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Characterization of a Serum Glycoprotein with Antibacterial Activity

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CHARACTERIZATION OF A SERUM GLYCOPROTEIN WITH ANTIBACTERIAL ACTIVITY

By

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DEDICATION

This dissertation is dedicated to the memory of my father, Vincent J. Lentino, who I am sure would have been proud; and to my mother, Vincie J. Lentino, who has affected my life more than she realizes or than I care to admit.

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INTRODUCTION

The primary concern of this study is a closer inspection of a serum glycoprotein with antibacterial activity in an effort to define adequately the characteristics of this agent.

Immunity to staphylococci is based on the interplay of cellular and humoral host defense mechanisms, but the significance and nature of these factors have not been adequately assessed (95). In a recent review, Ekstedt (28) describes studies pertaining to the phagocytosis and humoral bactericidal action of both germ free and conventional mouse blood. He emphatically stated that resistance to staphylococci was due to more than antibody and leukocytes. It is the nature and properties of those other ancillary factors that require elucidation.

The nature of immunity against microbial infections has been the focus of intense controversy for over 80 years. Antimicrobial substances have been sought in normal tissues and fluids in attempts to elucidate mechanisms of natural immunity. Hankin (42), in 1891, first observed that leukocyte extracts exhibited an antibacterial action against the anthrax bacillus. In 1904, Wright and Douglas (110) demonstrated that serum proteins promoted the phagocytosis of bacteria. Since normal adult sera contain opsonins for a wide variety of microorganisms, this broad spectrum of reactivity suggested that many natural opsonins may be nonspecific in their action (85). On the other hand, many serum opsonins are expressions of acquired resistance in the serum of convalescent animals in the form of specific immunoglobulins. Mudd et al. (68) have demonstrated the efficacy of such immune sera in promoting phagocytosis of the microbial parasite.

The presence of serum factors therefore has been shown to increase the efficiency of the phagocytic process and/or to act directly on the microbial cell; in either instance eliciting a deleterious effect upon the parasite. Our primary interest is non-antibody natural serum factors (i.e. non-acquired) and the effectiveness of such factors in maintaining the host-defense system. The non-immunoglobulin serum factors which have demonstrated antibacterial activity are readily classified by any of the following characteristics: 1) dependence on immunoglobulin; 2) origin from either cellular or humoral elements of the blood; 3) mechanism of action.

Because of the diverse nature of these substances, we shall concentrate on those factors which have been shown to effect primarily Gram positive bacteria. In addition we shall confine our discussion to factors which act independently of serum immunoglobulins. However, any discussion of serum bactericidal components should, for the sake of completeness, include the complement system. Complement, first observed to be a heat-labile component of serum by Nuttall in 1888 (75) was discovered to possess bactericidal as well as opsonic and hemolytic activities (12). Ehrlich recognized that complement's activities were dependent on interaction with an antibody serum fraction (24).

More recent reviews by Boyden (11) and Michael (64) confirmed that natural antibodies and complement were effective primarily versus Gram negative organisms. In his review, Michael clearly disputed the delineation of natural antibody on the basis of thermal stability. Instead, he stated that differences in heat lability reflect differences in physiocochemical

properties of antibodies. According to Michael, antibodies were not limited to the γ globulin class. The question still remained however, whether natural antibodies were formed in response to increased exposure of exogenous antigenic material or by the physiological ripening of an innate immune system. Boyden separated the two possible types of antibodies as antigen-induced antibody and spontaneously produced antibody. He was quick to state the possibility of a dual origin of natural antibody. Although the overall picture today remains one of confusion, to say that natural non-specific globulin antibodies are a definite factor in the maintenance of the host-defense system, regardless of their origin, would not be erroneous.

There is a second antimicrobial system which is dependent on complement interaction for its biological activity. Pillemer et al. (82) first isolated the immune protein which they named properdin. Its mechanism of action was similar to antibody, in that both substances react with complement to form immune complexes. Properdin however was much less specific than antibody. Further studies by Wardlaw and Pillemer (103) and Pensky et al. (79) have characterized properdin as a unique entity which was neither immunoglobulin nor any component of complement. Properdin required the interaction of other humoral substances such as complement to exert its bactericidal effect.

Leukocytic extracts have been a source of antibacterial agents for a variety of workers. Among the substances acquired from polymorphonuclear leukocytes have been lysozyme (101), leukin (94) and phagocytin (46). Hirsch (46) originally described phagocytin, following extraction from rabbit PMN leukocytes with an aqueous buffer, as effective solely against Gram negative organisms. In a subsequent report, Hirsch (47) noted that phagocytin extracted

in dilute organic acids was also bactericidal for Gram positive organisms. In his original paper Hirsch stated that active metabolic processes e.g. protein synthesis were most susceptible to phagocytin activity. However, the mechanism of action of this substance is largely unknown.

Serum lysozyme is usually sequestered in PMN leukocyte granules (53) and released spontaneously upon cell lysis into the extracellular milieu. This free lysozyme may be found in saliva and tears in addition to plasma. Since the discovery of lysozyme by Fleming (32) in 1922, a variety of microorganisms have been tested for susceptibility to the bacteriolytic enzyme. The mechanism of action of lysozyme has been elucidated by studies on the structural configuration of the molecule (81). In a series of reports, Glynn and Milne (37, 38) as well as Feingold et al. (30, 31) indicated that lysozyme induced damage to the Gram negative bacterial cell wall by disruption of the peptidoglycan structure. Furthermore, Glynn (39) suggested that lysozyme not only lysed the bacterial cell following complement : antibody fixation but that it augmented the bactericidal effect of such complement : antibody complexes. Few reports however have indicated lysozyme to be active against Gram-positive organisms under normal conditions of growth. Certain strains of Bacillus anthracis have been found to be susceptible to lysozyme under artificially high concentrations of bicarbonate and carbon dioxide (36). B. megaterium was disrupted by lysozyme following formalin exposure but not after heating (107). At high concentrations (3 mg/ml) lysozyme was shown to reduce the density of the peripheral cell wall of S. aureus as determined by electron microscopy (60); earlier reports (36) indicated both S. aureus and Clostridium welchii lost their Gram stain stainability following lysozyme exposure. In a

recent report, Neu et al. (74) indicated that human lysozyme had little effect on the growth of Gram positive organisms at concentrations 10 fold higher than physiological concentration (1).

Although the majority of leukocytic extracts were considered to be specific for either Gram positive or Gram negative bacteria, almost all extracts were subsequently shown to be effective against both types of bacteria. Skarnes and Watson in 1956 (94) described a bactericidal PMN leukocyte extract effective against Gram positive bacteria. More than a decade later, Skarnes (96) characterized leukin as a leukocytic granular extract rather than the nuclear extract he originally proposed. He based this change on the classic work of Hirsch and Cohn (48) who unequivocally demonstrated the degranulation process within the phagocyte as a direct response to bacterial engulfment. Skarnes further demonstrated that leukin, following Sephadex gel fractionation, possessed activity against Gram negative organisms. While there were multiple similarities between leukin and phagocytin, there was no conclusive evidence they were one and the same entity.

While many workers have extracted the PMN leukocyte for bactericidal factors, none have been able to adequately define the mechanism by which these factors exert their bactericidal effect; with the exception of lysozyme the precise mechanism of action of all these bactericidal factors remains unknown. Working with a distilled water extract from horse or rabbit platelets, Amano and his coworkers (2) have described a highly bactericidal substance which they have named plakin. Affecting only Gram positive organisms, plakin demonstrated a disruptive action against Bacillus species and not against S. aureus. Changes in the organism subsequent to plakin exposure included

decreased stainability of cell wall and cell surface lipoids with attendant decreases in the respiration of affected organisms. Electron microscopy revealed cell wall changes not compatible to bacteriolysis but to a phenomenon the authors termed "ghost-cell" formation. Further studies by Amano et al. (3) revealed increased loss of glutamate by washing the bacteria, increased vital stain uptake, and lack of plasmolysis by 25% NaCl solution. These results indicated disruption of the integrity of the semipermeable membrane as the mode of action of plakin.

Amano et al. (4, 5) extended these findings with B. megatherium, investigating the fate of respiratory enzymes. They found plakin completely inactivated cyctochrome C oxidase and depleted all endogenous dehydrogenase activity. This effect was dependent on nicotinamide adenine dinucleotide (NAD) and antagonized by adenosine triphosphate (ATP). Higashi et al. (44, 45) determined plakin to contain phospholipase-A activity responsible for the dissolution of the cytoplasmic membrane with consequent destruction of the oxidative phosphorylation enzyme system - thereby paralysing respiration of the bacteria.

In 1964 Donaldson, et al. (21) reported the separation and purification of a serum bactericidal substance. Because of the similarities between this bactericidal serum substance and other lethal serum substances active against Gram-positive organisms as described by Pettersson (80), the name β-lysin was retained. Beta lysin from normal human serum was heat stable; independent of specific antibody; especially active against Bacillus species; and dependent on blood clotting mechanisms for release. Donaldson et al. (22) reported on the serological relationship of β-lysin to other serum substances specifically

active against Gram positive microorganisms. While they could not demonstrate any cross-reaction between β-lysin and leukin, their work clearly indicated that plakin and β-lysin were very similar in nature. Their conclusions were based on both Ouchterlony gel diffusion studies and cross neutralization of activity by heterologous antisera. Subsequent work by Jensen *et al.* (53) strongly implicated that platelets were the source of β-lysin, this was confirmed subsequently by Tew *et al.* (101). Tew and coworkers observed that β-lysin release *in vivo* was accompanied by a decrease in the number of circulating platelets, a decrease in β-lysin per unit platelet and extensive morphologic damage of platelets. Matheson and Donaldson (63) had reported that β-lysin did not act as a cell wall lytic enzyme and was not a proteolytic activator for latent wall autolytic enzymes but instead irreversibly damaged the cytoplasmic membrane of *Bacillus* protoplasts. There was an apparent morphological difference between protoplasts damaged by β-lysin and protoplasts ruptured by osmotic shock. The authors suggested that β-lysin-induced membrane damage resulted in subsequent release of a wall-lytic enzyme.

Johnson and Donaldson (55) while investigating the effect of coagulase on β-lysin discovered a second β-lysin system active against staphylococci in rabbit serum. Using purified rabbit β-lysin they demonstrated an increase in staphylocidal activity over normal rabbit serum. This increased activity was clearly due to ancillary staphylocidal constituents in the serum other than β-lysin, since β-lysin and normal serum had been shown to affect *B. subtilis* with equivalent results in prior experiments. The authors differentiated the staphylocidal β-lysin from β-lysin active against *B. subtilis* on the following results: 1) neutralization by normal guinea pig serum as well as by specific

guinea pig anti-β-lysin antisera, 2) occurrence of staphylocidal β-lysin was limited to rabbits, as compared to the widespread distribution of β-lysin active against B. subtilis, 3) inactivity of platelet extracts against staphylococci as opposed to B. subtilis susceptibility; 4) inhibition of staphylocidal β-lysin by sodium citrate, sodium oxalate, heparin, ethylene diaminetetraacetate (EDTA) and protein solutions in addition to inactivation by cephalin, lecithin and phosphotidylserine which affected B. subtilis active β-lysin. Although staphylocidal β-lysin was found to be in greater concentrations in serum than in plasma or plasma-serum, the absence of activity in both platelet and PMN leukocyte extracts argued against a cellular origin for the molecule. Attempts to determine the source of staphylocidal β-lysin have met with equivocal results. The authors indicated the mechanism of action of both β-lysin systems may be to damage the cellular membrane.

In contrast to the multitude of bactericidal extracts from both PMN leukocytes and platelets, relatively few substances lethal for bacteria are found to be independent of cellular elements in the blood. One of the most throughly investigated serum bactericidal preparations is the serum bactericidin active against B. subtilis reported by Myrvik and Weiser (69). The authors clearly distinguished between serum bactericidin and complement by the increased heat stability of serum bactericidin. Lysozyme differed from serum bactericidin in that it could be extracted from PMN leukocytes while serum bactericidin could not be extracted. In addition the response of lysozyme to BCG vaccination in rabbits as compared to the total lack of response by serum bactericidin indicated the two substances to be independent

of each other. They characterized the serum bactericidin as a cationic, bentonite-adsorbable protein which was reversibily inactivated by citrate ions. The distribution of serum-bactericidin was widespread, however the highest titers were obtained in rabbit and rat sera as well as in the sera of humans in an acute phase of illness. Because of this last finding, Myrvik and Weiser proposed a site of tissue injury as the source of the molecule. All attempts to extract the serum bactericidin from tissues were unsuccessful. Myrvik (70) later expanded the bactericidin system and included a similar or identical system effective against S. epidermidis but not against S. aureus. Paradoxically the serum bactericidin was not antagonized by coagulase.

Because the acute phase rise of serum bactericidal substances was dependent on the presence of calcium (50), Myrvik et al. (71) investigated the ion requirements of the serum bactericidin system. Their results indicated the presence of both calcium and bicarbonate was necessary for activity in the human system, while rat and rabbit bactericidin required only bicarbonate ions. The authors postulated that the ions were required as co-factors of the bactericidin systems. Naff et al. (73) demonstrated serum bactericidin levels increased in various types of disease states such as myocardial infarction, neoplasia, and pneumonia whereas properdin levels remained stable. Myrvik and Leake (72) were able to separate two non-dialyzable components in the bactericidin system neither of which possessed complement activity. They were able to reconstitute an intact system by mixing rabbit component I with human component II, which suggested a close relationship between the individual systems.

Although Myrvik was unable to demonstrate coagulase reversal of the B. subtilis bacteriocidin system, Ekstedt and Nungester (25) successfully demonstrated the ability of coagulase positive staphylococci to grow in serum while coagulase negative staphylococci were inhibited. Furthermore, by the addition of exogenous coagulase, they were able to confer a protective effect on those organisms which were previously serum susceptible. The authors could detect no beta toxin in the coagulase preparation and inactivated alpha toxin by heating at 65°C for 30 minutes. Ekstedt (26, 27) observed that the serum antibacterial factor altered serum susceptible staphylococci so as to make them incapable of subsequent growth in coagulase-inactivated serum. The serum antibacterial factor was indicated to be non-specifically absorbed by heat-killed bacterial cells; dependent on calcium for activity; digested by pepsin with subsequent loss in activity; and neutralized upon addition of coagulase-contaminated cellular protein but not by staphylococcal polysaccharides. Yotis and Ekstedt (111) discovered that normal serum inhibited the growth and respiration of coagulase negative strains of staphylococci but that coagulase positive strains resisted this antibacterial activity. The authors indicated the bactericidal fraction to be a water soluble globulin which demonstrated activity only in aqueous suspensions or in buffers below 10^{-4} M. Activity was equivalent at pH values of pH 6 to pH 9 and was stable to heating at 70°C for 30 minutes. The serum pseudoglobulin was effective against B. subtilis and M. lysodeikticus as well as the staphylococci. Prior or concomitant treatment with coagulase protected B. subtilis and the staphylococci, but did not afford protection to M. lysodeikticus. Fletcher

(33) confirmed Ekstedt's observation that serum inhibited the growth of coagulase-negative staphylococcal cells. He demonstrated the influence of serum on staphylococci by growing the bacteria in the absence of serum at pH values up to pH 9.0 whereas in the presence of serum the organisms failed to grow at pH 8.3. In addition, Fletcher observed the inhibitory effect to be proportional to the concentration of serum in the growth medium.

Yotis (112) isolated the antibacterial serum factor in the supernatant following 62% ammonium sulfate saturation of the water-soluble globulin fraction. He demonstrated that mice injected intracerebrally with staphylococci exposed previously to the serum antibacterial factor had a 60-90% lower mortality rate than those mice injected with the same concentration of unexposed staphylococci. This protective effect was reversed by preincubation of the serum antibacterial agent with coagulase. Yotis (113) further indicated that the antibacterial agent (ABA) was specifically absorbed to those organisms which were affected by its antirespiratory and bactericidal properties. Absorption of ABA was markedly influenced by salt concentration and temperature which exert their action by altering the physical characteristics of the ABA molecule. Coagulase on the other hand inhibited absorption most probably by blocking the receptor site of the bacteria for ABA. The observation that phenol partially inhibited ABA activity indicated that the lipid containing cytoplasmic membrane of the cell may be the site of ABA action. Yotis and Ortiz (115) found the ABA from rat serum to be insensitive to calcium ions unlike the human ABA system. The authors indicated that rat ABA was inhibited by ferric ions at concentrations (1500 μg) 10 times greater than the physiological concentration, (120 μg) while the antibacterial factor

for B. subtilis was inhibited by 39 μg of ferric ions (9).

Cybulska and Jeljaszewicz (15) were unable to demonstrate the antibacterial effect of serum on staphylococci by intravenous infection of rabbits, nor were they able to demonstrate coagulase reversal of antistaphylococcal activity in whole human or rabbit serum. They concluded that the role of antistaphylococcal activity in the host defense mechanism is open to investigation.

Ehrenkranz et al. (23) described a serum bacteriostatic system, antagonized by coagulase which they found to be related to the immunoglobulin fractions of serum. Staphylococcal agglutinins, lysozyme, β-lysin, C-reactive protein and transferrin were not responsible for the S. aureus serum bacteriostatis.

The primary concern of this study is a closer inspection of a serum glycoprotein with antibacterial activity in an effort to define adequately the characteristics of this agent.

We can readily conclude from the substantial body of information available to us that the concept of a host defense mechanism mediated by innate microbicidal systems will be the subject of intense controversy in biology and medicine for years to come.

MATERIALS AND METHODS

Bacterial Cultures. S. aureus serotypes IX - XIII (ATCC 12606-12610) were obtained from American Type Culture Collection. S. aureus E33, a coagulase producing organism and S. aureus E33V, a noncoagulase producing mutant were obtained from Dr. F. Kelly of the University of Oklahoma, Oklahoma City, Oklahoma; Dr. V. T. Schuhardt of the University of Texas, Austin, Texas kindly provided the lysostaphin sensitive strains S. aureus FDA 209P and PS54; S. aureus H and S.aureus H52A5, a ribitol teichoic acid-free mutant, were the kind gift of Dr. James Park of Tufts University, Boston, Massachusetts. S. epidermidis kulba strain was generously provided by Dr. Jeljasewicz, State Institute of Hygiene, Warsaw, Poland. Upon receipt, all strains were identified as staphylococci by the gram stain, catalase test and glucose fermentation; species identification was by other appropriate biochemical tests (7). These strains were maintained at 4°C on Trypticase Soy Agar (TSA, Baltimore Biological Laboratories) slants in screwcapped tubes and transferred every 4 weeks. At each transfer the cultures were checked by coagulase titration, and anaerobic glucose and mannitol utilization by the method of Baird-Parker (7).

Bacterial Growth. All cultures were grown to mid-logarithmic or late logarithmic phase as specified in either Tryptic Soy Broth (TSB, Difco) or Brain Heart Infusion (BHI, Difco) in 50 ml or 100 ml on a rotary shaker at 37°C. After harvesting by centrifugation at 10,000 rpm at 4°C for 10 min,

the cells were washed twice with sterile 0.85% saline and suspended in either the appropriate sterile buffer or sterile deionized, distilled water (pH 6.8). A homogeneously smooth suspension was prepared by mixing the cells on a Vortex Junior Mixer for 2 to 4 min. The cells were adjusted to the specified cellular density in the appropriate medium with the aid of a Klett Summerson photoelectric colorimeter equipped with a blue (No. 42) filter for most experiments and a green (No. 54) filter in experiments with S. aureus FDA209P and S. aureus PS54. In all manometric experiments 1 ml of a 500 Klett unit (K.U.) suspension of cells was used, corresponding to 1.43 mg dry wt/ml. Most other experiments used either 1.0 ml of a 100 K.U. suspension or 0.5 ml of a 200 K.U. bacterial suspension.

Reagents. Rabbit β lysin and guinea pig anti-β lysin antisera were kindly provided by Dr. D. Donaldson of Brigham Young University, Provo, Utah. Cohn's serum fractions, IgA, IgG, IgM, and their respective antisera were purchased from Hyland Laboratories, Los Angeles, California. Human β lipoprotein, haptoglobin, thyroglobulin, C-reactive protein, ceruloplasmin and fibrinogen were purchased from Schwarz/Mann, Orangeburg, New York. Fetuin was obtained from Grand Island Biologicals, Grand Island, New York. Rabbit anti sera to human α_1-glycoprotein, human α_1-T glycoprotein, human α_1-B glycoprotein, human α_1-antichymotrypsin, human α_1-antitrypsin, human inter α-trypsin inhibitor, human α_2 HS-glycoprotein, human Zn α_2-glycoprotein and human GC-globulin were obtained from Behring Diagnostics, Inc., Woodbury, New York, as were horse anti human whole serum antisera, human α_1-antitrypsin and neuraminadase (Vibrio cholera).

General chemicals for the analysis of the composition of the antibacterial agent (ABA) were obtained from J.T. Baker Chemical Co., Phillips-

burg, New Jersey; Merck and Co., Rahway, New Jersey; and E. H. Sargent and Co., Chicago, Illinois. All chemicals were of reagent grade quality. Resorcinol and 2-thiobarituric acid for the sialic acid assays were purchased from Eastman Organic Chemicals, Rochester, New York. Prior to use, resorcinol was recrystallized from acetone. Sodium meta periodate was obtained from G. Frederick Smith Chemical Co., Columbus, Ohio. n-Butyl acetate, purchased from Matheson Coleman and Bell, Norwood, Ohio was redistilled and only the fraction collected at boiling point 124°-125°C was retained for use in the sialic acid assay. Crystalline anthrone was acquired from Sigma Chemical Co., St. Louis, Missouri; p-dimethylaminobenzaldehyde for use in the hexosamine assays was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin.

Standard sugar preparations used in both the qualitative and quantitative assays were obtained from the following sources: D-glucosamine hydrochloride and fucose, Pfanstiehl Chemical Co., Waukegan, Illinois; mannose, General Biochemicals, Chagrin Falls, Ohio; galactose, Matheson Coleman and Bell, Norwood, Ohio; N-acetyl neuraminic acid, Pierce Chemical Co., Rockford, Illinois; glucose, J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Uniformly labeled ^{14}C D-glucose was secured from Tracer-lab, Waltham, Massachusetts. The chemicals for the scintillation fluor: anisole, p-dioxane, and 1,2-dimethoxyethane were obtained from Eastman Organic Chemicals, Rochester, New York. Crystalline 2,5-diphenyl oxazole (PPO) and 1,4-bis 2-(5-phenyl oxazolyl)-benzene (POPOP) were obtained from Packard Instrument Company, Downers Grove, Illinois.

Reagents for acrylamide gel electrophoresis purchased from Canalco, Rockville, Maryland included: acrylamide, N, N´-methylenebis acrylamide

(Bis), N, N, N′, N′ tetramethylethylene diamine (TEMED) and riboflavin. Ammonium persulfate was obtained from Baker Chemical Co.; potassium ferricyanide was purchased from Merck & Co. Ampholytes for isoelectric focusing were acquired from LKB Produkter AB, Bromma, Sweden. Aminex resins used for amino acid analyses were a product of Bio-Rad, Richmond, California. Molecular weight studies were performed with Sephadex gel from Pharmacia Fine Chemicals, Piscataway, New Jersey. Amberlite MB-3 ion exchange resin was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. Lysostaphin (Schwarz/Mann) was used in the preparation of spheroplasts. Adenosine 5' triphosphate (Pabst Laboratories, Milwaukee, Wisconsin) was added to glucose in all manometric experiments. Ribonuclease, deoxyribonuclease and trypsin (Sigma) as well as pepsin (Cal Biochem, Los Angeles, California) were used in the preparation of whole cell walls.

Sera. Human blood and platelet rich plasma were obtained from Chicago Blood Donors, Inc., Chicago, Illinois. Freshly drawn blood was collected in glass bottles either with or without anticoagulants. Occasionally outdated blood was provided by the hospital blood bank. Animal sera were collected from rats, rabbits, and guinea pigs by intracardial puncture. Blood drawn in this manner was allowed to clot at 4°C overnight before serum was separated.

Serum Fractionation. Serum obtained from sources described above was cooled to 2°C and diluted with an equal volume of chilled distilled, deionized water (pH 6.8). Cold ammonium sulfate, finely ground, was added with constant stirring by a magnetic stirrer over a period of 15 to 20 min to raise the concentration of ammonium sulfate to 25%. The amount of ammonium sulfate added was calculated with the assumption that, at 0°C, 70g per 100 ml

represented 100% saturation. The precipitate was allowed to form for 2 to 4 hr at 0°C (pH 7.2 ± 0.2), separated by centrifugation at 5000 rpm at 0°C for 30 min (as with all fractions) and dissolved in 50 to 75 ml of ice cold distilled water and dialysed against cold running tap water (pH 7.6) at 6-12°C for 15-20 hours. Following dialysis the 25% ammonium sulfate fraction separated into water-soluble and water-insoluble portions. The water-insoluble portion was removed by centrifugation and discarded. The water-soluble supernatant (pH 6.8 ± 0.2) was fractionated further by the addition of ammonium sulfate to 33% saturation; 4.62 g of ammonium sulfate per 20 ml were added over a period of 10-15 min at 0° with constant stirring. The bulky white precipitate (pH 6.5 ± 0.2) formed after 2 hr was removed by centrifugation. To the supernatant of the 33% fraction, 2.68 g of ammonium sulfate per 20 ml was slowly added with stirring to raise the concentration to 50% (pH 6.3 ± 0.2). A light precipitate formed after 2 hr and was separated by centrifugation. The addition of 1.68 g of ammonium sulfate per 20 ml of the supernatant raised the concentration to 62%. After standing for 90 min the yellow-pink precipitate was removed and the supernatant dialysed against cold running tap water (pH 7.6) overnight and lyophilized. This fraction originally designated as R62 by Yotis (112) was the fraction known as ABA. Average yield per 100 ml of serum was 50-60 mg of dry protein.

Because of the extensive dialysis requiring cold running tap water the preparation of ABA was limited to the winter months. To overcome this drawback an alternative method was designed to yield a product similar in biological activity and physical properties to ABA. The ABA was isolated by ethanol fractionation of Cohn's serum fraction F-IV-1 (14) prepared as

described below: to 1 liter of whole serum 77 ml of 53.3% ethanol(-5°C)and 1 ml of 0.8 M sodium acetate buffer was added adjusting to pH 7.2. The ethanol-serum mixture was centrifuged at 10,000 rev/min for 10 min at 0°C, and the resultant precipitate discarded. To the supernatant an additional 601 ml of 53.3% ethanol/1, plus 0.88 ml of 10 M acetic acid, 0.44 ml of 4 M sodium acetate/1 and 2.30 ml of 95% ethanol/1 were mixed in slowly. The resultant precipitate corresponded to Cohn's fraction F-II, III and was dialysed and lyophilized. F-II, III supernatant was diluted with 311 ml of distilled water (0°C) and 78 ml/1 of 0.8 M sodium acetate buffer (0°C) and adjusted to pH 5.2. This mixture was stirred continuously with a magnetic stirrer for 1 hr at -5°C and allowed to settle for 6 to 8 hr at -5°C. The resultant precipitate harvested by centrifugation was the lipid rich alpha globulin fraction F-IV-1. Resuspended in distilled water and lyophilized, fraction F-IV-1 was the initial preparation used in the ethanol precipitation of ABA. One gram of lyophilized F-IV-1 was dissolved at 4°C into 400 ml of 0.0025 M sodium acetate buffer (pH 4.65) and stirred for 15 min. While this solution was stirred 200 ml of absolute ethanol (-10°C) was added quickly and the resultant precipitate permitted to settle for 30 min at -20°C. After centrifugation at 10,000 rpm for 30 min at -5°C the precipitate was discarded and the supernatant was dialysed against running tap water at 12-17°C (pH 7.35) or against chilled distilled water at 4°C (pH 6.8). Average yield was 75 mg per 100 ml of serum.

Unit of Activity. In a biological system, it is necessary to define a working unit of activity. We arbitarily have defined one unit of activity as the amount of protein (dry weight) necessary to cause a 5 µl reduction in the oxygen consumption of exposed staphylococci as compared to unexposed

staphylococci at a constant concentration of 500 K.U. of S. aureus XII (1.43 mg dry wt./ml) in an hour. Our units of activity were expressed as mg (dry wt.) ABA/mg (dry wt.) bacteria/5 µl-hr. For example a 2 mg sample of ABA causing an absolute decrease of 50 µl O_2 consumed/hr between treated and unexposed cells was considered to contain 10 units of activity.

Calibration of Respirometers. Warburg flask constants were calculated by the formula

$$K = \frac{Vg \frac{273}{T} + V_f \alpha}{Po}$$

where Vg was the volume of the gas in a closed system and equal to the sum of the flask and manometer volumes, T was the temperature in°K (37°C = 310°K); V_f was the volume of the fluid phase (2.2 ml) and α is the solubility of oxygen at a given temperature (0.027 at 37°C). Po is a constant value (10,000) reflecting the density of Brodie's manometer fluid at one atmosphere pressure as compared to mercury.

For calibration purposes, Vg was determined by weighing a volume of mercury equal to that of the gaseous phase in the manometer and flask and dividing by the density of the mercury at that temperature.

