THE PROPERDIN SYSTEM IN HOST RESISTANCE

by

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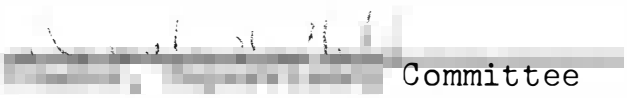

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INTRODUCTION

The ability of a host to resist infection can be broadly ascribed to humoral and cellular factors active against the invading organisms. Phagocytosis is one process by which the host is able to limit and destroy foreign matter introduced into the body. At present there is an abundance of information concerning the ingestion process and effects of various compounds on the phagocytic ability of phagocytes. In contrast, there is much less information concerning the fate of the ingested matter (cytopepsis). The lack of information concerning cytopeptic activity of phagocytes may be due primarily to unsuitable techniques for quantitating the digestion process.

The rate of ingestion of particulate matter by phagocytes has been shown to be increased in the presence of thermostable opsonin and thermolabile complement. Furthermore, properdin (Pillemer et al., 1954) which has been implicated as a humoral substance active in natural host defense against infectious disease, requires the participation of whole complement for its activation. The intimate relationship of properdin and complement, and the ability of these substances to participate in host resistance phenomena, led to the hypothesis that these humoral factors enhanced cytopeptic as well as phagocytic activities in addition to their other reported functions.

The studies to be reported have been conducted in an effort to investigate quantitatively the participating roles

of cellular and humoral factors in host resistance. Emphasis has been placed on the elucidation of the relationship between properdin and complement in the cytopeptic as well as the phagocytic process by employing isotope counting techniques.

REVIEW OF THE LITERATURE

In this thesis, the symbols of Pillemer *et al.* (1956) and Kabat and Mayer (1948) are employed as shown in Table 1.

TABLE I

SYMBOLS AND MEANINGS EMPLOYED IN COMPLEMENT AND PROPERDIN INVESTIGATIONS

Symbol	Definition
P	Properdin
RP	Serum deficient in properdin
Z	Zymosan
PZ	Properdin-zymosan complex
C'	Complement
C'1	First component of C'
C'2	Second component of C'
C'3	Third component of C'
C'4	Fourth component of C'
R1	Serum deficient in C'1
R2	Serum deficient in C'2
R3	Serum deficient in C'3
R4	Serum deficient in C'4

I. PROPERDIN

In 1953, Pillemer and co-workers at Western Reserve University reported that zymosan, an insoluble residue of yeast cells, required Mg^{++}, heat-labile factors resembling C'1 and C'2 and a hydrazine-sensitive factor resembling C'4 for the inactivation of C'3. Subsequent experiments were carried out in an attempt to isolate C'3 by eluting this heat-stable lipid or lipoprotein (Raffel, 1953) from zymosan. The experiments were unsuccessful; however, additional information was obtained concerning the interaction of zymosan with C'3. Pillemer et al. (1954) reported that zymosan readily inactivated C'3 at 37°C but not at 15°C. If fresh serum was incubated with zymosan at 15°C and if the insoluble phase was removed, C'3 could no longer be inactivated by zymosan at 37°C. It was further reported that the insoluble product obtained by 15°C incubation was a complex between zymosan and a heat-labile factor; additional evidence that C'3 was not involved in the complex. This complex could be dissociated at 37°C in buffer solutions of high ionic strength to yield zymosan and the heat-labile factor. This new factor was provisionally called properdin, a term derived from the Latin perdere, meaning to destroy (Elberg, 1956).

A. Physical and Chemical Properties

Properdin has been found to be present in all mammalian sera tested although in different amounts. Pillemer et al. (1954) reported that the rat has the highest titer and the

guinea pig the lowest while the human is intermediate. Normal human serum contains 4-8 units of properdin per ml, but no properdin activity has been shown to be present in human spinal fluid, ascitic fluid, pleural fluid, colostrum, milk and extracts of white blood cells or platelets.

The physical and chemical properties of properdin of human origin have been determined by Pillemer's group. Human properdin has been purified by dissociating the zymosan-properdin complex with buffers of high ionic strength, followed by dialysis, solution and differential centrifugation (Pillemer et al., 1954; Pillemer et al., 1956). The purified protein is a euglobulin with an isoelectric point between pH 5.5 and 5.8. It is stable between pH 4.8 and 8.4 at ionic strength of 0.15. The euglobulin contains lipid, carbohydrate and phosphorus, comprises not more than 0.03 per cent of the total serum proteins, and has a sedimentation constant, in the purified form, of 24 to 30 S. This large sedimentation value suggests a large molecule with a molecular weight over 1,000,000 or 8 times that of gamma globulin. A unit of properdin has been reported to represent not more than 0.5 ug of protein nitrogen (Pillemer et al., 1956). In serum, properdin is stable to heating at 48°C; there is slow destruction of activity at 50°C; complete inactivation occurs at 56°C for 30 minutes. In contrast, purified properdin is stable at 66°C for 30 minutes, but rapidly inactivates in 5 minutes at 100°C. Storage of properdin at -70°C results in destruction of activity but storage temperatures of 0° or -20°C result in indefinite retention of activity

(Pillemer, 1957). When the sedimentation constant decreases to 3 S, the euglobulin is inactive. Properdin in serum or the purified state is stable to freezing and thawing. Properdin in serum is not destroyed by hydrazine or trypsin or by removal of divalent cations, but is inactivated by traces of detergents, soaps, and carbon dioxide. Purified properdin does not contain any complement components or blood clotting factors. In addition, no protease, lipase, amylase, esterase, or phosphatase activities have been ascribed to the purified euglobulin.

Properdin has been reported (Pillemer et al., 1954; Pillemer et al., 1956) to differ from antibody in many respects. Properdin does not have serological specificity; it is always present in normal serum; the euglobulin requires C'1, C'4 and Mg^{++} for complexing; it is inactivated by complexing with the third component of complement; in serum, properdin resides in Fraction III-1. In contrast to the above, Skarnes and Watson (1957) presented arguments suggesting that properdin and natural antibody are likely the same substance. These same authors do not subscribe to the naming of the antibacterial system as the "properdin system" since properdin itself represents only one of at least six components known to participate in the bactericidal process. They suggest also that the terminology of normal or natural antibody is ambiguous and should be discarded in favor of the term "properdin" unless it can be proved that antibody and properdin are distinct substances.

B. Interactions of the Properdin System

1. Polysaccharides. Originally, human properdin was recognized by its ability to complex with zymosan, an insoluble polysaccharide mixture derived from yeast cell walls. Under controlled conditions this properdin-zymosan (PZ) complex was able to combine quantitatively with and to inactivate C'3. Two distinct stages have been found to occur (Pillemer et al., 1954; Pillemer, 1956). In the first stage there is stoichiometric combination of properdin with zymosan. Cofactors required for this reaction include C'1, C'4 and Mg^{++}. Pillemer (1956) reported that this reaction proceeds slowly, if at all, at 1°C, but rapidly at 15°C. Kinetic studies by Leon (1956) on the reaction between properdin and zymosan showed that PZ was formed from either human or bovine serum at 4°C although the reaction time required was 18 hours as contrasted to 1 hour if the reaction was carried out at 15°C. The second reaction involves the PZ combination with C'3 and thus, inactivation of the third component of complement occurs at temperatures above 20°C in the presence of Mg^{++}. Pillemer (1956) reported that this reaction appeared to be catalytic, that is, the reaction would not occur in the absence of Mg^{++} but the ion was not incorporated into the end product. Leon (1957) showed that the formation of PZ proceeded optimally at Mg^{++} concentrations of 0.001 M - 0.005 M; however, the resulting PZ inactivated C'3 in the absence of divalent cations. The conflicting data was suggested to be due to a difference between C'3

assayed with R3 and C'3 assayed with $EAgpC'_{1,4,2}$ or $EAhuC'_A$ (the complexes between sensitized sheep cells (EA) and guinea pig C' showing the activity of C'1, C'4, and C'2, and the complex between EA and human C' showing the activities of C'1, C'2 and C'4, respectively).

Wardlaw and Pillemer (1956) reported that of 22 metal ions of the alkali, alkaline earth and heavy metal groups tested, only Mg^{++} restored bactericidal activity of resin-treated serum in physiologic concentrations. Cobalt, manganese and iron also restored bactericidal activity in concentrations between 10^{-2} - 10^{-3} M; however, these concentrations were much greater than those occurring normally in serum and the possibility of these ions participating in the properdin system were considered remote. Further studies by Wardlaw, Blum and Pillemer (1958) showed that only Mg^{++}, Co^{++} and Mn^{++} were able to restore bactericidal activity due to the properdin system of serum depleted of cations by ion exchange procedures. Mg^{++} was reported to be quantitatively the most effective, i.e., twice as active as Co^{++} and 5 times as active as Mn^{++} on a molar basis. Ca^{++} was shown to be completely inactive. When Mg^{++}, Co^{++} or Mn^{++} was added to resin-treated serum at concentrations that restored the bactericidal activity, it was shown that the ability of the serum to form PZ at 17°C was also restored. However, only Mg^{++} or Co^{++} was effective in restoring to the resin-treated serum the property of rendering C'3 inactive in the presence of zymosan and at 37°C. Mn^{++} was ineffective for the latter reaction. As Pillemer (1956) stated "...the interactions of

the properdin system are indeed complex, and their significance must await further studies along these lines." Thus, resolution of the conflicting reports as to the divalent requirements of the properdin system need further experimentation.

Zymosan is not unique in its ability to form the PZ complex. Zymosan preparations isolated by DiCarlo and Fiore (1958), employing the procedure of Pillemer et al. (1956), have been classed as types A and B on the basis of serological activity. Composition studies of the two types revealed that zymosan is composed, on the average, of 54.7 per cent glucan, 18.8 per cent mannan, 14.5 per cent protein, 6.6 per cent fat, 3.2 per cent inorganic material, and 0.8 per cent chitin on the basis of accounting for 98.6 per cent of the hydrolyzed material. The analytical differences between type A and type B were no greater than the variations noted among different type A preparations. These authors concluded that the difference in serological activity of types A and B cannot be due to gross chemical composition, and they suggested that surface structures and charges determine zymosan activity.

Pillemer et al. (1955) investivated the mechanisms of action of properdin with zymosan and of properdin with various bacteria and other agents. It was found that all polysaccharide-properdin complexes did not inactivate C'3; however, the majority of the reactions resulted in the agent complexing with properdin. Among some of the agents tested were bacterial cell walls, endotoxins and lipopolysaccharides, dextrans and levans from bacterial filtrates, and lipopoly-

saccharides, polysaccharides and mucins of mammalian tissue origin. These substances were all highly branched and had molecular weights of 10^7 or greater. No correlation was shown to exist between the toxicity, pyrogenicity and antigenicity of these substances and their ability to interact with properdin. Pillemer (1956) stated that no correlation existed between the activities of these substances and any physical or chemical property. It was shown that polysaccharides of very similar structure and identical polymer units exhibited widely different activities. The active materials contained both α and β linkages, pyranosidic and furanosidic units, and a variety of interhexose linkages within the same compound. The various activities were present regardless of the presence or absence of lipids or proteins. In vivo experiments by Landy and Pillemer (1956) have shown that as little as 1 to 10 ug of bacterial lipopolysaccharides caused a marked elevation in properdin titers in mice. Similarly mammalian polysaccharides caused elevated properdin levels in very small amounts. It was suggested that serum properdin levels may be the result of stimulation or depletion of properdin by both bacterial and host products.

2. Bactericidal reactions. Ross et al. (1955) and Pillemer and Ross (1955) reported that the intravenous injection of zymosan into mice resulted in a marked initial decrease in serum properdin levels that was followed by an increase that was dose-dependent. The initial decrease in properdin levels was proportional to the dose of zymosan administered, however, the subsequent rise in titer was

inversely proportional to the dose, i.e., the smallest dose resulted in a very pronounced elevation while the largest dose resulted in a return of properdin titers to less than original levels present over a 10 day test period. Rowley (1955, 1956) showed that polysaccharides derived from E. coli cell walls, as well as zymosan, resulted in altered resistance of mice to subsequent E. coli challenge. It was suggested that since E. coli polysaccharides would react with properdin in vitro, similarly to zymosan, that the decreased levels of properdin caused by the injection of E. coli polysaccharide in vivo could account for the susceptibility of the mice to the bacterial challenge.

The bactericidal activity of the properdin system was investigated by Wardlaw and Pillemer (1956). Some 44 strains of a variety of species of bacteria were tested. It was shown that twenty strains were killed in fresh serum and by serum deficient in properdin (RP) to which properdin was added. These strains were viable in RP alone and classified as susceptible. Fourteen strains were viable in serum and RP plus properdin and were classified as resistant. Ten strains were killed in RP alone, as well as in serum and RP plus properdin. These ten strains were viable in properdin alone and were classified as unknown. The strains tested included Shigella sp., Salmonella sp., E. coli, A. aerogenes, Proteus sp., Pseudomonas sp., Paracolobactrum sp., and B. subtilis. Properdin proved to be inactive against Staphylococcus aureus. The presence of specific agglutinins in serum interfered with the bactericidal activity of pro-

perdin against Shigella dysenteriae. The bactericidal activity of the properdin system was shown to occur optimally in concentrations between 1 and 10 units per ml RP in vitro. As little as 0.01 unit per ml of RP showed definite bactericidal effects. Concentrations of properdin above 10 units per ml RP were inhibitory, i.e., a concentration of 60 units of properdin per ml showed less bactericidal activity than a concentration of 1 unit per ml.

Blattberg (1956) was able to increase the bactericidal activity of rabbit and guinea pig serum for E. coli B by a series of subcutaneous injections of zymosan suspended in egg-white and incorporated in Freund adjuvants. These animals responded to intracutaneous zymosan injections by exhibiting the Arthus phenomenon. Since zymosan absorbed the bactericidal activity of the sera without affecting the hemolytic activity, it was proposed that the properdin fraction of the serum contained the substances which destroyed the bacteria.

Landy and Pillemer (1956a) observed a transient increase in properdin levels of mice treated with small amounts of purified lipopolysaccharide derived from Salmonella typhosa. If the mice were injected with small amounts (1-10 ug) of the lipopolysaccharide (intraperitoneally or intravenously) 24 hours before challenge with certain gram negative organisms the mice were non-specifically protected, whereas the controls showed decreased properdin levels and died. Rowley (1955) had previously shown this protective effect by injecting E. coli cell walls into mice prior to challenge. Rowley noted that a highly susceptible state existed within 2 hours

after injection of the cell walls, but an increased resistance to infection resulted if the challenge of organisms was given 24 hours later. Rowley stated that no alteration of serum complement levels occurred during this time.

Ross (1958) reported that serum properdin levels of mice were markedly decreased and that death resulted when the mice were injected intraperitoneally with large numbers of viable E. coli. He was also able to show increased resistance to K. pneumoniae challenge if the mice were pretreated with zymosan. This nonspecific increase in resistance persisted in the mice for as long as 3 months after a single zymosan injection. Such treatment resulted in immediate decrease in properdin levels to a minimum in 2 hours, followed by an above normal level which lasted about 10 days. Increased resistance persisted despite the return of properdin levels to normal. It was also shown that purified human properdin administered intravenously into mice 2 or 4 hours before intraperitoneal challenge with K. pneumoniae resulted in marked protection. Of interest was the fact that the heterologous properdin was rapidly destroyed in vivo in 2 to 24 hours. In an addendum to the original paper, Ross stated that he was able to protect mice against K. pneumoniae infection with both heated (100°C, 30 min.) and undenatured properdin solutions.

Landy and Pillemer (1956b) were able to correlate the maintenance of, or increase in, serum properdin levels with increased resistance of animals to gram negative bacterial challenge doses, i.e., small doses of lipopolysaccharide

completely protected the animals when the challenge dose was administered 6 to 24 hours later. This correlation was not always exact, and in a few instances the properdin levels were merely maintained in test animals that showed increased resistance. It was concluded that other mechanisms of defense, in addition to properdin were active following lipopolysaccharide injections. More recently Rowley (1956) showed that a correlation between increased resistance of mice to infection and the bactericidal power of their serum existed following administration of E. coli O antigen. The pathogenic strains of E. coli were shown to be more anti-complementary than the nonpathogenic strains because of the increased content of O antigen. It was supposed that a substance like properdin could be involved in the mechanisms.

Alteration of properdin levels has been shown to occur with mammalian tissue polysaccharides as well as bacterial products (Pillemer, Landy and Shear, 1957). Resistance to infection was again correlated with properdin levels.

Recently Wood and Ono (1957) and Wood, Ono and Bessey (1958) described a method whereby antibacterial activity of sera on the test organism, B. subtilis, could be measured using the Warburg apparatus. It was found that components other than antibody were responsible for inhibitory effects. Inactivation of complement by heating the serum (56°C) removed part of the total inhibitory property. Subsequent readdition of complement restored the complete inhibitory properties of the serum. It was suggested that the complement dependent system was properdin.

Dubos and Schaedler (1956) observed an increased capacity of mice to resist challenge of gram positive organisms if lipopolysaccharides were administered prior to challenge. With the exception of one strain of B. subtilis (Wardlaw and Pillemer, 1956) gram positive organisms are resistant to the properdin system in vitro, and Dubos and Schaedler concluded that other defense mechanisms were activated by injection of the material. These same investigators (Schaedler and Dubos, 1956) have shown that increased susceptibility to infection resulted when the host is in a starved state. They concluded that the starvation period did not lower properdin levels and thus an active infection was initiated even in the presence of bactericidal concentrations of properdin. These authors used mice in their experiments; however, since the properdin system requires Mg^{++} and the four components of complement for its activation, and since the mouse serum has been reported to lack certain complement components (Brown, 1943; Rice and Crowson, 1950; Pillemer, 1956) and also exhibits no bactericidal effect (Marcus et al., 1954) then it is conceivable that an infection could originate in the mouse in spite of bactericidal concentrations of properdin in the serum. Landy (1956) showed that increased resistance following administration of lipopolysaccharide extended only to gram negative bacteria while changes in susceptibility to three species of gram positive organisms could not be demonstrated.

These observations of non-specific protection against infection by administration of killed organisms or injection

of extracts are not new. As early as 1893 Klein first observed that injection of various killed heterologous bacteria, either intraperitoneally or subcutaneously prior to challenge with lethal doses of cholera bacteria, protected the animals. Issaeff (1894) and Pfeiffer and Issaeff(1894) were able to protect guinea pigs against lethal doses of cholera organisms if the animals were treated previously with intraperitoneal injections of broth, peptone or urine. A marked leucocytosis was observed in the body cavity at the time of challenge, and these investigators thought the increased resistance was due to the release of bactericidal material from the leucocytes. Bedson (1915) injected autolyzed bacteria intravenously into animals and found that within a 2 hour period following injection there occurred a marked increase in opsonic activity as well as leucocytosis. This increased opsonic activity was non-specific in its action, was destroyed by heating the serum at 56°C, and disappeared from the serum after 24 hours. Hiss (1908) injected leucocytic extracts intraperitoneally into animals and found that this treatment protected the animals against challenge with both gram positive and gram negative organisms. A massive leucocytic infiltration was observed in the protected animals. When Wright (1931) injected animals with vaccines prepared from gram positive bacteria, an increase in bactericidal and phagocytic activities was observed. Furthermore, when these vaccines were added to normal serum in vitro, an increase in bactericidal power was noted. These changes were attributed to the release of opsonin from the damaged leucocytes.

It seems evident that injection of nonspecific materials stimulates many host defenses.

3. Viruses. In 1949, Ginsberg and Horsfall demonstrated that inhibition of influenza, mumps and Newcastle disease viruses by normal serum required a heat-labile factor, all components of complement and a divalent cation (thought to be Ca^{++} at that time). Further investigation showed that the unknown heat-labile factor was probably properdin and the divalent cation to be magnesium (Wedgwood, Ginsberg and Pillemer, 1956; Ginsberg and Wedgwood, 1956; Wedgwood and Pillemer, 1958). Experimental results showed that the properdin system can be equated to the heat-labile factor by the following criteria: (1) corresponding temperature dependence, (2) adsorption of properdin or heat-labile factor without significant complement titer reduction, and (3) reactivation of virus by reducing the availability of magnesium to the system.

Newcastle disease virus inhibition, within limits, was directly proportional to the properdin concentration. Optimal inhibition occurred between 2.5 and 5 units of properdin per ml of RP. Properdin concentrations of 10 units per ml of RP or greater appeared to be detrimental to the virus inhibition reaction (Wedgwood, Ginsburg and Pillemer, 1956).

According to Wedgwood and Pillemer (1958) all viruses are not susceptible to the properdin system. The polio virus was markedly resistant. However, it has been reported that bacterial viruses are inactivated

Van Vunakis, Barlow and Levine, 1956; Barlow, Van Vunakis and Levine, 1958a, 1958b). Recently the herpes simplex virus has been shown to be sensitive to a heat-labile inhibitor found in normal animal serum by Finkelstein, Allen and Sulkin (1958). These authors found that serum depleted of properdin had no effect, but became virucidal upon addition of purified properdin to the properdin-free serum. Complement was found to be essential for the reaction.

4. Properdin and irradiation. The effects of whole body irradiation on serum properdin levels of rats and mice were studied and reported by Ross et al. (1955) and Ross (1956). It was found that total body irradiation of rats (550 r) resulted in serum properdin levels rapidly falling to less than 30 per cent of pre-irradiation levels. The levels gradually returned to near-normal levels within a few weeks depending on the dosage of irradiation originally administered. During the same period whole complement titers and complement component titers were elevated, with the exception of C'1 and C'2. The C'3 titer increased the most in magnitude, i.e., 300 per cent above normal. Linder (1957) reported that rats exposed to 500 r whole body irradiation showed decreased serum properdin levels of 93 per cent of normal on the first post-irradiation day, 52 per cent of normal on the third post-irradiation day, and 34 per cent of normal on the eighth post-irradiation day. After the minimum level was reached on the eighth post-irradiation day, the level rose to 60 per cent of normal by the thirteenth post-irradiation day.

This rapid decrease in properdin levels can be partially explained by the fact that properdin only comprises 0.02 per cent of the total serum proteins and has an *in vivo* half life of 48 hours (Keller, Isliker, and Aebi, 1957). Since a cell is necessary for production of antibody and also properdin, then depression of activity or total inactivation of the cell as a result of irradiation, can make the explanation obvious.

