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THE EFFECT OF VARIATIONS IN TEMPERATURE ON *IN VITRO* PHAGOCYTOSIS USING GUINEA-PIG LEUCOCYTES

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PART ONE--REVIEW OF THE LITERATURE

The word phagocytosis is derived from two Greek words: phago, I eat and kytos, cell. According to Olga Metchnikoff (1921), Elie Metchnikoff first applied the term in 1883. The phenomenon of phagocytosis has been variously defined but usually refers to the ingestion of a particle by a living cell; some definitions include further the fate of the particle ingested.

I. Biological significance of phagocytosis

A. Widespread distribution. Zinsser, Enders, and Fothergill (1939) have remarked upon the wide biological distribution of the phenomenon for purposes of nutrition in the lower forms, protection in the higher forms. Mudd, McCutcheon, and Lucke (1934) have compared Rhumbler's observations on the mechanism of food-intake in the amoeba with Lewis' (1931) findings regarding the mechanism of phagocytosis in mammals. Of the four types of intake in the amoeba, two have been definitely observed in mammalian leucocytes. In the latter, ingestion seems to be limited to a sinking of the particle to be ingested into the leucocytic protoplasm or to a flowing of leucocytic protoplasm around the particle to be ingested.

Within the mammalian body, the process of phagocytosis is of widespread distribution and significance. Zinsser, Enders, and Fothergill (1939) cite original work to support its importance in the removal of disintegrated red blood cells, the return of the pregnant uterus to normal, whitening of hair, and the formation of bile pigment.

With this broad biological significance in mind, further discussion of phagocytosis will be confined to the activity of the leucocytes of man and common laboratory animals.

B. Early concepts of phagocytosis. Although Hayem (1870a,b) observed the phenomenon, he considered the process one of spreading infection rather than one of resistance to infection. It was Panum (1874) who suggested that such ingestion might serve a protective function. The first experimental observations were reported by Metchnikoff in 1883 (Metchnikoff, 1921) with a crustacean, daphnia. In the course of these observations, he introduced the terms microphage and macrophage. These terms are still in use, although their connotations have changed.

C. Role of phagocyte in mammalian resistance to infection. In Metchnikoff's original classification, microphages (polymorphonuclear leucocytes) were considered to ingest only bacteria and other living agents of infection. The term macrophage, on the other hand, included all other phagocytes (mononuclear leucocytes, fixed tissue phagocytes); these cells were considered to be scavengers, ingesting cellular debris as well as non-living irritants.

On the basis of his early observations, Metchnikoff (1905) established a cellular concept of immunity, an animal being able to resist an infection insofar as its phagocytes were active against the infective agent. Although the correlation between degrees of phagocytosis and resistance has been confirmed (Bordet, 1891; Wright, 1927; Frisch, 1938), other factors of resistance have been observed.

That such correlations may not extend to all hosts and all pathogens is suggested by observations of other workers. Gay and Morrison (1923), in a study of pleural and peritoneal streptococcic infections in rabbits found resistance to be inversely related to the number of polymorphonuclear leucocytes at the site of injection although directly related to the number of clasmatocytes present. Goodpasture and Anderson (1937) reported that in the chick embryo an intracellular environment favored spread of infections with Streptococcus viridans, Aerobacter aerogenes, Eberthella typhosa and Brucella abortus but limited those with Staphylococcus aureus, Streptococcus hemolyticus, Corynebacterium diphtheriae.

D. Inflammation. An obvious role of phagocytes and a role in which they have been often studied is that of localizing irritants. Such phagocytic response and activity is but a part of a more complex physiologic response, inflammation.

Inflammation has been studied since the time of Hippocrates (460-377 B.C.), but the part of leucocytes was not recognized until the time of Cohnheim (1839-1884). Virchow (1821-1902) considered inflammation degenerative, leucocytes immigrating because of nutritive attraction of the degenerating tissue.

In the concept of Menkin (1940), the primary effect in an inflammatory response is an increased capillary permeability followed by formation of a fibrin network blocking the vessels which drain the area. Polymorphonuclear leucocytes now infilter to ingest the injurious particles. Whether these cells actually destroy the ingested particles or merely prevent their spread to other areas remains obscure.

A discussion of inflammation is beyond the scope of this paper, but mention should be made of factors giving rise to diapedesis of polymorphonuclear leucocytes. Historically, many substances have been observed to attract these cells: "phlogosin" in dead staphylococcus cultures by Leber (1879); disintegration products of leucocytes by Massart and Bordet (1891); protein from Friedlander's bacillus, glycine, and leucine by Buchner (1890); and the calcium ion by Wolf (1921). Following the work of Grand and Chambers (1937) who found a thermolabile, positively chemotactic substance in tissue which had been mechanically injured, Menkin (1937) was able to obtain from exudates a crystalline substance itself capable of increasing capillary permeability and attracting polymorphonuclear leucocytes. The material was obtained by extraction of cell-free exudate with either pyridine or dioxane, followed by acetone, and then butyl alcohol. The material thus obtained was further purified to obtain a crystalline substance resembling a polypeptide. That is, it was not a protein and was dialyzable but contained amino and carboxyl groups and an indole nucleus, and was precipitated by concentrated ammonium sulfate.

The problem of differentiating the functions of polymorphonuclear leucocytes and mononuclear phagocytes is complicated by the sequence of an exudative process. The macrophages tend to follow the microphages so that it is difficult to say which is responsible for actually destroying the injurious agent.

Considerable controversy has arisen in regard to the factors giving rise to this sequence. The phenomenon was reported by Borrel (1893) and

again by Durham (1897). Opie (1906) found that the proteolytic enzyme of the polymorphonuclear leucocyte was inactive in an acid reaction and subsequently (1907) attempted to correlate the reaction of a pleural exudate with its proteolytic activity over a period of five days. The reaction remained slightly alkaline throughout the experiment, but Menkin suggests failure to prevent loss of carbon dioxide from the exudate as a possible source of error. Weiss (1939) has found that enzymes from polymorphonuclear leucocytes but not those from mononuclears were capable of the following hydrolyses: of casein and gelatin at pH 8.0 and of d- or l-alanyl-glycine at pH 5.0. Muller and Jochmann (1906) have denied the existence of a protease in monocytes.

It was Menkin (1934) who first attempted a correlated study of cell picture and pH in an exudative process. In a composite graph of twelve experiments, he showed that the percentage of polymorphonuclear cells decreased following a drop in pH over a period of four days. In charts of individual animals (dogs), it was seen that a drop below pH 7.0 was usually followed by a decrease of polymorphonuclear cells to less than fifty percent and that a high average pH was accompanied by a high average polymorphonuclear range.

Menkin and Warner (1937) in a study of factors predisposing to this local acidosis reported that disturbance of carbohydrate metabolism by inflammation resulted in the production of lactic acid and depletion of alkali reserve. By inhibiting anaerobic glycolysis in an inflammatory process and thus maintaining a pH greater than 7.0, they were able to demonstrate in dogs a constant cellular picture, predominantly polymorphonuclear.

By *in vitro* studies, Menkin (1939) was able to demonstrate definite damage to polymorphonuclear leucocytes at pH 6.6, macrophages undamaged at pH 6.5. Observations, however, had shown (Menkin, 1934) that the per-cent of microphages dropped to less than fifty at pH below 7.0. Granting the differences in optimum pH for activity of their respective enzymes (Opie, 1907), one need not correlate this factor with survival of the cell unless he attributed to the particle ingested nutritive value for the phag-ocyte. Another possibility remains: Since Menkin observed higher pH ranges of damage *in vivo*, it is probable that under circumstances where the cells are actively phagocytic, they are more exposed to the detrimental properties of the environment.

The importance of pH in determining the cellular picture of an exudate has been challenged by Steinberg and his co-workers (1938a,b,c). The bases for their criticism of Menkin's work (1934) are that determinations made outside the animal body do not present a true picture and that colorimetric determinations on such material can not be accurate. As evidence for the first criticism, they reported aberrant results obtained for a given sample *in vivo* and at intervals over a thirty-minute period after removal of the exudate from the peritoneal cavity; factors involved, they felt were tem-perature and gaseous changes. In answer to these criticisms, Menkin (1939) suggested keeping the exudate under a film of oil and reported that he had obtained good checks between colorimetric and electrometric determinations. He further expressed doubt as to determinations by blind introduction of an electrode into a cavity, not insuring contact with sufficient exudate and criticized presentation of data as averages only (Steinberg and Dietz, 1938).

The findings of Steinberg and Dietz were essentially, "The hydrogen

ion concentration showed no relationship to the cell predominant in the exudate, to the type of bacterium or toxin used, or to the duration of the inflammatory process." Using the technique indicated above and anesthetizing the animals, these investigators followed over a period of at least five days, usually seven, the peritoneal exudates resulting in rats and in dogs from the injection of diverse materials: forty-eight hour broth culture of _Staphylococcus aureus_ at pH 8.57; forty-eight hour broth culture of pneumococcus, type I, pH 4.90; and twenty-four culture of diphtheria bacillus, pH 5.36. Botulinus toxin was tested with similar results. An inflammatory response was insured by the injection of tragacanth along with these materials.

Because of conflicting reports as to the role of phagocytes, Steinberg and Martin undertook a study of a variety of animals and infectious agents. Dogs, rats, rabbits were employed with the following infectious agents: pneumococcus, type I; _Escherichia coli_, _Staphylococcus aureus_, hemolytic streptococcus, and _Corynebacterium diphtheriae_. At frequent intervals over periods of several days, exudate was taken: culture tests, differential white cell counts, and determinations of percent phagocytosis were made. In the rat, and to a lesser extent in the rabbit, clasmatocytes (mononuclears) tended to appear with the disappearance of free bacteria. In the dog, few mononuclears appeared under any circumstance. For this reason, the authors felt that the mononuclears were actually scavengers and that the first line of defense consisted of polymorphonuclear cells. When polymorphonuclear cells from dogs were introduced into the peritoneal cavities of rats and rabbits, the mononuclear response occurred more readily. Admitting the possibility stated by these writers that some factor in the polymorphonuclears either stimulated the production of mononuclears or attracted them

to the inflammatory field, it seems more consistent with the data of the same investigators to assume that when these extra leucocytes were present, the free bacteria merely disappeared more rapidly, presenting the condition apparently concommitant with the appearance of mononuclears. Similar evidence favoring a protective function for the polymorphonuclear leucocyte has been presented by Steinberg (1938) His observations showed that an otherwise fatal peritoneal infection (induced by injection of large numbers of colon bacilli) in dogs could be overcome by transferring exudative leucocytes from another dog. More specifically, all fourteen of the untreated controls died, while eight of nine treated animals survived. The leucocytes were taken at intervals from three hours to fourteen days following injection of a sterile irritant, exudates showing a minimum of eighty-six percent polymorphonuclears on direct smear. No protection was afforded by leucocytes which had remained outside the animal body more than two hours. Although positive bacterial cultures were obtained seven to ten days following administration of leucocytes, comparison of bacterial counts with those of untreated controls showed a consistent decrease of a factor approximating 10^3. Three other control groups treated respectively with whole exudate, supernatant, and extract of disintegrated leucocytes indicated that whole leucocytes were essential for the protective effect.

D. Anaphylactic type of inflammation. A similar but more pronounced inflammatory response is often obtained in animals which have had previous contact with the causative agent. Where the agent is a pyogenic bacterium there seems to be little doubt that this heightened response represents increased resistance (Wadsworth, 1904).

But, in regard to the similar allergic reaction occurring on second contact with protein of the tubercle bacillus, there is less agreement.

The observations of Opie (1929) indicated that the histological picture was one of polymorphonuclear infiltration during the first forty-eight hours, followed then by mononuclears. Dienes and Mallory (1932), on the other hand, found that the reaction differed from other inflammations by its early and late infiltration of mononuclears, polymorphonuclears entering in intermediate stages, apparently when necrosis occurred. A protective function of the hypersensitivity reactions was claimed by Krause and Willis (1924). Other investigators represented by Rich *et al*. (1932, 1934) have found equal localization in desensitized animals indicating that the allergic inflammatory reaction is but an early manifestation of developing immunity. But it should be remembered, as Topley and Wilson (1937) have pointed out, that desensitization probably involves more than mere loss of allergic hypersensitivity. An ultimate result of the reaction is one of damage to the cells in the area; this effect, Opie (1929) felt, was one of protection of vital organs at the expense of local tissue.

The reaction termed anaphylactic inflammation differs from the tuberculin type primarily in that the former has been passively transferred. Other immunologic differences are listed by Dienes and Mallory (1932), who also noted histological differences in the two types of skin reactions in guinea pigs. Polymorphonuclears predominated during most of the anaphylactic type of reaction, mononuclears arriving late. It was observed that tuberculous animals gave a more mononuclear response whatever the inciting agent; polymorphonuclears also were present in the necrotic stages of tuberculous reactions. According to Medlar and Sasano (1933), polymorphonuclears are found in a tuberculous lesion in numbers proportional to the amount of tissue damage. Allergic condition of the animal or age of the lesion affect the cell picture only insofar as they determine the amount of tissue damage.

Apparently, no one is willing to commit himself on the precise activity of the cells involved, although Opie (1924) has shown positive chemotaxis of specific antigen-antibody combination for polymorphonuclear leucocytes and has postulated intracellular digestion. Opie's antigen in this study was horse serum. Stewart, Long, and Bradley (1926) studied the tuberculin reaction in the pleural cavity of guinea pigs. In the presence of tuberculo-protein and its antibody, polymorphonuclears died while mononuclears lived; the interpretation of these investigators was that polymorphonuclears were sensitized, antigen-antibody combination occurring on their surfaces. These authors admitted the possibility of generalized resistance at the expense of local injury as later presented by Opie (1929) in the anaphylactic type of reaction. The former investigators, however, were reluctant to attribute a protective value to this type of localized response.

E. Phagocytosis in the bloodstream. Phagocytosis within the bloodstream is frequently observed in cases of septicemia (Denys and Kaisin, 1893; Wright, 1927), and whole blood has been used in phagocytosis experiments (Leishman, 1902; Cottingham and Mills, 1943). Although an inflammatory response is frequently associated with leucocytosis, certain infections, as, for example, typhoid fever, may be may be accompanied by leucopenia. According to Best and Taylor (1943), the latter condition may result from a relocation of the phagocytes rather than an absolute reduction in number. Leucocytosis usually presents an increased percentage of immature polymorphonuclear cells with relatively insignificant rises in the number of mature neutrophiles.

Such observations have given rise to the hypothesis that the factors disposing to leucocytosis have their primary effect upon the hemopoietic tissues. McCutcheon (1942) found evidence that the resulting cells moved into the bloodstream as a path of least resistance, moving by means of an alternation of sol-gel phases.

Leucotaxine, the substance which Menkin (1938) had found to be effective in eliciting an inflammatory response, had no apparent effect on the number of leucocytes in the bloodstream (Menkin, 1940), although it did increase capillary permeability and attract phagocytes. Menkin (1940) further observed that exudates from localized inflammation in dogs incited an increased white cell count and that an exudate from an animal having a relatively low leucocyte count (bloodstream) had also a relatively low capacity for inducing leucocytosis. These findings suggested that some fraction of the exudate might possess leucocytosis-promoting properties, and a heat-labile (60°C.), non-dialyzable protein substance seemed to be responsible. Further studies (Menkin, 1944) showed that such a substance could be concentrated from the supernatant of a one-third-saturated solution of ammonium sulfate.

II. Humoral factors affecting phagocytosis

A. Bactericidal effects of body fluids. Of historical interest is the role which leucocytes (especially polymorphonuclears) were considered to play in the production of bactericidal substances. Pfeiffer's (1894) observations on the extracellular disintegration of cholera vibrios constituted a severe threat to Metchnikoff's phagocytic theory. Subsequent studies (1895, 1899) led Metchnikoff to the opinion that

humoral immune factors were derived from disintegration of the leucocytes. It was Bordet, a student of Metchnikoff, who (1895) noted that in Pfeiffer's phenomenon two factors were functional: a heat-stable immune antibody and a heat-labile "complement". The latter, Bordet maintained, was of leucocytic origin.

The more modern concept of humoral immune factors dates from the work of Denys and Kaisin (1893) who found that bactericidal activity of blood could be reduced by the addition of dead organisms and that the bactericidal power of rabbit blood increased as an experimental infection progressed. While these observations suggested that the point of attack was upon the organisms, there was still the idea that leucocytes were important in the production of antibody. Denys and LeClef (1895) found that, while immune serum alone was inhibitory to growth (normal serum was not), the addition of leucocytes increased the _in vitro_ bactericidal power in proportion to the number in which they were added. This phenomenon occurred whether the leucocytes were from a normal or an immune animal. Slides made from such preparations showed phagocytosis ih proportion to inhibition and no evidence of extracellular disintegration of the bacteria. Evidence against the leucocytic origin of antibody was found in the observation that exposure of blood serum to leucocytes did not enhance the bactericidal activity of the serum. Microscopic examinations suggested that leucocytes were always actively mobile, not further stimulated by immune serum.

B. _Stimulins_. Metchnikoff, in his early work (1905), found serum to have a stimulating effect on activity of leucocytes, which effect

was not increased by active immunization. The factors in serum responsible for this activity he called "stimulins". Sawtchenko (1902) studied phagocytosis of red blood cells and the effect of immune serum. It was his opinion that the antibody should be called an intermediate body being adsorbed by both leucocytes and the particle to be ingested and thus altering chemotaxis. Wright and Douglas (1903) found that leucocytes which had been exposed to serum and subsequently washed in saline exhibited no increased phagocytic activity. Since the serum used was fresh normal serum and since leucocytes had already been in a similar environment, it would seem that such a test fails to rule out the possibility that some antibody was adherent to the leucocytes. Sellards (1908) found that washed sensitized cells were always ingested slightly less readily than bacteria remaining suspended in the corresponding serum; this phenomenon he attributed to a "stimulin" effect of the serum medium upon the mobility of the leucocytes.

C. Opsonins. Following the observations of Denys and LeClef (1895), Wright and Douglas (1903) emphasized the effect of humoral factors upon the bacteria and coined the term "opsonin" to describe such factors. These workers were the first to report the necessity of a heat-labile factor in the opsonic activity of normal serum. Comparisons of this substance with hemolytic and bactericidal complement as described by Bordet (1895) will be discussed later. Hektoen and Ruediger (1905) noted that opsonins of one species were effective with leucocytes of another species, lending further weight to the effect of serum upon bacteria.

