infection

It may be recalled that during the course of an interaction experiment, slips with attached infected exudate leukocytes were removed at intervals of 0, 1, 3.5, and 5 hrs. These slips were fixed, stained, and leukocyte counts were conducted as described in Section E Part 6 of Materials and Methods. The method consisted essentially of total counts of all leukocytes in 60 uniformly distributed oil immersion fields.

a. Number of exudate leukocytes attached to glass slips after infection

The method used in counting was considered to sample sufficient random areas of a slip to permit valid interpretations of the data. The data in Table 11 were derived from experiments in which each m was done in a separate experiment. The data do permit some degree of comparison between individual experiments, but because

the method of counting was not strictly quantitative, the data within a single experiment are more comparable than data between separate experiments. This is primarily because counts were done for only one experiment on a particular working day, and it was not practical to maintain the microscope used for counting exclusively for this purpose. It was necessary, prior to each counting period, to outline the counting area on the eyepiece. This step alone accounted for enough variation in technique to recommend only intra-experimental comparisons of data.

The data in Table 11 indicate that infection at low or high m did not apparently affect the number of infected leukocytes attached to the slips over a 5 hr period of incubation.

b. Differential counts of infected exudate leukocytes

Not only was it necessary to ascertain whether infected leukocytes detached from slips, but it was also necessary to ascertain if leukocyte population ratios, as determined by differential counts, shifted with time after exudate leukocyte infection and incubation.

Table 11 displays data indicating negligible shifts in differential PMN and MN counts. It appeared, therefore, that the infection of exudate leukocytes did not cause a shift in population ratio, that is, preferential detachment of one type of leukocyte.

With these problems resolved, it was possible to proceed with the interaction experiments which are described below.

2. Exudate leukocyte-meningococcus interactions

The condensed protocol for interaction experiments was as follows: Washed exudate leukocytes were settled onto glass slips for 1.5 hrs. The leukocytes were then infected for 1.0 hr at various m's. A one hr infection time was used because preliminary experiments indicated this time adequate for good phagocytosis of the meningococci (Table 10) as reflected in the number of intracellular bacteria. A longer time period was not used because PMN are end-cells, that is, they do not reproduce and possess a relatively short *in vivo* life span of approximately 24 hrs, and probably a considerably shorter *in vitro* survival (Sieracki, 1955). It was desirable to reduce treatments, prior to the actual measurement of the infected state of the leukocytes, to as brief a time period as was possible. After the leukocytes were infected for 1.0 hr, they were washed, and placed with fresh medium. Control slips were included in these experiments. The control slips were merely slips without attached exudate leukocytes, which had been treated precisely as the slips with attached exudate leukocytes. Three preparations were made at this time, and the designation of these preparations will be used in reporting the results of the interaction experiments. The preparations were as follows:

1. Homogenate - A homogenization of infected exudate cells was carried out. Plate counts were conducted at zero time, and the homogenate incubated. At intervals of 1, 2, 3.5, and 5 hrs, samples were removed and plate counts conducted.

2. Infected leukocytes - Slips with infected exudate leukocytes were incubated, and at 0, 1, 2, 3.5, and 5 hrs

following infection, a single slip was sacrificed, homogenized and a plate count conducted.

3. Control - Slips without exudate leukocytes were incubated, and at 0, 1, 2, 3.5, and 5 hrs, a single slip was sacrificed and homogenized.

Four variations of exudate leukocyte-meningococcus interaction experiments were carried out. These variations were as follows:

VARIATON 1. Exudate leukocytes settled from suspensions containing 1.0 X 10^6 cells per ml were infected at the following m's:

a. M-1027 - m's of 5, 14, or 25.

b. M-1628 - m's of 4, 7, 21, or 30.

VARIATION 2. Exudate leukocytes settled from suspensions containing 2.0 X 10^6 per ml were infected at the following m's:

a. M-1027 - m's of 5 or 7.

b. M-1628 - m's of 2 or 9.

VARIATION 3. Exudate leukocytes settled from suspensions containing 1.0 X 10^6 cells per ml were used in making the following preparations:

a. Homogenates of infected exudate leukocytes which were incubated, sampled, and viable counts conducted at 0, 1, 2, 3.5,

and 5 hrs.

b. Meningococci in suspension were added to infected exudate leukocytes and immediately homogenized. The homogenate was incubated and plate counts conducted as a.

c. Meningococci in suspension were added to uninfected leukocytes and immediately homogenized. The homogenate was incubated and plate counts conducted as a.

d. Homogenates of bacteria and glass slips without leukocytes were incubated and plate counts conducted as a.

Parts a, b, and d were coupled in separate experiments carried out with M-1027 at $\underline{m}$ = 5 and M-1628 at $\underline{m}$ = 12. Parts b, c, and d were coupled in separate experiments carried out with M-1027 at $\underline{m}$ = 24 and M-1628 at $\underline{m}$ = 13.

VARIATION 4. This variation of the interaction experiment was merely the sequential coupling of two separate interaction experiments. The technical considerations involved in carrying out these experiments were more complex than in the usual interaction experiment, for the time scheduling of operations had to be closely estimated and followed in order

to permit successful merging of the separate experiments.

The results of the interaction experiments of variations 1, 2, and 4 will be presented in the order of the designations homogenate, infected leukocytes, and controls. The results of variation 3 experiments will be presented as discussion of the preparations used in these experiments. Results will be presented in the order of increasing m.

a. Interaction of M-1027 and exudate leukocytes settled from a suspension of 1.0×10^6 cells per ml

m = 5

Figure 10 displays data indicating that death of the meningococci took place in the homogenates. At 3.5 hrs, the homogenate count was essentially zero, after a drop in bacterial numbers of approximately 2 logs. The infected leukocyte counts remained virtually constant for the first 2 hrs of incubation and then showed a steady decline of about 1 log over the next 3 hr. The control counts over the 5 hr period remained at approximately 1×10^3, except for the one abberant count of 1×10^1 at 1 hr. The zero-time counts for all three preparations were about 1×10^3 per ml.

m = 14

The data in Figure 10 indicate that maximum death in homogenate occurred at 2 hrs, after about a 1 log drop, and

and counts remained constant thereafter. The infected leukocyte counts declined about 1 log relatively slowly over the entire 5 hr period to about 1 X 10^3 per ml. The control counts, following an 0.1 log drop at 1 hr, appeared to increase thereafter about 0.2 log over the 5 hr period. It was noted that the zero time counts for all three preparations were very similar, and approximately 1 log greater at *m* = 14 than at *m* = 5.

m = 25

The data in Figure 11 indicate that death in homogenate was relatively rapid; the viable counts dropped 1 log over a 3.5 hr period and then rose about 0.2 log at 5 hrs. The infected leukocyte counts appeared nearly identical to those of the homogenate, at least over the first 3.5 hr, in declining to a low value 1 log less at 5 hrs. The control counts varied about the value of about 2.5 X 10^4 for the first 3.5 hr and then declined to a value of 1 X 10^4. The zero time counts for all three preparations were approximately 2 X 10^4 per ml.

A comparison of the results at the 3 different *m*'s indicates the following: Death in homogenate in *m* = 5 resulted in a 2 log drop whereas death in homogenate *m*'s = 14 and 25 resulted in only a 1 log drop over the 5 hr period. Death in the infected leukocytes at all three *m*'s resulted in about a 1 log drop. Control counts at *m* = 5 and *m* = 25 remained constant, or showed a slight decline over 5 hrs, whereas the control count at *m* = 14 appeared to increase over the 5 hr period. Infection of the exudate leukocytes at *m*'s= 14 and 25 resulted in zero time counts for all three preparations 1 log higher than at *m* = 5.

b. Interaction of M-1628 and exudate leukocytes settled from a suspension of 1.0×10^6 per ml

m = 4

Data in Figure 12 indicate that homogenate counts declined relatively slowly and steadily over the 5 hr period to essentially zero, about a 1 log decrease. Infected leukocyte counts appeared to decline over the 5 hr period only about 0.4 log. Control counts dropped at 1 and 2 hrs to essentially zero, then rose to 1×10^2 at 3.5 hrs, and then at 5 hrs dropped again to 6×10^1. The relative smoothness of the homogenate curve and the infected leukocyte curve are in striking contrast to the variability of the control counts. It was noted that all 3 preparations gave zero time counts about 2×10^2 per ml.

m = 7

The data on homogenate samples in Figure 12 indicate that counts declined relatively slowly and steadily about 1 log over the 5 hr period. Infected leukocyte counts appeared relatively constant for the first 3.5 hrs, and then rose to 4×10^3, a 1 log increase, over the last 1.5 hr. Control counts rose about 0.2 log at 2 and 3.5 hrs, and then dropped virtually to zero at 5 hrs, a decrease of approximately 2.5 logs. The zero time counts for homogenate and infected leukocytes were about 4×10^2 but the control count at zero time was nearly one log higher at 2.5×10^3 per ml.

$\underline{m} = 21$

Data on homogenate samples in Figure 13 indicate that death was relatively slow and steady, and resulted in about a 1 log decrease in counts over 5 hrs. The infected leukocyte counts appeared to slowly increase over the first 3.5 hrs by 0.1 log, and then increased nearly 1 log over the last 1.5 hrs. Control counts were relatively steady at about 8×10^3 for 3.5 hrs and then decreased about 1 log at 5 hrs. Zero time counts for homogenate and infected leukocytes were approximately 2×10^3 but the controls were 0.4 log higher.

$\underline{m} = 30$

Data in Figure 13 indicate that homogenate counts increased slightly at 1 hr, and then steadily and relatively slowly declined over the 4 hr period about 1 log. Infected leukocyte counts appeared to steadily increase 0.4 log, after no change for the first hr. Control counts were steady at about 1.5×10^4 for the first 2 hrs, decreased to 6×10^4 at 3.5 hrs, and then rose nearly 1 log in the last 1.5 hrs. Zero time counts for homogenate and infected leukocytes were about 1.5×10^3, but again the controls were 1 log higher.

A comparison of the results of the 4 different $\underline{m}$'s indicates the following: Death in homogenates at all 4 $\underline{m}$'s resulted in about a 1 log decrease in counts. In the infected leukocyte counts, death appeared at only $\underline{m} = 4$, and counts appeared to increase at $\underline{m}$'s = 7, 21, and 30. The results of control counts were not clear cut, but for $\underline{m}$'s = 4, 7, and 21, counts appeared to decrease, whereas for $\underline{m} = 30$, the counts appeared to increase. At an $\underline{m} = 4$, the zero time counts

were about the same for all three preparations. At m's = 7, 21, and 30, the zero time counts for homogenate and infected leukocytes were the same, but control counts were nearly 1 log higher. The zero time counts for homogenate and infected leukocytes were about 1 log higher at m's = 21 and 30 than at m's = 4 and 7.

A comparison of the results of the experiments using M-1027 and M-1628 indicates the following: Homogenate counts appeared to decrease about 1 log in all preparations at all m's over the 5 hr period, save for M-1027 m = 5 which decreased 2 logs. All infected leukocyte counts for M-1207 decreased about 1 log over 5 hrs, whereas only at m = 4 for M-1628, did counts appear to decrease. At the other 3 m's of M-1628, the counts increased over the 5 hr incubation period. Control counts for M-1207 at m = 5 and m = 25 appeared to remain relatively constant, with minor fluctuations but did increase for m = 14. Control counts for M-1628 at m = 4, m = 7, and m - 21, appeared to decrease, and at m = 30 to increase. Zero time counts for homogenate and infected leukocytes were approximately 1 log less at the same m for M-1628 and for M-1027.

c. Interaction of meningococci and exudate leukocytes settled from a suspension of 2.0×10^6 cells per ml

All of the previous interaction experiments were done using exudate leukocytes settled from suspensions containing 1.0×10^6 cells per ml. Interaction between phagocytes and bacteria is a complex process. The work of Hanks (1940) and others suggested that increase in leukocytes per unit area might influence outcome of the infection. With this rationale, exudate leukocytes settled from

suspensions containing 2.0 X 10^6 cells per ml were infected at low *m*. Low *m* were used because such high numbers of bacteria had to be used in the infecting bacterial suspensions that adhering meningococci might have badly obscured the results of the plate counts.

1. M-1027 interaction with exudate leukocytes

m = 5

Data for homogenized samples in Figure 14 indicate that counts decreased about 1 log in the first 2 hrs, and then slowly declined about 0.2 log to 2 X 10^2 per ml. The infected leukocyte count decreased steeply about 0.9 log in the first hr, then remained constant for 2.5 hrs, and then decreased 1.4 logs in the last 1.5 hrs. Control counts remained relatively constant about 1.5 X 10^3 per ml. Zero time counts for all three preparations varied between from 1 to 4 X 10^3 per ml.

m = 7

Figure 14 data indicate that homogenate counts decreased a total of about 0.8 log, of which about 0.7 log decrease occurred in the first 2 hrs. Infected leukocyte counts decreased 0.5 log over the first 2 hrs, apparently increased to about 0.5 log at 3.5 hrs, and then decreased 0.2 log at 5 hrs. Control counts increased from about 4 X 10^1 at zero time to 1 X 10^4 at 1 hr and then to 7 X 10^4 at 5 hrs. Zero time counts for homogenate and infected leukocytes were about 5 X 10^3, but the control count was unusually low, that is, 4 X 10^1 per ml.

2. M-1628 interaction with exudate leukocytes

m = 2

In Figure 15, homogenate counts steadily decreased about 2 logs over 5 hrs to essentially zero. Infected leukocyte counts decreased steadily and relatively uniformly about 1 log in 5 hrs. Control counts also decreased about 1 log over the 5 hr period. Zero time counts for homogenate and infected leukocytes were 1.5×10^3, but higher for the control at 8×10^3 per ml.

m = 9

In Figure 15, homogenate counts decreased relatively slowly about 0.6 log in the first 3.5 hrs, and then decreased to zero at 5 hrs, nearly a 2 log decrease. Infected leukocyte counts relatively steadily increased over the 5 hr period. Control counts were 6×10^3 at zero time, constant until 2 hrs, and then increased to about 1.5×10^4 for the last 1.5 hrs. Zero time counts for homogenate and infected leukocytes were about 8×10^2, but again the control was higher at 7×10^3 per ml.

Table 12 presents a summary of the changes in viable bacterial counts, for comparison with the results of these interaction experiments carried out using exudate leukocytes settled from suspensions containing 1.0×10^6 and 2.0×10^6 cells per ml.

d. Effect on viable counts after addition of meningococci to infected or uninfected exudate leukocytes

The following experiments were carried out to determine whether homogenates of infected leukocytes would show a greater or lesser killing effect upon meningococci than would homogenates of uninfected leukocytes. In brief, the experiments were done by preparing infected or uninfected exudate leukocytes as for the usual interaction experiment. At the point in the usual interaction protocol where zero time sampling operations and homogenizations were normally initiated, a slip, with either infected or uninfected leukocytes, was broken in half in a homogenization chamber, additional meningococci added as a Medium 199-1:8-PO_4 suspension, and homogenization carried out as in all interaction experiments. The homogenates were centrifuged and sampling was done at 0 hr, the tubes incubated, and sampled again at 1, 2, 3.5, and 5 hrs, and plate counts conducted as in the usual interaction experiment. Controls consisted merely of glass slips without attached leukocytes and the same volume of bacteria as used as additions to infected or uninfected leukocytes.

1. Comparisons of additions of M-1027 and M-1628 to homogenates of infected exudate leukocytes

M-1027 $m = 5$

In Figure 16, counts of homogenates

of infected exudate leukocytes without added meningococci decreased rapidly to zero at 3.5 hrs, a 2.4 log decrease from a zero time count of 4×10^3; these results were quite similar to those obtained earlier with the same multiplicity (Figure 10). The counts of homogenate of infected exudate leukocytes with added bacteria indicated rather steady, and relatively slow, decline from 8×10^3 at zero time to 7×10^2 at 5 hrs, nearly a 1 log decrease. Counts of controls decreased steadily, to zero at 5 hrs.

M-1628 $\underline{m} = 12$

In Figure 16, counts of homogenates of infected leukocytes without added bacteria declined rapidly to zero at 3.5 hrs, a 2 log decrease from a zero time count of 1×10^3 per ml; this was much more rapid killing than had been observed at $\underline{m} = 7$ or $\underline{m} = 21$, in earlier experiments (Figures 12 and 13). The counts of homogenate of infected leukocytes with added bacteria indicated a relatively steady decrease over 5 hrs, with about a 1.8 log decrease. Counts of control homogenate declined rapidly to zero at 5 hrs, about a 3.3 log decrease.

The interpretation of the results of these experiments depended upon the survival of the meningococci in control homogenate. It was observed that control counts rapidly declined to zero (Figure 16), both for M-1027 and M-1628. These controls differed from the usual interaction experiment controls in that the bacteria were homogenized with glass slips and then incubated for 5 hrs while samplings were conducted. Controls in the usual interaction experiments consisted of incubated intact slips with residual attached

meningococci, and homogenization was done only at intervals, and plate counts were immediately conducted. These experiments were repeated several times with similar results, in that control counts rapidly decreased to zero. Two lots of Medium 199 were examined to determine if a certain lot, then in use, was at fault, but control counts declined in either lot tested. The data in both experiments (Figure 16) indicate that homogenate of infected leukocytes plus meningococci provided some protection of bacterial viability, absent from both homogenates of infected leukocytes without added bacteria, and certainly absent from control homogenates. Since the reason for death in the controls is unknown, the nature of the protective effect is also unkown.

2. Comparison of additions of M-1027 and M-1628 to homogenates of uninfected exudate leukocytes and infected exudate leukocytes

M-1027 m = 24

In Figure 17, counts of homogenates of infected exudate leukocytes with added bacteria declined steadily, with about a 2.5 log decrease in counts by 5 hrs. The counts of homogenate of uninfected leukocytes with added bacteria rapidly declined for 3.5 hrs, and then reached zero at 5 hrs, a 3.4 log decrease in counts. Control homogenate counts decreased relatively steadily over the 5 hr period of incubation to zero.

M-1628 m = 43

In Figure 17, counts of homogenate of infected exudate leukocytes with added bacteria declined steadily over the 5 hr period, with about a 3 log drop in counts. The counts of homogenate of uninfected leukocytes with added bacteria declined little over 5 hrs. Control homogenate counts appeared to increase over the first 2 hrs by about 0.4 log, and then decreased to the zero time value.

