ANTIMICROBIAL FACTORS OF NORMAL TISSUES AND FLUIDS

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INTRODUCTION

Observations by many investigators during the last fifteen years of the 19th century demonstrated the existence of antimicrobial substances in blood, leucocytes and lymphatic tissues. Since the turn of the century many tissues have been shown to contain factors inimical to microorganisms. No doubt many of the recent findings represent rediscoveries of agents which previously had been inadequately defined. A great deal of confusion has arisen regarding their identities and their role in natural resistance to infectious processes.

This review represents an attempt to discuss and classify many of the antimicrobial agents obtained from normal tissues and body fluids. The subject matter is divided into two broad groups, based upon the antibacterial selectivity of the various factors toward gram negative or gram positive bacteria. It should be noted that only certain of the factors have been established as showing a definite predilection toward one group of bacteria or the other, while others are active against representatives of both groups or have not been adequately differentiated. In spite of these limitations, the general predisposition of these agents towards either gram negative or gram positive forms is patent and affords a convenient division. Other aids used in the classification of these antimicrobial substances are tissue or fluid source, heat stability and, if known, chemical composition.

ANTIMICROBIAL SUBSTANCES SELECTIVE FOR GRAM NEGATIVE BACTERIA

In 1888 Nuttall observed that normal dog or rabbit defibrinated blood lost its bactericidal property for gram negative bacteria after being heated at 52 to 55 C for ½ to 1 hr (129). The next year Buchner (24) repeated this observation on cell free normal serum heated at 55 C for ½ hr or 52 C for 6 hr. He attempted to identify the protein material by chemical methods but found it readily inactivated by the simple procedure of dialysis against distilled water (25). Buchner named this serum bactericidal substance "alexin" (Greek, to ward off) in 1891 (26). Since these early reports many investigators have added to the knowledge of this material.

In 1905–6 Ehrlich proposed the name "complement" in place of his earlier term "addiment" to apply to the heat labile normal serum substance active in hemolytic systems (41). He claimed that serum contained a multiplicity of normal hemolysins which consisted of thermostable interbodies (not inactivated at 55 C) and the thermolabile complement. He supported the view that Buchner's originally described alexin was actually a dual system of interbody and complement. Since this time alexin has generally been regarded as a thermolabile substance which required for its bactericidal action the participation of another serum factor, normal or natural antibody.

On the Identity of Alexin, Opsonin and Complement

The early controversy regarding the similarities or differences between alexin and complement has been resolved in more recent years. Among those who have reviewed and worked with the subject extensively (57, 116, 130, 187), the concensus is that they are identical.

The term "opsonin" (Greek, to cater for) was introduced by Wright and Douglas in 1903 (182) to denote the thermolabile phagocytosis-stimulating substance in normal serum. We agree with Raffel (145) that the term opsonin be restricted to Wright's original description to differentiate it from those specific antibodies, the bacteriotropins, which also enhance phagocytosis. Osborne (130) discussed the investigations which led to the provisional theory that opsonin represented complement without the fourth component. Citing the work of Maltaner (105) which indicated that leucocytes contained C_4', the theory contended that phagocytes ingest microorganisms buttered with opsonin and that the fourth component is added intracellularly. An excellent literature survey of the role of complement components in opsonization is found in Chapter 52 of the Topley-Wilson textbook (180).

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Through the development of unique methods, Maaløe (102) demonstrated that all four components of complement were necessary for the manifestations of bactericidal and opsonic activity in normal serum. Thus it appears that complement, in conjunction with normal antibody produces the bactericidal, hemolytic and phagocytosis-promoting effects of normal serum. Maaløe has suggested that a single new term be introduced to replace alexin, complement and opsonin. Recently, Tullis and Surgenor isolated by paper electrophoresis two normal serum proteins which proved stimulatory to phagocytosis (169). Although both of the phagocytosis-promoting factors were thought to be distinct from complement and properdin, further studies will be required to establish this.

Source of Alexin or Complement

A controversy developed concerning the source of alexin and complement and although the problem has been clarified somewhat, no definitive conclusions are yet possible. In 1894 Buchner (27) and Denys and Havet (32) reported that leucocyte extracts were much more active against gram negative bacteria than the corresponding serum from the same experimental animals. They found the substance in leucocytes to be heat labile and thus believed that serum alexin was derived from injured leucocytes. It should be noted that the testing of leucocyte extracts was performed in the presence of serum, a fact which probably accounted for the observed heat lability. Many subsequent workers also reached the conclusion that leucocytes represented the source of alexin or complement, usually as a result of finding greater antibacterial activity in leucocyte extracts than in corresponding sera (1, 22, 53, 62, 108). In some of these early reports it was shown that leucocyte extracts were active against gram positive as well as gram negative bacteria. These results indicated the presence of other antibacterial factors in leucocytes which were unrelated to alexin or complement.

Several investigators reported the absence of alexin or complement activity in carefully washed leucocytes (91, 112, 133, 152, 153, 186). Schattenfroh (152) tested extracts of washed leucocytes in saline rather than serum and found that they were active only against gram positive bacteria. Furthermore, since the extracts required temperatures of 75 to 80 C for inactivation in the absence of serum, he concluded that alexin did not originate from leucocytes. Later Korschun showed that rabbit leucocyte extracts were bactericidal for typhoid organisms and that the active material was only slightly inactivated in ½ hr at 72 C (91). However, if the extracts were heated in the presence of serum the activity was lost in ½ hr at 56 C. These early observations implied that leucocytes contained at least two thermostable antibacterial factors.

Gengou (53) found that uncoagulated cell free plasma, obtained without the use of anticoagulants, was not bactericidal whereas the serum from the same blood was active. In careful experiments Fuchs (50) showed that the clotting of blood must begin before hemolytic complement activity is manifested. He also noted that in blood to which anticoagulants had been added hemolytic complement action was immediately apparent. He postulated that, until the clotting process was initiated, complement was not free to act in circulating blood. Maltaner (105) showed that extracts of carefully washed leucocytes, while devoid of full complement activity, reactivated ammonia-treated serum, thus indicating that the leucocytes furnished the C_4' component of complement. This finding may explain the results obtained by Gengou and Fuchs regarding the lack of bactericidal action in normal uncoagulated plasma. The leucocyte injury which occurs as a consequence of coagulation, or after the addition of anticoagulants, could result in the release of C_4', thus providing full complement activity. This suggests that complement exists in a precursor state in circulating blood and is not activated until leucocyte injury occurs.

Normal Antibody

In 1899 Moxter observed that alexin required an additional normal serum component to manifest bactericidal action (113). Wright and Windsor (181) adsorbed normal human and rabbit sera with small quantities of killed cholera or typhoid cultures and found that either organism removed the bactericidal property, while bacteria not susceptible to the killing action of serum did not do so. Steinhardt (161) found that the bactericidal action for both typhoid and dysentery bacilli was removed from normal serum by dead cultures of either organism but was partially restored upon the addition of normal serum which had been heated to 55 C to

destroy complement. She ascribed the result to the action of a common, naturally occurring immune body which was adsorbed by the killed bacteria, resulting in a concomitant nonspecific reduction in complement.