Manometric Assay. Bacteria were grown to mid-logarithmic phase at 37°C and harvested by centrifugation, washed twice with sterile saline and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0). 500 K.U. of bacteria were exposed to ABA (2 mg/ml) for 1 hr at 4°C and shaken manually every 12 min. We have found the ABA concentration of 2 mg/ml to approximate 10 units of activity. Following exposure to ABA for 1 hr, 1 ml of the cell suspension

was pipetted into the main vessel of a Warburg flask along with 1 ml of 0.055 M glucose + 3 μ M ATP in 10^{-4} M sodium phosphate buffer (pH 7.0). In the center well a folded strip of filter paper was saturated with 0.2 ml of 40% potassium hydroxide to absorb CO_2 produced. The manometers were equilibrated at 37°C for 20 min before the initial readings were made. Readings were taken every 30 min for 2 to 3 hr and the average μl of oxygen consumed per hour was calculated.

Preparation of Coagulase. Coagulase was prepared by the method of Tager (100). Cells were separated from the crude coagulase supernatant by centrifugation and discarded. The supernatant was acidified to pH 4.0 with 4 N HCl and the 24 hr precipitate was recovered and suspended in M/15 phosphate buffer (pH 8.2). The supernatant was adjusted to pH 8.5 - 9.0 and consecutively, precipitated with 95% ethanol and 8-12% ammonium sulfate. The final product was dialyzed extensively, lyophilized and stored at -20°C. Coagulase activity was demonstable at a titer of 1:500,000.

Preparation of Bacterial Cell Wall. Two separate methods were used to prepare staphylococcal cell walls. The Morse (66) method was employed for cell walls used in absorption studies, while a modification of the Salton (86) method yielded cell walls for peptidoglycan isolation. In the Morse method cells grown overnight at 37°C on tryptic soy agar were washed into a 10% phenol solution and harvested by centrifugation at 5000 rpm for 10 min (4°C). Phenol-killed cells were washed twice with saline and then once more with acetone. Suspended in a minimal volume of acetone cells were dried in a dessicator in vacuo for 2 days, finely crushed and stored at -20°C. To insure

disintegration of cells, 200 mg (dry wt.) of cells, 4 ml of No. 12 Ballotini glass beads and 5 ml of sterile distilled water were agitated for 3 hr with a Mickle tissue disintegrator (H. Mickle Millworks, Surrey, England). The glass beads were separated with a fine sintered glass filter and the filtrate centrifuged at 12,800 g (10,000 rev/min) for 10 min at 4°C. Following two washings with 0.1 M sodium phosphate buffer (pH 7.8), the cell wall pellet was suspended in 2.5 ml of a toluene solution containing 100 μg of ribonuclease/ml and 200 μg of trypsin/ml for 24 hr at 37°C. The cell wall preparation was then washed 3 X with distilled water and resuspended in 2.5 ml of 0.01 N HCl containing 100 μg of pepsin/ml for 3 hr at 37°C. After pepsin digestion, the cell wall fraction was washed 4 X with distilled water and lyophilized.

For the Salton method a 10 ml aliquot of a fresh cell suspension adjusted to 750 K.U. was disrupted by 10 g of No. 12 Ballotini beads by Mickle disintegration for 3 hr. Cellular homogenates were sintered glass filtered and centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was retained and spun at 10,000 rpm for 10 min (4°C) which precipitated the cell wall fraction. After repeated washings (5-6X) with distilled water the cell wall preparation was lyophilized. In both methods cells were judged to be completely disrupted by both the gram stain reaction and loss of refractility under the phase contrast microscope. The average yield of cell wall was 35 to 45 mg dry wt., i.e., 10% of the dry weight of the bacteria.

Peptidoglycan was extracted by the method of Park and Hancock (77) with hot TCA (10%). In 10 ml of 10% TCA, a 50 mg sample of cell wall was heated

to 95°C for 15 min to remove all ribitol teichoic acid which left only muramyl and glutamyl residues in the peptidoglycan moiety. The residue was washed 3 X with distilled water and dialysed extensively to remove the TCA; 45% of the starting material was recovered as peptidoglycan.

Paper Chromatography of S. aureus H and S. aureus H52A5 Cell Wall.

Cell walls were prepared as described above by the Salton method to prevent excessive loss of ribitol teichoic acid. These cell wall preparations (5 mg/ml) were hydrolysed for 3 hr and 18 hr at 100°C in vacuo in 4N H_2SO_4. The hydrolysates were neutralized with barium hydroxide; the resulting barium sulfate was precipitated by centrifugation and the supernatant was taken to dryness over sodium hydroxide pellets in a dessicator in vacuo. The dried hydrolysates were taken up in 0.1 ml of distilled water and spotted for paper chromatography on Whatman No. 1 chromatography paper. Descending flow chromatography was performed using the solvent of Bourrillon and Michon (10) which contains n-Butanol: pyridene 0.1 N HCl (5:3:2). The chromatogram were run for 20 hr and 40 hr to insure adequate separation of all sugar spots. Neutral and amino sugars with reducing properties were detected by sequential spraying with a 2% silver nitrate solution in methanol containing 1% NH_4OH followed by sodium hydroxide (2%) dissolved in methanol as well as by aniline phthalate reagent in water saturated n-Butanol. Phosphoric esters were determined by spraying with the following solution: 5 ml 60% perchloric acid, 10 ml 1 N hydrochloric acid, 25 ml of 4% ammonium molybdate, water to 100 ml. The paper was heated for 7 min at 85°C and phosphate esters appear as yellow-blue spots against a light blue background (41a). Sugar alcohols and other non reducing sugars were detected by the method of Buchanan et al. (11b). A solution of 2% aqueous sodium metaperiodate was sprayed over the paper chromatogram and oxidation was allowed

to proceed for 7 min at 60°C in an atmosphere of air. The chromatogram was placed in a large (4 liter) beaker and a stream of sulfur dioxide (SO_2) gas was passed into the beaker to neutralize the periodate until most of the liberated iodine was consumed. The chromatogram was then sprayed with Schiff's reagent which was prepared by dissolving 250 mg p-rosaniline (Hartman-Leddon Co., Philadelphia, Pennsylvania) in 50 ml of distilled water, decolorizing with DARCO activated carbon (Atlas Chemical Industries, Inc., Wilmington, Delaware) and diluting to a final volume of 250 ml. The necessary sulfur dioxide (SO_2) gas was generated by the chemical reation elicited by mixing concentrated HCl with sodium bisulfite as follows:

$$Na\ HSO_3 + HCl \longrightarrow NaCl + H_2SO_3$$

$$H2\ SO_3 \longrightarrow H_2O + SO_2\uparrow \qquad (59a).$$

Ninhydrin spray reagent (Sigma) was used to detect amino acids and amino sugars.

Fractionation Studies. Bacteria grown to mid-logarithmic phase in 100 ml of TSB on a rotary shaker at 37°C were centrifuged, washed and suspended in sterile distilled water as described above. One ml of a cell suspension (600 K U to 800 K U dependent on the strain used) designed to yield 100 K U on dilution to 50 ml was incubated with ABA at a concentration of 5 mg/ml for 1 hr at 4°C. This suspension was diluted to 50 ml with sterile 0.1 M sodium phosphate buffer (pH 7.0) in a nephrolometric flask; 0.5 ml of ^{14}C labelled glucose 2 X 10^6 cpm/ml was added and flasks were placed on a rotary shaker for 30-60 min at 37°C. After incorporation of the radioactive substance the cells from a 15 ml volume were harvested by centrifugation and washed 2 X with ice-cold saline. The cellular pellet was then fractionated by the method of Park and

Hancock, dissolved in 2.5 ml of 5% TCA (4°C) for 10 min, the cells were centrifuged into a pellet and the supernatant was retained. The cellular pellet was treated with 2.5 ml of 75% ethanol (pH 3.0) at 45°C for 10 min and harvested by centrifugation. Following ethanol extraction 2.5 ml of 10% TCA at 90°C for 10 min was used to remove cellular teichoic acid and nucleic acid in the fractionation procedure. The cellular pellet remaining was digested with 2.5 ml of trypsin (1 mg/ml) in a solution containing 0.5 M NH_4HCO_3, 0.005 N NH_4OH, (pH 8.2) for 2 hr at 37°C. The resultant residue was suspended in 1.0 ml of distilled water. Aliquots of 0.1 ml of each extraction supernatant and the residue suspension were placed in a scintillation vial to which 10 ml of scintillation fluor was added. The scintillation cocktail included p-dioxane: anisole: 1,2-dimethoxyethane (900:150:150) in which 18 g of PPO and 60 mg of POPOP were dissolved. Vials were dark adapted and cooled for 12 hr prior to determination of ^{14}C-activity in a Packard Tri Carb liquid scintillation spectrophotometer model 3320. In this system, ^{14}C activity was counted with an 85% efficiency.

Radioisotopic Assay for Antibacterial Activity. To follow the leakage of radioactive compounds from cell suspensions, staphylococci were grown aerobically in TSB for 18 hr at 37°C and the cell density adjusted to 200 Klett units with a blue (No. 42) filter of a Klett-Summerson photoelectric colorimeter. One ml of uniformly labelled ^{14}C-glucose (1×10^7 counts/min/ml) was added to each 100 ml of cell suspension, the cells were incubated for 1-3 hr at 37°C on a rotary shaker and harvested by centrifugation, washed twice with chilled (4°C) saline and resuspended in an equal volume of chilled distilled water. After adding ABA at predetermined concentrations (heat inactivated ABA

was used as control) the cell suspensions were incubated anaerobically under CO_2, 1.5 - 2.0 lb/in^2, at 37°C for 0.5-18 hr. The cells were then transferred to conical centrifuge tubes and the release of radioactive ^{14}C cytoplasmic substance in the supernatant liquid was determined as described above.

Viability Counts. Bacteria were treated as in the radioisotopic assay procedure without the addition of radioactive glucose. ABA was sterilized by passage through Millipore filters of 0.22 μ pore diameter (Millipore Corporation, Bedford, Massachusetts). Following exposure to ABA 0.5 ml of cells were pipetted into 4.5 ml of sterile saline and a 10 fold serial dilution was prepared. Pour plates of Tryptic Soy Agar (TSA, Difco) were made in quadruplicate with 0.1 ml of each dilution per plate. Incubated at 37°C overnight, plates were scanned for colony formation on an American Optical Spencer illuminator and counted with the aid of an electric colony counter (American Optical, Buffalo, New York).

Coagulase Reversal of ABA. Late logarithmic phase cells were harvested, washed and suspended as described for manometric assay. Prior to ABA exposure cells were exposed to exogenous coagulase in appropriate concentrations for 30 min at 25°C. It had been previously shown that the addition of coagulase together with, or after ABA exposure was less effective in reversing the ABA activity. Following coagulase exposure, cells were mixed with ABA to a final concentration of 2 mg/ml and prepared for manometric assay.

In addition to the action of exogenous coagulase, the relationship of coagulase production on the reversal of ABA was demonstrated using S. aureus strains E33, E33V, and XII by assaying for leakage of radioactive cytoplasmic substance as described above under radioisotopic assay procedures.

Preparation of Osmotically Fragile Staphylococci. S. aureus FDA 209P and S. aureus PS54 were grown in 100 ml BHI at 37°C for 14 hr, harvested by centrifugation and washed 2 X with 10^{-4} M sodium phosphate buffer (pH 7.0) in 9% sodium chloride solution. Cellular density was adjusted to 500 Klett units in 9% saline-buffer solution with a Klett Summerson photoelectric colorimeter with a green (No. 54) filter. Each ml of cells was exposed to 10 units of lysostaphin for 3 hr at 37°C after which osmotic fragility was determined by dilution of a sample of lysostaphin-exposed cells with distilled water. Both fragile and control cells were exposed to ABA (heat-inactivated ABA served as control) in the presence of 9% saline buffer solution. Glucose solution was made up in 9% saline-buffer solution to prevent any dilution which would cause osmotic rupture of the fragile cells. All cells were prepared for manometric assay as described.

Preparation of Osmotically Fragile L-forms of Staphylococci. S. aureus Smith L-form (ATCC 19640) was grown on Heart Infusion Agar (0.75%) containing 3.5% sodium chloride, 10% heat-inactivated horse serum, and 1000 units of Benzyl penicillin/ml for 48 hr at 37°C in a candle jar. The L-forms were washed from the agar surface with 10^{-4}M sodium phosphate buffer (pH 7.0) containing 9% sodium chloride and 10 mM magnesium chloride, filtered through cheese cloth and harvested at 10,000 xg for 20 min at 4°C. The L-forms were resuspended in saline-buffer and adjusted to between 100-200 Klett units. After washing, the L-forms were prepared for manometric assay as described.

Qualitative Carbohydrate Analyses of Antibacterial Agent. All samples of ABA (30 mg/mg) used for paper chromatography were hydrolyzed for 4-6 hr in 4 N H_2SO_4 *in vacuo* at 110°C. The hydrolysates were neutralized and passed

over a column of Amberlite MB-3 ion exchange resin (Mallinckrodt Chemical Works, St. Louis, Missouri) 50 cm by 1.0 cm diameter (inner) in 200 ml of distilled H_2O. The column was washed with 100 ml N HCl to insure all neutral sugars were eluted off the resin. The washings were pooled and flash evaporated at 40°C to a thick syrup. This was taken up in 0.1 ml of H_2O and spotted on the chromatograph. Descending flow paper chromatography was performed in two solvent systems: n-Butanol : acetic acid : water (52:32:16) according to the method of Putnam (83) and n-Butanol:pyridine: 0.1 N HCl (5:3:2) according to the method of Bourrillon and Michon (10) on Whatman No. 1 chromatography paper, W. and R. Balston Ltd., England. Aniline hydrogen phthalate was used to detect neutral reducing sugars as well as reducing amino sugars. Aniline (Mallinckrodt Chemical Works, St. Louis, Missouri) was distilled until clear. A sample of 0.91 ml of aniline was added to 1.66 g of phthalic acid dissolved in 100 ml of water-saturated n-butanol. Amino sugars were better detected however by the Elson-Morgan reaction (97). After allowing the chromatography solvent to evaporate, the chromatographs were sprayed with the respective reagents and either heated at 105°C for 5 min. when using aniline phthlate or heated to 80°C in the first step of the Elson-Morgan procedure (5% potassium hydroxide in 80% ethanol : 1% acetylacetate in ethanol (1:10). After spraying with 10% p-dimethylaminobenzaldehyde in concentrated HCl mixed with ethanol (1:9) the chromatographs were dried with a cool-air hair dryer to complete the Elson-Morgan test for amino sugars.

Quantitative Analysis of Carbohydrate Composition of Antibacterial Agent. The results of paper chromatography indicated a variety of sugars to be present in the unknown serum fraction. Protein-bound hexoses were determined by both

the anthrone method of Shetlar (92) and the tryptophan method of Shetlar, et al. (93). Hexosamines were determined by the Rimington (84) modification of the Elson-Morgan reaction (29) as well as by comparison with recoveries from the analyses of amino acid content. Sialic acid was assayed by the thiobarbituric acid - periodate method of Warren (104) and by the resorcinol method of Svennerholm (99). Fucose was determined by the cysteine method of Dische and Shettles (17) using a 10 min boiling time instead of the originally reported 3 min period.

Protein Determination of Antibacterial Agent. The protein content of ABA was determined by a variety of established methods: the method of Lowry et al. (62); the biuret method as modified by Weichselbaum (106) and Dittlebrandt (19); and the micro-Kjeldahl total nitrogen assay (Kabat and Mayer, 58) were all utilized to determine the protein content of ABA. Crystalized bovine plasma albumin (Armour Pharmaceutical Co., Kankakee, Illinois) was the standard protein used in all three assay methods.

Antisera Preparation. To induce the production of antibody to ABA, pooled human ABA was used. Four New Zealand white male rabbits were injected intramuscularly on the right hind leg with 1 ml of antigen solution containing 7.5 mg of ABA in 1 ml of sterile saline and emulsified with an equal volume of Freund's complete adjuvant. Injections were made on the 1st and 10th days. Immune sera was collected from the marginal ear vein by vacuum with the aid of a rabbit ear bleeder (Bellco, Vineland, New Jersey) on the 14th, 28th and 42nd day after primary challenge. Pre-immune rabbit serum served as a control. Serum was separated from the whole blood collected after clotting for 18 hr at 4°C by centrifugation.

Immunoelectrophoresis. Immunoelectrophoresis was by the method of Scheidegger (89) in a 1.5% Noble agar gel (Difco) with an LKB 6800A Immunophor apparatus for 60 min at 250 v in 0.1 M barbital buffer (pH 8.6). Antigen concentrations ranged 0.5 - 24.0 mg/ml. Antisera troughs were filled with either rabbit antihuman ABA antisera or commercial antisera prepared against whole serum or various serum protein fractions. The slides were incubated in moist chambers for 48 hr to allow adequate time for diffusion of the reagents. Two saline (1%) washes were used to halt the reaction, followed by extensive rinsing with distilled water and overnight drying with lintless filter paper strips at room temperature. The slides were then stained with 0.5% solution of Aniline Blue (Allied Chemical and Dye Corporation, New York, New York) in a (45:45:10) methanol:water:glacial acetic acid mixture. The same solution without the dye was used to destain the gels.

Immunodiffusion. Ouchterlony immunodiffusion (76a) was performed to determine the degree of cross reactivity between ABA and other serum proteins. In order to elicit the presence of any contaminating substances in the ABA preparation, serial dilutions of both the suspected antigen and ABA were tested against antisera to the ABA, and against antisera to the antigen. Antiwhole human serum was used as a control in all Ouchterlony technique experiments. The eccentric immunodiffusion method of Feinberg (29b) was also used.

Disc Electrophoresis. Polyacrylamide gel electrophoresis was performed following the method of Davis (16) using a Canalco Model 1200D electrophoresis apparatus. Gels were formed in open-end glass tubes in three consecutive phases. The following stock solutions were used in the preparation of the various gel phases:

a) 48 ml of 1N hydrochloric acid, 36.3 g of tris buffer, 0.46 ml of TEMED and water to 100 ml pH 8.9.

b) 48 ml of 1 N hydrochloric acid, 5.98 g of tris buffer, 0.46 ml of TEMED and water to 100 ml pH 6.7.

c) 30.0 g of acrylamide, 0.8 g of methylene-bisacrylamide, 0.015 g of potassium ferricyanide and water to 100 ml.

d) 10.0 g of acrylamide, 2.5 g of methylene-bis-acrylamide and water to 100 ml.

3) 4 mg of riboflavine in 100 ml of water.

These solutions are stable in the dark at 4°C for at least a month. The separating (lower) gel solution contained 1 part a: 2 parts c: 1 part water plus an equal volume of 0.14% ammonium persulphate solution. Both the stacking gel and sample (upper) gel solutions contained 1 part b: 2 parts d: 1 part c: 4 parts water. As the gels formed, they were loaded with 200-300 µg/ml of protein in the sample gel solution. The anionic buffer solution used in both upper and lower compartments was tris-glycine buffer (pH 8.5) which contained 6.0 g of tris buffer and 28.8 g of glycine and distilled water to 1000 ml.

Disc electrophoresis was also performed in a cationic buffer system according to the method of Reisfeld et al. (83a). The following stock solutions were used in the preparation of the various gel phases

a) 48 ml of 1N potassium hydroxide, 17.2 ml of glacial acetic acid, 4.0 ml of TEMED and water to make 100 ml pH 4.3.

b) 48 ml of 1N potassium hydroxide, 2.87 ml of glacial acetic acid, 0.46 ml of TEMED and water to 100 ml pH 4.8.

c) 30.0 g of acrylamide, 0.8 g of methylene-bisacrylamide and water to 100 ml.

d) 10.0 g of acrylamide, 2.5 g methylene-bis-acrylamide and water to 100 ml.

e) 4 mg of riboflavin to 100 ml of water.

These solutions are as stable as the solutions used in the anionic gel system. The separating gel solution is prepared in the same proportions as the anionic gel - 1 part a: 2 parts c: 1 part water plus an equal volume of 0.28% ammonium persulfate solution. The cationic stacking and sample gels are prepared identically as their anionic counterparts were. The cationic buffer (pH 4.5) contained: 31.2 g β-alanine and 8.0 ml of glacial acetic acid and distilled water to 1000 ml. Gels were run electrophoretically towards the cathode in the bottom buffer tray as opposed to the anodic migration toward the bottom in the anionic system. In addition various concentration of acrylamide other than 7.5% were used for the preparation of the separating gels.

Electrophoresis was carried out with the glass tubes held vertically at a constant current of 5 m amp per gel at 250 volts for 1 hr or until the indicator dye (bromphenol blue) moved to within 0.5 cm of the end of the gel. Separating gels were removed by reaming tubes with a syringe in ice cold water. The gels were fixed in 7.5% acetic acid and stained in 0.25% Aniline Blue Black (Canalco, Rockville, Md.). Destaining of gels is performed in a Canalco Quick Gel Destainer with 7.5% acetic acid as the solvent at 1 volt and 0.5 m amp. for 20 min.

Isoelectric Focusing. Determination of the isoelectric point of ABA was performed in an LKB Model 8101 isoelectric focusing column using the technique described by Svensson(99a) and Vesterberg and Svensson(102a). A sucrose density-pH gradient was established using LKB ampholine carrier ampholytes in ranges pH 3-10, pH 3-6, and pH 4-6. For each column 2.5 ml of a 40% solution

of ampholytes was diluted to 10 ml and distributed 3:1 between dense and light solution. The dense solution contained 28 g of crystalline sucrose dissolved in a volume of 42 ml to which 7.5 ml of ampholyte solution had been diluted. One-fourth of the original ampholyte solution diluted to 60 ml with distilled water composed the light solution. Preparation of the density gradient was manually performed as described in the preliminary instruction sheet and its addendum supplied by LKB instruments.

The anode and cathode solutions were reversible with regards to their position within the density gradient. The dense electrode solution contained 12 g of sucrose dissolved in 14 ml of distilled water plus an appropriate volume of either 85% phosphoric acid (0.1 ml anode at top, 0.2 ml anode at bottom) or 85% ethylene-diamine (0.4 ml cathode at bottom; 0.2 ml cathode at top) while the light electrode was the acid or base solution diluted with 10 ml of water. In most experiments, the anode solution was placed on the bottom of the column and 30 mg of ABA was dissolved in 2 sucrose fractions close to the cathode solution. Whenever the anode solution was placed at the top of the column ABA would be dissolved in middle layers of the gradient. After 48-96 hr at 500 volts, or until the amperage was stable, the column was drained and 2.5 ml fractions collected. Temperature was held constant at 0°C by a circulating refrigerated ethylene glycol bath, Forma Scientific, Inc., Marietta, Ohio. Absorbance at 280 n m was determined for each fraction in a Beckman DB-G spectrophotometer (Fullerton, California) as well as the pH (Leeds and Northrup, North Wales, Pennsylvania). Protein fractions were dialyzed extensively at 4°C in distilled water to remove all traces of ampholytes and sucrose, lyophilized and tested by manometric assay for bactericidal activity.

SDS Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gels were prepared as described by Weber and Osborn (105). The protein samples to be run were dissolved in 1% mercaptoethanol, 6 M urea and 1% SDS at a concentration of 2 mg/ml and incubated at 45°C for 30-60 min prior to the electrophoreses run. Gels are prepared by mixing 15 ml of gel buffer and 13.5 ml of acrylamide solution (10%) with 1.5 ml of fresh ammonium persulfate (15 mg/ml) and 0.045 ml of Temed. The gel buffer contained: 7.8 g monobasic sodium phosphate, 38.6 g of dibasic sodium phosphate, 2 g of sodium dodecyl sulfate per liter of water; while the acrylamide solution was composed of 22.2 g of acrylamide and 0.6 g of bis per 100 ml of water. Gels were run for 6 hr at 5 ma/tube, however only four to six tubes could be run at a time. The gels were then removed from the tubes and stained with Coomassie Brilliant Blue R-125 (1.25 g in 554 ml of 50% methanol and 46 ml of glacial acetic acid) for a minimum of 2 hr and destained electrophoretically in a solution containing 50 ml of methanol, 75 ml of glacial acetic acid and 875 ml of distilled water. Prior to staining, the length of the gels and the distance of indicator dye migration were recorded. Following destaining the length of the gel and the farthest protein migration was recorded. These values were used to calculate the mobility of the various proteins in the gels by the following formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale. The use of SDS-gel electrophoresis to determine molecular weight was originally conceived by Shapiro et al. in 1967. These investigators also utilized 5% acrylamide gels within which they had incor-

porated 50 μg of protein sample to be electrophoresed (91a).

Molecular Weight Determination with Sephadex Gel Chromatography.

Sephadex G-200 superfine gel was soaked in distilled deionized water (pH 6.8) for 96-120 hr at 4°C and washed extensively to remove the fractured gel beads. A column 35 cm X 2.54 cm (inner diameter) was filled with water and the volume carefully measured to determine the total bed volume (V_t equal to $\pi r^2 h$). The column was packed with the gel overnight and equilibrated with distilled water by gravitational flow for an additional 24 hr. Using upward flow adaptors, the direction of flow through the column was reversed and the pressure was maintained at 10 cm of H_2O throughout the entire experimental procedure. The void volume (V_o) of the packed column was determined by multiple passage of Blue Dextran 2000 (1 mg/ml) until two consecutive V_o values were identical. A molecular weight calibration kit (Pharmacia Fine Chemicals) was employed to determine the elution volume (V_e) of proteins of known molecular weight. Included in the kit were aldolase (M.W. 158,000), ovalbumin (M.W. 45,000), chymotrypsinogen (M.W. 25,000) and ribonuclease A (M.W. 13,700). Separation of a mixture of two of these standards during passage through the column into distinct fractions was repeated until the elution volumes were constant. Aldolase : chymotrypsinogen and ovalbumin: ribonuclease were the mixtures employed in concentrations of 5-10 mg per ml. In addition, thyroglobulin (M.W. 800,000), was used to determine the void volume (V_o) of the packed column, while fetuin (M.W. 48,000), ceruloplasmin (M.W. 160,000), and pseudocholinesterase (M.W. 300,000) were used to calibrate the column for standard glycoprotein molecular weight determinations. The use of such glycoproteins more closely approximated the conditions during

Sephadex filtration of the ABA molecule. The molecular weight of the unknown was calculated by plotting K_{av} values against molecular weights on semi-logarithmic paper. K_{av} were determined by the following formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_e = elution volume of the protein

V_o = void volume of Blue Dextran 2000 or thyroglobulin

V_t = total bed volume

ABA was passed through the calibrated column three times per experiment to determine the average molecular weight of the glycoprotein. This procedure is based on the outstanding work of Andrews (5a) who utilized gel filtration to determine the molecular weight of a variety of proteins.

Amino Acid Analyses. Quantitative amino acid analyses were performed by the method of Spackman et al. (98) using a Beckman Model 120 C Amino Acid Analyzer. Samples of ABA 2 mg/ml were hydrolysed in vacuo in 6 N HCl for 18 hr at 100°C. After hydrolysis the samples were desicated over sodium hydroxide pellets. Rehydrated in 1.0 ml of 0.20 N sodium citrate buffer (pH 2.2), the hydrolysate was filtered through a fine sintered-glass filter and equal volume aliquots were applied to both short and long resin columns in sequence. The buffer and resin system used were modified from those recommended by Beckman to allow full recovery of amino sugars and hydroxy amino acids. Basic amino acids were assayed on a column (23.0 cm X 0.9 cm) of aminex A-5 (BioRad, Richmond, California) with 0.35 N sodium citrate buffer (pH 5.18). Natural and acidic amino acids were passed through aminex A-4 (69.0 cm X 0.9 cm) in 0.2 N sodium citrate buffers (pH 3.41 and pH 4.14). All samples were applied

under N_2, 20 lb/in^2, as were the buffer washes following the samples. Standard amino acid preparation were purchased from the Beckman Co. (Palo Alto, California).

Isolation of α_1 Antitrypsin. Antitrypsin was isolated from serum by the method of Bundy and Mehl (12a). Using 400 ml of outdated acid citrated plasma, the α_2 inhibitor of trypsin was precipitated by the addition of an equal volume of saturated ammonium sulfate. After 4 hr. at 5°C the precipitate was removed by centrifugation at 17,000 rpm for 45 min and discarded. The supernatant was raised to 70% ammonium sulfate saturated by the additon of solid ammonium sulfate and allowed to stand overnight at 5°C. The precipitate was separated as above and dissolved in 30 ml of distilled water and dialysed overnight. To the dialyzed solution an equal volume of saturated ammonium sulfate was added and the pH adjusted to 4.6 with N.H_2SO_4. The precipitate was immediately separated at 24,000 r.p.m. for 15 min. and discarded. The clear supernatant was adjusted to pH 7.35 with N NaOH and d alyzed overnight. The dialyzed solution was passed over Dowex 1-X4 and eluted by a stepwise increase in buffer concentrations employing the following sequence 0.02 M, 0.05 M, and 0.1 M Tris buffer pH 7.25. Following overnight dialysis, the residue was lyophilized. This lyophilized protein was the furthest step to which the purification of α_1 antitrypsin was taken. All manometric studies for antirespiratory activity were made with this preparation as were the trypsin inhibitor assays.