The interpretation of the results from the above two experiments also depended upon the survival of the meningococci in control homogenates. It was observed that M-1027 control homogenate counts rapidly decreased but M-1628 control homogenate counts remained relatively constant over the 5 hr period of incubation. The data in Figure 17, for M-1628, indicate that at relatively high m, homogenates of uninfected exudate cells showed less killing of added meningococci than the homogenates of infected leukocytes. The difference in killing effect could possibly be due to the fact that infection of phagocytic leukocytes appears to result in an increase in the intracellular enzyme activity with lysis of lysosomal granules (Dannenberg, Walter, and Kapral, 1963a), so that homogenates of such leukocytes are more enzymatically active than homogenates of uninfected leukocytes. Another point concerning the data in these experiments (Figure 17), indicates that for M-1027, the apparent protective effect of homogenate of infected exudate leukocytes was less than indicated in Figure 16, in which protection was afforded both M-1027 and M-1628.

e. Exudate leukocyte-meningococcus interaction using meningococci recovered from infected exudate leukocytes

These experiments were carried out using exudate leukocytes settled from suspensions containing 1.0 X 10^6 cells per ml. The rationale for these experiments was as follows: It was considered possible that infected leukocytes selectively phagocytized bacteria from a population of meningococci presented to the cells. It was also considered possible that only certain phagocytized bacteria proliferated or survived within the leukocytes, and then only in particular cells, and that such bacteria might be endowed with greater virulence characteristics. In order to test these possibilities, two linked sequential interaction experiments were carried out for each meningococcal strain. The plan of these experiments was as follows: The procedure for the usual interaction experiment was carried out either using M-1027 or M-1628 at an $\underline{m} = 6$ or $\underline{m} = 7$ respectively. In the first experiment of the two linked experiments, only intact infected leukocytes on slips were incubated. In Table 13, this first of the two linked interaction experiments was designated Infection 1, $\underline{m} = 6$ or $\underline{m} = 7$, as was the case. After 7 hrs incubation, the infected leukocytes were homogenized (2 glass slips in each of two homogenization chambers containing 4.5 ml of 199-IX-HB per chamber), the homogenates pooled, and centrifuged at 100 $\underline{g}$ for 1.0 min, and diluted to 32 ml with Medium 199-1X-HB. A plate count was done on this homogenate, and the homogenate designated infecting bacterial suspension for the second of

the two linked experiments. The m for the second interaction experiment was calculated from the plate count results of the diluted homogenate of Infection 1 experiment. Thus, the second interaction experiment received the designation Infection 2, m = 0.0003 or m = 0.001, respectively for M-1027 and M-1628. The timing of the two linked experiments was so arranged that immediately following the preparation of the infecting bacterial suspension for Infection 2, infection of exudate leukocytes in Infection 2 was effected. The usual protocol for an interaction experiment was then followed in its entirety. The data from these experiments are presented in Table 13. The m of M-1027 decreased from m = 6 in Infection 1, to m = 0.0003 in Infection 2. In the case of M-1628, the m of Infection 1 was 7 and fell to an m = 0.001 in Infection 2. The very low values of the second infections served to rather rigorously test whether meningococci possessing unusual properties were selected by Infection 1. Though the m's meant that fewer bacteria were available to be phagocytised in Infection 2, they also permitted an ample opportunity for the meningococci actually phagocytized, to exercise any unusual properties. It was in effect a situation of meningococcal do-or-die. As the data in Table 13 indicate, the outcome of the experiments was "die". The viable counts in all Infection 2 experiments were essentially zero, that is, counts were zero for homogenates, infected leukocytes, and controls. Results of these sequentially linked interaction experiments indicated that bacteria, phagocytized from a large population of meningococci, did not possess unique properties for intracellular growth.

DISCUSSION

A study of the interaction of exudate leukocytes and meningococci, in essence, constitutes a host-parasite problem. The point has been made that, in studies of host-parasite relationships, attention has been focused primarily on one protogonist and, too often, little consideration is given the other party in this "marriage" (Dubos, 1954). The relationship between host and parasite is truly a "marriage", if perhaps a bizarre union, in the sense that it takes two to make it "work". In the discussion of the results, attempts will be made to give suitable recognition to each party involved, namely the exudate leukocyte and the meningococcus. However, meningococcal involvement will be stressed over that of the exudate leukocyte, for this study was conducted primarily from the viewpoint of the effect of exudate leukocyte-meningococcus interaction upon the meningococcus.

The discussion will be developed in the order of bacterial studies, exudate leukocyte studies, acid phosphatase studies, and finally exudate leukocyte-meningococcus interaction studies. After covering these topics, the discussion will turn to a consideration of the results in relation to fulfilling the objectives of this study and in answering the questions posed in the statement of purpose of this thesis. All four of the above studies are closely interrelated and it should be noted that there will be a certain amount of overlap in the following discussion.

A. Bacterial studies

A prominent aspect of these studies, and one which certainly bears

on the interpretation of the interaction experiments, was the results indicating certain differences between the two meningococcal strains used in this study.

The fact that M-1027 and M-1628 were more than merely serologically distinct was observed by Hoesly (1962) in his report on the role of nutritional dependence in the pathogenicity of N. meningitidis. One difference was expressed in virulence for 12-day-old chick embryos. In a test system in which Neisseria catarrhalis was the reference strain for avirulence, M-1027 was found to be as benign for chick embryos as was N. catarrhalis. However, M-1628 was the most virulent of all 7 strains tested in his study. It may be recalled at this point that the differing virulence of M-1027 and M-1628 was considered in choosing these strains for this study.

In this study, certain relatively elementary observations are worthy of notice in so far as they serve to reinforce the argument that M-1027 and M-1628 are quite different meningococcal strains.

Early in these studies, it was noted that outgrowth of colonies of M-1027 to a countable size (1 mm diameter) required about 36 hrs incubation in a candle jar. Outgrowth of colonies of M-1628 to a comparable diameter, required only about 24 hrs incubation. It was apparent, that the generation time for M-1027 was longer than for M-1628 under these conditions. A second difference was noted in the consistency of colonial growth. The difference in the consistency of growth was particularly evident when suspensions of bacteria were prepared. Growth of M-1027 was both easily removed from "spread plates" using a sharpened wood applicator stick and emulsified and suspended in any medium used in this study. Growth of M-1628, on the other hand, was difficult to remove

from "spread plates", and difficult to emulsify and suspend in any medium used in this study. The primary reason for these difficulties seemed to be that growth of M-1628 was more mucoid than growth of M-1027.

The growth experiments (Figure 3) were directed toward the selection of a dilution of Medium 199 in phosphate buffered saline in which M-1027 or M-1628 would show little or no growth. The results of these experiments again indicated differences in growth under the conditions of the experiments. In all cases in which there were data, the fold changes, in 5 or 6 hr plate counts, compared to zero time plate counts, were greater for M-1628 than for M-1027. This difference in growth response was particularly significant and will be again mentioned in the discussion of the interaction experiments.

These differences in growth between the two strains were not mentioned by Hoesly (1962). Hoesly found that both M-1027 and M-1628 could be placed in the same nutritional group, for both strains grew in Frantz medium on initial wire-loop transfer from stock dextrose starch agar (formulation of dextrose starch broth but without dipotassium phosphate, and with 3 g disodium phosphate and 10 g of agar per liter).

Homogenization was perhaps the most vigorous procedure to which M-1027 and M-1628 strains were subjected. The results (Figure 4) of these studies indicated a welcomed similarity and an unexpected difference between the strains. The bacteria were in effect commanded to "run the gauntlet" of homogenization because this was the procedure selected to disrupt the infected exudate leukocytes. The meningococci were similar in that viability of neither strain appeared to be affected by homogenization in Medium 199-1:8-PO_4 with glass slips as compared to

homogenization without glass slips followed by immediate viable plate counts. Data in Figures 16 and 17 indicate that in controls of bacteria plus glass slips, homogenization with subsequent incubation did result in loss of viability of M-1027 in both experiments in Figure 16 and in one of the two experiments in Figure 17. Such killing upon incubation was of little immediate concern since plate counts were always conducted immediately following homogenization. Another difference between M-1027 and M-1628 was revealed when homogenization with sucrose was accomplished. The rationale behind such a maneuver was as follows: By analogy with the work of others (Hatten and Sulkin, 1966; Dienes et al., 1964), intracellular forms of the meningococcus could conceivably have been L-forms or spheroplast forms or merely meningococci with "weakened" cell-wall structure. The complexity of the manipulations and time intervals involved in these experiments made it most sensible to consider intracellular meningococci as "normal" or at most as with "weakened" cell-wall structure. It was considered that addition of sucrose to a final concentration of 0.25 per cent to the homogenization Medium 199-1:8-PO_4 might result in increased protection for the meningococci. This increased protection would be reflected in substantially higher viable counts after homogenization in medium with 0.25 per cent sucrose as compared to homogenization in medium without sucrose. The data from these experiments indicate that homogenization with sucrose drastically reduced viability of M-1628 and may have increased the viable counts for M-1027. The difference in plate counts of M-1027 homogenized with and without sucrose was not sufficient to justify a definite statement of increase in the viable count. It could be definitely stated, however, that homogenization with sucrose did not decrease viability of M-1027.

It is considered to have been amply demonstrated that M-1027 and M-1628 possessed a number of distinct characteristics. Probably the differences noted in the previous pages were merely a few of those actually existing between these strains. Before a discussion of possible differences in the virulence of M-1027 and M-1628 for exudate leukocytes can be considered, a number of observations must first be made of the exudate leukocytes and the test system used in the interaction experiemtns.

B. Exudate leukocyte studies

The mouse was selected as the experimental animal in which exudate leukocytes were prepared primarily because of costs of purchasing, maintenance, ease of handling, and small housing space required. The use of mice also made it possible to use a larger number of animals per experiment than would have been practical if larger animals such as rabbits or guinea pigs had been used.

Some workers have indicated that the exudate leukocyte may be inferior to the blood leukocyte as concerns phagocytic activity in vitro(Lerner, 1956). That is, exudate leukocyte PMN may phagocytize fewer bacteria than blood leukocyte PMN in a particular experiment. However, the design of experiments, such as those on interaction, which required a high proportion of PMN in the population, are difficult to carry out using blood leukocytes. Methods are available to effect some degree of separation of cell types such as PMN and MN using differential centrifugation (Archer and Hirsch, 1963), column separation (Garvin, 1961), or even iron powder and a magnet (Hastings et al., 1961). These procedures, in the main, are complex, require

treatments or centrifugation which is considered damaging to PMN, and are somewhat difficult to carry out in an aseptic manner. Not least of all, these procedures require a relatively long time during which the PMN might be declining in viability.

Peritoneal exudate leukocytes are, on the contrary, well suited for interaction studies. By injection of 12 per cent sodium caseinate, intraperitoneally, and by harvesting exudate at the proper time thereafter, it is possible to easily prepare a peritoneal exudate containing 85-90 per cent PMN or 90 per cent MN.

Blood PMN may be superior phagocytizing cells, under certain conditions, than exudate PMN. However, this effect may be more apparent than real and due to experimental conditions, for exudate PMN are considered to originate directly from blood, as are some lymphocytes and free histiocytes (Goodman, 1964). The heterogeneity of the populations of exudate leukocytes is recognized at even 90 per cent PMN for many intergraded types of cells are present (Dannenberg et al., 1963b). In the interaction experiments, exudate cells were permitted to attach to glass slips. The reasons for this choice of technique will be covered in the discussion of the interaction experiments.

Exudate leukocytes exhibit the particularly annoying property of clumping (Allison and Lancaster, 1961). Heparin has been used to prevent clumping by interfering with fibrin formation, but it appears that heparin possesses other than anticoagulant activity (Allison and Lancaster, 1961); (Lindahl and Roden, 1964); (Jindal, Patel, and Patel, 1964). Some workers indicate that clumping is not causally related to the fibrinogen-fibrin system (Allison and Lancaster, 1961). In our experiments, clumping occurred as readily with heparin in concentration

as high as 10 U. S.P. Units per ml as without heparin, and, therefore, with a view also of other possible actions in the system, heparin was not used in the interaction experiments in preparations of exudate leukocytes. The clumping propensity of the peritoneal exudate PMN may in part reflect the fact that it is present in exudates elicited by irritants which produce an inflammatory reaction in the peritoneal cavity (Allison, Lancaster, and Crosthwaite, 1963). It has been considered that clumping may also be affected by the irritant used to elicit exudate, for exudate leukocytes elicited by different agents apparently show differing ability to phagocytize identical bacteria (McElree and Downs, 1961). The considerations in the preceding sentence point out a problem in using elicited exudate leukocytes. Various agents have been used as irritants to elicit peritoneal exudates. Peptone and glycogen were used by McElree and Downs (1961), sodium caseinate by Stählin et al., (1956), and warm mineral oil (Berk and Nelson, 1962). These agents and others have been used in various animals, and for eliciting populations predominating in either PMN or MN. It is quite possible that exudate leukocytes, elicited by any of these agents, may have phagocytized some of the irritant. The intracellular localization of many of the irritants, such as peptone, would be troublesome to demonstrate. However, in the case of mineral oil as irritant, intracellular droplets of oil can be easily visualized microscopically within the collected exudate leukocytes. The effect of the irritant on the intracellular economy of the leukocyte has not been thoroughly defined, and this aspect is not often mentioned.

Sodium caseinate was used in this study to elicite peritoneal exudates in mice. The concentrations used and preparations were

modified after that of Stähelin et al.,(1956). Other irritants were used in preliminary experiments but were not as thoroughly examined as was 12 per cent sodium caseinate. It was found that in our system, an exudate population which predominated in PMN (about 85-90 per cent) was achieved at approximately six hours following injection of the sodium caseinate. This particular time, unfortunately, made the carrying out of interaction experiments more difficult because injections of mice had to be done generally at midnight, 6 a.m. or at noon, in order to complete the experiment in one day in a total time of about fifteen hours.

The PMN leukocyte is considered to be an end-cell, that is, it does not reproduce. Therefore, in vitro, one merely attempts to provide environmental conditions to extend survival time to a time more comparable to the short, (about 24 hr) in vivo life span (Sieracki, 1955). It is possible to assess the in vitro viability of exudate leukocytes by use of trypan blue (McLimans, 1957). The technique consists merely in mixing 0.5 ml of 0.5 per cent trypan blue, in saline for example, with 1 ml of cell suspension. The preparation is placed in a hemacytometer and incubated for about 1 minute. A direct count is made of the total number of viable (unstained) cells. This method serves to indicate, at least, changes in membrane permeability to the dye. The method does not indicate whether other cell functions, such as phagocytic ability, are equally affected. It would appear that the viability of the leukocyte is more than mere cell permeability to trypan blue or to other dyes, such as eosin or acridine orange (Hathaway, Newby, and Githens, 1964). The life processes of the leukocyte comprise many interrelated individual processes,

impairment of one process affecting the other processes to various degrees. Work using metabolic inhibitors such as that of Sbarra and Karnovsky (1959) indicated that impairment of one leukocyte process does not necessarily involve another. These workers found that iodoacetate and fluoride, glycolysis inhibitors, interfered with phagocytosis. Cyanide and dinitrophenol, inhibitors of aerobic respiration, did not inhibit phagocytosis. Staining for leukocytes viability was not done in our work because actually such procedures appear to add only meagre information pertinent to the "life-status" of the leukocyte. A different method was used to determine the viability of infected exudate leukocytes used in our study. The method will be discussed later in the section on interaction experiments.

The problem of enumerating the total number of exudate leukocytes attached to glass slips was encountered early in this work. When using suspensions of cells, it is a simple matter to determine the total number. One merely counts an aliquot of cell suspension by hemacytometer count. In enumerating the number of cells on a glass slip, one is obliged to enumerate directly all cells, or only a portion of the cells with extrapolation to a value for the total number of cells, or to select a measurement of a leukocyte parameter which is related to cell numbers. Clumping of leukocytes has been previously mentioned. This undesirable, though quite real, attribute of exudate leukocytes, made direct cell counts difficult. Centrifugation at low g forces during the washing procedures did tend to remove many cell clumps but all cell clumps were not sedimented and thus removed from suspension. Direct counting procedures of even a statistical design (Woolf, 1950) would have been undesireably influenced by the cell-clumps and direct

counts of all leukocytes on a slip having perhaps several hundred thousand cells, was impractical. The most sensible way to approach this problem appeared to be by assaying exudate cells for a particular enzyme. The enzyme selected for investigation was acid phosphatase.

C. Acid phosphatase studies

Acid phosphatase was selected because it is a well-studied enzyme for which assay protocols are readily available. A foremost consideration in choice of acid phsphatase lay in the fact that exudate PMN contain substantial amounts of acid phosphatase activity (Dannenberget al., 1963b; Dannenberg, Walter, and Kapral, 1963a; Weissman, 1964). The acid phosphatase activity appears to be located primarily in the lysosomal granules in PMN. The lysosomal granules are considered to contain both antimicrobial agents (Hirsch, 1960; Spitznagel and Chi, 1963; Zeya, Spitznagel, and Schwab, 1966) and various digestive enzymes (Weissman, 1964). Degranulation has been shown to accompany phagocytosis (Karnovsky and Sbarra, 1960; Cohn and Hirsch, 1960; Sbarra et al., 1961). If certain PMN, after phagocytizing meningococci, disrupted and/or leaked acid phosphatase into the cell-bathing medium, it was considered that the assay of levels of acid phosphatase in the medium could indirectly communicate information indicating leukocyte damage (Cohn and Wiener, 1963a; Cohn and Wiener, 1963b). The preceding rationale served as the basis for these acid phosphatase studies.

The assay for acid phosphatase involved a relatively simple procedure. One merely determined the spectrophotometric values for levels of p-nitrophenol released by action of acid phosphatase on

p-nitrophenylphosphate substrate (Bergmeyer, 1963). In order to measure the acid phosphatase content of exudate leukocytes, it was necessary to rupture the cells to release lysosomal contents. Various methods were available for rupturing leukocytes such as use of saponin (Rosner and Lee, 1965), a membrane disrupting agent, by passage of cells through a fine mesh screen (Archer and Hirsch, 1963), and freezing and thawing (Cohn et al., 1963). It was decided not to use a reagent such as saponin to rupture the cells, for the viability of the meningococcus could be affected. A second consideration was also involved. This was that the determination of acid phosphatase was to be made on leukocytes attached to glass slips. Cohn and Wiener (1963a) used homogenization with a teflon pestle for this purpose and it was considered that homogenization would, similarly, be especially effective in breaking the leukocytes because the bits of glass generated from the slips would act as a grinding compound. Experiments disclosed that homogenization in Medium 199-1:8-PO_4, with immediate viable plate counts, did not result in the death of meningococci, but did result in excellent leukocyte breakage. It was not determined what effect the homogenization had on the integrity of the lysosomal granules. These are of sub-microscopic size and the use of electron microscopy would have been necessary to visualize the granules. It was considered that the homogenization procedure probably did destroy integrity of many granules for the gentle procedures of cell homogenization generally recommended for granule isolation result in undesirable lysosomal rupture (DeDuve, 1963b). The first experiments on acid phosphatase (subsequently referred to as "enzyme") assay using homogenized leukocytes, indicated that homogenization, which resulted in apparent complete leukocyte

breakage, did not release all enzyme activity into solution for centrifugation at 3500 g for 5 minutes sedimented nearly 50 per cent of total homogenate enzyme activity. This brief relatively low g centrifugation should not have sedimented intact granules, which are reported to sediment at about 12,000 g for 10 minutes (DeDuve, 1963b). Loss of enzyme activity following centrifugation was considered to possibly have been due to the following: 1) Intact cells on particles of glass were sedimented. 2) Enzyme activity was not released from the ruptured cells but remained associated with cell material. 3) Enzyme activity was adsorbed to glass or cell debris following release from lysosomal granules. Sonication of homogenate was tested as a means of "shaking" free additional enzyme activity, to assure breakage of all leukocytes which might have survived homogenization, and to shatter any intact lysosomal granules remaining after the homogenization procedure. Additional enzyme activity was not released by sonication of uncentrifuged though settled (5 minutes) homogenates. In fact, sonication rather decreased enzyme activity, perhaps by enzyme denaturation. Loss of enzyme activity following centrifugation of ruptured exudate leukocytes appeared related to a similar effect noted by Rosner and Lee (1965) in which alkaline phosphatase activity was removed by centrifugation of disrupted blood leukocytes. Rosner and Lee (1965) reported that the addition of saponin to intact blood leukocytes resulted in a 2 to 4 fold increase in alkaline phosphatase activity levels. They used a protocol calling for 9 ml of 1 per cent saponin solution and 1 ml of leukocyte suspension with a contact time of 15 minutes at 0 C. This concentration of saponin yields a final concentration of 900 mg/per ml. The resultant mixture was termed the saponin lysate which

was then centrifuged at 8200 g for 15 minutes at o C. The sedimented pellet was resaponized as above to release additional alkaline phosphatase activity. Valentine and Beck (1951) used a preparation which contained a final concentration of saponin of 100 mg per ml. Saponin was therefore tried in our experiments to release additional enzyme activity. Saponin concentrations at 200 mg per ml were found to not increase enzyme activity. Some of the effect may have been due to denaturation of enzyme for a 200 mg per ml saponin solution produced a great deal of foam when shaken. A reduction in saponin concentration to 80 mg per ml appeared to release additional enzyme activity from homogenates, though not 2 to 4 times the amount as without saponin. In these experiments, homogenization with saponin was impractical because of excessive foaming. The removal of the top of the Omni-Mixer chamber from such a homogenized preparation gave an effect very similar to that noted upon removal of the cap from a well-shaken bottle of warm beer.