Ehrlich described the complex nature of normal serum hemolysins (41). He observed that when a normal serum, which dissolved certain unsensitized foreign erythrocytes at 37 C, was mixed with these cells at 0 C and centrifuged, the supernatant lost its ability to lyse red cells at higher temperatures. This property could be restored to the centrifuged serum by the addition of a quantity of normal serum which had been heated to 55 C for ½ hr. He referred to the substance adsorbed at 0 C as interbody and the heat labile substance as complement.

Muir (115) reported the removal of bactericidal activity of normal guinea pig serum by adsorption with killed homologous or heterologous bacteria without appreciable impairment of hemolytic activity against sensitized erythrocytes. When normal serum was adsorbed with the same species of bacterium against which it was tested, a greater decrease in bactericidal effect was observed than if the adsorption step had been performed with heterologous species. These experiments suggested that normal serum contained one or a few closely related molecular species of a nonspecific substance having a differing affinity for various bacteria. Since this time, the substance has come to be known most commonly as normal or natural antibody. The investigations of many workers have since confirmed and extended these early reports. Pettersson (140) introduced the term, "*alpha*-lysin," to describe the two-component alexin system consisting of the thermolabile substance and normal antibody.

Gordon and Wormall (57) showed that heat-killed dysentery bacilli removed the bactericidal power of normal guinea pig serum when incubated for 1 hr at 37 C although the hemolytic property toward sensitized red cells was only slightly diminished. The bactericidal power could be reinstated by the addition of normal serum heated to 56 C for ½ hr. If large amounts of adsorbing bacteria were employed, the specific hemolytic action was also lost. The authors referred to the bactericidal factor removed at 0 C as normal immune body.

In 1928 Dunlop (40) found that many strains of *Salmonella typhosa* caused the fixation of guinea pig complement as a result of the interaction with natural (normal) antibody. He found that bacteria, exposed to normal serum at 0 C for 1 hr, became sensitized to subsequent complement action at body temperature. The cold adsorption of normal antibody was nonspecific since many typhoid strains as well as charcoal and powdered glass removed it from serum. The natural antibody resembled immune antibody in its ability to fix complement but differed in its nonspecificity and greater thermolability. Dunlop found a variation in the heat stability of natural antibody, depending upon the serum source and the strain of bacteria against which it was tested. In some sera it appeared to be more labile than complement.

Mackie and Finkelstein (103, 104) studied the heat labile antibacterial normal serum system and found it to be active only against gram negative bacteria of the typhoid-dysentery-vibrio groups. Antibacterial action was dependent upon two serum components, complement and a more heat stable constituent which they referred to as sensitizing or intermediary antibody. Complement activity was lost in heated serum after ½ hr at 55 C while the sensitizing antibody (natural or intermediary antibody) required temperatures of 57 to 60 C to be inactivated in ½ hr (104). The intermediary antibodies could be adsorbed out by viable or killed typhoid organisms at 0 to 2 C, and they sensitized susceptible bacteria to the killing action of complement, the latter not being adsorbed out by the cells in the cold. These authors suggested that normal serum contained specific naturally occurring antibodies which were increased upon immunization. They pointed out that the complexity of natural antibody specificities was affected by the serum source and the species or strain of bacteria used in adsorption studies. Gordon and Carter (58) adsorbed normal guinea pig serum with washed bacterial suspensions at 0 C, which removed nonspecifically the bactericidal power for a number of gram negative bacteria. Variations in this activity of normal serum against different organisms depended upon the sensitivity of these bacteria to the nonspecific serum component (normal antibody). In 1946 Maaløe concluded that normal serum probably contained only one or a few fairly nonspecific normal antibodies (102). In discussing the differences between true normal antibodies and classical antibodies, Land-

steiner suggested (94*a*) that there may exist in serum a great variety of globulin molecules which, "by virtue of accidental affinity to certain substrates are picked out as (specific) antibodies."

Recent experiments by Adler (2) offer a clarification of the issue of normal antibody specificity. It is well known that enteric bacteria share common antigens and the widespread occurrence of the typhoid antigens, XII and IX, among enterics suggests that subdetectable levels of antibody could be produced to these antigens, whatever their bacterial source. Thus, the adsorption of "normal" serum by a particular enteric strain may result in the removal of specific antibody with consequent reduction in serum bactericidal power toward that bacterium. Gaines and Landy reported a highly specific antibody to *Pseudomonas aeruginosa* present in "normal" sera at levels undetectable by conventional methods (51). Undoubtedly, some of the confusion regarding specificity of normal antibody can be laid to the presence of ordinarily undemonstrable levels of specific antibody in apparently normal sera. This seems particularly likely when we consider that these specific antibodies are directed towards members of the normal enteric flora of most animal species.

A simple way to differentiate between normal antibody and specific antibody is to measure their respective heat stabilities, but unfortunately this was not done in the two papers just cited. Adler found that the bactericidal antibodies were able to survive 56 C for ½ hr (2), but no further heat stability studies were undertaken. Most experimenters have shown that normal antibody is inactivated after ½ hr between the temperatures of 55 and 60 C, while classical antibodies require temperatures of 65 to 70 C for inactivation. Normal antibodies from various animal sources reasonably can be expected to vary somewhat in thermostability and as Jacox noted (80), stored serum may vary significantly in pH, a factor which also may alter the heat stability of serum components. In the future a more critical study of the heat stability of normal antibody would aid in its proper classification and avoid confusion with the more stable specific antibodies which may be present in subdetectable amounts in "normal" serum.

It is generally accepted that in experiments where hemolysis of nonsensitized erythrocytes, bactericidal reaction, or phagocytosis enhancement occurs in the absence of specific antibodies, serum normal antibody represents a second necessary component in these systems.

The role of alexin or complement in immune systems has been elucidated by Bordet (22) and others (41). *In vitro* studies by many investigators have shown that the addition of small amounts of heated specific serum to normal serum resulted in marked increases in the bactericidal activity against gram negative bacteria. Exceptions to this phenomenon have been observed where certain bacteria survived or grew profusely in fresh immune serum while being inhibited or killed in normal serum from the same animal (108, 125, 172). At least a partial explanation for these seemingly contradictory findings is apparent from the early experiments of Neisser and Wechsberg (125) and more recently, of Maaløe (102). The former authors showed that the addition of very small amounts of heated immune serum to normal serum enhanced the bactericidal effect while the addition of larger amounts of the specific serum caused a reversal of antibacterial action. Observations such as these led Mechnikov to become more strongly imbued with the cellular theory of immunity. He reasoned that it was the agglutinating action of specific antibody, resulting in greatly increased phagocytosis, which heightened the resistance of immunized animals (108). Maaløe found that subdetectable amounts of specific agglutinins enhanced the bactericidal action of normal serum whereas a two- or threefold increase in agglutinin concentration did not further increase this effect; in fact, high levels of the specific antibody completely reversed bactericidal action (102). Maaløe also noted that high concentrations of normal serum interfered with antibacterial action presumably because of the presence of excess normal antibody. Just recently Muschel and Muto demonstrated that the bactericidal action of normal mouse serum was dependent upon full complement, normal antibody, and Mg^{++} ions (117).