It was obvious from the work of Wright and Douglas (1903), however,

that not all the opsonic activity of normal serum was destroyed by inactivation temperatures. The demonstration of a thermostable opsonin in normal serum involved more than the observation that some phagocytosis occurred in heated normal serum as Wright and Douglas (1903) had done. Cowie and Chapin (1907) were able repeatedly to combine heated normal and diluted fresh normal sera (each alone incapable of opsonization) to obtain a mixture with a high opsonic index.
Ward and Enders (1933), while unable to repeat this sort of observation, found that the same was true if fresh infant serum were substituted for diluted fresh normal serum.

D. Bacteriotropins. Sawtchenko (1902) had demonstrated a thermostable immune opsonin for red blood cells, when Neufeld and Rimpau (1904, 1905) demonstrated similar antibodies for streptococci and pheumococci and introduced the term "bacteriotropin". The specificity of such antibodies for red blood cells and for bacteria was confirmed by Russel (1907).

The question naturally arose as to whether thermostable normal and immune antibody were the same, differing only in quantity. The classic method of study became the testing of normal sera absorbed with a given organism against both the same organism and others. Arguments for specificity of the normal thermo-stable opsonin were presented in the work of Bulloch and Western (1906), Bull and McKee (1921), Sia (1926), and Ward and Enders (1933). Arguments against such specificity were presented in the work of Simon, Lamar, and Bispham (1906); Russell (1907); and Ledingham (1908). It seems significant that Ward and Enders were able to use infants' sera as a source of heat-labile substance without intro-

ducing the "normal" heat-stable opsonin, which was present in the serum of "normal" adults; this observation suggests that the so-called "normal" heat-stable specific opsonin may differ from the similar substance in immune serum only in quantity, the normal antibody resulting from either subclinical contact with the organism in question or with the same antigen elsewhere in nature. Simon, Lamar, and Bispham (1906) found normal opsonins in euglobulin, the fraction in which Strumia _et al_. (1930) found the immune antibody responsible for opsonization.

E. _Relation of normal thermo-labile opsonin to hemolytic complement_. The nature of the thermo-labile component has presented a problem equal to that of the normal thermo-stable component. That is, it has an inactivation temperature (55°C. to 60°C.) comparable to that of Bordet's complement and is apparently coexistent with hemolytic complement in nature: Zinsser, Enders, and Fothergill (1939) point out that both are reduced in phosphorous poisoning, absent from normal aqueous humor, present in aqueous humor following injury. Muir and Martin (1906) found that specific antigen and antibody combinations took out "normal opsonin" as they removed hemolytic complement but these workers declined to speculate as to the identity of the two substances. On the other hand, Bulloch and Atkin (1905), Russell (1907), and Sellards (1908) found that normal opsonin was absorbed at 0°C., while complement is ordinarily not removed at this temperature. In Sellard's observations, materials were cooled before mixing and were filtered while cool; controls showed that the low temperature of itself had no destructive effect on the heat-labile component. Welch, Brewer, and Hunter (1940) found that antiseptics destroyed

opsonic complement without destroying hemolytic complement. It would seem that while the thermolabile opsonin of normal serum is closely related to complement of hemolysis and bacteriolysis, the two substances are probably not strictly identical.

F. Role of the heat-labile factor. The mechanism whereby the heat-labile factor takes part in opsonizing bacteria has been a problem difficult to solve. The work of Wright and Douglas (1903) suggested that it acted only as a catalyst. That is, once it had aided in the union of normal heat-stable antibody and antigen, inactivation temperatures failed to desensitize the bacteria. Hektoen and Ruediger (1905) not only failed to confirm this observation but found that bacteria thus treated could not be opsonized subsequently. Sellards' (1908) findings agreed more closely with those of Wright and Douglas, although he was able to deopsonize by heating if he first washed the opsonized cells as Hektoen and Ruediger had done. In any case, it hardly seems conclusive that the heat-labile factor was only a catalyst. If, as the work of Muir and Martin (1906) suggests, it is bound as a result of such opsonizations, its heat-labile portion may, by such union, be protected from heat destruction. The more recent work of Ward and Enders (1933) was done with immune antibody and the heat-labile factor. These authors found that the complement-like substance was not capable of enhancing the final degree of opsonization but only of attaining the final level in less time.

Similar observations with opsonic complement and the so-called "normal" heat-stable factor would be further evidence of the identity of this factor and the immune opsonin (or tropin).

An observation of this nature, although not strictly quantitative, was made by Rosenthal (1909).

III. Physical and chemical considerations in phagocytosis

A. Surface effects. The mechanism whereby opsonins promote phagocytosis is primarily one of surface phenomena. Sellards (1908) found evidence for adsorption of opsonins on bacterial and leucocyte surfaces in the observation that chemically inert carbon particles removed opsonins. He maintained, however, that serum had some stimulating effect on mobility of the leucocytes.

According to Mudd, McCutcheon and Lucke (1934), the first formulation for surface effects in phagocytosis was made by Rhumbler (1914), who studied food ingestion in the amoeba. Other formulations were made on the basis of Rhumbler's, the most generally accepted being that of Fenn (1922b). Mudd and Mudd (1933), found the following conclusions implied by Fenn's theory: (1) that quantitative correlation should exist between phagocytosis and surface properties of the particle ingested, (2) that phagocytosis is essentially a phenomenon of spreading of phagocyte surface of the particle to be ingested, and (3) that partial ingestion should occur either at equilibrium of various forces or when surface forces would lead to ingestion but the phagocyte is inert.

Evidence for the first of these conclusions dates from the observations of Mudd, Lucke, McCutcheon, and Strumia (1929) and of Lucke *et al.* (1929). Their findings indicated that along with good opsonization of

acid-fast bacteria occurred also increased cohesiveness, decreased surface electrical potential, and decreased wettability with oil. The view that one factor was responsible for all these effects was strengthened by the findings of the same group (Mudd et al., 1930) that all effects were produced by immune serum and its fractions in the following decreasing order of effectiveness: whole serum, globulins, and albumen. However, a slight discrepancy was observed in the relative effectiveness of euglobulins and pseudoglobulins for opsonization and agglutination, on the one hand, and for changing cataphoretic properties on the other. Strumia et al. (1930), by studying cross-reactions among the acid-fast group and their respective antisera, found evidence that following the specific union of antigen and antibody on the surface of the organism, the surface properties of the resulting complex were largely those of the serum globulins in proportion as the antigen (organism) had adsorbed them.

Their second conclusion Mudd and Mudd (1933) confirmed by direct microscopic observations. The mechanisms of ingestion observed, even those involving great distortion, showed no vacuole surrounding the ingested particle. Furthermore, weakly sensitized particles adhered to the phagocyte but did not enter.

This latter observation helped to confirm the third implied conclusion--that partial ingestion may occur when surface forces are at equilibrium. This view, of course, only assumes that unmeasured forces are at equilibrium; under similar conditions more strongly sensitized particles were completely ingested. The other condition leading to partial ingestion (inertia of the phagocyte) these workers observed only

with macrophages and oil droplets; small droplets were readily ingested, but the larger ones were too much for the macrophages, which resisted deformation.

It is interesting that Hamburger (1912) observed that such surface depressants as iodoform, chloroform, and ethyl alcohol enhanced in vitro phagocytosis of carbon particles. His results were reported as number of phagocytes active. Graham (1911) found that ether caused a decrease in opsonic index, apparently by inhibiting sensitization. Although this effect was alleviated by the addition of lecithin, lecithin alone had no apparent effect on the process. Similarly, Arkin (1913) found alcohol, chloroform, ether, and chloral hydrate to inhibit phagocytosis but he attributed this effect to an inhibition of oxidation.

B. Ionic effects. The effect of ions has received considerable attention but much controversy remains as to whether the effect is one of surface factors or of penetration into the phagocyte.

Hamburger (1912) found isotonicity to be optimum for phagocytosis. Departures toward either extreme were more inhibitory with NaBr than with NaCl. In Hamburger's series, $CaCl_2$ apparently increased the rate of phagocytosis in either saline or serum. Tunnicliff (1931) reported an increase in phagocytic power of rabbit's blood following injections of calcium gluconate and found similar results when the material was added in vitro. Radsma (1919-20) found that calcium salts enhanced phagocytosis only when calcium had been removed from blood by means of citrate or oxalate. Hektoen and Ruedger (1905), on the other hand, found that serum treated with M/8 $CaCl_2$ was unable to opsonize streptococci. The authors interpre-

ted this result as an effect directly upon the serum, but an effect on the process of opsonization seems more likely. Mudd _et al._, (1934) gave little support to Hamburger's hypothesis that the beneficial effect of calcium is the replacing of that lost from the cell or that similar effects of other salts are due to penetration of the cell. Rather, they attributed these effects to changes in interfacial tension at the surface of the phagocyte.

The effects of pH, also, Mudd _et al._ (1934) have considered as primarily surface effects--this time affecting adsorption of opsonins onto the bacterial surface. They admitted, however, that Evans' (1922) findings indicated penetration of the cell. Briefly, Evans' findings were that pH between the range of 4.0 and 8.0 had little effect on opsonization of streptococci and that organic acids were more inhibitory to phagocytosis than mineral acids at the same pH, organic acids being able to penetrate the cell more readily than are mineral acids (Jacobs, 1924). Moreover, Evans found that the effect of citric acid at a given pH was proportional to the length of time and number of times the leucocytes had been exposed to the acid environment; _i.e._, the effect was cumulative.

C. _Temperature_. The physico-chemical factor with which we are most concerned is temperature. Although it may be studied as such _in vitro_, experimental evidence suggests that, _in vivo_, it is more than just that.

Considering the _in vitro_ system, Ledingham (1908) found phagocytosis to increase over a temperature range of 18°C. to 43°C. The animal species from which he obtained leucocytes is not stated. He was unable to calculate temperature coefficients because the rate of increase did not remain constant. Using _Staphylococcus aureus_ and fresh normal serum, Ledingham

preceded phagocytosis by a period of opsonization. Altogether, his findings suggest that increased temperature enhances both opsonization and phagocytosis but primarily the former. Furthermore, the effect on opsonization is apparently one of increasing the rate of the reaction rather than raising its end-point, longer time intervals and light bacterial suspensions tending to reduce differences. In regard to earlier work along these lines, two other papers should be mentioned. Bulloch and Atkin (1905) found in two experiments that staphylococci had completely adsorbed the opsonin from fresh normal serum in as little as ten minutes, whether the adsorption temperature be 0°C. or 37°C. In both these experiments, supernatants were tested; in the second experiment, the sensitized cells also were tested by adding leucocytes. Dean (1905) used heated normal human serum for sensitizing _Staphylococcus aureus_ over a thirty-minute period. Temperatures for sensitization were 6°C. to 8°C. and 37°C. Leucocytes were then added to the sensitized bacteria and phagocytosis recorded. The phagocytic index for bacteria sensitized at the higher temperature was ten times as large as that for bacteria sensitized at ice-box temperature. Dean's assumption that the effect was on rate of opsonization hardly seems justifiable, since only one time interval was investigated and only one experiment reported. Sellards (1908) tested the tendency of bacteria to be phagocyted by rabbit leucocytes following sensitization with fresh normal serum for fifteen minute intervals at 0°C. and at 37°C. In his two experiments, he found distinctly more phagocytosis following sensitization at 37°C. Ledingham (1908) investigated the disappearance of opsonin from fresh normal serum incubated at 0°C. and 37°C. with dead tubercle bacilli; whether the incubation time was thirty minutes or one

hour and forty-five minutes, his three experiments indicated that more opsonin disappeared at 37°C. than at 0°C.

Of interest is the work of Madsen and Wulff (1919), who found that the optimum temperature for phagocytosis by phagocytes of a given animal species was the "normal" body temperature of the animal: 37°C. for human leucocytes, 39°C. for guinea-pig leucocytes, and 41°C. for leucocytes of pigeons and chickens. Leucocytes from cold-blooded animals (frogs) ingested bacteria as well at 0°C. as at higher temperatures. Even more remarkable, these authors found leucocytes from febrile patients to exhibit an optimum at the temperature exhibited at the time the leucocytes were taken. They did not, however, indicate in what manner body temperatures of the patients were determined.

Ledingham's (1908) data showed higher phagocytic indices at 42°C. than at 37°C. in five out of six experiments; it is assumed that he used human leucocytes. Ellingson and Clark (1942) found that guinea-pig leucocytes were actively phagocytic over the range of 39°C. to 41°C. , while human leucocytes exhibited an optimum of 38°C. to 40°C. This difference in species optima lends support to the findings of Madsen and Wulff (1919), although Ellingson and Clark have reported only one experiment with leucocytes of each species. Ono (1928) reported the optimal range for phagocytosis of starch granules by rabbit leucocytes to lie between 25°C. and 30°C., higher temperatures inhibiting ameboid movement of leucocytes. Both he (1928) and Chadani reported that a rise in temperature increased phagocytosis by frog leucocytes.

In vivo experiments of Ellingson and Clark (1942) showed that artificial fever maintained from the time of injection (intracutaneous) of

pneumococcus until the infection itself caused fever in unheated controls had no marked effect on the outcome of such infections. Neither survival of the animals nor blood counts showed significant effect on resistance.

In the same article reference is made to the work of Rolly and Meltzer (1908), who found that placing animals in a hot box very slightly increased resistance. Besides the fact that the number of animals was small, body temperatures of the animals were not determined.

Cottingham and Mills (1943) have investigated the effect of environmental temperature and diet on _in vivo_ phagocytosis in mice. Smears of peritoneal exudate were reported by the authors to be variable in cell picture, but they reported two series, one with graded amounts of thiamin in the diets, the other with graded amounts of choline in the diets. The method included keeping animals on prescribed diets either in a hot room (90°F, to 91°F., 60 to 70 percent. relative humidity) or in a cool room (68°F.) for three weeks before testing for phagocytosis. Highest levels of phagocytosis were reported for animals in the hot room and on diets including either thiamin or choline in excess for animals kept at the lower temperature. The implication is that increased temperature increased the vitamin requirements but that, once these requirements were satisfied, phagocytosis was enhanced. Such high vitamin levels inhibited both growth and phagocytosis in animals at 68°F. It is unlikely that the temperature of the hot room was sufficient to give the animals fever. Ellingson (1939) found it necessary to employ hot-box temperatures of 94°F. to 105.8°F. to induce rises of 4°F. in rabbit temperatures (rectal). It might be asked whether short periods at such high temperatures might also require an

altered diet.

Cottingham and Mills (1943) and Mills and Cottingham (1943) tested their animals by still another technique. Extending the diet studies to include different levels of other vitamins of the B group, ascorbic acid and protein, they bled rats and guinea pigs which had been kept either in a hot or in a cool room for several weeks and fed a specified diet. Phagocytosis in heparinized blood was then tested _in vitro_. In the choline series, results were similar to those for the _in vivo_ mouse series. In ascorbic acid series, the highest level reported did not inhibit phagocytosis; however, the heated animals' phagocytes showed a higher degree of phagocytosis than did those of animals kept at 68°F. Heated animals apparantly required a higher level of protein; but, once these animals were supplied this extra requirement, their phagocytes were more active than those of animals kept in the cold. The differences due to temperature were small, but the regularity with which they occur in the various diets compared suggests that the differences are real. While no conclusion can be drawn from these experiments regarding the effect of body temperature on _in vivo_ phagocytosis, the results may help to explain negative results reported for artificial infections in heated animals.

It would seem, then that the effect of body temperature on _in vivo_ phagocytosis is an unexplored field. The only microscopic determinations of _in vivo_ phagocytosis reported fail to take into account more than environmental temperature. On the other hand, the reports of body temperature in relation to resistance fail to include microscopic determination of phagocytosis.

IV. Mathematical considerations

The various data published on the effect of temperature on phagocytosis fail to answer conclusively the question how and to what extent increased temperature increases phagocytosis. It seems fairly definite that there is an increase in the number of bacteria ingested as temperature increases between 0°C. and 37°C.

A. Attempts to derive formulae. Mudd et al. (1934) insisted that determinations of temperature effects be made at more than one time interval before expressing results as temperature coefficients. That is, there should be some conception of the rate of the reaction at each of the temperatures to be compared.

Madsen and Wulff (1919), using only one time interval and determining phagocytic indices, found four to ten times as much phagocytosis at 37°C. as at 18°C. These authors attempted to calculate expected phagocytic indices at higher temperatures by substituting values obtained at lower temperatures in the equation for the Van't Hoff-Arrhenius law, but their calculated values were consistently too low.

Madsen and Watabiki (1919) plotted time curves for data obtained at different temperatures. In the lower temperature ranges, the rate of the reaction suggested that it might be a monomolecular one. The formula for this reaction states that the rate of the reaction is proportional to the rate of disappearance of reagents: $K=\frac{1}{T}\log\frac{A}{A-x}$, where K=velocity constant, A=total amount of a substance (e.g. particles being ingested), x=amount of substance disappearing in time T. But Fenn (1922a) has pointed out that in

attempting to apply this equation, Madsen and Watabiki have taken A not as the total number of bacteria present but as the maximum number ingested at the temperature being considered. He further criticized that not only were there always more bacteria than the total ingested but also that the maximum ingested varied with the temperature. Although Fenn (1921a,b) had found the formula for a monomolecular equation to fit phagocytosis of carbon and quartz particles by rat leucocytes, he re-analyzed the data obtained by Madsen and Watabiki to show that the same principle did not apply to phagocytosis of bacteria. Having established the time necessary for maximum phagocytosis at each temperature, Fenn then calculated the bacteria per leucocyte per minute for the first half of the reaction at each temperature. In this manner, Fenn found ten-degree-Centigrade temperature coefficients approximating 2.0 in a temperature range from 0°C. to 35°C.

B. Complexities of the reaction. But this temperature coefficient, according to Fenn, represents at least two reactions besides phagocytosis proper: The first occurs in a latent period necessary to prepare the bacteria for ingestion and the phagocytes for activity. Ledingham (1908) had observed this latent period and had found it shorter at higher temperatures. In fact, Ledingham's data suggested that the speeding of this preparatory reaction was the principal effect of increased temperatures. The third reaction observed by Fenn was a "lethal" reaction resulting in a shorter active period of phagocytes at higher temperatures. Fenn reasoned that the temperature coefficient of this reaction was lower than that for phagocytosis, since increase in temperature continued to increase the phagocytic index. Calculation of Q_{10} of the "lethal reaction" on the basis of

time required to complete the total reaction substantiated Fenn's hypothesis. The "lethal reaction" was considered by Fenn as one which injured the leucocytes directly.