The experiments of Valentine and Beck (1951) and Rosner and Lee (1965) were done using intact leukocytes so the results of these experiments were not directly comparable. However, our results using homogenates of intact cells, indicated that the relatively high concentration of saponin at 200 mg per ml, decreased rather than increased enayme activity. In view of the results using high saponin concentrations and homogenates, and having in mind the fact that intact leukocytes had been used by others, it was decided to test the effect of low saponin concentrations on intact leukocytes. The results of these experiments did indicate, in some agreement with Rosner and Lee (1965), that at the low concentration of 0.0125 mg per ml, saponin released

considerable enzyme activity from intact leukocytes. It was possible to construct a curve indicating a linear relationship between enzyme activity and cell number at the saponin concentration of 0.0125 mg per ml, though at 0.025 mg per ml saponin, the curve appeared to be non-linear at lower cell numbers. The effect of saponin on enzyme activity appeared to indicate that the enzyme was adversely affected by relatively low concentrations of saponin. It should be noted that Bohringer standard enzyme activity was decreased by the same concentrations of saponin as was the enzyme activity from homogenates of leukocytes. This method for enumerating total exudate leukocytes on a glass slip appeared to hold some promise but experiments to further refine the relationship of enzyme activity to cell number were deferred until it could be determined whether enzyme retained activity in the cell-bathing medium. Experiments were carried out testing enzyme activity of homogenates and Boehringer standard enzyme, with time of incubation in Medium 199-1X-HB at 37 C. Unfortunately, enzyme activity in both preparations rapidly declined. At the time these studies were conducted, Medium 199-1X-HB was being used as the leukocyte-bathing medium. Medium 199-1:8-PO_4 was later used as bathing-medium for exudate leukocytes on glass slips. Therefore, activity of enzyme in Medium 199-1:8-PO_4 versus time was not determined. There were a number of difficulties attendant upon use of enzyme assay in this particular study. These were as follow: 1) The standard deviation for the data used to relate enzyme activity to leukocyte number was 1.18. It would be possible to use the curve to relate enzyme activity to leukocyte number. However, only major loss of leukocytes from a slip during an experiment would give $OD_{400\ m\mu}$ values large enough to be significant. In other words, the

sensitivity of the method for detecting changes in cell numbers was relatively low. Had the exudate leukocytes contained higher assayable enzyme activity, the sensitivity of the method in detecting changes in cell numbers would have been increased. 2) It was not known whether enzyme activity in infected leukocytes decreased with time following infection. Such a phenomenon could be interpreted erroneously as a decrease in leukocytes remaining on the slip. 3) Enzyme activity appeared to decline rapidly in leukocyte-bathing medium use. Therefore quantitation of enzyme in the medium at various periods of time following infection was not possible. Since this decline occurred in a solution of Medium 199, namely Medium 199-1X-HB, it was considered that similar results would have been obtained using another solution of Medium 199, 199-1:8-PO_4.

The method was not further investigated in this thesis for it appeared that a number of refinements were necessary before quantitatively useful data could be obtained. The method also was not further developed in order to avoid channeling the content of the thesis into a prime consideration of the effect of the meningococcus on the leukocyte, rather, than as previously stated, the effect of the interaction on the meningococcus.

D. Exudate leukocyte-meningococcus interaction studies

In vitro phagocytosis experiments using exudate leukocytes are commonly carried out using leukocytes suspended in medium (Sbarra and Karnovsky, 1959; Li, Mudd, and Kapral, 1963) or attached to a surface such as a glass slip (Howard, 1959; McElree and Downs, 1961). Certain advantages attend using suspended leukocytes.

Some of these may be listed as follows: 1) Leukocyte counts during any phase of the experiments are simple to conduct merely by hemacytometer counts. 2) The multiplicity of infection can be easily and accurately determined. 3) Plate counts of bacteria in extracellular medium can be rather easily determined, after centrifugation, by plate counts on the supernatant. Certain disadvantages also attend such experiments and some of these may be listed as follows: 1) Clumping of leukocytes presents a problem in counting leukocytes, especially after leukocytes have phagocytized, for they then show even a greater tendency to clump (Allison et al., 1963). 2) Efficient phagocytosis requires active mixing of leukocytes and bacteria to increase contact (Lerner, 1956). 3) Extracellular bacteria adhere to the leukocyte surface and may distort plate count values.

The rationale for choosing to work with exudate leukocytes attached to glass slips was as follows: Wood (1946) reported on the increased phagocytosis of pneumococci when blood leukocytes were permitted to phagocytize from a surface as opposed to phagocytosis from suspension. It was assumed that Wood's experiments were correct and conflicting points of view (Lerner, 1956) did not contradict Wood's basic premise. The phagocytizing cell has been observed to carry out this activity from a fixed position by a number of workers (Knisely, Bloch, and Warner, 1948; Gözsy and Kátó, 1957). Permitting an exudate leukocyte to phagocytize from a fixed position, on a glass slip, may serve to more nearly approximate the in vivo situation than permitting phagocytosis in suspension.

One disadvantage to the use of exudate cells attached to glass slips has already been pointed out, namely, difficulty in the quantitation

of leukocyte number attached to the glass slip at various times during and experiment.

A method for enumerating the total cells on a slip was not used in our study. However, a method was developed and used to determine whether leukocyte differential populations changed following infection or whether the number of cells attached to slips decreased with time following infection. The data from these experiments indicated only that no gross changes occurred in the populations on the slips, for the method used was not strictly quantitative. Because of the fact that leukocytes did not detach following meningococcal infection at various multiplicities of infection (up to 30), it was possible to follow the infection by homogenizing the leukocytes to release intracellular bacteria. More on this aspect will be mentioned later in this section.

The medium used for bathing infected leukocytes was a compromise choice, for the medium had to: a) prevent or minimize bacterial growth, without loss of viability when homogenization of the leukocytes was done, and b) maintain leukocytes in good morphological condition. Medium 199-1:8-PO_4 was selected as the leukocyte bathing medium. No serum additions were used in any medium preparations in the final interaction experiments. Experiments were done to determine the effect of serum on phagocytosis of M-1027 as compared to phagocytosis without serum and these will be discussed later in this section. A number of additional problems arise when using serums such as non-specific phagocytosis which appears to be enhanced by alpha-1 and beta globulins (Sbarra and Karnovsky, 1959), and effect of serums on cell attachment (Nordling et al., 1963). Preliminary data in this work had indicated

that phagocytosis of meningococci did occur in the absence of mouse serum and to much the same extent. Certain studies of others have indicated that animal cells may tolerate non-serum bathing media better than is generally appreciated (Tribble and Higuchi, 1963; Higuchi, 1963). The Medium 199-1:8-PO_4, used for bathing infected leukocytes in our work, was perhaps not optimal and certainly more extensive experiments using other buffers and media may have effected a superior compromise medium. The Medium 199-1:8-PO_4 had to prevent or minimize meningococcal bacterial growth because no antibiotics were used in the bathing medium to control multiplication of extracellular bacteria. Antibiotics are commonly used to control the multiplication of extracellular bacteria (Nelson and Becker, 1959) and to control growth of contaminants. However, there is substantial evidence that phagocytizing cells ingest a certain amount of the external medium during the phagocytic act (Karnovsky and Sbarra, 1960; Sbarra et al, 1962). If the external medium contains an antibiotic, then ingestion of medium will also result in ingestion of antibiotic with possible deleterious effects on intracellular bacteria. It is, of course, possible that a pinocytotic vacuole containing medium with antibiotic may not liberate its contents into the intracellular environment where ingested bacteria are located. It was decided to obviate the problems associated with antibiotics by using no antibiotics in the studies reported in this thesis. Therefore, a different approach was required for controlling extracellular growth of meningococci. To control extracellular growth of bacteria, one can use a continuously flowing bathing medium to wash away extracellular bacteria (Baker, 1954), or use a bathing medium which minimizes bacterial growth.

The latter method was selected in choosing Medium 199-1:8-PO_4. To avoid contamination in these procedures without added antibiotics, it was necessary to exercise diligence and very careful aseptic technique. However, the added burden of aseptic manipulations was not without good purpose, for evidence of intracellular death of bacteria in experiments not using antibiotics is more forceful than in experiments using antibiotics.

The interpretation of the interaction experiments in this thesis depended considerably on the viable plate counts of controls. It has been repeatedly mentioned that it was not possible to remove all bacteria which adhered to leukocytes and glass slip surfaces by washing procedures. It is also apparent from the experiments (Figure 3) from which data Medium 199-1:8-PO_4 was selected, that it was not possible to prevent growth of M-1027 and M-1628 entirely even by using such diluted Medium 199. Two points to be kept in mind are that: a) both strains appeared to grow in Medium 199-1:8-PO_4 to a small extent and b) that M-1628 appeared to grow a few fold more than M-1027 over 5 or 6 hours. In considering the counts of controls in experiments using exudate leukocytes settled from suspension containing 1.0×10^6 cells per ml, it was noted that the control values were irregular, in that they rose and fell, especially in experiments in which meningococci were used at low multiplicities of infection. The irregular counts were probably not due to bacterial growth but most likely to the fact that the very thorough washing procedure did not remove bacteria from all glass slips to the same extent. The large irregularities were nearly limited to low multiplicities and at high multiplicities, counts were less irregular. Unfortunately, because of the

irregularities of the control counts, it was not possible merely to use control counts as a measure of the number of extracellular meningococci. Even had the control counts been regular, they would not necessarily have been the best estimate of extracellular bacteria for several reasons. The exudate leukocytes do occupy space on the surface of the slip on both sides. Therefore, there is less area on a leukocyte-occupied slip for attachment of bacteria. Furthermore, the tendency of bacteria to adhere to leukocytes, slips, or both, may have been identical or different to varying degrees. It can be said that the control counts indicated that bacterial growth was not excessive. The results of the control plate counts for M-1628 at multiplicities of infection of infection of 21 and 30, and M-1027 at 25, indicate that perhaps both strains adhered to the glass slips to a similar degree. If this were correct, then one might anticipate that M-1027 and M-1628 adhere to the exterior surfaces of leukocytes also to a similar degree and that any difference between zero time counts of leukocytes infected at similar multiplicities with M-1207 or M-1628 was due to the fact that one strain was more easily phagocytized than the other. This may be the case for M-1027 at a multiplicity of 14 and at a multiplicity of 25, for zero time infected leukocyte counts were approximately 1 log higher than zero time infected leukocyte counts for M-6128 at multiplicities of 21 and 30.

A word of caution must be included in the consideration of plate count values for N. meningitidis. The colony counted as one in a plate counting procedure is assumed to arise from a single bacterial cell. When using bacteria which do not form chains, tetrads, or other clusters, and do not clump in the dilution medium, then a single colony

may validly be considered to arise from a single bacterial cell. In quantitating N. meningitidis by plate count, the correspondence between colony and the number of bacteria which grew to form the colony cannot be considered to be one. The reason for this deviation is, of course, because meningococci exist in suspending media as both single cells, diplococci and occasionally chains. The plate count value on a suspension of meningococci in dextrose starch broth, for example, was probably less than the true count of individual cells. In these interaction experiments, plate count values are also probably less than the true count. However, the effect of intracellular residence may be to sever diplococci into single cells, which when released by homogenization and plated, yield two colonies from what was a single-colony-forming diplococcus. In this manner, it is possible to have an apparent increase of intracellular bacteria with no growth having occurred.

One consistent result in all the interaction experiments is that death occurred in homogenate for both strains and at all multiplicities of infection. The exudate leukocytes did apparently possess the armament to kill these strains in homogenates. This killing effect may not have been purely due to homogenate but may have been partially due to the homogenization procedure, for it may be recalled that both M-1027 and M-1628, after homogenization with glass slips in Medium 199-1:8-PO_4 and incubation, tended to die rapidly (Figures 16 and 17). The data for killing in homogenates of infected leukocytes appear to indicate that M-1027 is killed at a more rapid rate than is M-1628 at similar multiplicities of infection. This information might indicate that M-1628 is more resistent to intracellular killing.

A second consistent result occurred in all the M-1027 interaction experiments. This was that M-1027 infected leukocyte counts decreased at all multiplicities of infection. A consideration of the infected leukocyte counts for M-1628 appeared to indicate death only at low multiplicities and counts at all other multiplicities appeared to increase with time of incubation. In the light of knowledge that M-1628 apparently grew better in Medium 199-1:8-PO_4 than did M-1027, the apparent intracellular growth of M-1628 might be ascribed to growth in the medium. Second thoughts on the matter, lead to the question of why did not M-1027 also increase in the medium since M-1027 also grew in Medium 199-1:8-PO_4 albeit to a lesser degree (Figure 3)? The question might be answered by postulating that M-1027 did grow in Medium 199-1:8-PO_4 as did M-1628, but M-1027 in perhaps being more easily phagocytized than M-1628, was continually phagocytized at a rate, in effect 1 log greater than M-1628. Unfortunately, comparative direct counts of intracellular M-1027 and M-1628 phagocytized at various multiplicities were not possible, since such experiments were done only with M-1027. The answer given was not completely satisfying, for as mentioned in Results Section Part B 2, morphology of exudate leukocytes in Medium 199-1:8-PO_4 did not indicate active phagocytosis in progress, that is, leukocytes exhibiting many pseudopodia and apparent diapedisis. In contrast, it may be recalled that the morphology of leukocytes in Medium 199-1X-HB, used for infecting bacterial suspension, was indicative of active phagocytosis in progress. It is probable that the result of counts of infected leukocytes for both strains at all multiplicities reflect processes of both multiplication and death, occurring simultaneously in populations

of intracellular meningococci. Superimposed on these intracellular events are the processes of continued phagocytosis of the few extracellular meningococci. The gross view of these interdigitated events indicates death of M-1027 in exudate leukocytes and multiplication of M-1628 in exudate leukocytes. The results of these experiments did not permit a clear cut distinction between the events of intracellular growth and death, continued phagocytosis of meningococci, and growth, however slight, in the extracellular medium.

The interaction experiments using leukocytes settled from suspensions containing 2.0×10^6 cells per ml yielded results which did not depart, to any great extent, from the results of the previously discussed experiments. These data do permit the conclusion that infecting twice the number of leukocytes on a slip at low multiplicities of infection did not affect the interaction events as compared to experiments using leukocytes settled from suspensions containing 1.0×10^6 cells per ml. These results did not, however, augment insight of intracellular events.

The results of the interaction experiments did not indicate a marked difference in the abilities of M-1027 and M-1628 to survive in exudate leukocytes, or in homogenates thereof, as one might expect if such survival is a prerequisite for virulence of N. meningitidis. Hoesly (1962) was able to demonstrate a striking difference between the virulence of M-1027 and M-1628 for chick embryos. He found that M-1027 was as avirulent as the reference avirulent strain of Neisseria catarrhalis for chick embryos, whereas strain M-1628 exhibited the greatest degree of virulence of all 7 strains tested in chick embryos. Hoesly maintained his stock cultures in dextrose

starch agar, and Frantz medium, a completely synthetic medium. Growth of M-1027 on either dextrose starch agar or Frantz medium prior to use for chick embryo inoculation, did not appear to affect virulence. However, M-1628 was more virulent when grown on Frantz medium than when grown on dextrose starch agar medium. The culture medium used in our work for growth of infecting bacteria was Mueller-Hinton agar, and it is possible that this medium for culture could alter the survival pattern of the organism in exudate leukocytes or possibly even alter virulence for chick embryos.

The question of the validity of comparing results of meningococcal infection of chick embryo with results of infections of mouse exudate leukocytes, should be considered. Hoesly (1962) infected 12 day old chick embryos by inoculating a suspension of meningococci onto the chorio-allantoic membrane. He reported finding intracellular and extracellular Gram negative diplococci (most likely meningococci), primarily in monocytes in allantoic fluid from embryos apparently killed by M-1027. It appeared that an inflammatory reaction was elicited in the embryos by the meningococci. These results were in agreement with those of Buddingh and Polk (1939), in which they reported that a slight amount of inflammation was noted in 12 day old chick embryos inoculated on the chorio-allantoic membrane with meningococci. Hoesly did not report the approximate amount of inflammatory exudate. He did note that the polymorphonuclear leukocytes present were in varying states of disintegration and that few polymorphonuclear leukocytes were found to contain more than one or two pairs of bacteria. With only this information, it is difficult to relate the status of the polymorphonuclear leukocyte in the dead embryos to the status of the polymorphonuclear

leukocytes in those embryos in the same series which survived infection, for a comparative examination of surviving embryos was not reported. Similar data on examinations of M-1628 killed and surviving embryos would have been valuable. Hoesly stated that endotoxin of M-1628 (prepared by boiling dilutions of meningococci for 10 min at 100 C) tested in chick embryos did not account for the killing effect. These results are in accord with the reports of Smith and Thomas (1956), and Finkelstein (1964), which indicate the reduced susceptibility to endotoxin of 12 day old chick embryos as compared to 10 day old and younger embryos.

Some workers such as Heilman (1965) and Thomas (1954), have indicated that endotoxin, such as E. coli lipopolysaccharide as used in the work of Heilman, has a marked slective toxicity for mammalian macrophages and a slight toxicity for granulocytes. Such observations may indicate that the mouse exudate polymorphonuclear leukocyte is more sensitive to endotoxin of the meningococcus than is the 12 day old embryo. It would have been useful in our experiments to have compared the infection of exudate leukocytes with M-1027 and M-1628 after treatment of infected leukocytes with the respective endotoxins (Heat killed meningococcal cells). If the mouse exudate polymorphonuclear leukocyte is indeed more sensitive to endotoxin than the 12 day old chick embryo, endotoxin effects may have served to modify and reduce the difference between intracellular growth of M-1027 and M-1628 in leukocytes as compared to the marked difference in the virulence of these two strains for the 12 day old chick embryo. In view of possible differing effects of endotoxin on the chick embryo and exudate polymorphonuclear leukocytes results of experiments comparing the virulence

of meningococci in 9 to 10 day old chick embryos with intracellular growth in exudate leukocytes, may be more comparable than experiments and results obtained with 12 day old embryos.