Properdin

Pillemer and co-workers (142) isolated a protein from normal human serum which required the presence of complement and Mg^{++} ions to exhibit bactericidal action against gram negative bacteria. It also neutralized certain viruses and was active in an unsensitized hemolytic system in the presence of complement and Mg^{++} ions.

This factor was named properdin (Latin, to destroy) and it was found to be a euglobulin, representing no more than 0.03 per cent of total serum proteins. It formed complexes with a yeast zymosan, bacterial products, large branched polysaccharides and charcoal (143) between the temperatures of 10 to 20 C, enabling its removal from serum without destruction of specific hemolytic complement. At temperatures above 20 C inactivation of hemolytic complement (C_3') occurred in most cases. The substance proved heat labile in serum at 56 C for ½ hr but was more stable in a purified state. It was not removed by antigen-antibody complexes, not involved in the serum clotting mechanism, and was assayed by its ability to inactivate C_3' in the presence of zymosan at 37 C. Properdin was isolated from the serum of several animal species (172).

Properdin proved to be active against certain strains of several gram negative bacteria but inactive for *Micrococcus pyogenes* var. *aureus*. The presence of specific agglutinins in serum interfered with its bactericidal action against *Shigella dysenteriae*. The re-addition of physiologic levels of properdin to properdin-deficient serum restored almost completely the bactericidal effect, but excess amounts of properdin reversed serum antibacterial activity (172).

Landy and Pillemer (96) injected small amounts of purified lipopolysaccharide into mice and observed a transient increase in properdin levels within 12 hr. If mice were treated with small amounts of lipopolysaccharide 24 hr prior to challenge with certain gram negative bacteria they were protected, whereas control mice exhibited decreased properdin levels and death. Rowley (147) had previously shown this protective effect with prior injections of cell walls of *Escherichia coli*. He noted an immediate highly susceptible state within 2 hr after injection of the cell walls but an increased resistance if challenge doses were administered 24 hr later. Serum complement levels did not increase after *E. coli* extracts were injected. Landy and Pillemer (97) correlated the maintenance of, or increase in, properdin levels with increased resistance of mice to challenge doses of gram negative bacteria. Small amounts of lipopolysaccharide (endotoxin) completely protected animals if challenge doses were given 6 to 24 hr later. The correlation of properdin levels and resistance to infection was not always exact, and in some cases properdin levels were merely maintained in test animals which exhibited increased resistance. The authors concluded that other defense mechanisms, in addition to properdin, were responsible for increased host resistance following administration of lipopolysaccharides. Recently, Rowley (148) reported a correlation between increased resistance of animals to infection and the bactericidal power of their serum following injection of O antigen of *E. coli*. He found that pathogenic strains of this organism were more anticomplementary than nonpathogenic strains because of their greater content of O antigen, and he supported the view that a substance like properdin could be involved.

Dubos and Schaedler (39) observed an increased resistance to gram positive infections following the administration of lipopolysaccharides. Since gram positive microorganisms are not affected by properdin *in vitro* it was concluded that other defense mechanisms were stimulated by injection of the foreign material. Landy (95) found that increased resistance following intraperitoneal injection of lipopolysaccharide extended only to gram negative bacteria while no change in susceptibility was demonstrated to three species of gram positive bacteria. The fact that different mouse strains were used by these investigators may explain the conflicting results, since other variables were similar in both studies.

In 1893 Klein (87) first observed that the intraperitoneal or subcutaneous injection of various killed bacteria prior to the administration of lethal doses of cholera organisms protected experimental animals. The next year Issaeff (78) and Pfeiffer and Issaeff (141) protected guinea pigs from lethal doses of cholera by a previous intraperitoneal injection of broth, peptone or urine. They observed a marked leucocytosis in the body cavity at the time of challenge and attributed the increased resistance of treated animals to the release of bactericidal material from the leucocytes. Ledingham and Bulloch (98) and Bedson (15) found that the intravenous injection of autolyzed bacteria was followed within 2 hr by a marked increase in serum opsonic activity and leucocytosis. The increased opsonic activity was nonspecific, was destroyed at 56 C and disappeared after 24 hr. Hiss (74) and Hiss and Zinsser (74*a*) observed that intraperitoneal

injection of leucocyte extracts protected animals against subsequent infections with both gram positive and gram negative bacteria. The authors reported a marked leucocytic infiltration in protected animals and found that protection was manifested in either intraperitoneal or septicemic infections. Wright (184) observed increased bactericidal and phagocytic activities of the serum of animals injected previously with gram positive bacterial vaccines. The addition of these vaccines to normal serum *in vitro* also brought about an increased killing power. Wright noted that both *in vivo* and *in vitro* antistaphylococcal activities were nonspecific and almost immediate in occurrence. He attributed the changes to the release of opsonin from damaged leucocytes.

It seems quite clear that the numerous observations of heightened resistance following the injection of various nonspecific materials are the result of a general stimulation of multiple host defenses. The marked leucocytosis commonly noted in many of these reports certainly implicates the leucocytes as important participants in the altered response of the host (Suter, 162).

Common Characteristics of Properdin and Normal (Natural) Antibody

Because of the strong similarities which became apparent between normal antibody and properdin, this section is devoted to a summary of this subject and is compiled from the two preceding sections. Both substances require full complement and Mg^{++} ions for the manifestation of bactericidal and unsensitized hemolytic activities. Both function in bactericidal and hemolytic systems in the absence of specific antibody. Their antibacterial action is directed almost exclusively toward gram negative bacteria. Both materials are regarded as being heat labile, *i.e.*, inactivation usually takes place at temperatures between 55 and 60 C in 20 to 30 min. Variations in heat stability are the result of factors such as the animal serum source, species and strains of microorganisms against which the heated sera are tested, pH of sera at time of heating and the complexity of the medium in which heating takes place. Both substances have been found in the normal serum of many mammalian species and both can be adsorbed nonspecifically from serum by similar materials.

Adsorption and removal from the serum of either substance at temperatures below 15 to 20 C causes little, if any, inactivation of complement as it functions in sensitized hemolytic reactions. At a temperature of 37 C the nonspecific adsorption results in destruction of the hemolytic complement power of the serum. Normal antibody has been shown to be adsorbed out in the temperature range of 0 to 37 C with killed bacterial suspensions and other materials but not with zymosan, *per se*. Pillemer and co-workers have reported that properdin cannot be adsorbed from serum below temperatures of 5 to 10 C. However, no data have been published to support this finding for adsorbing materials other than zymosan.