Since bacteremia is one of the greatest causes of death in whole body irradiated animals, the value of properdin as a therapeutic agent was investigated by Ross *et al.* (1955), and Ross (1956). The intravenous injection of purified bovine and human properdin into whole body irradiated rats and mice was reported to significantly protect the animals against $LD_{100/30}$ day and $LD_{100/6}$ day doses of irradiation. The degree of protection was dependent on the dose, route, and time of administration of properdin. Animals treated on any post-irradiation day but the fifth day exhibited survival rates similar to or more accelerated than the controls. Animals treated on the fifth day showed a four-fold increase in survival rate. Properdin-treated mice exposed to 820 r whole body irradiation were reported to have gained weight and remained healthy up to 3 months after exposure. Ross *et al.* (1955) used zymosan in other irradiation experiments. It was reported that the intravenous injection of zymosan into rats and mice caused an initial reduction in serum properdin levels followed by a return to normal or above normal levels. Zymosan injected into animals before or

after exposure to irradiation increased or decreased the lethal effects of irradiation, depending on the time, dose and route of injection. Other high-molecular weight polysaccharides also partially protected the whole body irradiated mice when administered prior to exposure. Conversely, Haley et al. (1956) could not demonstrate the beneficial effects of zymosan in treatment of irradiation injury. These authors concluded that zymosan in the doses and modes of administration employed, was not beneficial to irradiated mice. Recently, Hardin et al. (1958) reported that survival of CFW mice exposed to whole body irradiation was enhanced by administration of human blood serum or saline. The results were attributed to supportive effects. Whole blood was reported to be no more effective than serum, and saline was found to be as effective as serum. The properdin system was reported not to enhance survival rate.

5. Miscellaneous reactions of the properdin system. Pillemer, Hinz and Wurz (1957) were able to prepare and characterize an anti-human properdin rabbit serum. The antibody was stable to heating at 56°C for 60 minutes; properdin was inactivated almost instantaneously by the antibody at either 37°C or 1°C; and the antiproperdin activity was removed by adsorption of the serum with PZ complexes, but not by either RP or zymosan. The authors suggested that in view of their results the use of properdin of heterologous origin for in vivo experimental work was undesirable because of the antigenic nature of properdin.

The relationship of the Toxoplasma antibody act-

ivator to the properdin system has been investigated by Gronroos (1955) and Feldman (1956). Six years before the discovery of properdin, Sabin and Feldman (1948) reported that a heat-labile serum component ("activator") was necessary in order for neutralizing antibodies to act against Toxoplasma gondii. Human serum was used as a source of activator since, in the absence of antibody, the serum was without effect upon the parasite. The role of various co-factors was investigated and it was shown that all four components of complement Mg^{++} were necessary for the Toxoplasma antibody to act on the parasite. After the properdin system was reported by Pillemer et al. (1954) the activator was restudied by Gronroos (1955) and Feldman (1956) from this point of view. Gronroos concluded that the activator is composed of properdin and C'2, C'3, and C'4. In addition, Mg^{++} was also assumed to be required. Feldman (1956) reported that complement or Mg^{++} alone did not enhance the activity of the antibody. Furthermore, it was reported that excess concentrations of properdin added to RP resulted in inhibition of the reaction, a situation similar to that reported for bactericidal and virus inhibition studies (Wardlaw and Pillemer, 1956; Ginsberg and Wedgwood, 1956). In view of these experiments it would appear that the heat-labile serum factor called "accessory factor" by Sabin and Feldman (1948) is similar to, if not identical with, the properdin system; however the role of C'1 remained obscure.

Recently the hypothesis was suggested that properdin

or properdin system components were nonspecific inhibitors of hyaluronidase in normal serum (NSI) by Hadidian, Mahler and Murphy (1957). A number of striking similarities existed such as: (1) NSI was thermolabile, protease sensitive and quantitatively precipitated from solutions of low ionic strength at pH 5.2-5.5; (2) the action of NSI on hyaluronidase required Mg^{++} or Co^{++}; and (3) NSI action on hyaluronidase was inhibited in solutions of high ionic strength. However, and contrary to expectations, experiments conducted with zymosan showed that NSI could not be identified with properdin. Furthermore, the serum from hyaluronidase injected rabbits (a procedure that removed NSI) showed no loss of C' activity; thus NSI could not be identified with any C' component. A similarity of NSI with properdin was noted when lipopolysaccharide was injected into guinea pigs. There was an increase in titer of NSI in the serum due to treatment known to increase properdin levels in serum.

In 1958, Tauber and Garson investigated the effect of cortisone, properdin and reserpine on N. gonorrhoeae endotoxin activity. Cortisone was reported to protect mice from death due to N. gonorrhoeae endotoxin. When relatively large doses of properdin were used, results similar to those with cortisone were obtained. However, reserpine significantly enhanced the endotoxin lethality under certain conditions.

In 1957, Rowley briefly reviewed the properdin system and its role in immunity. In the discussion

Lawley stated: "It is entirely possible that properdin may possess 'opsonic' activity and increase the rate of uptake of bacteria by phagocytic cells, or that it may play a part in intracellular digestion." In the same year Rutenburg and Fine (1957) published the results of experiments designed to determine the effect of properdin on *in vitro* phagocytic function. It was concluded that phagocytic and bacteriostatic activities of phagocytes *in vitro* was not influenced by the properdin in plasma from normal or hemorrhagically shocked rabbits. Recently a report concerning the identity between the thermolabile fraction of opsonin and properdin was published by Nanni (1958). It was found that a marked decrease in opsonic activity occurred in serum treated with zymosan at 17°C (RP). The extent of decrease resembled that obtained when the serum was heated at 56°C for 30 minutes. If properdin was added to the RP or heated serum, normal opsonic activity was restored. If properdin was added to fresh serum no increase in opsonic power was noted, indicating that the relationship is qualitative rather than quantitative. Mg^{++} always brought about a marked increase in opsonization when added to fresh serum; however when used alone, Mg^{++} was ineffective. The results confirmed the accepted hypothesis that opsonin is composed of a thermolabile and thermostable fraction. The probable identity of properdin with the thermolabile fraction was based on the fact that the thermolabile fraction was destroyed by zymosan, and that activity could be restored by addition of properdin

to properdin depleted serum.

C. The Properdin System and Disease.

The possibility that the properdin system could be involved in human disease was reported by Hinz, Jordan and Pillemer (1956a), Hinz, Weisman and Hurley (1956b), and Hinz (1956). These investigators found that Mg^{++}, factors resembling complement components, and properdin were required for the *in vitro* hemolysis of abnormal erythrocytes from patients with a form of chronic hemolytic anemia known as paroxysmal nocturnal hemoglobinuria (PNH). No antibody was required for this reaction, indicating that the red blood cell is defective. Hinz *et al.* (1956a) reported that normal human serum hemolyzed the PNH cells, but serum lacking properdin (RP) or serum lacking any of the complement (C') components failed to hemolyze the PNH cells. The addition of properdin to RP restored the hemolytic activity of the serum, but properdin added to serum lacking C' components did not restore the hemolytic ability. The requirement for Mg^{++} was demonstrated by addition of different cations to serum that was passed through cation exchange resins. Ca^{++} did not restore the hemolytic activity at 1.5×10^{-3} M while 5×10^{-4} M Mg^{++} concentration resulted in complete restoration of the hemolytic activity. Excess Mg^{++} resulted in hemolytic inhibition, possibly because of an increase in total ionic strength. Recently, Hinz, Abraham and Pillemer (1957) reported that normal human red blood cells could be

hemolyzed *in vitro* with human serum if pretreated with tannic acid (TA). Although similarities existed between this reaction and the hemolytic reaction of PNH cells, some differences were apparent. The cofactors required for hemolysis of TA cells or PNH cells include properdin, Mg^{++} and C' components. In contrast hemolytic reactions involving isohemolysins and panhemolysins required specific antibody and C', but were stated to not require properdin or magnesium (Hinz *et al.*, 1957). The differences between TA cells and PNH cell hemolytic reactions included: (1) TA cells agglutinated spontaneously whereas PNH cells did not; (2) TA cells spontaneously hemolyzed after exposure to low temperatures but PNH cells were stable in the cold; and (3) TA cells lysed optimally in normal serum at a pH of 7.4 while PNH cells lysed optimally at pH 6.8. The degree of TA cell hemolytic reaction was found to be directly proportional to the amount of purified properdin added to RP; however, when whole serum was added to RP varying amounts of hemolysis resulted. Thus, while the TA cell hemolytic reaction could be used to assay purified properdin, it was not a reliable quantitative test of properdin in serum.

Recently Crosby and Benjamin (1958) were able to inhibit the *in vitro* hemolysis of PNH cells by the addition of dextran to the serum. Although the inhibition was not marked, it was accomplished with clinically reasonable concentrations of dextran. One mechanism of action suggested by the investigators was that the properdin preferred the dextran molecule over the PNH cells and thus was unavailable for the

hemolytic reaction. Stratton, Wilkinson and Israels (1958) employed dextran infusions on 2 patients with PNH. In both cases the treatment was successful in that the hemoglobinuria ceased. The results indicated that more clinical experimentation be attempted since PNH occasionally stops spontaneously.

Hinz (1956) reported that normal properdin levels have been obtained from patients with chronic recurrent infections in which gamma globulin levels are normal and also in other afflictions such as bronchiectasis, hepatic cirrhosis, acute leukemia, diabetes, nephrosis, and agammaglobulinemia. With regard to agammaglobulinemia, Barandun and Isliker (1956) and Soulier and Menache (1958) have reported that low properdin values were found in patients afflicted with this disease. However, the former authors stated that their assay techniques may be imperfect and thus may be responsible for the large variations in properdin levels observed. Furthermore, it was reported by Hinz (1956) that steroid treatment did not change the properdin levels of patients. Other patients in whom normal properdin levels were observed included: (1) patients who were markedly malnourished, (2) those who had myocardial infarctions, and (3) patients afflicted with tuberculosis and rheumatoid arthritis of acute and chronic nature. In contrast to the results reported by Hinz for the last named disease, Laurell and Grubb (1958) reported that sera from 8 patients with rheumatoid arthritis assayed less than 1 unit of properdin, while prozones were observed in the other 5 samples tested. These authors stated that their results confirm the work

of Eyquem and Tullis (1957) that properdin titers are frequently low or non-determinable due to prozone phenomena in the zymosan assay procedure. Perhaps the different properdin levels obtained by Laurell and Grubb (1958) and those reported by Hinz (1956) can be attributed to technical details.

Hinz (1956) reported that low properdin levels were found in patients infected with gram negative organisms, PNH, and pneumococcal pneumonia. He also stated that normal levels of properdin were found in patients with various localized and metastatic malignancies including both lymphoma and carcinoma. In contrast, serum properdin level studies by Rottino and Levy (1957), Southam and Pillemer (1957), and Rottino, Levy and Conte (1958) had shown low concentrations in patients with epidermoid cancer, adenocarcinoma, and sarcoma; normal amounts were reported in patients with Hodkin's disease, chronic lymphatic leukemia or lymphosarcoma.

Properdin level studies have been carried out by several investigators in other fields. Data by Benson et al. (1955) showed that properdin levels are not influenced by anesthesia or uncomplicated surgery. No changes were observed following splenectomy or adrenalectomy. An interesting study was carried out on patients who received localized radiotherapy to various parts of the body with doses as high as 3000 r. In each case no properdin level changes occurred as a result of treatment.

Wiss, Weber and Isliker (1957) reported that serum

properdin levels of the rat were markedly decreased as a result of pantothenic acid deficiency but the level remained uninfluenced by vitamin B_1 deficiency. The deficiency symptoms (reduced growth, disturbed acetylation ability, decrease coenzyme A concentration in the liver, and low properdin levels) were intensified by the addition of a pantothenic acid antagonist called sodium DL-ω-methylpantothenic acid.

In cases of hemorrhagic shock, one of the causes of death has been attributed to endotoxemia or bacteremia. Properdin levels in dogs were determined before and after induced hemorrhagic shock by Frank, Fine and Pillemer (1955). The dogs showed a distinct decrease of properdin in their serum within 1 to 2 hours after the induced bleeding. The reduction was more marked later in shock. The properdin titer remained at a minimal level despite blood volume deficit corrections later in the shock period. No significant changes in C' titers were evident.

A study by Lowbury and Ricketts (1957a, 1957b) of the properdin system in patients who had been burned showed that the properdin concentration is reduced in the serum. However the bactericidal capacity of the sera against a properdin sensitive Shigella organism from patients with no detectable properdin was in the same range as the sera from normal controls containing definite amounts of properdin. Several strains of gram negative bacteria isolated from burn wounds were found to be resistant to the bactericidal action of serum. It was suggested that

due to the prevalence of properdin-resistant organisms in burn wounds, properdin was not a significant factor in protecting burns against gram negative bacterial infection. It was quite possible that the lower serum properdin level of burn patients increased their susceptibility to infection by organisms normally killed by the properdin system. These authors stated that since it appeared that the properdin system could not kill the bacterial flora in established burn infections, it would not be useful as a therapeutic agent although properdin probably is an important co-factor in the defense mechanisms of uninfected tissue.

Weld and Kean (1958) reported that Trichomonas vaginalis were killed when injected intravenously into rabbits. Subsequent in vitro experiments showed that the blood itself was trichomonicidal although the reticulo-endothelial system was probably active in ridding the blood of many organisms in vivo. The in vitro study was conducted on 54 serum specimens obtained from humans as well as from guinea pigs, rats, hamsters, dogs and rabbits. In each case the serum specimens had the capacity to kill and lyse the organism although the time required varied. The rat and rabbit sera completely lysed the orgainism within 3 minutes whereas 4 to 5 minutes were required by the human, rabbit, guinea pig and hamster sera. The activity of the sera was destroyed by exposure to a temperature of 56°C for 30 minutes. Implication of the properdin system in this trichomonicidal reaction was discussed since the rat and rabbit have considerably higher

properdin levels than the other species sera tested (Pillemer et al, 1954; Pillemer et al., 1956).

Recently DeWitt (1958) studied the differential effect of hog gastric mucin on properdin and host resistance to infection. Although considerable variation in infection-promoting ability and in vitro antiproperdin activity was observed among different mucin samples, all the samples examined possessed uniformly high in vivo antiproperdin activity. Concomitant with the antiproperdin activity, distinct changes in host resistance to infection were noted, but the relationship was not causally related. It was suggested that the ability of hog gastric mucin to increase the virulence of certain infectious agents can be ascribed to the "...result of a complex interaction of numerous effects directed primarily against the host." (DeWitt, 1958)

Herbut and Kraemer (1958a) extended and substantiated the work originally reported by Kidd (1953) concerning a tumor inhibitory factor present in guinea pig serum. The factor was found to be active against lymphosarcoma 6C3HED in C3H mice and lymphoma II in strain A mice while the appearance of Murphy-Sturm lymphosarcoma in Wistar rats was delayed. The guinea pig serum was administered intravenously daily for six days or 3 times a week 4 days after tumor implantation (tumor approximately 8 mm in diameter at this time). Guinea pig serum treated mice showed complete regression of tumor while, in the amounts employed, rat, chicken, dog, bovine, horse, calf, rabbit sera and human or sheep plasma did not exhibit tumor in-

hibitory properties. Properdin levels of serum from treated mice showed that the levels were low when the tumors were large, and normal or elevated when the tumor regressed. Thus it would appear that the properdin system of the host was involved in controlling the tumor growth or at least in reflecting its activity. The tumor inhibitory factor was concluded not to be properdin since all of the sera tested possessed properdin titers equal to or greater than guinea pig serum and none showed the activity of the guinea pig serum.

More recent studies on the tumor inhibitory factor of guinea pig serum by Herbut, Kraemer and Pillemer (1958) have shown that complete regression of lymphosarcoma 6C3HED in C3H mice occurred with (1) normal guinea pig serum, (2) guinea pig R1, (3) guinea pig R3, (4) guinea pig R4, (5) guinea pig RP, (6) heated guinea pig serum (56°C for 20 and 60 minutes; 66°C for 30 minutes), and (7) with supernatant from centrifuged guinea pig serum (35000 g, 2°C, 3 hours). No tumor inhibitory effect was noted with guinea pig R2 and the lipopolysaccharide pellet from ultracentrifuged serum. The results indicated that the inhibitory factor is not whole complement, lipopolysaccharide properdin. Preparations of properdin exhibited no adverse tumor inhibition even when used in conjunction with new inhibitory amounts of guinea pig serum and Mg^{++} (Herbut and Kraemer, 1958a).

The effect of zymosan in stimulating the host defense mechanisms against Sarcoma 180 in mice was re-

ported by Bradner, Clarke and Stock (1958). Intraperitoneal injection 24 hours after tumor implantation of zymosan in low doses (5-20 mg/kg) in divided daily doses for 7 days (total of 13 injections) or 2 daily doses were shown to promote a significant degree of tumor loss. In contrast, zymosan doses of 80 to 160 mg/kg were non-effective. The zymosan was suspended in 0.5 per cent carboxymethyl-cellulose-normal saline diluent. The diluent control showed no inhibitory action by itself. Zymosan was tested against Sarcoma 180 and normal mouse connective and epithelium tissues in tissue culture. No direct cytotoxic effect was observed as assessed by mitotic counts and numbers of degenerate nuclei. Therefore the tumor loss phenomenon appeared to be entirely mediated by the host as an immune reaction. Correlation studies between the properdin levels and tumor growth indicated that progressive tumor growth was attended by a significant decrease in serum properdin levels. In this connection it was reported that carboxymethylcellulose interfered with the properdin assays as performed by Dr. L. Pillemer's laboratory. Recently Mankowski et al. (1957) reported on the activity of zymosan in promoting recovery of mice bearing Sarcoma 37. Similar results were obtained by Bradner, Clarke and Stock (1957), who found that zymosan caused regression of Sarcoma 180 in mice.

The conditioning of rats with injections of small doses of zymosan to a transplantable human carcinoma of the colon (HR132) was reported by Herbut and Kraemer (1958b).

These investigators did not carry the heterologous cancer transplant for more than one generation. In most instances serial propagation of heterotransplanted cancers have failed in hosts conditioned by irradiation and cortisone. In an attempt to serially propagate heterologous tumors in rats treated with zymosan, Jude and Schaten (1958) showed that the intravenous administration of zymosan was ineffective in decreasing resistance to heterologous tumor growth. Among the tumors tested were Sarcoma 37, Krebs ascites, Sarcoma 91, lymphosarcoma C3HBA, and HeLa. Zymosan alone was ineffective and the combination of zymosan (150 mg/kg) and cortisone (3 mg) was as effective as cortisone alone indicating that cortisone by itself is the agent responsible for host resistance alterations. In 1956 Mathe and Bernard were able to transplant mouse leukemia 1210 in the hamster if they pretreated the hamster with zymosan, cortisone or x-irradiation. Therefore the role of zymosan in conditioning of animals in heterotransplants needs further investigation.

Levan had been reported by Pillemer *et al.* (1955) to alter properdin levels *in vivo*, and Brenner, Wentz and Ecker (1958) investigated the effect of levan on transmitted mouse leukemia. Available evidence indicates that mouse leukemia is caused by a virus, and the properdin system has been reported by Pillemer *et al.* (1956) to neutralize and inactivate certain viruses. Mice (C58) treated with 5 mg of levan intraperitoneally

were not able to alter the course of transmitted leukemia, and CFW mice treated intravenously or intraperitoneally with levan failed to inhibit or enhance the establishment of a solid phase Erhlich ascites tumor given subcutaneously. The properdin system apparently was not activated by the levan treatment or had no effect on the two diseases investigated.

The role of properdin and renal homografts was investigated by Hubay and Persky (1957). Zymosan in doses of 5-10 mg/kg administered intravenously to mongrel dogs depressed the properdin levels to 3 units per ml or less. A renal homotransplantation was then carried out in the neck by direct anastomosis of the carotid and renal arteries, and the renal and jugular veins. The length of survival of the renal homografts varied from 24 hours to 8 days. The properdin levels were satisfactorily depressed during the life of the homotransplant. It was concluded that the preliminary experiments did not permit any conclusive statement concerning the acceptance of renal homografts and the properdin system, and further work was necessary for further clarification on the role of the properdin system in the injection of tissue homotransplants.

D. Isolation of Properdin

In order to adequately study the physico-chemical and biological interactions of properdin, it was desir-

able to obtain as pure a preparation as possible. Originally properdin was isolated by Pillemer et al. (1954). The procedure involved the adsorption of properdin to zymosan in the presence of other serum factors and Mg^{++}. Elution of properdin from the zymosan-properdin complex was accomplished by buffers of high ionic strength. Subsequent dialysis and differential centrifugation would yield a product containing 2000 units per mg nitrogen (Pillemer, 1956). Modification of the procedure by Todd and Pillemer (1958) resulted in properdin solutions of comparable activity, however, the amount of reagents employed at certain steps in the preparation were decreased.

Since different zymosan preparations exhibited non-uniform activity relative to properdin adsorption (Pillemer et al., 1956), the activity of purified properdin solutions varied considerably. Furthermore the cost of zymosan prohibited any large scale operations. Also since human plasma was more readily available than human serum, isolation of properdin from plasma was successfully attempted by Pennell et al. (1957). The Mg^{++} ion concentration necessary for zymosan adsorption of properdin from plasma was adequately insured by addition of Mg^{++} to a concentration of 10^{-3} M to resin-collected plasma, or by saturating the citrate ions with added Mg^{++} which led to the liberation of Ca^{++} ions (the association constant for calcium citrate complex being smaller than the constant for magnesium citrate). The liberated Ca^{++} ions

were prevented from participating in the coagulation process by prompt adsorption of prothrombin onto $BaSO_4$. Furthermore, these authors were able to recover properdin from Fraction I without the use of zymosan in pilot experiments. Extending these findings, Spicer et al. (1958) prepared large quantities of purified properdin by analysis of the various fractions of the Cohn process, particularly Fraction I. The properdin activity was found to be present in a glycine-buffer extract which was ordinarily discarded. Further fractionation of this extract yielded properdin of purity comparable to that obtained using the zymosan method of Pillemer et al. (1956). Briefly, the method consisted of (1) concentration by zinc precipitation, (2) the addition of sodium tetrametaphosphate for removal of inert proteins, (3) alcohol precipitation or dialysis.

In addition to alcohol fractionation procedures for obtaining purified properdin Kent, Toussaint and Hook (1957) reported that they were able to precipitate properdin by the addition of nine volumes of 0.0027 N HCl to one volume of serum at 0°C. The sediment was removed by centrifugation (200 g, 10 minutes) in the cold. The precipitate was not as pure as that previously reported (Pillemer, 1956; Fennell et al., 1957; Spicer, et al., 1958) because the procedure precipitated all the euglobulins present as well as the properdin fraction.