Assay for Trypsin Inhibitory Activity. Trypsin activity was determined by the method described in the Worthington Enzyme Manual (109a) in which the rate of hydrolysis of p-toluensulfonyl-L-arginine methyl ester (T.A.M.E.) is measured by the increase in absorbancy at 247 n.m. T.A.M.E. was obtained from Worthington Biochemical Corp., Freehold, New Jersey, and soybean trypsin inhibitor was purchased from Cal. Biochem, Los Angeles, California. When demonstrating the inhibitory activity of α_1 antitrypsin or ABA as measured by the decrease in tryptic activity, an aliquot of either serum protein inhibitor was mixed with trypsin prior to trypsin hydrolysis of T.A.M.E. Soybean trypsin inhibitor was used as a control for antitrypsin activity measurements. T.A.M.E. substrate was used at a concentration of 0.01 M in distilled water, trypsin enzyme was prepared in 0.001 M HCl and the buffer used was 0.05 M Tris pH 8.1 containing 0.01 M $CaCl_2$.

Manometric Studies with α_1 Antitrypsin. The supernatant fraction resulting from acid precipitation of serum was separated into four fractions for studies on the antirespiratory activity of α_1-antitrypsin. Following dialysis the supernatant was separated by centrifugation at 17,000 r.p.m. for 45 min. into supernatant (No. 1) and precipitate (No. 2) fractions. The supernatant was lyophilized to reduce its volume and passed over Dowex 1-X4 in a 20 ml volume of 0.02 M Tris buffer pH 7.25. The eluant from the Dowex column was separated into supernatant (No. 3) and precipitate (No. 4) fractions by centrifugation at 17,000 r.p.m. for 45 min. These four fractions were tested independently by manometric respirometry for anti-staphylococcal activity against S. aureus serotypes XI and XII, the two strains most sensitive to ABA.

Neuraminidase Removal of Sialic Acid from ABA. Sialic acid was removed from ABA through the use of neuraminidase (Vibriocholera). The enzyme preparation had an activity of 500 neuraminidase units per ml and was dissolved in 0.05 M sodium acetate buffer (pH 5.5) with 9 mg of sodium chloride and 1 mg of calcium chloride per ml. Following incubation of 25 mg of ABA with 0.5 ml of neuraminidase at 37°C for 1 hr the solution was dialyzed extensively at 4°C in distilled water to remove the enzymatically degraded sialic acid. Neuraminidase enzyme protein was subsequently separated from ABA by passage of the lyophilized dialysis residue over a Sephadex G-200 gel column (20 cm x 2.54 cm). ABA minus neuraminidase passed through the column fastest and was collected with a fraction collector. This preparation of ABA minus sialic acid was tested in preliminary studies for antirespiratory activity by manometric technique. In addition a comparison of the electrophoretic mobility of ABA both with and without sialic acid was performed by SDS gel electrophoresis.

Controls. Autoclaved heat-inactivated ABA was shown to possess no activity against the respiration or viability of staphylococci, in concentrations as high as 10 mg/ml. Cells suspended in the complete absence of exogenous heat-inactivated protein respired at equivalent rates and were no less viable than those cells which were exposed to heat-inactivated ABA. Both heat-inactivated ABA exposed cells and unexposed cells were controls for ABA activity studies.

RESULTS

Effect of Serum Fractions on Staphylococci. The effect of well described commercially available serum fractions (4 mg/ml) on the oxygen consumption and viability of staphylococci was investigated by both manometric technique and viability counts as described previously. The results presented in Table 1 are from a single representative strain (one of the five randomly selected hospital-acquired strains). These data indicated the only serum fraction which had any detectable antibacterial activity and also reacted with antibody to human ABA was Cohn's fraction IV corresponding to serum α globulins. Thrombin inhibited the oxygen consumption but not the viability of staphylococci. To determine the relationship of human ABA to animal ABA as well as to those human serum proteins tested for bactericidal activity and to the antibacterial substance, β lysin, described by Donaldson et al. (21) immunoelectrophoretic studies were performed. Antiserum to human ABA prepared in rabbits reacted with α globulin, plasma bovine transferrin, rat and rabbit ABA, but not with orosomucoid prepared by Dr. Winzler (Fig. 1). Human ABA did not react with rabbit antisera to human: α_1 glycoprotein, α_1 T-glycoprotein, α_1 B-glycoprotein, α_1 antichymotrypsin, inter α trypsin inhibitor, α_2 HS-glycoprotein, ZN α_2 glycoprotein, GC-globulin, β lipoprotein, haptoglobin, transferrin, ceruloplasmin, thyroglobulin, fibrinogen, C-reactive protein (CRP), IgA, IgG, IgM, Cohn's serum fractions IV-5, 6 and III-1 or with guinea pig antiserum to rabbit β-lysin but did react to antisera prepared against α globulin and α_1 antitrypsin. As a control whole human serum was immunoelectrophoresed on the same slide as ABA

[a] S. aureus were grown to late logarithmic phase in TSB, harvested, washed and suspended in 10^{-4} M sodium phosphate buffer, (pH 7.0) at a cell density of 500 Klett units for the manometric assay and 200 Klett units for the viability count assay. Cells were exposed to 4 mg/ml of one of the commercially available serum fractions or ABA for 1 hr at 4°C. Following exposure 1 ml of cells (500 K.U. suspension) was placed into the main flask of a Warburg vessel and oxygen consumption measured. From the 200 K.U. suspension 0.5 ml of cells were added to 4.5 ml of sterile saline, a 10 fold serial dilution prepared and 0.1 ml of each dilution was mixed into pour plates of TSA in quadruplicate. Counts were made after 24 hr at 37°C. Data are from 1 representative strain out of 5 hospital acquired strains tested.

TABLE 1

Effect of commercially prepared serum fractions on oxygen consumption and viability of staphylococci.[a]

Serum Fraction	µl O_2 consumed/hr		No. of viable organisms (x 10^7)	
	Exposed to serum fraction	Control	Exposed to serum fraction	Control
Thrombin	32 ± 2	79 ± 6	126	114
Plasmin	72 ± 6	74 ± 9	94	107
Cohn's F-I	94 ± 7	97 ± 2	76	98
Cohn's F-II	67 ± 1	69 ± 7	220	255
Cohn's F-II, III	58 ± 3	61 ± 6	104	118
Cohn's F-IV	43 ± 5	61 ± 4	65	201
Cohn's F-V	73 ± 4	74 ± 5	64	79
Transferrin	57 ± 1	62 ± 6	114	126
Trypsin	64 ± 2	59 ± 8	160	190
Cholinesterase	48 ± 6	54 ± 9	120	106
ABA	21 ± 2	68 ± 3	31	151

Figure 1. Immunoelectrophoresis of ABA and selected plasma proteins: (a), human ABA (2 mg/ml) vs. anti-whole human serum; (b), topwell, IgG (10 mg/ml), bottom well, IgM (10 mg/ml) vs. antihuman ABA; (c), human ABA (3 mg/ml) vs. anti-human ABA; (d), rabbit ABA (24 mg/ml) vs. anti-human ABA; (e), rat ABA (24 mg/ml) vs. anti-human ABA;

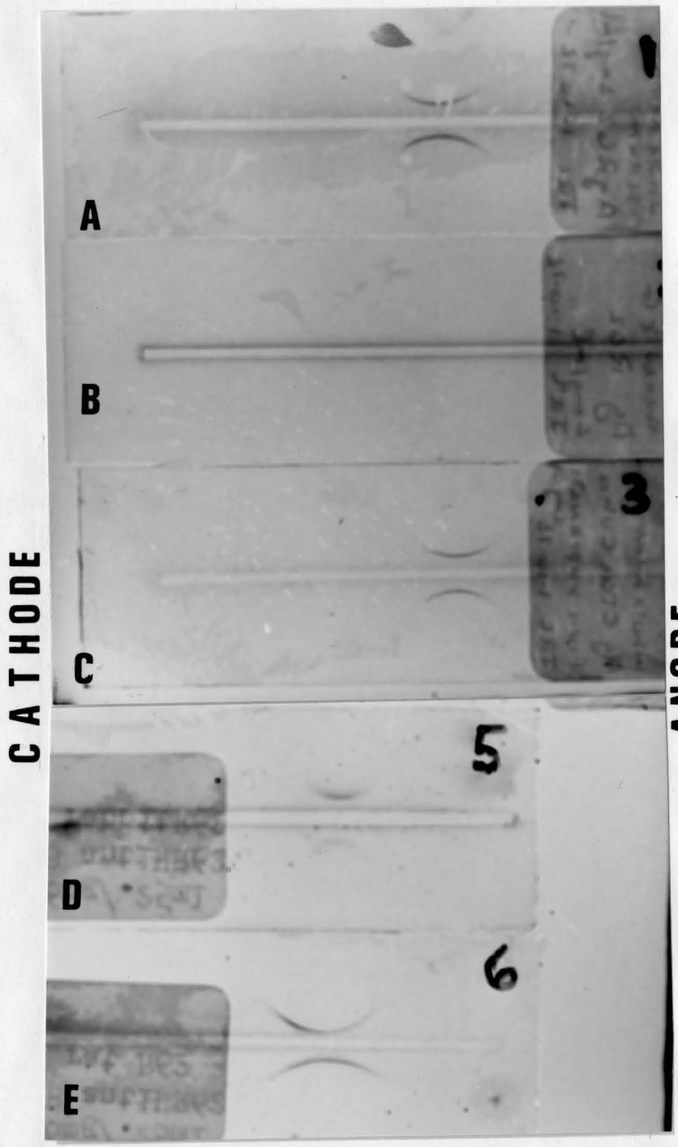


Figure 1. (contd.) (f), top well, human ABA (10 mg/ml), bottom well, human transferrin (20 mg/ml) vs. anti-whole human serum; (g), top well, human ABA (10 mg/ml), bottom well, human transferrin (20 mg/ml) vs. anti-human ABA; (h), top well, human ABA (10 mg/ml), bottom well, human transferrin (20 mg/ml) vs. anti-human transferrin; (i), top well, human ABA (10 mg/ml), bottom well, orosomucoid (20 mg/ml) vs. anti-human ABA.

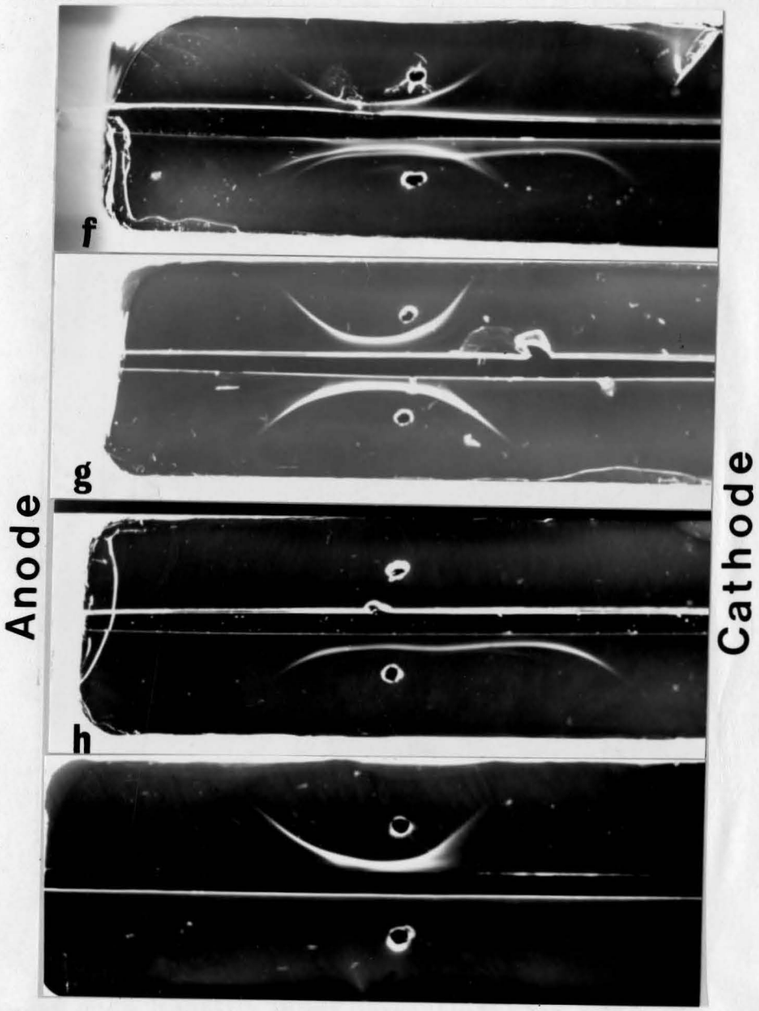


Figure 1. (Contd.) Immunoelectrophoresis of human ABA and selected human plasma proteins. (j), bottom well, whole human serum, top well, human ABA (2 mg/ml) vs. horse anti-whole human serum; (k), top well, whole human serum, bottom well, human ABA (2 mg/ml) vs. rabbit anti-human ABA. In Fig. 1 l-t antigens are top well, human ABA (2 mg/ml), bottom well, whole human serum. Antisera are the following: (l), antihuman α_1 antitrypsin; (m), antihuman α_1 acid-glycoprotein; (n), anti-α_2 HS-glycoprotein; (o), antihuman α_1 T-glycoprotein; (p), antihuman ZN α_2 glycoprotein; (q), antihuman α_1 B-glycoprotein; (r), antihuman GC-globulin; (s), antihuman α_1 antichymotrypsin; (t), antihuman inter α trypsin inhibitor. All antisera were produced in rabbits except for anti whole human serum antisera which was obtained from horses.

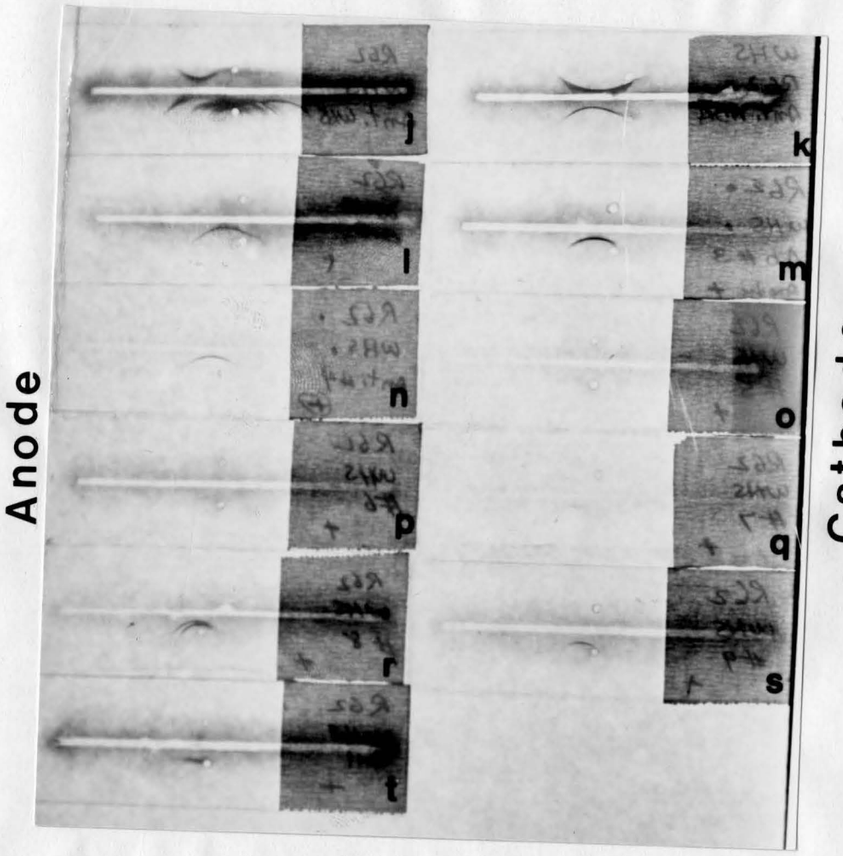
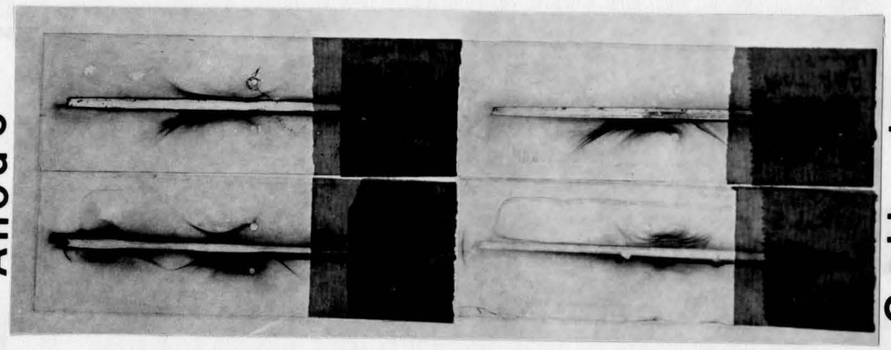


Figure 1 (Contd.) Immunoelectrophoresis of animal sera ABA and whole serum from selected species. (u), top well, rat ABA (10 mg/ml), bottom well, whole rat serum vs. rabbit anti-whole rat serum antisera; (v), top well, rat ABA (1.5 mg/ml), bottom well, whole rat serum vs. rabbit anti-whole rat serum antisera; (w), top well, rabbit ABA (10 mg/ml), bottom well, whole rabbit serum vs. donkey anti-whole rabbit serum antisera; (x), top well, whole rabbit serum, bottom well, rabbit ABA (1.5 mg/ml) vs. donkey anti-whole rabbit serum antisera.



and the reaction with the various antisera tested was allowed to proceed. As can be seen in Fig. 1 anti α_1 antitrypsin reacted with the ABA. These data are presented in complete detail in Table 2. Those slides for which no reaction occurred have been omitted from Fig. 1. Because rat and rabbit sera contained ABA we tested these preparations against antisera to either whole rat serum (produced in rabbits) or whole rabbit serum (produced in donkeys). The whole unfractionated serum from each animal was used as a control to determine the presence of exogenous serum proteins in the animal sera ABA preparations (Fig. 1).

Because the spectrum of activity of β lysin was comparable to that of ABA, inhibiting both the staphylococci and Bacillus subtilis, it was necessary to further distinguish the two antibacterial substances. For this reason, serum was Seitz filtered (to remove the β lysin) prior to the preparation of ABA. In addition ABA prepared from plasma (to prevent β lysin release from platelets which occurs during blood clot formation) was demonstrated to be effectively bactericidal.

Table 3 presents the data from leakage experiments which measured the efficacy of the various ABA preparations to induce the release of radic-labelled ^{14}C cytoplasmic substances from staphylococci. These data expressed as counts per minute per ml of supernatant fluid clearly indicated all preparations of ABA tested to cause the release of ^{14}C in proportion to the concentration of ABA present. Supernatant fluids were tested for radioisotope leakage after 18 hr of cell exposure to ABA under CO_2 (1-2 lbs/in^2) at 37°C. It is noteworthy that with the radioisotopic method concentrations of ABA 20

Immunoelectrophoresis was by the method of Scheidegger (89) in 1.5% noble agar gel with an LKB 6800 Immunophor apparatus for 60 min at 250 V in 0.1 M barbital buffer (pH 8.6). Antigen concentrations ranged 0.5 - 24.0 mg/ml. Antisera troughs were filled with either rabbit antihuman ABA or commercial antisera prepared against whole serum or various serum protein fractions. Guinea pig antirabbit β-lysin antisera (obtained from Dr. Donaldson) was used in the appropriate samples. Antiserum to whole human serum was prepared in horses. Antiserum to whole rabbit serum was produced in donkeys.

TABLE 2

Immunoelectrophoresis of antibacterial agent.

Samples analyzed		No. of precipitin arcs (and location)	
Antigen	Antibody		
Human ABA	Anti-rabbit β lysin	0	-
Rabbit β lysin	Anti-rabbit β lysin	1	anodic
Human ABA	Anti-human ABA	2	anodic
Rabbit β lysin	Anti-human ABA	0	-
Human ABA	Anti-human Cohn's Fraction IV-5,6	0	-
Human ABA	Anti-human Cohn's Fraction III-1	0	-
Human ABA	Anti-human β lipoprotein	0	-
Human ABA	Anti-human haptoglobulin	0	-
Human ABA	Anti-human fibrinogen	0	-
Human ABA	Anti-human C-R-P	0	-
Human ABA	Anti-human thyroglobulin	0	-
Human ABA	Anti-human ceruloplasmin	0	-
Human ABA	Anti-human IgA	0	-
Human ABA	Anti-human IgG	0	-
Human ABA	Anti-human IgM	0	-
Human ABA	Anti-human whole serum	1	anodic
Human ABA	Anti-human α_1 glycoprotein	0	-
Human ABA	Anti-human α_1 T-glycoprotein	0	-
Human ABA	Anti-human α_1 B-glycoprotein	0	-
Human ABA	Anti-human α_1 anti-chymotrypsin	0	-
Human ABA	Anti-human inter α trypsininhibitor	0	-
Human ABA	Anti-human Zn α_2-glycoprotein	0	-
Human ABA	Anti-human α_2HS-glycoprotein	0	-
Human ABA	Anti-human GC globulin	0	-
Human ABA	Anti-human α_1 antitrypsin	1	anodic
Human ABA	Anti-human transferrin	0	-
Whole human serum	Anti-human whole serum	Numerous	
Whole human serum	Anti-human ABA	2	anodic
Whole human serum	Anti-human α_1 glycoprotein	1	anodic
Whole human serum	Anti-human α_1 T-glycoprotein	1	anodic
Whole human serum	Anti-human α_1 B-glycoprotein	1	anodic
Whole human serum	Anti-human α_1 anti-chymotrypsin	1	anodic

TABLE 2 (Contd.)

Samples analyzed		No. of precipitin arcs (and location)	
Antigen	Antibody		
Whole human serum	Anti-human inter α trypsin inhibitor	3	anodic
Whole human serum	Anti-human Zn α_2 glycoprotein	1	anodic
Whole human serum	Anti-human α_2 HS-glycoprotein	1	anodic
Whole human serum	Anti-human GC-globulin	2	anodic
Whole human serum	Anti-human α_1 antitrypsin	2	anodic
Human transferrin	Anti-whole human serum	3	(anodic (cathodic
Human transferrin	Anti-human transferrin	2	cathodic
Human transferrin	Anti-human ABA	1	anodic
Human α globulin (F-IV)	Anti-human ABA	1	anodic
Human β globulin (F-III)	Anti-human ABA	0	-
Plasma bovine transferrin	Anti-human ABA	1	anodic
Orosomucoid	Anti-human ABA	0	-
Rat ABA	Anti-human ABA	2	anodic
Rat ABA	Anti-whole rat serum	2	anodic
Rat ABA	Anti-whole rabbit serum	1	anodic
Rabbit ABA	Anti-human ABA	1	anodic
Rabbit ABA	Anti-human rat serum	1	anodic
Rabbit ABA	Anti-human rabbit serum	2	anodic
Whole rat serum	Anti-human ABA	0	-
Whole rat serum	Anti-whole rat serum	Numerous	
Whole rat serum	Anti-whole rabbit serum	1	anodic
Whole rabbit serum	Anti-human ABA	0	-
Whole rabbit serum	Anti-whole rat serum	0	-
Whole rabbit serum	Anti-whole rabbit serum	Numerous	
Guinea pig ABA	Anti-human ABA	0	-

[a] S. aureus serotype XII ATCC 12609 was grown to late logarithmic phase in TSB at 37°C harvested, washed and the cellular density adjusted to 200 Klett units. To each 100 ml of cell suspension 1 ml of ^{14}C-glucose (u.l) and cells were incubated for 1-3 hr at 37°C on a rotary shaker. Labeled cells were harvested, washed twice with chilled (4°C) saline and resuspended in an equal volume of distilled water (4°C). ABA was added in the appropriate concentration and cells were incubated anaerobically under CO_2, 1.5 - 2.0 lb/in^2, at 37°C for 18 hr. After centrifuging in conical tubes 1 ml of supernatant fluid was placed in 9 ml of scintillation fluor and the release of ^{14}C cytoplasmic substance was determined in a Packard Tri-Carb liquid scintillation spectrometer model 3320. Activity is expressed as counts/min/ml. Data are mean values from three experiments performed in duplicate.

TABLE 3

Alteration in staphylococcal permeability by the ABA derived from filtered and unfiltered body fluids.[a]

Source of ABA	Counts/min/ml of supernatant fluid with varying concentrations of ABA (μg/ml)				
	0	10	50	100	500
Rabbit, unfiltered serum	1093	1074	1423	1953	3757
Rabbit, filtered serum		1157	1556	1889	3795
Rat, unfiltered serum	1113	1061	1482	2082	3329
Rat, filtered serum		1003	1649	2284	3395
Guinea pig, unfiltered serum		1158	1787	2143	4128
Guinea pig, filtered serum		1095	1472	2010	3677
Human, unfiltered serum	978	946	1425	1936	3291
Human, filtered serum		1004	1511	2003	3054
Human, unfiltered plasma		993	1644	2187	2962
Filtered human plasma		1083	1777	2073	3086
Platelet-rich, unfiltered human plasma		1129	1613	1991	2800
Human, hypoglobulinemic serum		1013	1231	1680	2728
Human, diabetic serum		981	1149	1795	3513
Human serum from persons with recurrent furunculosis		1002	1468	1966	3111

to 40 times lower than those required with the manometric method were necessary to achieve an equivalent effect. Although ABA was found in filtered, unfiltered, pathological, and platelet rich body fluids in approximately similar concentrations, no definite correlation could be established between the susceptibility of the host to staphylococcal infection and the concentration of ABA in serum. The rabbit is most susceptible to staphylococci, the rat is highly resistant while the guinea pig is intermediate in resistance. The *in vitro* effect of ABA was demonstrated to be inversely proportional to the concentration present in the serum (Table 3); while the data presented in Table 4 indicated the concentration of ABA in mg protein per ml body fluid.

Since a single 18 hr period of exposure of cells to ABA cannot provide a very accurate picture of the potency of various preparations of antibacterial agent from the sera of hosts of varying susceptibility to staphylococcal infections, time course and concentration effect studies were performed. Table 5 illustrates the progressive leakage of radioactive substances from *S. aureus* serotype XII exposed aerobically at 37°C for 0.5, 1, 2, 4 and 8 hr to 50 μg/ml of ABA prepared from unfiltered human, rat, rabbit and guinea pig serum. As it would be expected the leakage of radioactive substances released from staphylococci increased with time. Antibacterial agent from guinea pig and rat serum was found to be slightly more active than ABA from other sources. Similar results were obtained when *S. aureus* serotype XII was exposed for 1 hr and 8 hr to 10 μg, 100 μg, and 500 μg of ABA/ml (Table 6). At a concentration of 100 μg of ABA/ml the amount of ^{14}C labelled cytoplasmic substance leakage produced after 8 hr was nearly twice as great as

TABLE 4

Average yield of ABA obtained from unfiltered or filtered (Seitz) body fluids of various animal species.[a]

Source of ABA	Yield (mg/ml) of body fluid
Rabbit, unfiltered serum	0.3 - 1.6
Rabbit, filtered serum	0.2 - 1.8
Rat, unfiltered serum	0.4 - 1.4
Rat, filtered serum	0.4 - 1.5
Guinea pig, unfiltered serum	0.2 - 0.7
Guinea pig, filtered serum	0.2 - 0.4
Human, unfiltered serum	0.4 - 2.0
Human, filtered serum	0.3 - 1.8
Human, unfiltered plasma	0.5 - 1.7
Filtered human plasma	0.4 - 1.3
Platelet-rich, unfiltered human plasma	0.7 - 1.9
Human, hypoglobulinemic serum	0.5 - 1.6
Human, diabetic serum	0.3 - 1.2
Human serum from persons with recurrent furunculosis	0.7 - 1.5

[a] Data are values obtained from 15 preparations of ABA.

TABLE 5

Time course leakage of radioactive cytoplasmic substance from staphylococci exposed to antibacterial agent.[a]

Source of Antibacterial Agent	Counts/min/ml of supernatant					
	Time of exposure in hr					
	0	0.5	1	2	4	8
Control	432	502	591	679	1001	1242
Rabbit serum		662	871	1217	1552	1981
Rat serum		699	919	1196	1591	2112
Guinea pig serum		715	992	1296	1929	2382
Human serum		599	782	1247	1521	2021

[a] Experimental conditions were identical to those of Table 3 except that cells were exposed aerobically at 37°C for the specified time to 50 μg/ml of ABA prepared from unfiltered human, rat, rabbit and guinea pig serum. Heat-inactivated human ABA served as control. Data are mean values of three experiments performed in duplicate.

TABLE 6

Effect of concentration on ABA leakage of radioactive cytoplasmic substance from staphylococci. [a]

Source of ABA	Counts/min/ml of supernatant fluid with ABA (μg/ml)							
	0		10		100		500	
	after		after		after		after	
	1 hr	8 hr	1 hr	8 hr	1 hr	8 hr	1 hr	8 hr
Rabbit serum	563	1229	523	1454	806	2626	1220	2954
Rat serum			681	1571	1172	2841	1760	3619
Guinea Pig serum			753	1699	1312	3031	1969	3837
Human serum			534	1449	892	2591	1657	3531

[a] Experimental conditions were identical to those of Table 3 except that cells were exposed aerobically at 37°C for 1 hr and 8 hr to 10 μg, 100 μg, and 500 μg of ABA/ml. Data are mean values of three experiments performed in duplicate.

that produced at 10 μg of ABA/ml for the same period. The optimal concentration used in this system however was 500 μg of ABA/ml; cells exposed for 8 hr at this concentration demonstrated a 280% - 310% increase in release of ^{14}C-labelled cytoplasmic substances relative to the leakage from unexposed control cells.