Properdin is nonspecific in its antimicrobial action toward various gram negative bacteria and certain viruses. Sensitivity of different bacterial species to properdin is variable, depending upon the age of the culture and the particular strain employed (96).

There seems to be some doubt as to the specificity of normal antibodies. Muir showed that serum adsorbed with killed suspensions of the same organism against which it was then tested (homologous strain) proved less active than if it were tested against a heterologous bacterial culture. However, by using a longer adsorption time or a larger number of adsorbing heterologous bacteria, all bactericidal action could be removed before greatly affecting hemolytic complement activity. Mackie and Finkelstein believed that normal serum contained a number of naturally occurring somewhat specific antibodies which were increased upon immunization. This confusion about normal antibody specificity may be explained, in part, on the basis of Adler's findings (2). One must differentiate between low levels of classical antibody in "normal" serum and the normal antibody ordinarily present in serum. Since both normal antibody and specific antibody can be adsorbed out of serum by a particular bacterium, the distinction between the two can be made on the basis of their differing heat stabilities.

Most investigators of this problem have concluded that normal or natural antibodies are nonspecific or only slightly specific. As noted on p. 275, Dunlop was able to remove the bactericidal action of serum toward several bacterial strains by first adsorbing normal serum with charcoal or powdered glass at low temperature. Gordon and Carter (58) were able to remove from serum the bactericidal action for several different bacteria by cold adsorption with a single killed

bacterial suspension. It is most reasonable to accept the view that serum contains one or at most only a few normal antibodies of rather nonspecific nature and that variable results can be explained on the basis of differing affinities of bacteria for this substance. As others have stated, it is difficult to imagine that normal serum could contain the myriads of naturally occurring specific antibodies necessary to sensitize great numbers of susceptible bacterial species and strains, each by its own specific antibody. However, it is reasonable to expect that heterophile reactions in serum explain, in part, those reported observations in which specificity seemed to be established.

Wardlaw and Pillemer (172) found the bactericidal action of properdin to be inhibited by specific antibody with *Shigella dysenteriae*. Furthermore, an excess of properdin itself caused a reversal of the antibacterial effect. As has been stated, both specific and normal antibody in excess quantities also have been shown to interfere with the thermolabile bactericidal system of normal serum.

It is concluded from this discussion of the numerous similarities shared by normal antibody and properdin that they are likely the same substance. The extensive and careful work of Pillemer and co-workers has added much specific information to our knowledge of the thermolabile bactericidal system in normal serum and their term, properdin, is more satisfactory than the ambiguous label of normal or natural antibody. It is suggested that the old terminology be discarded unless it can be proved that normal antibody and properdin are distinct substances.

We cannot subscribe to the naming of the complete antibacterial system as the "properdin system." Since properdin itself represents only one of at least six components known to play a requisite role in bactericidal action, this term seems rather overweighted. Furthermore, the large amount of work which began with the researches of Nuttall, Buchner and Moxter laid the foundation for what has since come to be regarded by many as the alexin system. These early important observations, as well as subsequent contributions, reveal that the alexin system bears close and, as yet, unconflicting similarities to the recently described "properdin system." For these reasons it is suggested that Buchner's original name, alexin, be retained to describe the thermolabile bactericidal system of normal serum, and that the term, properdin, be used in place of normal or natural antibody.

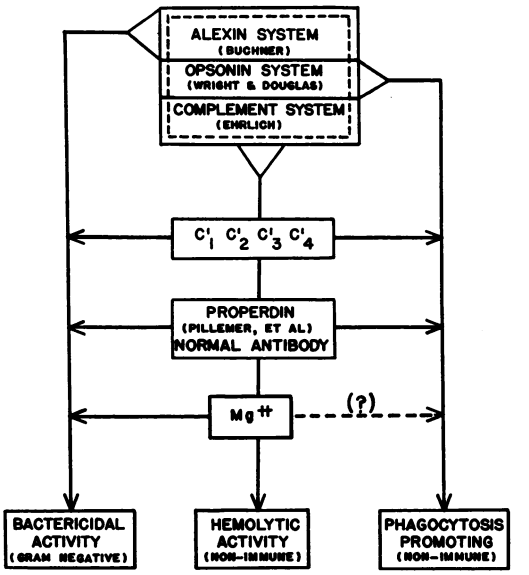

Figure 1. The thermolabile antimicrobial and hemolytic systems of normal serum.

Buchner's term, alexin, to describe his original important observation, did not envisage a complex bactericidal system in serum. He developed the concept that normal serum possessed a bactericidal property which was thermolabile. Since his time investigators have demonstrated that the antibacterial action of alexin required the participation of other factors. We feel it is unfortunate that today alexin, opsonin and complement have become identified, through usage, as one substance. Since the bactericidal, hemolytic and phagocytosis-promoting properties of normal serum have been found to depend upon the interaction of full complement, properdin (normal antibody) and Mg^{++} ions, we prefer to regard alexin, opsonin and complement as similar *systems* expressing the manifestations of bactericidal action, phagocytosis-promotion and hemolysis, respectively. Figure 1 is a schematic presentation of the antimicrobial and hemolytic activities of normal serum and is offered as a convenient way of expressing certain general information.

Antibacterial Substances from Leucocytes

Among the earliest reports of the presence in leucocytes of antibacterial materials active against gram negative bacteria were those of Buchner (27) and Denys and Havet (32) in 1894.

They found the bactericidal substance to be heat labile at 55 C but it was heated in the presence of serum which probably accounted for their identification of this material with serum alexin. Korschun (91) prepared rabbit leucocyte extracts by the freeze-thaw technique and reported a substance which killed typhoid and cholera organisms. The material proved heat labile if heated in the presence of serum but was only slightly inactivated at 72 C for ½ hr in the absence of serum. Zinsser (186) also found an antityphoid substance in rabbit leucocytes to be relatively heat stable, requiring a temperature of 75 C for ½ hr for inactivation. Manwaring (106) prepared leucocyte extracts in water and demonstrated an antityphoid factor which was heat stable and nondialyzable. Amano and coworkers (8, 9, 11) have recently reported substances from leucocytes, one of which bears resemblance to these antibacterial factors.

Hirsch (72, 73) has demonstrated bactericidal material present in rabbit, human and guinea pig neutrophils that proved active against several species of gram negative bacteria. The factor, which was ineffective against gram positive pathogens, was associated with a globulin fraction in the cytoplasm of the leucocytes. It lacked properdin activity and was relatively heat stable at acid pH, being only slightly inactivated after 2 hr at 65 C. It was not dependent upon divalent ions for its action and exhibited a bactericidal pH optimum in the acid range. The substance has been named phagocytin by Hirsch and, as he suggested, it is probably similar or identical to the poorly defined substances recorded in the early literature. Whether the phagocytins from white cells of different animals are a single substance or a group of closely related substances is not known.