E. Assay of Properdin

The assay of properdin was originally reported by Pillemer et al. (1954). Zymosan (Z) at 15°C complexed with properdin (P) to form an insoluble complex (PZ). The PZ was capable of inactivating the third component of complement (C'3) at 37°C but not at 15°C. The C'3 in serum deficient in properdin (RP) was not affected by Z at either temperature but was inactivated at 37°C if properdin and zymosan both were added. The difference of behavior of RP and normal serum was the basis for the zymosan assay of properdin. Pillemer et al. (1954) defined the unit of properdin as "...the smallest amount of sample that, in the presence of zymosan under controlled conditions, reduced the C'3 titer of an RP from an initial value of 120 units per ml to zero." Briefly the method consists of: (1) incubating decreasing amounts of sample with a constant amount of RP and zymosan for 60 minutes at 37°C, (2) centrifugation of the tubes to remove the zymosan particles, and (3) titration of the residual C'3 activity in the supernatant with serum deficient in C'3 (R3), sheep red blood cells and rabbit hemolysin. The amount of hemolysis is compared visually with standards prepared by lysing red cells with saponin, and the 50 per cent hemolysis end point determined.

McNall (1957) first reported one of the many modifications of the zymosan assay procedure. Instead of employing human serum for the preparation of RP and R3,

McNall used guinea pig serum. The advantages were reported to be that (1) only 10-20 per cent of human sera are suitable for RP or R3, (2) guinea pig serum has the lowest properdin content of any experimental animal, and (3) the guinea pig serum has C'3 as its limiting component. Furthermore, McNall used inulin instead of zymosan for binding of properdin because it was found that zymosan frequently adsorbed out other complement components while inulin did not. The amount of hemolysis present was determined by spectrophotometric recording. Hunter and Hill (1958) applied McNall's procedure to routine clinical determinations of properdin levels and reported that the procedure was "relatively simple" and practical.

A slight modification in reagent preparation by Kent et al. (1957) helped overcome the problem reported by Pillemer et al. (1956) that the reaction of zymosan with properdin of certain animal species was inhibited in the presence of human RP. The problem was solved by precipitating the properdin from the test serum as euglobulin, then reconstituting the properdin in R3 or end piece. Two fold dilutions were prepared and 0.25 ml was added to a series of tubes containing a constant amount of RP and zymosan. After incubation for 60 minutes at 37°C, the tubes were centrifuged and the supernatant fluid assayed for residual C'3 activity. In addition to the above modification, it was found that addition of RP after the PZ complex had formed, instead of simultaneously mixing all the reagents, resulted in successful

assaying of difficult sera.

Reproducible properdin assay results have been reported by Soulier, Menache and Larrieu (1957) who did not centrifuge the zymosan out of suspension before adding the hemolysin and sheep red blood cells. The tubes were centrifuged just prior to reading the amount of hemolysis in the supernatant in a spectrophotometer.

The possibility that bactericidal and virucidal tests could be employed as properdin assay procedures as well as tannic acid treated human cells was suggested. Wardlaw and Pillemer (1956) showed that a relationship between bactericidal activity and properdin concentration existed. Studies were conducted to determine if a bactericidal test for assay of properdin was possible. The difficulties involved included (1) preparation of an RP completely free of any properdin because 0.01 units per ml RP was significantly bactericidal, (2) the test organism had to be carefully chosen, and (3) a means of determining if antibody or bactericidal activities of serum other than the properdin system might be involved in the reaction.

The adaptation of a bacteriophage neutralization test for the assay of properdin was reported by Van Vunakis, Barlow and Levine (1956). Further studies (Barlow, Van Vunakis and Levine, 1958a, 1958b) indicated that the neutralizing activity of human serum using $T2_r^+$ phage (active against E. coli B) as indicator yielded precise and reproducible results in

terms of the 50 per cent neutralizing unit. Evidence was presented that the phage-neutralizing activity might be a reflection of properdin levels. However, Cowan (1958) questioned the phage neutralization technique for interpretation of properdin levels. Several different sera were assayed using T2, T6, and T7 phage types. It was proposed that different degrees of neutralization would be observed, but if the susceptibility of the phages were due to a single substance, the ratio of serum titers with the three different phages would approximately remain constant from one serum to another. If no correlation occurred then it would suggest that the neutralizing substance(s) had some degree of specificity and would be present in different sera in varying amounts. No constant ratio was observed by Cowan, and the question of the true nature of properdin was discussed. It was suggested that properdin may have some degree of specificity suggestive of antibody, a hypothesis upheld by Skarnes and Watson (1957) and Nelson (1958).

The use of tannic acid treated human erythrocytes for assay of properdin was attempted by Hinz, Abraham and Pillemer, (1957). Although purified properdin could be reliably assayed, the quantitative measurement of properdin in serum was not reproducible.

Many procedures for assaying properdin are available, but no technique is less complex than the others. In fact, discrepancies in properdin titers arise when different techniques assay similar serum samples. It may

be, as Cowan (1958) suggested, that different substances are being measured by the different techniques; "Thus, one is drawn to the conclusion that either there are several different properdins, or that properdin is a family of cross-reacting antibodies capable of combining with zymosan."

MATERIALS AND METHODS

I. Animals

Adult albino mice (Mus musculus) and adult albino rats obtained from a local source were employed as test animals in radiation experiments. The mice were also used as a source of peritoneal phagocytes for the in vitro phagocytosis and cytopepsis experiments.

II. X-irradiation

X-irradiation of the test animals was accomplished by means of a Westinghouse Quadrocondex x-ray machine. The technical factors were: 250 KVP; 15 ma; 1.0 mm aluminum and 0.5 mm aluminum and 0.25 mm copper; target distance, 16 inches. The mice were irradiated in groups of 10 in a cylindrical container with a diameter of 18.5 cm and a height of 3.0 cm. The rats were irradiated in groups of 4 in a cylindrical container with a diameter of 23 cm and a height of 5.5 cm. Guinea pigs were irradiated in groups of 4 in a wooden box with dimensions of 8 x 16 x 7.5 inches. All animals received total body exposure. The average dose rate as measured with a Victoreen r meter was 90 r per minute when recorded in the center of a lifelike wax phantom. Variation in radiation dose did not exceed 2 r per minute when measured in any position in the containers.

III. Organisms

Histoplasma capsulatum, strain G17M, isolated in 1955 from an accidentally induced superficial skin lesion and Candida guilliermondi ATCC 6260 were employed in the in vitro phagocytosis and cytopepsis experiments.

IV. Media

The H. capsulatum and C. guilliermondi organisms were grown and/or maintained on the following media:

1) Solid. The organisms were maintained by repeated sub-culturing on media consisting of tryptose phosphate broth (Difco), containing 20 per cent human blood and 2 per cent agar. In addition penicillin and streptomycin were added to final concentrations of 25 units and 25 ug per ml respectively.

2) Liquid. When organisms were needed for vaccine production or metabolically incorporated P-32, the following liquid medium was employed:

Tryptose phosphate broth (Difco)....	29.4 gm
Yeast extract.....	4.0 gm
Maltose.....	10.0 gm
Cystine.....	0.5 gm
Distilled H_2O.....	1000 ml

3) Phagocyte buffer solution. The macrophage maintenance fluid employed in the experiments was composed of the following:

a. Earle's Balanced Salt Solution (BSS)

Phenol red	0.02 gm
NaCl	6.80 gm
KCl	0.40 gm
$MgSO_4 \cdot 7H_2O$	0.20 gm
$NaHPO_4 \cdot H_2O$	0.14 gm
Glucose	1.00 gm
$CaCl_2$	0.20 gm
Distilled H_2O	1000 ml

The $CaCl_2$ was added last to avoid precipitate formation. This stock solution was made up 10 times concentrated and sterilized in the autoclave for 15 minutes at 15 pounds pressure. Working solutions were obtained by diluting the stock solution 1:10 with sterile distilled water on the day of use.

b. Bicarbonate Solution

$NaHCO_3$	50.0 gm
Phenol red	0.02 gm
Distilled H_2O	1000 ml

This solution was sterilized in the autoclave for 15 minutes at 15 pounds pressure.

The diluted Earle's BSS was prepared and mixed with penicillin and streptomycin (100 units and 100 ug per ml of each antibiotic respectively). The pH of the solution was adjusted to 7.2 - 7.4 by the addition of $NaHCO_3$ (5 per cent solution).

V. Immunization

The experimental animals were injected with formalin killed yeast phase H. capsulatum vaccine prepared in the following manner: A 1.0 ml broth suspension of yeast phase organisms was added to 800 ml of liquid culture medium contained in a low-form culture flask of 2500 ml capacity. The flask was incubated at 37°C with adequate agitation to insure maximum growth within 3 to 7 days. After maximal growth was obtained, formalin was added to a final concentration of 0.5 per cent, and the flasks were re-incubated overnight. As demonstrated by culture on antibiotic containing blood agar media, the procedure had inactivated the yeast phase organisms. The cells were concentrated and washed with saline by centrifugation procedures. After the final wash, the cells were resuspended to a concentration of 10^7 organisms per ml with saline and used as vaccine.

The mice were immunized by intraperitoneal injection of 0.5 ml of the formalin-killed suspension. The injection schedule was 3 injections spaced one week apart. Rabbits were immunized by intravenous injection of 0.25 ml of the vaccine. The injection schedule was 3 injections per week and a total of 12 injections.

VI. Phagocytes

Peritoneal exudation was induced in normal and immunized mice by intraperitoneal injection of 1 ml of 0.002 per cent (20 ug) glycogen-saline solution. Forty-eight hours later, when the majority of the peritoneal cellular population were macrophages the mice were decapitated.

The peritoneal cavities were washed with 5 ml of Earle's BSS and the washings and cells were collected in one pool in silicone-coated tubes. The cells were concentrated by low speed centrifugation (150-200 g), washed three times in Earle's BSS, and resuspended in a small known volume of Earle's BSS. An aliquot of cells was counted in a hemocytometer chamber, and the desired concentration of the suspension was obtained by dilution. The cells were allowed to attach to the bottom of 50 ml glass stoppered flasks. Sufficient phagocyte maintenance fluid was added to maintain the proper pH environment. Attachment of the cells was allowed to proceed for 5 hours at 37°C.

Another procedure involved the attachment of phagocytes to cover slips placed in steel counting planchets for correlation studies between radioactivity counts and microscopic evidence of phagocytosis and cytopepsis. The details of this procedure are given on the section of phagocytosis and digestion quantitation.

VII. Serum

Serum was obtained from normal and immunized rabbits by cardiac puncture after ether anesthesia. After the bleeding, the animals were given intravenous solutions of commercially obtainable glucose-salt solution.* This procedure enabled the animals to overcome the hemorrhagic shock

*This solution was obtained from Allied Pharmacal Company, Inc., Centerville, Utah, and marketed under the trade name of Multisal.

and remain viable. The antibody titer of the serum was determined by complement fixation (Micro-Kolmer) using a cell-free extract preparation as antigen.

Whole bovine blood was obtained from a local slaughterhouse. The serum was recovered by centrifugation and stored at -20°C until used.

Healthy adult guinea pigs were bled by cardiac puncture under ether anesthesia. The blood was allowed to clot and the serum recovered by centrifugation. The residual cells and bacteria were removed by passing the serum through a Seitz filter under positive pressure. The whole complement titer was unchanged as a result of the filtration process. The bacteria-free serum was sealed in glass ampules and stored at -65°C until used.

The whole C' titer of the serum from normal and immunized rabbits and from normal guinea pigs was determined by the Micro-Kolmer technique (Kolmer, Spaulding and Robinson, 1951).

The properdin content of the rabbit serum was determined by the zymosan assay procedure of Pillemer et al. (1956).

VIII. Zymosan Adsorption

The rabbit serum used in certain experimental procedures was absorbed with zymosan to deplete it of properdin. The serum was placed in a tube and equilibrated to 15° ± 1°C in a water bath. For each ml of serum, 0.3 ml of a 15 mgm per ml zymosan suspension (Pillemer et al. 1956) was added.

The mixture was maintained at 15° ± 1°C for 75 minutes with occasional mixing to resuspend the zymosan. After the incubation period the mixture was centrifuged at 1000 g for 60 minutes in a refrigerated centrifuge. The supernate was assayed for properdin, complement and antibody titer. In all cases, the properdin content was less than 1 unit per ml, the whole complement titer did not decrease appreciably, and the antibody titer remained the same.

IX. Properdin

The purified human properdin used in the experiments was obtained from the late Dr. L. Pillemer, Western Reserve University, and from Dr. B. E. Sanders, Merck Institute of Therapeutic Research. The preparations were assayed in this laboratory and confirmation of the original titers was obtained. Partially purified bovine properdin solutions prepared as described in the experimental results were also employed.

X. Labeling of Organisms

Yeast phase cells used in these experiments were labeled with phosphorus-32 (P-32) by employing metabolic incorporation techniques. The cells were grown in liquid culture medium containing between 10 and 20 microcuries of P-32 per ml (Hill, 1958). The cultures were agitated continuously for 3 days at 37°C. At the end of the growth period, the cells were washed four times with sterile saline

and transferred to another flask containing sterile 0.5 per cent glucose solution. The cells were re-incubated at 37°C with shaking for one hour so that the yeast phase organisms could rid themselves of the easily exchanged P-32 and stabilize the remaining isotope more firmly within the cell. Cells treated in this manner showed a range of specific activity of from 800 to 1500 counts per minute per 10^6 cells which is comparable to results obtained by Hill (1958).

XI. Phagocytosis and Digestion Quantitation

Radioactivity measurements and microscopic examination were two techniques employed for quantitating phagocytosis and digestion of yeast phase H. capsulatum cells by normal and "immune" mouse phagocytes. The radioactivity measurement technique is as follows:

A constant number of P-32 labeled cells were added to a 50 ml glass stoppered flask which contained phagocytes attached to the bottom and overlayed with maintenance fluid (20 ml). The various serum factors to be tested were added concomitantly with the addition of the P-32 labeled yeast phase organisms. The reaction flask was incubated at 37°C on a shaker. The oscillation rate was 25-30 cycles per minute with an amplitude of 2.5 cm. At various time intervals an aliquot of the supernatant fluid was removed and transferred to a steel counting planchet.

Phagocytosis and cytopepsis of labeled organisms by phagocytes was also correlated microscopically employing a

technique modified from Hill (1958). A predetermined number of washed phagocytes contained in 1.5 ml of maintenance fluid was added to siliconed, steel counting planchets which contained 22 mm round cover slips. The planchets were enclosed in petri dishes. The cell suspension was overlayed with 1 ml of sterile mineral oil (Braun-Knecht-Heimann, No. 9). Mineral oil was added to prevent CO_2 loss from the small volume of fluid. The phagocytes were allowed to attach to the cover slips for 5 hours at 37°C. The labeled organisms were added with a sterile syringe (0.1 ml). At suitable time intervals, a planchet was removed and gently agitated to suspend the non-phagocytized labeled particulate matter. The cover slip was removed, thoroughly rinsed in buffer and placed in absolute methanol to fix the phagocytes. The radioactivity of the cover slip was determined (phagocytosis), then the cells were stained with Giemsa's stain. The degree of phagocytosis and digestion observed microscopically was then correlated with the radioactivity counts.

Other experiments were conducted to determine the number of viable yeast phase organism at various time intervals when exposed to phagocytes from normal and immunized mice. Aliquots of the supernate fluid from the reaction flask were transferred to antibiotic containing blood agar medium and spread over the surface with the aid of a bent glass rod. The petri dishes were sealed with parafilm and incubated at 37°C for 7-14 days. Colony counts were done employing the Quebec colony counter.

XII. Radioactivity Measurement

The supernatant fluid samples were transferred to counting planchets, evenly distributed over the bottom surface, and allowed to air dry. Following this procedure, the sample-containing planchet was placed in a dry heat oven at a temperature of 90°C for 2 hours in order to destroy any viable H. capsulatum organisms present and avoid contamination of the radioactive isotope counting equipment. The samples were counted in a Nuclear Chicago Gas Flow isotope counter (Model D47), and the relative activity of the samples was determined. All samples were counted during the same day, therefore no decay correction factor was applied to the counts obtained. The efficiency of the equipment was periodically checked with a carbon-14 standard.

The counting error in all radioactive measurements was calculated to be 1.4 per cent.

XIII. Other Materials and Methods

The foregoing methods and materials are considered standard. Modifications of the procedures are specified in the section on experimental results and have been placed there for the purposes of continuity.

EXPERIMENTAL RESULTS

It is currently accepted that radiation-induced injury (LD_{50} range) enhances the susceptibility of the exposed host to infection. Properdin has been reported (Ross et al., 1955; Ross, 1956) to protect laboratory animals against the effects of radiation injury. In the experimental results to be reported, the role of properdin in host resistance mechanisms was investigated. It was proposed that if properdin was found to be effective in protecting animals against the effects of whole body ionizing x-irradiation, this protection could be related to the enhancement of the activities of phagocytes by properdin.

I. ISOLATION OF PARTIALLY PURIFIED PROPERDIN BY COLD ETHANOL FRACTIONATION

A procedure for the isolation and concentration of properdin substance was described by Pillemer et al. (1956). The protocol involved the adsorption of properdin to zymosan and subsequent elution under suitable conditions. The use of the zymosan adsorption technique generally yielded final properdin solutions of variable activity since zymosan preparations varied in activity.

Pillemer et al. (1954) reported that properdin is found in serum Fraction III using the separation procedure described by Deutsch et al. (1946). Pennell et al. (1957) used Method 6 of Cohn et al. (1946), and reported that 90-95 per cent of

the total properdin activity of plasma was recovered in a subfraction of Fraction I, and the remaining activity appeared in Fraction III. Linder (1957) has shown that the majority of the properdin activity of human serum resides in the gamma globulin eluate when the serum constituents are separated electrophoretically.

Experiments designed to determine the value of properdin as a therapeutic agent following the exposure of animals to whole body x-irradiation made necessary the preparation of properdin solutions of high activity per unit volume in order that adequate concentrations of material could be administered intravenously or intraperitoneally without causing physiological stress in mice and rats. A Cohn ethanol fractionation (modified Method 6) procedure was employed to obtain partially purified properdin solutions without using the variable and costly zymosan. The results of a direct ethanol fractionation procedure are presented in this section.

Whole blood of healthy adult cows was collected in sodium dichromate-sulfuric acid cleaned glass receptacles and allowed to stand at room temperature for 2 hours. The clots were then rimmed and the blood stored overnight at 2°C. The serum was separated by centrifugation at high speed in a refrigerated centrifuge. The clear serum was used immediately in the fractionation procedure.

For each liter of serum the following ethanol buffer solution was prepared:

601 ml of 53.3 per cent ethyl alcohol at -5°C

0.88 ml of 10 M acetic acid at 25°C

0.44 ml of 4 M sodium acetate at 25°C

2.30 ml of 95 per cent ethyl alcohol at -5°C

The above ingredients were measured out at the specified temperature and thoroughly mixed together. The entire mixture was allowed to equilibrate at -5°C before addition to the serum.

A pH 7.4 barbital buffer was prepared according to the directions of Kabat and Mayer (1948).

A constant temperature bath was prepared in the following manner: A double jacketed stainless steel container was constructed. The smaller inner compartment contained a solution of sodium chloride with a freezing point depression of -5°C to -6°C. The larger outer compartment contained the freezing mixture of calcium chloride and ice.

The fractionation procedure was as follows: The glass container holding the clear bovine serum was placed into the NaCl solution. The serum was stirred slowly with a mechanical agitator to hasten equilibrium conditions and prevent ice crystal formation. When the temperature of the serum was 0°C, the precooled (-5°C) ethanol buffer mixture was added. The rate of addition through a capillary jet was 5-6 ml per minute. The overall addition time per liter of serum was about 2 hours. The entire mixture was adequately stirred during the addition of the ethanol buffer mixture in order to prevent high ethanol concentrations near the jet tip which could cause irreversible protein changes. The final concentration of ethanol was 20 per cent v/v. After addition of the ethanol buffer solution the mixture was continually stirred for another 60 minutes.

Care was exercised to keep the serum-ethanol mixture at a temperature of $-5^{\circ} \pm 0.5^{\circ}C$. The resulting precipitate was centrifuged out at high speed in a refrigerated centrifuge ($0^{\circ}C$). The supernate was discarded, and the centrifuge tubes were inverted 10 minutes in the refrigerator to drain the excess fluid.

The precipitate was redissolved in pH 7.4 barbital buffer, (250 ml buffer/liter of original serum used). The redissolved protein fraction was transferred to a filtering flask, and negative pressure was applied to remove the residual alcohol. This operation was carried out at room temperature. The protein solution was cooled to $0^{\circ}C$ for 24 hours during which time a precipitate appeared. The mixture was centrifuged and the residue collected. The supernate was discarded. The residue was treated with pH 7.4 barbital buffer (3 ml buffer per ml of residue). Suspension of the residue was obtained by gentle agitation at room temperature. The final product was a stable turbid suspension and was stored at $0^{\circ}C$.

The titers of the properdin mixtures at various steps in the isolation are shown in Table 2.

It is seen that considerable increase in activity, on a volume basis was achieved, i.e., from 15 to 100 units per ml. However, the isolating procedure was carried out at a net loss, i.e., from 15000 units to 6000 units or a net recovery of about 40 per cent. The value of the procedure derives from the increased concentration per unit volume.

Anticomplementary titers of the products were determined

TABLE II

PROPERDIN ACTIVITY OF VARIOUS FRACTIONS OBTAINED DURING ISOLATION PROCEDURE

Material	Amount (ml)	Properdin* units/ml	Mg N per ml
Bovine serum	1000	15	18.7
↓			
Redissolved ethanol precipitation fraction	250	100-200	27.5
↓			
Supernate from cold separation centrifugation	235	2-4	12.7
Residue from cold separation centrifugation	15	400	37.6
↓			
Final suspension	60	100	9.4

*Zymosan assay procedure of Pillemer *et al.* (1956).