The data presented in Tables 1-6 indicated ABA activity to be independent of the antibacterial substance known as β lysin as well as all other serum fractions tested with the exception of serum α globulins. These results were obtained by a variety of assay systems under a variety of conditions. The effect of time and concentration of ABA was directly releated to the leakage of cytoplasmic substance while the potency of ABA seemed to be inversely related to the average yield of ABA in mg protein per ml serum.

Because the relationship between ABA and serum α globulins was strongly indicated, an attempt to prepare α globulins from normal human serum was made. Any such protein isolated should possess equal bactericidal activity to ABA in addition to chemicophysical similarities. Table 7 presents the data obtained by manometric assay of staphylococcal respiration following exposure to either ABA or the ABA prepared by ethanol fractionation of Cohn's fraction IV-1 (C-E ABA). The Cohn-ethanol fraction was indicated to effectively inhibit the respiration of thestaphylococci. As compared to their respective unexposed control cell counterparts, ABA exposed cells consumed 65% less oxygen and C-E ABA exposed cells decreased oxygen utilization by 75%. The chemico-physical properties of both preparations were compared and the molecules were demonstrated to possess similar electrophoretic properties as shown

a

S. aureus serotype XII was grown to mid-logarithmic phase in TSB at 37°C, harvested, washed, and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0) at a cellular density of 500 K.U. (1.43 mg dry wt/ml). Staphylococci were exposed to 2 mg/ml of either preparation for 1 hr at 4°C prior to manometric respirometry. Values expressed as µl O_2 consumed per hr were obtained in duplicate samples for each experiment. The relative % difference is defined as $100 - \left(\frac{\text{Native ABA}}{\text{Control}} \times 100\right)$.

TABLE 7

Comparison of antibacterial effect of ABA and Cohn-Ethanol ABA.[a]

Exp. No.	µl O_2 consumed/hr				µl O_2 consumed/hr			
	Native ABA	Heated ABA	Control cells	Relative % differ.	Native CE ABA	Heated CE ABA	Control cells	Relative % differ.
1	23	69	75	69	20	66	72	72
	27	65	73	63	25	63	70	64
2	33	78	85	61	10	54	66	85
	31	71	77	60	15	62	69	78
3	29	61	74	61	14	70	68	79
	21	75	82	74	22	50	69	68
Average				65				75

by immunoelectrophoresis and isoelectric focusing as is shown in Figs. 5 and 6. The chemical composition of CE ABA is shown in Table 34.

Effect of ABA on the Incorporation of ^{14}C-glucose into Various Staphylococcal Cellular Fractions. Having established the efficacy of ABA under various conditions of growth, time of exposure, environment and concentration of ABA, the incorporation of ^{14}C-glucose by various staphylococcal strains was investigated by the fractionation method of Park and Hancock (77). Cells grown to mid-logarithmic phase were harvested, washed and exposed to ABA or heat inactivated ABA as described in Materials and Methods. Following the incubation of cells for 1 hr with ^{14}C-glucose (u.l.), excess label was removed by washing two (2) times with saline, and the extraction procedure was performed. The extraction procedure and the characterization of the five fractions obtained are outlined in Table 8. The data presented in Table 9 indicated not all strains to be equally susceptible to ABA; while S. aureus serotypes IX and X were hardly affected by ABA, serotypes XI and XII exhibited a 13% and 30%, respectively, relative increase in leakage of radiolabeled cytoplasmic substances following ABA exposure. Those strains which were affected significantly demonstrated a reduction in the amount of ^{14}C-glucose incorporated into the ethanol soluble fraction(Table 10,11). This fraction was comprised of cellular lipid and very small amounts of alcohol soluble protein. The results presented in Tables 10 and 11 indicated that ^{14}C-glucose incorporation into the ethanol soluble cellular fractions of serotypes XI and XII was reduced by ABA to an extent of 55%-60%.

a Adapted from Park and Hancock (77).

b Cellular pellet (from 15 ml of 100 K.U. suspension) of washed organisms was suspended in 2.5 ml of 5% TCA (0°C). After 10 min cell suspension was centrifuged at 4000 g for 10 min and the extract supernatant was decanted and saved.

c Residue from cold TCA was suspended in 2.5 ml of 75% ethanol (pH 3.0) at 45°C for 10 min and the extract was separated as described above.

d Residue was suspended in 2.5 ml of 5% TCA at 90°C for 10 min, the extract was separated as described above.

e Residue was suspended in 1.5 ml of 0.05 M NH_4HCO_3 containing 0.005 N NH_4OH to which crystalline trypsin was added at a concentration of 1.0 mg/ml. The suspension was incubated at 37°C for 2 hr and the extract was separated as described above.

f The residue was suspended in 1.0 ml of distilled water.

TABLE 8

Fractionation of staphylococci.[a]

Fraction	Contents of fraction
Cold TCA[b]	All low molecular weight compounds soluble in 5% TCA
75% ETOH[c]	Ethanol soluble protein and lipid
Hot TCA[d]	Breakdown products of nucleic acid and teichoic acids
Trypsin[e]	Trypsin-degraded protein
Residue[f]	Mucopeptide of wall

[a] S. aureus serotypes IX-XIII were grown to mid-logarithmic phase at 37°C in TSB, harvested, washed and adjusted to 100 K.U. cellular density in 10^{-4} M sodium phosphate buffer(pH 7.0). Cellular suspensions were labeled as described in Table 3 following exposure for 1 hr at 4°C to 100.0 µg ABA/ml. Suspensions were filtered by membrane filtration (0.45 micron pore size, 12 mm diameter). The filters were washed with 5 volumes of cold buffer, air dried, and placed directly into 10 ml of scintillation fluor. Radioactivity was determined as described in Table 3. The values represent the data from two experiments performed in duplicate. The relative % difference is defined as $100 - \left(\frac{\text{Native ABA}}{\text{Control}} \times 100 \right)$.

TABLE 9

Alteration of permeability of various strains of staphylococci.[a]

Serotype	Counts/min/ml			
	Native ABA	Heat-inactivated ABA	Unexposed cells	Relative % difference
IX	644	610	664	4%
X	420	435	439	5%
XI	1557	1756	1776	13%
XII	1344	1765	1893	30%
XIII	424	469	464	9%

[a] Bacteria were grown to mid-logarithmic phase in TSB at 37°C, harvested, washed and suspended in sterile distilled water. One ml of a cell suspension designed to yield a 100 K.U. suspension on dilution to 50 ml was incubated with ABA(5 mg/ml)for 1 hr at 4°C. Following exposure the cell suspension was diluted to 50 ml with sterile 10^{-4} M sodium phosphate buffer (pH 7.0) and labeled with 0.5 ml ^{14}C-glucose (u.l. 2 x 10^{6} cpm) for 1 hr at 37°C on a rotary shaker. Cells were centrifuged, washed twice with cold (4°C) saline and resuspended in 2.5 ml of cold 5% TCA (4°C) and fractionated according to the method of Park and Hancock (77). Aliquots of 0.1 ml of each extraction supernatant and the residue suspension were placed in 10 ml of scintillation fluor and radioactivity was determined. Data are the mean values of two experiments performed in duplicate. The relative % difference is defined as $100 - (\frac{\text{Native ABA}}{\text{Control}} \times 100)$.

[b] To ^{14}C-glucose values represent the amount of radioactivity in 1 ml of the labeled cell suspension immediately upon addition of the radiolabeled glucose.

[c] T_{60}-^{14}C-glucose values represent the amount of radioactivity in 1 ml of the labeled cell suspension following a 60 min incubation period during which staphylococcal uptake of ^{14}C-glucose occured.

[d] Washed cells values reflect the amount of ^{14}C-radioactivity in 1 ml of cells taken from the 15 ml sample to be fractionated.

[e] The mean amount of ^{14}C activity in each fraction. Values are based on the fractionation of the remaining 14 ml of radiolabeled cells. 0.1 ml of each fraction was placed in 10 ml of scintillation fluor and radioactivity determined.

TABLE 10

Effect of ABA on the incorporation of glucose by *Staphylococcus aureus* serotype XI.[a]

Fraction	Counts/min/ml ± S.D.			
	Native ABA	Heat-inactiv. ABA	Control Cells	Relative % differ.
To ^{14}C-glucose [b]	80,332 ± 4266	78,655 ± 4797	65,506 ± 5286	22.6
T_{60} ^{14}C-glucose [c]	102,902 ± 1101	114,334 ± 1613	107,746 ± 1585	5.0
Washed cells [d]	91,600 ± 773	94,258 ± 1155	93,526 ± 1192	3.0
Cold TCA [e]	134,988 ± 1543	125,343 ± 1733	123,802 ± 1662	9.0
75% ETOH [e]	7,066 ± 382	18,647 ± 605	17,378 ± 718	60.0
Hot TCA [e]	32,211 ± 561	36,295 ± 524	29,085 ± 425	10.0
Trypsin [e]	2,775 ± 372	4,391 ± 308	2,434 ± 266	14.0
Residue [e]	20,821 ± 215	24,825 ± 255	25,274 ± 219	18.0

TABLE 11

Effect of ABA on the incorporation of glucose by *S. aureus* serotype XII.[a]

Fraction	Counts/min/ml ± S.D.			
	Native ABA	Heat-inactiv. ABA	Control cells	Relative % differ.
T ^{14}C-glucose [b]	88,215 ± 3042	92,574 ± 3341	87,529 ± 2545	0.7
T ^{14}C-glucose [c]	113,262 ± 743	115,540 ± 726	119,410 ± 1064	6.0
Washed cells [d]	101,171 ± 432	104,082 ± 541	105,440 ± 489	5.0
Cold TCA [e]	144,461 ± 1566	156,064 ± 1745	147,749 ± 2188	3.0
75% ETOH [e]	6,852 ± 856	13,211 ± 725	14,933 ± 759	55.0
Hot TCA [e]	55,259 ± 385	50,165 ± 237	46,732 ± 271	18.0
Trypsin [e]	4,133 ± 280	5,091 ± 371	6,069 ± 304	32.0
Residue [e]	33,160 ± 304	42,594 ± 423	38,324 ± 341	14.0

[a] As in Table 10.

[b] As in Table 10.

[c] As in Table 10.

[d] As in Table 10.

[e] As in Table 10.

Delineation of the Site of Action of ABA. Mickle disintegration of staphylococci was performed until a uniformly smooth homogenate, as judged by the gram stain and phase contrast microscopy, was obtained. The homogenate as well as whole cells from the same culture were exposed to ABA and tested for susceptibility by manometric technique. The data presented in Table 12 indicated that cellular structural integrity was not necessary for ABA to exert pronounced respiratory inhibition. Respiration of intact cells was markedly reduced in the presence of ABA as compared to the level of oxygen consumption by the corresponding heat-inactivated ABA-exposed controls. Since ABA was demonstrably active in the presence of ruptured cells as well as in the presence of intact cells, it was of interest therefore to determine the susceptibility of staphylococcal spheroplasts which would possess an intact cytoplasmic membrane. To this end, lysostaphin-induced spheroplasts of staphylococci were prepared by incubating 500 Klett units of S. aureus PS 54 with 10 units of lysostaphin per ml at 37°C for 3 hrs in a buffered 9% saline solution. The results in Table 13 indicated that the osmotically fragile cells exhibited a slight increase in susceptibility over the whole cells under identical experimental conditions. The respiration of ABA-exposed osmotically fragile staphylococci was inhibited to a greater degree (59%-72%) than was the respiration of ABA-exposed whole cells (50%-58%). Staphylococci suspended in 9% saline-buffer both in the presence and absence of heat-inactivated ABA served as experimental controls.

The lack of cell wall after enzymatic degradation did not inhibit ABA activity but seemed to potentiate the bactericidal effect. The increased levels of oxygen consumption by the control osmotically fragile cells was

TABLE 12

Effect of ABA on staphylococcal homogenates.[a]

Exp. No.	µl O_2 consumed/hr ± S.D.			
	Homogenates		Whole cells	
	Native ABA	Heat-inactiv. ABA	Native ABA	Heat-inactiv. ABA
1	5 ± 3	60 ± 4	3 ± 1	75 ± 7
2	3 ± 2	64 ± 5	2 ± 1	55 ± 4
3	12 ± 4	74 ± 2	4 ± 2	67 ± 2

[a] S. aureus serotype XII was grown to mid-logarithmic phase, harvested, washed, and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0) at a cellular density of 500 K.U. (1.43 mg dry wt/ml). Homogenates were prepared by Mickle disintegration of 5 ml of a 1000 K.U. suspension of staphylococci with 4g of No. 12 ballontini glass beads for 3 hr at 4°C. Following homogenization the glass beads were removed by sintered-glass filtration and the homogenate density adjusted to 500 K.U. Both whole cells and homogenates were exposed to ABA (2 mg/ml) for 1 hr at 4°C prior to manometric respirometry. Data are mean values for duplicate samples in each experiment.

TABLE 13

Effect of ABA on lysostaphin induced osmotically fragile spheroplasts.[a]

Exp. No.	µl O_2 consumed/hr ± S.D.					
	Osmotically fragile spheroplasts			Whole cells		
	Native ABA	Heat-inactiv. ABA	Relative % differ.	Native ABA	Saline Control	Relative % differ.
1	34 ± 6	118 ± 22	72	41 ± 3	96 ± 11	58
2	39 ± 2	94 ± 14	59	39 ± 2	86 ± 17	55
3	44 ± 10	123 ± 15	65	44 ± 6	88 ± 14	50
4	33 ± 3	117 ± 21	72	41 ± 5	96 ± 10	58

[a] S. aureus PS54 was grown to mid-logarithmic phase in BHI at 37°C, harvested, washed and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0). Cellular density was adjusted to 500 K.U. in 9% saline buffer with a green (No. 54) filter. Cells were exposed to 10 units of lysostaphin/ml for 3 hr at 37°C and spheroplasts harvested by centrifugation. Prior to manometric respirometry both whole cells and spheroplasts were exposed to ABA (2 mg/ml) for 1 hr at 4°C. Data are the mean values for duplicate samples in each experiment. The relative % difference is defined as $100 - (\frac{\text{Native ABA}}{\text{Control}} \times 100)$.

understandable when considering the increased free enzyme level following cellular degradation.

The use of an L-form of the Smith strain of S. aureus allowed us to study the cellular membrane as the site of ABA activity in the complete absence of cell wall material. With the addition of human ABA, the respiration of the L-forms was shown to be decreased by 38.4% within 10 min of exposure (Table 14). As the length of exposure increased the % inhibition rose proportionally. After 20 min the L-forms consumed 50% less oxygen, following 30 min of exposure, the level of respiration was only 25% of the control unexposed L-forms. All previous studies with whole cells had required a 60 min incubation period before ABA would significantly affect staphylococcal respiration. The minimal amount of time required for L-form susceptibility to become evident suggested that in the absence of cell wall, direct access of ABA to the cell membrane resulted in the rapid bactericidal effect. Without the benefit of osmotic protection, L-forms did not respire to a significant degree. L-forms exposed to heat-inactivated ABA were shown to respire at a level greater than that of the unexposed L-forms. This finding was assumed to reflect the increased availability of nutrient material present in the form of free amino acids provided by the inactivated protein molecule.

Based on the results of the above experiments, the cytoplasmic membrane was indicated to be the primary site of action of ABA. Cell wall absorption however was not precluded as a possible factor in the bactericidal process. In order to demonstrate that the ABA activity was disassociated from cell wall interaction, the experiments presented in Tables 15-19 were performed. The

TABLE 14

Effect of ABA on S. aureus Smith L-form.[a]

Time of Exposure (min)	µl O_2 consumed/hr ± S.D.				
	Native ABA	Heat-inactiv. ABA	Control L-forms	Osmotically Shocked L-forms	Relative % differ.
10	45 ± 2	109 ± 12	73 ± 7	9 ± 0	38.4
20	13 ± 4	57 ± 6	26 ± 3	4 ± 0	50.0
30	4 ± 1	28 ± 4	17 ± 2	2 ± 0	76.5

[a] S. aureus Smith L-forms (ATCC 19640) were grown on 2.5% heart infusion containing 0.75% Bacto Agar, 3.5% soidum chloride, 10% heat-inactivated horse serum and 1000 u of Benzyl Penicillin/ml for 48 hr at 37°C in a candle jar. L-forms were harvested from the agar surface with 9% saline buffer, filtered through a double layer of cheese cloth to remove agar particles and centrifuged at 10,000 x g for 20 min at 4°C. The resulting pellet was suspended in 10-11 ml of 9% saline-phosphate buffer containing 10 mM magnesium chloride and exposed to 2 mg of ABA/ml for the reported time intervals at room temperature (25°C). Respirometry was performed in the presence of 0.11 M glucose in 9% saline buffer. Data are mean values obtained from triplicate or duplicate samples in each experiment. The relative % difference is defined as $100 - \left(\frac{\text{Native ABA}}{\text{Control L-forms}} \times 100\right)$.

data in Table 15 indicated that high concentrations of exogenous cell wall material was able to inhibit ABA, presumably by adequate adsorption, only after 2 hr of exposure. Under the conditions for manometric studies most cells were exposed to ABA for only 1 hr, in the absence of exogenous cell wall material, which allowed for sufficient damage to the cell membrane. Adsorption therefore does not appear to occur quickly enough to prevent the membrane damaging effects yet cannot be disregarded as a possible prerequisite step in the bactericidal process. Peptidoglycan preparations from which 99.9% of ribitol teichoic acid had been removed by boiling cell walls for 15 min in 10% TCA (77) were shown to adsorb ABA after 2 hr of exposure and inhibited ABA activity against whole cells (Table 16). The rise in oxygen consumption by cells after exposure to peptidoglycan was puzzling, however it was reasoned that because of the degradation of the cell wall by TCA with the release of constituent amino acids, there were increased nutrients available to the cell to foster enhanced oxygen utilization. However the addition of such amino acids, as are found in the staphylococcal cell wall, in excessive quantities did not serve to increase oxygen consumption significantly in the presence of ABA. ABA was added to whole cell suspensions both consecutively with or following the amino acid solutions. The results shown in Table 17 indicated that ABA retained nearly 100% potency in the presence of the amino acids and inhibited the staphylococcal respiration significantly. Such experiments as described above were dependent on the addition of exogenous whole cell wall or cell wall constituents. Through the use of a ribitol teichoic acid-free mutant S. aureus H52A5, we investigated the influence of a defect in the endogenous cell wall on ABA activity. The data presented in Tables 18 and 19

TABLE 15

ABA inhibition following cell wall adsorption.[a]

Adsorption Time (hr)	μl O_2 consumed/hr ± S.D.						
	Cell wall adsorbed ABA				Unadsorbed ABA	Heat-inactiv. ABA	Relative % differ.
	Supernatant	Relative % differ.	Precipitate	Relative % differ.			
0.5	36 ± 4	44	63 ± 3	1	25 ± 3	64 ± 5	61
2.0	90 ± 6	11	72 ± 2	12	46 ± 4	81 ± 6	44
12.0	85 ± 7	2	74 ± 5	11	27 ± 4	83 ± 9	68

[a] *S. aureus* serotype XII was grown to mid-logarithmic phase in TSB at 37°C, harvested, washed and suspended in 10^{-4} M phosphate buffer (pH 7.0) at a cellular density of 500 K.U. (1.43 mg dry wt/ml). ABA (2 mg/ml) was adsorbed with 15 mg of *S. aureus* serotype XII cell wall/ml for the stated time intervals. Following adsorption, 1 ml of cell suspension was exposed to 1 ml of ABA solution for 1 hr at 4°C. Data are mean values of duplicate samples in each experiment. Relative % difference is defined as $100 - \left(\frac{\text{ABA (Native or Adsorbed)}}{\text{Heat-inactivated ABA}} \times 100\right)$.

TABLE 16

Effect of ABA adsorption by cell wall peptidoglycan.[a]

Exp. No.	μl O_2 consumed/hr $\pm$ S.D.				
	Peptidoglycan Adsorbed ABA				
	Supernatant	Precipitate	Native ABA	Heat-inactiv. ABA	Relative % differ.
1	56 $\pm$ 7	64 $\pm$ 6	6 $\pm$ 1	25 $\pm$ 4	76
2	76 $\pm$ 11	67 $\pm$ 6	13 $\pm$ 3	43 $\pm$ 7	70

[a] The experimental procedures were identical to those described in Table 15 except that ABA solution (2 mg/ml) was adsorbed for 2 hr at 4°C with 20 mg of S. aureus serotype XII peptidoglycan/ml.

TABLE 17

Effect of staphylococcal cell wall amino acids adsorption on ABA activity.[a]

Exp. No.	µl O_2 consumed/hr ± S.D.					
	ABA & Alanine[b]	ABA & Lysine[c]	ABA & Glutamic Acid[d]	Native ABA	Heat-inactiv. ABA	Relative % differ.
1	41 ± 6	33 ± 7	36 ± 4	30 ± 2	132 ± 10	78
2	49 ± 5	51 ± 3	49 ± 12	68 ± 10	141 ± 15	52

a Experimental procedures were identical to Table 15 except that ABA solution (2.5 mg/ml) was adsorbed by 25 mg of any one amino acid/ml for 2-4 hr at 4°C.

b Alanine 0.280 M

c Lysine 0.137 M

d Glutamic Acid 0.170 M

Appendex for Tables No. 15, 16, 17

Effect of ABA on S. aureus serotype XII [a]

ABA Prep. No.	μl O_2 consumed/hr		
	Native ABA	Heat-inactiv. ABA	Control cells
8	56 ± 6	95 ± 7	101 ± 6
	76 ± 12	126 ± 5	108 ± 1
10	39 ± 4	93 ± 7	97 ± 15
	19 ± 7	55 ± 7	53 ± 5
13	20 ± 8	72 ± 12	78 ± 4
	22 ± 1	65 ± 15	68 ± 2

[a] Each fraction was tested for activity against 500 K.U. of S. aureus serotype XII harvested after 10-16 hr of aerobic growth in 100 ml TSB at 37°C on a rotary shaker. Manometric assay was performed in the presence of 10^{-4} PO_4 buffer (pH 7.0) and 0.055 M-glucose (1%).

TABLE 18

Effect of ABA on S. aureus H and S. aureus H52A5.[a]

Exp. No.	Strain	µl O_2 consumed/hr ± S.D.			
		Native ABA	Heat-inactiv. ABA	Control cells	Relative % differ.
1	H	35 ± 1	60 ± 4	62 ± 2	44
	H52A5	34 ± 1	27 ± 2	44 ± 3	23
2	H	35 ± 3	47 ± 3	55 ± 3	36
	H52A5	58 ± 5	71 ± 4	66 ± 5	12

Average % difference S. aureus H 40.0%

Average % difference S. aureus H52A5 17.5%

Rel. % Differ. 57%

[a] S. aureus H and H52A5 were grown to mid-logarithmic phase in TSB at 37°C, harvested, washed and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0) at a cell density of 500 K.U. After exposure to ABA (2 mg/ml) for 1 hr at 4°C, respirometry was performed. Data are the mean values of duplicate samples in each experiment. Relative % difference is defined as $100 - (\frac{\text{Native ABA}}{\text{Control}} \times 100)$.

TABLE 19

Effect of ABA on S. aureus serotype XII and S. aureus H52A5.[a]

Exp. No.	Strain	µl O_2 consumed/hr. ± S.D.			
		Native ABA	Heat-inactiv. ABA	Control cells	Realtive % differ.
1	XII	50 ± 3	120 ± 4	99 ± 5	49
	H52A5	51 ± 3	65 ± 2	52 ± 1	2.0
2	XII	47 ± 4	120 ± 6	105 ± 6	55.2
	H52A5	38 ± 3	41 ± 7	36 ± 2	5.0

Average % difference S. aureus XII 52%

Average % difference S. aureus H52A5 4%

Relative % differ. 93%

[a] Experimental conditions were identical to those described in Table 18 except S. aureus serotype XII was substituted for S. aureus H.

compared the effect of ABA on S. aureus serotype XII, a susceptible unrelated strain and S. aureus H52A5 as well as between S. aureus H, a related strain and the mutant S. aureus H52A5. In Table 18 a 57% difference in inhibition between S. aureus H and S. aureus H52A5 was demonstrated, while in Table 19 the results indicated a 93% difference in susceptibility between the two unrelated strains. These results apparently were contradictory to previous findings which indicated cell wall constituents to play only a minor role in ABA susceptibility. The data demonstrated that cells which contained ribitol teichoic acid were apparently more susceptible to ABA than those mutant cells which had no ribitol teichoic acid. It could easily be conjectured therefore that ribitol teichoic acid was a requisite component for cellular susceptibility to ABA.

The data in Table 20 indicated S. aureus H52A5 grew more slowly than the parent strain S. aureus H. We sought to determine if any change in oxygen consumption would be present as a result of the altered growth rate. A variety of substrates were utilized to investigate the respiratory capabilities of the two strains. S. aureus H52A5 was indicated to be more active than the non-mutated strain S. aureus H in the presence of all four substrates (Table 21). The largest difference (43%) in oxygen consumption by the two strains was shown to occur with glucose as the substrate.

Paper chromatography of S. aureus H and S. aureus H52A5 cell wall hydrolysates indicated the mutant strain to contain similar components as S. aureus H with the exception of ribitol and an unidentified phosphate ester. The results of these qualitative studies indicated both strains contained large amounts of alanine and glucosamine. S. aureus H52A5 appeared to contain

TABLE 20

Growth rate of S. aureus H and S. aureus H52A5.[a]

Strain	Mean Klett Reading at Various Times (hr)											
	0	4	5	6	7	8	9	10	11	13	15	24
S. aureus H	5	15	31	66	136	221	347	413	415	435	451	463
S. aureus H52A5	6	16	23	46	83	136	226	249	296	340	390	411

[a] S. aureus H and H52A5 were grown to mid-logarithmic phase in 5 ml of TSB, harvested, washed and suspended to a cellular density of 30 K.U. 0.5 ml of either of these cellular suspensions was transferred to 100 ml TSB in a nephrolometric flask and placed on a rotary shaker at 37°C. Data are the mean values obtained from 4 experiments.

TABLE 21

Oxygen consumption per hr by S. aureus H and S. aureus H52A5 in the presence of various substrates. [a]

Substrate	µl O_2 consumed/hr in the presence of various substrates ± S.D. STRAINS		Relative % differ.
	Strain H	Strain H52A5	
Glucose	77 ± 6	135 ± 14	43
Tryptic Soy Broth	314 ± 30	394 ± 29	21
Pyruvate	35 ± 6	47 ± 18	26
Mannitol	29 ± 4	42 ± 7	31

a

S. aureus H and H52A5 were grown to mid-logarithmic phase in TSB at 37°C, harvested washed and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0). Cellular density was adjusted to 450 K.U. Glucose (0.055 M), pyruvate (0.091 M) and mannitol (0.055 M) were used as substrates. 3% TSB was used undiluted. Data are the mean values from 2 experiments run in triplicate for each substrate tested. The relative % difference is defined as $100 - (\frac{H}{H52A5} \times 100)$.

ribose, this was confirmed by quantitative analysis using Bial's orcinol reagent according to the method of Dische (17a); while S. aureus H did not contain appreciable quantities of ribose it was none-the-less present in the cell wall hydrolysate. The presence of two unknown spots close to the line of origin was noted. They reacted with the spray reagents for non-reducing sugars, amine groups, and also phosphate esters for S. aureus H cell wall hydrolysates. The results of these experiments are shown in Table 22.

The Effect of Variable Factors on the Activity of Antibacterial Agent. It had been shown earlier (112) that the bactericidal and antirespiratory activity of ABA derived from unfiltered human serum could be reversed by prior exposure of staphylococci to coagulase. The ability of exogenous coagulase to decrease cellular susceptibility was readily apparent. The relationship of coagulase production to reversal of ABA was investigated by a comparative study in which S. aureus E33 a coagulase-producing virulent organsim, S. aureus E33V a non-coagulase-producing virulent mutant, and S. epidermidis an avirulent noncoagulase-producing organism were used. The results indicated that exogenous coagulase was less effective in neutralizing ABA activity against strain E33 than against either strain E33V or S. epidermidis (Table 23). The relative "percent" reversal of ABA due to the addition of coagulase was 10%, 36%, and 49.5% for S. aureus E33, E33V and S. epidermidis respectively. These data indicated that S. aureus E33 was afforded less protection due to exogenous coagulase and suggested that inherent coagulase production may play a greater role in the breakdown of the host defense mechanism thus accounting for the decreased susceptibility of S. aureus E33

TABLE 22

Paper chromatography of S. aureus H and S. aureus H52A5 cell wall.