Ledingham (1912) found that increased bacteria-to-leucocyte ratios resulted in increased phagocytic indices but that the latter increase did not keep pace with the former. This decreased efficiency he could not account for solely on the basis of reduced amount of opsonin available. Hanks (1940) confirmed these findings of Ledingham and found that the percent of phagocytes active likewise increased but at a slower pace than the bacteria-to-leucocyte ratio. He rejected the hypothesis of Fenn (1922a) that the "lethal reaction" was due to unneutralized bacterial products on the ground that equally sensitized bacterial suspensions still exhibited the phenomenon. But it seems likely that, in diluting bacterial suspensions following sensitization, one is also diluting adsorbed, unneutralized anti-phagocytic substances which may have accumulated. Furthermore, the bacteria thus sensitized and diluted are still alive and probably capable of producing anti-phagocytic substances; it is logical that a larger number of bacteria would produce more of such a substance. Hanks' (1940) explanation for the decreased efficiency of a system with a higher bacteria-to-leucocyte ratio is that surface energy relations are altered.

Where the formula for a monomolecular reaction applies, as in the ingestion of carbon and quartz particles (Fenn, 1921a,b), it would be expected that temperature changes could not change the end-point of the reaction. By changing the chances for contact per unit time, an increase in temperature might, nevertheless, increase the rate at which the end-

point was reached. Fenn's analysis (1922a) of Madsen's and Watabiki's (1919) data for phagocytosis of bacteria shows that increased temperature does change the end-point.

C. Reporting results. Most of the earlier work is reported in average numbers of bacteria per leucocyte (Leishman, 1902). Hamburger (1912) introduced the use of percent of phagocytes active. A mathematical relation was found to exist between the two determinations (McKendrick, 1913-14): The amount of work done (i.e., the phagocytic index) was a logarithmic function of the number of inactive phagocytes, assuming an even distribution of bacteria in the leucocytes. Hanks (1940), with the number of bacteria as his only variable, found that the phagocytic index increased as a linear function of increasing numbers, the percent phagocytes active to increase as a logarithmic function. Neither determination, however, revealed the effectiveness of the system, unless the total numbers of bacteria and leucocytes were known.

V. Experimental methods

A. In vivo methods. As widely varying as methods of reporting results and interpreting them have been methods of study. It is interesting that some of the earliest work--that of Metchnikoff--was done with in vivo techniques. According to Olga Metchnikoff (1921), he used the Crustacean daphnia, which is transparent, with yeast cells as his test organism. In vivo techniques lend themselves less readily to experimental control and have received less attention and development than have in vitro techniques. Durham (1897) studied the course of peritoneal infections with special

reference to phagocytes and phagocytosis by sacrificing animals at various intervals following infections. Steinberg et al. (1938) have made similar studies without sacrificing their animals. In some cases, leucocytes were added from other animals; in others, only natural cellular response was studied for comparison. Cottingham and Mills (1943) have studied intraperitoneal phagocytosis of pneumococci by sacrificing the animals four hours following infection; cells were only those in response to the infectious agent.

Denys and Kaisin (1893) studied disappearance of colon bacilli from the bloodstream as an index of phagocytosis. They assumed such disappearance to be due to phagocytosis, since direct smears showed phagocytosis with no evidence of extracellular disintegration of the bacteria. Wright (1927) used the rate of disappearance of pneumococci from the bloodstream as an index of resistance of rabbits. Zinsser, Enders, and Fothergill (1939) pointed out that this technique, in so far as it is to be applicable to phagocytosis studies, must be limited to the use of organisms resisting extracellular lysis.

B. In vitro methods. In vitro methods have been used much more commonly than in vivo ones. Denys and LeClef (1895) studied blood leucocytes in the presence of antiserum; estimates of the degree of phagocytosis were made by subsequent plate counts. Leishman (1902) devised a film method of incubation, followed by examination of stained smears. The method was far from perfect, but a control of normal blood to parallel each test served to minimize several variables. Comparison of the average number of staphylococci per leucocyte in the patient's blood with that of the control

suggested importance of phagocytosis in determining the patient's condition. These studies were made in connection with the use of therapeutic staphylococcic vaccines which was a common practice at that time.

Wright and Douglas (1903) suggested the use of blood decalcified with either citrate or oxalate. Wright (1921) introduced many new techniques into the study of phagocytosis. Perhaps his most outstanding contribution was the use of a single suspension of leucocytes with several normal sera as well as with the patient's serum. Stained smears were examined to determine the average number of bacteria ingested per leucocyte (phagocytic index). The significant determination was that of opsonic index--*i.e.*, the ratio of the phagocytic index obtained with patient's serum to the average phagocytic index obtained with normal sera. A larger number of cells was considered than was the case with Leishman's technique.

Wright's method constituted a practical application of the hypothesis of Denys and LeClef (1895) that variations in phagocytic activity were due to variations in the capacity of different sera to sensitize bacteria for ingestion. Although the opsonic test has not been widely employed as a clinical laboratory test, it still finds its place as an aid in the diagnosis of Brucellosis (Tovar, 1944). The usual technique has slipped back to the use of whole blood with its varying numbers of leucocytes, a practice criticized by Hanks (1940). The citrate (according to Tovar, 1944) is employed so as to inhibit normal opsonins. Where no inhibition is desired, the use of heparin as an anticoagulant has been recommended by Boerner and Mudd (1935). Heparin has been thus employed by Westerfeld and Senekjian (1937) and by Cottingham and Mills (1943).

Another advance which has been made in the field of investigation and which has failed to take hold in routine laboratory tests is the use of a uniform shaking or rotating device. According to Fleming (1931), it was Parry who first suggested the use of uniform mixing, but the earliest report in the literature is that of Rosenow (1906), who favored this method for simulating in vivo conditions. It is doubtful whether any biological system ever made 120 to 150 vibrations per minute through a three mm. stroke, but the technique did serve to standardize chance contacts so that more accurate determinations could be made of differences in opsonic activity. Subsequent modifications of the mixing technique have been made so that a smoother, slower rotation has been the trend in more recent work: Fenn (1921a), nineteen revolutions per minute about a horizontal axis; Robertson and Sia (1924), eighteen to twenty revolutions per minute and eight to ten oscillations per minute through a 10° to 11° arc; Todd (1927), twenty revolutions per minute about a horizontal axis; Ward (1930), six revolutions per minute about a horizontal axis; Ellingson (1939), twenty revolutions per minute about an axis 20° from the longitudinal axis of the tube; Kass and Seastone (1944), two revolutions per minute about the longitudinal axis.

Hotopp and Kahn (1936) have applied the single-cell technique to phagocytic studies. By isolating and inoculating onto suitable media single active phagocytes at various time intervals, the fate of the ingested particle can be studied.

PART TWO--EXPERIMENTAL

I. Technique

5 to 10 cc. of aleuronat-starch paste* mixed with an equal volume of tryptose broth (total of 10-20 cc.) was injected intraperitoneally into guinea pigs eighteen to twenty-four hours before leucocytes were desired. Leucocytes were obtained from the resulting peritoneal exudate. Except in the runs designated 1 through 10 in Group C, the animal was exsanguinated and the leucocytes harvested in saline. After brief, slow centrifugation, the leucocytes were resuspended in saline; no attempt was made to wash the leucocytes, since serum from the same animal was to be used. In Group C, Experiments 1 through 10, only a small amount of blood was taken and peritoneal exudate was aspirated without killing the animal.

The *in vitro* system consisted of 0.1 cc. each of leucocytes, serum from the same animal, and bacteria mixed in tubes of 8 mm. diameter.

Since Group A was for the purpose of standardization of procedure, details for that group will be presented along with the results. The following description applies to Groups B, C, and D only.

The leucocyte suspension was standardized so that there were 25,000 to 30,000 cells per c.mm. as determined by direct count (Neubauer haemocytometer. The test organism was an old stock strain of *Staphylococcus aureus*. Plain agar cultures were harvested after twenty hours' growth (*twenty-four*-hour broth culture used for inoculum). The bacteria were

* 3 percent potato starch in distilled water boiled, aleuronat added to make 5 percent.

harvested in saline, shaken until only single cells and a few small clumps remained. After being diluted to the turbidity of McFarland Nephelometer standard number one, the bacterial suspension was stored in the refrigerator to be used over a period not exceeding six weeks; pH and gram-staining characteristics showed little change during this period. At the time of use, a portion of the suspension was diluted with an equal volume of saline so that the final suspension used was half the turbidity of McFarland standard number one. Direct count (Petroff-Hausser bacteria counter) showed 1,200,000 organisms per c.mm., while a total plate count gave 1,510,000 per c.mm. The bacteria-to-leucocyte ratio was between 40:1 and 60:1.

The test organism showed the following characteristics (August 17, 1944):

hemolysis, moderate
coagulase, positive at two hours
gelatine liquefaction, negative at twenty-four hours, positive at forty-eight hours
mannite fermentation, doubtful at forty-eight hours, positive at seventy-two hours.

5 cc. of sterile Seitz filtrate from a suspension of a twenty-four-hour agar-slant culture injected intraperitoneally into a guinea pig caused death in less than eighteen hours.

At the time of the test (usually beginning within two hours after bleeding of the animal), the components were mixed, bacteria being added last except where a period of "opsonization" was to precede phagocytosis. Amounts were delivered with 0.2 ml pipettes graduated in thousandths. Both tubes and pipettes were boiled in distilled water and dried before use; paraffined corks were used to stopper the tubes. Rotation was accomplished in a constant-temperature water bath by a rotator making two and

one-half complete revolutions per minute about an axis 20° from the long axis of the tube.

After the allotted period of opsonization and phagocytosis, smears were made as quickly as possible. 0.02 cc. of the mixture was placed on each slide with pipettes such as those used in mixing. Since smears made with cigarette papers showed a more even distribution of leucocytes and less distortion of the cells than those made with glass slides, the former method was employed routinely.

The staining technique was worked out separately for each series in order to allow for differences in affinity for stains among leucocytes from various animals. Usually, the slides were fixed for thirty seconds to one minute in methyl alcohol and stained five to twenty seconds in a dilute methylene blue. They were then allowed to dry in air and their descriptive labels covered with adhesive tape. Serial numbers were then used to identify the slides until examination of the slides was completed. Taping the descriptive labels reduced to a minimum personal prejudice in interpreting results.

Examination consisted of classifying 200 polymorphonuclear leucocytes as negative (having ingested no bacteria), positive with few bacteria (having ingested one to five bacteria), and positive with many bacteria (having ingested more than five bacteria). In counting the 200 cells, only the middle one-third of the smear was considered.

In all groups, results reported are averages; the number of individual determinations is indicated by the number in parentheses, following the temperature. In Group B, the number of determinations considered in arriving at the total average (last column of tables) is approximately

twice the number in parentheses. Although almost all determinations for a given temperature fall within a five percent range, no result has been excluded from the average, save under the following conditions: When there were at least four determinations, and when the deviation of the doubtful result from the average of the other results was greater than the product of the average deviation of the others multiplied by the total number of determinations, the doubtful result was then excluded and the average of the remaining ones accepted. For example, in Experiment 8 of Group D, determination number three was excluded from the values obtained for 22°C.

determination	none	few	many	total
1	95.5	4.5	0.0	4.5
2	93.5	6.5	0.0	6.5
3	80.5	19.5	0.0	19.5
4	93.0	7.0	0.0	7.0

The average negative result for determinations one, two, and four is 94.0 percent.

determination	deviation from the average
1	1.5
2	0.5
4	1.0
Average	1.0 x 4 = 4.0
3	13.5

Since the product of four (the total number of determinations) times the average deviation of the other three determinations (1.0) is less than the deviation of determination number three (13.5), determination number three may be excluded.

II. Presentation of results

Group A

In this group, all three components of the system were mixed (bacteria added last) and rotated for ten minutes at the indicated temperatures. For the following reasons, the results of Group A must be regarded as of less significance than those of other groups:

1. The machine was adjusted to make forty complete rotations per minute--enough to cause some disintegration of the leucocytes. Nevertheless, the settling to the bottom of the tube suggested incomplete mixing, a reasonable possibility since the tubes were so placed as to rotate about an axis 20° from their horizontal axis.

2. Pipettes used to deliver the materials were graduated in tenths only.

3. Smears were made from estimated amounts (e.g., one drop delivered from a capillary pipette)

4. Rather than employing constant amounts of bacteria and leucocytes, varying determined amounts were used to find in what bacteria-to-leucocyte ratio temperature effects could be most easily detected.

5. Descriptive labels were visible at all times.

6. Only one person counted the slides and only one or two determinations were made for each temperature.

Further details of procedure, when available, will be presented with individual experiments.

In all tables for all experiments, the first column represents

temperature in degrees Centigrade; the second, the percent of phagocytes negative; the third, the percent of phagocytes positive with few bacteria; the fourth, the percent of phagocytes positive with many bacteria; and the fifth, the total percent of phagocytes active. In Group A only, phagocytic indices (average number of bacteria ingested per leucocyte) were determined. These numbers are presented in the sixth column.

In the graphs of Group A only, the plain line represents the numbers in column five, the segmented one representing numbers in column six. The abscissa gives temperature in degrees Centigrade; the right-hand ordinate gives phagocytic indices; the left-hand ordinate shows total percent of 200 leucocytes active. This method of plotting is employed merely to conserve space and is not intended to imply an absolute relationship between percent phagocytes active and phagocytic index.

Experiment 1:

Table I

temperature	none	few	many	total	ph. index
22 (2)	61.2	36.8	2.0	38.8	1.01
27 (2)	34.2	53.0	12.8	65.8	2.48
32 (2)	29.7	48.8	21.5	70.3	3.50
37 (2)	30.2	53.8	16.0	69.8	2.76
42 (2)	30.7	49.3	20.0	69.3	3.28

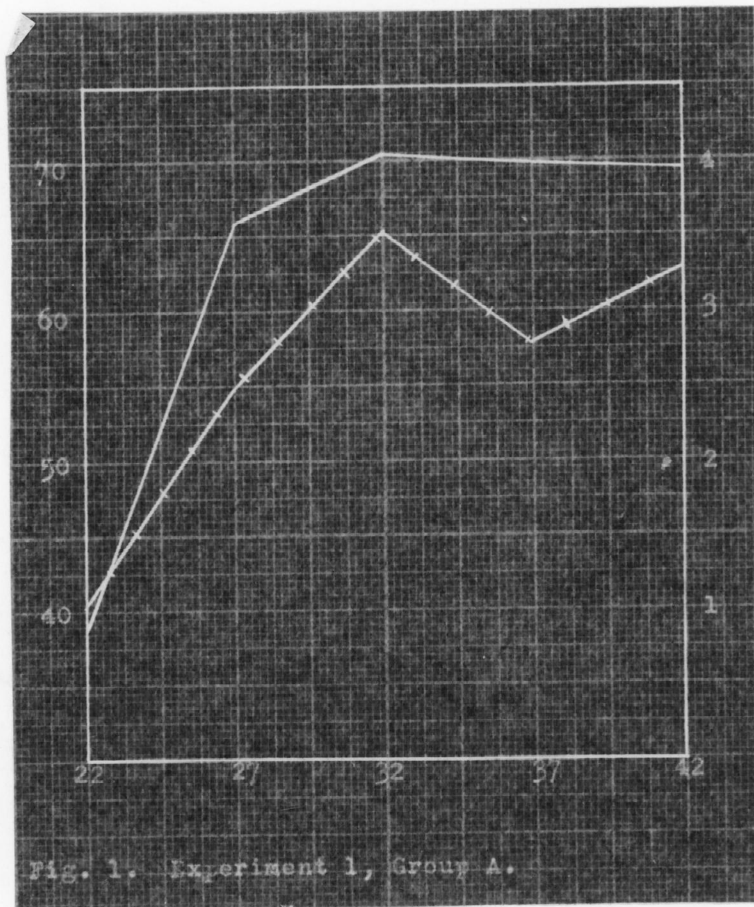

Fig. 1. Experiment 1, Group A.

Experiment 2:

Table II

temperature	none	few	many	total	ph. index
32 (2)	3.0	20.5	76.5	97.0	11.39
37 (2)	4.5	25.5	70.0	95.5	9.23
42 (2)	2.7	21.8	75.5	97.3	10.05

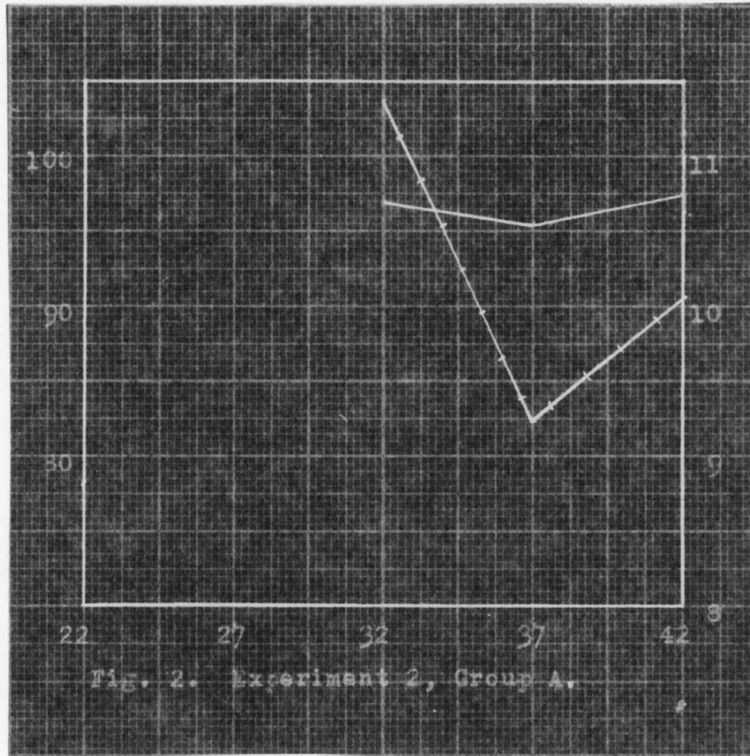

Fig. 2. Experiment 2, Group A.

Since almost 100 percent of the phagocytes were active at all temperatures, it seemed that the ratio of bacteria to leucocytes was too high. Therefore, an attempt was made to determine relative numbers in subsequent experiments.