Lysozyme as an Antibacterial Agent for Gram Negative Bacteria

Fleming (46), in his original paper on the discovery of lysozyme, reported that it was active against certain gram negative pathogenic bacteria as well as several saprophytic forms. In his classic review, Thompson (165) found little evidence that lysozyme in a purified state was active against pathogens. He cited literature which showed that the antibacterial activity of saliva and tears was due to the presence of other factors in these fluids; for example, a heat labile factor has been shown to participate.

The recent researches of Amano and his coworkers (5, 7, 9) and others (77) have brought to light a new role for the participation of lysozyme in certain antibacterial reactions involving gram negative bacteria. It was found that purified egg white lysozyme and lysozyme from leucocytes greatly accelerated the lysis of several gram negative pathogenic bacteria in the presence of specific antiserum and complement. An acceleration of the action of the immune bacteriolytic system was also observed in the presence of plakin, acid extracts of leucocytes or a basic polypeptide of pancreatic origin. In none of the numerous experiments were normal serum controls substituted in place of specific antiserum; thus the participation of other factors in immune serum has not been eliminated. However, this criticism may be minimized in view of the high dilutions of immune serum used in the experiments. The following scheme represents the interaction of lysozyme or other materials in the immune bacteriolytic system.

$$\left[\begin{array}{l}\text{complement and}\\ \text{specific antibody}\end{array}\right] + \left[\begin{array}{l}\text{lysozyme}\\ \text{plakin}\\ \text{basic peptide}\\ \text{leucocyte extract}\end{array}\right] \rightarrow \begin{array}{l}\text{accelerated}\\ \text{antibacterial}\\ \text{action}\end{array}$$

ANTIMICROBIAL SUBSTANCES SELECTIVE FOR GRAM POSITIVE BACTERIA

Beta-lysin

Fodor (48) was among the first to direct attention to the anthracidal action of defibrinated blood in 1887. The next year Behring (16) reported that the anthracidal power of rat serum withstood heating at 60 C for ½ hr. The heat stability of this serum anthracidal factor was also confirmed shortly after by Pane (132), Bail (12) and Sawtchenko (151). Pirenne (144) noted that the thermostable rat serum anthracide proved bactericidal for various gram positive bacteria but was inactive against gram negative species.

In a series of papers (135–137, 139, 140) Pettersson reported studies of a thermostable bactericidal substance from human, horse and dog serum, which he named *beta*-lysin. He found that *beta*-lysin, in contrast to alexin, acted principally against gram positive bacteria, required temperatures of 64 to 75 C for ½ hr to

inactivate, withstood dialysis and extraction with fat solvents, and was somewhat active at low temperature. Like alexin, it was reputed to be a dual system composed of a heat stable "activating" factor and a less heat stable "activable" factor which did not adsorb onto susceptible cells in the absence of the former substance. Pettersson claimed that the addition of small amounts of fresh serum to heat-inactivated serum (64 C) caused a reactivation of the bactericidal power. That *beta*-lysin is a two-component system has been supported by Ostenfeld (131), but most investigators either have not observed this or have been unable to confirm the duality of the serum *beta*-lysins (104, 120).

In 1932 Mackie and Finkelstein (104) reported a thermostable substance (it withstood ½ hr at 56 C) in normal serum which was active only against gram positive bacteria. Braun described a relatively heat stable normal serum factor which was active against *Brucella abortus* and *Micrococcus pyogenes* var. *aureus* and was found to be associated with serum globulins (23). Fishman and Shechmeister (45) also reported a heat stable substance in normal serum which was active against staphylococci.

Myrvik and Weiser (118) investigated a serum bactericidin active against *Bacillus subtilis* which was relatively heat stable. Ekstedt (42, 43) worked with a normal serum antistaphylococcal factor which appeared to be similar to *beta*-lysin. It was relatively heat stable and active against avirulent and weakly coagulase-positive strains of staphylococci but not active toward good coagulase producing strains, an observation reported earlier by Spink and Vivino (159). It was thought that coagulase neutralized the serum bactericidin thus enabling coagulase positive strains to survive the antibacterial action. Calcium ions were thought to be required for antibacterial action. Myrvik (120) was unable to confirm the role of coagulase in the neutralization of this serum bactericidin. Unlike Ekstedt, he found that the addition of crude coagulase to test sera did not impair bactericidal action toward susceptible bacterial strains. Furthermore, his observations indicated that calcium ions may not be required in the antibacterial action but rather that the addition of citrate to serum increased ionic strength, accounting for inactivation of the bactericidin.

Several investigators have reported a nonspecific increase in the bactericidal power of serum during the acute phase of various illnesses with subsequent diminution upon recovery (75, 80, 118, 166, 167, 185). The active serum substance or substances were relatively heat stable, withstanding 56 C for periods of at least ½ hr, and were most potent against gram positive bacteria. Jacox (80) and Myrvik and Weiser (118) have shown that citrate inactivated the bactericidin. It is probable that these reports concerned substances related or identical to the serum *beta*-lysin of Pettersson. In some instances, gram negative antibacterial activity was also observed to increase during the acute phase of illness. It is possible that substances other than *beta*-lysin are also elaborated under these conditions of stress to account for increased antibacterial action against these forms.

Whether these various poorly characterized substances, described as being relatively heat stable and usually more active against gram positive bacteria, are identical or closely related to the *beta*-lysins is not ascertainable at present. Until more information is made availabie they will be regarded here as a group of closely related substances in the category of *beta*-lysins.

The *beta*-lysins were originally believed to have been derived from leucocytes, but their source is still undetermined. The presence of other antibacterial factors in leucocytes has contributed to the confusion about the source of *beta*-lysins. Pettersson believed that *beta*-lysins were different from the endolysins of leucocytic origin (later named leukins) in that the latter material was more heat stable and exhibited a somewhat different antibacterial selectivity against gram positive organisms (138). Mackie and Finkelstein (104) also differentiated the serum *beta*-lysins from leukins on the basis of the greater heat stability of the latter. In Pettersson's last paper on this subject (140) he concluded that it would be difficult to differentiate between *beta*-lysin and leukin because of their similar properties. To attempt to distinguish between these two substances on the basis of heat stability may reveal a difference more apparent than real. The heat stability of *beta*-lysin was of necessity tested in serum where the pH might vary anywhere from 7.5 to 8.5, depending upon serum storage time. An alkaline pH has been shown to decrease the heat stability of *beta*-lysin (104). On the other hand, leucocyte extracts are normally acidic and this could account for the

apparently greater heat stability of leukins when heated.