Substance	R_f value with detection by developing spray reagents			
	Silver nitrate	Periodate: Schiff's	Ninhydrin	Molybdate
Standards				
Glucose	0.579	-	-	-
Glucosamine	0.430	-	0.430	-
Ribose	0.684	-	-	-
Ribitol	0.632	0.650	-	-
Alanine	-	-	0.256	-
Unknowns				
Strain H				
Spot # 1	-	0.040	0.060	-
Spot # 2	0.079	0.110	0.110	0.106
Spot # 3	-	-	0.261	-
Spot # 4	0.450	-	0.446	-
Spot # 5	0.674	0.654	-	-
Spot # 6	0.741	-	-	-
Strain H52A5				
Spot # 1	-	0.040	-	-
Spot # 2	0.079	0.110	0.110	-
Spot # 3	-	-	0.251	-
Spot # 4	0.420	.516	0.423	-
Spot # 5	0.549	-	-	-
Spot # 6	0.679	-	-	-

TABLE 23

Interaction between ABA and coagulase on staphylococcal permeability.[a]

Culture	Conc. of ABA (μg/ml)	Counts/min/ml of supernatant fluid with coagulase (μg/ml)		
		0	0.5	1.0
S. aureus E33	0	932	981	980
	100	2750	2661	2209
	500	4622	4117	4225
S. aureus E33V	0	948	792	842
	100	3492	2449	2049
	500	4871	2962	3341
S. epidermidis	0	1322	1005	1152
	100	2561	1832	1991
	500	4114	2294	1919

[a]Staphylococci were grown to late logarithmic phase in TSB at 37°C, harvested washed and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0). Cellular density was adjusted to 200 K.U. and cells were labeled as described in Table 3. Coagulase was added in the appropriate concentrations and the cellular suspension was incubated at 25°C for 30 min. Cells were harvested by centrifugation and resuspended in ABA:buffer solution for 8 hr at 37°C under CO_2 1.5-2.0 lb/in.^{2}. Following ABA exposure, cells were removed by centrifugation and 1.0 ml of supernatant fluid was mixed with 9 ml of scintillation fluor to measure the leakage of ^{14}C cytoplasmic substance from the cell. Data are the mean values of three experiments.

to ABA.

Significant reversal of human ABA was demonstrated at low concentrations of coagulase and was in agreement with manometric studies previously reported (113). In contrast, the antirespiratory activity of ABA prepared from animal sera required a 2-4 fold increase in the concentration of coagulase necessary to demonstrate a reversal effect similar to that observed with the human serum preparation (Tables 24 and 25). In the data presented in Tables 24 and 25 a comparative study of the degree of respiratory inhibition between cells exposed to animal sera ABA and cells exposed to heat-inactivated animal sera ABA was made. These results clearly are based on the assumption that heat-inactivated ABA possesses little if any antibacterial effect. That this assumption was correct was a matter of experimental observation after performing numerous such experiments. However, so that such data is not discounted as a matter of conjecture, the results presented in the Appendix for Tables 24, 25 clearly indicated the ABA preparations used were active in their native state and inactive following autoclaving. In both situations insoluble material was separated by centrifugation before the staphylococci were exposed to supernatant portions. To supplement the studies presented in the Appendix further experiments were performed with an additional variety of control factors, e.g., cells exposed to coagulase only and cells unexposed to any exogenous substance except for buffered 1% glucose. These results have been incorporated into Tables 24 and 25.

Based on the amount of ABA necessary to inhibit significantly the respiration of staphylococci, this represented a 12-16 fold increase in the potency of rat and rabbit ABA. Myrvik had previously reported a serum

TABLE 24

Interaction of coagulase and ABA of rat origin.[a]

ABA Prep. No.	Exp. No.	µl O_2 consumed by staphylococci exposed to RAT ABA and varying concentrations of coagulase (µg/ml)						Coagulase (µg/ml)	Heat-inactiv. ABA ± S.D.	Control cells ± S.D.	Relative % differ.
		0	25	100	125	150	250	500			
2	1	35	-	-	41	-	74	70	66 ± 6	71 ± 5	51
	2	27	-	-	49	54	-	53	50 ± 5	57 ± 6	53
3	3	28	-	31	49	-	-	-	52 ± 2	-	47

[a] S. aureus serotype XII was grown to mid-logarithmic phase in TSB at 37°C, harvested, washed and suspended in 10^{-4} M sodium phosphate buffer (pH 7.0) at a cellular density of 500 K.U. (1.43 mg dry wt/ml). Cells were exposed to coagulase for 30 min at 25°C with intermittent manual shaking, prior to the addition of ABA (0.75 mg/ml). ABA exposure was for 1 hr at 4°C prior to respirometry. Data are mean values obtained from duplicate samples in each experiment. Relative % difference is defined as $100 - \left(\frac{\text{ABA without coagulase}}{\text{Heat-inactiv. ABA}} \times 100\right)$. A dash indicates experiment was not performed.

TABLE 25

Interaction of coagulase with ABA of rabbit origin.[a]

ABA Prep. No.	Exp. No.	µl O_2 consumed by staphylococci exposed to Rabbit ABA and varying conc. of coagulase (µg/ml)				Coagulase (µg/ml)	Heat-inactiv. ABA ± S.D.	Control cells	Relative % differ.
		0	250	350	500	500			
1	1	47	75	-	98	-	94 ± 6	-	50
	2	49	-	61	-	-	77 ± 7	-	37
	3	30	50	-	65	-	67 ± 7	-	56
2	4	27	27	-	93	80	80 ± 7	65 ± 7	58
	5	35	39	-	108	87	90 ± 2	93 ± 2	62

[a] Experimental conditions were identical to those described in Table 24, except that rabbit ABA was used in place of rat ABA. Data are the mean values obtained from duplicate samples in each experiment. Relative % difference is defined as $100 - \left(\frac{\text{ABA without coagulase}}{\text{Heat-inactiv. ABA}} \times 100\right)$. A dash indicates experiment was not performed.

Appendex to Tables No. 24, 25

Effect of ABA of animal oxygen on S. aureus serotype XII.[a]

ABA Prep. No.	µl O_2 consumed/hr		
	Native ABA	Heat-inactiv. ABA	Control cells
Rat No. 2	28 ± 4	53 ± 2	57 ± 3
Rat No. 2	38 ± 8	62 ± 2	79 ± 1
Rabbit No. 2	34 ± 5	80 ± 1	82 ± 1
Rabbit No. 2	39 ± 1	82 ± 1	86 ± 2

[a] Each fraction was tested for activity against 500 K.U. of S. aureus serotype XII harvested after 10-16 hr of aerobic growth in 100 ml TSB at 37°C on a rotary shaker. Manometric assay was performed in the presence of 10^{-4} M PO_4 buffer (pH 7.0) and 0.055 M-glucose (1%).

bacteriocin active against both staphylococci and B. subtilis which he found to be most potent in the sera of rats and rabbits. These findings are in agreement with our results. However, Myrvik was unable to demonstrate coagulase neutralization of the serum bacteriocin with cell free culture supernatants. We used a highly concentrated and purified form of coagulase in concentrations 3-4 fold greater than that previously used (113). We were able to reverse the antirespiratory effects of rat ABA by prior treatment of staphylococci with 250 μg of coagulase/ml. Rabbit ABA activity however was not significantly neutralized until the concentration of coagulase was raised to 500 μg/ml. We were further able to demonstrate that the antirespiratory effect of ABA of human origin, directed against cell wall deficient forms of staphylococci was not neutralized, even in the presence of 500 μg of coagulase/ml. The inability of coagulase to reverse ABA inhibition of respiration was evident. From our studies with spheroplasts, we demonstrated that respiration of the osmotically fragile forms was decreased by 50% - 89% even in the presence of coagulase; while L-forms showed a similar degree of susceptibility, their respiration was inhibited by 45% - 83% following pretreatment with coagulase (Tables 26, 27). These results strongly indicated that the main site of ABA activity, the cell membrane, was not afforded protection by coagulase in the cell wall deficient forms. Cell wall adsorption of coagulase with consequent alteration or masking of cell surface receptor sites for ABA appeared to be the mechanism by which coagulase reversal of ABA occurred as originally postulated by Yotis (113).

With the knowledge that ABA activity was dependent on ionic strength as was the activity of the serum bacteriocin described by Myrvik, we attempted

TABLE 26

Effect of coagulase on ABA activity against osmotically-fragile staphylococcal spheroplasts.[a]

Exp. No.	µl O_2 consumed/hr by spheroplasts exposed to ABA and varying conc. of coagulase (µg/ml) ± S.D.					
	0	500	osmotically shocked spheroplasts	Heat-inactiv. ABA	Control spheroplasts	Relative % differ.
1	4 ± 2	10 ± 1	1	30 ± 4	35 ± 1	89
2	3 ± 1	7 ± 3	1	30 ± 4	27 ± 6	89
3	27 ± 2	25 ± 5	0	49 ± 7	53 ± 2	50

[a] Experimental conditions were identical to those described in Table 13 except that staphylococci were exposed to 500 µg of coagulase/ml for 30 min at 25°C prior to ABA exposure. Spheroplasts used in Experiment No. 3 were exposed to lysostaphin for only 1 hr instead of 3 hr at 37°C. The relative % difference is defined as $100 - (\frac{\text{Native ABA}}{\text{Control}} \times 100)$.

TABLE 27

Effect of coagulase on ABA activity against staphylococcal L-forms.[a]

Exp. No.	µl O_2 consumed/hr by L-forms exposed to ABA and varying conc. of coagulase (µg/ml) ± S.D.			Heat-inactiv. ABA	Control L-forms	Relative % differ.
	0	500	shocked L-forms			
1	3 ± 1	2 ± 1	0	18 ± 2	12 ± 3	83
2	5 ± 1	7 ± 3	0	14 ± 3	14 ± 4	65
3	19 ± 1	14 ± 3	0	34 ± 6	25 ± 3	45

[a] Experimental conditions are identical to those of Table 14 except that L-forms were exposed to 500 µg of coagulase/ml for 30 min at 25°C prior to ABA exposure. The relative % difference is defined as $100 - \left(\frac{\text{Native ABA}}{\text{Heat-inactiv. ABA}} \times 100\right)$.

to uncover further similarities between the two preparations. Myrvik et al. (71) reported that serum bacteriocin was dependent on both calcium and bicarbonate ions for activity. Table 28 presents the data from viability studies performed in the presence of additional bicarbonate or calcium ions and ABA. Both these experiments and the leakage experiments presented in Table 29 indicated the presence of bicarbonate ions alone inhibited ABA activity most significantly, while calcium ions either alone or with bicarbonate inhibited ABA to a lesser degree. Bicarbonate ions reduced ABA activity by 30.8% while calcium ion containing solutions reduced the ABA effect on staphylococcal viability by 13%. In the leakage experiments, the bicarbonate ion effect was dominant even in the presence of calcium ions. These findings are in agreement with Yotis and Ortiz (115) who determined that calcium ions were unable to restore ABA activity to rat serum once it had been inactivated with sodium citrate.

Partial Characterization of the Physical and Chemical Properties of Antibacterial Agent. Under the experimental conditions of density gradient isoelectric focusing in the pH 3-10 range (Fig. 2), antibacterial activity was found in the single protein peak obtained from the 110 ml column eluant. After siphoning off the dense electrode solution the remaining density gradient was collected in fractions of 2.5 ml volume. The pH and ultraviolet absorbance (280 nm) were measured for each fraction obtained. These data were plotted on rectilinear graph paper: the pH and absorbance (280 nm) on the ordinate ("y" axis), the number of the corresponding fraction on the abcissa ("x" axis). Of the more than 40 fractions collected from the column only

TABLE 28

Viability of staphylococci exposed to ABA and various ions.[a]

Ions	Colony forming units of staphylococci per 0.1 ml (10^{-5} dilution)		
	Heat-inactivated ABA	Native ABA	Relative % differ.
None	91	22	75.9
HCO_3^- [b]	61	50	45.1
Ca^{++} [c]	53	34	62.7
HCO_3^- [b] & Ca^{++} [c]	45	35	62.6

TABLE 29

Effect of various ions on activity of ABA.[a]

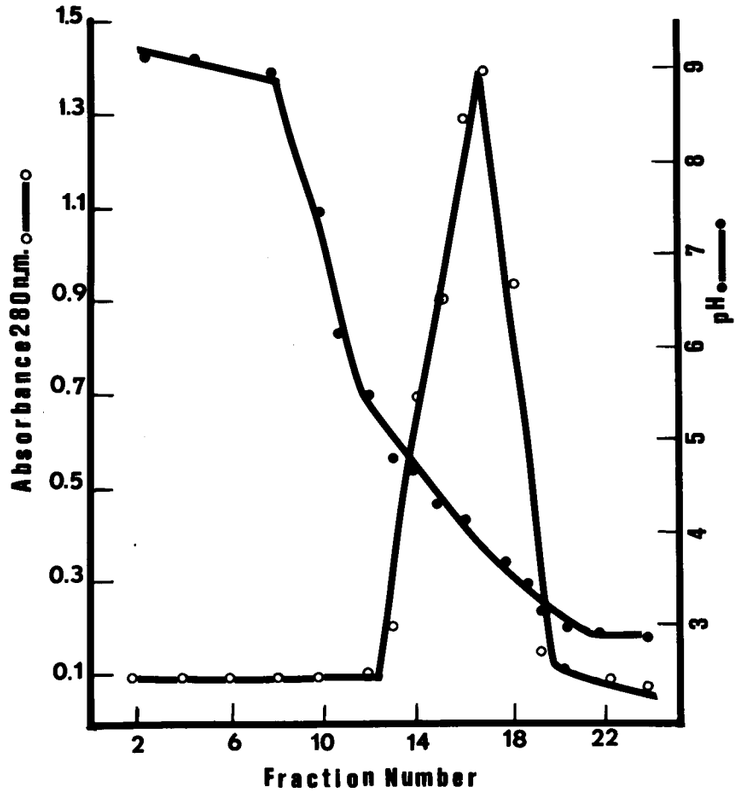
Ions	Counts/min/ml of supernatant fluid with ABA and various ions		
	Heat-inactiv. ABA	Native ABA	Relative % differ.
None	1120	788	29.7
HCO_3^- [b]	1027	934	16.7
Ca^{++} [c]	1036	856	23.6
HCO_3^- [b] & Ca^{++} [c]	1135	956	14.7

[a] Experimental conditions for growth and labelling of cells were as described in Table 3 except that cellular density was adjusted to 100 K.U. ABA (100 µg/ml) exposure was for 18 hr at 37°C under CO_2, 1.5 - 2.0 lb/in.^{2}. Data are mean values obtained from 3 experiments performed in duplicate. The relative % difference is defined as $100 - \left(\frac{\text{Native ABA}}{1120} \times 100\right)$.

[b] HCO_3^- was present as sodium bicarbonate at a 0.006 M concentration in double distilled deionized water, with final distillation over permanganate.

[c] Calcium concentration was 10^{-4} M as calcium chloride in permanganate distilled water.

Figure 2. Isoelectric focusing of human ABA in the pH 3-10 range. Absorbance measured at 280 n.m. (open circles) was plotted against pH (solid circles). Protein peak indicated the isoelectric point of ABA to be pH 4.80



those in which protein absorbance (280 nm) was measured were plotted on the graphs. Figure 2 shows a protein peak which spreads from fraction #14 to #18 inclusive, a total of 12.5 ml or 11.3% of the 110 ml sucrose density gradient volume. According to Catsimpoolas (12b) sufficient diffusion can occur within the hour's time necessary to drain the column following the cessation of isoelectric focusing so as to spread the peak over an area more than 2-3 times greater than that which was occupied within the electrical field during isoelectric focusing. Thus ABA could have concievably occupied only 3-4% of the column's total volume and this value is within the limits for a homogeneous protein (N. Catsimpoolas - personal communication). The experimental results indicated a highly purified ABA protein was subjected to the isoelectric focusing experiment presented in Figure 2. Through the use of narrower pH ranges (pH 3-6 and pH 4-6) the resolution of the isoelectric focusing method was substantially increased. ABA was demonstrated to have a mean isoelectric point (pI) of 4.73 (Fig. 3) when determined from five separate experiments all within 0.15 pH units of the mean value in the range pH 3-6. In the range pH 4-6, ABA had a mean isoelectric point of 4.80 (Fig. 4) determined from three separate experiments. All values were within 0.13 pH units. This fluctuation in the isoelectric point was a reflection of the high carbohydrate content of ABA. In all experiments (Fig. 3) 30 mg of ABA was focused for 48 hr to 72 hr at 500 volts with decreasing amperage throughout the experiment. The elution pattern obtained from experiments in the range pH 3-6 (Fig. 3) was obtained by fractionating the column into 44 portions of 2.5 ml each. The base of this protein peak was rather wide,

Figure 3. Isoelectric focusing of human ABA in the pH 3-6 range. Absorbance measured at 280 n.m. (open circles) was plotted against pH (solid circles). Mean isoelectric point of ABA was pH 4.73.

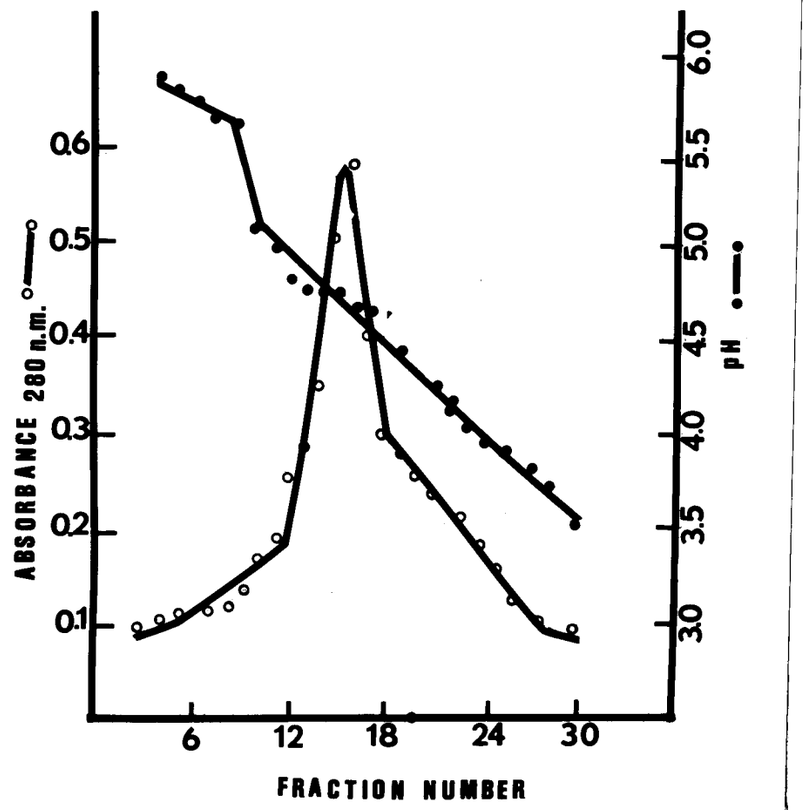
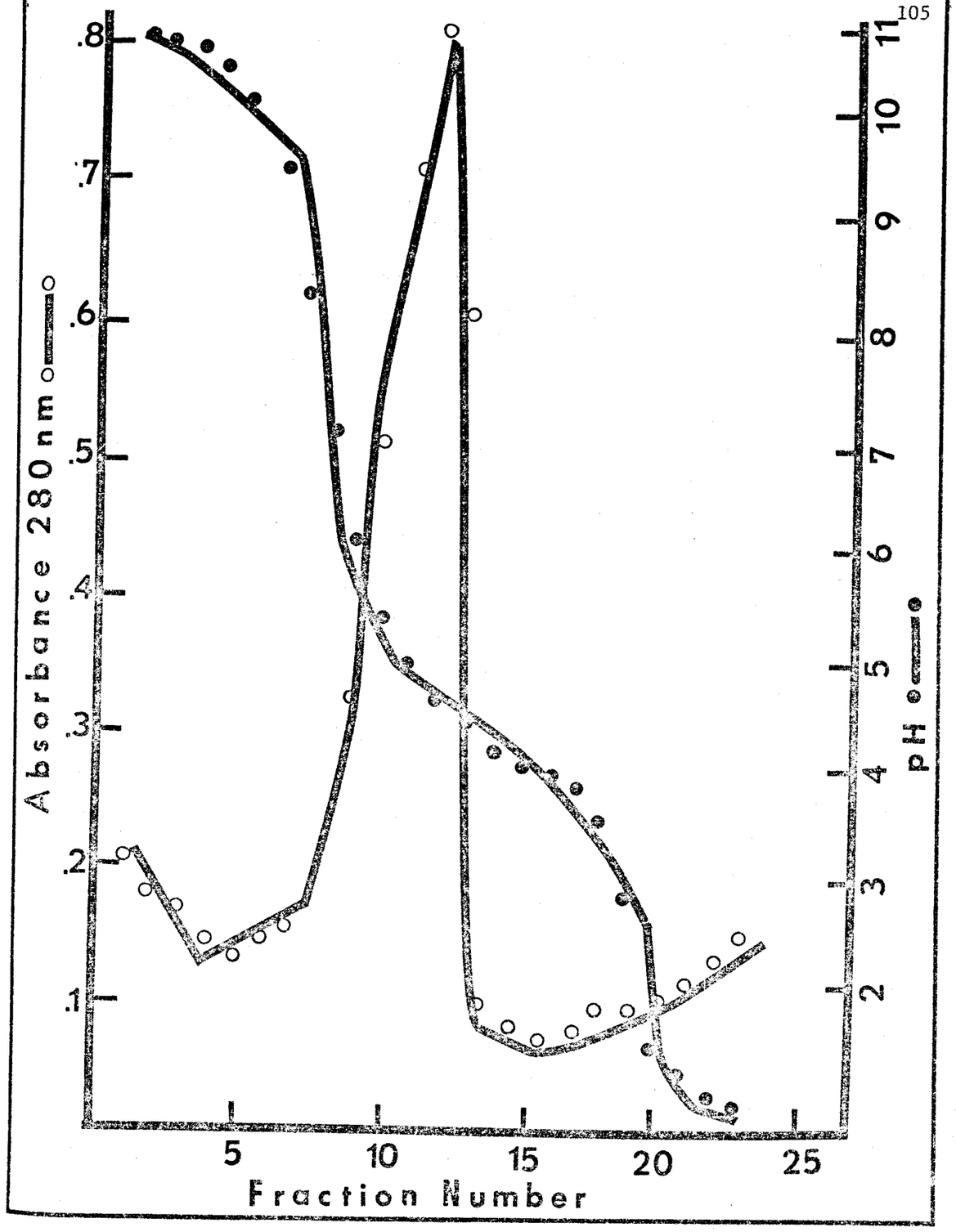


Figure 4. Isoelectric focusing of human ABA in the pH 4-6 range. Absorbance measured at 280 n.m. (open circles) was plotted against pH (solid circles). Mean isoelectric point of ABA was pH 4.80.



extending from fractions #12 to #24 inclusive. However the major protein peak was eluted within a 15 ml volume or 13.6% of the column capacity. Due to diffusion we can realistically assume a 2 to 3 fold increase in the area occupied over that of the focused protein. Therefore the major peak most probably occupied less than 5% of the column. Due to the presence of the long trail we can assume that ABA was a heterogeneous protein or a single protein which was present in multiple forms similar to an isozyme. To further resolve the exact isoelectric point of ABA, experiments were performed in the range pH 4-6 (Fig. 4). The resultant figure is a composite of three experiments during which isoelectric focusing of 25 mg of ABA was performed for 56 hr at a constant voltage of 500 v. The column was maintained at 4°C, as in all experiments, during the course of the run. Following fractionation into 24 portions each 4.8 ml the pH and absorbance at 280 nm were determined. The single protein peak obtained eluted in a volume of 24 ml between fractions #9 and #14. Taking into account the diffusion factor within an hour, the area originally occupied by the protein was approximately 9 ml or 8.1% of the column capacity. These results suggested that ABA may be a heterogeneous protein since the upper limits of the area occupied by a homogeneous protein is closer to 5%. Similar experiments with the ethanol soluble portion of Cohn's fraction IV-1 indicated that this bactericidal preparation (Table 7) could be resolved into two peaks by isoelectric focusing between pH 3-6, 3-10 (Fig. 5). The peak at pH 4.85 yielded 11.0 mg of lyophilized protein after dialysis, whereas the peak at pH 4.35 yielded only 2.5 mg of lyophilized protein when a 30 mg sample was applied to the column. Manometric studies demonstrated that both fractions possessed antirespiratory activity and each

Figure 5a. Isoelectric focusing of Cohn-Ethanol ABA in the pH 3-10 range. Absorption measured at 280 n m (open circles) was plotted against pH (solid circles). Resolution of the protein into two peaks (A) pH 6,10, and (B) pH 4.85 was demonstrated by isoelectric focusing.

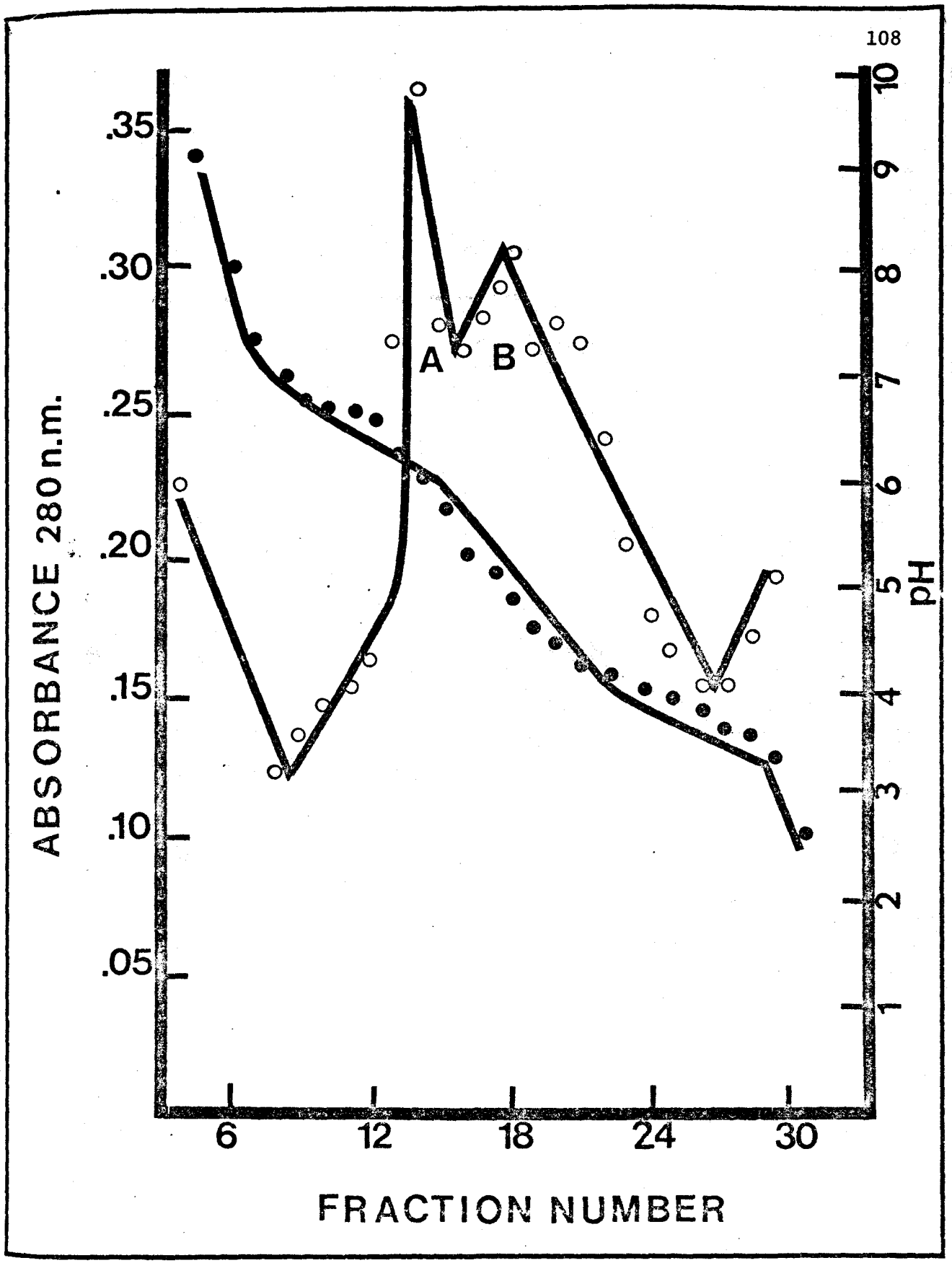

ABSORBANCE 280 n.m.
.35
.30
.25
.20
.15
.10
.05
A
B
6
12
18
24
30
FRACTION NUMBER
pH
1
2
3
4
5
6
7
8
9
10

Figure 5b. Isoelectric focusing of Cohn-Ethanol ABA in the pH 3-6 range. Absorption measured at 280 n.m. (open circles) was plotted against pH (solid circles). Resolution of the protein into two peaks (a) pH 4.85, and (b) pH 4.35 was demonstrated by isoelectric focusing.