Experiment 3:

Table III

temperature	none	few	many	total	ph. index
22 (3)	48.5	37.5	14.0	51.5	2.39
32 (3)	22.3	25.2	52.5	77.7	8.94
37 (3)	16.8	24.5	58.7	83.2	8.49
42 (3)	19.8	28.4	51.8	80.2	8.38

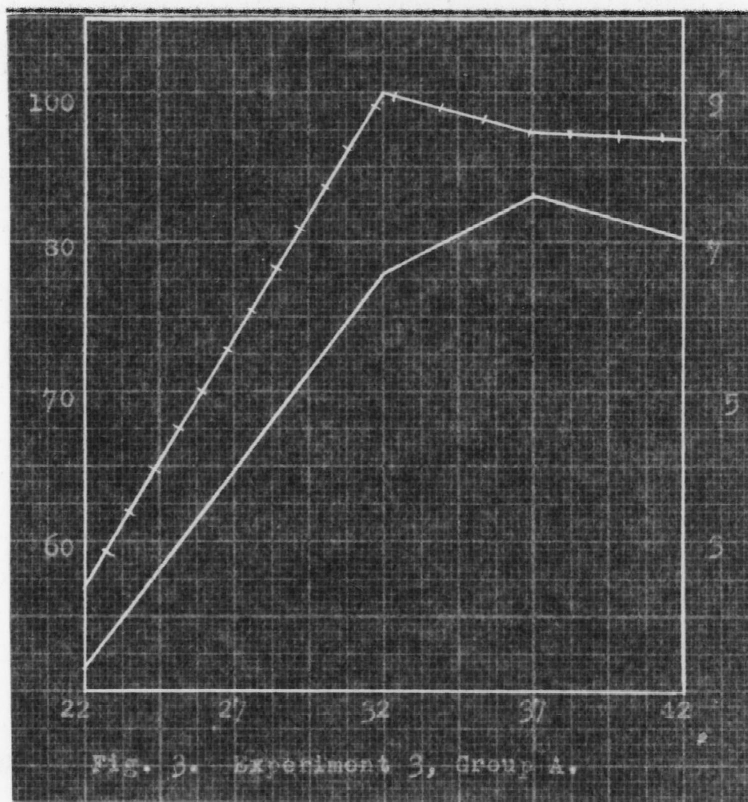

Fig. 3. Experiment 3, Group A.

By direct count, it had been determined that the leucocyte suspension used in Experiment 3 contained 40,000 cells per c.mm. and the bacterial suspension, 1,800,000 cells per c.mm. Since maximum phagocytic index had been reached at 32°C., it was considered possible that the bacteria-to-leucocyte ratio was still too high.

Experiment 4:

Leucocytes were determined to be 45,000 per c.mm. A suspension of a broth culture (washed once in saline) was compared with one of a slant culture. Their absolute numbers were slightly different, but probably not significantly so: slant, 1,040,000 per c.mm. and broth, 1,140,000 per c.mm.

a. with slant suspension

Table IV

temperature	none	few	many	total	ph. index
22 (1)	45.5	39.0	15.5	54.5	2.33
32 (1)	19.0	22.5	58.5	81.0	8.69
37 (1)	20.5	34.0	45.5	79.5	6.28
42 (1)	5.5	23.5	71.0	94.5	10.67

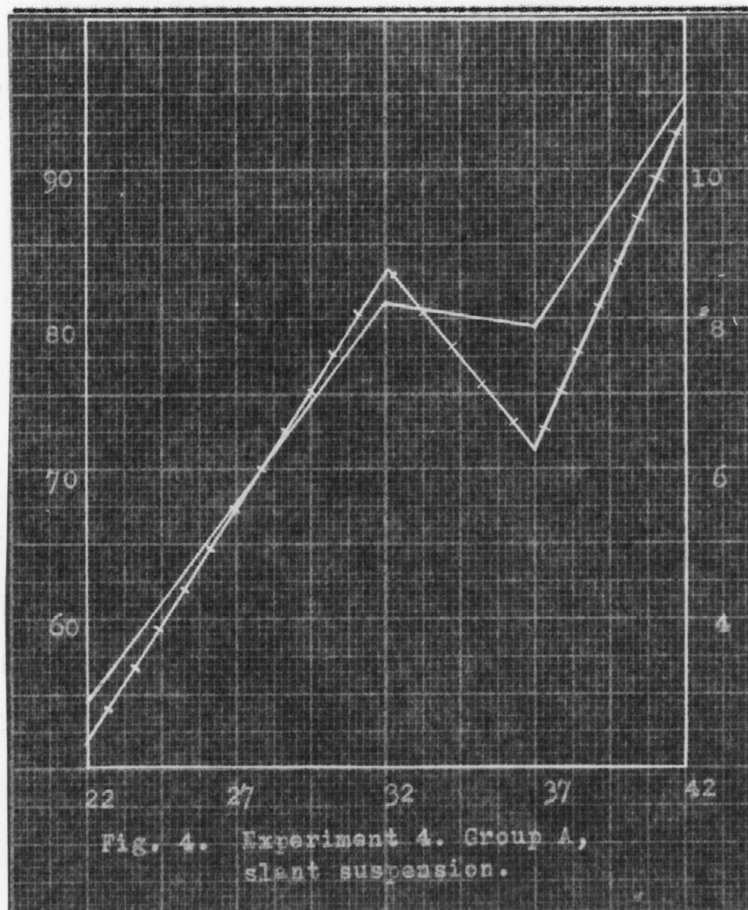

Fig. 4. Experiment 4. Group A, slant suspension.

b. with broth suspension

Table V

temperature	none	few	many	total	ph. index
22 (1)	52.5	42.0	5.5	47.5	1.44
27 (1)	32.5	40.5	27.0	67.5	3.52
32 (1)	25.5	41.0	33.5	74.5	4.95
37 (1)	19.5	32.0	48.5	80.5	5.79
42 (1)	19.0	33.5	47.5	81.0	5.74

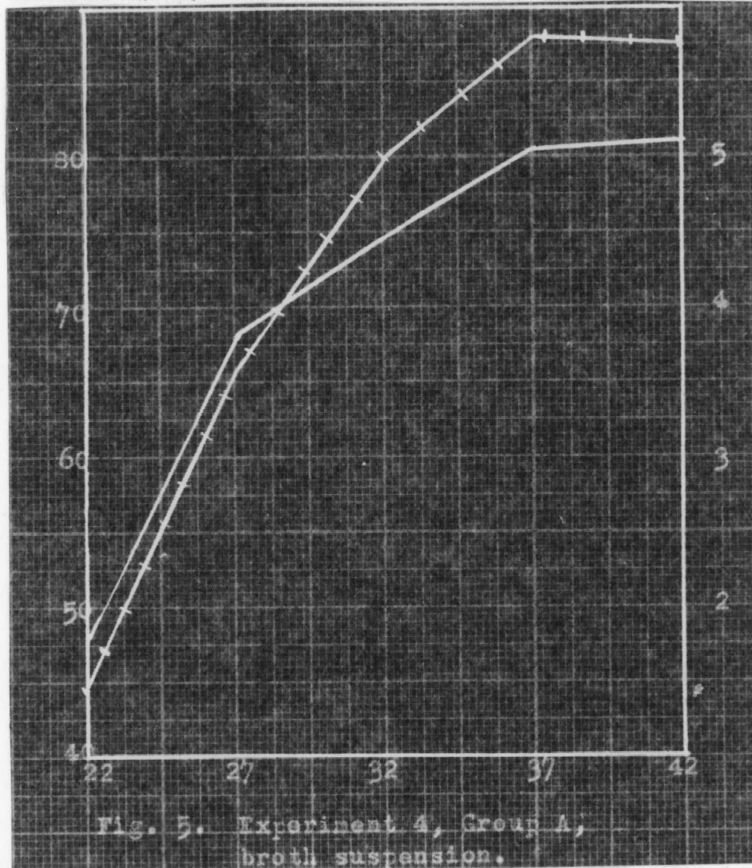

Fig. 5. Experiment 4, Group A; broth suspension.

The bacteria-to-leucocyte ratio (about 24:1) seemed satisfactory for demonstrating the effect of temperature. And, even though the slant suspension was ingested more readily, the two suspensions seemed to lend themselves equally well to a determination of the effect of temperature. In all subsequent runs, slant cultures were used because of their greater convenience.

Experiment 5:

A still lower bacteria-to-leucocyte ratio was tried for comparison: leucocytes, 40,000 per c.mm.; bacteria, 600,000 per c.mm.; bacteria-to-leucocyte ratio, 15:1. Since Experiment 4 had shown consistent rises in phagocytosis between 22°C. and 32°C., this experiment emphasized the range between 32°C. and 42°C.

Table VI

temperature	none	few	many	total	ph. index
32 (1)	46.5	47.0	6.5	53.5	1.71
35 (1)	42.0	49.0	9.0	58.0	1.95
37 (1)	34.0	59.0	7.0	66.0	1.84
39 (1)	41.5	52.5	6.0	58.5	1.45
42 (1)	28.0	67.0	5.0	72.0	2.03

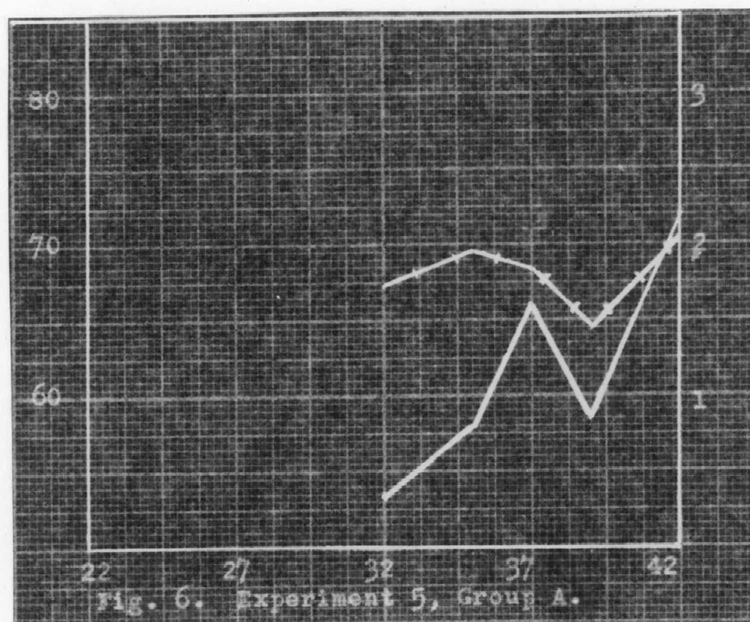

Fig. 6. Experiment 5, Group A.

Group B

The following twelve experiments test the over-all effect of temperature on an *in vitro* phagocytic system. Mixtures were rotated for ten minutes in a water bath at the temperature indicated.

It should be restated that in this group of experiments (as compared with those of Group A) more accurate pipettes were employed, a larger number of determinations was made for each temperature, constant numbers of bacteria and leucocytes were used, descriptive labels were masked during examination of the slides, and two persons (CZ and DH) examined each slide. Only my own determinations are presented except in the last column of each table where the average of all determinations for total percent of phagocytes positive is given. Numbers in parentheses indicate the number of my own determinations, the number whose average appears in the last column being approximately two times as large.

In the graphs, abscissae again represent temperature in degrees Centigrade, ordinates the total percent of phagocytes active. Plain lines represent my own determinations, segmented ones the average result.

Experiment 1:

Table VII

temperature	none	few	many	total	total av.
22 (2)	66.5	32.7	0.8	33.5	33.1
27 (2)	50.2	44.5	5.3	49.8	48.7
32 (3)	42.5	50.8	6.7	57.5	59.5
37 (3)	37.5	52.5	10.0	62.5	65.0
42 (3)	22.3	61.7	16.0	77.7	76.7

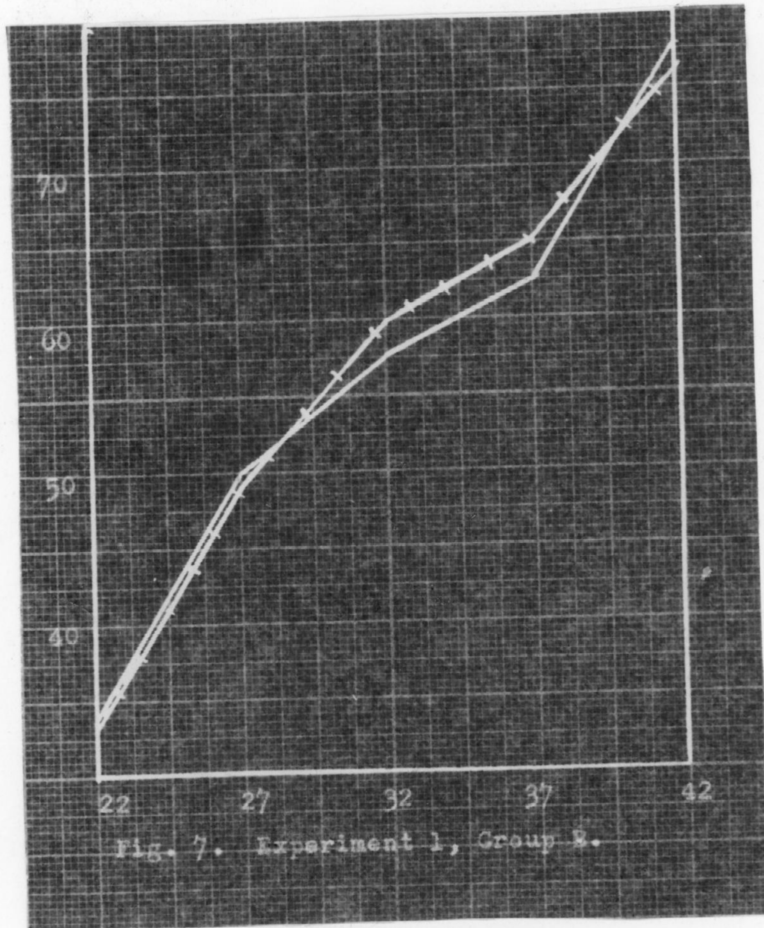

Fig. 7. Experiment 1, Group E.

Experiment 2:

Table VIII

temperature	none	few	many	total	total av.
22 (3)	72.6	25.2	2.2	27.4	28.9
27 (2)	45.3	46.5	8.2	54.7	53.8
32 (3)	59.3	32.3	8.4	40.7	40.9
37 (3)	36.2	48.8	15.0	63.8	61.3
42 (3)	35.9	48.2	15.9	64.1	64.3

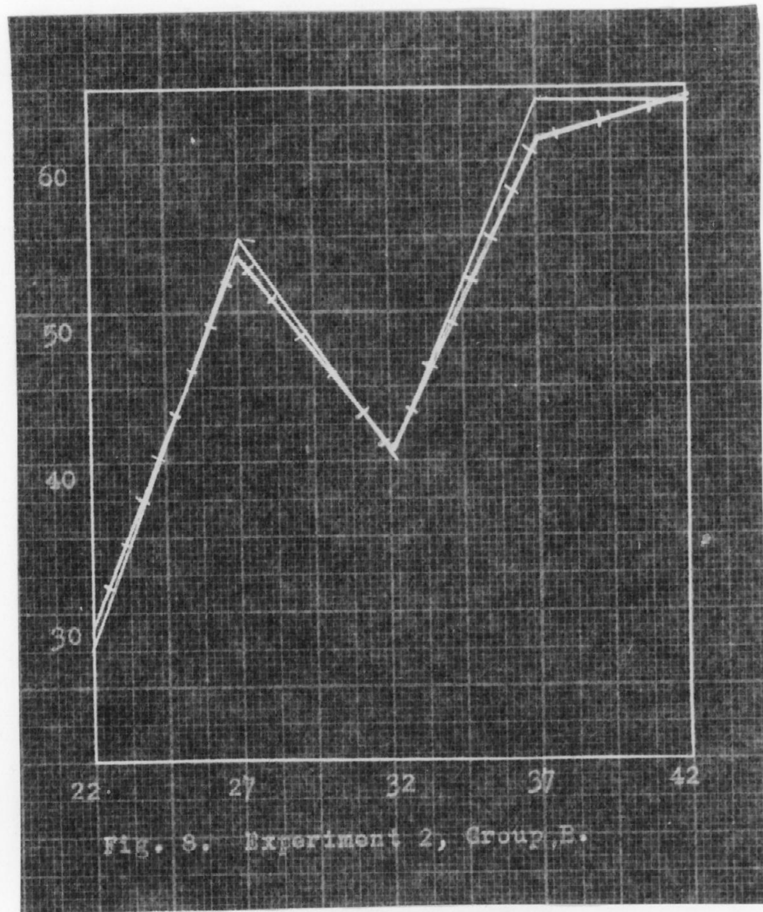

Fig. 8. Experiment 2, Group E.

Experiment 3:

Table IX

temperature	none	few	many	total	total av.
22 (2)	65.2	33.5	1.3	34.8	35.5
27 (2)	45.7	51.8	2.5	54.3	57.1
32 (3)	30.7	62.3	7.0	69.3	70.0
37 (3)	20.4	64.8	14.7	79.6	80.8
42 (2)	16.4	53.3	30.3	83.6	83.8

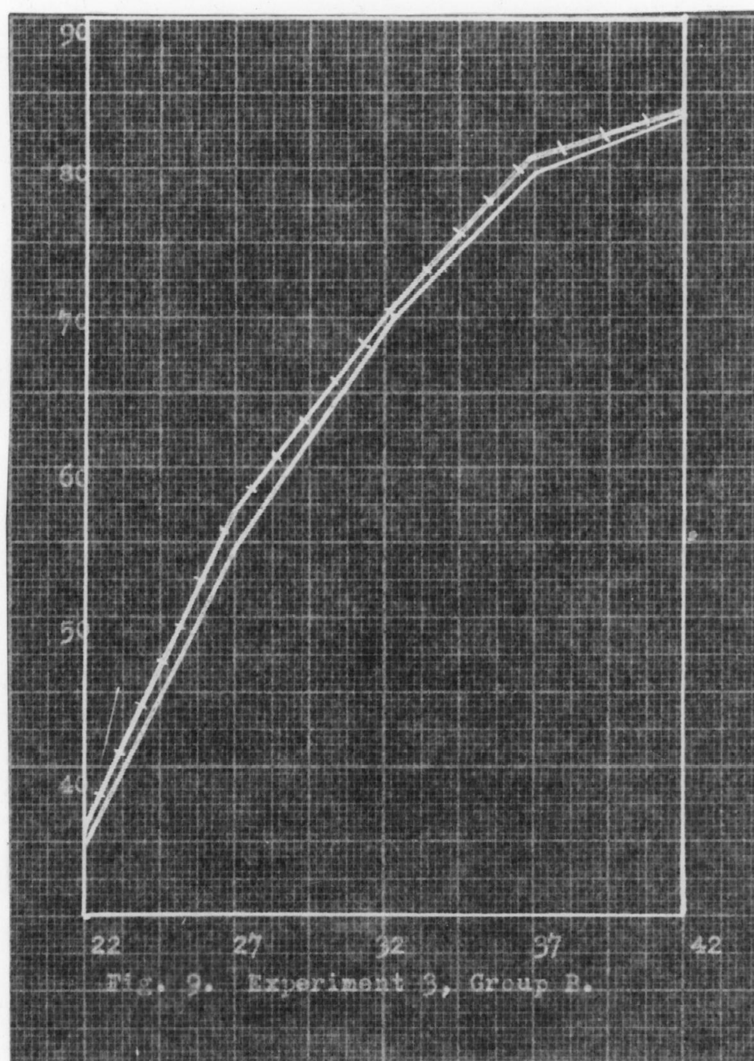

Fig. 9. Experiment 3, Group B.