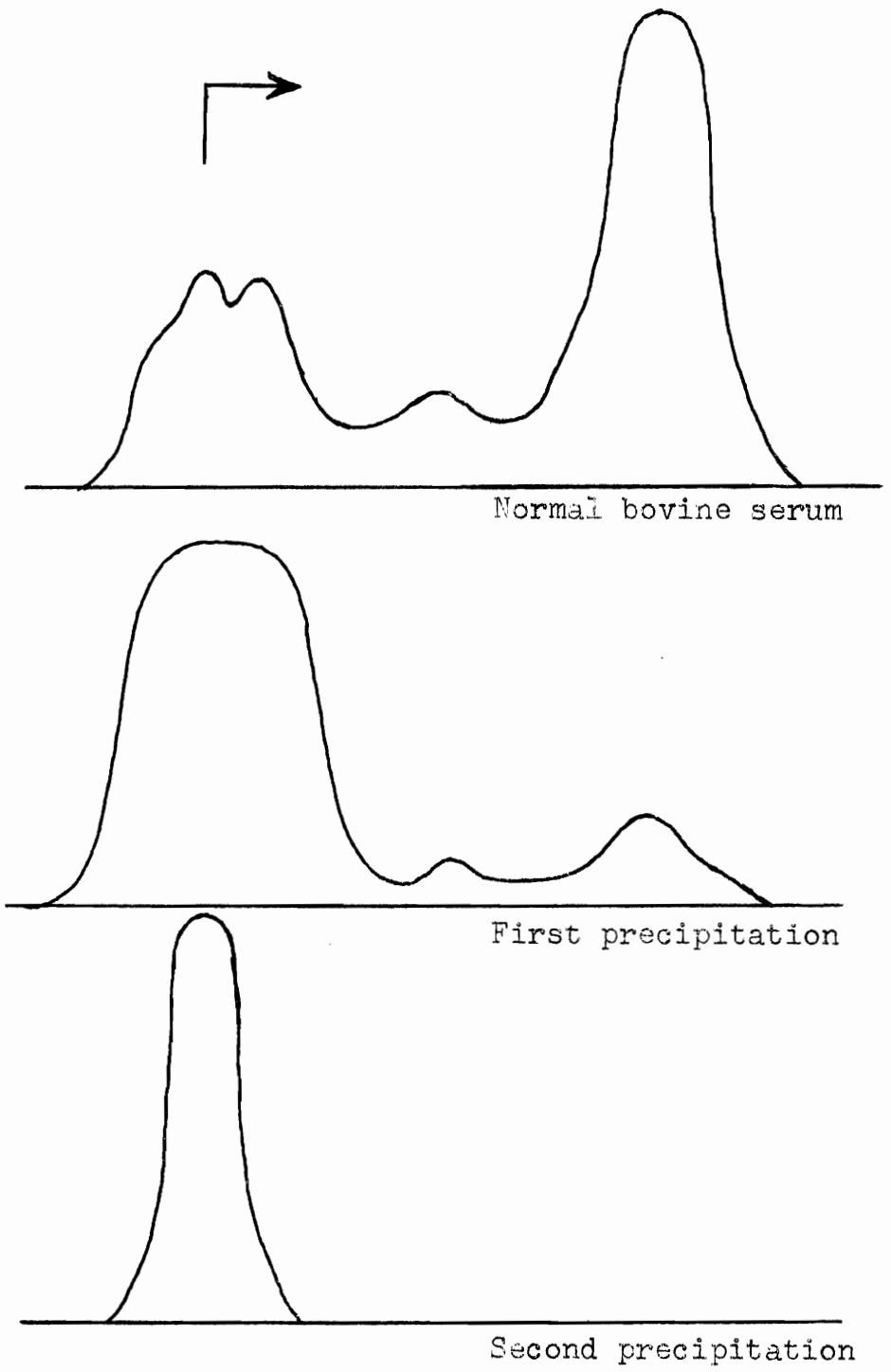

Figure 1. Electrophoretic pattern of partially purified properdin prepared by Method 6 modification of Cohn ethanol procedure.

in conjunction with the properdin assays and in each case the product was shown to be non-anticomplementary. The final product had paper electrophoretic characteristics of gamma globulin as shown in Figure 1.

II. EFFECT OF PROPERDIN ON WHOLE BODY IRRADIATED MICE AND RATS

The parenteral administration of properdin of heterologous origin into rats and mice has been reported to protect significantly against $LD_{100/30}$ day and $LD_{100/6}$ day doses of whole body x-irradiation (Ross et al., 1955; Ross, 1956). The degree of protection was reported to be dependent on the dose, route and time of administration of properdin. Ross (1956) observed that mice exposed to 600 r and treated with 50 units of partially purified bovine properdin administered intravenously on the fifth post-irradiation day showed a four-fold greater 30 day survival rate than the untreated controls. However, mice treated with properdin on the second, third, fourth, seventh or tenth post-irradiation days showed similar or accelerated death rates as compared to the controls. In order to further assess the role of properdin in protection of animals against the effects of whole body x-irradiation, experiments were conducted to determine the value of properdin as a therapeutic agent.

The properdin solutions were prepared from fresh bovine serum according to the zymosan adsorption technique of Pillemer et al. (1956), and also by a modified Cohn

ethanol fractionation procedure. In addition, purified human properdin was used in the experiments. The titers of the partially purified properdin (PPP) solutions and the human properdin (HP) were determined by the zymosan assay technique.* The route and time of administration of the properdin solutions is given in each experimental design.

A. Effect of intravenous injection of properdin in x-irradiated mice. Mice were exposed to a single 550 r total body dose of x-irradiation and randomly divided into groups. Animals of the first group of 11 mice were injected intravenously with 50 units of PPP on the first post-irradiation day. The next three groups of 11 mice each were treated with 50 units of intravenously administered PPP on the third, fifth and seventh post-irradiation days, respectively. The fifth group of 40 mice was used as untreated controls. The mortality curves following this irradiation and treatment procedure are shown in Figure 2. It is seen that the intravenous injection of PPP did not afford any protection to the x-irradiated mice regardless of the time of administration. However, there appeared to be a trend toward delayed mortality if treatment was initiated early following total body x-irradiation.

In another experiment, the first group of 10 mice each received 50 units of HP intravenously on the first post-

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*The original RP and R3 assay reagents were kindly supplied by the late Dr. Louis Pillemer and Mr. Earl W. Todd of the Institute of Pathology, Western Reserve University, Cleveland, Ohio. Portions of the supplied reagents were used to standardize subsequent assay reagents prepared in this laboratory.

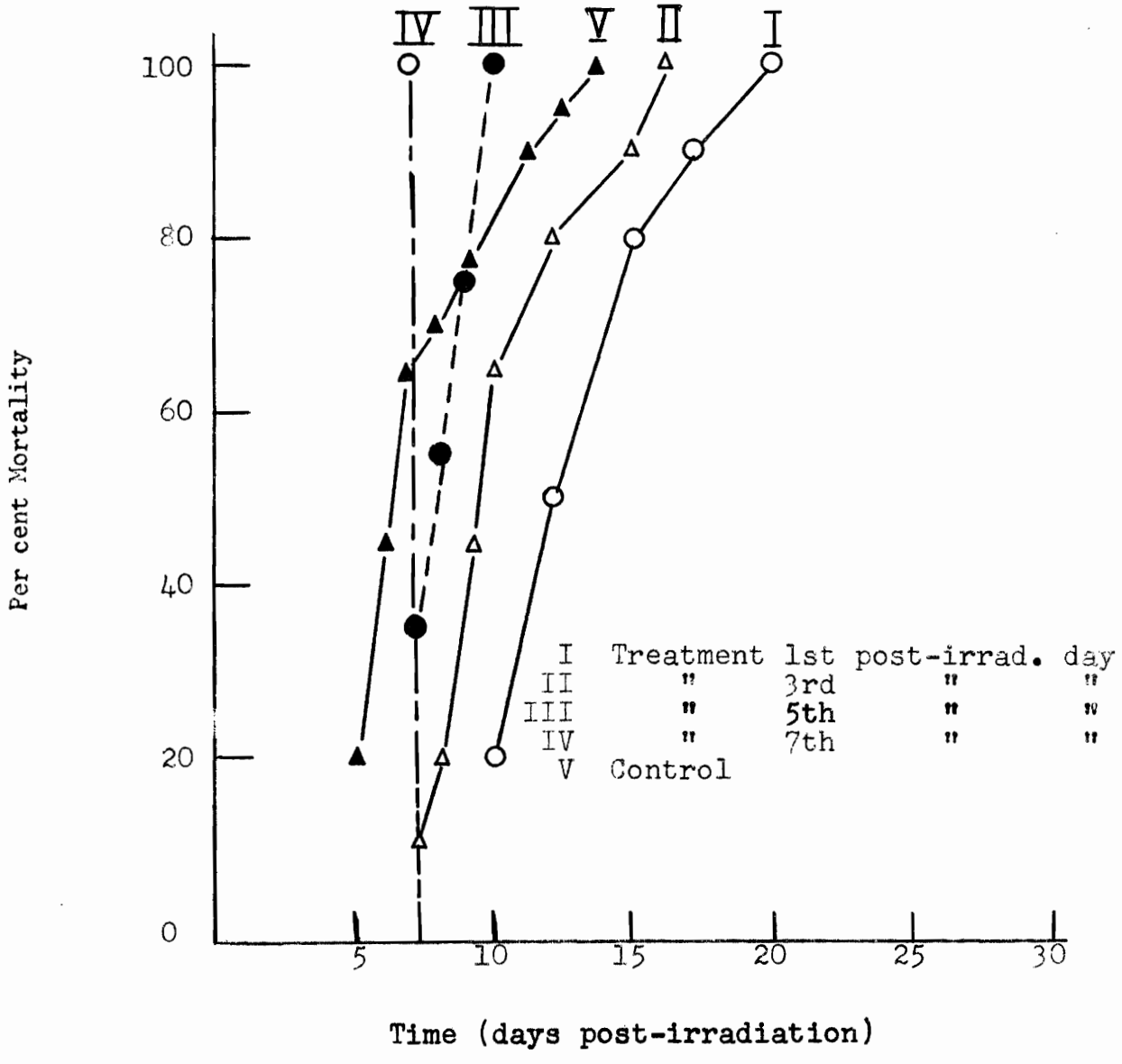

Figure 2. Mortality of adult albino mice treated intravenously with 50 units of partially purified bovine properdin (PPP) following 550 r total body x-irradiation.

TABLE III

SURVIVAL OF ADULT ALBINO MICE TREATED INTRAVENOUSLY WITH 50 UNITS OF PURIFIED HUMAN PROPERDIN (HP) FOLLOWING 550 r TOTAL BODY X-IRRADIATION

Treatment	Treatment day	Number of mice	Median survival day
HP	1 PID*	10	6.0
HP	3 PID	10	4.5
HP	5 PID	10	4.5
Control		33	7.5

* PID, post,irradiation day.

irradiation day following a single exposure to 550 r of total body x-irradiation. A similar number of mice received the same treatment on the third post-irradiation day and a third group of 10 mice each received 50 units of HP intravenously on the fifth post-irradiation day. A fourth group of 33 mice were used as untreated controls. The median survival days are shown in Table 3. It is seen that post-irradiation treatment with 50 units of HP administered intravenously did not yield any protection to the whole body x-irradiated mice regardless of the time of administration.

B. Effect of intraperitoneally injected properdin in x-irradiated mice. It is difficult to obtain properdin with high activity per unit volume. In order to increase the host systemic concentration of properdin, it was suggested that the intraperitoneal injection of large doses of properdin solution would enable the host to absorb more properdin than could be administered by the intravenous route. This increased level of properdin might then be effective in protecting against the effects of whole body x-irradiation. To test this hypothesis, mice were subjected to 600 r of whole body x-irradiation and randomly separated into 5 groups. The first group of 20 mice received 200 units of PPP intraperitoneally on the third post-irradiation day; the second and third groups of mice (20 mice per group) each received 200 units of PPP injected intraperitoneally on the fifth and seventh post-irradiation days respectively. The fourth group of 20 mice received a total of 600 units of PPP administered intraperitoneally at a dosage of 200 units per day given on the

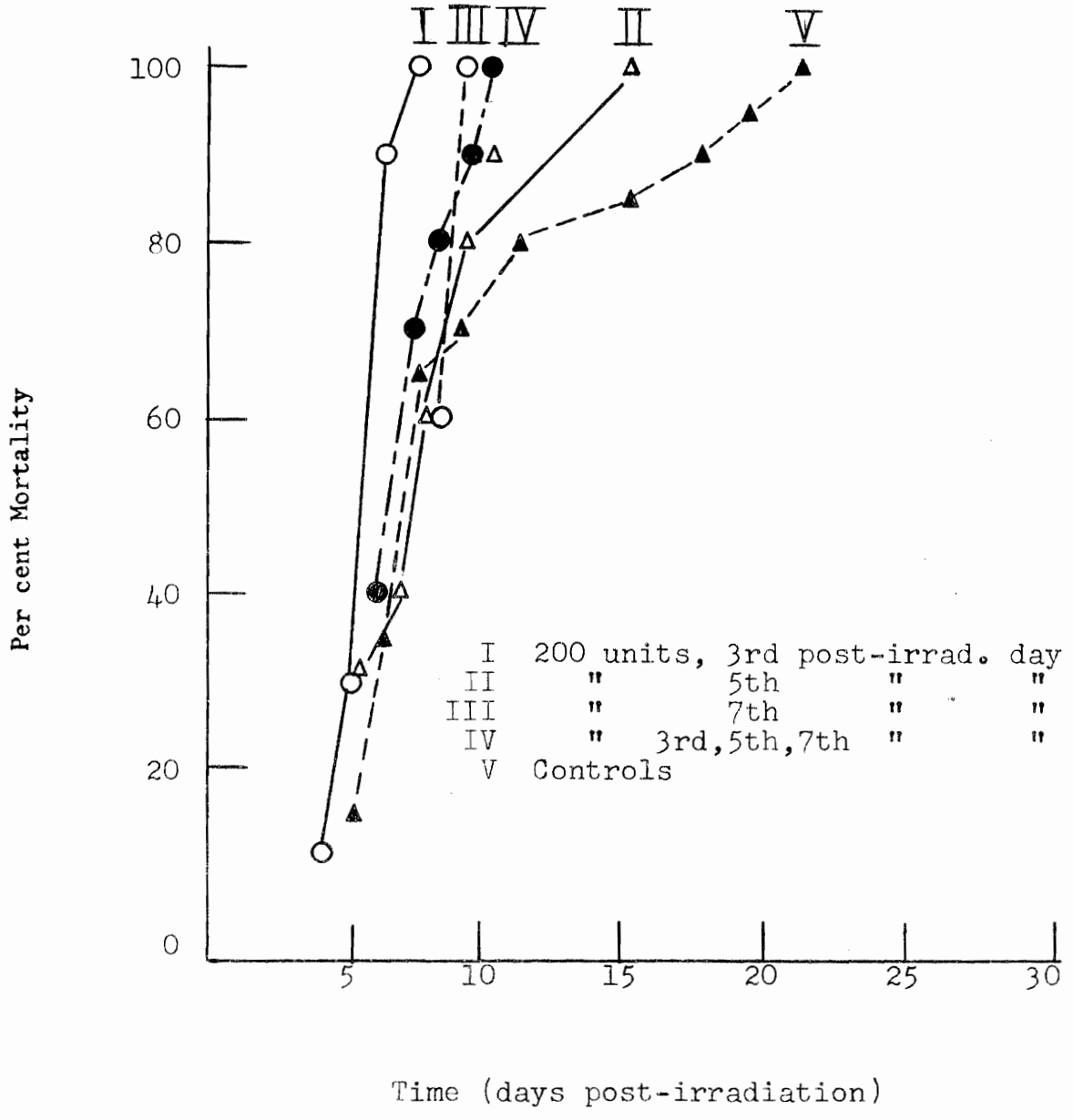

Figure 3. Mortality of adult albino mice treated intraperitoneally with partially purified bovine properdin (PPP) following 600 r total body x-irradiation.

third, fourth and fifth post-irradiation days. The 25 mice of the fifth group were used as controls. The mortality curves of this irradiation and treatment experiment are shown in Figure 3. It is seen from the results that properdin was not effective as a therapeutic agent against the effects of whole body x-irradiation even in concentrations as high as 600 units when given by the intraperitoneal route, regardless of the time of administration.

C. Effect of complement injected concomitantly with properdin in x-irradiated mice. Various investigators have reported that mouse serum is deficient in complement components (Brown, 1943; Rice and Crowson, 1950; Pillemer, 1956). The lack of bactericidal activity of mouse serum reported by Marcus et al. (1954) could be explained as a result of deficient complement components. Since the properdin system has been reported by Pillemer et al. (1954) to require the four components of complement and Mg^{++} for its activation, the failure of properdin to protect mice against the effects of whole-body x-irradiation might be explained on this basis. An experiment was carried out in order to determine if the properdin system could be activated in vivo in the mouse by concomitant injection of guinea pig complement with properdin. Groups of mice were exposed to 500 r of total body x-irradiation and treated with 200 units of PPP and 33 units of guinea pig complement (GPC'). The mixture was administered intraperitoneally rather than intravenously since a total volume of 1.1 ml was injected. The results are shown in Table IV. It is seen that x-irradiated mice were not protected even though all

TABLE IV

SURVIVAL OF ADULT ALBINO MICE TREATED INTRAPERITONEALLY WITH 200 UNITS OF PARTIALLY PURIFIED BOVINE PROPERDIN (PPP) AND 33 UNITS OF GUINEA PIG COMPLEMENT (GPC') FOLLOWING 550 r TOTAL BODY X-IRRADIATION

Treatment	Treatment day	Number of mice	Median survival day
PPP + GPC'	1 PID*	10	4.0
GPC'	1 "	5	7.0
PPP + GPC'	3 "	10	4.5
GPC'	3 "	5	15.0
PPP + GPC'	5 "	10	5.0
GCP'	5 "	5	4.5
PPP + GPC'	7 "	10	4.0
GPC'	7 "	5	5.0
Control		20	6.5

* PID, post-irradiation day.

components of complement were present. The median survival day of the treated mice was, in all cases, accelerated as compared to the control group. The CPC¹ was not toxic in the concentrations employed.

D. Effect of intraperitoneally injected properdin in x-irradiated rats. The hypothesis was proposed that mouse serum inactivates guinea pig complement in vivo. The failure of properdin to protect x-irradiated mice might be explained on this basis. It has been reported (Marcus and Donaldson, 1953; Donaldson and Marcus, 1954) that x-irradiation does not affect rabbit complement titers. Furthermore Ross (1956) reported that complement component titers of the rat were not affected by x-irradiation. Therefore in subsequent experiments rats were used.

Groups of rats were subjected to 720 r of whole body x-irradiation and randomly divided into 7 groups. The first group of 15 rats received 200 units of PPP injected intraperitoneally on the third post-irradiation day. The next two groups of 15 rats per group each received 200 units of PPP intraperitoneally on the fifth and seventh post-irradiation days, respectively. The fourth group of 15 rats received a total of 1000 units of PPP intraperitoneally at a dosage rate of 200 units per day for 5 consecutive days starting with the third post-irradiation day. The fifth group of 15 rats received a total of 500 units of PPP administered intraperitoneally at a rate of 100 units per day for 5 consecutive days starting with the third post-irradiation day. The sixth group of 14 rats received a total of 250 units of PPP intra-

peritoneally injected at a rate of 50 units per day for 5 consecutive days commencing with the third post-irradiation day. The seventh group of 20 rats was untreated and held as controls. The results of this experiment are shown in Table V. It is seen that properdin did not protect rats from the effects of whole body x-irradiation, even in doses as high as 1000 units when administered by the intraperitoneal route. The median survival day of the groups receiving multiple injections is seen to be similar to or slightly accelerated compared to the control group and groups receiving a single treatment. It is also seen that a trend toward delayed mortality appears to occur if a single injection of properdin is given to rats early in treatment following whole body x-irradiation.

III. STUDIES WITH CELL-FREE SPLEEN EXTRACTS

It is a current hypothesis that the effectiveness of post-irradiation treatment with hematopoietic containing tissues is a function of "cellular repopulation" and/or "transitory activity" of the injected cells pending recovery of recipient marrow rather than any elusive humoral factor which stimulates recovery. Experimental evidence has demonstrated the replacement of circulating erythrocytes, platelets and leukocytes in x-irradiated recipients by parenterally introduced cellular components from non-irradiated donors (Ford et al., 1957; Nowell et al., 1957). Jacobson (1956) has reported that at least 50,000 spleen cells are required

TABLE V

SURVIVAL OF ADULT ALBINO RATS TREATED INTRAPERITONEALLY WITH PARTIALLY PURIFIED BOVINE PROPERDIN (PPP) FOLLOWING 720 r TOTAL BODY X-IRRADIATION

Treatment PPP	Treatment day	Number of rats	Median survival day
200 units	3 PID*	15	15.0
200 "	5 "	15	6.5
200 "	7 "	15	6.5
200 "	3,4,5,6,7 "	15	6.0
100 "	" "	15	6.5
50 "	" "	14	6.5
Control		20	7.5

* PID, post-irradiation day.

to enhance survival of irradiated mice. On the other hand, Ellinger (1956, 1957) has reported protection of mice and guinea pigs against death following exposure to whole body x-irradiation by injection of cell-free mouse spleen extract. The results of experiments conducted to determine the value of cell-free mouse spleen extracts employed as a protective agent for mice and guinea pigs exposed to whole body x-irradiation are presented in this section. It was proposed that if protection was observed, then the effects might be due to properdin.

The cell-free extract was prepared in the following manner. Spleens from 350 normal albino mice of mixed gender, age and size were removed following ether anesthesia. The spleens were placed in a precooled mechanical blender containing 350 ml of 0.15 M saline and homogenized in two periods of one minute each with cooling in between. The homogenate was alternately frozen and thawed three times for maximum cellular disruption. The mixture was then placed in a refrigerator for 24 hours after which it was centrifuged in a refrigerated centrifuge at high speed. The supernate was passed through several layers of Whatman No. 1 filter paper in a Buchner funnel under light negative pressure. Microscopic examination of numerous fields of the resulting filtrate failed to show any intact cells, therefore the extract was assumed to be cell-free. The extract was then lyophilized and stored at -20°C. The entire procedure was conducted under aseptic conditions at low temperatures.

Forty-five mice similar to those used for the prepara-

tion of the splenic extract were exposed to 600 r total body x-irradiation. The dosage corresponded to a $LD_{100/10}$ days. The mice were randomly divided into two groups of 20 and 25; the larger group served as controls. The lyophilized spleen extract was resuspended in 0.15 M sterile saline to give a concentration corresponding to four spleens per ml. This extract was injected intraperitoneally, 0.1 ml immediately following exposure the day of irradiation and 0.6 ml administered at a dose of 0.2 ml per day on the first, second and third post-irradiation days for a sum total of 0.7 ml.

Thirty-three guinea pigs of mixed gender, each weighing about 600 g were exposed to a total body dose of 450 r. The dosage corresponded to a $LD_{85/10}$ days under these conditions. These animals were randomly divided into two groups of 20 and 13 each, the smaller group serving as controls. The treated animals were injected intramuscularly with 0.5 ml of the spleen extract immediately after irradiation and 2.0 ml was administered at a dose of 0.5 ml per day given on the first, second, third, and fourth post-irradiation days for a sum total of 2.5 ml.

The results of the experiments are shown in Figure 4. It can be seen that post-irradiation treatment of whole body x-irradiated mice and guinea pigs with cell-free spleen extracts under the conditions noted, did not result in protection against post-irradiation mortality. In fact, treated animals died at an accelerated rate when compared to the death rate of controls.