The results of experiments to determine killing effect of homogenates of infected and uninfected leukocytes on meningococci were disappointing. The value of the data of these experiments lay in comparison to control counts of homogenates of glass slips and bacteria. Though the experiments were repeated 5 times using M-1027, and 4 times using M-1628, it was not possible to consistently obtain control counts which did not rapidly decrease upon incubation of the homogenate. Only in Figure 17, M-1628 at a multiplicity of infection of 43, did the results justify a conclusion. From these results, it may be concluded that at a high multiplicty of infection, homogenate of uninfected leukocytes less effectively killed M-1628 than homogenate of infected leukocytes. This conclusion was predicted on the basis that phagocytosis results in degranulation and release of more available enzyme within the leukocyte. If these experiments were conducted so that control bacteria survived, it would be predicted that both M-1027 and M-1628 would show better survival in homogenates of uninfected rather than infected leukocytes. These experiments were performed by including added meningococci immediately before homogenization of the particular preparation. If bacteria were added after homogenization, death in control counts may have been reduced.

The final interaction experiments posed perhaps the most interesting question in this thesis. This was, did certain meningococci, out of the population phagocytized by exudate leukocytes, possess unusual abilities for intracellular growth following residence in

leukocytes? In the interaction experiments discussed in the first part of this section, presence of certain small populations of so-called "unique" meningococci, with unusual abilities, could have been easily masked by the relatively vast number of "non-unique" meningococci. These final interaction experiments were designed to better permit meningococci, in a minority population, to in effect, express themselves. The results of these experiments indicated that certain meningococci, out of the population phagocytized by exudate leukocytes, did not possess unusual abilities for intracellular growth following residence in leukocytes. A conclusion such as the above is not firm, of course, for the design of the experiments, while making conditions more ideal for expression of activities of a few meningococci, also reduced the chances of selection of these few meningococci from a population, since the infecting suspension, in Infection 1 (Figure 17), was at a relatively low multiplicity of infection. To warrant a firm conclusion, these experiments would have to be repeated a number of times to better permit the selection of possible "unique" meningococci.

E. Results in relation to questions posed for the thesis problem and objectives.

Four questions were posed to be answered by the results of this investigation. As stated in the Introduction, these were as follows:

1. Do certain strains of N. meningitidis survive after phagocytosis?
2. Do certain strains of N. meningitidis proliferate within PMN after phagocytosis?

3. In an *in vitro* system, what are some parameters of certain experimental conditions which may provide answers to questions 1 and 2?
4. What experimental evidence from the above studies may be applied to the problem of pathogenicity of *N. meningitidis*?

The statement has often been made that rules are made to be broken and, in reference to the above questions, it might be similarly stated that questions are posed to be unanswered. The results of these studies do not sum to a total answer to all four of the above questions. The results do present information concerning the application of several techniques to the problem of pathogenesis of *N. meningitidis*. An attempt to study intracellular events following infection of exudate leukocytes *in vitro*, has not previously been reported for *N. meningitidis*, to the best of our knowledge. It appears that the techniques presented must be further refined and developed to a more sophisticated level before definitive answers may be secured.

The attempt has been made in the interpretation of the results, to not deduce unwarranted conclusions from the data. In a few words, the results of this thesis have provided information to partially answer questions 4 and 3, to permit a tentative answer to question 2, and to permit a speculative answer to question 1. The contents of this paragraph will be expanded to be more specific in the following Summary and Conclusions.

TABLE 1

A HISTORY OF

NEISSERIA MENINGITIDIS STRAINS USED IN THIS STUDY

ATCC Number	13077	13102
Branham strain number	M-1027	M-1628
Serological group	A	C
Date isolated	4 - '37	11 - '49
Place isolated	Chicago, Illinois	Hartford, Connecticut
Received from	C. P. Miller, M.D.	F. L. Mickle, M.D.
Source	Spinal fluid	Spinal fluid
Comments	Suggested neotype strain for Group A and species.	Suggested neotype strain for Group C

TABLE 2

DEXTROSE STARCH BROTH

CONSTITUENT	g/L
Proteose peptone No. 3	15
Bacto-Dextrose	2
Soluble starch, Difco	10
Sodium chloride	5
Disodium phosphate	2
Dipotassium phosphate	1
Bacto-gelatin	20

Final pH of medium at 25 C is 7.3

TABLE 3

MUELLER-HINTON MEDIUM

CONSTITUENT	g/L
Infusion from beef	300
Bacto-Casmino acids, Technical	17.5
Starch	1.5
Bacto-agar	17

Final pH of medium at 25 C is 7.4

TABLE 4

MEDIUM 199

Component	Gms/L
NaCl	8.000
KCl	.400
$MgSO_4 \cdot 7H_2O$	.200
$Na_2HPO_4 \cdot 7H_2O$	.090
KH_2PO_4	.060
Dextrose (anhyd.)	1.000
Phenol Red	.020
$CaCl_2$ (anhyd.)	.140
$NaHCO_3$	.350

Component	Mgs/L
L-arginine	70
L-histidine	20
L-lysine monohydrochloride	70
DL-tryptophan	20
DL-phenylalanine	50
DL-methionine	30
DL-serine	50
DL-threonine	60
DL-leucine	120
DL-isoleucine	40
DL-valine	50
DL-glutamic acid monohydrate	150
DL-aspartic acid	60
DL-alpha-alanine	50
L-proline	40
L-hydroxyproline	10
Glycine (aminoacetic acid)	50
L-glutamine	100
Sodium Acetate Trihydrate	50
L-cystine	20

Component	mgs/L
L-tyrosine	40
L-cysteine hydrochloride	0.1
Adenine	10
Guanine	0.3
Xanthine	0.3
Hypoxanthine	0.3
Uracil	0.3
Thymine (5-methyl uracil)	0.3
Disodium Alpha Tocopherol Phosphate	0.01
Thiamine	0.01
Pyridoxine Hydrochloride	0.025
Riboflavin	0.01
Pyridoxal Hydrochloride	0.025
Niacin	0.025
Niacinamide	0.025
Calcium Panthothenate	0.01
i-inositol	0.05
Ascorbic Acid	0.05
Folic Acid	0.01
p-Aminobenzoic Acid	0.05
Ferric Nitrate	0.1
Biotin	0.01
Menadione	0.01
Glutathione	0.05
Vitamin A	0.1
Calciferol	0.1
Cholesterol	0.2
Tween 80	5.0
Adenosinetriphosphate (sodium salt)	1.0
Adenylic Acid	0.2
Desoxyribose	0.5
D-ribose	0.5
Choline Chloride	0.5

TABLE 5

CONFIRMATION OF STRAIN SEROLOGICAL TYPE		
Antiserum Dilution	*N. meningitidis* Strain	
	M-1027	M-1628
Anti-A		
1:2	+	0
1:4	+	0
1:8	+	0
1:16	+	0
Anti-C		
1:2	0	+
1:4	0	+
1:8	0	+
1:16	0	+
Saline control	0	0

+ = Agglutination

0 = No agglutination

TABLE 6

Effect Of Various Preparations Of Medium 199 On Growth Of M-1027 And M-1628

Preparation of Med. 199	Exp. No.	No. Strain	CFU* per ml 0 Hr.	CFU* per ml 6 Hr.	Fold Change
1X-H_2O	1	M-1027	2.3×10^5	2.3×10^6	+ 10
		M-1628	5.8×10^5	1.5×10^7	+ 26
	2	M-1027	2.2×10^5	4.2×10^5	+ 2
		M-1628	2.5×10^5	1.1×10^7	+ 44
1X-PO_4	1	M-1027	1.5×10^5	-	-
		M-1628	8.8×10^5	3.6×10^5	- 3
	2	M-1027	1.6×10^5	2.1×10^4	- 8
		M-1628	2.8×10^5	2.6×10^6	+ 9
1:2-PO_4	1	M-1027	2.4×10^5	1.8×10^4	- 13
		M-1628	7.3×10^5	6.4×10^5	- 1
	2	M-1027	2.8×10^5	6.4×10^5	+ 2
		M-1628	2.9×10^5	2.4×10^7	+ 83
1:4-PO_4	1	M-1027	2.0×10^5	1.1×10^5	- 2
		M-1628	7.5×10^5	2.2×10^6	+ 3
	2	M-1027	3.2×10^5	2.7×10^6	+ 8
		M-1628	2.9×10^5	4.4×10^6	+ 15
1:8-PO_4	1	M-1027	1.7×10^5	1.5×10^5	- 1
		M-1628	6.8×10^5	1.6×10^6	+ 2
	2	M-1027	3.1×10^5	3.1×10^6	+ 10
		M-1628	2.9×10^5	4.4×10^6	+ 15
			0 Hr.	5 Hr.	
1X-H_2O	3	M-1027	-	-	-
		M-1628	-	-	-
	4	M-1027	1.0×10^2	9.0×10^1	- 1
		M-1628	9.3×10^3	8.0×10^4	+ 9
1X-PO_4	3	M-1027	5.5×10^3	8.4×10^2	- 7
		M-1628	4.8×10^4	2.8×10^5	+ 6
	4	M-1027	5.0×10^2	1.9×10^2	- 3
		M-1628	9.4×10^3	5.0×10^4	+ 5
1:2-PO_4	3	M-1027	1.1×10^4	9.7×10^3	- 1
		M-1628	7.2×10^4	2.2×10^6	+ 31
	4	M-1027	2.0×10^2	1.5×10^3	+ 8
		M-1628	8.9×10^3	1.4×10^5	+ 16
1:4-PO_4	3	M-1027	1.1×10^4	5.0×10^4	+ 5
		M-1628	3.7×10^3	1.4×10^6	+ 38
	4	M-1027	6.0×10^2	2.3×10^3	+ 4
		M-1628	9.5×10^3	1.8×10^5	+ 19
1:8-PO_4	3	M-1027	5.8×10^3	4.5×10^4	+ 8
		M-1628	-	-	-
	4	M-1027	7.0×10^2	2.8×10^3	+ 4
		M-1628	1.4×10^4	9.7×10^4	+ 7

* CFU - Colony forming units.

TABLE 7

DIFFERENTIAL POPULATIONS OF SODIUM CASEINATE ELICITED PERITONEAL EXUDATE LEUKOCYTES

Hr Exudate Collected Post-injection	Mouse[1] Number	Percentage[2]		
		PMN		MN
3	1	83		17
	2	63		37
	3	80		20
	4	71	$\bar{X}$ = 74	19
6	1	87		13
	2	88		12
	3	87		13
	4	90	$\bar{X}$ = 88	10
9	1	67		33
	2	75		25
	3	70		30
	4	69	$\bar{X}$ = 70	31
12	1	46		54
	2	56		44
	3	51		49
	4	46	$\bar{X}$ = 50	54
18	1	56		45
	2	63		37
	3	51		49
	4	37	$\bar{X}$ = 51	63

1 = 4 Different mice used in each experiment
2 = Total of 400 leukocytes counted per slip

TABLE 8

EFFECT OF INCUBATION OF EXUDATE LEUKOCYTE HOMOGENATE IN MEDIUM 199-1X-HB ON ACID PHOSPHATASE ACTIVITY

Tube Number[1]	Sample Number	Contents of Leighton tube	Incubation Time at 37C Hrs	OD 400 mu
1	1	Uncentrifuged homogenate	0	0.467
-	2	Supernatant from tube 1	2.3	0.104
-	3	Remainder of tube 1 contents after removal of all supernatant	2.3	0.194
2	4	Supernatant as sample 2 but from tube 2	5.0	0.000
-	5	Remainder of tube 2 contents after removal of all supernatant	5.0	0.066

1 = Replicate tubes

TABLE 9

PHAGOCYTOSIS OF M-1027 BY MN AND PMN

PMN

m	Slip Number	Number of Exudate Leukocytes With Intracellular Bacteria		
		Intracellular	Zero	Doubtful
50	1	152	20	28
	2	164	14	22
	3	132	26	42
	4	116	56	28
	5	110	43	47
	6	135	19	46
Arithmetic mean		134	30	36
Per cent of 200 cells counted		67	15	18

MN

m	Slip Number[1]	Number of Exudate Leukocytes With Intracellular Bacteria		
		Intracellular	Zero	Doubtful
50	1	53	40	7
	2	66	29	5
	3	37	49	14
	4	41	47	12
	5	46	42	12
	6	49	38	13
Arithmetic mean		49	41	10
Per cent of 100 cells counted		49	41	10

m = Multiplicity of infection
1 = Same slips as used for PMN counts

TABLE 10

EFFECT OF NORMAL MOUSE SERUM ON PHAGOCYTOSIS OF M-1027 BY EXUDATE LEUKOCYTES

Normal Mouse Serum	m	Number of Exudate Leukocytes with Intracellular Bacteria[1]					Per cent Leukocytes with Intracellular Bacteria
		Zero[2]	Doubtful	1-5	6-10	>10	
+	3	129	17	44	9	2	29
		122	9	47	16	7	
		123	14	50	12	2	
		154	6	29	10	1	
		$\bar{X}$=132	$\bar{X}$=12	$\bar{X}$=43	$\bar{X}$=12	$\bar{X}$=3	
	100	10	8	37	53	91	89
		10	18	50	49	83	
		15	6	47	34	108	
		27	12	58	37	66	
		$\bar{X}$=16	$\bar{X}$=11	$\bar{X}$=48	$\bar{X}$=43	$\bar{X}$=87	
-	3	140	11	45	3	1	22
		135	11	47	5	2	
		152	11	37	1	0	
		153	10	35	2	0	
		$\bar{X}$=145	$\bar{X}$=11	$\bar{X}$=41	$\bar{X}$=3	$\bar{X}$=1	
	100	19	3	53	41	84	86
		18	9	63	50	61	
		16	17	75	48	44	
		27	13	59	57	45	
		$\bar{X}$=20	$\bar{X}$=11	$\bar{X}$=63	$\bar{X}$=49	$\bar{X}$=49	

m = Multiplicity of infection
1 = Total of 200 cells counted per slip
2 = Individual slips
$\bar{X}$ = Arithmetic mean

TABLE 11

DIFFERENTIAL POPULATION AND NUMBER OF EXUDATE LEUKOCYTES ATTACHED TO GLASS SLIPS AFTER INFECTION

Bacterial Strain	Duration of Infection Hrs.	Total Exudate Leukocyte count[1]	Percentage[2]	
			PMN	MN
M-1027 m = 5	0	723	83	17
	1	831	-	-
	3.5	770	-	-
	5.0	613	90	10
M-1027 m = 25	0	932	84	16
	1	1105	-	-
	3.5	738	-	-
	5.0	976	86	14
M-1628 m = 4	0	692	87	13
	1	649	-	-
	3.5	492	-	-
	5.0	588	90	10
M-1628 m = 30	0	834	88	12
	1	672	-	-
	3.5	680	-	-
	5.0	758	86	14

m = Multiplicity of infection

1 = Total number of all cell types in 60 uniformly distributed oil immersion fields per slip

2 = Total of 400 cells counted per slip

TABLE 12

COMPARISON OF RESULTS OF INTERACTION EXPERIMENTS USING EXUDATE LEUKOCYTES SETTLED FROM SUSPENSIONS CONTAINING 1.0×10^6 or 2.0×10^6 CELLS PER ML

m	1.0×10^6 cells per ml						2.0×10^6 cells per ml					
	M-1027			M-1628			M-1027			M-1628		
	H	I	C	H	I	C	H	I	C	H	I	C
2										-2	+1	-
4				-1	-.4	=						
5	-2	-1	=				-1	-2	=			
7				-1	+1	+	-1	=	+			
9										-2	+ .5	+
14	-1	-1	+									
21				-1	+1	-						
25	-1	-1	-									
30				-1	+.4	+						

+ or - number = Log change from zero time count
= = No change from zero time count
+ = Increase from zero time count
- = Decrease from zero time count

m = Multiplicity of infection
H = Homogenate
I = Infected leukocytes
C = Control

TABLE 13

EXUDATE LEUKOCYTE - MENINGOCOCCUS INTERACTION USING MENINGOCOCCI RECOVERED FROM INFECTED EXUDATE LEUKOCYTES

M -1027

Infection Number	Preparation	Colony forming units/ml				
		0 Hr.	1 Hr.	2 Hr.	3.5 Hr.	5 Hr.
Infection 1 $\underline{m}$ = 6	NOT DONE	N O T D O N E				
Infection 2 $\underline{m}$ = 0.0003	Homogenate	1	0	0	0	0
	Infected Leukocytes	0	0	0	0	0
	Control	0	0	0	0	0

M-1628

Infection Number	Preparation	Colony forming units /ml				
		0 Hr.	1 Hr.	2 Hr.	3.5 Hr.	5 Hr.
Infection 1 $\underline{m}$ = 7	NOT DONE	N O T D O N E				
Infection 2 $\underline{m}$ = 0.001	Homogenate	0	1	1	1	2
	Infected Leukocytes	0	1	0	0	0
	Control	4	0	4	0	2

$\underline{m}$ = Multiplicity of infection

FIGURE 1

PROCEDURE FOR ELICITATION, HARVESTING AND WASHING OF PERITONEAL EXUDATE LEUKOCYTES

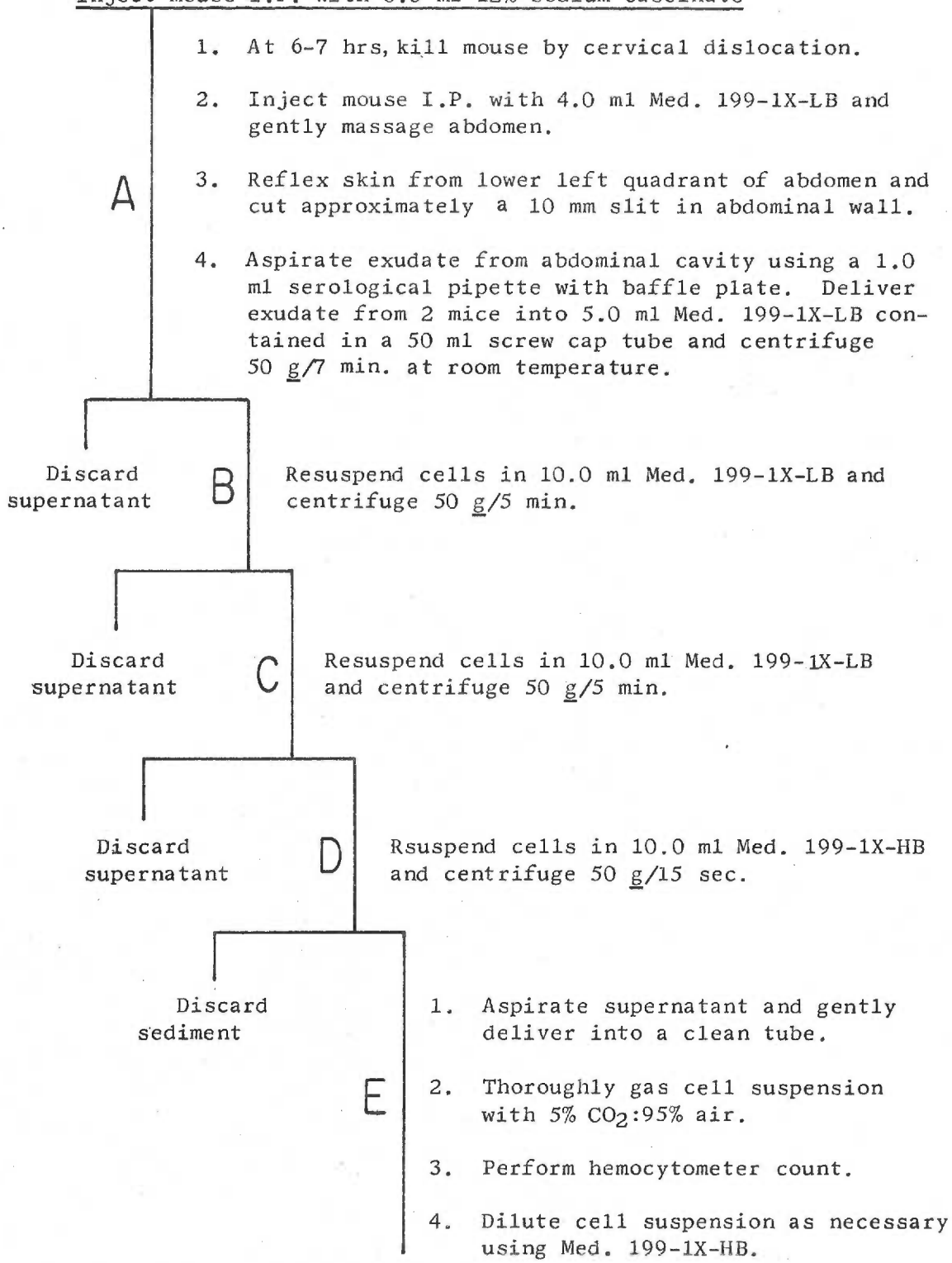


FIGURE NO. 2 VIABLE COUNTS OF M-1207 AND M-1628 AFTER SUSPENSION IN DEXTROSE STARCH BROTH. Bacteria were suspended in DSB medium and held at the various dilutions for 3 hrs at room temperature.