Experiment 4:

Table X

temperature	none	few	many	total	total av.
22 (1)	61.5	34.5	4.0	38.5	36.7
27 (5)	50.0	46.2	3.8	50.0	52.8
32 (5)	35.9	56.7	7.4	64.1	65.7
37 (5)	24.3	59.8	15.9	75.7	79.5
42 (7)	23.7	57.9	18.4	76.3	79.2

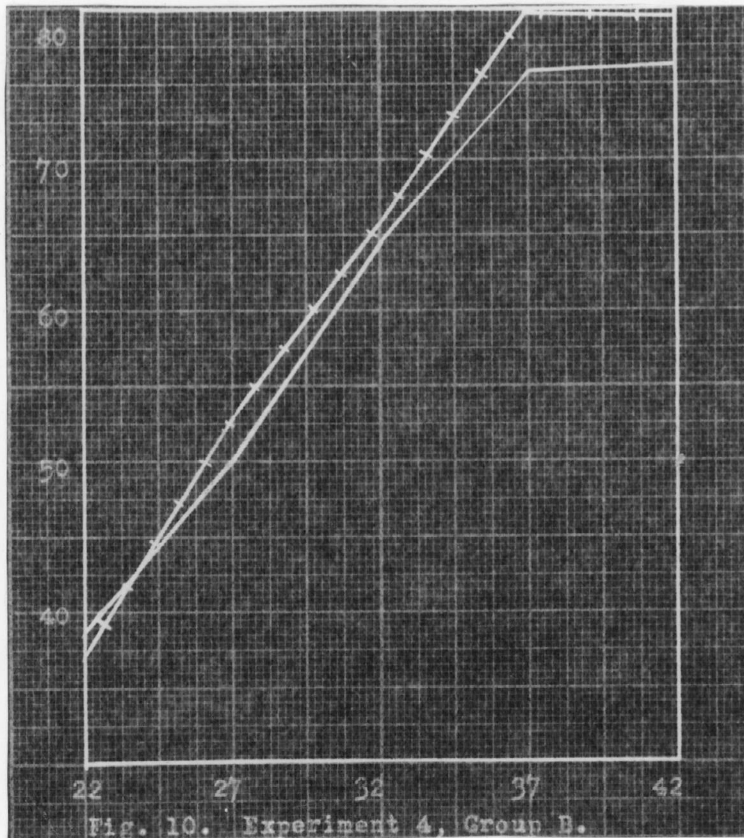

Fig. 10. Experiment 4, Group B.

Experiment 5:

Table XI

temperature	none	few	many	total	total av.
22 (3)	72.7	25.0	2.3	27.3	21.8
27 (3)	72.5	23.8	3.7	27.5	26.7
32 (5)	59.0	32.5	8.5	41.0	40.3
37 (5)	60.5	33.8	5.7	39.5	39.2
42 (5)	73.0	22.8	4.2	27.0	27.1

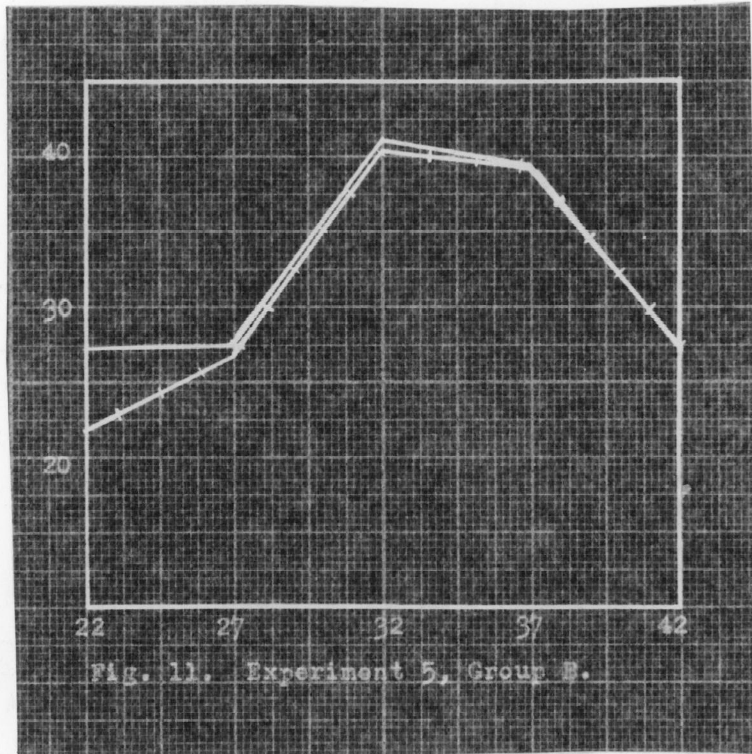

Fig. 11. Experiment 5, Group B.

<u>Experiment 6</u>:

Table XII

temperature	none	few	many	total	total av.
22 (3)	86.5	12.3	1.2	13.5	13.0
27 (2)	87.5	10.2	2.3	12.5	12.7
32 (2)	70.8	23.7	5.5	29.2	28.9
37 (2)	61.3	33.5	5.2	38.7	39.3
42 (4)	38.0	42.0	20.0	62.0	60.4

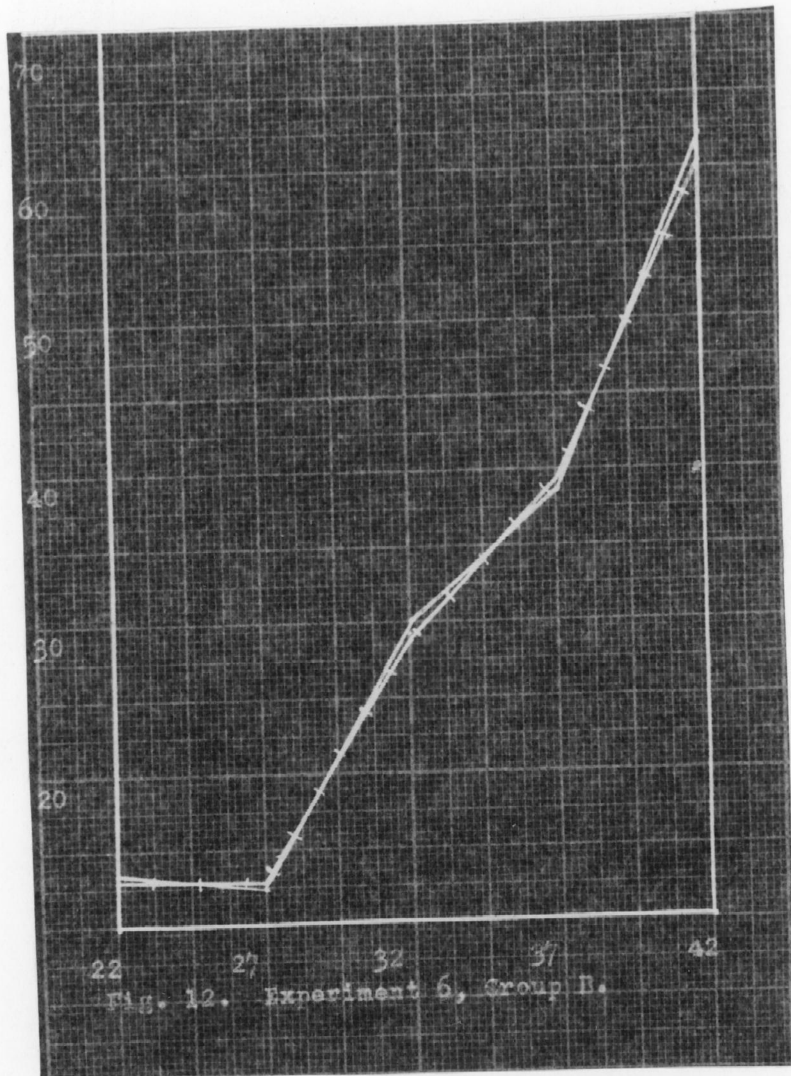

Fig. 12. Experiment 6, Group B.

Experiment 7:

Table XIII

temperature	none	few	many	total	total av.
22 (2)	83.2	15.3	1.5	16.8	16.2
27 (2)	67.4	26.8	5.8	32.6	31.9
32 (2)	53.5	31.0	15.5	46.5	44.0
37 (2)	43.0	37.2	19.8	57.0	57.7
42 (2)	38.7	43.8	17.5	61.3	63.5

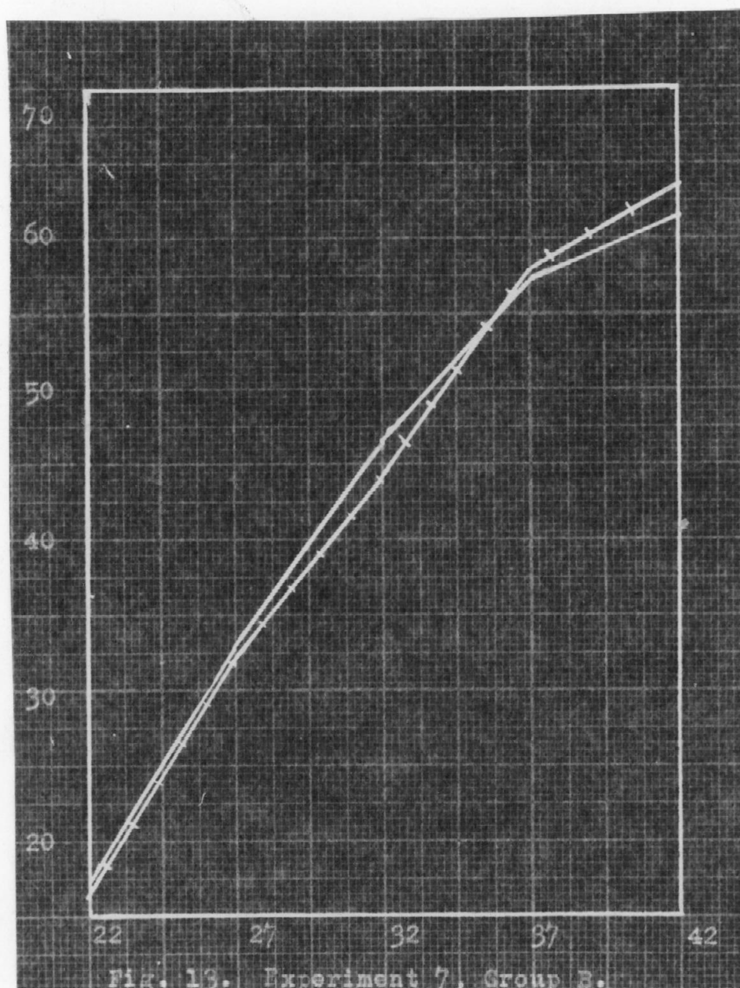

Fig. 13. Experiment 7, Group B.

Experiment 8:

Table XIV

temperature	none	few	many	total	total av.
22 (2)	88.0	11.5	0.5	12.0	14.7
27 (2)	76.2	19.8	4.0	23.8	22.0
32 (2)	69.3	27.4	3.3	30.7	30.8
37 (2)	73.4	21.3	5.3	26.6	26.7
42 (2)	38.7	43.8	17.5	61.3	63.2

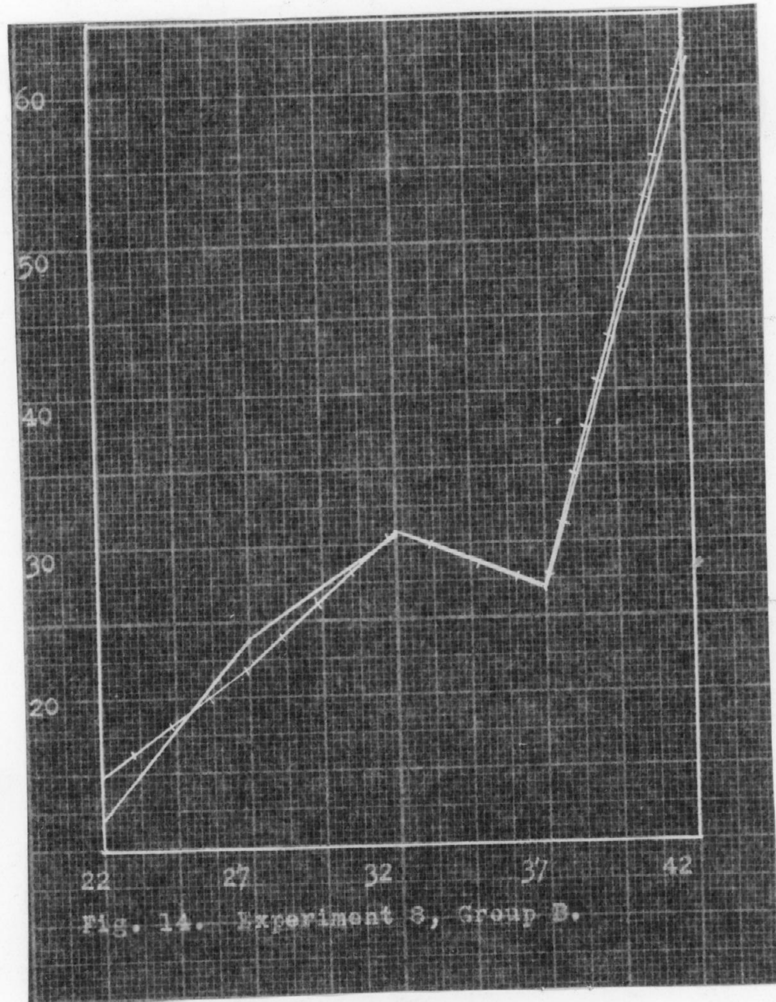

Fig. 14. Experiment 8, Group B.

Experiment 9:

Table XV

temperature	none	few	many	total	total av.
22 (2)	82.3	15.5	2.2	17.7	14.9
27 (2)	80.8	14.0	5.2	19.2	16.3
32 (2)	75.5	18.3	6.2	24.5	22.9
37 (2)	73.2	21.0	5.8	26.8	25.9
42 (2)	68.5	22.5	9.0	31.5	30.2

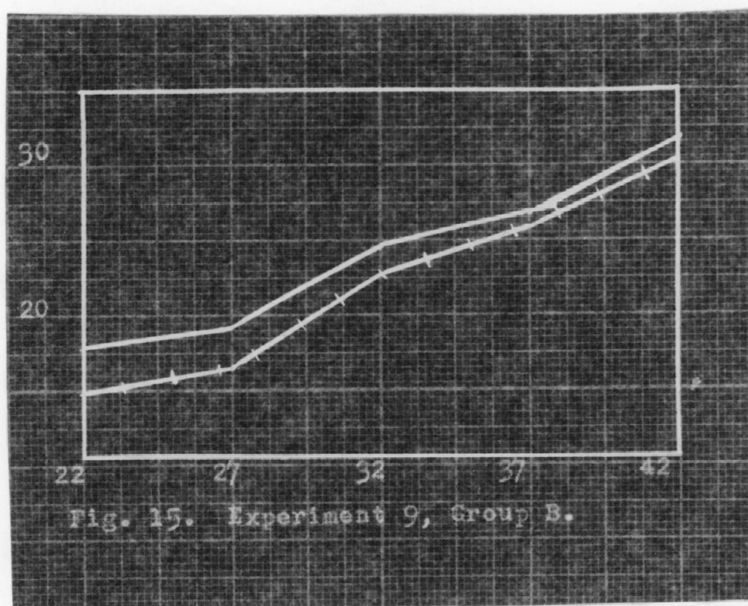

Fig. 15. Experiment 9, Group B.

Experiment 10:

Table XVI

temperature	none	few	many	total	total av.
22 (2)	83.0	15.3	1.7	17.0	16.5
27 (2)	79.0	17.5	3.5	21.0	20.4
32 (3)	79.0	17.0	4.0	21.0	18.8
37 (2)	65.5	27.5	7.0	34.5	34.1
42 (2)	63.2	28.5	8.3	36.8	37.4

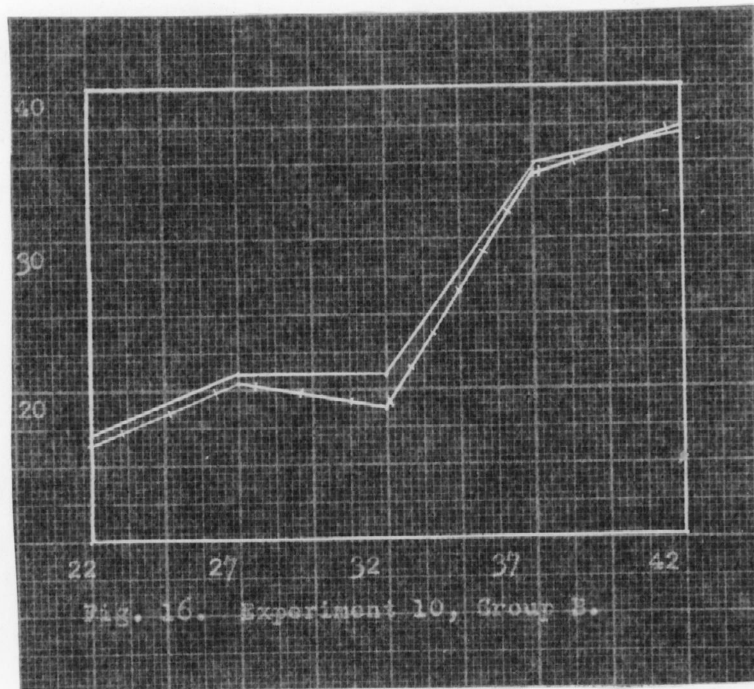

Fig. 16. Experiment 10, Group B.