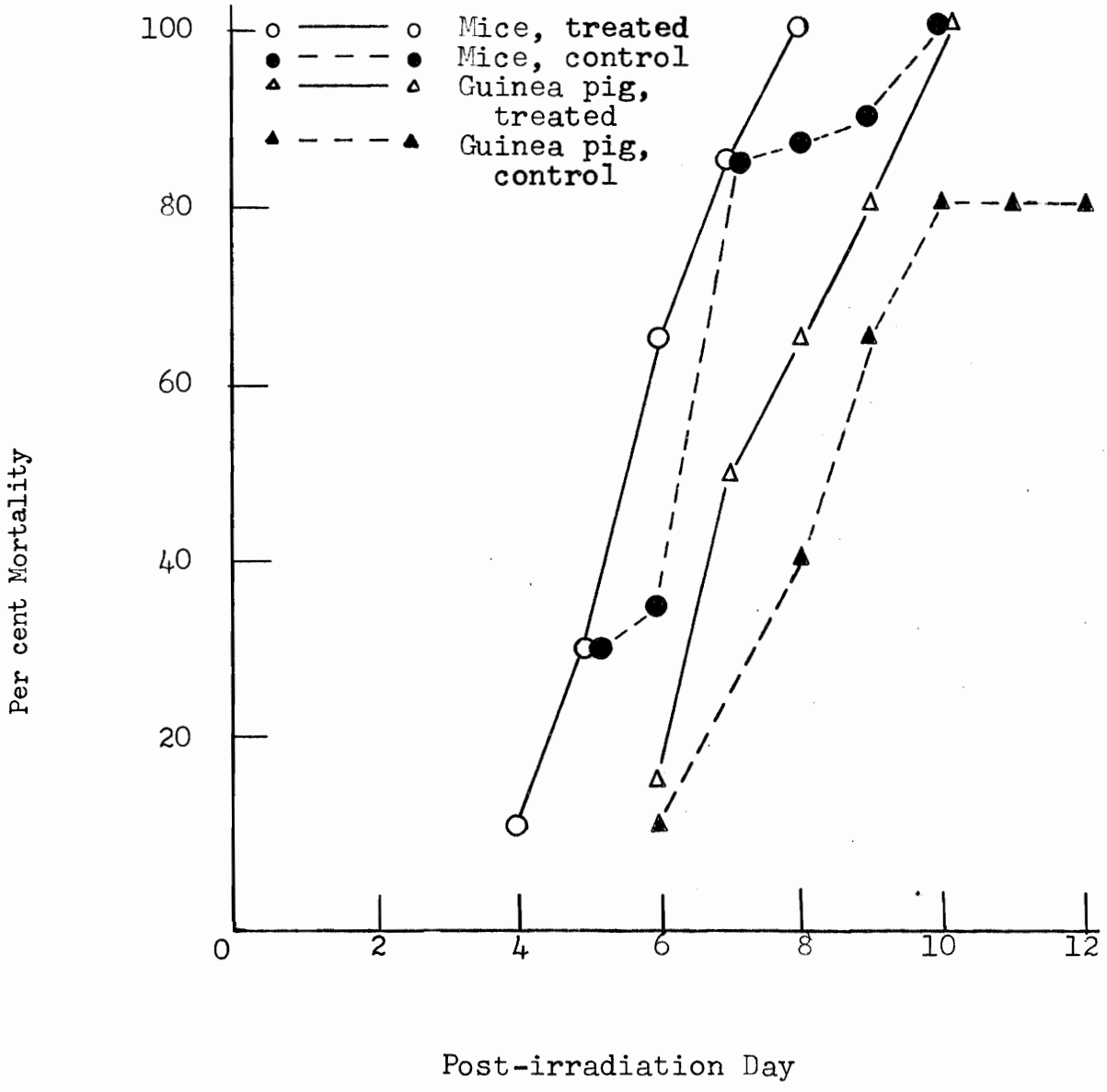

Figure 4. Mortality of adult albino mice treated intraperitoneally and guinea pigs treated intramuscularly with cell-free spleen extract following 600 r and 450 r total body x-irradiation, respectively.

IV. EFFECT OF COMPLEMENT AND PROPERDIN ON *IN VITRO* PHAGOCYTIC AND CYTOPEPTIC ACTIVITIES OF NORMAL AND "IMMUNE" PHAGOCYTES

Cellular as well as humoral factors must be considered in any investigation designed to study the role of various factors in host resistance to infectious diseases. It is well established that antibacterial antibodies (bacteriotropins) possess the properties of thermostability and specific opsonizing capacity. Evidence has been provided by Cowie and Chapin (1907), Dean (1907), and Egger (1908) that the effect of thermostable opsonin is increased by the addition of complement. Further, the rate of phagocytosis of heat-killed encapsulated pneumococci has been shown by Ward and Enders (1933) to be increased in the presence of antibody and complement as compared to the effect of antibody alone. The hypothesis can be derived from this work that the thermolabile factor (complement) did not increase the absolute capacity of antibody to promote phagocytosis, but that the increased reaction rate observed in the presence of antibody and complement was a function of an increase in the velocity with which the organisms were prepared for ingestion.

The process by which the host's cellular defenses are able to destroy or inactivate parenterally introduced foreign particulate matter can be divided into two stages: (1) ingestion or phagocytosis and, (2) intracellular digestion or cytopepsis. Antibody and complement have been shown to

promote phagocytosis. The hypothesis examined was that antibody and/or complement are also necessary to promote optimal cytopeptic activity of phagocytes. The contributing role of the recently discovered serum protein, properdin, (Pillemer et al., 1954) in phagocytosis and cytopeptic action of phagocytes was also investigated.

This section of the thesis is concerned with results relevant to the problem of quantitating the effects of various substances on the ingestive and digestive activities of mouse peritoneal phagocytes by isotope counting techniques employed in an in vitro system.

A. Isotope technique. Peritoneal exudate cells were allowed to attach to the bottom of glass stoppered flasks, and a known number of P-32 labeled C. guilliermondi cells were added. The supernatant fluid was sampled at various time intervals and transferred to a counting planchet. At the same time, using the samples of the same phagocyte suspension, counts were obtained from cover slip preparations which had attached phagocytes exposed to P-32 labeled C. guilliermondi cells. As shown in Figure 5, the counts obtained are inversely related. That is, the activity of the cover slip preparations increased initially while the supernatant fluid samples decreased. Microscopic examination of stained cover slip preparations showed that the yeast cells were ingested by phagocytes and not merely attached to the cover slip itself. Therefore, decreasing activity of supernatant fluid aliquots strongly suggest that phagocytosis is occurring.

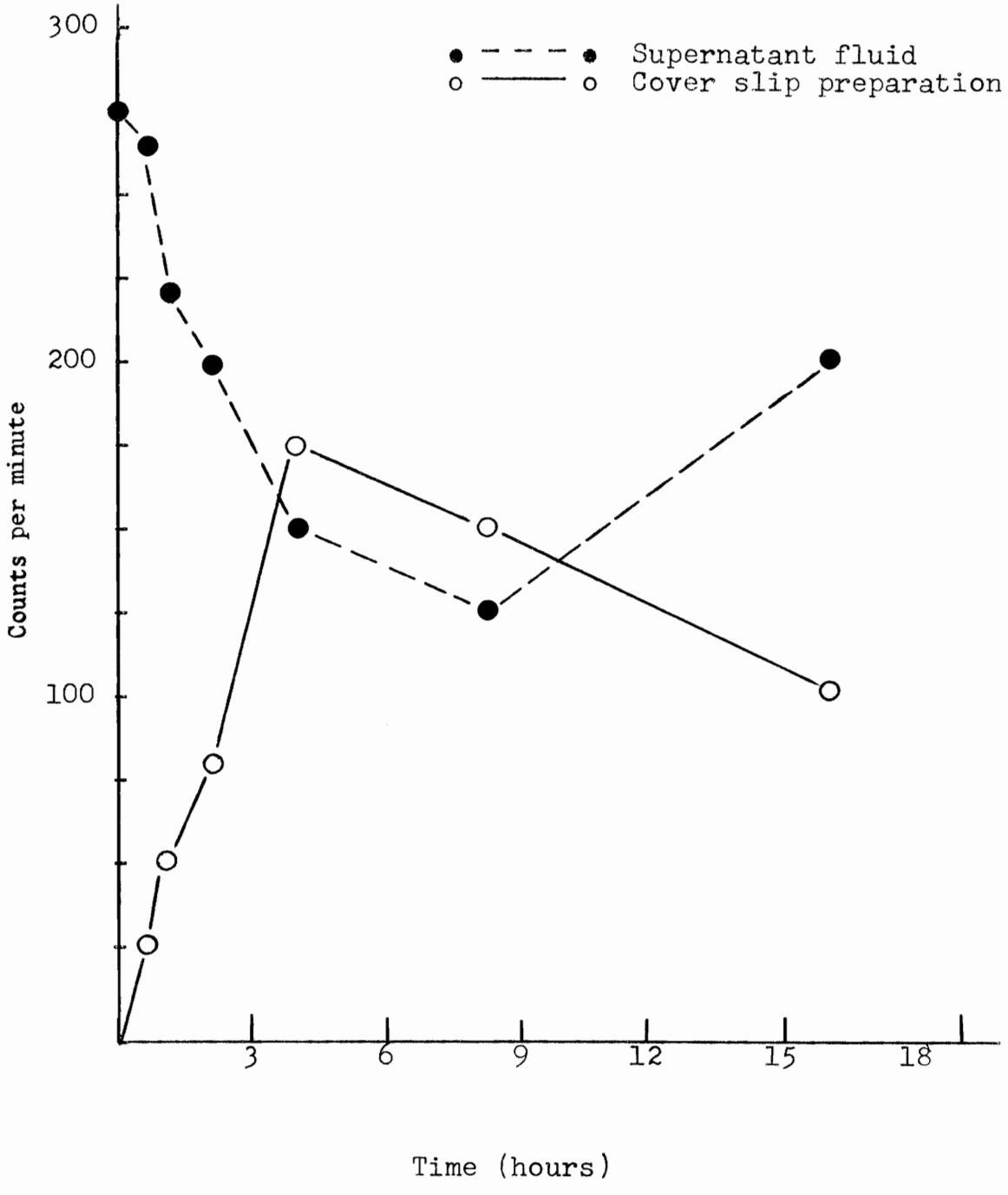

Figure 5. Phosphorus-32 activity of cover slip preparations and supernatant fluid of normal mouse phagocytes after exposure to labeled Candida guilliermondi.

B. Determination of optimal complement (C') concentration. In order to determine the concentration of guinea pig complement that would be optimal for phagocytosis of C. guilliermondi by mouse phagocytes, the following experiment was conducted. Normal mouse peritoneal phagocytes were allowed to attach to the bottom of 50 ml glass-stoppered flasks. Three different concentrations of guinea pig C' (133, 66, and 16 units per ml of buffer) were added with the P-32 labeled yeast cells. The supernatant was sampled as described previously and the radioactivity determined. It is seen in Figure 6 that C' concentrations of 133 and 66 units per ml of buffer closely paralleled each other in the rate of removal of the yeast from the supernatant while C' concentrations of 16 units per ml appear to be slower. In terms of serum concentrations, 133, 66 and 16 C' units per ml of buffer corresponded to 25, 12.5 and 2.5 per cent and showed a phagocytic rate comparable to 2.5 per cent (16 C' units per ml buffer) of unheated guinea pig complement. As expected, the phagocytic rate in the absence of serum components was considerably slower. The complement concentration chosen for future experiments was 66 units of C' per ml of buffer. If the guinea pig serum had a whole C' titer of 500 units per ml, then a 12.5 per cent serum concentration was used.

The whole C' titer of the different preparations were determined at various time intervals to see the extent of inactivation of the complement by the different components of the in vitro phagocytosis system. It is seen in Table VI that inactivation of complement occurs very slowly at 37°C

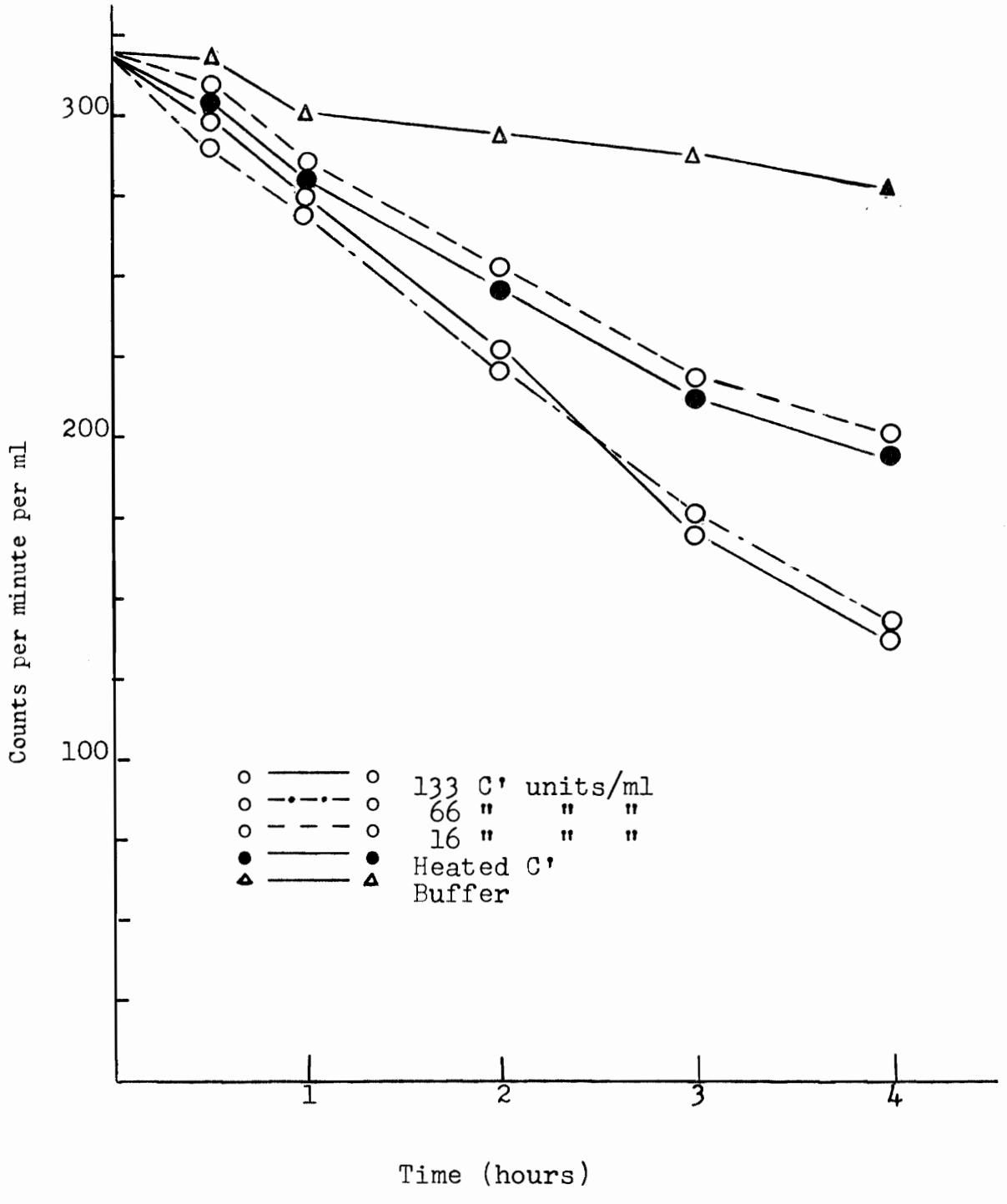

Figure 6. Effect of varying concentrations of guinea pig complement (C') on phagocytosis of P-32 labeled Candida guilliermondi by normal mouse peritoneal phagocytes.

TABLE VI

EFFECT OF COMPONENTS OF IN VITRO PHAGOCYTOSIS EXPERIMENTS ON INACTIVATION OF GUINEA PIG COMPLEMENT (GPC')

Components	C' units/ml					
	0*	2	4	8	16	24
25% g.p. serum + phagocytes + Candida	133	133	133	133	133	100
12.5% " " "	66	66	66	66	66	50
2.5% " " "	16	16	16	13	10	8
2.5% heated g.p. serum + phagocytes + Candida	0	-	-	-	-	-
25% g.p. serum + Candida	133	133	133	133	133	100
25% " " + phagocytes	133	133	133	133	133	100

* time in hours.

in guinea pig serum concentrations of 25 and 12.5 per cent. However, guinea pig serum concentrations of 2.5 per cent show a loss of 50 per cent C' activity at the end of 24 hours. It is also seen that the mouse phagocytes and yeast cells did not inactivate the guinea pig complement during the incubation period.

The in vitro technique for quantitating rates of phagocytosis and cytopepsis was found to be reproducible when Candida guilliermondi cells labeled with P-32 were used as labeled particles. The following sections present further evidence for the reliability of the technique and include studies conducted employing P-32 labeled Histoplasma capsulatum, a chronic infectious disease agent, as the labeled particle.

C. Elution rate of P-32. Experimental work during the course of this investigation had shown that the yeast phase cells of H. capsulatum can be labeled by employing metabolic incorporation techniques. The results of Hill (1958) had been reproduced, that is, maximum incorporation of P-32 into the cells occurred when the cells were grown in liquid medium containing between 10 and 20 microcuries of P-32 per ml. The specific activity was found to be 900-2000 counts per minute per 10^6 organisms. The isotope appeared to be firmly bound since the radioactivity of the supernatant from the yeast cell washings was equal to the background count after 4 repeated washings. The washed cells were resuspended in Earle's BSS after the glucose solution incubation and an aliquot containing known activity was transferred to a 50 ml

glass stoppered flask containing 20 ml of Earle's BSS. At various time intervals an aliquot was removed and centrifuged at high speed. A sample of the supernatant fluid from the centrifuged specimen was transferred to a counting planchet and air-dried. The P-32 did not elute from the labeled yeast phase H. capsulatum to any significant extent during the period of the experiment with the numbers of labeled cells employed.

D. Effect of Earle's BSS. Peritoneal phagocytes obtained from normal and immunized mice were tested for their ability to phagocytize and digest P-32 labeled H. capsulatum yeast phase cells in the presence of Earle's BSS alone. The results are shown in Figure 7. It is seen that phagocytosis of the labeled cells by phagocytes from normal or immunized mice proceeds at the same rate as evidenced by a decrease in the activity of the supernatant fluid. Maximum phagocytosis occurs at 8 hours after which time the curves remain constant. The interpretation assigned is that little digestion of the labeled particles is occurring. Microscopic examination of cover slip preparations of phagocytes from the same phagocyte suspensions revealed that these cells appeared to be disintegrating gradually from 16 hours to the termination of the experiment at 48 hours. In contrast, microscopic examination of cells which were suspended in Earle's BSS containing 20 per cent rabbit serum (heated or non-heated) revealed minimal morphological changes during the same period. Therefore, it appears that some protein is necessary in order that the phagocytes maintain their normal morphological character-

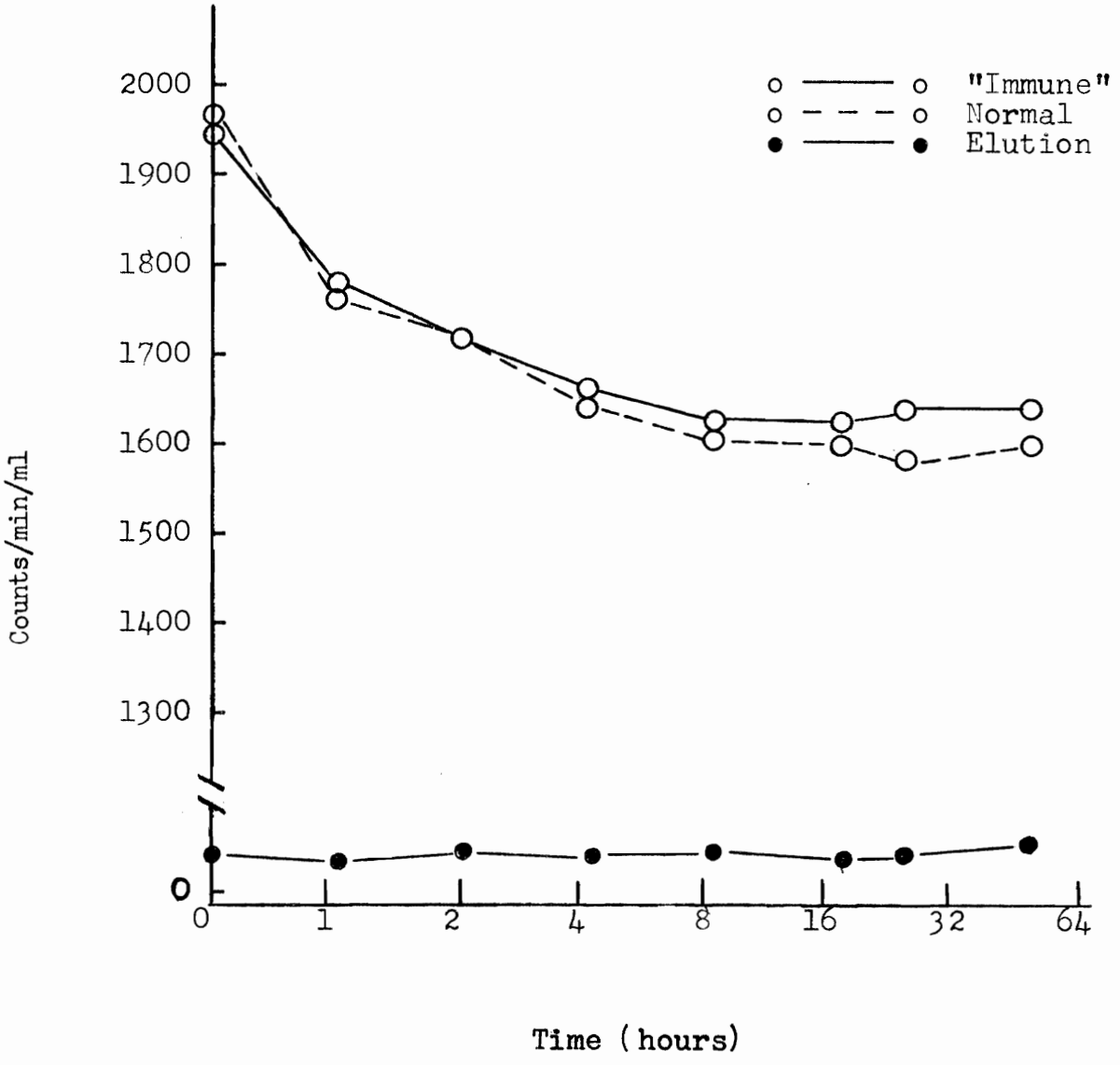

Figure 7. Effect of Earle's BSS on *in vitro* phagocytosis and cytopepsis of P-32 labeled *H. capsulatum* yeast phase cells by peritoneal phagocytes from normal and immunized mice.

istics for the time period employed in experiments such as those described here.

The bottom curve seen in Figure 7 represents the P-32 elution rate from labeled H. capsulatum. It is seen that P-32 does not elute from the organisms during the time of the experiment and does not contribute significantly to the radioactivity of the supernatant fluid.