M-1027 M-1628

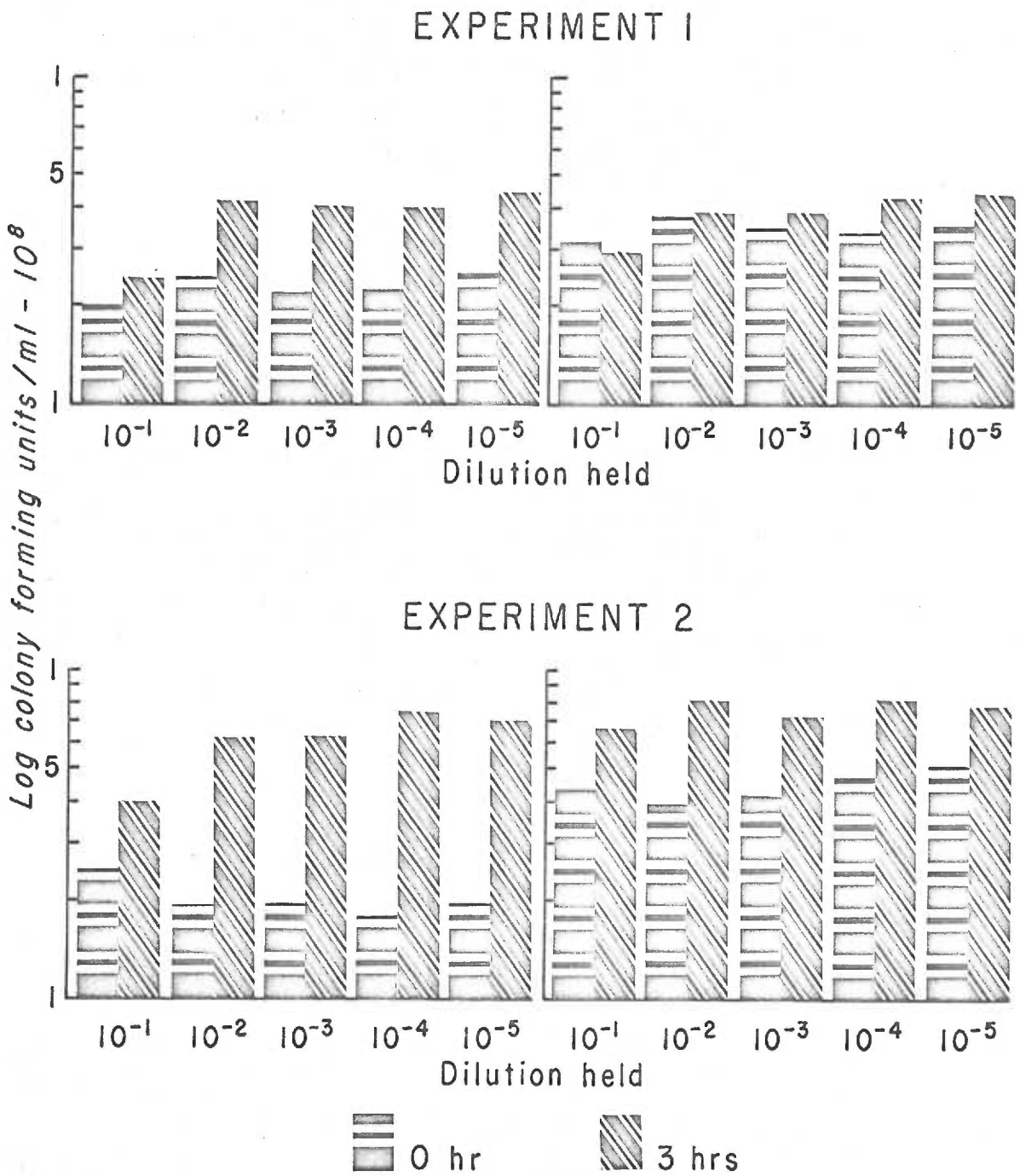

EXPERIMENT 1
Log colony forming units/ml - 10⁸
1
5
1
10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵
Dilution held
EXPERIMENT 2
1
5
1
10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵
Dilution held
0 hr
3 hrs

FIGURE NO. 3. VIABLE COUNTS OF M-1027 AND M-1628 AFTER SUSPENSION IN VARIOUS DILUTIONS OF MEDIUM 199 IN PHOSPHATE-BUFFERED SALINE. Bacteria were suspended in 0.02 M K_2HPO_4:KH_2PO_4 pH 7.0 with 0.85 per cent NaCl at 37 C for either 5 or 6 hrs as indicated. The numerical values for zero time counts, 5 or 6 hr counts, and fold change, are given in Table 6. Unwashed bacteria were used in experiments 1, 2, and 3. Washed bacteria were used in experiment 4.

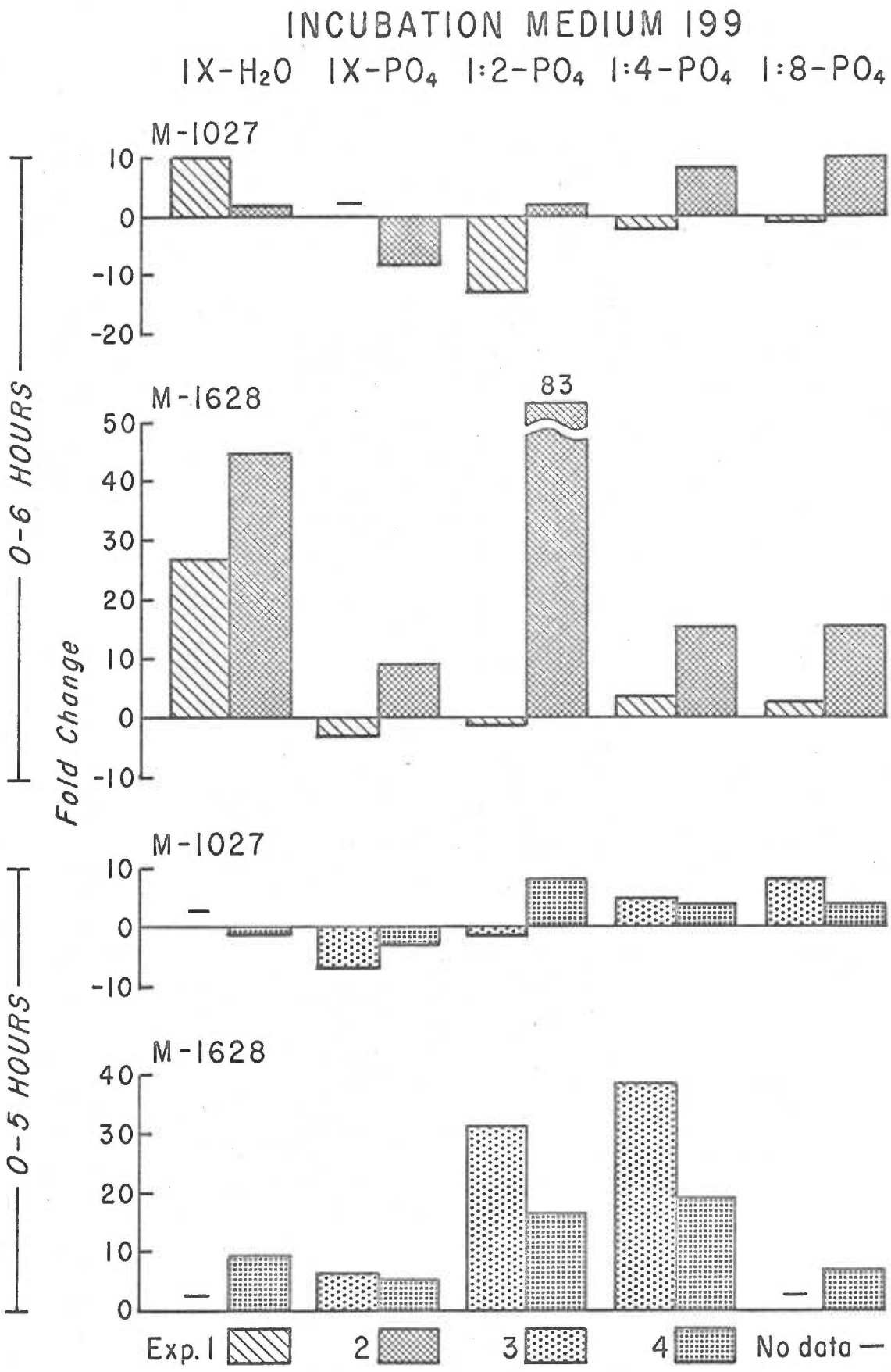
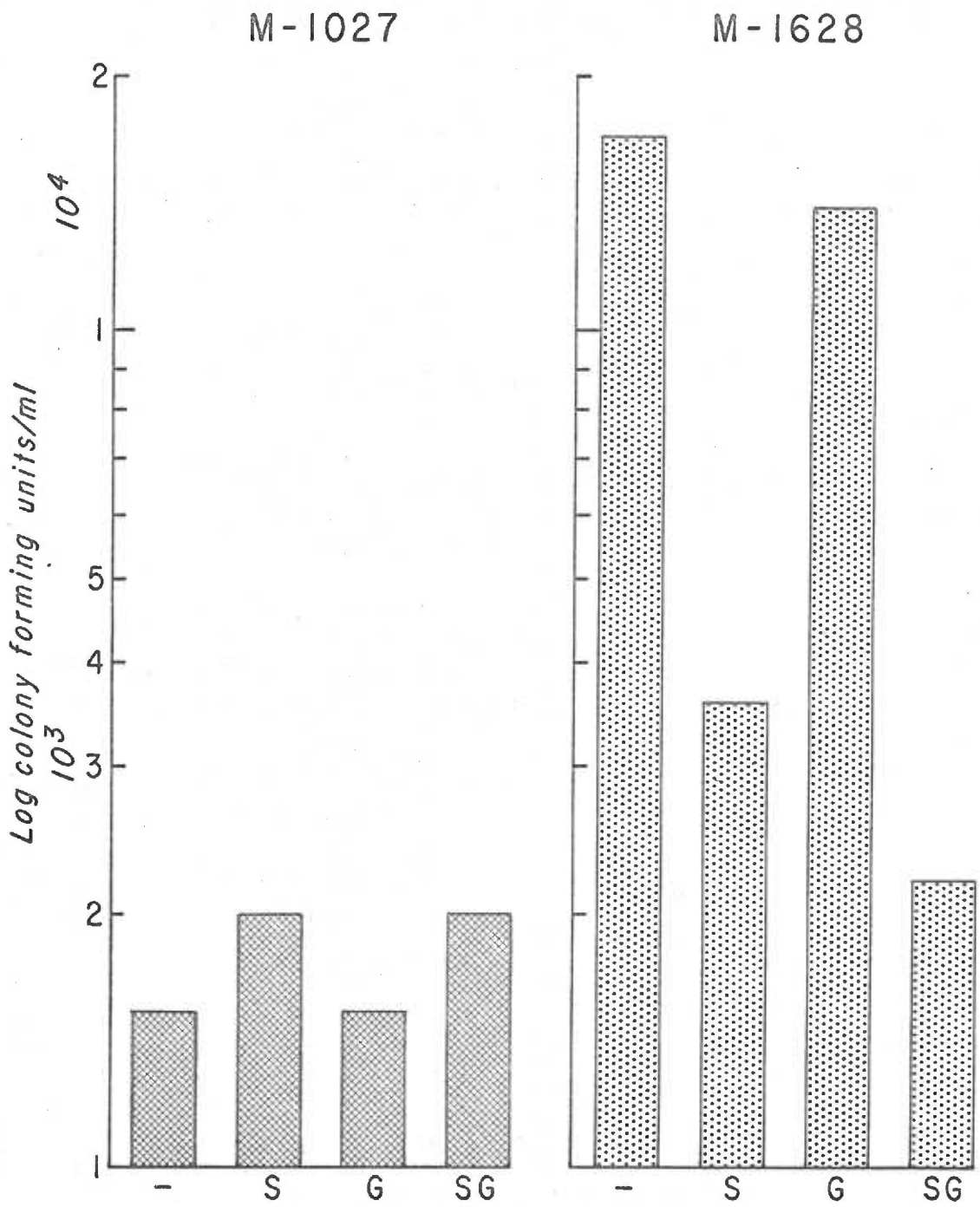

INCUBATION MEDIUM 199
1X-H2O 1X-PO4 1:2-PO4 1:4-PO4 1:8-PO4
M-1027
M-1628
83
M-1027
M-1628
Fold Change
0-6 HOURS
0-5 HOURS
Exp. 1
2
3
4
No data

FIGURE NO. 4. EFFECT OF HOMOGENIZATION ON VIABILITY OF M-1027 AND M-1628 IN MEDIUM 199-1:8-PO_4. Homogenization was conducted at a Powerstat setting of 140 for 2.0 min. All homogenates were centrifuged at 100 g for 1.0 min before samples were plated for viable counts.



BAR	GLASS SLIP	0.25% SUCROSE
–	–	–
S	–	+
G	+	–
SG	+	+

FIGURE NO. 5. EFFECT OF pH ON ACID PHOSPHATASE ACTIVITY OF HOMOGENATE OF EXUDATE LEUKOCYTES AND BOEHRINGER'S STANDARD ACID PHOSPHATASE ENZYME. A 120 min incubation was used for the assay with homogenate of mouse peritoneal exudate cells. A 30 min incubation was used for the assay with Boehringer's standard acid phosphatase enzyme.

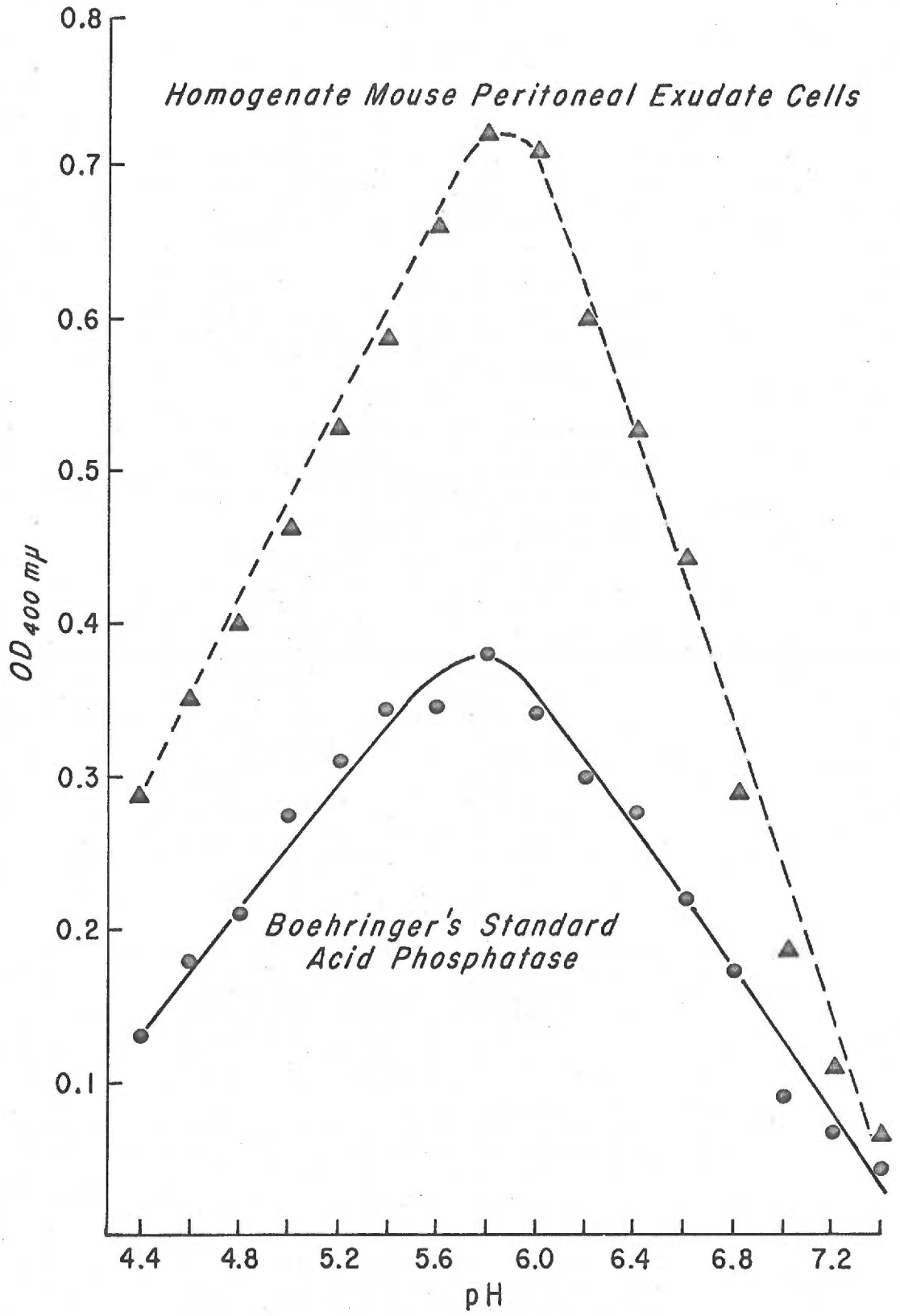

Homogenate Mouse Peritoneal Exudate Cells
Boehringer's Standard
Acid Phosphatase
OD 400 mμ
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
4.4
4.8
5.2
5.6
6.0
6.4
6.8
7.2
pH

FIGURE NO. 6. EFFECT OF HOMOGENIZATION WITH GLASS SLIPS AND SUBSEQUENT CENTRIFUGATION ON ACID PHOSPHATASE LEVELS OF EXUDATE LEUKOCYTES. Homogenization was conducted at a Powerstat setting of 90. Centrifugation was carried out at 3500 g for 5 min at 2 C.