Experiment 11:

Table XVII

temperature	none	few	many	total
37 (3)	58.2	37.3	4.5	41.8
39 (3)	59.6	36.7	3.7	40.4
41 (3)	59.7	36.8	3.5	40.3

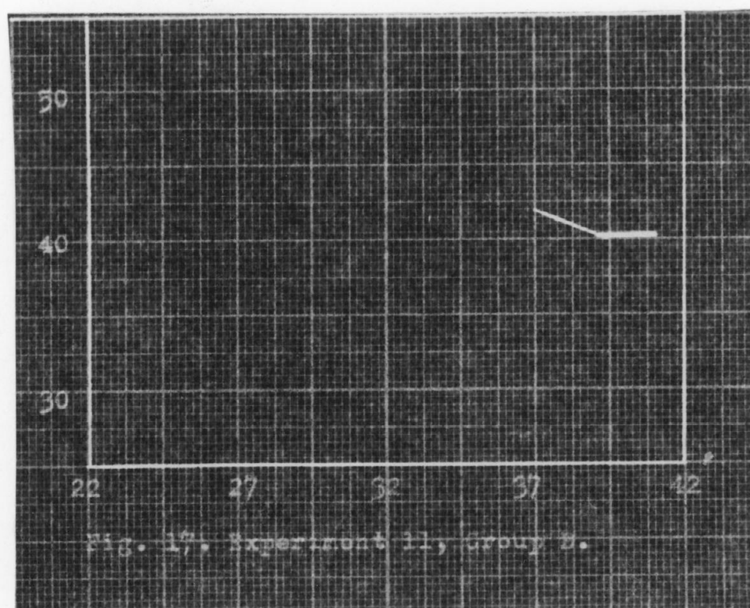

Fig. 17. Experiment 11, Group B.

Experiment 12:

Table XVIII

temperature	none	few	many	total	total av.
37 (2)	41.7	53.0	5.3	58.3	58.5
39 (2)	50.5	45.0	4.5	49.5	47.1
41 (2)	51.5	46.0	2.5	48.5	48.3

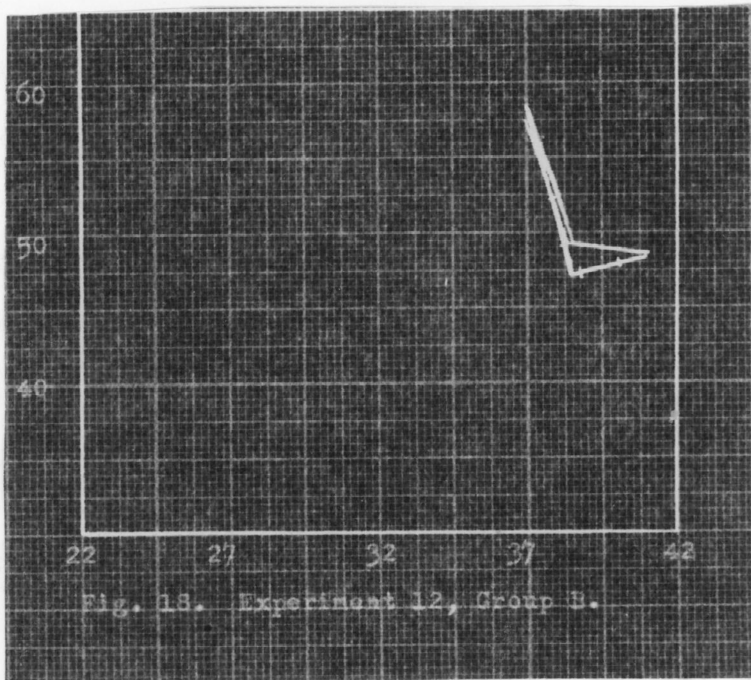

Fig. 18. Experiment 12, Group B.

Group C

Since the effect of temperatures in the range of 37°C. to 42°C. was less consistent than other temperatures tested (see Group A, Experiments 1 and 3 and Group B, Experiments 5, 6, and 12), it was considered possible that opsonization was favored by higher temperatures, while the phagocytes themselves might be susceptible to damage by such temperatures. Variations in different experiments might accordingly reflect differences in susceptibility of phagocytes from individual animals.

To test this hypothesis, the following group of experiments has been carried out. In reporting results, the temperature of "opsonization" is given first; that of "phagocytosis" second. "Opsonization" was carried out in tubes as described above containing 0.1 cc. each of normal guinea-pig serum and bacterial suspension. These tubes were incubated vertically, without rotation, in a water bath at the temperature indicated for thirty minutes. Immediately thereafter, 0.1 cc. phagocyte suspension was added. Phagocytosis was carried out as in the experiments of Group B but for five minutes only.

Experiment 13:

Table XIX

temperature	none	few	many	total
37-37 (3)	51.0	35.8	13.2	49.0
37-40 (2)	55.5	34.0	10.5	44.5
40-37 (2)	46.0	37.5	16.5	54.0
40-40 (2)	38.0	47.2	14.8	62.0

Experiment 14:

Table XX

temperature	none	few	many	total
37-37 (2)	75.0	21.3	3.7	25.0
37-40 (2)	51.5	37.0	11.5	48.6
40-37 (2)	57.5	36.0	6.5	42.5
40-40 (2)	58.2	35.5	6.3	41.8

Experiment 1:

Table XXI

temperature	none	few	many	total
37-37 (2)	77.0	21.8	1.2	23.0
37-40 (2)	78.0	20.2	1.8	22.0
40-37 (2)	76.0	22.8	1.2	24.0
40-40 (2)	68.5	29.3	2.2	31.5

Experiment 2:

Table XXII

temperature	none	few	many	total
37-37 (2)	51.7	42.8	5.5	48.3
37-40 (2)	50.3	45.3	4.5	49.8
40-37 (2)	55.8	39.0	5.3	44.3
40-40 (2)	54.8	39.8	6.0	45.3

Experiment 3: Guinea pig's rectal temperature, 102.4°F. (39.1°C.)

Table XXIII

temperature	none	few	many	total
37-37 (3)	33.0	52.3	14.7	67.0
37-40 (2)	32.7	54.8	12.5	67.3
40-37 (2)	40.5	50.5	9.0	59.5
40-40 (3)	30.5	52.2	17.3	69.5

Experiment 4: Guinea pig's rectal temperature, 103.4°F. (39.7°C.)

Table XXIV

temperature	none	few	many	total
37-37 (2)	46.0	48.8	5.2	54.0
37-40 (2)	39.0	54.3	6.7	61.0
40-37 (3)	47.5	45.0	7.5	52.5
40-40 (3)	37.3	52.7	10.0	62.7

Experiment 5: Guinea pig's rectal temperature, 102.6°F. (39.2°C.)

Table XXV

temperature	nohe	few	many	total
37-37 (3)	44.8	47.8	7.4	55.2
37-40 (4)	23.0	51.9	25.1	77.0
40-37 (3)	44.5	46.7	8.8	55.5
40-40 (4)	36.5	50.6	12.9	63.5

Experiment 6: Guinea pig's rectal temperature, 101.4°F. (38.6°C.)

Table XXVI

temperature	none	few	many	total
37-37 (3)	66.8	32.9	0.3	33.2
37-40 (3)	54.5	43.7	1.8	45.5
40-37 (2)	56.7	42.5	0.8	43.3
40-40 (2)	44.0	53.5	2.5	56.0

Experiment 7: Guinea pig's rectal temperature, 101.4°F. (38.6°C.)

Table XXVII

temperature	none	few	many	total
37-37 (4)	60.9	37.3	1.8	39.1
37-40 (4)	58.4	38.7	2.9	41.6
40-37 (3)	56.2	42.6	1.2	43.8
40-40 (3)	47.6	49.2	3.2	52.4

Experiment 8: Guinea pig's rectal temperature, 101.0°F. (38.2°C.)

Table XXVIII

temperature	none	few	many	total
37-37 (2)	56.0	42.5	1.5	44.0
37-40 (2)	55.7	42.0	2.3	44.3
40-37 (4)	59.4	38.9	1.7	40.6
40-40 (4)	48.6	48.5	2.9	51.4

The negative result obtained in the effect on opsonization strengthened suspicions as to the technique: did more opsonization take place in the five minutes of "phagocytosis" than occurred in the thirty minutes during which the tube was stationary? The fact that the percent phagocytosis for 37-40 was as great as that for 40-40 in five out of ten experiments (14, 2, 3, 4, and 5) suggested that in further work rotation should be employed during the "opsonization" period also.

Group D

In undertaking to rule out the technical errors of Group C, the following changes in technique were employed:

1. A wider range of temperatures was used to make differences in percent phagocytosis easier to detect. (These findings for 22°C. and 37°C. can not, of course, be interpreted as strictly those which might be found in a higher temperature range.)

2. Rotation was carried out during "opsonization" as well as during "phagocytosis".

3. In Experiments 2 through 8, controls subjected to "phagocytosis" only were run with each experiment, one for each of the temperatures. It was intended that these controls should show to what extent a previous period of "opsonization" increased phagocytosis.

4. Shorter periods of both "opsonization" and "phagocytosis" (fifteen minutes and two minutes, respectively were used in Experiments 7 and 8. Half the usual amount (0.01 cc. instead of 0.02 cc.) was used for

the smear; in this manner, drying time was shortened in order to reduce the amount of phagocytosis on the slide.

Experiment 1:

Table XXIX

temperature	none	few	many	total
22-22 (2)	67.5	24.0	8.5	32.5
22-37 (2)	50.5	39.7	9.8	49.5
37-22 (2)	70.5	22.3	7.2	29.5
37-37 (2)	50.7	44.0	5.3	49.3

Experiment 2: Guinea pig's rectal temperature, 98.8°F. (37.1°C)

Table XXX

temperature	none	few	many	total
22 (2)	86.0	14.0	0.0	14.0
22-22 (2)	72.2	21.5	6.3	27.8
22-37 (2)	52.0	39.3	8.7	48.0
37-22 (3)	79.5	14.5	5.8	20.3
37-37 (2)	72.8	21.0	6.2	27.2
37 (2)	54.5	44.3	1.2	45.5

Experiment 3: Guinea pig's rectal temperature, 101.1°F. (38.2°C)

Table XXXI

temperature	none	few	many	total
22 (2)	71.0	28.8	0.2	29.0
22-22 (4)	64.9	20.0	15.1	35.1
22-37 (2)	47.3	33.2	19.5	52.7
37-22 (4)	72.5	18.5	9.0	27.5
37-37 (4)	64.6	22.5	12.9	35.4
37 (2)	34.0	59.2	6.8	66.0

<u>Experiment 4</u>: Guinea pig's rectal temperature, 101.1°F. (38.2°C.); weight, 700 gm.

Table XXXII

temperature	none	few	many	total
22 (2)	69.8	29.7	0.5	30.2
22-22 (2)	56.8	29.2	14.0	43.2
22-37 (2)	39.8	40.5	19.7	60.2
37-22 (5)	68.7	21.2	10.1	31.3
37-37 (5)	58.2	32.1	9.7	41.8
37 (2)	22.5	69.7	7.8	77.5

<u>Experiment 5</u>: Guinea pig's rectal temperature 99.9°F. (37.7°C.); weight, 580 gm.

Table XXXIII

temperature	none	few	many	total
22 (2)	68.5	31.3	0.2	31.5
22-22 (5)	49.4	33.6	17.0	50.6
22-37 (5)	51.0	36.2	12.8	49.0
37-22 (2)	76.0	16.0	8.0	24.0
37-37 (2)	64.3	22.5	13.2	35.7
37 (2)	31.8	63.5	4.7	68.2

<u>Experiment 6</u>: Guinea pig's rectal temperature, 104.0°F. (40.0°C.); weight, 420 gm.

Table XXXIV

temperature	none	few	many	total
22 (3)	79.8	20.0	0.2	20.2
22-22 (3)	65.0	24.0	11.0	35.0
22-37 (3)	59.0	26.8	14.2	41.0
37-22 (3)	78.7	15.7	5.6	21.3
37-37 (4)	62.1	20.6	17.3	37.9
37 (2)	54.0	42.2	3.8	46.0

Experiment 7: Guinea pig's rectal temperature, 105.1°F. (40.5°C.); weight, 475 gm.

Table XXXV

temperature	none	few	many	total
22 (2)	79.2	20.5	0.3	20.8
22-22 (3)	51.2	35.0	13.8	48.8
22-37 (5)	41.0	42.5	16.5	59.0
37-22 (2)	58.2	28.5	13.3	41.8
37-37 (4)	51.5	26.5	22.0	48.5
37 (2)	66.2	33.5	0.3	33.8

Experiment 8: Guinea pig's rectal temperature, 103.8°F. (39.9°C.); weight, 370 gm.

Table XXXVI

temperature	none	few	many	total
22 (3)	94.0	6.0	0.0	6.0
22-22 (4)	74.5	21.5	4.0	25.5
22-37 (2)	54.7	35.3	10.0	45.3
37-22 (2)	61.3	24.5	14.2	38.7
37-37 (4)	55.5	25.6	18.9	44.5
37 (2)	67.0	33.0	0.0	33.0

III. Analysis of results

Groups A and B

The following tables have been compiled on the basis of total percent phagocytes active. Table XXXVII represents the results presented in columns labeled "total" in the presentation of results. Table XXXVIII deals with total average results and is limited to Group B.

The total percent positive obtained for 22°C. has in each experiment been taken as unity. Values for each of the other temperatures of that experiment have been calculated by dividing their respective total percents positive by that of 22°C. The numbers so obtained represent the extent to which phagocytosis at a given temperature is increased over that obtained at 22°C. Averages of such values have been made and plotted in accompanying graphs (Fig. 19 for Table XXXVII and Fig. 20 for Table XXXVIII). In the row designated "Inc.", the numerator shows the number of experiments in which an increase was observed, the denominator the total number of experiments considered. The percent of increase over each 5°C. range has been calculated by dividing the average of the higher temperature by that of the lower and is expressed in the tables as "Q_5".

Table XXXVII

group	exper.	22°C.	27°C.	32°C.	37°C.	42°C.
A	1	1.00	1.72	1.81	1.80	1.79
	3	1.00	----	1.51	1.61	1.56
	4a	1.00	----	1.49	1.46	1.72
	4b	1.00	1.42	1.57	1.69	1.70
B	1	1.00	1.49	1.72	1.87	2.32
	2	1.00	2.00	1.49	2.32	2.34
	3	1.00	1.57	2.00	2.30	2.40
	4	1.00	1.30	1.67	1.96	1.98
	5	1.00	1.01	1.50	1.45	0.99
	6	1.00	0.93	2.16	2.86	4.60
	7	1.00	1.94	2.76	3.40	3.66
	8	1.00	1.98	2.56	2.21	5.10
	9	1.00	1.07	1.38	1.52	1.78
	10	1.00	1.19	1.19	1.95	2.16
	Av.	1.00	1.47	1.78	2.03	2.44
	Inc.		11/12	10/12	10/14	11/14
	Q_5		1.47	1.21	1.14	1.20

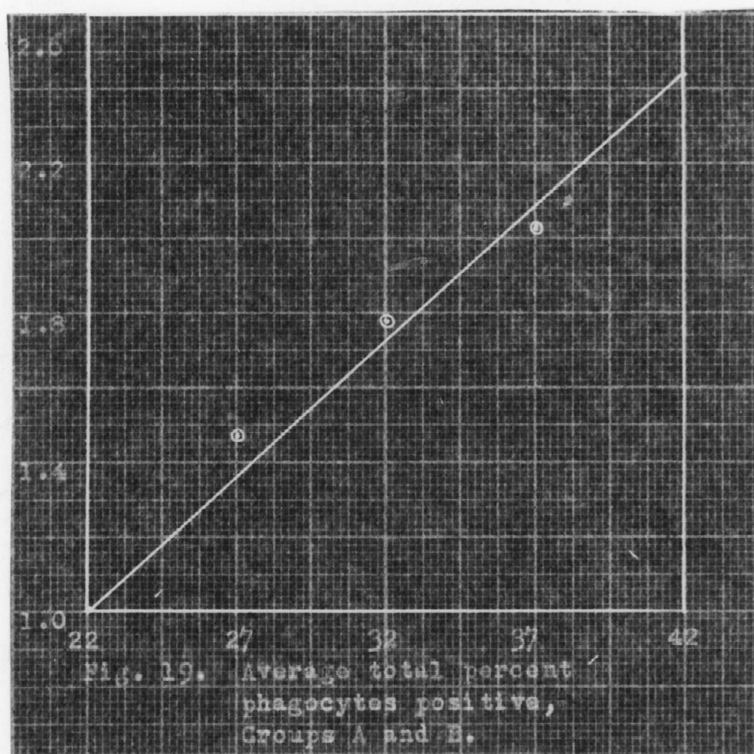

Fig. 19. Average total percent phagocytes positive, Groups A and B.

Table XXXVIII

exper.	22°C.	27°C.	32°C.	37°C.	42°C.
1	1.00	1.46	1.80	1.96	2.32
2	1.00	1.86	1.41	2.12	2.22
3	1.00	1.61	1.97	2.27	2.36
4	1.00	1.44	1.79	2.17	2.16
5	1.00	1.23	1.85	1.80	1.24
6	1.00	0.98	2.22	3.02	4.65
7	1.00	1.97	2.72	3.56	3.92
8	1.00	1.40	2.09	1.82	4.30
9	1.00	1.09	1.54	1.74	2.04
10	1.00	1.23	1.14	2.06	2.26
Av.	1.00	1.44	1.85	2.25	2.75
Inc.	9/10	8/10	8/10	8/10	
Q_5	1.44	1.29	1.22	1.22	

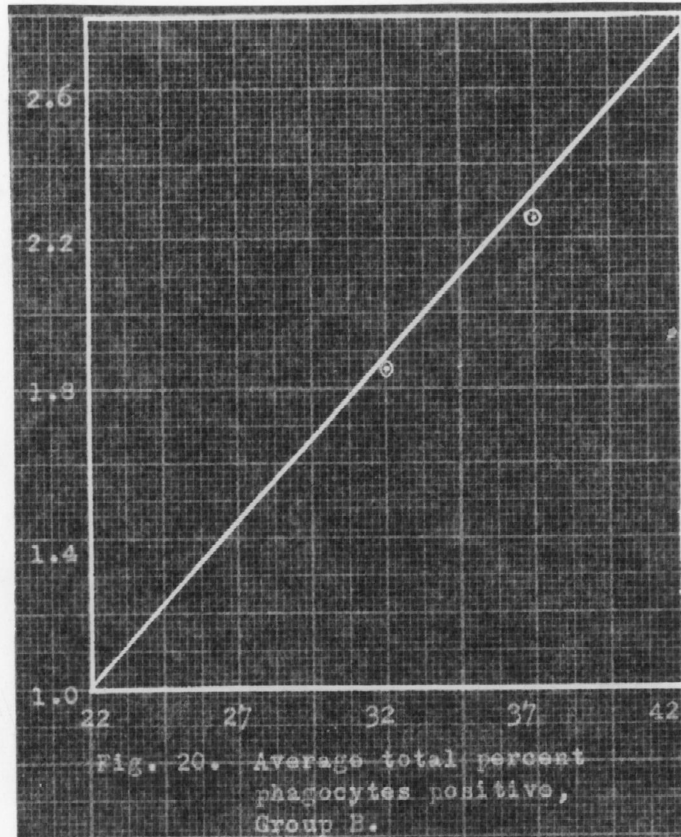

Fig. 20. Average total percent phagocytes positive, Group B.