E. Effect of normal serum. Serum was obtained from rabbits in the manner previously described. Serologic assay of the serum for complement, properdin, and specific antibody for H. capsulatum revealed that the serum contained 60-100 units per ml of whole complement, 4-8 units per ml of properdin, and no complement fixing antibody for H. capsulatum.

Peritoneal phagocytes obtained from normal and immunized mice were allowed to attach to the bottom of the reaction flasks. After attachment, the normal rabbit serum was added to Earle's BSS to a final concentration of 20 per cent. The final complement and properdin concentrations were calculated to be 12 units per ml and 0.8 - 1.6 units per ml respectively. The P-32 labeled yeast phase cells were added, and the supernatant fluid was sampled periodically for radioactivity determinations as previously described. It is seen in Figure 8 that phagocytosis of the labeled particles proceeds at the same rate whether the phagocytes are from normal or immunized mice. Maximum phagocytosis occurs at 4 hours after which time digestion proceeds as evidenced by an increase in the radioactivity of the supernatant fluid. It is apparent that digestion of the labeled particles proceeds

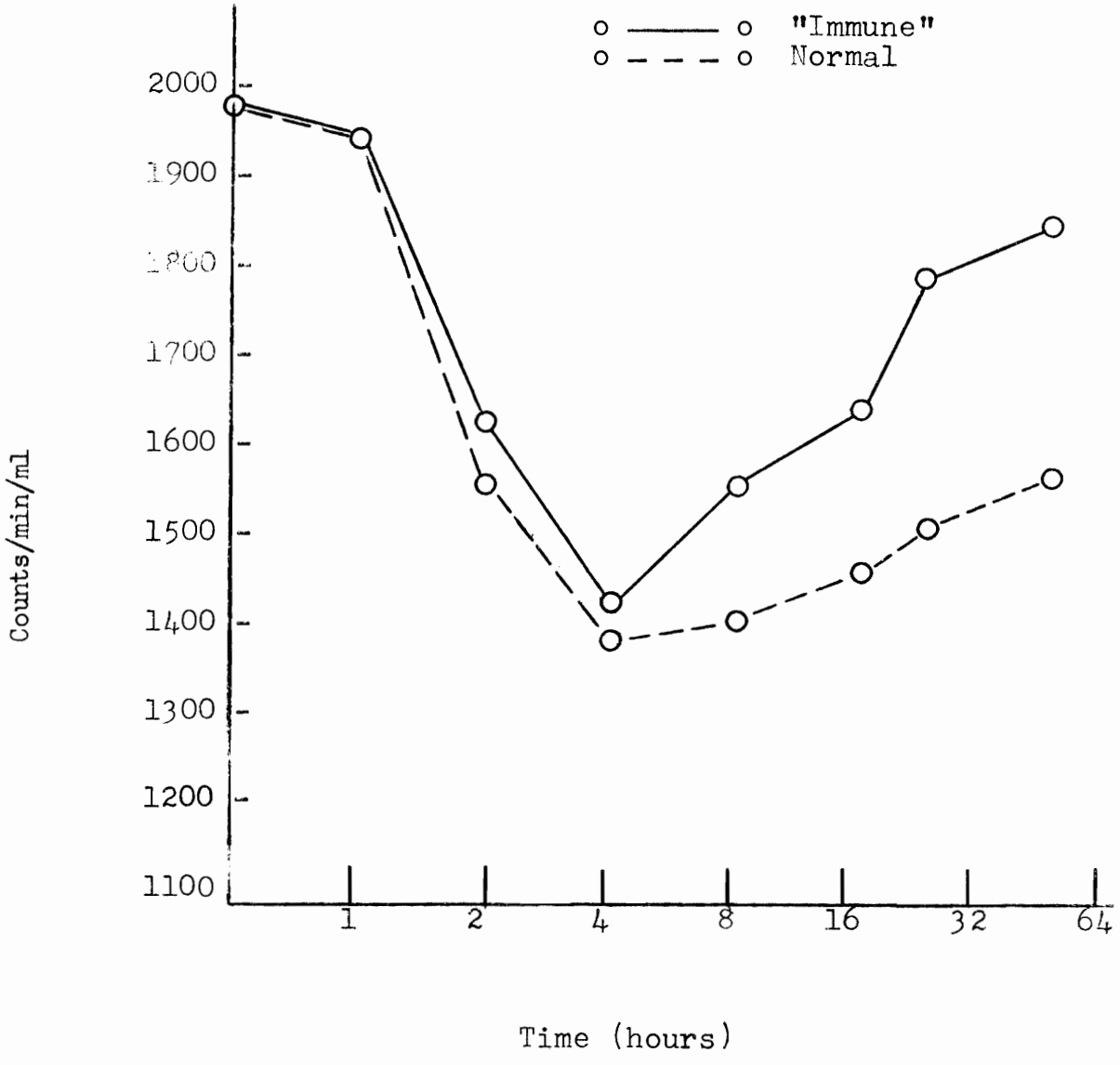

Figure 8. Effect of normal rabbit serum on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

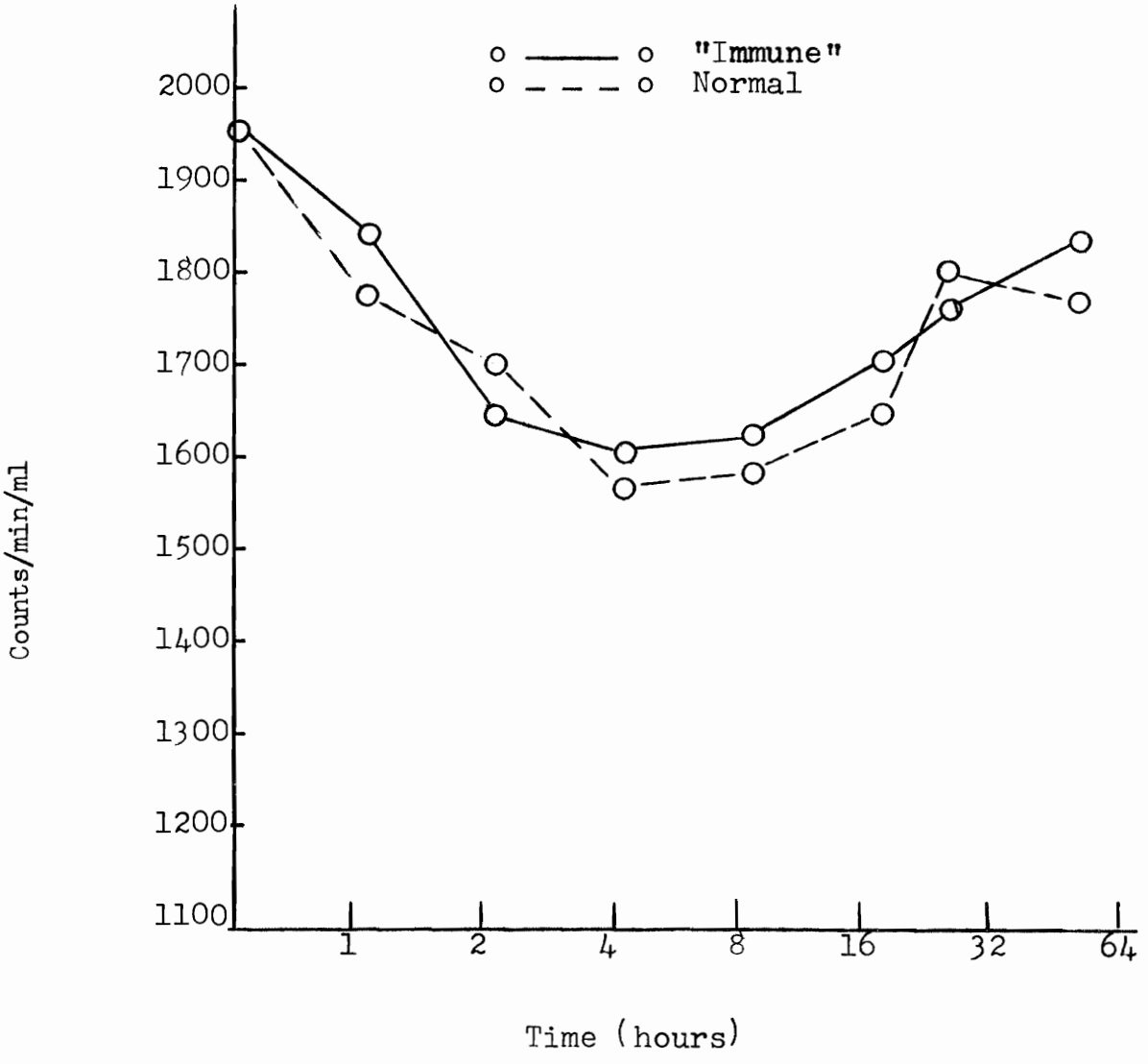

Figure 9. Effect of heated normal rabbit serum on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

at a more rapid rate in the "immune" phagocyte preparation than in the normal preparation. This difference in digestive rates if significantly increased with each increment of time and is most pronounced at the termination of the experiment.

As a control, normal rabbit serum was heated at 56°C for 30 minutes, and the same amounts were used as employed with the unheated serum. The results are shown in Figure 9. Again, the phagocytic rates of the normal and "immune" phagocytes are not significantly different when compared with each other; maximum phagocytosis occurs at 4 hours after which time digestion occurs. It is seen that differences in digestive rates are not as obvious as in the case of the rates in non-heated normal rabbit serum (Figure 8). In addition the magnitude of phagocytosis is less than in the presence of non-heated serum; for example, there is a decrease in supernatant radioactivity of 390 counts per minute in 4 hours compared to a decrease of 630 counts per minute in 4 hours for the unheated serum. These results suggest that a heat-labile factor(s) is necessary for optimal phagocytic rates by either normal or "immune" phagocytes. In addition a heat-labile component appears to be necessary for the increased enhancement of the digestive rates of "immune" phagocytes as compared to the normal phagocytes.

F. Effect of serum from immunized rabbits. Serum from rabbits immunized against yeast phase H. capsulatum cells was serologically assayed for whole complement, properdin and specific antibody content. The results showed that the C' titer was 60-100 units per ml, the properdin content was

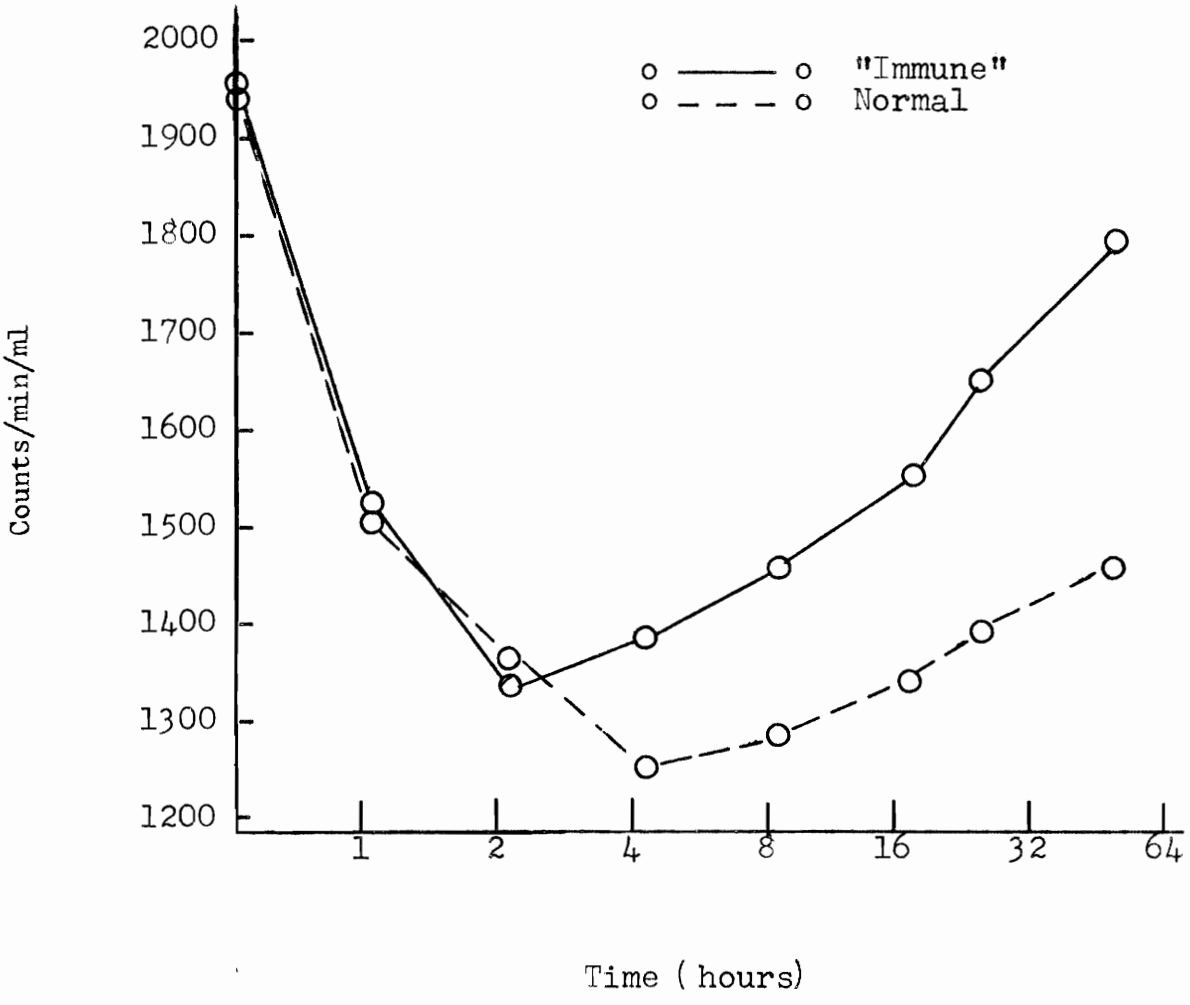

Figure 10. Effect of serum from immunized rabbits on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

4-8 units per ml, and the complement fixing antibody titer was 1:80. The antibody titer was determined by complement fixation test employing a cell-free extract of H. capsulatum as antigen (Hill, 1958).

Peritoneal phagocytes were obtained from normal and immunized mice and allowed to attach to the bottom of 50 ml glass-stoppered flasks in the presence of 20 ml of Earle's BSS. At the end of the attachment period, the "immune" serum was added to a final concentration of 20 per cent. The P-32 labeled H. capsulatum cells were added, and the supernatant fluid was sampled as previously described. The experimental results are shown in Figure 10. The results indicate that phagocytosis of the P-32 labeled organisms by either normal or "immune" phagocytes occurs at the same rate. Maximum phagocytosis by the normal phagocytes occurred at 4 hours while the "immune" phagocytes reached a maximum at 2 hours. This indicates that the "immune" phagocytes begin to digest the labeled material at a rate which is equal to the ingestion rate, whereas the normal phagocytes have an ingestive rate much greater than the digestive rate until the fourth hour is reached. Evidence to support the above observations is gained from analysis of the digestion curves. It is seen that digestion of the labeled organisms by the "immune" phagocytes occurs faster as evidenced by the significant differences in the amounts of P-32 released back to the supernatant fluid.

When the "immune" serum was heated at 56°C for 30 minutes, to rid the serum of heat-labile components (C' and properdin) but not antibody, and tested for effects on

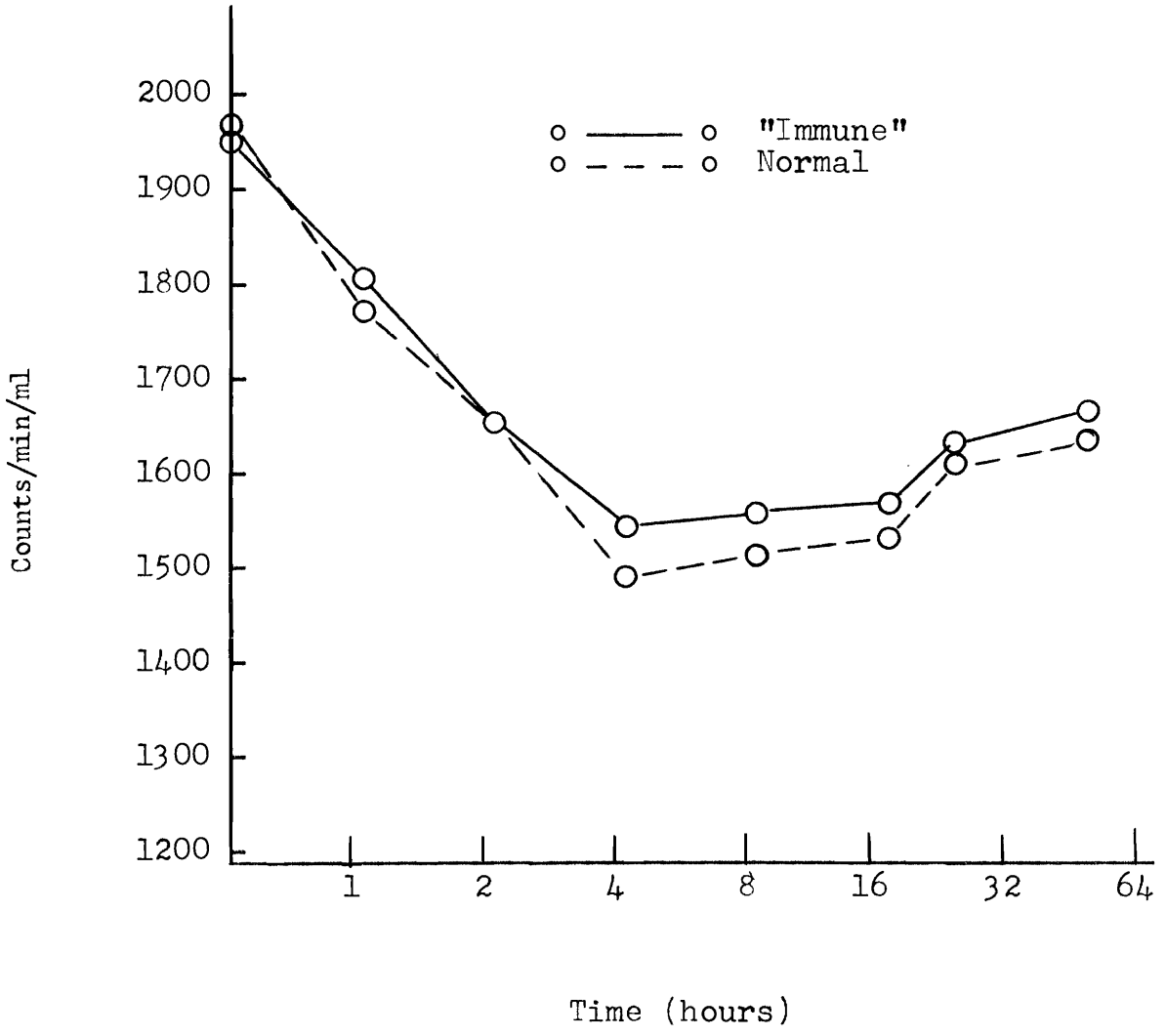

Figure 11. Effect of heated serum from immunized rabbits on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

phagocytic and cytopeptic activities of phagocytes from normal and immunized mice, a different picture was obtained as shown in Figure 11. Phagocytic and digestive activities of phagocytes (normal or "immune") are not significantly different. Further, it is seen that phagocytosis is slower and of lesser magnitude than in the case of the unheated "immune" serum experiment. In fact, the curves are not significantly different from those plotted with data obtained when heated normal serum was used as a variable (Figure 9). As in the case of normal serum, a heat-labile component(s) appears to be necessary for enhanced digestion rates by "immune" phagocytes when compared to the digestion rates of the normal phagocytes. In addition a heat-labile component(s) appears to enhance the phagocytic rates of both the normal and "immune" phagocytes.

G. Effect of 5 per cent gelatin. In order to further clarify the observations seen in the experiment with heated "immune" serum, that is, that heated "immune" serum (antibody) behaves similarly to heated normal serum, an experiment was conducted employing 5 per cent gelatin solution as a variable. The results are shown in Figure 12. It is seen that phagocytosis rates of the normal and "immune" phagocytes are not significantly different when compared to each other. Very little digestion appears to occur in this menstruum. The curves appear similar to that seen with Earle's BSS alone; however, a trend toward increased phagocytic rate appears when compared to the rate seen in Earle's BSS alone. This phagocytic rate is not significantly different when compared

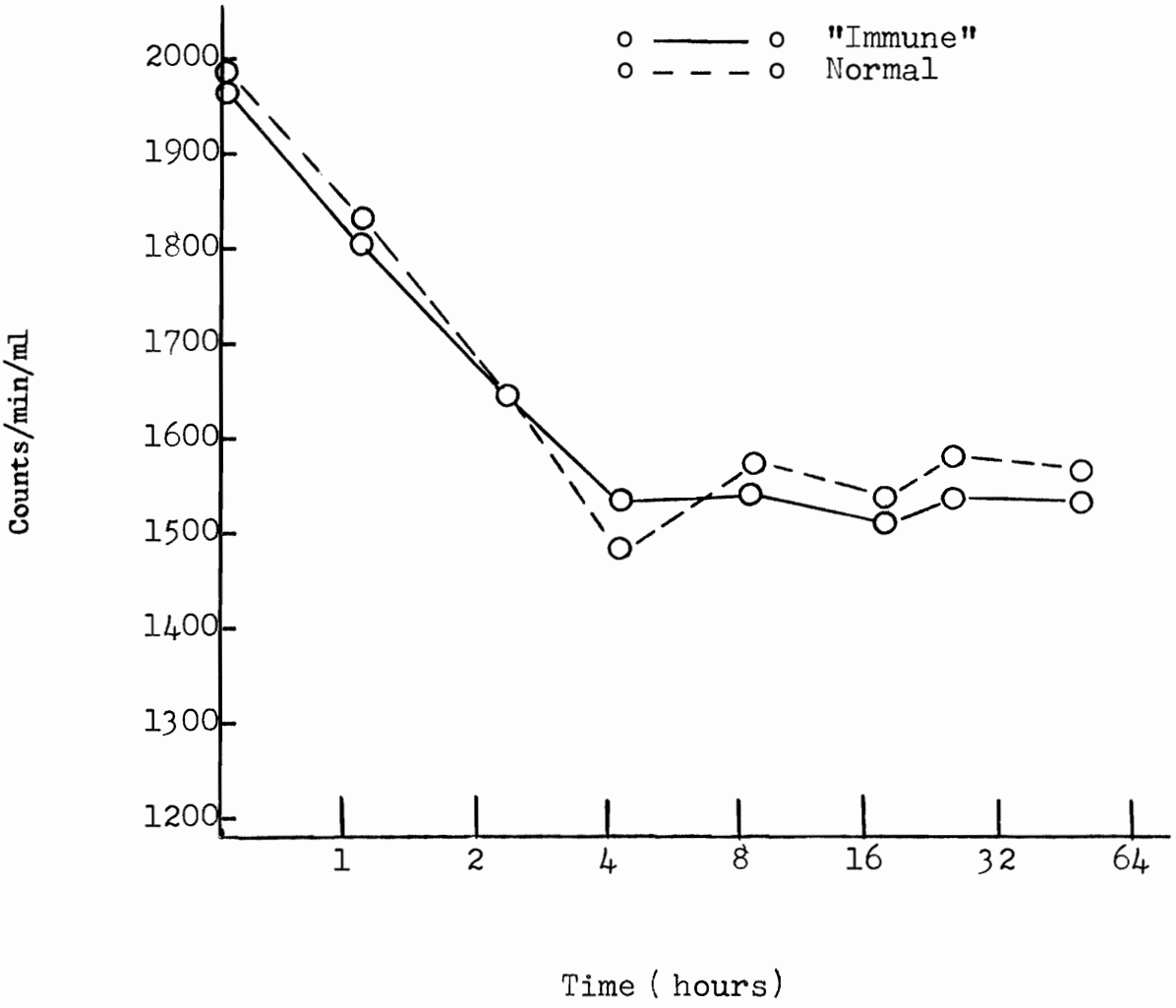

Figure 12. Effect of 5 per cent gelatin solution on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

to the phagocytic rates in heated normal or heated "immune" serum. Thus, it appears that non-specific "stickiness" of gelatin acts similarly to serum, and that antibody alone does not affect the phagocytic rates.