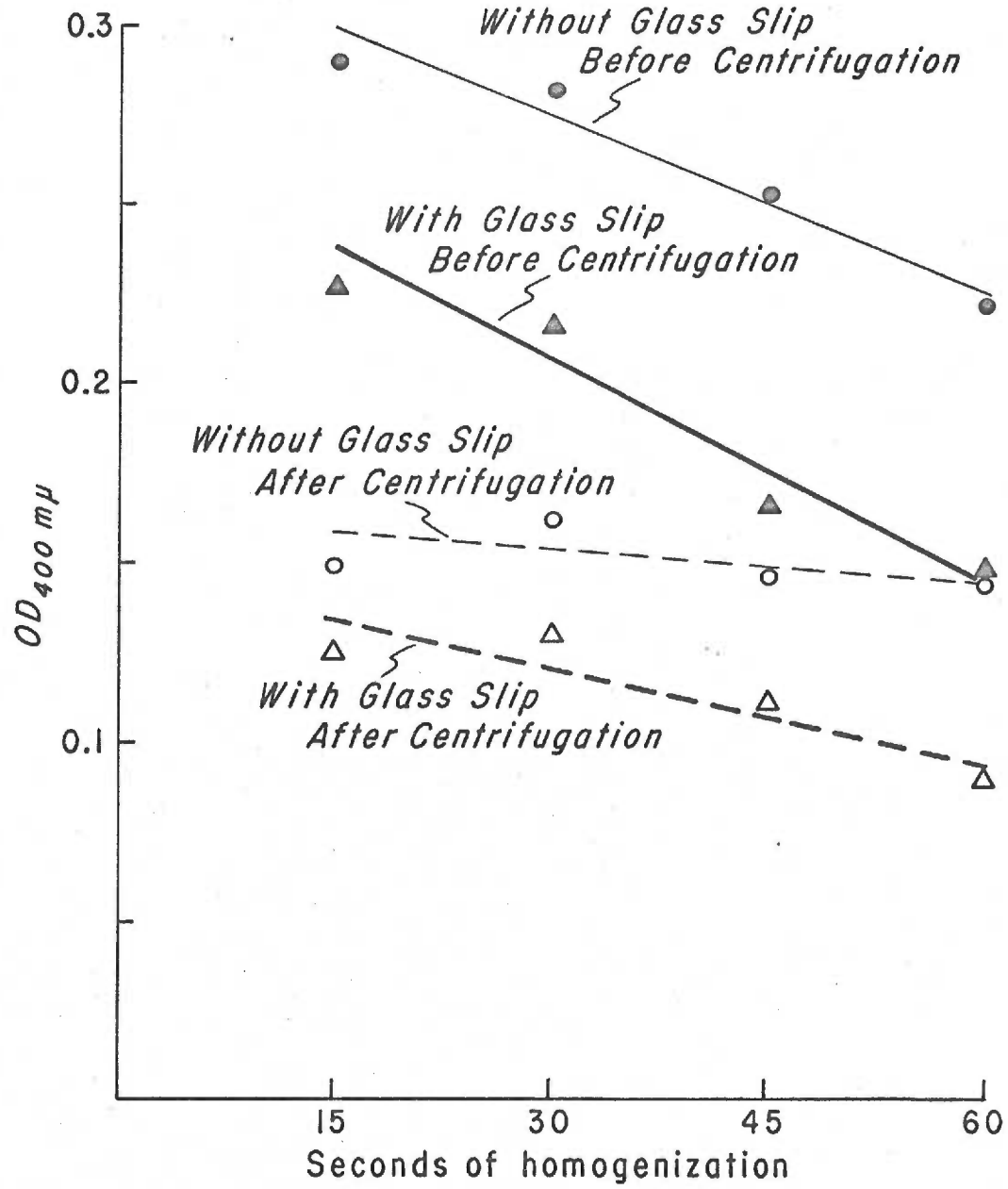

0.3
0.2
0.1
$OD_{400\ m\mu}$
Without Glass Slip
Before Centrifugation
With Glass Slip
Before Centrifugation
Without Glass Slip
After Centrifugation
With Glass Slip
After Centrifugation
15
30
45
60
Seconds of homogenization

FIGURE NO. 7. EFFECT OF SONICATION AND CENTRIFUGATION ON ACID PHOSPHATASE LEVELS OF UNCENTRIFUGED HOMOGENATES OF EXUDATE LEUKOCYTES. Homogenate was prepared at a Powerstat setting of 90 for 30 sec and settled for 5 min in ice to removed most glass particles. Sonication was carried out at 1.25 Amps. Centrifugation was conducted at 3500 g for 5 min at 2 C.

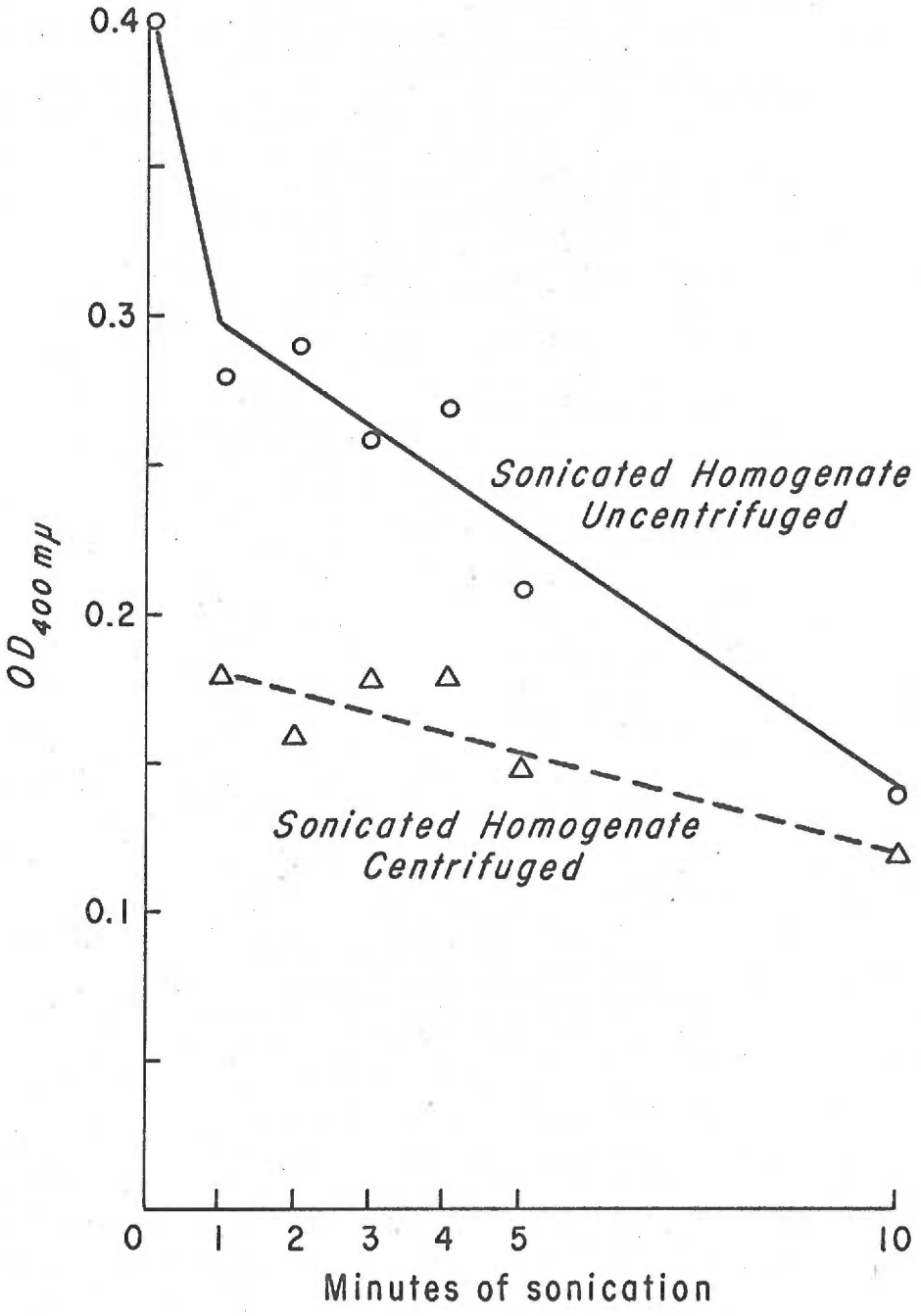

0.4
0.3
0.2
0.1
$OD_{400\,m\mu}$
Sonicated Homogenate
Uncentrifuged
Sonicated Homogenate
Centrifuged
0
1
2
3
4
5
10
Minutes of sonication

FIGURE NO. 8. EFFECT OF SAPONIN TREATMENT AND CENTRIFUGATION ON ACID PHOSPHATASE LEVELS OF HOMOGENATES OF EXUDATE LEUKOCYTES. Final concentration of saponin was 80 mg per ml. Preparations labeled "Untreated" and " + Saponin" remained at room temperature for 30 or 60 min, as indicated, before centrifugation at 3500 g for 5 min at 2 C.

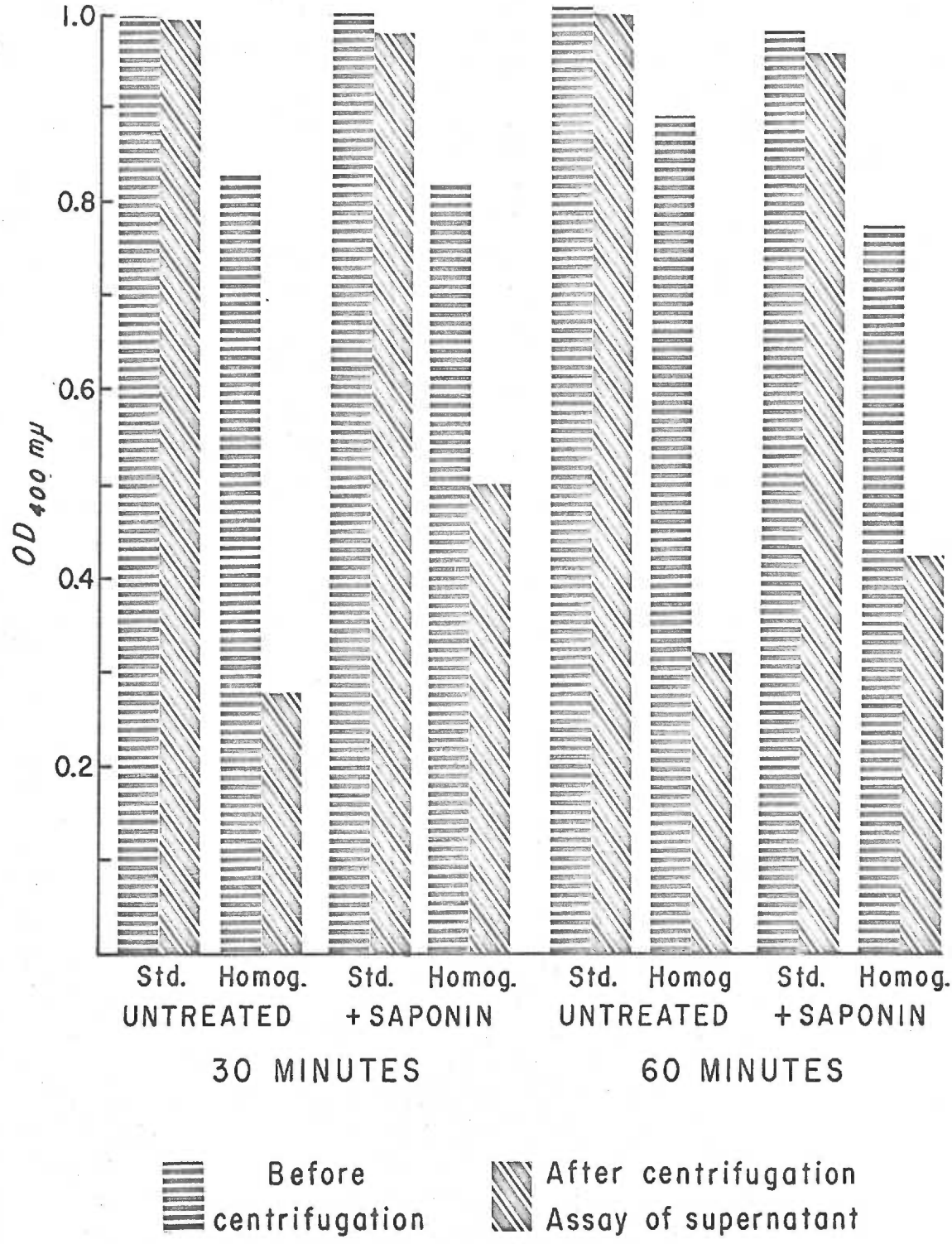

1.0
0.8
0.6
0.4
0.2
OD 400 mμ
Std.
Homog.
Std.
Homog.
Std.
Homog
Std.
Homog.
UNTREATED
+SAPONIN
UNTREATED
+SAPONIN
30 MINUTES
60 MINUTES
Before centrifugation
After centrifugation
Assay of supernatant

FIGURE NO. 9. RELATIONSHIP OF ACID PHOSPHATASE ACTIVITY TO THE NUMBER OF EXUDATE LEUKOCYTES. Final concentration of saponin was 0.0125 mg per ml.

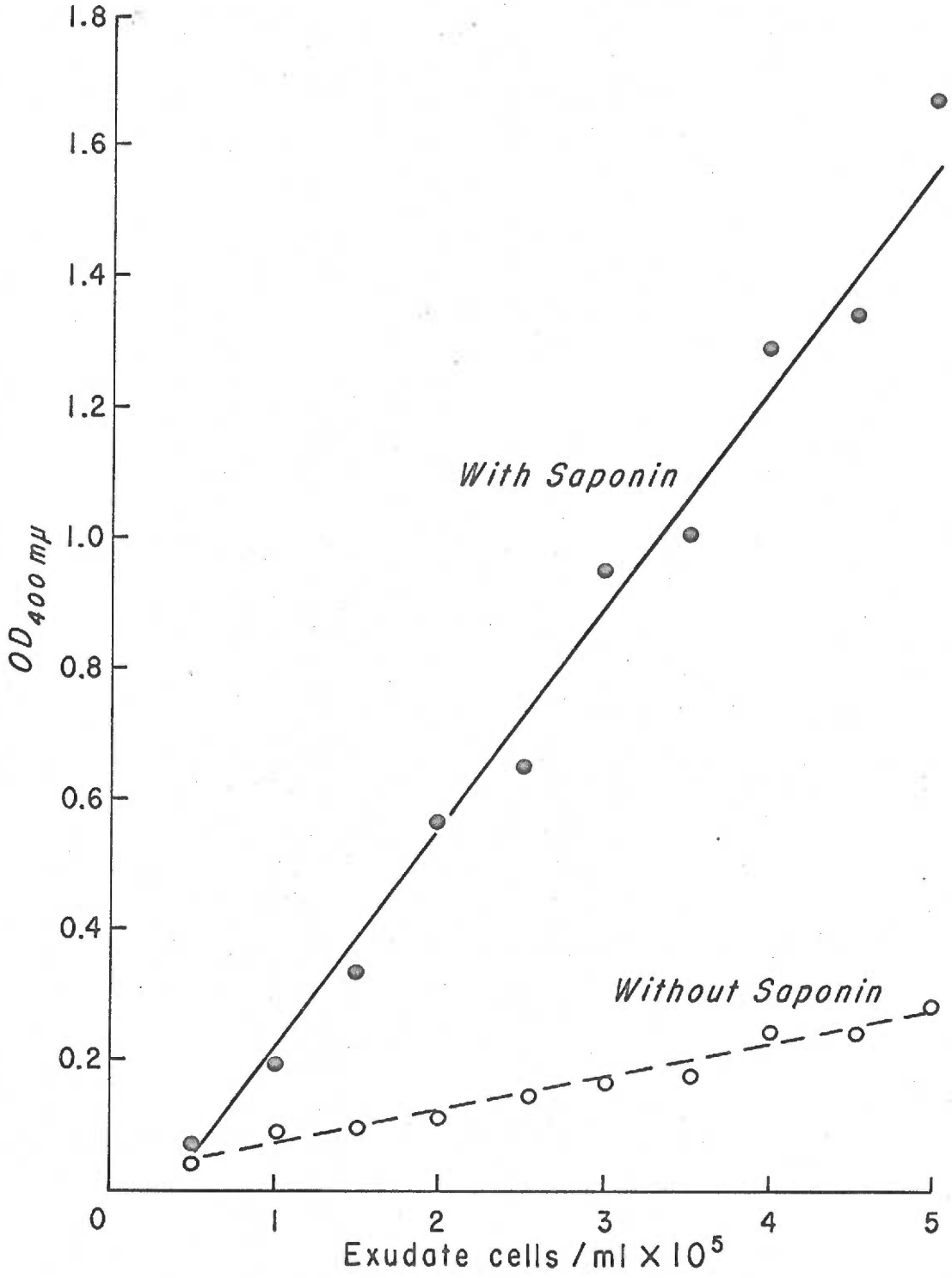

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
$OD_{400\,m\mu}$
With Saponin
Without Saponin
Exudate cells / ml X 10^5

FIGURE NO. 10. EXUDATE LEUKOCYTE - M-1027 INTERACTION AT m = 5 AND m = 14. Exudate leukocytes used were settled from suspensions containing 1.0×10^6 cells per ml. All plate counts were conducted at 0, 1, 2, 3.5, and 5 hrs. A - A single infected exudate leukocyte:glass slip was homogenized at each of the above time intervals. B - Two infected exudate leukocyte:glass slips were homogenized at zero time and samples removed at the above time intervals. C - A single washed control slip with only residual attached bacteria was homogenized at each of the above time intervals.