Most of the antibacterial substances to be discussed have been identified as basic proteins, polypeptides or polyamines which have been derived principally from cellular elements. They are generally most active against gram positive bacteria, exhibiting little action against most gram negative forms, except in higher concentrations.

Lysozyme

As mentioned above, lysozyme, *per se*, has not been shown to be active against pathogenic microorganisms (165). Recently, however, Gladstone and Johnston (56) found purified lysozyme to act against certain encapsulated anthrax strains, in the presence of high levels of HCO_3^- and CO_2. This finding represents the first well-defined example wherein lysozyme has been shown to inhibit a pathogenic microorganism. Attempts by the authors to inhibit other gram positive pathogens under conditions of high CO_2 concentration were not successful.

The role of lysozyme in defense against bacterial invaders should not be minimized, particularly in view of its synergistic action with complement and immune antibody as reported above. Furthermore, the suggestion by Dubos (35) that potentially pathogenic bacteria may be prevented from establishing themselves in a host because of their lysozyme sensitivity is credible. The basic nature and properties of lysozyme have been reviewed (150*a*, 165). Amino acid analysis has shown it to contain the three basic amino acids, being particularly rich in arginine (49).

Other basic proteins of enzymic nature which have been reported to possess antimicrobial properties are ribonuclease (3, 17, 86), deoxyribonuclease and hyaluronidase (47). It is possible that the antibacterial property of these enzymes, active only in high concentrations, was due to the presence of active impurities, as suggested by the work of Fletcher *et al.* (47).

Nucleins, Histones and Protamines

Antibacterial nucleins, which are complexes of nucleic acids and simple proteins such as histones and protamines, were reported to be active against gram positive species by Vaughan *et al.* in 1893 (171) and by Kossel in 1896 (93). The first histone was discovered and named by Kossel, who extracted it from nuclei of avian red blood cells (92). Its antimicrobial properties have recently been confirmed (110, 124, 176). In 1869 Miescher extracted a nuclein from the nuclei of human neutrophils and he later characterized and named the basic portion of the salt-like nuclein, protamine (109). Protamines from various sources have since been shown to exhibit antimicrobial action against a virus (123), a trypanosome (146), a yeast (107) and several species of bacteria (19, 110, 123, 124, 176). Gram positive bacteria generally appeared to be more sensitive to the protamines.

The basic nature of the histones and protamines is well established. The histones possess large amounts of the two basic amino acids, lysine and arginine (30), whereas the protamines contain large concentrations of arginine and are usually low in lysine (94).

Tissue Basic Polypeptides

Bloom *et al.* (17) succeeded in extracting a nondialyzable anthracidal material from the thymus, pancreas and caecum of different animal species. The factor from calf thymus was found to be an acid and heat stable basic polypeptide containing approximately 30 per cent lysine and 3.5 per cent arginine and having an isoelectric point of pH 11.2. The active substance was thought to have been derived from the nuclear thymus histone (176). Basic tissue polypeptides have also been derived from spleen (20, 21) and thyroid tissue (18). A mycobactericidal peptide from calf thymus was studied by Dubos and Hirsch (37, 70, 71). This substance was basic, possessing large amounts of lysine and arginine, and having an isoelectric point between pH 10 and 11.

We have further characterized the thymus peptide of Bloom and co-workers and showed that it was derived from a histone fraction of the calf thymus (156) as originally thought. An amino acid analysis proved it to be identical to the histone Fraction A, analyzed by Crampton, *et al.* (30). This histone fraction was shown to be most active at alkaline pH and proved to be active predominantly against gram positive bacteria.

It has been postulated that the antibacterial properties of the basic tissue peptides are due to the presence of large amounts of the basic amino acid, lysine (160, 173, 176). Watson and Bloom

(173) pointed out the significance of the high lysine content (29 per cent) of the antibacterial thymus peptide. In comparing a synthetic polylysine and thymus peptide they found that polyamino lysine was about four times more active than thymus peptide on a weight basis. They concluded that antimicrobial activity resided within the lysine portion of the peptide molecule. The work with synthetic monoamino polypeptides of lysine (28, 83, 160, 173) and arginine and ornithine (83) lends strong support to this concept. The basic polyamino acids proved very active against both viruses and bacteria while neutral and acidic polyamino acids exhibited no antimicrobial action at high concentrations (83). Monomers of the basic amino acids were inactive, indicating that molecular weight was also important for activity of these peptides, a conclusion previously offered by Massart in his study of an antimicrobial protamine (107).

Several investigators have suggested that antimicrobial basic proteins and polypeptides combine with cell nucleoproteins or other negatively charged surface constituents of bacteria or viruses, thus disrupting important cell functions (19, 83, 85, 160, 176). The union of the basic substances with negatively charged cell surfaces is believed to occur through electrostatic bonding.

Bloom and Blake (18) observed that bacteria clumped by the tissue polypeptide could be redispersed by agitation, and after several washings these cells were again clumped upon the addition of ribonucleic acid. It was concluded that the first clumping resulted from the attraction of the basic linear peptide to oppositely charged cell surfaces while the second clumping indicated that the peptide, which was in firm union with cell surfaces, combined with the ribonucleic acid by mutual discharge of electrostatic bonds.

The fact that nucleic acids and long chain polysaccharides neutralize the antimicrobial properties of basic proteins or peptides (19, 83, 84, 154, 156, 173, 176) offers indirect evidence supporting the view that the basic materials exert their deleterious effects by union with cell nucleoproteins. It is thought that the positively charged ammonium groups of the linear basic substances unite with negatively charged acidic groups on the surfaces of susceptible microorganisms. The reversal of antibacterial action by large acidic macromolecules likely results from the inability of the basic materials to combine with microorganisms, since their positively charged groups had reacted preferentially with the free acidic polymers (83). The role of mucin and other large acidic molecules in the lowering of natural resistance may be partly the result of neutralization of the basic antibacterial agents in tissues, as previously suggested (122, 154).

The cause of death or inhibition of microorganisms, once an antimicrobial factor becomes fixed upon the cell surface, is not clear. Few and Schulman (44) showed that the basic peptide, polymyxin E, was adsorbed in large amounts upon the cell walls of susceptible bacteria. The result of this adsorption was a disorganization of the cell components taking part in the maintenance of osmotic equilibrium. Amano *et al.* (4) reported that the bactericidal peptide, plakin, adsorbed onto bacterial surfaces causing damage to membrane permeability. Since molecular weight, and perhaps linearity, was shown to be a requisite for antibacterial action of certain basic polypeptides, it is possible that surface steric stresses result after combination with susceptible bacteria, thus altering membrane integrity. This subject has been reviewed recently by Newton with respect to the polymyxin peptides (126).