From these data, it would seem that phagocytosis, considered as it was by our particular method, exhibits a slightly decreasing, almost steady rate of increase with temperatures from 22°C. to 37°C. so that the average percent of phagocytes positive plotted against temperature in degrees Centigrade appears to be a straight line. It is to be remembered, however, that only a ten-minute interval has been examined and that nothing can be said as to the rate or end-point of the reaction itself at various temperatures. That a steady increase would continue at higher temperatures is improbable because of susceptibility of leucocytes to higher temperatures and destructibility of heat-labile opsonins at higher temperatures.

Group C

For the data of this group, a method of analysis similar to that for Groups A and B has been used. In Group C, however, various values have been taken as unity, depending upon the reaction to be considered: "opsonization" or "phagocytosis". For example, with the percent phagocytes positive at 37-37 taken as unity, the relative number obtained for 37-40 would represent the effect of increased temperature on "phagocytosis"; similarly, the relative number obtained for 40-37 would represent the effect of increased temperature on "opsonization". Table XXXIX shows the effect on "opsonization", giving separate columns for values obtained by phagocytosis at 37°C. and by phagocytosis at 40°C. Table XL deals with the effect on "phagocytosis". One row of each table (labeled "Inc.") gives the number of instances in which an increase was observed; the denominator represents the total number of observations.

Table XXXIX

exper.	37-37	40-37	37-40	40-40
13	1.00	1.10	1.00	1.39
14	1.00	1.70	1.00	0.86
1	1.00	1.04	1.00	1.43
2	1.00	0.93	1.00	0.91
3	1.00	0.89	1.00	1.03
4	1.00	0.97	1.00	1.03
5	1.00	1.01	1.00	0.83
6	1.00	1.30	1.00	1.23
7	1.00	1.12	1.00	1.26
8	1.00	0.93	1.00	1.16
Av.	1.00	1.10	1.00	1.11
Inc.	6/10		7/10	

Table XL

exper.	37-37	37-40	40-37	40-40
13	1.00	0.91	1.00	1.15
14	1.00	1.94	1.00	0.98
1	1.00	0.96	1.00	1.31
2	1.00	1.03	1.00	1.02
3	1.00	1.00	1.00	1.17
4	1.00	1.13	1.00	1.19
5	1.00	1.40	1.00	1.14
6	1.00	1.37	1.00	1.29
7	1.00	1.06	1.00	1.19
8	1.00	1.01	1.00	1.27
Av.	1.00	1.18	1.00	1.17
Inc.	7/10		9/10	

The results seem to indicate that both "opsonization" and "phagocytosis" are favored by an increased temperature in the range of 37°C. to 40°C., "phagocytosis" more consistently and to a greater extent than "opsonization". The question might well be asked whether during the first few seconds of phagocytosis, the tubes opsonized 40°C. have a

higher temperature than the corresponding ones opsonized at 37°C. If so, it follows that the apparent increase in opsonization may represent an actual increase in the first few seconds of phagocytosis. Another question, which has already been pointed out, is whether more opsonization occurs in five minutes of rotation than in thirty minutes of stationary incubation. In such a case, the designation of "phagocytosis" to the last five minutes of the test is inaccurate.

The most consistent result in this group was an increase in the over-all result from "opsonization" and "phagocytosis" at 37°C. (37-37) to "opsonization" and "phagocytosis" at 40°C. (40-40). This comparison is presented in Table XLI. In nine out of ten experiments, the result was an increase. This result is interesting in view of the lack of significant increase in the 37°C. to 40°C. range of Experiments 1, 2, and 3 in Group A and of Experiments 2, 4, 5, 11, and 12 in Group B. The comparison is not strictly valid, however, since the conditions of the experiments in Groups A and B were different from those in Group C.

Table XLI

exper.	37-37	40-40
13	1.00	1.27
14	1.00	1.67
1	1.00	1.37
2	1.00	0.94
3	1.00	1.04
4	1.00	1.16
5	1.00	1.15
6	1.00	1.69
7	1.00	1.34
8	1.00	1.17
Av.	1.00	1.28
Inc.	9/10	

Group D

The results of this group have been summarized as were those of Group C. The effect of increased temperature on "opsonization" is presented in Table XLII, on "phagocytosis" in Table XLIII. The over-all effect is shown in Table XLIV; the first pair of figures represents systems subjected to "opsonization" before phagocytes were added; the second pair represents those subjected to the "phagocytosis" treatment only. The results of Experiments 7 and 8 are not included in averages or in considering the number of increases, since time intervals were shorter in these experiments. Otherwise, the technique was comparable; therefore, they are included in the summarizing tables.

Table XLII

exper.	22-22	37-22	22-37	37-37
1	1.00	0.91	1.00	1.00
2	1.00	0.73	1.00	0.57
3	1.00	0.78	1.00	0.68
4	1.00	0.77	1.00	0.69
5	1.00	0.48	1.00	0.73
6	1.00	0.61	1.00	0.94
Av.	1.00	0.71	1.00	0.77
Inc.	0/6		0/6	
7	1.00	0.86	1.00	0.83
8	1.00	1.52	1.00	0.98

Table XLIII

exper.	22-22	22-37	37-22	37-37
1	1.00	1.52	1.00	1.67
2	1.00	1.72	1.00	1.34
3	1.00	1.49	1.00	1.29
4	1.00	1.39	1.00	1.33
5	1.00	0.97	1.00	1.49
6	1.00	1.18	1.00	1.78
Av.	1.00	1.38	1.00	1.48
Inc.	5/6		6/6	
7	1.00	1.21	1.00	1.16
8	1.00	1.78	1.00	1.15

Table XLIV

exper.	22-22	37-37	22	37
1	1.00	1.52	----	----
2	1.00	0.98	1.00	3.20
3	1.00	1.01	1.00	2.28
4	1.00	0.97	1.00	2.57
5	1.00	0.73	1.00	2.15
6	1.00	1.08	1.00	2.28
Av.	1.00	1.05	1.00	2.50
Inc.	3/6		5/5	
7	1.00	0.99	1.00	1.61
8	1.00	1.75	1.00	5.50

From these data, it would seem that, while "phagocytosis" is favored by increased temperature, "opsonization" is impaired by it. But, by comparing results obtained at the two temperatures of "opsonization" with those obtained with no previous "opsonization" at all (Table XLV), it becomes evident that it is not opsonization alone that takes place in the preliminary incubation period of organisms and serum.

This table brings out three points:

1. When "phagocytosis" is at 37°C., higher results are obtained

with no previous "opsonization" than with previous "opsonization" at either 22°C. or 37°C. This observation may be considered partly a matter of the method of counting, since with only five minutes' phagocytosis at 37°C.

a) a large value for total percent phagocytes positive is correlated with a relatively low figure for percent phagocytes ingesting many bacteria (Experiments 2, 3, 4, 5, and 6);

b) a distinct decrease in phagocytic index was obtained at 37°C. in five of six experiments of Group A, when ten minutes' phagocytosis was studied.

2. When "opsonization" time is cut to fifteen minutes and "phagocytosis" time to two minutes as in Experiments 7 and 8 of Group D, the total percent phagocytes positive is still disproportionately greater than the number of cells ingesting "many" bacteria. But previous opsonization can now be demonstrated to contribute to increased phagocytosis.

3. Even longer periods of "opsonization" and "phagocytosis" (Experiments 2 through 6) show previous "opsonization" at 22°C. (and, to a lesser extent, that at 37°C.) to increase the amount of phagocytosis at 22°C. But in this case, also, shorter periods of "opsonization" and "phagocytosis" better demonstrate the effect of previous "opsonization".

Table XLV

exper.	22	22-22	37-22	37	22-37	37-37
2	1.00	1.99	1.45	1.00	1.06	0.60
3	1.00	1.20	0.95	1.00	0.80	0.54
4	1.00	1.44	1.04	1.00	0.78	0.54
5	1.00	1.60	0.77	1.00	0.71	0.52
6	1.00	1.75	1.06	1.00	0.89	0.82
Av.	1.00	1.59	1.05	1.00	0.85	0.60
Inc.		5/5	3/5		1/5	0/5
7	1.00	2.34	2.00	1.00	1.75	1.47
8	1.00	4.25	6.45	1.00	1.38	1.36

These results suggest that in addition to opsonization and phagocytosis, there is an inhibitory reaction in progress. Increased temperatures or prolonged incubation at any temperature tends to give this reaction predominance over opsonization. Whether or not this reaction proceeds more rapidly at 40°C. than at 37°C. can not be definitely determined from the data obtained. It seems possible, however, that this inhibitory reaction is one of metabolism and would decrease in rate at temperatures above the optimum of the test organism.

It is obvious from the results of Group D that all reactions relating to phagocytosis are very rapid and that better differentiation among them is obtained by using shorter periods of incubation. It is likely that with immune serum, one would be able to demonstrate more readily the effect of temperature on opsonization.

IV. Discussion of results

The data of Groups A and B are in accord with those of Ledingham (1908), who found that phagocytosis was more marked at higher temperatures up to and including 42°C. The data of Madsen and Wulff (1919), on the other hand, indicates that 42°C. was high enough to inhibit phagocytosis by both human and guinea-pig leucocytes. The two experiments of Ellingson and Clark (1942) showed about equal phagocytosis at 37°C. and 42°C. The optimum for guinea-pig leucocytes (about 40°C.) was two Centigrade degrees higher than the optimum for human leucocytes (about 38°C.). In view of these apparent species differences, our results are less striking than would be similar ones for human leucocytes. Ledingham (1908) did not state the animal species from which he obtained leucocytes.

The data of Madsen and Watabiki (1919) as analyzed by Fenn (1922a) showed an increase in both rate and end-point of phagocytosis with increased temperature, although the time of phagocytosis was decreased. These data take into account only those temperatures between 5°C. and 35°C. It should be pointed out that all the work mentioned above was reported in phagocytic indices.

The matter of the effect of temperature on opsonization is somewhat more difficult to study. According to two experiments of Bulloch and Atkin (1905), opsonin is completely gone from the supernatant following absorption of fresh normal serum with *Staphylococcus albus* for as little as ten minutes at either 0°C. or 37°C. In the second

experiment, the bacteria used for absorption were tested with leucocytes with the result that those opsonized for fifteen minutes at 0°C. were taken up as easily as those opsonized the same length of time at 37°C. Ledingham (1908) found in three experiments that, while dead tubercle bacilli adsorbed more opsonin from fresh normal serum at 37°C. than at 0°C., the bacteria thus sensitized were equally well ingested.

But, when Ledingham (1908) employed staphylococci, he found that after long periods of sensitization at 37°C., bacteria (still suspended in the sensitizing serum) were equally well ingested at phagocytosis temperatures from 18°C. to 37°C. Bacteria sensitized for corresponding periods at lower temperatures (*e.g.*, 7°C. or 18°C.) were less readily phagocyted than those sensitized at 37°C. and phagocyted at comparable temperatures. The time allowed for phagocytosis (fifteen to thirty minutes) was sufficient to rule out the possibility that a tube opsonized at a low temperature failed to reach its supposed phagocytosis temperature.

Dean (1905) sensitized *Staphylococcus aureus* with heated normal serum. He found that bacteria (centrifuged in the cold) which had been sensitized at 37°C. were ingested to ten times the extent of those sensitized at 6°C. to 8°C. Only one experiment was reported; the time of sensitization was thirty minutes.

Sellards' (1908) two experiments tested ingestion of bacteria sensitized at 0°C. and 37°C., respectively, for fifteen minutes and subsequently heated to inactivation temperatures before adding leucocytes.

In agreement with Dean (1905) and Ledingham (1908), he found greater ingestion of bacteria sensitized at 37°C. In fact, opsonization at 0°C. was not significantly greater than that in serum heated immediately after adding bacteria.

In Group D of our experiments, we find indirect evidence that opsonization at 37°C. is more rapid than that at 22°C. While a previous period of "opsonization" was capable of increasing phagocytosis for five minutes at 22°C., the five minutes of "phagocytosis" at 37°C. gave sufficient opsonization so that previous incubation even reduced the amount of phagocytosis. When "phagocytosis" time was reduced to two minutes, a previous period of "opsonization" increased phagocytosis at 37°C. in all four comparisons (Table XLV). Tests with "phagocytosis" for two minutes at 22°C. still showed greater "need" for previous opsonization than did corresponding ones phagocyted at 37°C.

It remains, then, to be explained why, if opsonization is more rapid at 37°C., a previous period of "opsonization" at 22°C. should consistently do more good or less harm than the corresponding incubation at 37°C. (Table XLVII). This observation held in three of four comparisons where "opsonization" period was only fifteen minutes. It would seem that the explanation lies in an inhibitory reaction which proceeds simultaneously with opsonization and that, while the rate of both opsonization an inhibition appear to be increased by temperature increase, the latter reaction is enhanced to a greater degree. Another possibility, and one in better accord with the hypothesis of immediate opsonization, is that opsonization is quickly completed but that the

inhibitory reaction continues with longer incubation and to a greater extent at 37°C. than at 22°C.

It should be pointed out that neither of these explanations is in agreement with Fenn's (1922a) calculations of temperature coefficients for the "preparatory reaction" (2.3 to 2.6) and for the "inhibitory reaction"(1.3 to 1.7). Fenn's "preparatory reaction", however, probably included both opsonization and what he called effect on amoeboid activity of phagocytes. The latter is, of course, not included where only bacteria and serum are incubated. Furthermore, one would expect the inhibitory reaction to vary with the number and virulence of the bacteria used. A personal hypothesis in regard to the inhibitory reaction is that metabolism of the organism in serum is sufficient to produce a substance inhibitory to phagocytosis. When such incubation precedes addition of phagocytes, not only are the phagocytes spared contact with unsensitized bacteria but also they are introduced into an environment where inhibitory products have accumulated. If, as in the system considered by Fenn (1922a), phagocytes are introduced along with the bacteria and serum, they might conceivably be able to ingest some organisms before an inhibiting concentration of metabolic products accumulated.

Thus far, there is no explanation of why, in thirteen of twenty comparisons in Group C, we were able to demonstrate better "opsonization" at 40°C. than at 37°C. (Table XXXIX). It is probable that since the optimum temperature for the test organism is 37°C., slower production of an inhibitory substance would occur at 40°C. At least, one would

expect no more rapid production at 40°C. than at 37°C.; thus, assuming opsonization itself to be favored by the three-degree-Centigrade rise, a final increase in the amount of phagocytosis could be effected by preliminary incubation of serum and bacteria at the higher temperature.

It must be admitted that there is no direct experimental evidence for this hypothesis. Further evidence in its favor might be obtained by detection of lactic acid or some other metabolic product known to inhibit phagocytosis in a system having undergone preliminary incubation.

That actual ingestion of organisms is favored by increased temperature is evident from the data of Table XLIII. In light of this evidence, we may also draw the same conclusion from Table XL, which would otherwise be inconclusive, since the system was rotated only after the addition of phagocytes.

V. Summary and conclusions

1. Over the temperature range from 22°C. to 42°C., an increased temperature increases phagocytosis of *Staphylococcus aureus* by guinea-pig leucocytes under the influence of fresh normal guinea-pig serum and for a ten-minute period. Fifteen tests with the same number of animals support this conclusion.

2. Comparing the effect of temperature increase as determined at five minutes and ten minutes, respectively, we find an insignifi-

cant difference. From the data obtained, this comparison can be made for 22°C. and 37°C. only. From Table XXXVIII, we find the ratio to be 2.25 at ten minutes (ten animals); and, from Table XLIV, we find the ratio to be 2.50 at five minutes (six animals). The comparison would be more significant if the determinations had been made on leucocytes from the same animals.

3. The rate of increase for a 5°C. rise in temperature as determined from data limited to a ten-minute interval, either remains constant or decreases slightly as we go up the temperature scale. A wider range of temperatures and time intervals would be desirable before reaching definite conclusions.

4. Maximum benefit from preliminary incubation of bacteria and fresh normal serum is apparently reached in less time at 37°C. than at 22°C. Whether opsonization is complete at this maximum can not be determined from the available data.

5. "Phagocytosis" of bacteria is actually a complex of reactions consisting of at least three separate reactions: sensitization of bacterial surfaces, ingestion of bacteria by leucocytes, and inhibition of the leucocytes by accumulating bacterial products.

6. The sensitization of the bacterial surface is apparently the process least markedly affected by a rise in temperature. Ingestion of bacteria is favored by an increase in temperature up to 40°C. The inhibitory reaction is greater at 37°C. than at 22°C. and probably greater at 37°C. than at 40°C.

7. The steady (or almost steady) increase in phagocytosis with

a temperature increase does not, then, indicate a simple reaction. Over a period of ten minutes, the various reactions affected by temperature changes between 22°C. and 42°C. are so interrelated that the over-all effect of each 5°C. increase is approximately the same relative increase in the number of phagocytes active.

As already mentioned, the data of Group B are the results of work done in collaboration with Doralea Harmon, whose thesis will appear at a later date.

BIBLIOGRAPHY

ARKIN, A. 1913 The influence of strychnin, caffein, chloral antipyrin, cholesterol, and lactic acid on phagocytosis. J. Inf. Dis., 13: 408-424.

BEST, C.H. and Taylor, N.B. 1943 The Physiologic Basis of Medical Practice, third edition, Williams and Wilkins Co., Baltimore, 1942 pp.

BOERNER, F. and Mudd, S. 1935 Determination of the phagocytic power of whole blood or plasma-leucocyte mixtures for clinical or experimental purposes. Am. J. Med. Sci., 189: 22-35.

BORDET, J. 1895 Les leucocytes et les proprietes actives du serum chez les vaccines. Ann. de l'Inst. Past., 9: 462-506.

BORREL, A. 1893 Tuberculose pulmonaire experimentale. Ann. de l'Inst. Past., 7: 593-627.

BUCHNER, H. 1890 Die chemisch. Reizbaskeit der Leukocyten und deren Beziehung zur Entzündung und Eiterung. Berlin Klin. Wchnschr., 47: 1084 (cited by Menkin, 1940).

BULL, C.G. and McKee, Clara M. 1921 Antipheunococcus protective substances in normal chicken serum. Am. J. Hyg., 1: 284-300.

BULLOCH, W. and Atkin, E.E. 1905 Experiments on the nature of opsonic action of the blood serum. Proc. Roy. Soc. Lond., 74: 379-387.