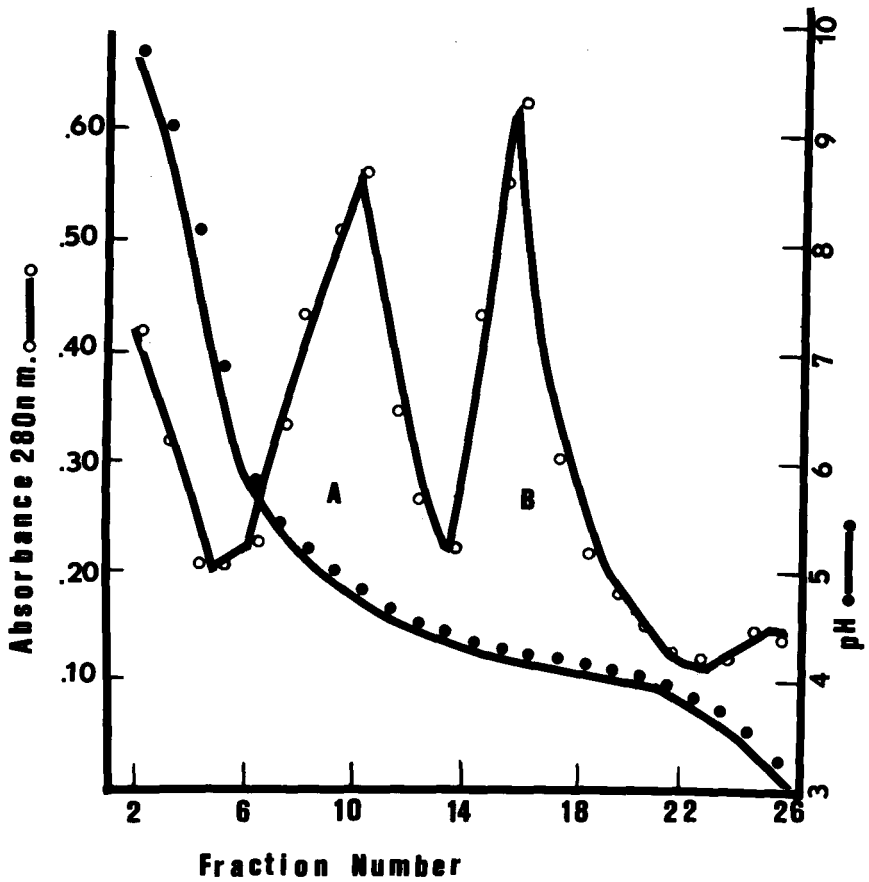

Absorbance 280nm.
.60
.50
.40
.30
.20
.10
A
B
pH
10
9
8
7
6
5
4
3
2
6
10
14
18
22
26
Fraction Number

Figure 6. Immunoelectrophoresis of Cohn-Ethanol ABA components: (a), CE - ABA (2 mg/ml) vs. anti-human ABA; (b), pH 4.85 peak (2 mg/ml, obtained from CE - ABA following isoelectric focusing) vs. anti-human ABA; (c) pH 4.35 peak (2 mg/ml, obtained from CE - ABA following isoelectric focusing) vs. anti-human ABA.

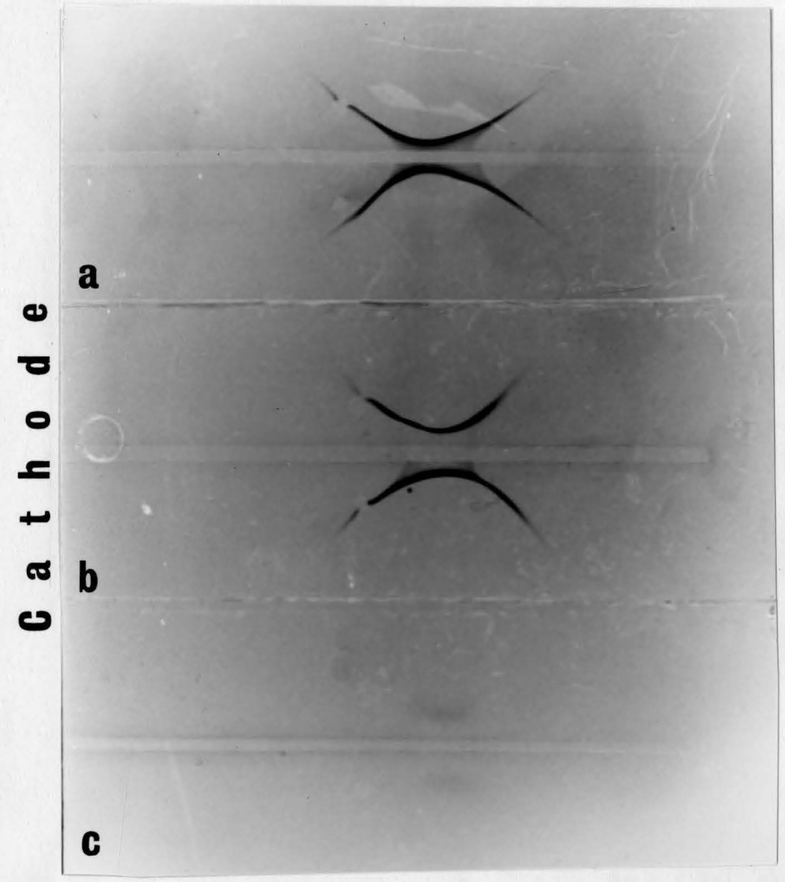


Figure 7. Polyacrylamide gel disc-electrophoresis: (a), whole human serum; (b), human ABA (200-300 µg/gel). Migration was anodic in tris glycine buffer (pH 8.5).

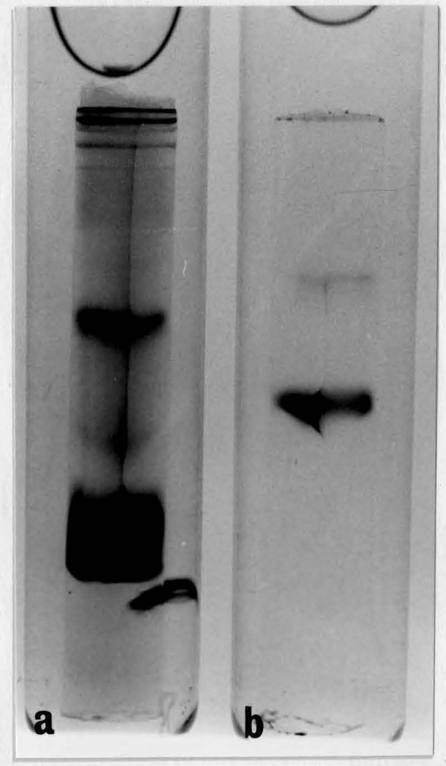
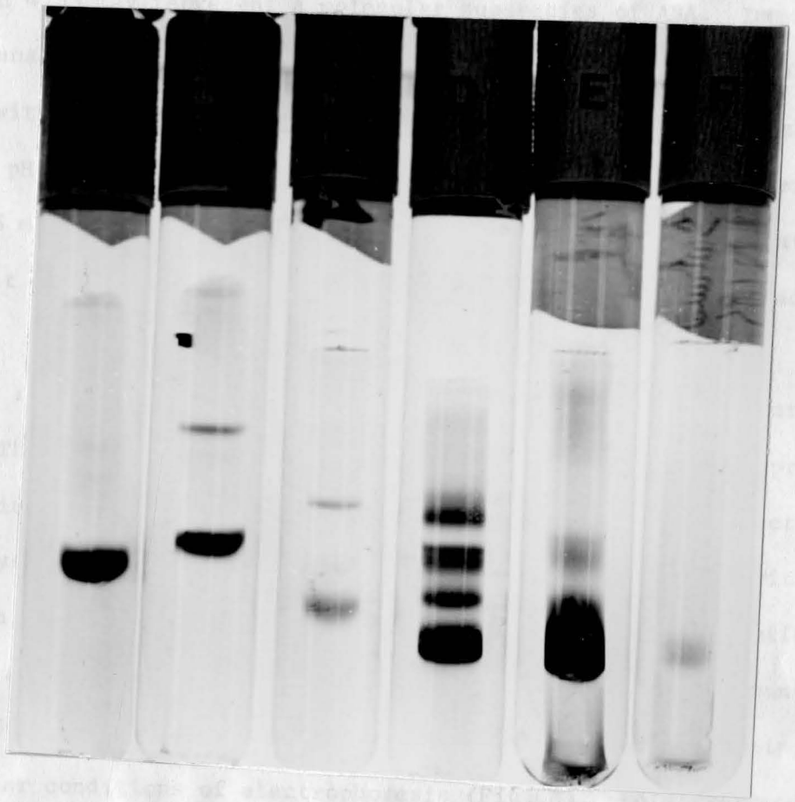


Figure 8. Disc electrophoresis of ABA and Cohn Ethanol ABA (CEABA). Gels were electrophoresed in the following buffer systems A and B anionic tris-glycine (pH 8.5). Gels C to F were run in cationic buffer system, β-alanine-acetic acid (pH 4.5). (A), ABA; (B), CEABA; (C), ABA; (D), CEABA; (E), pH 4.85 peak from CEABA isoelectric focusing (see Fig. 5b); (F), pH 4.35 peak from CEABA isoelectric focusing (see Fig. 5b). All gels in one buffer system were run simultaneously at 5 m amp/gel for 1.5 - 2.0 hr or until the marker dye was 0.5 cm from end of gel column. Protein concentration per gel was 250 μg/ml.



one required 2 to 3 mg of protein per ml to produce a 50% inhibition in oxygen consumption in the assay system. Consequently it appeared that the peak at pH 4.85 corresponded closely to ABA which was shown to have an isoelectric point of pH 4.73 in the range pH 3-6 and pH 4.80 in the range pH 4-6. The peak at pH 4.35 may represent a molecular subspecies of ABA. Immunoelectrophoretic analyses of the two components indicated the peak at pH 4.85 reacted strongly with rabbit antihuman ABA antisera as did the unfractionated CE-ABA, while the pH 4.35 peak gave only a weak response (Fig. 6). Antigen concentration (5 mg/ml) was chosen to give optimal precipitin line formation with the weakest antigen (pH 4.35 peak). Antisera was undiluted and had a titer of 1:64.

The results of polyacrylamide gel discelectrophoresis appear in Figs. 7 and 8. The antibacterial agent appeared to be a heterogeneous protein demonstrating 1 major and 1 minor protein band in the anionic electrophoretic system at pH 8.5 after 1 to 2 hours at 5 m amp per gel with an increase in voltage from 50 v to 150 v (Fig. 7). The cationic buffer system (β-alanine and glacial acetic acid pH 4.5) separated an equal amount of ABA protein (250 μg/gel) into 2 major protein bands and 1 minor protein component under similar conditions of electrophoresis (Fig. 8). Polyacrylamide separating gels were 7.5% acrylamide while stacking gels and sample gels contained 2.5% acrylamide.

Polyacrylamide disc-electrophoresis of CE-ABA under identical experimental conditions in an anionic buffer system indicated that CE-ABA resolved into an identical pattern as ABA with an increase in the density of the minor band closer to the cathode (Fig. 8). In the cationic buffer system

CE-ABA yielded more than 7 distinct protein bands. Clearly then CE-ABA appeared to be a heterogeneous mixture of serum proteins.

Previous studies in this laboratory (114) have indicated ABA to be immunologically related to α globulin. The electrophoretic mobility of ABA correlates with that of an α globulin protein and supports the previous immunoelectrophoretic studies.

The heterogeneous pattern, obtained with ABA on disc electrophoresis indicated the presence of exogeneous substances in the preparation. Because other serum components were present in trace amounts in the ABA preparation, Ouchterlony immunodiffusion was performed to distinguish ABA from those serum proteins suspected to be contaminants of ABA. Transferrin and orosomucoid were distinguished from ABA (Figs. 9 and 10), even though traces of both substances were seen by immunoelectrophoresis. Precipitin arc formation due to reactions between ABA and antisera to either transferrin or orosomucoid was attributed to the possible contamination of antigens used in the preparation of commercial antisera. Ouchterlony analysis of α_1 antitrypsin at a concentration of 0.8 mg/ml and ABA at an equal concentration against antihuman ABA antisera by the method of Feinberg (29b) indicated that ABA and α_1 antitrypsin were similar proteins (Fig. 11).

Qualitative carbohydrate analyses were performed by descending flow paper chromatography. Adequate separation of the two unknown hexose spots did not occur unless the chromatograms were allowed to run for between 36 to 40 hr (Fig. 12). In Fig. 12 separation of the carbohydrate content of an ABA hydrolysate revealed the presence of three distinct spots. These unknown sugars corresponded to the known standards of galactose, mannose and fucose.

Figure 9. Ouchterlony immunodiffusion. Top row: (a), anti-human ABA; (b), anti-human transferrin. Middle row: (c), human ABA (10 mg/ml); (d), human transferrin (10 mg/ml); (e) human ABA (10 mg/ml). Bottom row: (f), whole human serum; (g), anti-whole human serum.

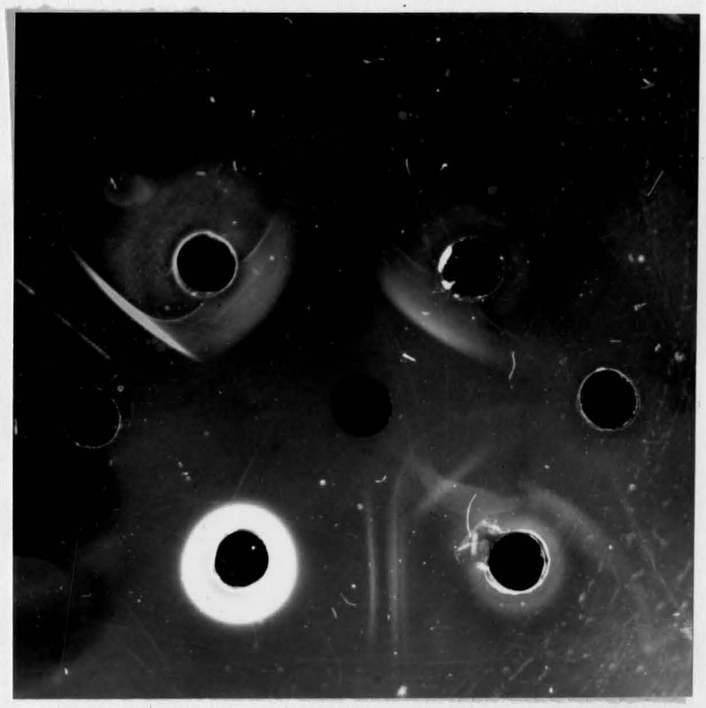


Figure 10. Ouchterlony immunodiffusion of selected serum proteins. Antigens in each plate from top clockwise: (1), whole human serum; (2), human urinary α_1 glycoprotein (10 mg/ml); (3), human ABA (10 mg/ml); (4), human ABA (5 mg/ml) and orosomucoid (5 mg/ml); (5), orosomucoid (10 mg/ml); (6) human ABA (10 mg/ml). Antisera in center wells: (A), top left, polyvalent anti-human α-globulin; (B), top right, antiwhole human serum; (C), bottom anti-human ABA.

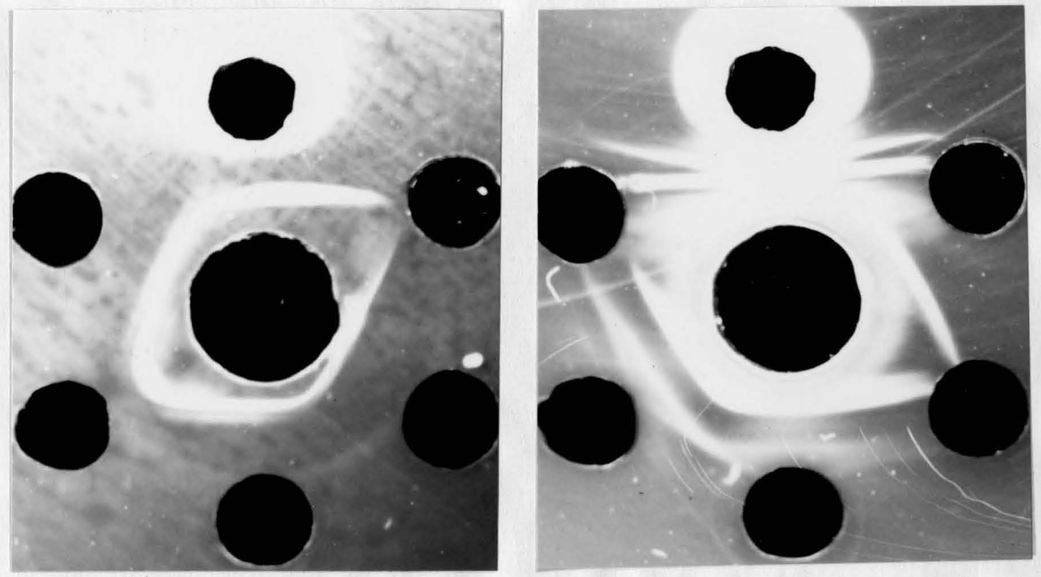


Figure 11. Ouchterlony immunodiffusion of ABA and α_1-Antitrypsin. Fig. 11a, Antigens are the same both top and bottom. They are (No. 1, 3) ABA (1 mg/ml), No. 2, 4) commercially prepared α_1-antitrypsin (0.88 mg/ml); top well; horse antisera to whole human serum, bottom well rabbit anti-sera to human ABA. Fig. 11b differs from 11a in that the α_1-antitrypsin was prepared by the method of Bundy and Mehl. Fig. 11c antigen ABA was diluted serially starting at 2 mg/ml (well No. 1) to 0.625 mg/ml (well No. 6) vs. rabbit antihuman ABA. Fig. 11d repeats the experiment using α_1-antitrypsin as the antigen No. 1 (0.88 mg/ml); No. 2 (0.32 mg/ml); No. 3 (0.16 mg/ml) vs. rabbit antihuman ABA.

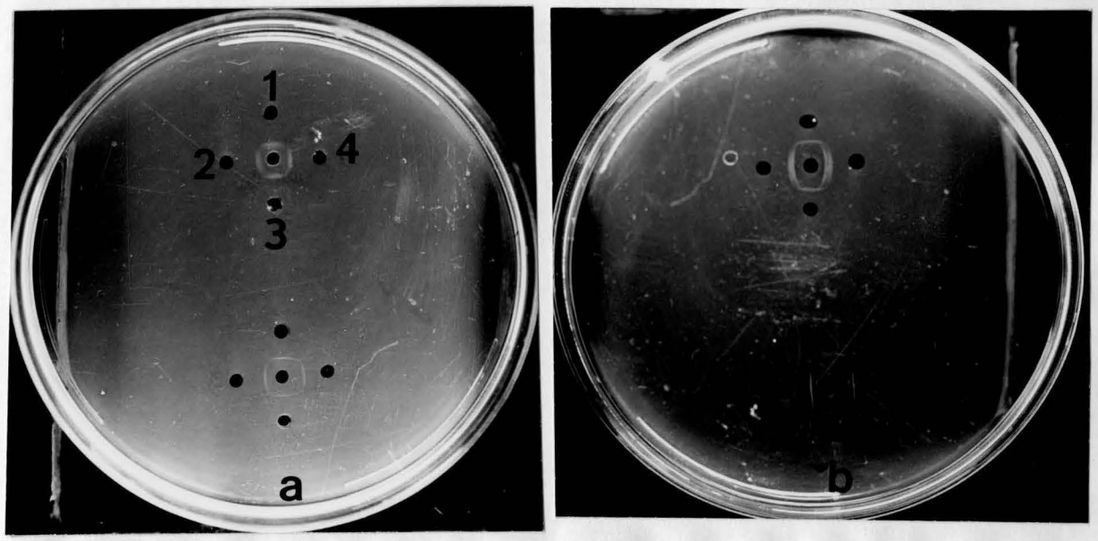


Figure 11 (Contd.)

Fig. 11e and f are identical experiments using rabbit antihuman α_1-antitrypsin(e) and rabbit antihuman ABA (f) antisera. The antigens are α_1-antitrypsin (AT) (0.88 mg/ml) in each of four wells on the left, while ABA (0.88 mg/ml) was placed in four wells on the right. Fig. 11g, α_1-antitrypsin at 3 concentrations (0.88 mg/ml, No. 1; 0.32 mg/ml, No. 2; 0.16 mg/ml, No. 3; and ABA 0.1 mg/ml, No. 4) vs. antisera to human α_1-antitrypsin. The bottom pattern repeats the experiment with ABA at 1.0 mg/ml concentration. Fig. 11h from top clockwise antigens are whole human serum No. 1; ABA (0.3 mg/ml), No. 2; commercially prepared α_1-antitrypsin (0.88 mg/ml), No. 3; α_1-antitrypsin (Bundy and Mehl method) (1.0 mg/ml); No. 4; ABA (1.0 mg/ml), No. 5; and α_1-antitrypsin (0.88 mg/ml), No. 6 vs. antisera to whole human serum.

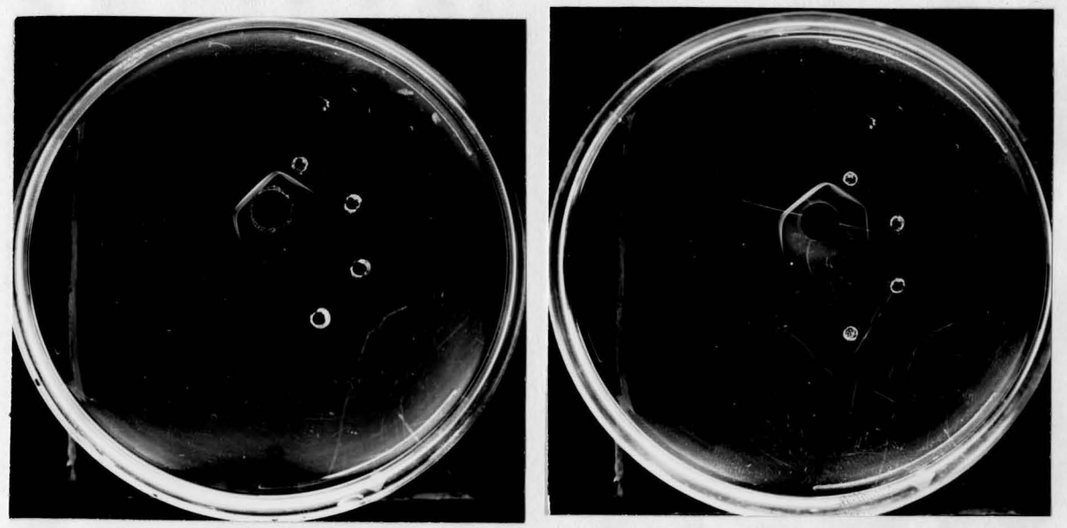


Figure 12. Paper chromatography of human ABA for carbohydrate analysis. Descending flow paper chromatogram was allowed to run for 40 hr in n-butanol:acetic acid: water solvent. ABA hydrolysate separated into 3 distinct spots in 40 hr time period. From left to right spots on line of origin are ABA (unk), glucosamine (gln), fucose, mannose (man), galactose (gal), and glucose (glc).

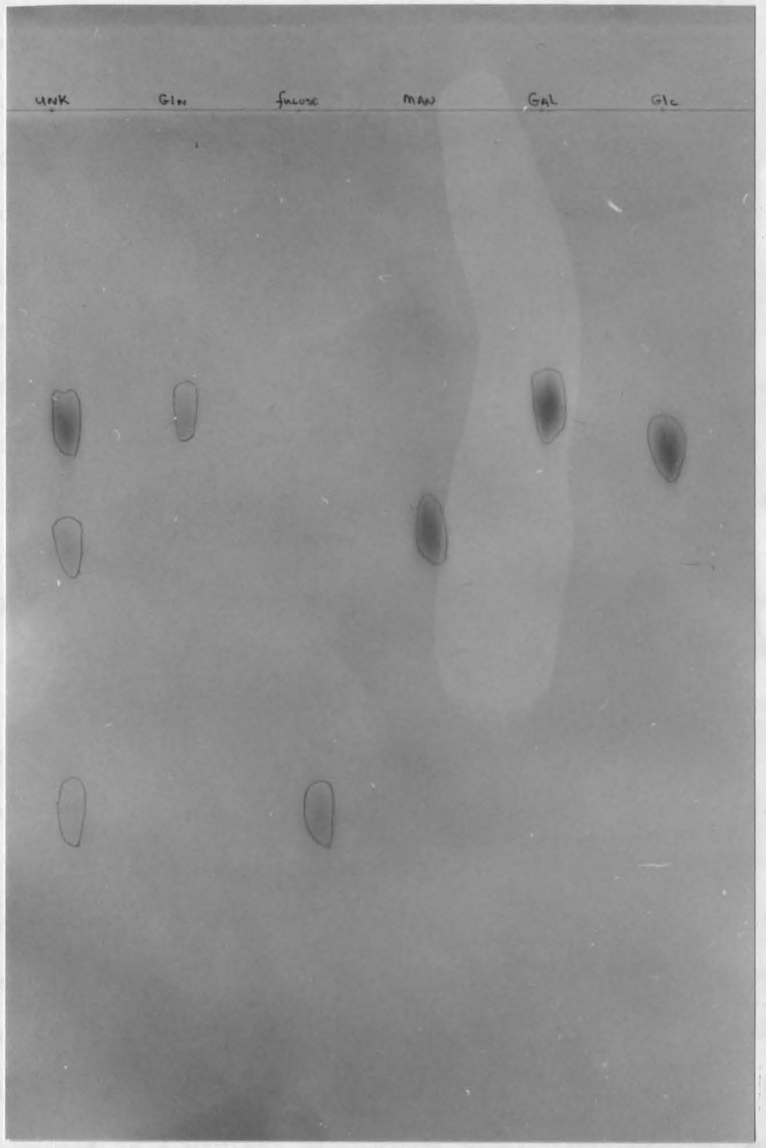


Table 30 presents the respective Rf values and Rglc values obtained in each of the two solvent systems employed. The results of the glucose oxidase test indicated that no glucose was present in ABA. The results of the glucose oxidase test are based on the colorimetric determination of the corresponding oxidation of the dye dianisidine. Glucose most likely is oxidized to glucono-1, 5-lactone and producing H_2O_2 in the procedure.

Chromatograms which were developed by the Elson-Morgan reaction yielded a bright red spot among the unknown spots which descended only a short distance from the origin. This spot reacted in a manner similar to that elicited with the glucosamine standard and was considered to be a hexosamine. No other sugars were detected qualitatively by paper chromatography in ABA hydrolysates.

The total carbohydrate content of ABA was 22.56%; this was composed of 11.08% of protein bound hexoses, essentially galactose and mannose in 3:1 ratio as determined by the measurement of light absorption of acetone extract of each spot. Hexosamines were found to be present at a concentration of 0.86%, this value is in agreement with that obtained by quantitative amino acid analyses. The remaining carbohydrates were found to be 6.3% fucose and 4.32% sialic acid (Table 31). The use of a 10 min boiling time in the fucose determinations allowed for maximal color development of the methyl pentose sugar in the presence of cysteine. The protein content of ABA accounted for the remaining 80% of the glycoprotein by weight. ABA protein content was determined by a variety of methods which included the Lowry method, 83.2%; the biuret method, 78.4%; and micro-Kjedahl total nitrogen assay, 12.8 mg N /ml or 80% protein (Table 31). This loss of

TABLE 30

Qualitative paper chromatography of ABA carbohydrate content.

Carbohydrate	Solvent A [a]		Solvent B [b]
	R_f [c]	R_{glc} [d]	R_f [c]
Glucose	0.143	1.000	0.221
Mannose	0.184	1.234	0.286
Galactose	0.133	0.872	0.187
Fucose	0.322	2.063	0.385
Glucosamine	0.123	0.872	0.128
ABA unknown			
Spot # 1	0.138	0.900	0.181
Spot # 2	-	1.276	0.210
Spot # 3	0.335	2.035	0.398

[a] N-butanol: acetic acid: water (52:32:16).

[b] N-butanol: pyridine: 0.1 N Hcl (5:3:2).

[c] R_f values obtained on 20 hr chromatograms.

[d] R_{glc} values refer from the center of standard glucose spot.

TABLE 31

Chemical composition of antibacterial agent.

Component	Experimental Procedure		Concentration mg/100mg
Carbohydrate	Hexoses		
	Anthrone	10.62)	11.08
	Tryptophan	11.54)	
	Hexosamine		0.86
	Fucose		6.30
	Sialic Acid		4.36
	Total CH_2O		22.56
Protein	Lowry		83.26
	Biuret		78.37
	Total N x 6.25 (Micro-Kjedahl)		80.00

carbohydrate material may account for the concomitant loss of antibacterial activity. In relation to these findings, the effective removal of sialic acid from ABA was accomplished through the use of neuraminadase (Vibrio cholera). Removal of sialic acid from ABA also effectively reduced its antimicrobial activity in preliminary manometric studies. One can assume sialic acid therefore plays a vital role in the biological properties of the molecule.

Calculation of amino acid residues by quantitative amino acid analyses indicated ABA to contain 77.26% protein and confirmed the preliminary protein determinations described above in Table 31. Amino acid analyses were performed with three separate preparations of ABA. The data presented in Table 33 has been compiled from the three analystical runs and represents the mean value of amino acid present in ABA. The data are expressed as μg of amino acid/100 μg sample and are based on a 99.90% recovery of total sample analysed.