H. Effect of properdin. In order to determine which heat-labile component, that is, complement or properdin, is necessary for increased phagocytic and digestive rates by normal and "immune" phagocytes, the following experiment was conducted. Purified human properdin was added to a final concentration of 8 units per ml of Earle's BSS. The P-32 labeled cells were added and the supernatant fluid sampled as reported in the section on materials and methods. It is seen in Figure 13 that phagocytosis of the labeled particles by normal and "immune" phagocytes does not significantly differ, but a trend toward significance occurs in the digestion rates. The "immune" phagocytes appear to be slightly more active in digestion of the yeast phase cells in this menstruum. The phagocytic rate appears to be similar with that obtained in Earle's BSS alone. This may be explained by the fact that the protein content of the added properdin was in the microgram range. The final protein content was calculated to be 8 ug per ml; therefore, for all purposes the menstruum can be considered similar to Earle's BSS alone. It should be noted that this small amount of protein did cause a trend toward enhanced digestion rates by the "immune" phagocytes over that seen with the normal phagocytes.

I. Effect of zymosan absorbed sera. Normal rabbit serum was absorbed with zymosan to remove endogenous properdin.

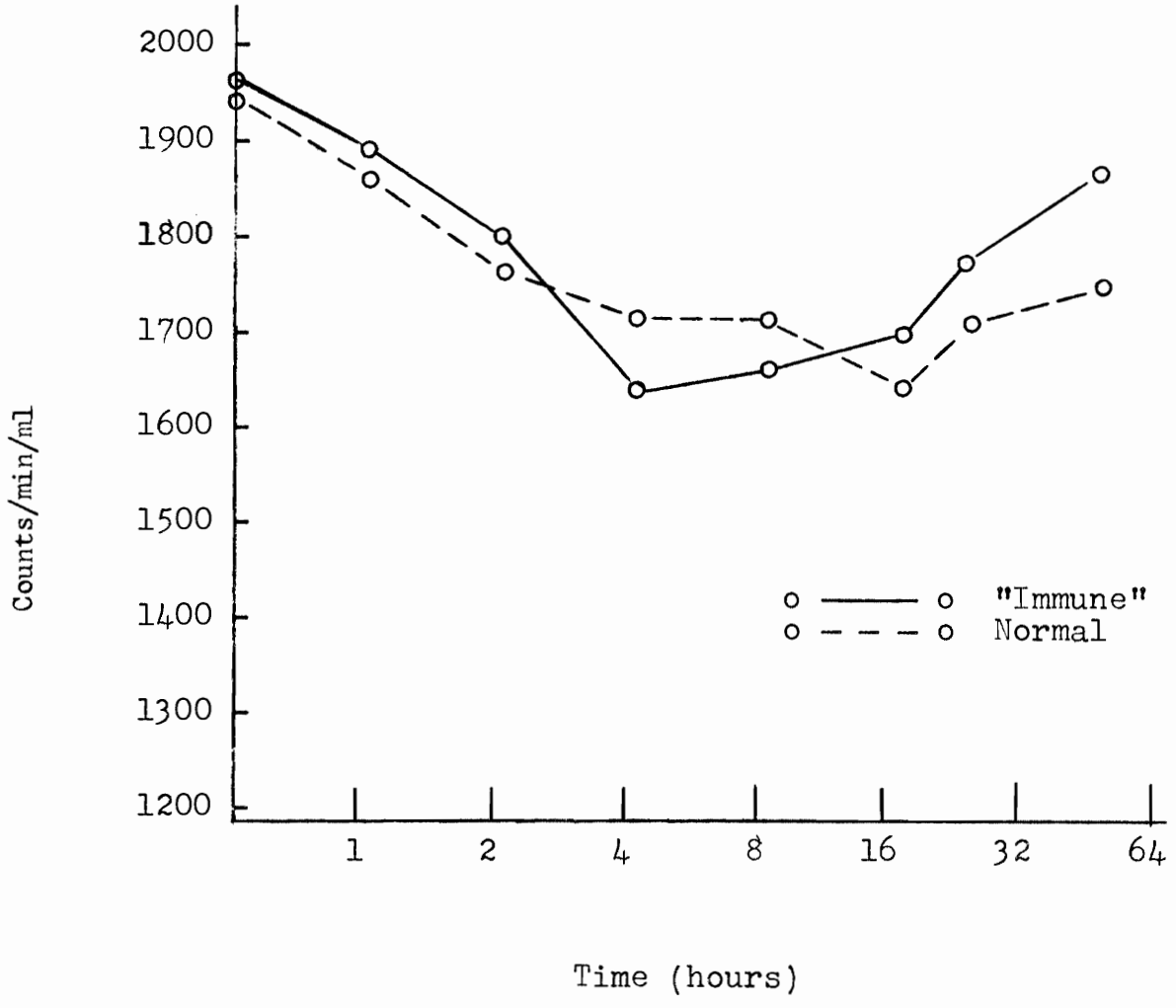

Figure 13. Effect of purified human properdin on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

The process did not decrease the whole C' titer appreciably, but lowered the properdin content to undetectable levels. This RP was used to test the effects of C' on phagocytic and digestive rates of phagocytes from normal and immunized mice. The results are shown in Figure 14. It is seen that the phagocytic rates of the normal or "immune" phagocytes are not significantly different. In both cases the maximum point is reached at 4 hours. However, a significant difference in digestion rates is seen which is pronounced at 48 hours. This situation is similar to the observed when normal serum (complement and properdin) was the menstruum (Figure 8). When purified human properdin was added to the normal RP to a final concentration of 8 units per ml with respect to the total volume of the medium, no significant changes in the phagocytic or digestive rate curves occur (Figure 15) when compared to the curves in Figure 8. The "immune" phagocytes digested the labeled particles significantly faster whether in C' alone (RP) or RP plus properdin. The small increase in digestive rates observed when properdin alone was tested (Figure 13) did not appear to be an additive factor or else the increment of increase was so small that it could not be detected in this system.

When zymosan absorbed "immune" serum was employed as a variable (C' plus antibody), the same significant increase in overall phagocytic rates by both normal and immune phagocytes was observed (compare Figure 16 with Figure 10). It appears that the lack of properdin does not influence the phagocytic rates significantly. As in the case of "immune" serum, the

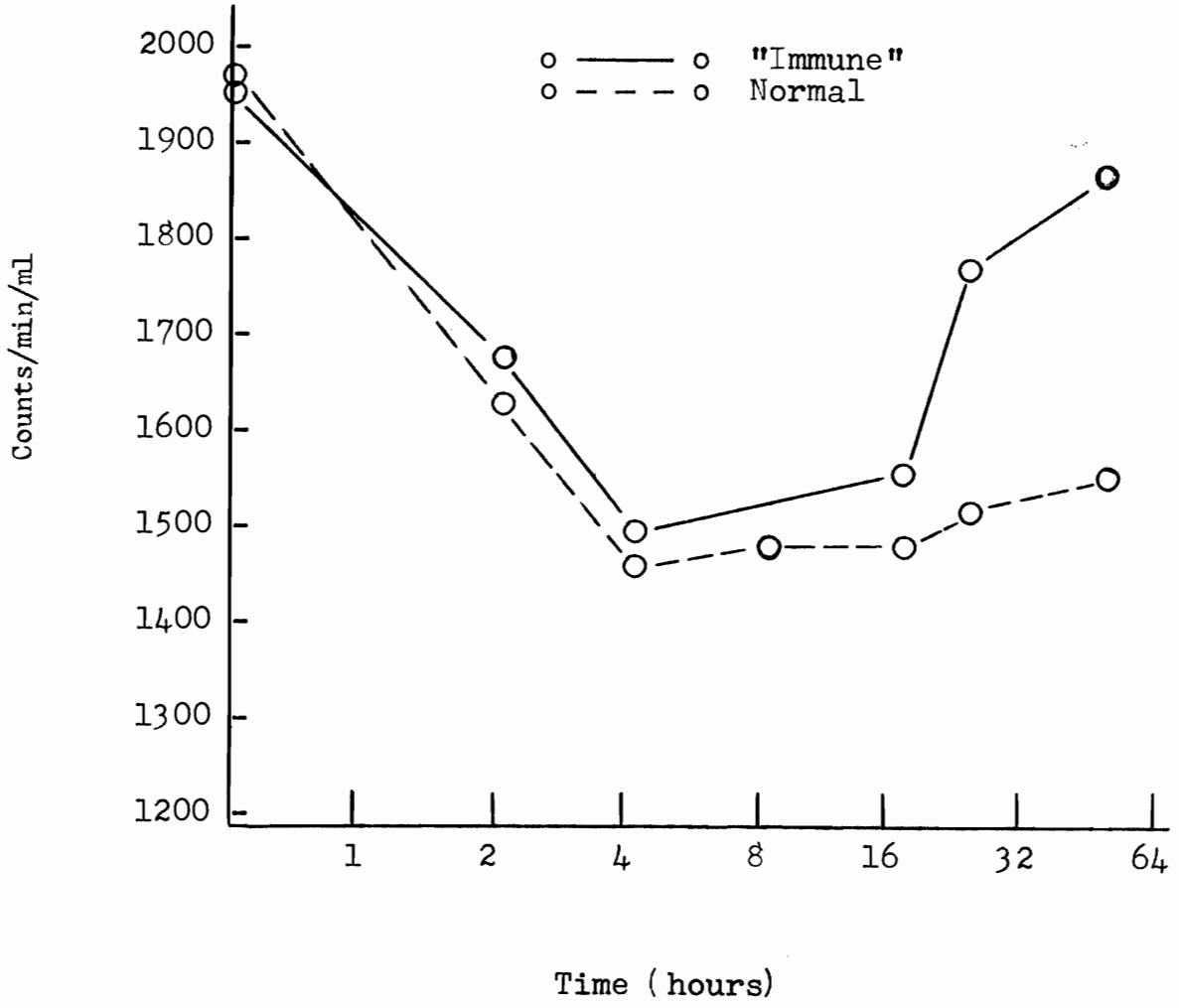

Figure 14. Effect of zymosan-absorbed normal rabbit serum on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

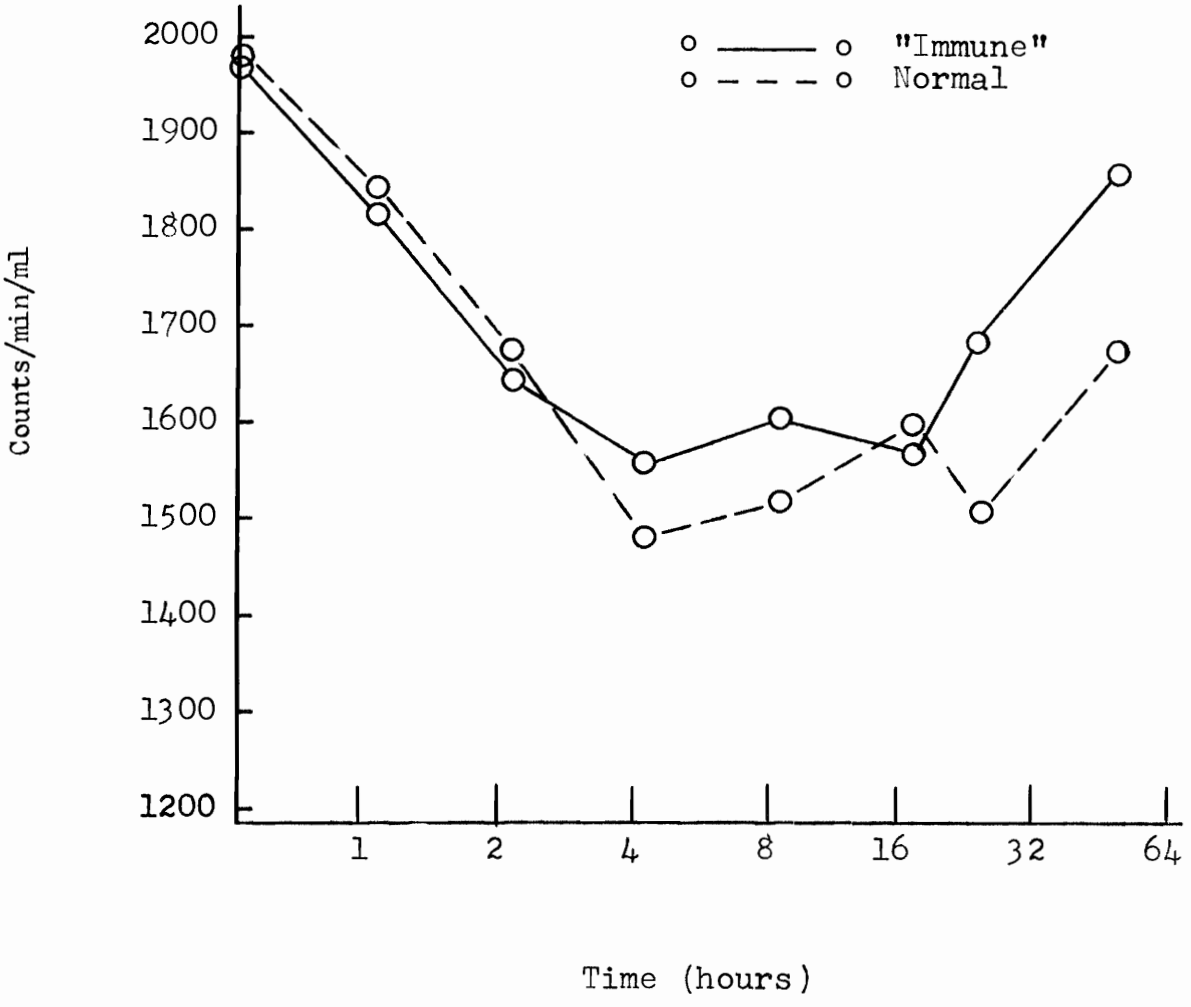

Figure 15. Effect of zymosan absorbed normal rabbit serum with added purified human properdin on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

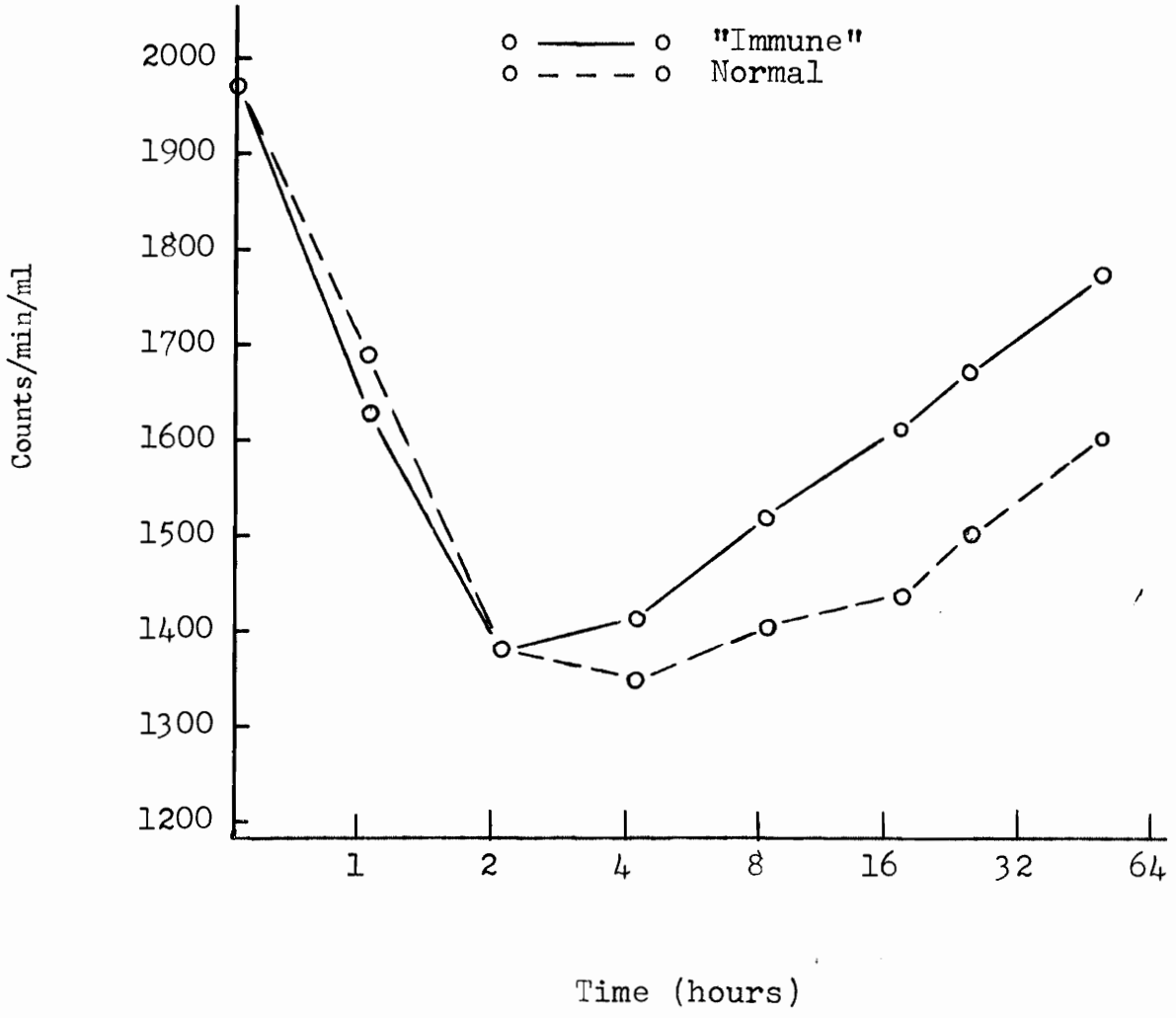

Figure 16. Effect of zymosan absorbed serum from immunized rabbits on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

magnitude of phagocytosis appeared to approach that observed when "immune" serum alone was used as menstruum (compare Figure 17 with Figure 10). It is seen from Figure 17 that the digestion rates of the "immune" phagocytes significantly exceeds that of the normal phagocytes.

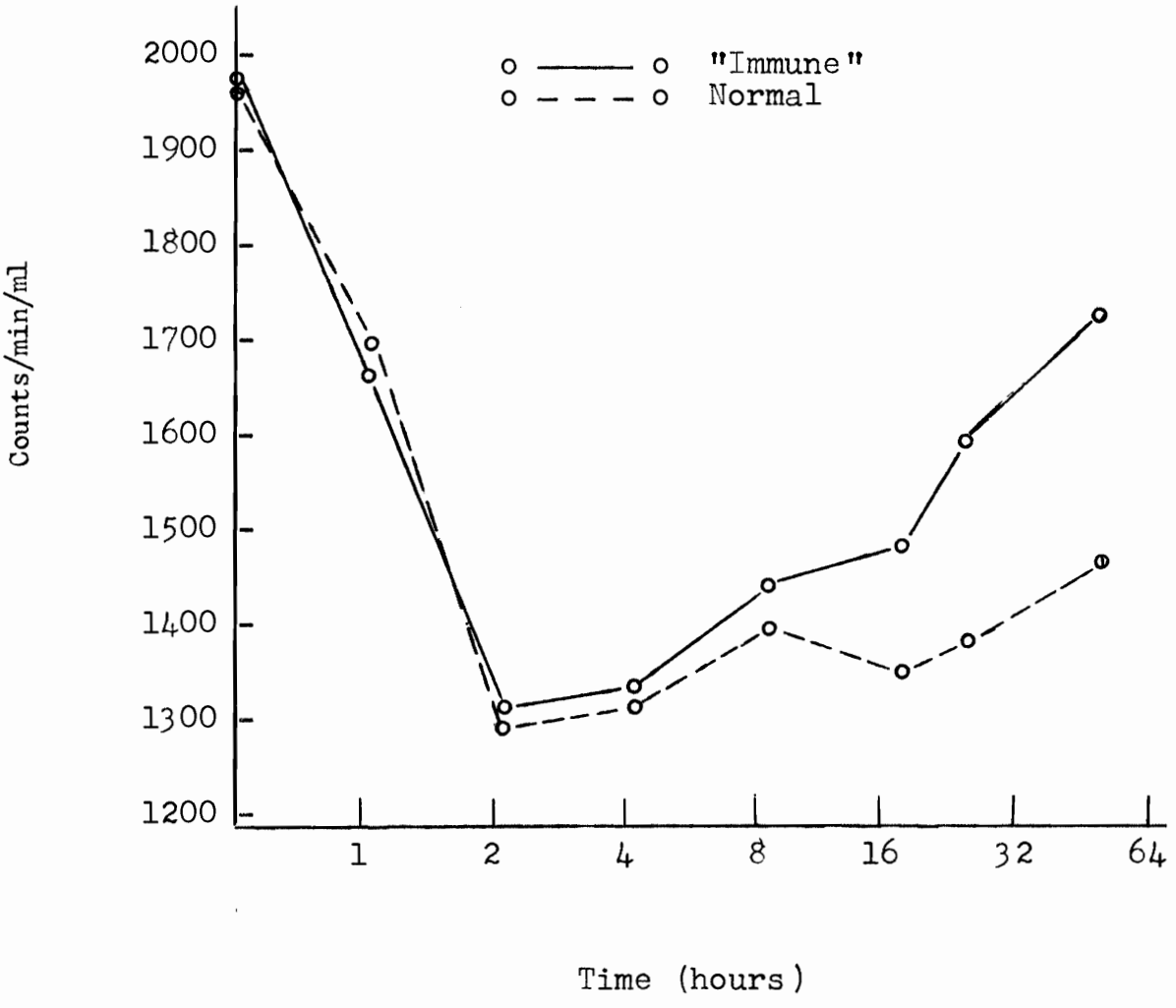

Figure 17. Effect of zymosan absorbed serum from immunized rabbits with added purified human properdin on phagocytosis and cytopepsis of P-32 labeled yeast phase H. capsulatum cells by peritoneal phagocytes from normal and immunized mice.