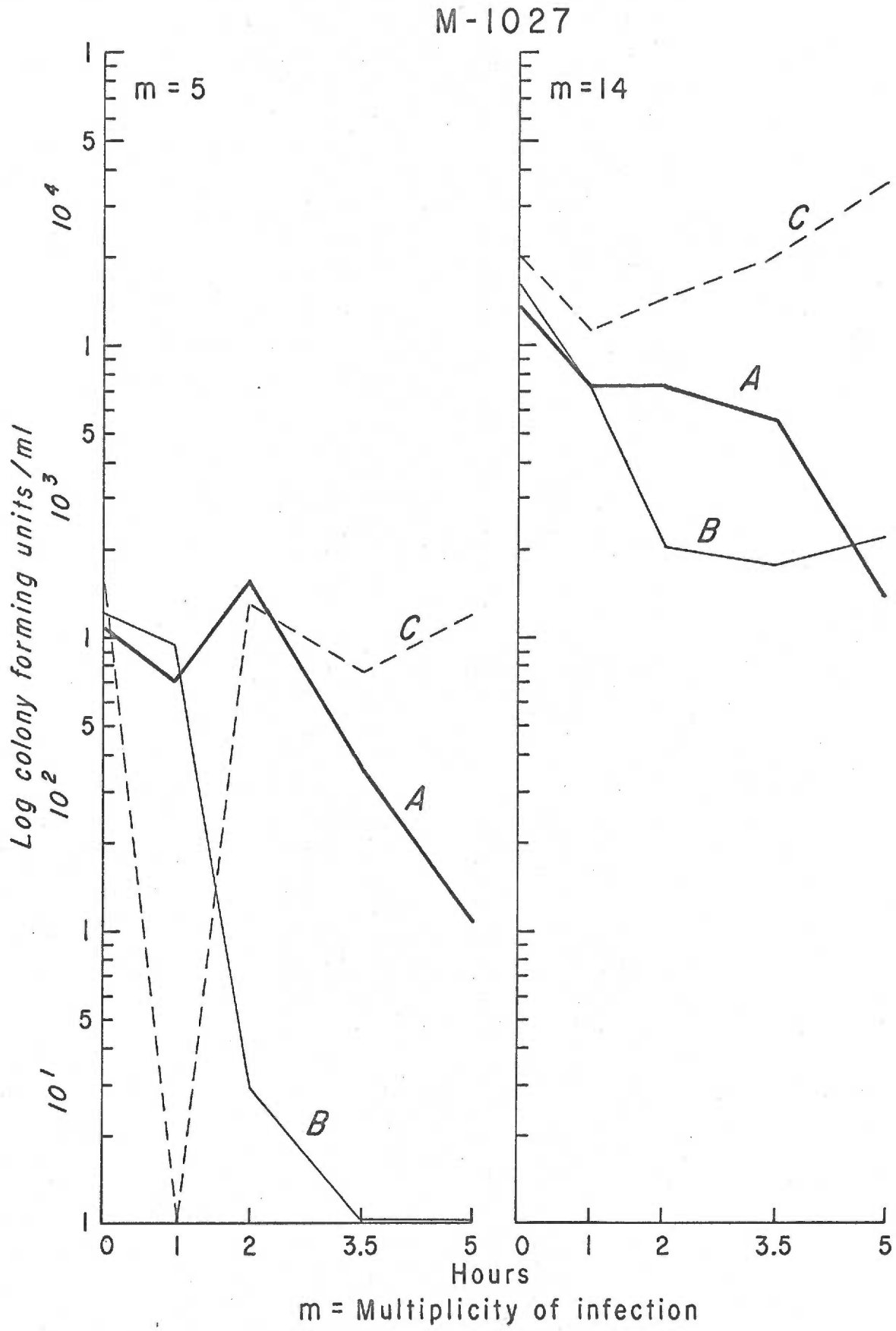
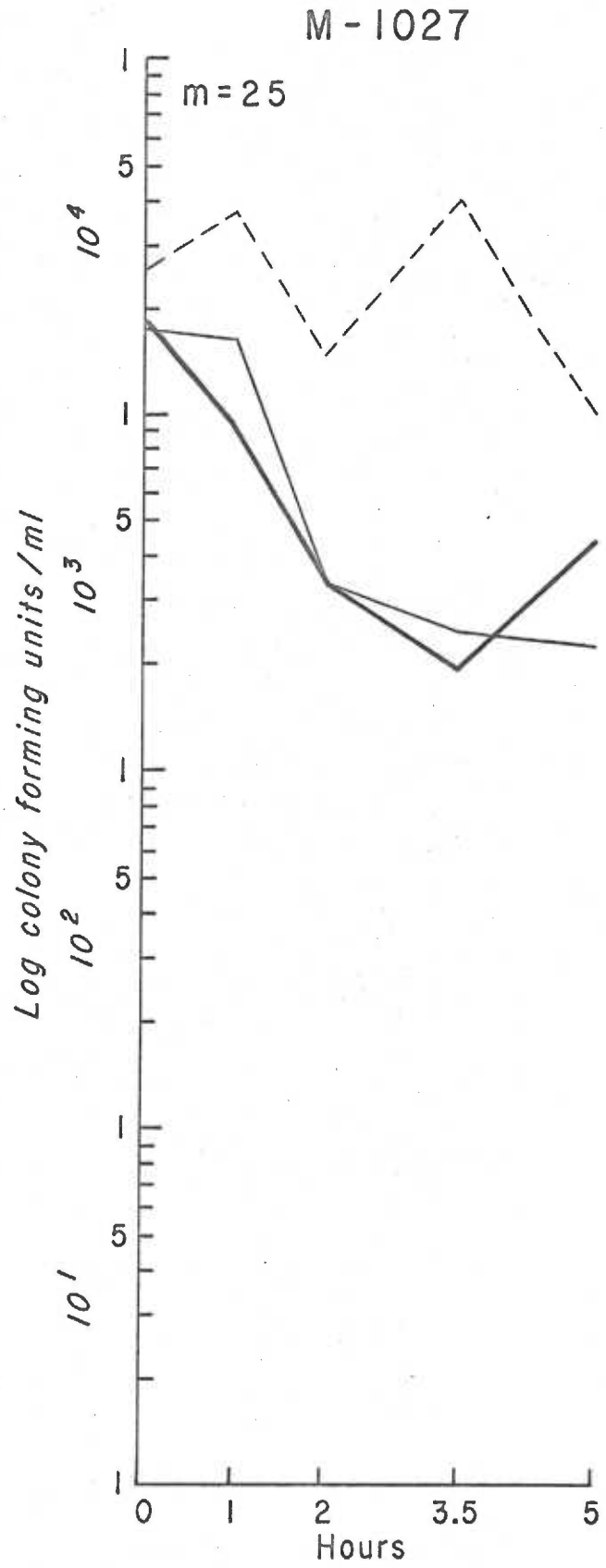

M-1027
m = 5
m = 14
Log colony forming units/ml
10⁴
10³
10²
10¹
1
5
A
B
C
0
1
2
3.5
5
Hours
m = Multiplicity of infection

FIGURE NO. 11. EXUDATE LEUKOCYTE - M-1027 INTERACTION AT *m* = 25. Exudate leukocytes used were settled from suspensions containing 1.0×10^6 cells per ml. All plate counts were conducted at 0, 1, 2, 3.5, and 5 hrs. *A* -A single infected exudate leukocyte:glass slip was homogenized at each of the above time intervals. *B* -Two infected exudate leukocyte:glass slips were homogenized at zero time and samples removed at the above time intervals. *C* -A single washed control slip with only residual attached bacteria was homogenized at each of the above time intervals.



m = Multiplicity of infection

FIGURE NO. 12. EXUDATE LEUKOCYTE - M-1628 INTERACTION AT m = 4 AND m = 7. Exudate leukocytes used were settled from suspensions containing 1.0 X 10^6 cells per ml. All plate counts were conducted at 0, 1, 2, 3.5, and 5 hrs. A - A single infected exudate leukocyte:glass slip was homogenized at each of the above time intervals. B - Two infected exudate leukocyte:glass slips were homogenized at zero time and samples removed at the above time intervals. C - A single washed control slip with only residual attached bacteria was homogenized at each of the above time intervals.

M-1628

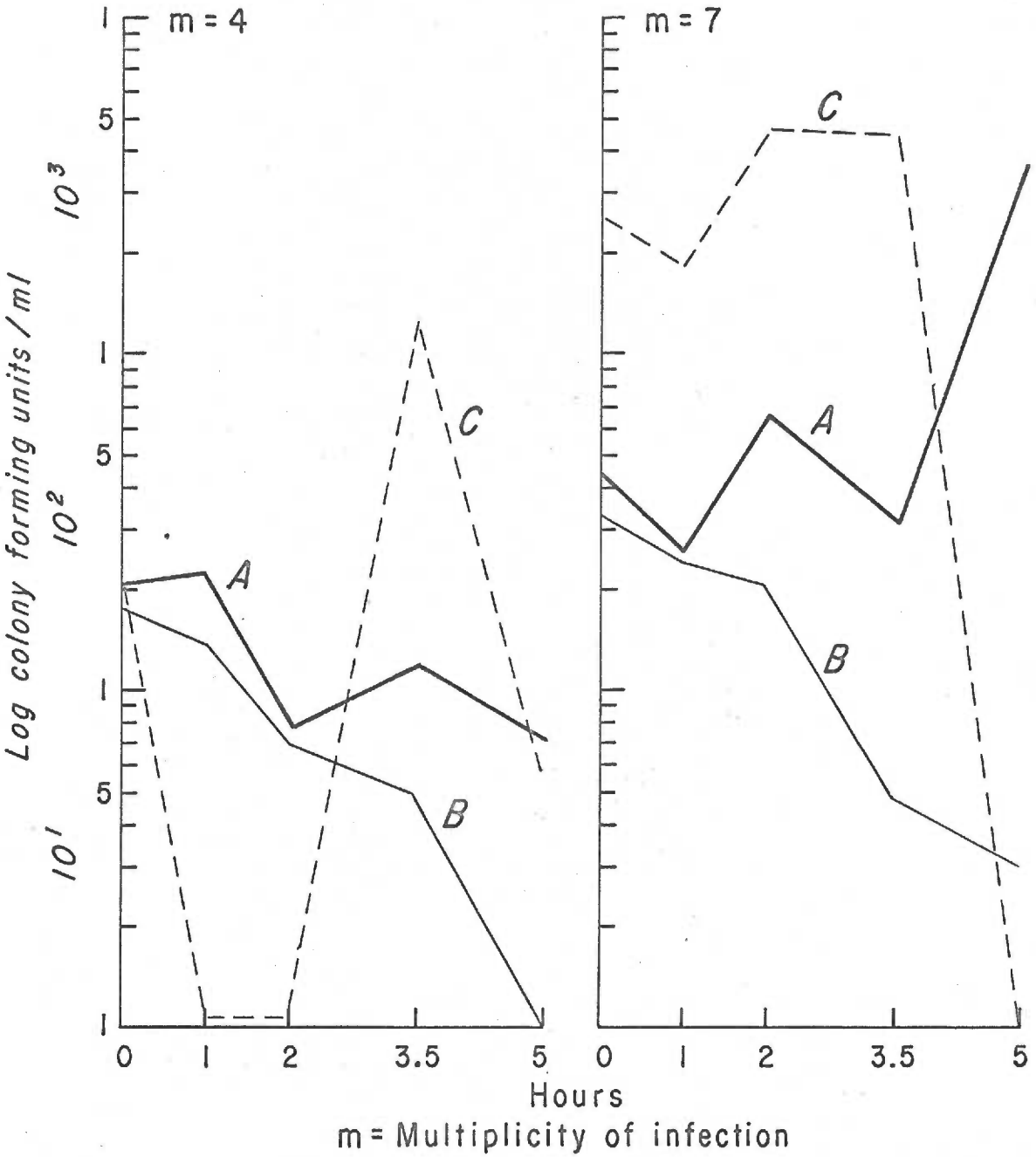

m = 4
m = 7
Log colony forming units / ml
1
5
10³
1
5
10²
1
5
10¹
1
A
B
C
0
1
2
3.5
5
Hours
m = Multiplicity of infection

FIGURE NO. 13. EXUDATE LEUKOCYTE - M-1628 INTERACTION AT *m* = 21 AND *m* = 30. Exudate leukocytes used were settled from suspensions containing 1.0 X 10^6 cells per ml. All plate counts were conducted at 0, 1, 2, 3.5, and 5 hrs. A - A single infected exudate leukocyte:glass slip was homogenized at each of the above time intervals. B - Two infected exudate leukocyte:glass slips were homogenized at zero time and samples removed at the above time intervals. C - A single washed control slip with only residual attached bacteria was homogenized at each of the above time intervals.

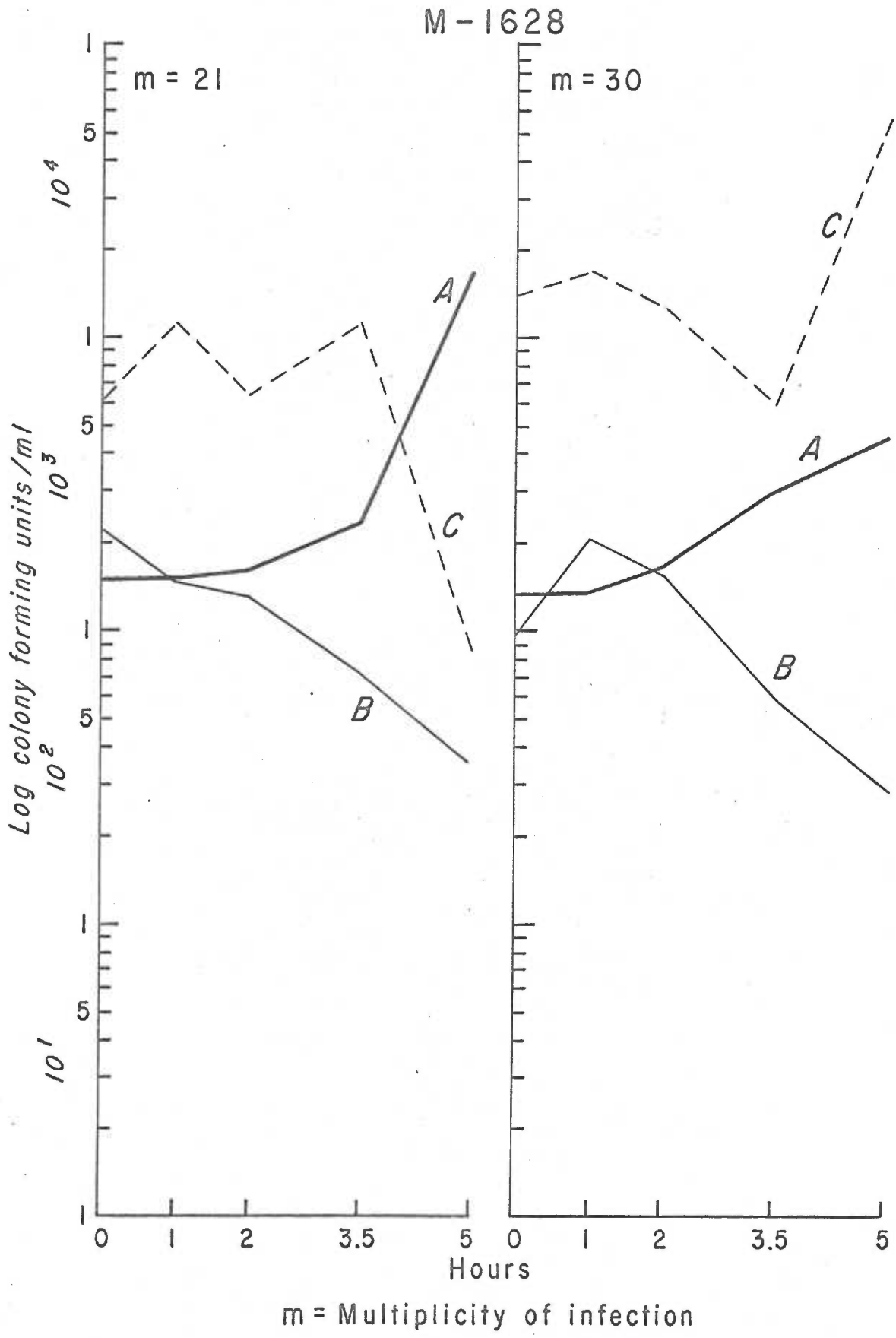

M-1628
m = 21
m = 30
A
B
C
Log colony forming units/ml
10^4
10^3
10^2
10^1
1
5
0
1
2
3.5
5
Hours
m = Multiplicity of infection

FIGURE NO. 14. EXUDATE LEUKOCYTE - M-1027 INTERACTION AT *m* = 5 AND *m* = 7. Exudate leukocytes used were settled from suspensions containing 2.0 X 10^6 cells per ml. All plate counts were conducted at 0, 1, 2, 3.5, and 5 hrs. *A* - A single infected exudate leukocyte:glass slip was homogenized at each of the above time intervals. *B* - Two infected exudate leukocyte:glass slips were homogenized at zero time and samples removed at the above time intervals. *C* -A single washed control slip with only residual attached bacteria was homogenized at each of the above time intervals.

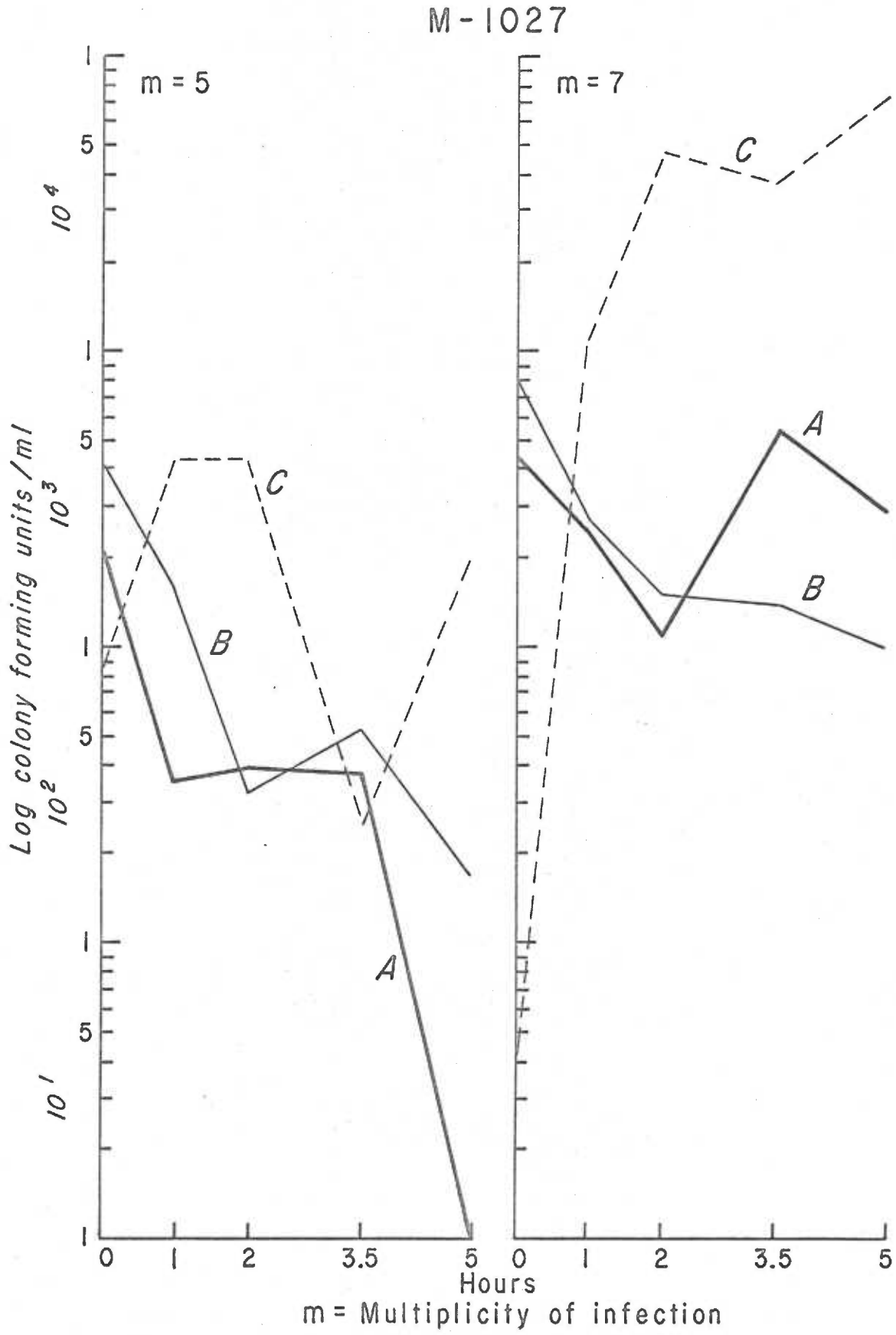
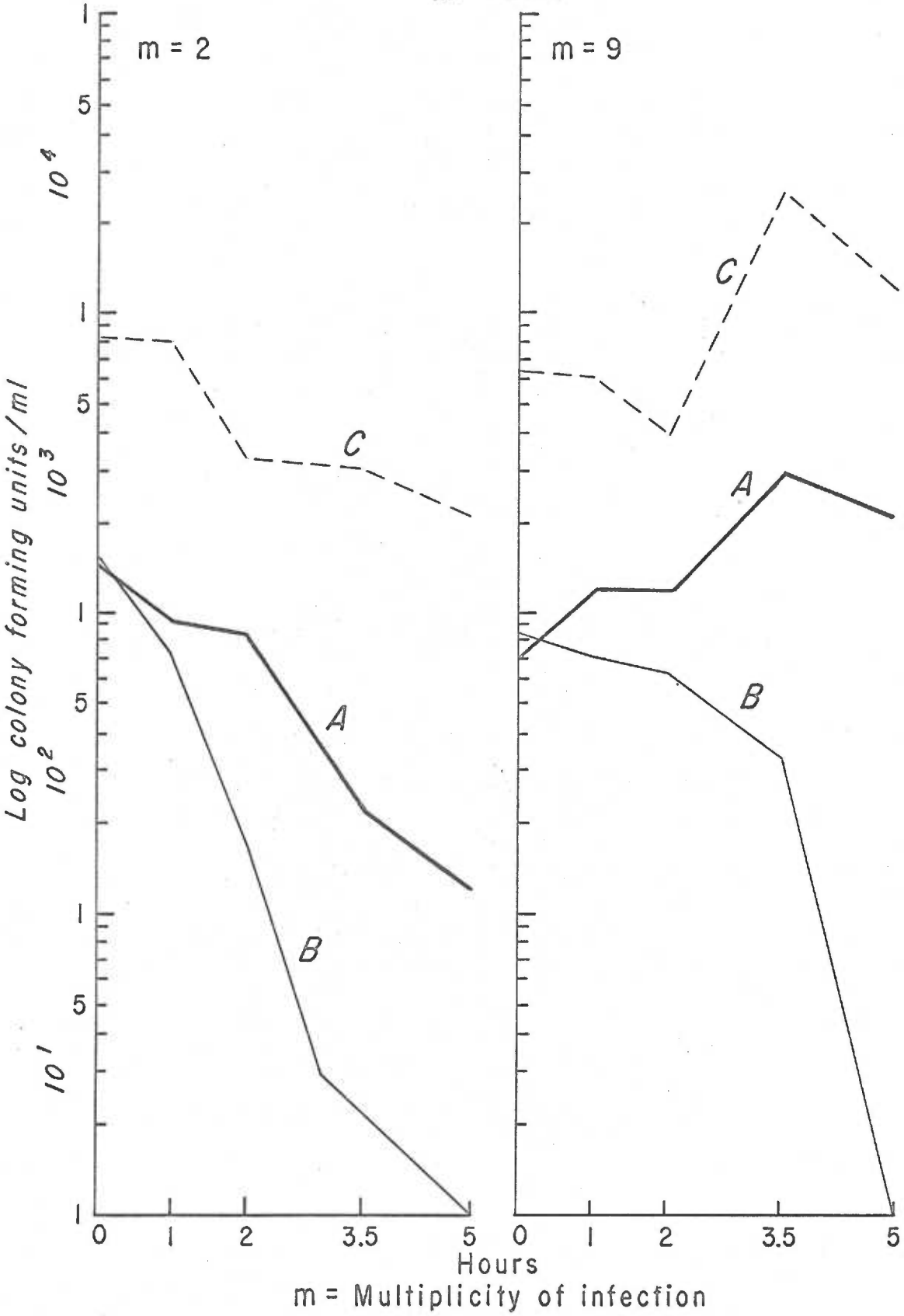