The acidic amino acid content while high also represents the glutamine and asparagine residues as well as glutamic acid and aspartic acid. The amino acid analyses were performed to determine the relative proportion of basic amino acids, which antimicrobial activity had been associated with in the literature (95). There seems also to be an increased concentration of valine and leucine over the other neutral amino acids. This is of interest

TABLE 32

Chemical composition of Cohn-Ethanol ABA

Component	Experimental procedure	Concentration mg/100mg
Carbohydrate	Hexoses	
	Anthrone	12.50
	Hexosamines	0.40
	Fucose	5.20
	Sialic acid	6.18
	Total CH_2O	24.28
Protein	Lowry	78.56
	Biuret	73.21

TABLE 33

Amino acid analyses of ABA

Amino Acid	µg residue per 100 µg protein	% Residue recovered per sample
Lysine	7.98	9.52
Histidine	2.22	2.78
Ammonia	-	1.17
Arginine	6.78	7.75
Aspartic acid	7.26	9.08
Threonine	3.18	4.18
Serine	2.87	4.12
Glutamic acid	11.86	14.40
Proline	4.22	5.94
Glycine	1.03	1.67
Alanine	3.97	5.51
Cystine 1/2	4.07	5.54
Valine	5.36	7.13
Methionine	0.62	0.68
Isoleucine	1.01	1.32
Leucine	6.86	8.56
Tyrosine	3.34	4.65
Phenylalanine	4.63	5.90
TOTAL	77.26	99.90

for it indicates the presence of non-polar side chains which give rise to hydrophobic bonds. The presence of such bonds in proteins would indicate the possibility of a folded structure rather than a linear or pleated sheet structure. More importantly the non-polar side chains of valine and leucine may serve as the point of ABA attachement to the lipid laden staphylococcal membrane.

Molecular Weight Determinations. By reverse flow column chromatography a substantial number of proteins or glycoproteins were applied to a Sephadex G 200 superfine gel bed in order to calibrate the column according to the method of Andrews (5a). Upon completion of the calibration, which was performed by the sequential separation of two mixtures of two proteins each in duplicate, ABA was passed through the gel bed to ascertain its approximate molecular weight. Only when the elution volumes of sequential passages were within 2.0 ml for each protein were they recorded as true values. The results in Table 36 and Figs. 16 and 17 indicated the molecular weight to be between 55,000 (on passage through the protein calibrated column) to 74,000 (on passage through the glycoprotein calibrated column). The latter values probably are a truer indication of ABA molecular weight. The gel filtration studies were considered to be only preliminary studies by which the approximate range of ABA molecular weight could be estimated.

The use of SDS gel electrophoresis to determine the molecular weight of ABA has been reported in the literature to be a highly reliable technique (Shapiro et al.91a). The Weber and Osborn (105) modification of Shapiro and coworkers original method afforded us a relatively easy and fast pro-

TABLE 34

Molecular weight determination of ABA.[a]

Exp. No.	Standard	V_e	K_{av}	M.W.
1	Aldolase	72.5	0.1471	158,000
	Ovalbumin	101.5	0.3540	45,000
	Chymotrypsinogen	125.0	0.5288	25,000
	Ribonuclease A	150.0	0.7080	13,700
	Antibacterial Agent	95.0	0.3055	55,000
2	Fetuin	92.5	0.3260	49,000
	Ceruloplasmin	67.5	0.1340	180,000
	Pseudocholinesterase	57.0	0.0530	300,000
	Antibacterial Agent	87.5	0.2690	74,000

[a] The data are mean values from two experiments.

cedure by which molecular weight values could be determined within a day. The data presented in Fig. 16 shows the molecular weight of five known glycoproteins plotted against their relative electrophoretic mobilities. The molecular weight value of ABA was extrapolated to be 62,000 . The actual gels used in this study are pictured in Fig. 17. In those instances where more than 1 polypeptide chain was present, the molibility of the most dense band was recorded as the electrophoretic mobility of the major substance in the protein. All the glycoproteins used were obtained from commercial sources and may be contaminated with other serum proteins. Still a reasonable estimate of molecular weight can be made based on the findings presented here.

Desialyzed ABA. The electrophoretic mobility of ABA in the presence and absence of sialic acid was shown to differ only slightly as can be seen in Fig. 18. The effective removal of sialic acid by neuraminidase from ABA has been shown to decrease the electrophoretic mobility of the anodic migrating protein when assayed by SDS gel electrophoresis. In addition the removal of the second polypeptide (slower) component as seen by SDS gel electrophoresis appeared to be affected along with the sialic acid removal. This however may be due to a reduction in the protein content of the molecule on a weight basis. With the loss of sialic acid the activity of ABA as measured by manometric technique has been greatly diminished. The desialyzed ABA did not reduce the oxygen consumption of the staphylococci significantly. While native ABA caused a 32% reduction in the oxygen consumption by staphylococci per hr, the desialyzed ABA caused less than a 6% reduction in the oxygen uptake of the bacteria.

Figure 13. Molecular weight determination of ABA on protein calibrated Sephadex G-200 superfine gel column. K_{av} value for ABA indicated its molecular weight to be 55,000.

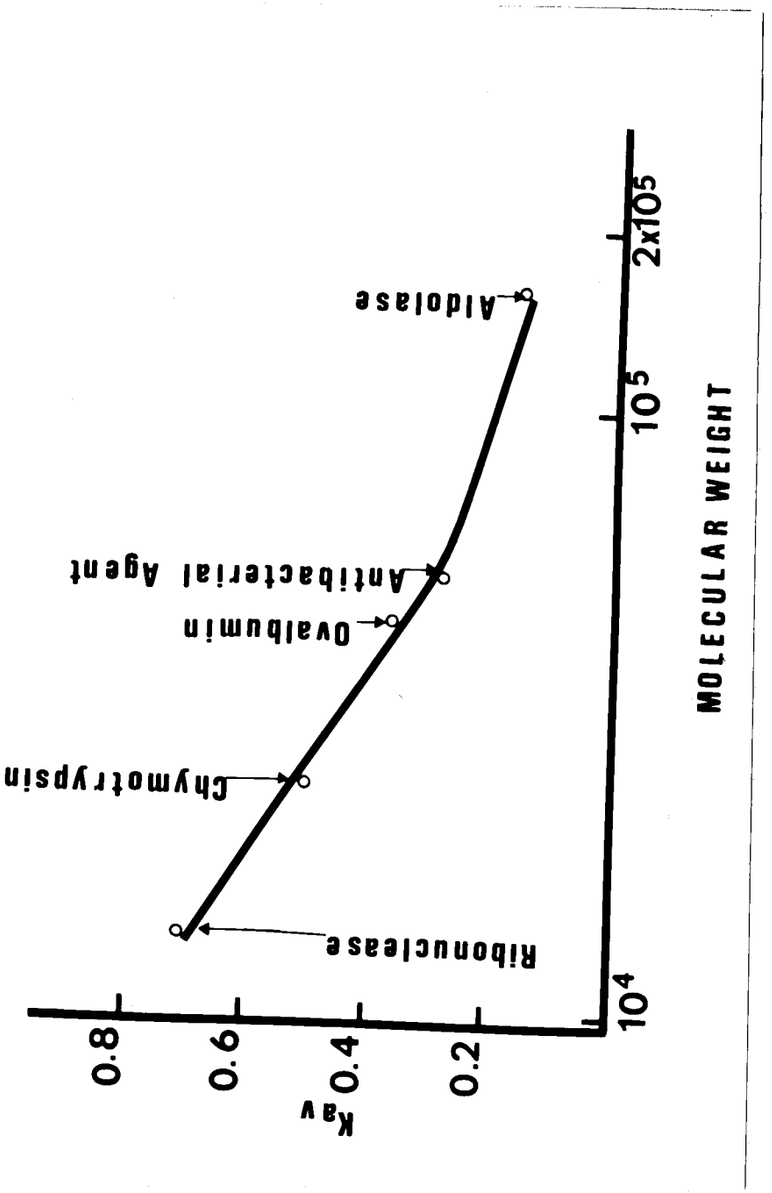
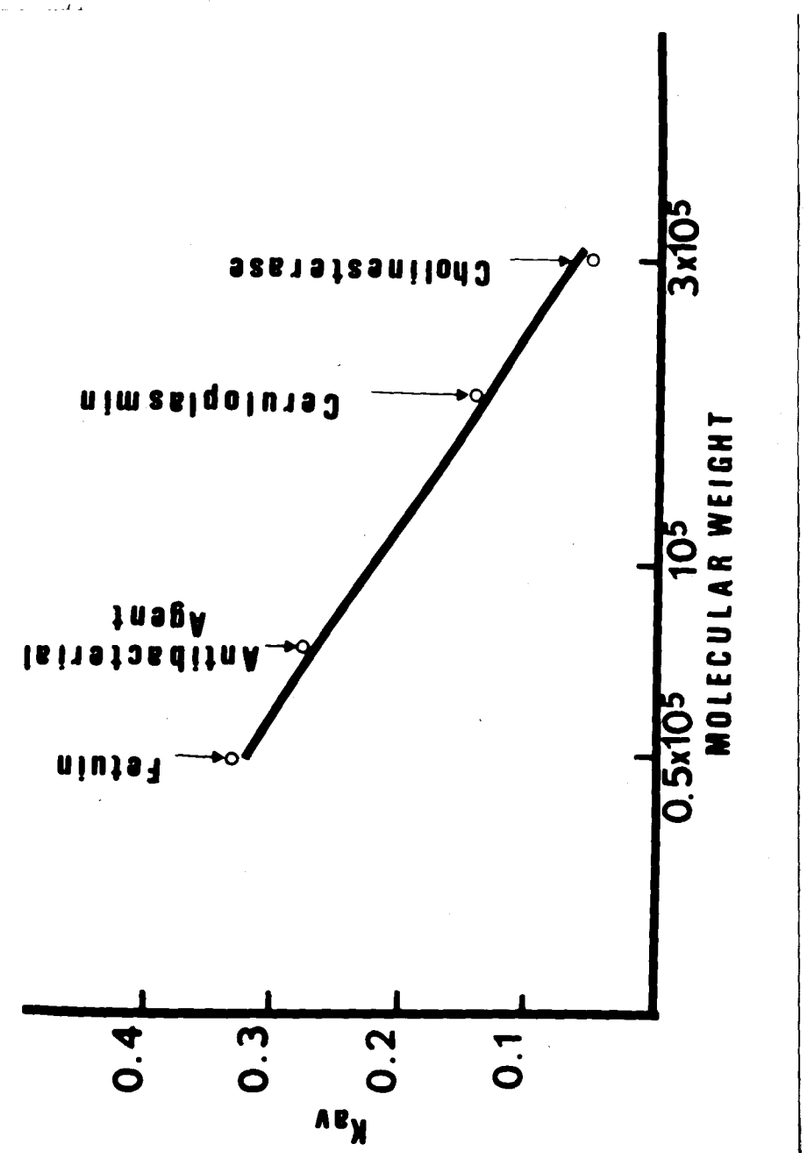


Figure 14. Molecular weight determination of ABA on glycoprotein calibrated Sephadex G-200 superfine gel column. K_{av} value for ABA indicated its molecular weight to be 74,000.



Comparative Analyses Between ABA and α_1-Antitrypsin. The immunological and electrophoretic patterns of ABA and α_1-antitrypsin shown in Figs. 11, 19, 20 illustrate the close similarity between the two preparations. Ouchterlony analysis (Fig. 11) strongly indicated that the ABA molecule may be α_1-antitrypsin. As can be seen in Fig. 11a, the commercial preparation of α_1-antitrypsin formed a line of identity with ABA when tested against horse antisera to whole human serum (top well) and rabbit antisera to human ABA (bottom well). Fig. 11b illustrates the reactions of α_1-antitrypsin (prepared by the method of Bundy and Mehl, 12a) and ABA versus the same antisera as above. Fig. 11c shows a serial dilution of ABA starting at a concentration of 2 mg/ml (No. 1) and rotating counterclockwise to 0.0625 mg/ml versus rabbit antihuman ABA. Fig.11d repeats the experiment in Fig. 11c using α_1-antitrypsin, the greatest concentration of which was 0.88 mg/ml (No. 1), 0.32 mg/ml (No. 2) and 0.16 mg/ml (No. 3); no precipitin lines are percievable beyond the No. 3 well.

Figs. 11e and f are identical experiments using rabbit antihuman α_1-antitrypsin (e) and rabbit antihuman ABA (f) antisera in the respective central antiserum wells. The antigens consisted of α_1-antitrypsin (0.88 mg/ml) in each of four (4) wells on the left, while ABA (0.88 mg/ml) was placed in the wells on the right hand side of the plate. Fig. 11g illustrates the reactions of α_1-antitrypsin at 3 concentrations (0.88 mg/ml, No. 1; 0.32 mg/ml, No. 2; 0.16 mg/ml, No. 3, and ABA, No. 4 (0.1 mg/ml) vs. antisera to human α_1-antitrypsin, the bottom well repeated the experiment using ABA at a concentration of 1.0 mg/ml. Fig. 11h tested from the top clockwise whole human serum; ABA (0.2 mg/ml); commercially prepared α_1-antitrypsin (0.88 mg/ml), α_1-antitrypsin (1.0 mg/ml, prepared by method of Bundy and Mehl, 12a); ABA (1.0 mg/ml) and α_1-antitrypsin 0.88 mg/ml). A line of identity can be seen between the innermost

Figure 15. Molecular weight determination of human ABA by SDS gel electrophoresis. Molecular weight of known glycoprotein standards are plotted against their electrophoretic mobility (E.M.). ABA molecular weight is then calibrated by the standard curve. Known molecular weight markers include pseudo-cholinesterase (M.W. 300,000, E.M. .0678); ceruloplasmin (M.W. 180,000, E.M. .0947); transferrin (M.W. 90,000; E.M. .1259); albumin (M.W. 69,000, E.M. .1502) and fetuin (M.W. 48,000, E.M. .1739). ABA electrophoretic mobility was .1569 which corresponded to a molecular weight of 62,000.

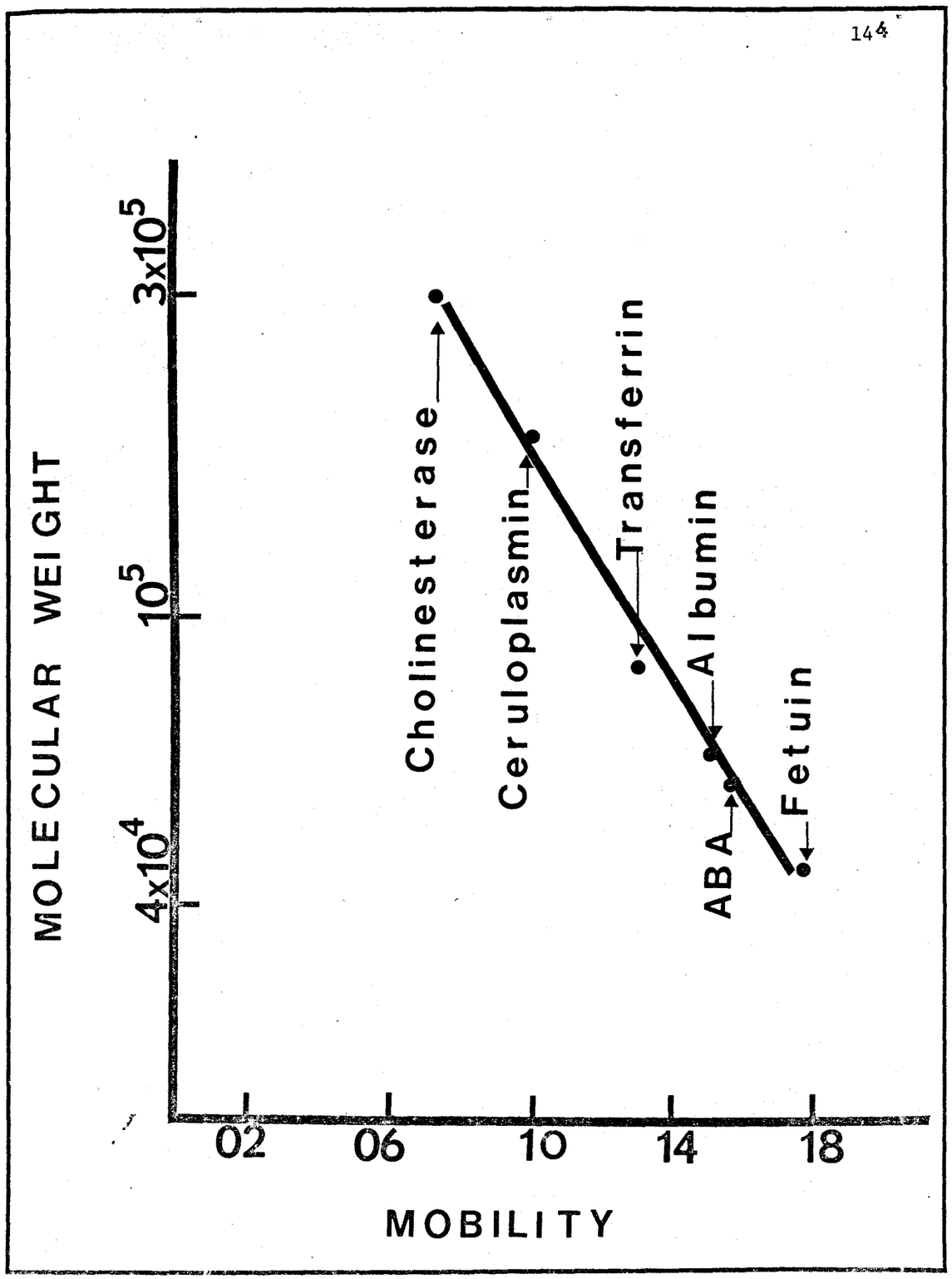
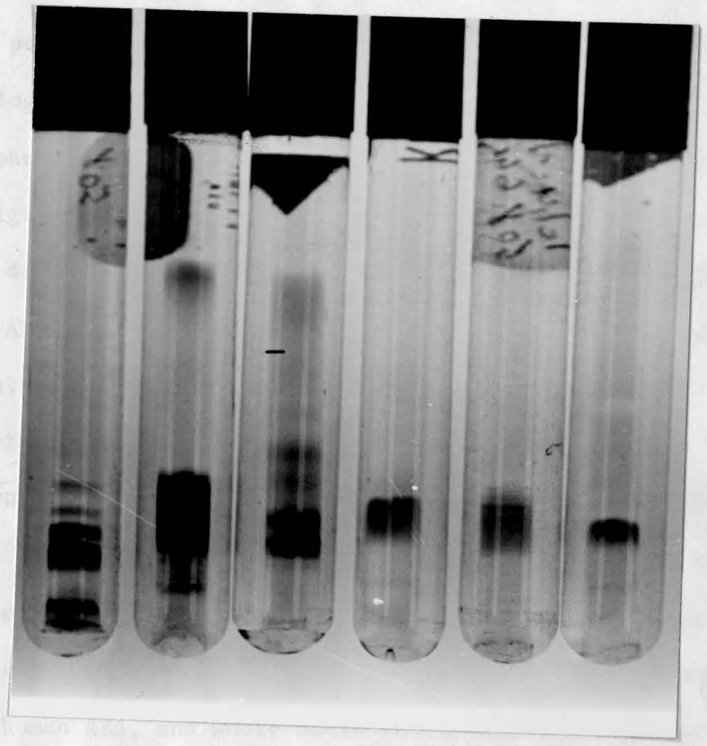


Figure 16. SDS gel electrophoresis of ABA and molecular weight markers. Glycoprotein markers are from left to right (A), pseudo-cholinesterase; (B), ceruloplasmin; (C), transferrin; (D), albumin; (E) fetuin; (F), ABA. Gels are inverted from position during electrophoresis. Migration was anodic or toward the top of the gel as seen in the photograph. Albumin was used as a protein standard reference marker.



component of whole human serum, α_1-antitrypsin, ABA and the Bundy and Mehl preparation of α_1 antitrypsin. These results strongly indicate that the ABA molecule may be immunologically similar to α_1 antitrypsin. These studies, however, only compare the immunochemical nature of the molecules; as an immunogen ABA could possibly be contained in α_1-antitrypsin or may contain similar groupings which present to the antisera forming animal as α_1-antitrypsin. The possibility remains however that both molecules may possess different biological activities.

Electrophoretically, ABA was shown to contain only 2 major components in a cathode migrating buffer system. While α_1-antitrypsin contained 3 and in one case 4 distinct protein bands. As can be seen in Fig. 19, the two components of ABA match perfectly with the fastest and slowest components of α_1-antitrypsin. Fig. 19d has an additional component which is slower-migrating than any others shown. All gels were loaded with 400 μg/ml of protein and all were subjected to electrophoresis simultaneously for 1.5 hr at 5 m amp/gel with a maximum voltage of 100 v. The results of these studies further strengthens the physiocochemical similarities between ABA and α_1-antitrypsin.

Immunoelectrophoretic analysis (Fig. 20) of all four α_1-antitrypsin preparations, human ABA, and whole human serum conclude the studies designed to indicate the similar physiocochemical nature of the two serum fractions. Fractions 1 (Fig. 20d, top well) and 4 (Fig. 20f, bottom well) most closely match the precipitin arc pattern of ABA when tested with rabbit antihuman ABA antisera. All antigen were present at a concentration of 2 mg/ml.

Antirespiratory activity of α_1-antitrypsin was not demonstrated with any of the four preparations isolated while the staphylococci were harvested

at various stages in the growth cycle, no one stage was any more susceptible to α_1-antitrypsin. Table 37 presents the results of these manometric studies and indicates that the antirespiratory activity associated with ABA could not be found in α_1-antitrypsin. This does not indicate the two preparations are different molecules, but rather that the different methods of preparation of the molecules resulted in a loss of biological activity from α_1-antitrypsin. At no time were we able to demonstrate activity in ABA if dialysis were carried out against chilled distilled water rather than cold running tap water when prepared by ammonium sulfate fractionation.

In a search for antitryptic activity in ABA, we were successful in demonstrating slight inhibition of trypsin hydrolysis of T.A.M.E. by ABA. Figure 21 illustrated the resultant curves for trypsin hydrolysis of T.A.M.E., soybean trypsin inhibitor of T.A.M.E. hydrolysis, ABA inhibition of trypsin and α_1-antitrypsin inhibition of trypsin. As can be readily seen in Fig. 21, ABA reduces the hydrolysis of T.A.M.E. very slightly, with the greatest decrease within the first three minutes. Soybean trypsin inhibitor greatly reduced the T.A.M.E. hydrolysis, while α_1-antitrypsin caused a significant but not as marked decrease in the activity of trypsin.

TABLE 35

Effect of α_1-Antitrypsin preparations on staphylococci.[a]

Prep. No.	Native Antitrypsin	Heat-inactivated Antitrypsin	Control Cells
1[b]	137	150	147
2[c]	84	85	80
3[d]	166	160	171
4[c]	88	84	94

a. S. aureus XII was grown for various periods of time (see below), harvested, washed and suspended in 10.4 M sodium phosphate buffer pH 7.0. Cells were exposed to the antitrypsin preparations for 1 hr at 4°C prior to respirometry, which was performed in the presence of 1% glucose (10 mg/ml). Data are the mean values of two experiments performed in duplicate for each preparation of antitrypsin.

b. Cells were grown to mid logarithmic phase harvested after 15 hr.

c. Cells were grown to late logarithmic phase harvested after 18 hr.

d. Cells were grown to early logarithmic phase harvested after 10 hr.

Figure 17. SDS gel electrophoresis of ABA (A) and desialyzed ABA (B). Both gels were run simultaneously for 4 hr at 8 m amp/gel. Protein concentration was 100 μg of either ABA or desialyzed ABA. Note the distinct decrease in electrophoretic mobility demonstrated by the desialyzed protein. Migration was toward the anode, as the gels are upside down the anodic end will be at the top of the photograph.

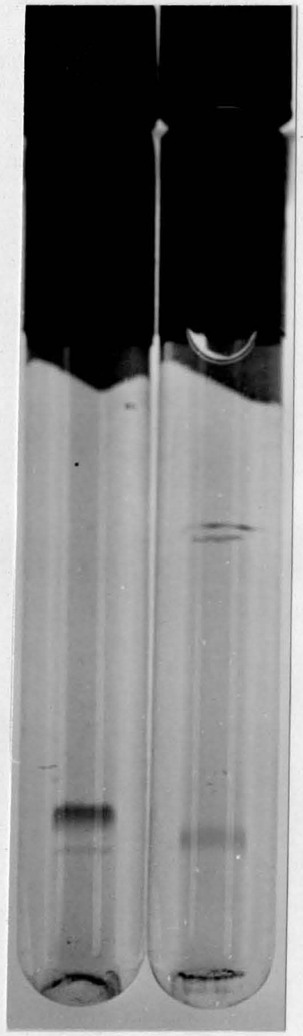


Figure 1 8. Cationic buffer system disc electrophoresis of ABA and α_1-antitrypsin. All gels were loaded with 400 µg/ml of protein and all were subjected to electrophoresis simultaneously for 1.5 hr at 5 m amp/gel with a maximum voltage of 100 v. Gels are (a), ABA, (b), α_1-antitrypsin (No. 1); (c), α_1-Antitrypsin, (No. 3); (d), α_1-antitrypsin, No. 4). For explanation of α_1-antitrypsin preparation numbers, see text. Cathodic end of gels is at bottom of photograph.

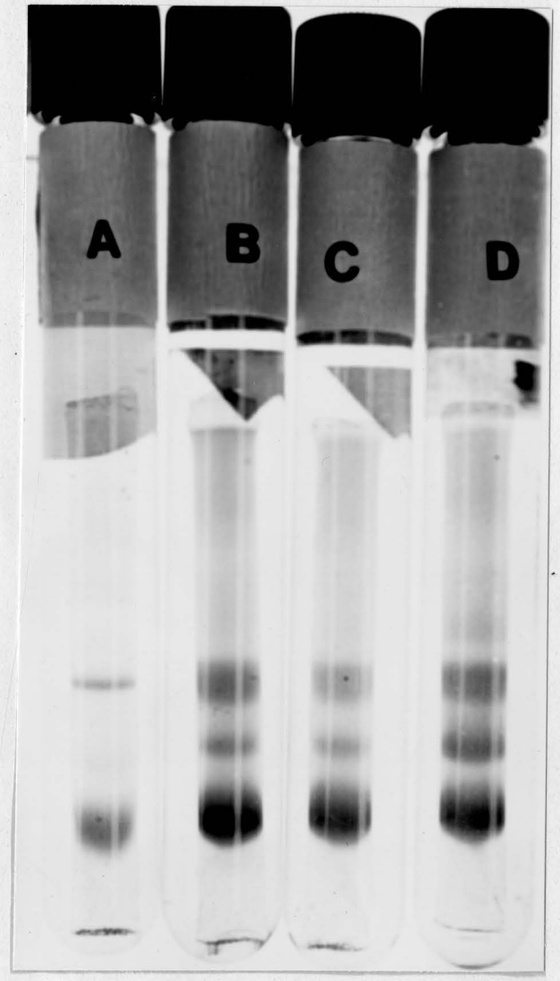


Figure 19. Immunoelectrophoresis of ABA, whole human serum and α_1-Antitrypsin. Antitrypsin was prepared by the method of Bundy and Mehl (12a). Fig. 20a, top well, whole human serum, bottom well, ABA (1.0 mg/ml) vs. horse anti-whole human serum antisera. Fig. 20b top well, ABA (1.0 mg/ml); bottom well, whole human serum vs. rabbit antihuman ABA. Fig. 20 c,d, top well α_1-antitrypsin preparation No. 1, (1.0 mg/ml); bottom well α_1-antitrypsin No. 2, (1.0 mg/ml) vs. anti whole human serum (c) and antihuman ABA (d). Fig. 20 e,f, top well, α_1-antitrypsin No. 3, (1.0 mg/ml); bottom well, α_1-antitrypsin No. 4 (1.0 mg/ml) vs. anti whole human serum (e) and anti human ABA (f).