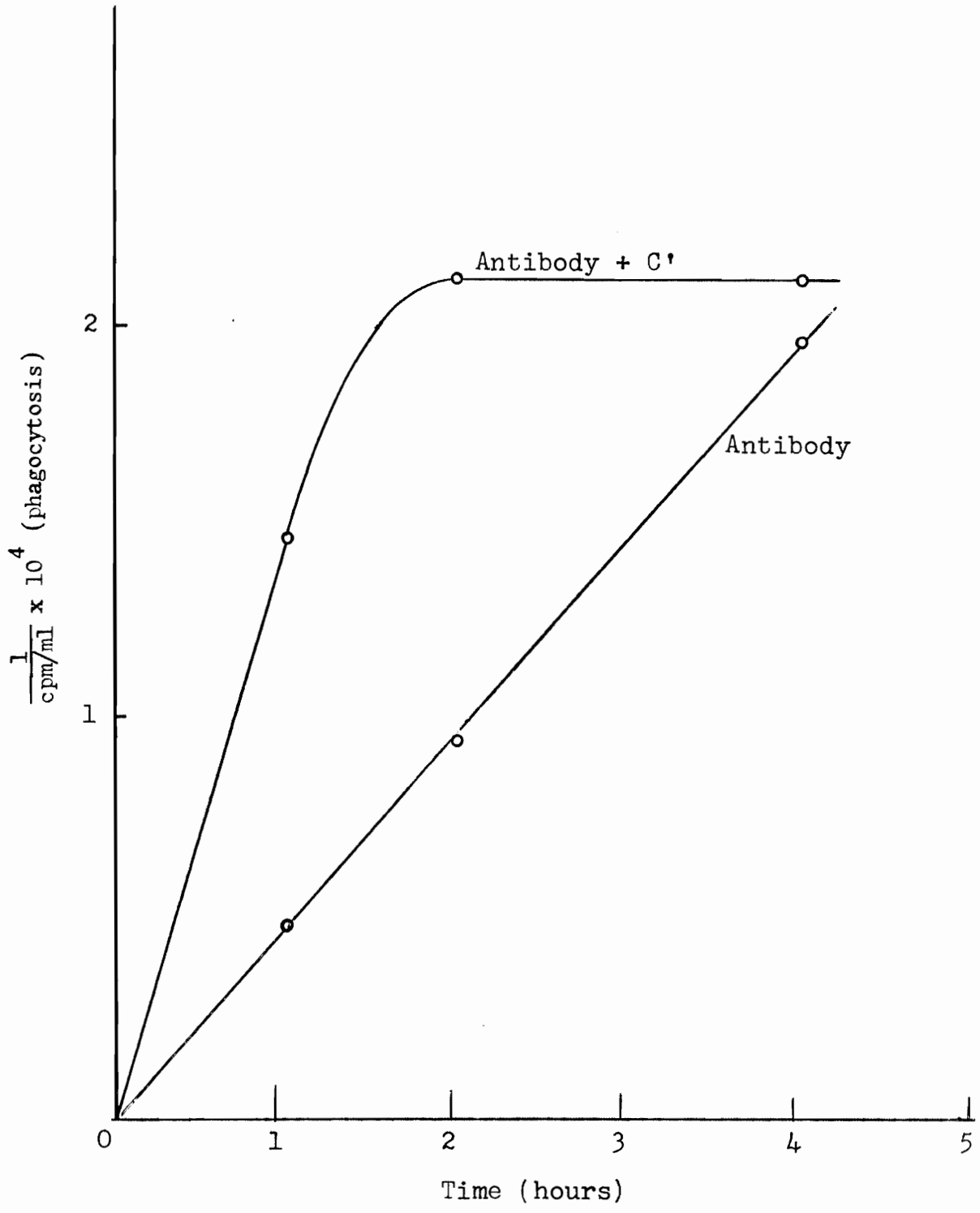

Figure 18. Transformation of experimental data to show similarity of curves describing phagocytosis obtained by isotope technique to that obtained by Ward and Enders (1933) using microscopic technique.

DISCUSSION

In order to conduct an investigation of the properdin system in host resistance, large amounts of properdin solutions were necessary. Several methods for isolation and purification of properdin from serum were attempted. These included ammonium sulfate fractionation, continuous curtain electrophoresis and whole yeast cell adsorption techniques. The ammonium sulfate and electrophoresis procedures were unsuccessful. The whole yeast cell adsorption-elution technique proved somewhat successful in that properdin solutions of low activity were obtained.

The cold ethanol fractionation procedure had certain advantages over the zymosan or whole yeast cell adsorption-elution procedures. For example, (1) the ethanol procedure was completed in one day in contrast to five days required for adsorption-elution techniques; (2) solutions with significantly greater increases in properdin activity per unit volume were obtained by this procedure; and (3) the divalent cation and C' component cofactor requirements were eliminated.

The partially purified bovine properdin (PPP) obtained by cold ethanol fractionation procedures contained considerable amounts of extraneous protein as compared to the purified properdin solutions of Pillemer (1956), i.e., the 2000-fold purified product of Pillemer's contained 0.5 ug protein nitrogen per unit of properdin while the PPP contained 94 ug of protein nitrogen per unit of properdin.

Large quantities of properdin solutions with high activity

per unit volume were necessary for the x-irradiation experiments. The high cost of zymosan prohibited any large scale isolation and purification operations; in addition products of variable activity were obtained due to variations in the different zymosan preparations used. Under these conditions the PPP solutions were considered adequate for the x-irradiation experiments; however, the highly purified properdin solutions were used in the experiments concerned with the *in vitro* quantitation of phagocytic and cytopeptic rates of phagocytes.

It is of interest to note that the purification step achieved by the inadvertent discovery of the insolubility of the properdin containing fraction at 0°C was apparently made possible by the prior concentration steps. The possible relation of "cryogenic" proteins (Lerner and Greenberg, 1946; Lerner and Watson, 1947; Lerner, Barnum and Watson, 1947) to the partially purified properdin, which acted similarly to described cryogenic globulin, remains to be determined.

It has been reported by Ross (1956) that properdin levels in rats and mice decrease to undetectable levels following whole body x-irradiation. Linder (1957) reported that the properdin levels of 500 r x-irradiated rats falls to 52 per cent of normal on the third post-irradiation day and reaches a minimum of 34 per cent of normal by the eighth post-irradiation day. The properdin level begins to rise and reaches 60 per cent of normal on the thirteenth post-irradiation day. It was observed in the present investigation that properdin levels of 720 r x-irradiated rats fell rapidly and

reached undetectable levels between the fifth and seventh post-irradiation days. Furthermore, the properdin levels of 500 r x-irradiated mice rapidly decreased to 10 per cent of normal levels by the fifth post-irradiation day and remained undetectable until all animals died on the fifteenth post-irradiation day.

If properdin has therapeutic value for whole body x-irradiated animals, it seemed apparent that treatment should be given at a time when the host endogenous levels are at a minimum. In all the experiments conducted during this investigation, the time of administration of exogenous properdin was varied in order to include the post-irradiation time during which some endogenous properdin is still present (first post-irradiation day) to when undetectable levels are found (fifth to seventh post-irradiation days). It was found that properdin did not afford any protection to the whole body x-irradiated animals in doses as high as 600 units given intraperitoneally for mice and 1000 units by this route for rats. It was observed, however, that time of administration of exogenous properdin appears to be an important factor. Trends toward delayed mortality were observed to occur if treatment was initiated early following exposure to whole body x-irradiation. In all cases, treatment on the seventh post-irradiation day accelerated the death rate considerably as compared to the controls. This observation was also reported by Ross (1956).

It had been very difficult to obtain properdin solutions of high activity per unit volume. Consequently, intravenous

injections of properdin of high activity could not be completed without introducing large fluid volumes. The choice of the intraperitoneal route of injection was made on the assumption that the properdin could be absorbed by the animal in doses greater than could be administered intravenously without causing physiological stress. It is of interest to note that in the bactericidal tests conducted with the sera of x-irradiated rats, no bactericidal activity was measurable when the properdin levels of the animals were undetectable. Perhaps the failure of properdin to protect the animals against the bacteremia that follows whole body x-irradiation might be explained on the assumption that sufficient levels of systemic properdin were not obtained. However, in vitro studies by Wardlaw and Pillemer (1956) showed that bactericidal activity of the properdin system occurred with a system containing as little as 0.01 units of properdin. However, extrapolation of in vitro observations to in vivo predictions must be guarded. Wardlaw and Pillemer also reported that concentrations of properdin above 10 units was inhibitory in the in vitro bactericidal process. Possibly a minimum of properdin would give maximal in vivo protection. In an initial experiment in this laboratory, use of properdin of low activity (1 unit/ml) yielded 60 per cent 30 day mortality among 500 r x-irradiated mice compared to 100 per cent mortality of the controls in 15 days. However, attempts to confirm this observation have been uniformly negative.

The negative results obtained when cell-free splenic extracts were tested as a therapeutic agent for animals exposed

to whole body ionizing radiation might be explained on the following basis. The cell-free spleen extract preparation used was similar to but different than that of Ellinger (1956, 1957), and the induced x-irradiation mortality was higher and more rapid with both guinea pigs and mice than in Ellinger's experiments. Irradiated animals died within 10 days whereas in Ellinger's experiments significant differences in mortality rates of spleen-treated animals and their saline controls were not evident prior to the fifteenth day after irradiation. Other deviations from Ellinger's technique were reconstitution of lyopilized suspensions to yield injectable material equivalent to 4 spleens rather than 5, the injection of this material for 3 or 4 days after irradiation rather than for 5 days, the use of repeated freezing and thawing of spleen cell suspensions to obtain cell-free extracts and the use of ether anesthesia to sacrifice the animals from which spleen extracts were prepared. Finally, the intraperitoneal rather than intramuscular route was employed for injection of mice although not guinea pigs. It is possible that the difference in preparation and administration of the cell-free extract as well as the method and time of storage may have been destructive to the "humoral spleen factor(s)" and/or that the higher dosage of irradiation was sufficient to mask the effects of the factor(s) is present. Under the experimental conditions employed protective humoral factors could not be shown to exist in cell-free mouse spleen extracts. These results suggest that strict adherence to Ellinger's technique and methods is critical.

No protection to the x-irradiated animals was observed as a result of cell-free splenic extract treatment; therefore, properdin content correlation studies were not carried out.

Since in vivo experiments could not elucidate the role of the properdin system in host resistance, it was decided that in vitro experiments be conducted in order to determine what effects properdin and other humoral factors had on the activities of phagocytes. The effects of humoral factors on phagocytic and cytopeptic activities of phagocytes were studied by employing a slight modification of the isotope technique of Hill (1958). The reliability of the technique and the results obtained by this method are discussed in the following section.

In the majority of the reported in vitro experiments concerned with phagocytosis, the authors have employed histochemical or manometric techniques. The use of radioactive substances have had their main application in in vivo phagocytic studies (Gyi and Marcus, 1957; Kabisch, 1957). The use of radioactive labeled substances for in vitro phagocytosis studies (Hill, 1958) enables the investigator to quantitate the results in a manner that is less laborious than microscopic techniques. Furthermore, the fate of the particle within the phagocyte can be quantitated in the same isolated system. As shown in Figure 5, the cover slip preparations were radioactively inversely related to the supernatant fluid. On examination of the stained cover slip preparations, it was observed as time progressed that the

ingested yeast cells showed various degrees of destruction as evidenced by loss of basophilic staining characteristics and by actual cell disruption.

The maximum digestion of the C. guilliermondi was seen by direct microscopy to occur within 4 hours. It was shown in Figure 5 that the radioactive counts of the cover slip preparations begin to decrease at that time. A working hypothesis to explain this phenomenon was that digestion of the P-32 labeled Candida caused a release of the isotope to the supernatant fluid. Evidence to support this contention was gained from the fact that the radioactivity of the fluid began to rise during the same increment of time. The fact that after 8 hours the supernatant fluid increased and the cover slip preparation activity decreased suggests that the phagocytes themselves are not appreciably labeled. The correlation study between the radioactive counts and microscopic examination indicated that phagocytosis and cytopepsis occurred in the in vitro system.

In the medium described it was shown that essentially no difference in phagocytic rates occurred at guinea pig complement concentrations of 133 or 66 units per ml of buffer. These C' unit concentrations corresponded to 25 and 12.5 per cent serum concentrations respectively. It was observed that C' in the above concentrations did not lose any appreciable activity when incubated at 37°C for 24 hours. However, the 2.5 per cent serum concentration corresponding to 16 units per ml of buffer showed a 50 per cent loss of C' activity in the same time period. This observation is in agreement with

Kabat and Mayer (1948) who stated "...that loss of complement activity which occurs during incubation (at 37°C) is more marked in higher dilutions." The mouse phagocytes and yeast cells were not anticomplementary in the concentrations used; however, previous experiments had shown that C. guilliermondi cells in concentrations of 10^8 or greater per ml of whole guinea pig serum inactivated the complement activity within 60 minutes at 37°C. Subsequent experiments were conducted with yeast cell concentrations of approximately 5×10^5 cells per ml of buffer, a concentration which was shown to be without effect on C' titer.

Evidence for the validity of the in vitro technique for quantitating rates of phagocytosis and cytopepsis by phagocytes was further substantiated by P-32 elution studies. No elution studies had been conducted because it was felt that the elution rate, if any, would be constant for the labeled yeast phase cells in the presence of either normal or "immune" phagocytes. However, the experimental work had shown that the elution rate of P-32 from labeled cells was not extensive during the time of the experiment; thus, leakage from the intact cells was a very small factor in contributing to the supernatant radioactivity. Therefore, any increase in P-32 activity in the supernatant fluid must have come from damaged and/or digested labeled yeast phase cells, which helped substantiate the original assumption made for digestion quantitation by this in vitro technique.

With respect to phagocytosis, one can use similar serum variables employed by Ward and Enders (1933), set up the

experiment by using this radioactive isotope procedure, plot the reciprocal of the counts per minute per ml versus time, and obtain curves which are very similar to that obtained by Ward and Enders (1933). Such an example is shown in Figure 18. The radio-isotope technique had additional value in that it was less laborious and more objective. For example, no prolonged microscopic examination of preparations was necessary, and a means of determining digestion rates in the same system was available.

A working hypothesis that phagocytes from immunized animals were endowed with an increased capacity to ingest and digest specific material in the absence of any humoral factor had been studied. In these experiments the constants were (1) peritoneal phagocytes from normal and immunized mice, (2) Earle's BSS, (3) P-32 labeled yeast phase H. capsulatum cells, and (4) the oscillation rates and incubation temperature. The variables were (1) normal serum (C' and properdin), (2) heated normal serum, (3) "immune" serum (C', properdin and antibody), (4) heated "immune" serum (antibody), (5) zymosan absorbed normal serum (C'), (6) zymosan absorbed "immune" serum (C' and antibody), (7) 5 per cent gelatin, and (8) purified human properdin.

From the results obtained by this technique, antibody appeared to play little or no role in enhancing phagocytic or digestive rates by peritoneal phagocytes from either normal or "immune" mice. In every instance where C' was present a significant increase in phagocytic rates by both normal and "immune" phagocytes was observed. Furthermore, the presence

of C' also caused a significant increase in digestion rates of the "immune" phagocytes as compared to the normal phagocytes. This increased digestive activity was destroyed by heating the serum. Therefore, it may be deduced that the phagocytosis and digestion promoting factor(s) could be C' or other heat-labile substances. Properdin showed slight digestion promoting activity as well as slight ingestion promoting activity. Considerable evidence that thermostable as well as thermolabile components are necessary for phagocytosis has been accumulated (Cowie and Chapin, 1907; Dean, 1907; Egger, 1908; Ward and Enders, 1933; Nelson and Lebrun, 1956). In the experiments reported here, antibody appeared to play little or no significant role in phagocytosis enhancement. The results suggested that a heat-labile component resembling complement and/or properdin was responsible for the enhanced phagocytic rates by both normal and "immune" phagocytes when a chronic infectious disease agent such as H. capsulatum was used as the labeled particle. In addition, the heat-labile component(s) appeared to endow the "immune" phagocytes with increased digestion capacity as compared to the normal phagocytes. The situation could be different in the case of an acute disease agent. Earlier workers employed acute disease agents in their experimental work and one must guard against extrapolation of data obtained with one type of disease agent to other situations. It is of interest to note the work of Tullis and Surgenor (1956) who studied plasma and serum proteins in an attempt to isolate and identify the factors responsible for phagocytosis acceleration. They grouped all protein factors

that stimulate natural non-immune phagocytosis under the term of phagocytosis promoting factors (PPF). These factors were found to be heat-labile, closely associated with complement and clotting components of plasma, and were suggested to act solely on the leucocytes. Perhaps these same factors were operative in the series of experiments reported herein.

SUMMARY

1. Under carefully controlled conditions bovine serum properdin was isolated and obtained in a partially purified state. The euglobulin was recovered in the serum fraction which was insoluble at pH 6.9, -5°C and at an ethanol concentration of 20 per cent v/v.

2. Partially purified bovine properdin and purified human properdin were employed as therapeutic agents against the effects of ionizing radiation in whole body x-irradiated mice and rats. No protection was observed when the solutions were administered intraperitoneally or intravenously; however, there appeared to be a relationship between delayed mortality and post-irradiation time of injection.

3. Post-irradiation administration of cell-free mouse splenic extract to whole body x-irradiated mice (intraperitoneally) and guinea pigs (intramuscularly) did not result in protection against post-irradiation death. The treated animals died at an accelerated rate when compared to the controls.

4. Candida guilliermondi cells labeled with P-32 were used in an in vitro system for quantitating phagocytosis and cytopepsis rates by mouse peritoneal phagocytes. In this system the optimal concentration of guinea pig serum was determined to be 12.5 per cent (66 C' units per ml buffer). Complement was not significantly inactivated at 37°C for 24 hours in serum concentrations of

25 and 12.5 per cent, but a loss of 50 per cent activity occurred when a serum concentration of 2.5 per cent was employed.

5. Yeast phase Histoplasma capsulatum cells labeled with P-32 were used in a series of experiments to study the effects of humoral factors on the in vitro ingestive and digestive activities of peritoneal phagocytes obtained from normal and immunized mice. Among humoral factors studied by this technique were complement, properdin and antibody. In all cases, a heat-labile component (complement and/or properdin) not only enhanced phagocytic rates by normal and "immune" phagocytes, but caused a significant enhancement of the digestive activities of the "immune" cells as compared to the normal phagocytes. Specific antibody did not appear to play any significant role in phagocytosis or cytopepsis activities of normal or "immune" phagocytes when a chronic disease agent, H. capsulatum, was used as the P-32 labeled organism.

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THE PROPERDIN SYSTEM IN HOST RESISTANCE

by

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An abstract of a thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

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ABSTRACT

Under carefully controlled conditions, bovine serum properdin was isolated and partially purified by employing cold ethanol fractionation procedures. The euglobulin was recovered in the serum fraction which was insoluble at pH 6.9, -5°C and at a final ethanol concentration of 20 per cent v/v.

The partially purified bovine properdin and purified human properdin were employed as therapeutic agents against the effects of ionizing radiation in whole body x-irradiated mice and rats. No protection was observed when the solutions were administered intraperitoneally or intravenously; however, there appeared to be a relationship between delayed mortality and post-irradiation time of injection.

Under the experimental conditions employed, post-irradiation administration of cell-free mouse splenic extract to whole body x-irradiated mice (intraperitoneally) and guinea pigs (intramuscularly) did not result in protection against post-irradiation death. The treated animals died at an accelerated rate when compared to the controls.

In vivo experiments could not elucidate the role of the properdin system in host resistance; therefore, in vitro techniques for investigating the effect of properdin on phagocytes were conducted. Yeast phase Histoplasma capsulatum cells labeled with P-32 were used in a series of experiments to study the effects of humoral factors on the in vitro ingestive and digestive activities of peritoneal phagocytes obtained from normal and immunized mice. Among humoral

factors studied by this technique were complement, properdin and antibody. In all cases, a heat-labile component (complement and/or properdin) not only enhanced phagocytic rates by normal and "immune" phagocytes, but caused a significant enhancement of the digestive activities of the "immune" cells as compared to the normal phagocytes. Specific antibody does not appear to play any significant role in phagocytic or cytopeptic activities of normal or "immune" phagocytes when a chronic disease agent, H. capsulatum is used as the P-32 labeled organism.

RESEARCH PROPOSALS

submitted

by

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in partial fulfilment of
the requirements for the
degree of

Doctor of Philosophy

Department of Bacteriology
University of Utah
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RESEARCH PROPOSALS

1. Complement is always defined in functional terms. A study of the effect of various enzymes on complement activity might aid in elucidating the constitutive nature of the protein complex.
2. Not all sera can be used for preparation of a serum reagent deficient in properdin (RP). What are the factors present or absent in sera which are suitable for use?
3. The role of metallic ions in complement action remains to be clarified. Employing newer techniques for depleting serum of divalent cations, a critical investigation of cation requirements of complement in opsonic, bactericidal and hemolytic systems should be conducted.
4. In chronic infectious disease states the host usually generates humoral antibody which exerts little or no apparent effect on acquired resistance. What factors are involved in the increased resistance that is apparent?
5. A common observation in whole body x-irradiated mice (LD_{50} range) is an overwhelming bacteremia; in contrast rabbits do not exhibit this effect when subjected to the same conditions. Is this a function of depressed activity of phagocytosis and/or cytopepsis in the mice and can these effects be quantitated? Why do not rabbits develop this bacteremia?
6. Evidence indicates that complement has opsonizing activity. To what extent does each complement component contribute to the opsonic reaction?

7. Inactivation of the third component of complement does not always occur when complement containing serum is mixed with polysaccharides although the polysaccharide-properdin complex is present. What factors are involved in these apparent exceptions?

8. The decreased resistance of animals to infection following whole body x-irradiation (LD_{50} range) is overcome by the survivors. What factors are involved in the return to normality?

9. Pathogenic bacteria produce substances which contribute to the virulence of the organism. To what extent can the virulence promoting characteristics of these substances be related to interference with humoral or cellular resistance mechanisms?

10. The source of protein exhibiting complement activity is unknown. What are the cellular origins involved in the synthesis of this protein?