M-1027
m = 5
m = 7
Log colony forming units / ml
10⁴
10³
10²
10¹
1
5
A
B
C
0
1
2
3.5
5
Hours
m = Multiplicity of infection

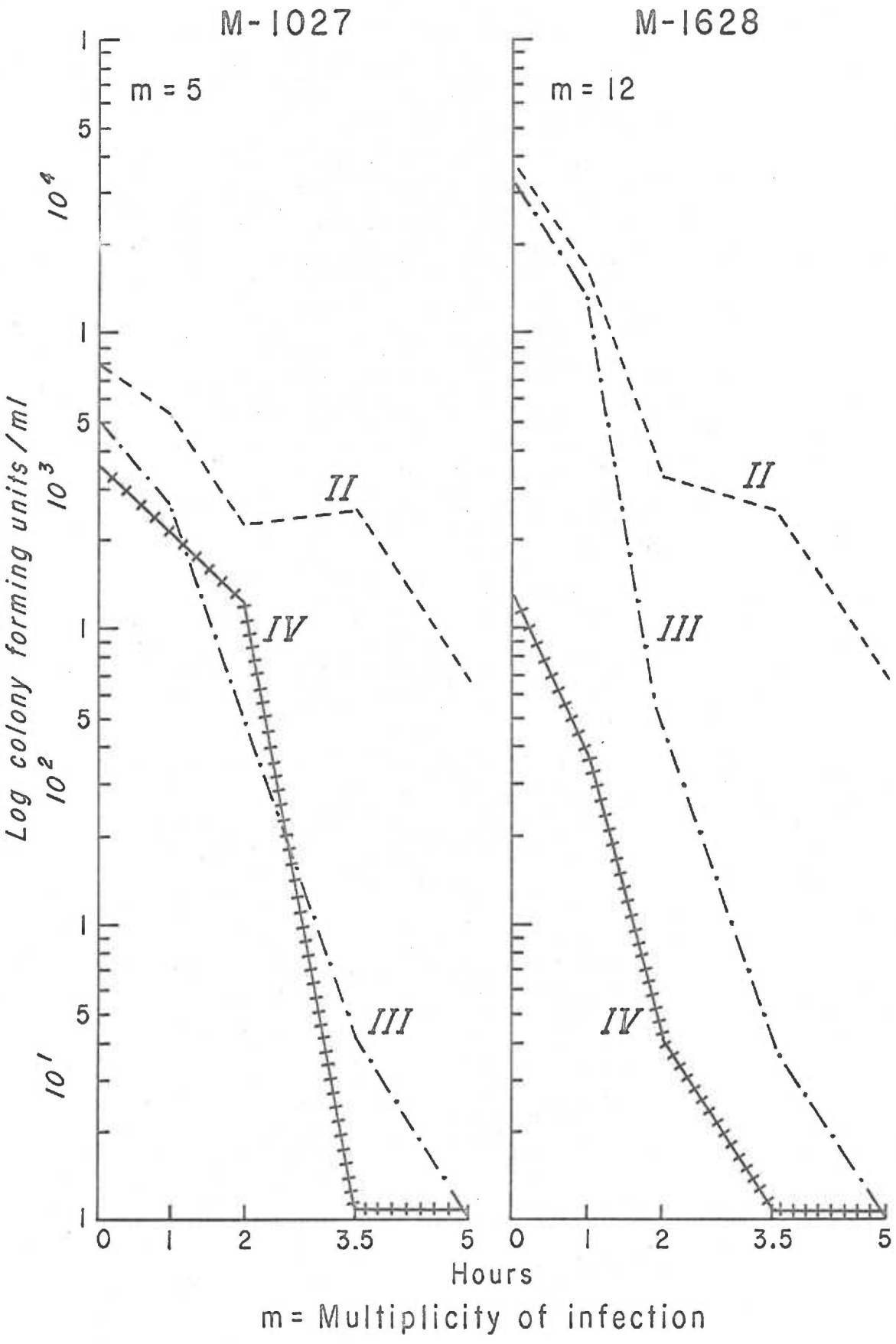
FIGURE NO. 15. EXUDATE LEUKOCYTE - M-1628 INTERACTION AT *m* = 2 AND *m* = 9. Exudate leukocytes used were settled from suspensions containing 2.0 X 10^6 cells per ml. All plate counts were conducted at 0, 1, 2, 3.5, and 5 hrs. *A* - A single infected exudate leukocyte:glass slip was homogenized at each of the above time intervals. *B* - Two infected exudate leukocyte: glass slips were homogenized at zero time and samples removed at the above time intervals. *C* - A single washed control slip with only residual attached bacteria was homogenized at each of the above time intervals.

M-1628



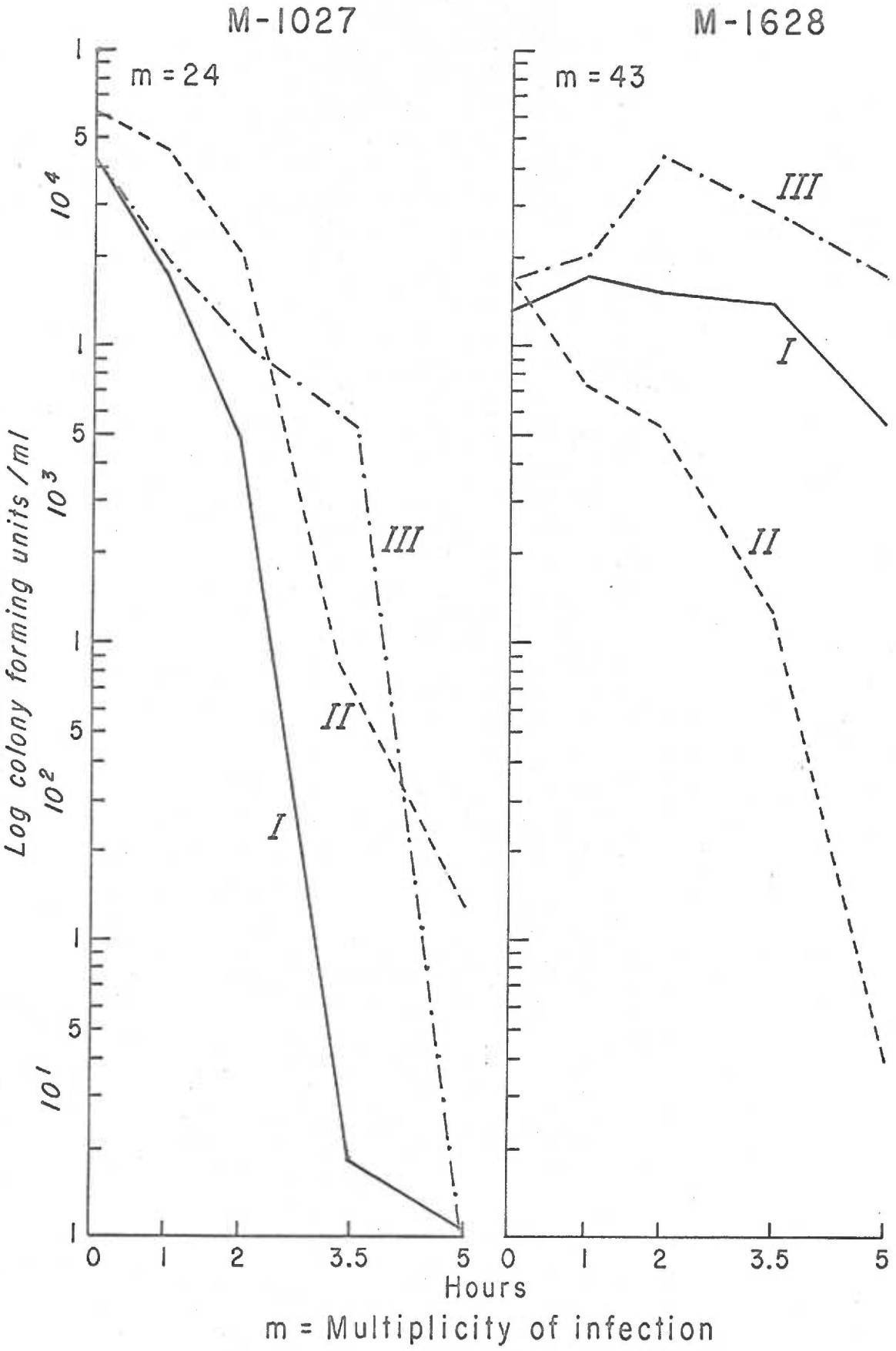
m = Multiplicity of infection

FIGURE NO. 16. EFFECT OF HOMOGENATES OF INFECTED EXUDATE LEUKOCYTES ON VIABILITY OF M-1027 AND M-1628. II - Homogenate of infected exudate leukocytes plus added bacteria. III - Homogenate of control slips which were merely glass slips plus added bacteria. IV - Homogenate of infected leukocytes.



m = Multiplicity of infection

FIGURE NO. 17. EFFECT OF HOMOGENATES OF UNINFECTED AND INFECTED EXUDATE LEUKOCYTES ON M-1027 AND M-1628. I - Homogenate of uninfected leukocytes plus added bacteria. II - Homogenate of infected exudate leukocytes plus added bacteria. III - Homogenate of control slips which were merely glass slips plus added bacteria.


M-1027
M-1628
m = 24
m = 43
Log colony forming units / ml
10⁴
10³
10²
10¹
I
II
III
0
1
2
3.5
5
Hours
m = Multiplicity of infection

SUMMARY AND CONCLUSIONS

I. Bacterial studies

Several differences in the characteristics of M-1027 and M-1628 were noted and are as follows:

A. The consistency of the growth of M-1027 and M-1628 on Mueller-Hinton medium differed, in that growth of M-1628 was more viscous and tenacious than growth of M-1027. This difference was noted particularly on emulsification of growth in the preparation of bacterial suspensions.

B. Observation of the time required for the outgrowth of colonies of countable size (about 1 mm in diameter) on Mueller-Hinton medium indicated that M-1027 required about 36 hrs and M-1628 required about 24 hrs incubation in candle-jars at 37 C.

C. Experiments were conducted to select a dilution of Medium 199 in phosphate-buffered saline which would prevent or minimize extracellular growth of both meningococcal strains. The results indicated that though it was possible to minimize growth of M-1027 and M-1628 by using Medium 199-1:8-PO_4 (a 1:8 dilution of Medium 199 in phosphate-buffered saline), growth could not be entirely prevented. Studies on the growth of M-1027 and M-1628 in various dilutions of Medium 199 suggested that M-1027 possessed a longer generation time than did M-1628. It was estimated that the generation time of M-1628 was 60 to 70

per cent that of M-1027 in Medium 199-1:8-PO_4.

D. Studies were done to determine whether viability of M-1027 and M-1628 was decreased in homogenization, as determined by viable plate counts on Mueller-Hinton medium. Bacteria were homogenized with or without glass cover slips in Medium-199-1X-HB (Medium 199 in distilled water plus 1.0 ml of 10 per cent sodium bicarbonate per 100 ml of medium), at a Powerstat setting of 90 for 15 sec. After glass particles were permitted to settle for 5 min, samples of the supernatant were removed from the homogenization chamber, and plate counts immediately performed. The results indicated that such conditions of homogenization did not affect viability of either strain.

E. Homogenization of M-1027 and M-1628 were also done in Medium 199-1:8-PO_4: a) with glass cover slips b) without glass cover slips, and c) with a final concentration of 0.25 per cent sucrose or d) without sucrose. Homogenization in this medium was done at a Powerstat setting of 140 for 2.0 min. After glass particles were sedimented at 100 g for 1.0 min, plate counts were immediately performed on the supernatants. The results indicated that homogenization of either strain with glass in this medium did not result in loss of viability as compared to homogenization without glass. In contrast to these results, homogenization in the presence of sucrose, with or without glass, resulted in a large loss of viability of M-1628, although not of M-1027. Homogenates of bacteria with

glass coverslips prepared at a Powerstat setting of 140 for 2.0 min, were immediately incubated at 37 C following centrifugation at 100 g for 1.0 min to sediment glass particles. Samples were removed from the incubated supernatants at various times for plate counts. The results indicated that M-1027 rapidly and consistently lost viability over a 5 hr period of incubation. M-1628 also showed a rapid loss of viability but results were not consistent and varied over a series of experiments.

II. Exudate leukocyte studies

Mice were injected intraperitoneally with 1.0 ml of 12 per cent sodium caseinate. The inflammatory peritoneal exudates were harvested at various intervals of time. The results indicated that exudates harvested at about 6 hrs after caseinate injection yielded populations of exudate leukocytes approximately 85 to 90 per cent polymorphonuclear leukocytes and 10 to 15 per cent mononuclear leukocytes. The proportion of mononuclear cells increased as the time of harvesting was delayed. The leukocytes from exudates harvested at 6 to 7 hrs were used in all experiments.

III. Acid phosphatase studies

A. In order to determine acid phosphatase content, exudate leukocytes were homogenized with glass cover slips for 15, 30, 45 and 60 sec, at a Powerstat setting of 90. Results indicated that homogenization did not result in complete

solubilization of acid phosphatase activity. Nearly 50 per cent of the enzyme activity was sedimented at 3500 g for 5 min at 0 C.

B. Homogenates of exudate leukocytes prepared with glass cover slips at a Powerstat setting of 90 for 15 sec were treated in attempts to completely solubilize acid phosphatase activity. Sonication of homogenate at 1.25 amperes for periods up to 10 min, did not result in the solubilization of enzyme activity above the level of unsonicated homogenate. Treatment of homogenate with a final concentration of saponin of 80 mg per ml for 30 or 60 min at 0 C, did not result in solubilization of enzyme activity above the level of untreated homogenate. This concentration of saponin did not affect the activity of Boehringer standard acid phosphatase as compared to untreated standard. All of the preceding results indicated that it was not possible to completely solubilize acid phosphatase of exudate leukocytes by homogenization or treatment of homogenate by sonication or saponin.

C. A method was worked out for enumerating the number of leukocytes attached to glass slips. The method was based on a linear relationship of acid phosphatase activity and number of leukocytes. Saponin at a final concentration of 0.0125 mg per ml resulted in a large increase in acid phosphatase activity over untreated cells. The method was, however, not sufficiently sensitive, without further refinements, to be

profitably used for this purpose.

D. Acid phosphatase activity in homogenates of leukocytes in Medium 199-1X-HB was rapidly lost at 37 C incubation over 5 hrs. In view of these results, assay of acid phosphatase in infected leukocyte bathing medium was considered an unlikely possibility.

IV. Exudate leukocyte-meningococcus interaction studies

A. Meningococci of strain M-1027 were phagocytized by mouse leukocytes attached to glass slips in Medium 199-1X-HB. The number of meningococci phagocytized per leukocyte was approximately the same in the presence or absence of normal mouse serum. Meningococcal strain M-1027 was phagocytized by both PMN and MN exudate leukocytes in Medium 199-1X-HB without serum.

B. Death of M-1027 and M-1628, at all multiplicities of infection tested, occurred following incubation of homogenates of infected leukocytes. These data indicate that leukocyte homogenates did possess the ability to kill both meningococcal strains.

C. Results on intact infected leukocytes indicated that M-1027 was killed at all multiplicities of infection and that M-1628 was killed only at low multiplicities of infection, but survived or grew at high multiplicities. Viable counts of extracellular bacteria, as determined using homogenized control

glass slips without attached exudate leukocytes, were irregular in that counts increased and decreased in a non-uniform way. The irregularity in counts indicated that the thorough washing of infecting bacterial suspension from the slips did not remove bacteria to the same extent from all slips even though the method was standardized. The irregularity in counts was probably due to the washing procedure and not to intracellular growth, but the very irregularity of counts make it difficult to assess whether extracellular bacterial growth did occur and to what extent.

D. Meningococcal strains M-1027 and M-1628 were homogenized at a Powerstat setting of 140 for 2.0 min: a) with uninfected leukocytes b) with leukocytes infected with the respective strain. The homogenates were centrifuged at 100 g for 1.0 min to sediment glass particles and incubated at 37 C. Samples were removed at intervals over a 5 hr incubation and viable plate counts performed. The results indicated that homogenates of infected leukocytes were more effective in killing added M-1628 than homogenates of uninfected leukocytes at the multiplicity of infection of 43 bacteria per leukocyte. In the case of M-1027, the death of bacteria in homogenate of bacteria and glass slips occurred nearly as rapidly as in homogenates of bacteria and infected or uninfected leukocytes. Thus, it was not possible to compare the effect of homogenates of infected and uninfected leukocytes on M-1027.

E. Results of sequentially linked interaction experiments indicated that meningococci, phagocytized from a relatively large population of bacteria and after residence within exudate leukocytes, did not possess unique properties for extracellular multiplication.

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