Leukins

In 1891 Hankin (63) obtained an anthracidal material from lymph nodes of the dog and cat which he characterized as a *beta*-globulin. He postulated that the active principle was derived from the damaged leucocytes in the lymphatic tissue. Since this initial observation many investigators have succeeded in extracting antibacterial substances from leucocytes, particularly neutrophils. In the majority of early reports leucocyte extracts proved to be most active against gram positive bacteria and relatively heat stable (88, 134, 152, 153, 175), withstood heating at 56 C but was destroyed in ½ hr at temperatures of 60 to 80 C. In 1909 Schneider named this group of antibacterial substances "leukins" and distinguished them from alexins on the bases of their greater heat stability, antibacterial selectivity for gram positive bacteria and their source (153). Later confirmations of the presence of these leukins in leucocyte extracts have been published (17, 52, 64, 138).

Conflicting results were occasionally reported concerning the heat stability and antibacterial

selectivity of the leukins. No doubt this confusion arose from the fact that there are at least two different bactericidal factors in leucocyte extracts: the leukins and the phagocytins. For example, Gengou (54) reported an acid-extractable leucocyte material which inhibited many gram negative species in high concentrations and was heat resistant at 80 C for ½ hr at acid pH but not at alkaline pH. Since Gengou did not test his material against gram positive species it is not possible to know whether he was working with leukin, phagocytin or a combination of both. Gay and Clark (52) demonstrated the presence of two different antibacterial substances in single leucocyte extract preparations. Unheated extract killed both gram negative and gram positive bacteria while heated extract proved active only against the latter.

Amano and co-workers (6, 9) and others (77) described acid extracts of leucocytes which proved active in accelerating immune bacteriolytic systems against gram negative bacteria. The authors named two factors from the acid extracts, "leucozyme A" and "leucozyme B" (10). The method of preparation and heat stability of these two substances suggests that they belong to the category of leukins. Furthermore, it is probable that the two names describe but one substance which manifests itself in two different ways, depending upon the presence or absence of calcium or magnesium ions. However, these findings may elucidate a new relationship of immunity whereby a substance active only against gram positive bacteria can also function against gram negative forms in the presence of specific antibody.

We have recently characterized a leukin from rabbit polymorphonuclear cells (155). Presumably this leukin was derived from nucleoprotein of the white cell and is a protamine split product with a definite predilection for gram positive pathogenic bacteria. The product proved to be active in low concentrations (2 to 5 μg/ml) *in vitro* and was very heat stable at acid or neutral pH, surviving 100 C for 2 hr. It was shown to contain a large amount of the basic amino acid, arginine, which likely was responsible for the antibacterial action. Large acidic polymers blocked its activity in the manner described for the basic polypeptides cited on p. 283. The apparently greater heat stability of this leukin as compared with leukins previously reported is probably a reflection of its further purification as compared with the early crude fractions.

Leukins or leukin-like products have been obtained from leucocytes of the human, rabbit, dog, guinea pig and rat, but whether these various fractions share similar properties is uncertain. It is reasonable to assume that they are protamine or histone fractions of nucleoprotein origin.

Few attempts have been made to obtain antibacterial substances from leucocytes other than the neutrophils. However, Bloom *et al.* reported an inhibitory action of rat mononuclear extract upon tubercle bacilli (21). While Gengou reported little or no antibacterial activity in macrophage cell extracts (53), Gay and Clark demonstrated antibacterial action against both gram positive and gram negative bacteria with macrophage extracts (52). It would not be surprising to find that the nuclei of all types of leucocytes contain leukin-like material obtainable by suitable extraction methods.

Plakin

Blood platelets represent another source of antibacterial material, as first demonstrated by Gruber and Futaki in 1907 (61). They obtained an anthracidal substance from blood platelets of the horse. Barreau found in the platelets of several animals a relatively heat stable substance which proved active against anthrax bacilli (14). More recently Amano and co-workers extracted from horse platelets a plakin which inhibited cell respiration of two gram positive saprophytes (3). The product was relatively heat stable in acid medium, requiring heating for ½ hr at 80 C for inactivation, but was less stable at neutral pH. Amano and his colleagues also found plakin to be operative in immune bacteriolytic systems (7). Although plakin did not attack gram negative bacteria directly, it caused an acceleration of the immune bacteriolysis. The relationship of plakin to leukin is unclear but the possibility exists that the former substance was derived from nucleoprotein of the platelets and consequently is similar to leukin.

Hematin, Mesohematin

In 1914, Kammerer observed that mesohematin was inhibitory to many gram positive saprophytes and pathogens, and that hematin was less active (81). Whitney *et al.* (178) extracted an antibacterial material from trypsin-treated

bovine red blood cells. The factor was stable to autoclaving for 15 min at alkaline pH only and the authors suggested that it was a peptide. The source and preparation of this factor made it more probable that antibacterial action was due to the presence of hematins, as suggested by van Heyningen (66). This investigator obtained from horse red blood cells hematin and mesohematin which, in very low concentrations inhibited a large number of gram positive saprophytic bacilli. Ivanovics and Koczka (79) found mesohematin bactericidal for many gram positive bacteria but not for gram negative forms, with the exception of two *Hemophilus* strains. The susceptible bacteria adsorbed large amounts of mesohematin whereas resistant species did not, unless pretreated with organic acids or ethylene glycol. The authors postulated that resistant gram negative bacteria possessed a polysaccharide layer at their cell surface which prevented penetration of mesohematin. The mechanism of action of the antibacterial heme compounds may be due to a competitive action of these porphyrins with those essential to metabolism of the cell, as suggested by Dubos (38).

Spermine, Spermidine

In 1951 Dubos (36) extracted a tuberculostatic factor from various animal tissues. Its action was later found to be dependent upon the presence of a bovine albumin fraction and it was identified as spermine (67). Hirsch further expanded this work and also found spermidine to be equally active against certain strains of *Mycobacterium tuberculosis* whereas other related polyamino compounds proved inactive (68, 69). The dependency of tuberculostatic action of the two basic polyamines upon the presence of an *alpha*-globulin fraction was explained on the basis of an enzymatic alteration which released the active principle. Rozansky *et al.* (149) demonstrated that spermine inhibited growth of several gram positive bacteria and two strains of *Neisseria*, although it was inactive against enteric microorganisms. The antibacterial power of spermine was seen to increase greatly as pH was changed from acid to alkaline, a finding in keeping with the results obtained with other basic antibacterial substances. Grossowicz *et al.* (60) reported that spermine and spermidine were not dependent upon the presence of the bovine albumin fraction for activity against a staphylococcus strain. This apparent conflict of results with those of Dubos and Hirsch may be explained on the basis of the different bacterial species used.

Lactenin

Hesse was the first to report the antibacterial effect of cow's milk against several bacterial species (65). Wilson and Rosenblum studied the bactericidal protein, lactenin, found in the whey of human, cow and goat milk. The substance in low concentrations was selectively active against Group A streptococci and was inactivated at 80 C (179).