BULLOCH, W. and Western, G.T. 1906 The specificity of the opsonic substances in the blood serum. Proc. Roy. Soc. Lond. B, 77: 531-536.

CHADANI, R. 1928 Studies on the phagocytic function of connective tissue cells taken from living or dead animals and human bodies. II. The influence of temperature thereon. Trans. Jap. Path. Soc., 18: 102-110 (abstracted in Biol. Abst., 8: 19743, 1934).

COTTINGHAM, Esther and Mills, C.A. Dec., 1943 Influence of environmental temperature and vitamin deficiency upon phagocytic functions. J. Imm., 77: 493-502.

COWIE, D.M. and Chapin, W.S. 1907 On the reactivation of heated normal human opsonic serum with fresh diluted serum. A contribution to the study of the structure of opsonins. J. Med. Res., 17: 57-75.

DEAN, G. 1905 An experimental enquiry into the nature of the substance in serum which influences phagocytosis. Proc. Roy. Soc. Lond. B, 76: 506-525.

DENYS, J. and Kaisin, A. 1893 Le pouvoir bactericide du sang. La Cellule, 9: 337-393.

DENYS, J. and LeClef, J. 1895 Sur le mecanisme de l'immunite chez le lapin vaccine contre le streptocoque pyogene. La Cellule, 11: 177-221.

DIENES, L. and Mallory, T.B. 1932 Histological studies of hypersensitive reactions. Am. J. Path., 8: 689-700.

DURHAM, H.E. 1897 The mechanism of reaction to peritoneal infection. J. Path. and Bact., 4: 338-382.

ELLINGSON, H.V. 1939 The Influence of Artificial Fever on Resistance to Infection. Ph.D. Thesis, Univ. of Wisc., 98 pp.

ELLINGSON, H.V. and Clark, P.F. 1942 The influence of artificial fever on mechanisms of resistance. J. Imm., 43: 65-83.

EVANS, Alice C. 1922 The toxicity of acids for leucocytes as indicated by the tropin reaction. J. Imm., 7: 271-304.

FENN, W.O. 1921a The phagocytosis of solid particles. I. Quartz. J. Gen. Phys., 3: 439-464.

FENN, W.O. 1921b The phagocytosis of solid particles. II. Carbon. J. Gen. Phys., 3: 465-482.

FENN, W.O. 1922a The temperature coefficient of phagocytosis. J. Gen. Phys., 4: 331-345.

FENN, W.O. 1922b The theoretical response of living cells to contact with solid bodies, J. Gen. Phys., 4: 373-385.

FLEMING, A. 1931 Phagocytosis, emigration of leucocytes and the bactericidal power of blood. A System of Bacteriology, 9: 212-222.

FRISCH, A.W. 1938 Sputum studies in lobar pneumonia. Phagocytosis and the effect of serum therapy. Proc. Soc. Exp. Biol. and Med., 39: 473-477.

GAY, F.P. and Morrison, L.F. 1923 Clasmatocytes and resistance to streptococcus infection. J. Inf. Dis., 33: 338-367.

√√ GOODPASTURE, E.W. and Anderson, Katherine 1937 The problem of infection as presented by bacterial invasion of the chorio-allantoic membrane of chick embryos. Am. J. Path., 13: 149-174.

GRAHAM, E.A. 1911 The influence of ether and ether anesthesia on bacteriolysis, agglutination, and phagocytosis. J. Inf. Dis., 8; 147-175.

GRAND, C.G. and Chambers, R. 1937 The chemotactic reaction of leucocytes to irritated tissue. J. Cell. and Comp. Physiol., 9: 165 (cited by Menkin, 1940).

HAMBURGER, H.J. 1912 Physikalisch-chemisch Untersuchungen über Phagozyten Wieshaden (cited by Mudd, McCutcheon, and Lucke, 1934).

HANKS, J.H. 1940 Quantitative aspects of phagocytosis as influenced by the number of bacteria and leucocytes. J. Imm., 38: 159-176.

HAYEM, M. 1870a Abces metastiques du foie. Compt. Rend. Soc. de Biol., 22: 84-86.

HAYEM, M. 1870b Leucocytes (circulation des) et depots dans les visceres a la suite d'une injection souscoutanee de cinabre. Compt. Rend. Soc. de Biol., 22: 115.

HEKTOEN, L. and Ruediger, G. 1905 Studies in phagocytosis. J. Inf. Dis., 2: 128-141.

HISS, P.H. 1908 Experimental and clinical studies on the curative action of leucocyte extracts in infections. I. The curative influence of extracts of leucocytes upon infections in animals. J. Med. Res., 14: 323-398.

HISS, P.H. and Zinsser, H. 1908a Experimental and clinical studies on the curative action of leucocyte extracts in infections. II. On the precipitation of bacterial extracts by extracts of leucocytes. J. Med. Res., 14: 399-410.

HISS, P.H. and Zinsser, H. 1908b Experimental and clinical studies on the curative action of leucocyte extracts in infections. IV. The curative influence of extracts of leucocytes upon infections in man. J. Med. Res., 14: 429-469.

HOTOPP, M. and Kahn, M.C. 1936 The fate of phagocytized acidfast bacteria as determined by single cell method. I. Polymorphonuclear leucocytes. J. Inf. Dis., 58: 324-330.

JACOBS, M.H. 1924 Permeability of the cell to diffusing substances, in E.V. Cowdry. General Cytology, Chicago (cited by Mudd, McCutcheon, and Lucke, 1934).

KASS, E.H. and Seastone, C.V. March, 1944 The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of Group A hemolytic streptococci. J. Exp. Med., 79: 319-330.

KLEBS, E. 1872 Beiträge zur pathologische Anatomie der Schusswunden, Leipzig, Vogel (cited by Zinsser, Enders, and Fothergill, 1939).

KRAUSE, A.K. and Willis, H.S. 1924 The dissemination of virulent tubercle bacilli after infection and reinfection. Trans. Nat. Assoc. Prev. Tub., 20: 277-280.

LEBER, T. 1888 Uber die Entstehung der Entzündung und die Wirkung der Entzündungserregenden Schädlichkeiten. Fortschr. D. Med., 6: 460 (cited by Menkin, 1940).

LEDINGHAM, J.C.G. 1908 The influence of temperature on phagocytosis. Proc. Roy. Soc. Lond. B, 80: 188-195.

LEDINGHAM, J.C.G. 1912 The mechanism of phagocytosis from the adsorption point of view. J. Hyg., 12: 320-359.

LEISHMAN, W.B. 1902 Note on a method of quantitatively estimating the phagocytic power of the leucocytes of the blood. Brit. Med. J., 1: 73-75.

LEWIS, W.H. 1931 Bull. Johns Hopk. Hosp., 49: 17 (cited by Mudd, McCutcheon, and Lucke, 1934).

LORD, F.T. 1919 The relation of proteolytic enzymes in the pneumonic lung to hydrogen ion concentration. An explanation of resolution. J. Exp. Med., 30: 379.

LUCKE, B., McCutcheon, M., Strumia, m. and Mudd, S. 1929 On the mechanism of opsonin and bacteriotropin action. II. Correlation between changes in bacterial surface properties and in phagocytosis caused by normal and immune serum. J. Exp. Med., 49: 797-813.

LYONS, C. 1937 Antibacterial immunity to Staphylococcus pyogenes. Brit. J. Exp. Path., 18: 411-422.

MADSEN, T. and Wulff, O. 1919 Influence de la temperature sur la phagocytose. Ann. Inst. Past., 33: 437-447.

MADSEN, T. and Watabiki, T. 1919 Medd. k. setakad. Nobelinst., V (cited by Fenn, 1922a).

MASSART, J. and Bordet, C. 1891 Le chimiotaxisme des leucocytes et l'infection microbienne. Ann. de l'Inst. Past., 5: 417-444.

McCUTCHEON, M. 1942 Chemotaxis. Arch. Path., 34: 167-181.

McKENDRICK, A.G. 1913-14 Sc. Prog. Twent. Cent., 8: 497 (cited by Fenn, 1921a).

MEDLAR, E.M. and Sasano, K.T. 1933 The effects of virulence of micro-organism on the histopathology of experimental pulmonary tuberculosis. Am. Rev. Tub., 48: 62-79.

MENKIN, V. 1934 Studies on inflammation. X. The cytological picture of an inflammatory exudate in relation to its hydrogen ion concentration. Am. J. Path., 10: 193 (cited by Menkin, 1940).

MENKIN, V. 1937 Isolation and properties of the factor responsible for increased capillary permeability in inflammation. Proc. Soc. Exp. Biol. and Med., 36: 164-167.

MENKIN, V. 1938 Studies on inflammation. XIV. Isolation of the factor concerned with increased capillary permeability in injury. J. Exp. Med., 67: 129 (cited by Menkin, 1940).

MENKIN, V. 1939 Inflammation. XVII. Direct effect of changes in the hydrogen ion concentration on leucocytes. Arch. Path., 27: 115-121.

MENKIN, V. 1940 Dynamics of Inflammation, McMillan Co., New York, 244 pp.

MENKIN, V. Sept., 1944 Further studies on the leucocytosis promoting factor and on necrosin in inflammatory exudates. Am. J. Med. Sci., 208: 290-293.

MENKIN, V. and Warner, C.R. 1937 Studies on inflammation. XIII. Carbohydrate metabolism, local acidosis, and the cytological picture in inflammation. Am. J. Path., 13: 25 (cited by Menkin, 1940).

METCHNIKOFF, E. 1895 Etudes sur l'immunite. Sixieme memoire sur la destruction extracellulaire des bacteries dans l'organisme. Ann. de l'Inst. Past., 9: 433-461.

METCHNIKOFF, E. 1899 Etudes sur la resorption des cellules. Ann. de l'Inst. Past., 13: 737-770.

METCHNIKOFF, E. 1905 Immunity in Infective Disease, translated by F.G. Binnie, Cambridge Univ. Press, 591 pp.

METCHNIKOFF, Olga 1921 Life of Elie Metchnikoff, 1845-1916, Houghton Mifflin Co., Boston, 297 pp.

MILLS, C.A. and Cottingham, Esther Dec., 1943 Phagocytic activity as affected by protein intake in heat and cold. J. Imm., 47: 503-504.

MUDD, S., Lucke, B., McCutcheon, M., and Strumia, M. 1929 On the mechanism of opsonin and bacteriotropin action. I. Correlation between changes in bacterial surface properties and in phagocytosis caused by sera of animals under immunization. J. Exp. Med., 49: 779-795.

MUDD, S., Lucke, B., McCutcheon, M., and Strumia, M. 1930 On the mechanism of opsonin and bacteriotropin action. VI. Agglutination and tropin action by precipitin sera, characterization of the sensitized surface. J. Exp. Med., 52: 313-329.

MUDD, S., McCutcheon, M., and Lucke, B. 1934 Phagocytosis. Physiol. Rev., 14: 210-275.

MUDD, E.B.H. and Mudd, S. 1933 The process of phagocytosis. The agreement between direct observations and deductions from theory. J. Gen. Phys., 16: 625-636.

MUIR, R. and Martin. W.B. 1906 On the combining properties of opsonins of normal serum. Brit. Med. J., 2: 1783-1785.

MULLER, F. and Jochmann, G. 1906 Münch. med. Woch., 53: 1507 (cited by Zinsser, Enders, and Fothergill, 1939).

NEUFELD, F. and Rimpau, W. 1904 Deut. med. Woch., 30: 1458 (cited by Zinsser, Enders, and Fothergill, 1939).

NEUFELD, F. and Rimpau, W. 1905 Z. Hyg., 51: 283 (cited by Zinsser, Enders, and Fothergill, 1939).

ONO, J. 1928 Studies on the survival length of blood cells in vitro, tested by their ameboid movements. II. The influence of temperature thereon and on phagocytosis. Trans. Jap. Path. Soc., 18: 92-102 (abstracted in Biol. Abst., 9: 2663, 1935).

OPIE, E.L. 1906 The enzymes in phagocytic cells of inflammatory exudates. J. Exp. Med., 8:410-436.

OPIE, E.L. 1907 Experimental pleurisy--resolution of a fibrinous exudate. J. Exp. Med., 9; 391-413.

OPIE, E.L. 1924 The fate of antigen (protein) in an animal immunized against it. J. Exp. Med., 39: 659-675.

OPIE, E.L. 1929 Inflammation and immunity. J. Imm., 17: 329-341.

PANUM, P.L. 1874 Virch. Arch. Path. Anat., 60: 301 (cited by Zinsser, Enders, and Fothergill, 1939).

PFEIFFER, R. 1894 Z. Hyg., 18: 1 (cited by Zinsser, Enders, and Fothergill, 1939).

RADSMA, W. 1918 Arch. neer. Physiol., 2: 301 (cited by Mudd, McCutcheon, and Lucke, 1934).

RHUMBLER, L. 1914 Ergebn. Physiol., 14: 474 (cited by Mudd, McCutcheon, and Lucke, 1934).

RICH, A.R. 1932 The nature of allergy in tuberculosis as revealed by tissue culture studies. Bull. Johns Hopk. Hosp., 50: 115-132.

RICH, A.R. and McKee, Clara M. 1934 A study of the character and degree of protection afforded by the immune state independently of the leucocytes. Bull. Johns Hopk. Hosp., 54: 277-311.

ROBERTSON, O.H. and Sia, R.P.H. 1924 Studies on pneumococcus growth inhibition. II. A method for demonstrating the growth inhibitory and bactericidal action of normal serum-leucocyte mixtures. J. Exp. Med., 39: 219-244.

ROLLY, F. and Meltzer 1908 Experimentelle Untersuchungen über die Bedeutung der Hyperthermie. Deutsches Arch. f. klin. Med., 94: 335 (cited by Ellingson and Clark, 1942).

ROSENOW, E.C. 1906 The role of phagocytosis in the pneumococcidal action of pneumonic blood. J. Inf. Dis., 3: 683-700.

ROSENTHAL, W. 1909 Centr. Bakt. Abt. 1, Ref. 42, Beih. 177 (cited by Zinsser, Enders, and Fothergill, 1939).

RUSSELL, F.F. 1907 Studies on the specificity of opsonins in normal and immune serum. Bull. Johns Hopk. Hosp., 18: 252-258.

SAWTCHENKO, J.G. 1902 Du role des immunisines (fixateurs) dans la phagocytose. Ann. Inst. Past., 16: 106-126.

SELLARDS, A.W. 1908 Note on the nature of opsonic activity. J. Inf. Dis., 5: 308-323.

SIA, R.H.P. 1926 Studies on pneumococcus growth ingibition. VI. The specific effect of pneumococcus soluble substance on the growth of pneumococci in normal serum-leucocyte mixtures. J. Exp. Med., 43: 633-645.

SIMON, C.E., Lamar, R.V., and Bispham, W.W. 1906 A contribution to the study of opsonins. J. Exp. Med., 8: 651-680.

STEINBERG, B. 1938 Inflammation of serous surfaces. Transfer of living leucocytes and the effect on acute infectious states. Arch. Path., 25: 785-791.

STEINBERG, B. and Dietz, A. 1938 Inflammation of serous surfaces. Hydrogen ion concentration in relation to cell type. Arch. Path., 25: 777-784.

STEINBERG, B. and Martin, R.A. 1938 Inflammation of serous surfaces. Factors modifying types of cell response. Arch. Path., 25: 792-801.

STEWART, F.W., Long, P.H., and Bradley, J.I. 1926 The fate of reacting leucocytes in the tuberculin and reinfection reactions. Am. J. Path., 2: 47-56.

STRUMIA, M., Mudd, S., Mudd, E.B.H., Lucke, B., McCutcheon, M. 1930 On the mechanism of opsonin and bacteriotropin action. V. Experimental test of a theory of tropin action. J. Exp. Med., 52: 299-313.

TODD, E.W. 1927 A method of measuring the increase or decrease of the population of hemolytic streptococci in blood. Brit. J. Exp. Path., 8: 1-5.

TOPLEY, W.W.C. and Wilson, G.S. 1937 The Principles of Bacteriology and Immunity, second edition, William Wood and Co., Baltimore, 1645 pp.

TOVAR, R.M. Oct., 1944 The opsonization test in brucellosis. J. Imm., 49: 203-207.

TUNNICLIFF, R. 1931 Promotion of phagocytosis by calcium gluconate, sodium iodide, dextrose and other substances. J. Inf. Dis., 48: 161-166.

WADSWORTH, A. 1904 Experimental studies on the etiology of acute pneumonitis. Am. J. Med. Sci., 127: 851 (cited by Menkin, 1940).

WALDEYER 1872 Arch. f. Gynaekol., 3: 293 (cited by Zinsser, Enders, and Fothergill, 1939).

WARD, H.K. 1930 Observations on the phagocytosis of pneumococcus by human whole blood. I. The normal phagocytic titre, and the anti-phagocytic effect of the specific soluble substance. J. Exp. Med., 51: 675-684.

WARD, H.K. and Enders, J.F. 1933 An analysis of the opsonic and tropic action of normal and immune sera based on experiments with the pneumococcus. J. Exp. Med., 57: 527-547.

WEISS, C. 1939 Proteinase and peptidase activity of polymorphonuclear leucocytes, monocytes, and epithelioid cells. Am. Rev. Tub., 39: 228-231.

WELCH, H., Brewer, M. and Hunter, A.C. 1940 Toxicity of antiseptics, experiments with hemolytic complement. J. Imm., 38: 273-282.

WESTERFELT, D.E. and Senekyian, H. 1937 On the relationship of opsonization to somatic and flagellar agglutination by the blood of vaccinated individuals. Am. J. Hyg., 26: 11-26.

WOLF, Elizabeth P. 1921 Experimental studies on inflammation. I. The influence of chemicals upon the chemotaxis of leucocytes in vitro. J. Exp. Med., 34: 375-396.

WRIGHT, A.E. and Colebrook, L. 1921 Technique of the Teat and Capillary Glass Tube, Constable and Co., London, 384 pp.

WRIGHT, A.E. and Douglas, S.R. 1903 An experimental investigation of the role of blood fluids in connection with phagocytosis. Proc. Roy. Soc. Lond., 72: 357-370.

WRIGHT, H.D. 1927 Experimental pneumococcal septicemia and anti-pneumococcal immunity. J. Path. and Bact., 30: 185.

ZINSSER, H., Enders, J.F., and Fothergill, L.D. 1939 Immunity Principles and Applications in Medicine and Public Health, fifth edition, McMillan Co., New York, 801 pp.

ZINSSER, H. and Hiss, P.H. 1908 Experimental and clinical on the curative action of leucocyte extracts in infections. III. Observations on the mechanism of protection by leucocytic extracts/ J. Med. Res., 14: 411-428.