There are many other reports in the literature not included in this review which deal with antimicrobial substances of tissue origin. No doubt some of these agents (89, 100, 119, 128, 158, 168) are related or identical to the basic factors already discussed but a current lack of information prevents their inclusion under any of the headings listed. The various antimicrobial substances discussed in this review are briefly characterized in table 1.

Antiviral Factors

In 1930 Douglas and Smith noted the viricidal action of a heat labile normal serum component (33). The next year Mueller attributed this antiviral activity to the alexin system of serum (114). Since this time many investigators have confirmed the virus neutralizing effect of normal serum (13, 55, 157), an effect which not only removed viral infectivity but hemagglutinating activity as well. Much evidence has accumulated pointing to complement as the heat labile viral inhibitor in serum (34, 76, 101, 111, 150, 177), and it was observed that complement power was heightened in the presence of specific viral antibody. McCarty and Germer (121) found what appeared to be two heat labile serum components necessary for virus neutralization. Recently this observation was enlarged by Wedgwood *et al.* (174) who defined the complete serum system operating in viral neutralization. They found that complement, properdin and Mg^{++} ions were required and that properdin probably combined directly with virus, the complement components and divalent ion acting as cofactors. Koprowski observed that a relatively heat stable serum component was responsible for the neutralization of yellow fever virus and certain other viruses of the encephalitogenic group (90). Casals and

TABLE 1

Tissue antimicrobial substances

Name	Common Source	Heat Stability	Chemical Class
I. Antibacterial selectivity: gram negative			
Complement*	Serum	Labile†	Euglobulin-carbohydrate-albumin
Properdin (normal) antibody	Serum	Labile to relatively stable	Euglobulin
Phagocytin	Neutrophilic leucocytes	Relatively stable	Globulin fraction
Lysozyme (special case)	Ubiquitous distribution	Stable	Small basic protein
II. Antibacterial selectivity: gram positive			
beta-Lysin	Serum	Relatively stable	Protein (?)
Lysozyme	Ubiquitous distribution	Stable	Small basic protein
Histone	Lymphatics (nucleoprotein)	Stable	Small basic protein
Protamine	Sperm cells (nucleoprotein)	Stable	Small basic protein
Tissue polypeptides	Lymphatics (nucleoprotein)	Stable	Linear basic peptides
Leukin	Neutrophilic leucocytes	Stable	Basic peptides (protamine)
Plakin	Blood platelets	Relatively stable	Peptide (?)
Hematin, mesohematin	Red blood cells	Stable	Iron porphyrins
Spermine, spermidine	Pancreas, prostate	Stable	Basic polyamines
Lactenin	Milk	Relatively stable	Protein (?)

* We do not apply the names alexin or opsonin to single substances; rather we consider them to denote similar systems which require full complement in addition to properdin (normal antibody).

† Labile, inactivated 56 C, ½ hr; relatively stable, resist 56 C, ½ hr but destroyed below 80 C, ½ hr; stable, resist 80 to 100 C, ½ hr or more. (pH at heating *ca.* neutral or acid.)

Olitsky reported a heat stable lipid fraction which inactivated neurotropic viruses and which was obtainable from normal sera of several animals (29). Utz prepared a lecithin-like fraction from normal sera which neutralized the infectivity of influenza and Newcastle disease viruses but did not alter hemagglutinating activity (170).

Antiviral action has also been reported for protamine (59, 123), thymus polypeptide (173) and synthetic polylysine (59, 160, 173). It was postulated that the basic ammonium groups on the polypeptide combine by ionic linkage to virus nucleoprotein in a manner analogous to that previously suggested for bacteria. Green *et al.* (59) reported *in vivo* activity of synthetic polylysine and protamine. Protection of chicken embryos resulted when both the antiviral agent and the virus were injected at the same site, indicating that the protective action was only local. It is doubtful whether protection would have resulted if the basic substances were injected by a different route where they would be rapidly neutralized by embryonic tissues before getting to the site of virus inoculation.

RELATIONSHIP OF ANTIMICROBIAL SUBSTANCES TO NATURAL RESISTANCE

In 1922 Ledingham cautioned against ascribing too much significance in immunity to the various bactericidal factors described in the literature, particularly since most such factors have been tested *in vitro* only (99). This caution is even more warranted today and has been reiterated and expanded recently by Wilson and Miles (180). In addition to the limitations inherent in the application of *in vitro* experimental results to the *in vivo* environment, another important qualification is apparent. Often, the preparative methods used for obtaining antimicrobial agents will give rise to artifacts which bear little resemblance to naturally occurring tissue products. However, this latter criticism is lessened in those

experimental procedures where harsh chemical extractions were not necessary to evidence antimicrobial action. For example, serum factors and crude preparations of leukins and phagocytins can be demonstrated quite simply.

Dubos has offered some tenable views regarding the significance of many of the antimicrobial agents described above (38). He has also discussed the influence on host resistance of antibacterial fatty acids, high CO_2 tension, and the accumulation of organic acids such as lactic acid in inflammatory sites. It is likely that many antimicrobial agents are released locally in damaged tissues and that their activities are augmented or enhanced by ensuing changes within the immediate environment of inflammatory loci (31, 72, 155, 173).

The role of antimicrobial tissue factors in resistance may represent one facet of the whole defense system. We do not suppose that these substances exist, as such, in normal tissues but that they arise in response to physiologic changes which accompany stress. They may act in the body to kill or to slow the growth of invading microorganisms, enabling other host defenses to operate more efficiently in the removal of inimical agents from the tissues. Allied mechanisms of natural resistance to infectious disease, with which the antimicrobial factors may be integrated, have been well reviewed by Nungester (127), Kass and Finland (82) and Suter (162).

CONCLUSIONS

This review, though incomplete, should aid in eliminating some of the confusion which has prevailed regarding the identities of many of the antimicrobial tissue and fluid substances reported in the literature. In certain instances we have generalized beyond present-day knowledge concerning the classification of these host factors but this oversimplification is used to facilitate a better general understanding of the subject.

Ideally it would be most desirable to determine exact chemical compositions of the various host factors to arrive at a more accurate classification. Unfortunately, as this is not yet possible, we must rely upon gross observations to define certain of these antibacterial products. Until such time as definitive characterizations become available, the following suggestions may prove fruitful in categorizing antimicrobial agents in the future.

When authors report measurements of heat stability, the experimental procedures should be well described. That is, heat stability can be examined under conditions of varying pH, varying concentrations of the active material, and in the presence of carefully defined media. The degree of purity of the particular factor being tested will also influence the observed heat stability. The determination of antibacterial selectivity is useful in classification; however, it is important to take cognizance of the variation in individual species and strain sensitivities. Such properties as coagulase production among staphylococcal strains (42, 159, 163) and the phase of shigella strains, whether rough or smooth (164), affect individual susceptibilities to the antagonizing agent and make classification more difficult. Measurements of potency, optimal pH of action, effects of inhibitors and ionic influences upon particular antimicrobial factors also may aid in their identification.

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