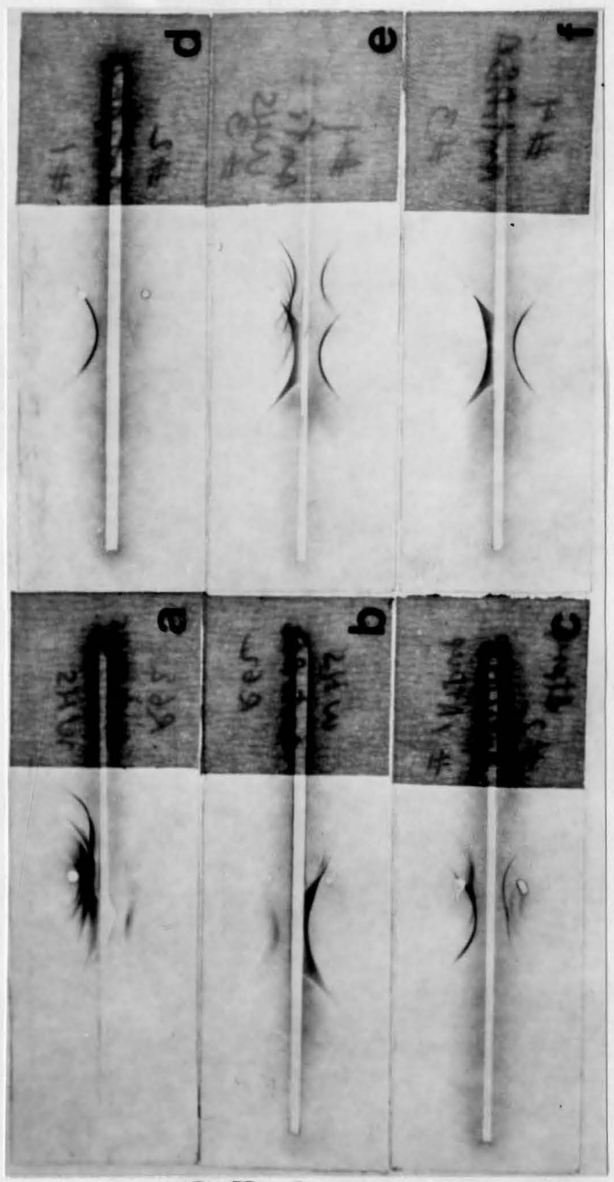
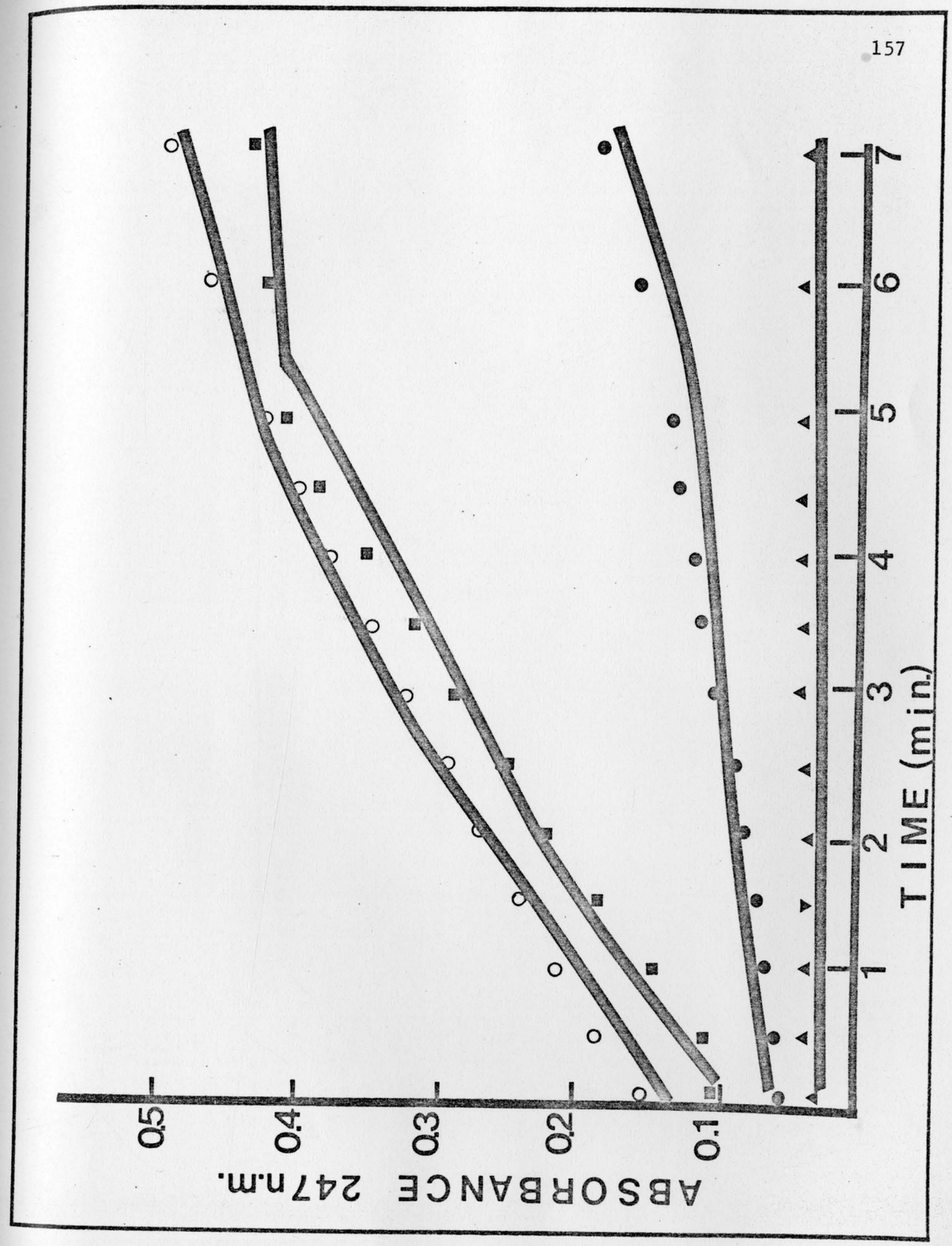


Figure 20. Inhibition of trypsin hydrolysis of T.A.M.E. Absorbance measured at 247 n.m. was plotted against time. In all experiments 2.6 ml of 0.05 M Tris buffer pH 8.1 containing 0.01 M $CaCl_2$ was pipetted into a 1 cm cuvette along with 0.3 ml of substrate (0.01 M T.A.M.E.). The effects of 0.1 ml of trypsin (40 μg/ml), open circles; 0.1 ml of trypsin (40 μg/ml) and 0.1 ml of soybean trypsin inhibitor (40 μg/ml), solid triangles; 0.1 ml of trypsin (40 μg/ml) and 0.1 ml α_1-antitrypsin (4 mg/ml), solid circles; and 0.1 ml of trypsin (40 μg/ml) and 0.1 ml of ABA (8 mg/ml), solid squares on the hydrolysis of T.A.M.E. are illustrated.



a Data is adapted from Yotis (112) and Yotis and Ekstedt (111).

b Protein concentration is the amount (dry wt.) necessary to achieve a 60 µl/hr reduction in the oxygen consumption of staphylococci.

c Units of activity are calculated by dividing $\frac{60\ \mu l\ O_2\ \text{consumed/hr}}{5\ \mu l/hr}$ as explained in Materials and Methods.

d Specific activity is calculated by dividing $\frac{\text{Units of Activity}}{\text{Protein concentration}}$ and is expressed as Units/mg/ml.

e $$\text{Purification} = \frac{\text{Specific activity of purified ABA}}{\text{Specific activity of original fraction}}$$

TABLE 36

Concentration of ABA from serum by ammonium sulfate fractionation. [a]

Ammonium sulfate fraction	Protein concentration (mg/ml) [b]	Units of activity/ml [c]	Sp. Act. [d]	Purification [c]
25% water soluble)) 33% water insoluble)	40 mg	12	0.30	00.00
33% water soluble)) 50% water insoluble)	30 mg	12	0.40	1.33
50% water soluble)) 62% water insoluble)	16 mg	12	0.75	2.50
62% water soluble)	2 mg	12	6.0	20.00

DISCUSSION

Several investigations reported in recent years were concerned with the antibacterial activity of serum (15, 23, 56). Other than the immunoglobulins, serum contains abundant antibacterial fractions such as plakin (2) leukin (94), β-lysin (21) and bactericidin (69). In recent years many of these and other factors have proven to be glycoproteins (65, 67). Recent reports indicate an increase in the concentration of α globulin glycoproteins subsequent to various microbial infections. Mitruka (65) demonstrated a 10-40% increase in the α globulin serum fractions of rats infected with Diplococcus pneumoniae. Murphy, Kaliner, and Clyde (67) reported a marked increase in the α_1 region of the glycoprotein electrophoretic pattern to be most characteristic of symptomatic malaria in human volunteers. Antibacterial agent (ABA) described by Yotis (112) in 1962 was demonstrated to inhibit bacterial respiration and viability, however the nature of the ABA molecule remained to be satisfactorily defined. For this reason the preliminary experiments were designed to further delineate ABA from known serum fractions.

The data presented in Table 1 indicated that of the ten serum fractions tested only Cohn's fraction IV possessed both bactericidal and antirespiratory properties similar to ABA. The electrophoretic mobility of Cohn's fraction IV placed it among the α globulins. In addition the effect of α_1 acid glycoprotein on the respiration of staphylococci was tested in

separate experiments to determine whether orosomucoid shared antibacterial properties with ABA. In preliminary manometric experiments utilizing orosomucoid prepared by Dr. R. Winzler (obtained through Dr. C. F. Lange) we could demonstrate no decrease in the oxygen consumption of those staphylococci exposed to the native protein as compared to unexposed cells or to cells exposed to the heat inactivated (autoclaved) orosomucoid. Therefore while orosomucoid may be a trace contaminant of ABA it is not responsible for the antirespiratory activity of the ABA molecule. There are certain features of ABA which distinguish it from any and all other serum fractions tested. For instance, ABA activity is limited to Gram positive organisms; in contrast to ABA, thrombin also inhibited the oxidation of glucose by Gram negative bacteria such as Escherichia coli, Pseudomonas aeruginosa, Proteus morgani and Aerobacter aerogenes. This inhibition was not reversed by coagulase and it could be demonstrated even in 0.1 M sodium phosphate buffer (pH 7.0) which alters the solubility of ABA and so negates its antirespiratory effect (111). The loss of biological activity in the presence of phosphate ions at a concentration $> 10^{-4}$ M and reversal of the inhibition with the addition of exogenous coagulase separate the ABA molecule from the other serum fractions tested, especially the β-lysin of Donaldson (21). In addition the electrophoretic patterns of α globulin and ABA are sufficiently similar to suggest a commonly shared antigen which does not react immunologically with β-lysin. The preparation of a highly active bactericidal factor from the α globulin fraction of serum served to confirm the relationship between ABA and α globulins (Table 7), as well as provided an alternate method of ABA isolation.

ABA was shown to reduce the level of respiration of staphylococci to less than 35% of that of control cells, while the ethanol-soluble α globulin preparation decreased staphylococcal respiration to less than 25% of the level attained by the corresponding control cells.

ABA demonstrated a 3-6 fold increase in specific activity above that of the commercially prepared α globulin fraction. The findings reported in Table 1 further indicated that only the α globulin fraction of serum, in addition to ABA, possesses antimicrobial activity against the staphylococci in the in vitro systems employed. Because ABA and α globulins shared similar electrophoretic mobility patterns a variety of available α_1 and α_2 globulin antisera were tested by immunoelectrophoresis for the development of a precipitin arc against both ABA and whole human serum (Table 2, Fig. 1). The results of these experiments verified that ABA was indeed an α globulin and more specifically may possibly be α_1 antitrypsin on the basis of immunoelectrophoretic (Fig. 1) and Ouchterlony double diffusion (Fig. 11) analysis. The lack of reactivity of ABA with the majority of the other antisera indicated that ABA was prepared in a highly purified condition. Human ABA only reacted with antisera to human ABA, whole human serum or human α_1 antitrypsin. On the other hand rabbit antisera to human ABA reacted to human ABA, whole human serum, human transferrin, human α globulin, plasma bovine transferrin, rat and rabbit ABA. Human transferrin and orosomucoid could be possible contaminants of human ABA, however they are distinct protein as demonstrated by Ouchterlony double diffusion (Fig. 9 and 10). In addition anti human transferrin antisera and anti human α_1 glycoprotein antisera does not react.

with human ABA on immunoelectrophoresis.

Whatever function ABA plays in affecting staphylococcal viability its antibacterial action depends on the concentration of the α globulin protein (Table 3). ABA prepared from both animal and human sera whether Seitz filtered or unfiltered possessed significant antibacterial activity at concentrations as low as 50 μg/ml. The fact that filtered ABA was still active helped to differentiate ABA from β-lysin which was retained by Seitz filtration and which could not be isolated from plasma.

Although whole cells of unrelated strains were shown to be susceptible to ABA under various conditions of time, concentration, and oxygen tension (Tables 1, 3, 5, 6), it was of major significance that those strains which were most susceptible to ABA demonstrated a decrease in the amount of radioactivity incorporated into their cytoplasmic membrane (Tables 10, 11). The amount of ^{14}C glucose found in the ethanol soluble fraction of ABA treated cells was 55% and 60% less than that in the corresponding control cells of serotypes XII and XI respectively. This observation confirmed our initial finding that serotypes XI and XII were most susceptible to ABA (Table 9); and invited speculation on the role of the cytoplasmic membrane in the ABA: bacterial cell interaction. The results demonstrated in these initial fractionation experiments led us to further studies designed to more conclusively implicate the cytoplasmic membrane as the site of action of ABA. The amount of ^{14}C-glucose incorporated into the staphylococci within 60 min at 37°C was determined prior to the fractionation of the organisms. We have demonstrated that both ABA exposed staphylococci and the corresponding con-

trol cells incorporated equivalent amounts of ^{14}C-glucose into the whole cells. Following washing of excessive exogenous ^{14}C-glucose, staphylococci containing similar concentrations of ^{14}C-glucose were fractionated. It was of importance to maintain the amount of radioactivity among the various washed cell preparations at equivalent values, in order that valid comparisons could be made following fractionation of staphylococci on the effect of ABA on the incorporation of glucose.

Cells treated with phenol were shown to have absorbed less ABA than untreated controls, an indication that alteration of the membrane lipid and protein content by phenol may have also resulted in the decreased absorption of ABA by the bacterial cell (113). To support the hypothesis that ABA primarily affected the cell membrane rather than the cell wall, various experiments utilizing cell wall deficient organisms were performed. The data presented in Table 12 demonstrates the degree of respiratory inhibition ABA exerted against a staphylococcal cellular homogenate. The mechanism of ABA action clearly does not depend on the integrity of the cell. The level of homogenate respiration following ABA exposure was decreased from that of the heat-inactivated ABA exposed staphylococcal homogenate. The absolute values for oxygen consumption by the unexposed homogenate preparations were higher than anticipated. However, these observations were understandable if we considered that rupture of the cell membrane by Mickle disintegration caused a sudden release of the cytoplasmic contents, allowing enzymes and substrates to come into contact and subsequently initiate oxidation reactions, e.g. glucose-6-phosphate to 6-phosphogluconate catalyzed by glucose-6-phosphate

dehydrogenase. The presence of a number of non-viable (i.e., cells which cannot replicate) yet intact cells, which may have survived the Mickle disintegration could also account for the amount of oxygen consumed. Nevertheless, the fact remains that ABA exposed cells or homogenates utilized less oxygen than their unexposed counterparts. In comparison to the oxygen consumption values recorded for control cells in other experiments, the control cells of the homogenate experiments used a relatively small amount of oxygen. This was understandable because cells were prepared in buffered solution and kept at 4°C without additional nutrients, while the homogenates were prepared. Consequently their metabolic rate was depressed beyond that of cells kept at 4°C for only 1 hr as in similar experiments.

These results lead to further studies in which ABA demonstrated enhanced antibacterial activity against cells deficient in cell wall. The experiments with lysostaphin induced osmotically fragile spheroplasts (Table 13) indicated the susceptibility of the enzymatically degraded cells was increased by 59% - 72% from the corresponding control spheroplasts; in comparison, ABA treated whole cells were inhibited by 50% - 58%. While osmotically fragile spheroplasts were shown to be markedly affected by ABA, L-forms of staphylococci demonstrated an even higher degree of susceptibility to ABA (Table 14). The level of respiration of L-forms exposed to ABA was inhibited by more than 75% from the control L-forms level. The observation that cell wall deficient organisms had significantly reduced their oxygen consumption following ABA exposure confirmed the hypothesis that the cell membrane was the primary site of ABA action. In addition, we have shown that ABA activity against cell wall deficient cells was not diminished from its

efficacy against whole cells. The rupture of the cytoplasmic membrane followed by the subsequent loss of cellular constituents was hypothesized to be a possible mechanism of the bactericidal activity exhibited by ABA. Clearly the biological activity of ABA was independent of cell wall destruction unlike lysozyme.

Recent reports have indicated the presence of antibacterial activities in the α globulin fraction of serum, however the mechanism of action of these antibacterial substances has rarely been discussed. Myrvik and Weiser (69) have described an electron microscopic study of B. subtilis following exposure to rabbit serum bactericidin. In their description of the resultant electron micrographs, the authors stated "the internal protoplasm shows extensive disintegration and a limited amount of granular debris is all that remains". Both the cell wall and the cell membrane appeared to be intact, although faintly outlined, following bactericidin exposure. It was evident that the serum bactericidin described by Myrvik and his co-workers caused cellular death by disruption of the internal protoplasmic pool containing all endogenous nutrients as well as the precursors for cellular structures. Myrvik and Leake (72) described the serum bactericidin as a growth inhibiting factor unrelated to properdin, lysozyme, and complement. Myrvik and his coworkers found rabbit and rat sera contained a more potent bactericidin than human, horse, bovine and guinea pig sera. The bactericidin activity was determined by measuring the turbidity of B. subtilis and S. epidermidis cultures grown in serial dilutions of serum (pH 7.2) at 37°C for 2 hr. The endpoint was expressed as the highest dilution of serum in which no visible

growth of B. subtilis was evident.

While antibacterial agent was also not related to any of these factors, it differed from the serum bactericidin in ion requirements (Tables 27, 28); ABA was clearly shown to be inactivated by the addition of bicarbonate ion and to a lesser degree in the presence of calcium ions. In addition the serum bactericidin system was shown to be inactivated in the presence of citrate, probably the result of salt formation rather than due to a lack of calcium ions (50). Donaldson and Marcus (20) extended this observation and stated that normal coagulation was a necessary step in the release of bactericidin activity, as heparin and citrate were incompatible with normal serum bactericidal activity. Myrvik and Leake (72) therefore concluded that their bactericidin system was very similar to the beta lysins described by Donaldson and Marcus (20) and Jacox (50) in that both bactericidin and beta lysins required calcium and bicarbonate ions; were shown to rise in "acute phase" serum; and were not related to either complement components C_1, C_2 or C_4 or to lysozyme.

In contrast to the findings of these investigators, we have described a bactericidal glycoprotein which can be prepared from both serum and plasma (Table 4) in equivalent concentrations. Furthermore, ABA failed to react with antisera to rabbit β-lysin protein, nor did anti-ABA antisera react with β-lysin (Table 2).

Consequently we can state that neither Myrvik and co-workers nor Donaldson and co-workers have described the same serum fraction that we have described as ABA.

In contrast to the Bacillus bactericidin system, the mechanism of which was not clearly elucidated, serum transferrin was shown to inhibit growth of microorganisms by binding essential iron (61, 88).

In a discussion of a paper presented by Ekstedt (28) at the New York Academy of Science, the question was raised as to whether the ABA was related to transferrin which has also demonstrated antistaphylococcal activity (88). It is now felt that the ABA as first described by Yotis and Ekstedt (111) and as a more purified ABA described by Yotis (112) is not transferrin. There are major differences between transferrin and the ABA reported in this study, for example, the mean isoelectric point of ABA was found to be 4.73, that of transferrin has been reported to be 5.45 and 5.80 for iron saturated and iron free molecules respectively (59). Isoelectric point values of 5.98 and 6.01 were reported for genetic variants of the transferrin molecule (54). The molecular weight of transferrin has been reported to be 90,000 (35). In comparison ABA was found to have a M.W. of 62,000. The carbohydrate content of transferrin was reported by Winzler (109) to be 5.5 percent while ABA has been found to contain 22.5 percent carbohydrate. Immunoelectrophoresis of ABA indicated the alpha globulin-like nature of the molecule (114) while transferrin has been characterized as a β_1-globulin (49). In addition we have shown that anti-ABA reacts with transferrin only at high concentrations (24 mg/ml) as opposed to alpha globulin which reacts with anti-ABA at low concentrations (2 mg/ml), (Fig. 1).

We have demonstrated that L-forms, spheroplasts and homogenates of staphylococci were susceptible to ABA; therefore, the cell wall was not essential for the bactericidal action of ABA to occur. However, much con-

sideration was given to the role of the bacterial cell wall as it related to ABA inhibition of whole cells. The results obtained indicated the cell wall to play a minor role in staphylococcal susceptibility to ABA. Yotis (113) previously had demonstrated that ABA was absorbed specifically by heat killed cells of susceptible organisms. In order to elucidate the role of the cell wall in the absorption of ABA we prepared purified whole cell wall preparations as well as the peptidoglycan fraction of cell wall from staphylococci. Classically the staphylococcal cell wall has been described as a lattice or net of N-acetyl glucosamine N-acetyl muramic acid polymers interconnected by pentaglycine bridges between the terminal carboxyl group of D-alanine in one tetrapeptide unit and the ϵ-NH_2 group of lysine in another (76). To this basic peptidoglycan backbone, ribitol teichoic acid has been postulated to be attached to the muramic acid residues by various modes of linkage (6, 34).

Our studies indicated that both whole cell wall preparations and the peptidoglycan fraction isolated from cell walls of the staphylococci only adsorbed ABA after two hours of incubation had elaspsed. The results in Tables 15 and 16 demonstrated that both cell wall and peptidoglycan, when present in a 10 fold greater concentration than ABA, could not adsorb ABA quickly enough for the cell wall to be the main site of activity. At no time were we able to adsorb ABA activity with either of these preparations in less than two hours. It should also be noted that after two hours, both the supernatant and residue fractions of the cell wall: ABA suspensions failed to inhibit staphylococcal respiration. The lack of activity in the cell wall residue was attributed to the strong affinity of the ABA molecule for the

cell wall of susceptible staphylococci which resulted in a stable ionic bond. The active regions of the ABA molecule are possibly either covered by cell wall or structurally altered so as to be inactivated. Once bound, ABA could not be separated from cell wall or peptidoglycan surfaces. The amino acids contained in the pentapeptide fraction of cell wall peptidoglycan did not decrease the ABA activity (Table 17). The values presented in Table 15 clearly indicate that ABA activity was present only in the supernatant fraction following 0.5 hr of adsorption by S. aureus serotype XII cell wall. Adsorption was nearly complete at 2 hr and seemed to be irreversible. No ABA activity could be shown in both the supernatant and precipitate fractions of peptidoglycan adsorbed ABA (Table 16). The increased amount of oxygen consumed in the presence of these fractions could be due to either a stimulatory effect of staphylococcal respiration by the extraneous peptidoglycan or it could reflect an oxidative reaction occurring between the peptidoglycan moiety and the staphylococci. Whatever the mechanism responsible for the increased oxygen consumption in the presence of peptidoglycan, the fact remains that these values are within the normal limits for staphylococci exposed to either inactivated ABA or to native ABA as presented in the Appendex for Tables No. 15, 16, 17. Those values in the Appendex are representative of the effect of three different ABA preparations on S. aureus serotype XII respiration at various stages of bacterial growth ranging from early logarithmic to late logarithmic phase.

As mentioned earlier, the cell wall in addition to peptidoglycan contains a ribitol teichoic acid moiety. The effect of ribitol teichoic

acid was investigated through the use of a ribitol teichoic acid-free mutant S. aureus H52A5. The lack of ribitol teichoic acid in the cell wall of S. aureus H52A5 was demonstrated qualitatively by comparitive paper chromatography of hydrolysates of cell wall preparations from both S. aureus H and S. aureus H52A5. The results presented in Table 22 indicated that only S. aureus H cell wall contained a molybdate reaction positive phosphate ester close to the origin of the chromatogram. Both cell wall hydrolysates contain large amounts of alanine and glucosamine; while only S. aureus H cell wall hydrolysate contains a spot which corresponded to the location of the ribitol standard upon development with periodate: Schiff's reagent. Both strains also possessed ribose in their cell wall hydrolysates. The presence of a number of undetermined spots for each cell wall hydrolysate was disturbing, however these unknown spots were not indicated to be crucial to the qualitative identification of either ribitol, ribitol phosphate, or ribitol teichoic acid in the cell wall of S. aureus H52A5. The one spot in the hydrolysate of this strain's cell wall which was most unclear had an R_f value of 0.516 and was detected only by the periodate: Schiff's reaction for non-reducing sugars. More probably this spot represents either glycerol or a glycerol containing compound, due to the rapid manner in which the periodate: Schiff's reaction occurred. A phosphate ester would not have migrated so far from the line of origin, and anhydroribitol should have either migrated further than ribitol or not reacted as readily with periodate: Schiff's reagent. However there is no proof as to the positive identity of this unknown spot other than it's reaction as a nonreducing sugar. Having qualitatively established that S.

aureus H52A5 does not possess in its cell wall ribitol teichoic acid, the results of experiments on bacterial susceptibility to ABA become more meaningful. The results of studies with this organism indicated it was less than half as susceptible to ABA as was S. aureus H, a related strain and one-tenth as susceptible as S. aureus serotype XII an unrelated strain. (Tables 18 and 19). Such a lack of activity on the part of ABA against strain H52A5 was puzzling and seemingly inferred that ABA's antirespiratory activity was dependent on ribitol teichoic acid. Excessive ABA absorption by the ribitol teichoic acid free mutant may be interfering with the activity of the bactericidal substance by precluding ready access to the cellular membrane; or the absence of the ribitol teichoic acid in the cell wall may have altered the configuration of the remaining peptidoglycan moiety such that ABA either was trapped physically or the steric alteration could have also affected functional enzymes necessary for transport across the cell wall. It was assumed that alterations of cellular metabolism may have impaired the cells ability to respond to ABA.

We had also noted that the pellet of cells formed during centrifugation took on an altered appearance for S. aureus H52A5. Rather than being compacted at the bottom of the centrifuge bottle, the cells were found to line the side of the bottle in a thin sheet which did not readily go back into suspension. It was assumed that such peculiar behavior was a direct result of cell wall alteration.

The induction of the ribitol teichoic acid free mutant by nitrosoguanidine also depressed the growth rate as well as altering the cell surface and structure of S. aureus H52A5; however, the respiratory rate of the

organism relative to S. aureus H was increased in the presence of four different substrates tested (Tables 20 and 21). While not multiplying as readily as S. aureus H, strain H52A5 did increase the mass:cell ratio. Chatterjee and co-workers (13) have described S. aureus H52A5 as a pleiotrophic mutant with altered growth characteristics comparable to those presented here. These investigators found the mutant tended to form clumps in liquid cultures; did not divide properly; and possessed a thickened cell wall. We sought to determine if any change in oxygen consumption would be presented as a result of these alterations. A variety of substrates were utilized to investigate the respiratory capabilities of the two strains. S. aureus H52A5 was indicated to be more active than its non-mutant counterpart S. aureus H in the presence of all four substrates (Table 21). These findings were interpreted to be a reflection of the mutagenic alterations and were not contradictory with the decreased growth rate; because the mutant strain did not divide properly and produced a thicker cell wall than S. aureus H, such an increase in substrate utilization may have reflected the mutant strain's attempt to maintain its growth rate prior to mutagenesis. It was found that the mutant strain did not accumulate nucleotide-bound cell wall precursors in the presence of penicillin, which supported the idea of a possible membrane system defect according to Chatterjee et al. (13). Indirectly therefore the results of these studies (Tables 18 - 22) hepled delineate the site of action of ABA to the cell membrane.

Steric configuration of the cellular external surface presented to ABA greatly influenced the activity of the molecule as evidenced by the

neutralization of ABA by prior treatment of the staphylococci with exogenous coagulase. Yotis and Ekstedt (111) and Yotis (112) described the neutralization of ABA prepared from human serum by coagulase and attributed the interference to a mechanical blockage of ABA receptor sites on the cell surface by the coagulase molecule. Yotis (113) had noted that the following various experimental treatments: hydrochloric acid, formaldehyde, sodium hydroxide or heat, had little effect on the cells ability to irreversibily bind ABA. Repeated washing did not remove the bound ABA from the cell surface. Indeed only pretreatment with phenol inhibited the binding of the ABA molecule to the staphylococcal surface (113). The ability therefore of coagulase to inhibit ABA appears to be related to alteration of the cellular surface area which acts as a specific receptor site for ABA. Myrvik (70) had indicated the cell free culture supernatant from coagulase positive staphylococci did not antagonize the bactericidin system in rabbit serum. We have demonstrated that ABA prepared from both rat and rabbit sera was reversed by prior exposure of cells to appropriate concentrations of coagulase (Tables 24, 25). Myrvik's conclusion that serum bactericidin systems were not reversible by coagulase can be readily explained, when we consider this system to be analogous to the β-lysin described by Donaldson et al. (21). At no time were we able to demonstrate an ABA precipitin reaction with β-lysin and presumably the serum bactericidin also would not react to ABA antisera either. In addition, preliminary experiments indicated that coagulase (at tenfold greater concentration than required with ABA) could not significantly negate the inhibitory effect of β-lysin on staphylococcal respiration. The

activity of coagulase then served as another criterion by which both β-lysin (21) and serum bactericidin (70) were distinguished from ABA. The results in Tables 24 and 25 indicated that animal sera ABA was significantly more potent than human sera ABA. In contrast to the level of coagulase which affectively inhibited human sera ABA as reported (112) the antirespiratory activity of ABA prepared from animal sera required a 2-4 fold increase in the concentration of coagulase necessary to demonstrate a reversal effect similar to that observed with the human serum preparation. Based on the amount of protein (dry weight) necessary to inhibit significantly the respiration of staphylococci, this represented a 12-16 fold increase in the potency of rat and rabbit ABA. The effective decrease in the respiration of ABA exposed staphylococci as compared to unexposed control cells was presented in the Appendex to Tables No. 24, 25. Autoclaved ABA was shown to be devoid of antirespiratory activity and those cells which were exposed to such heat-inactivated ABA could be considered to respire as well as unexposed staphylococci. In addition to both unexposed and heat inactivated ABA exposed staphylococci, cells exposed only to coagulase (at a concentration of 400 μg/ml) were included as controls in the manometric experiments. Coagulase alone did not alter the respiration of staphylococci from the level attained by unexposed controls. Numerous preparations of ABA from both rat and rabbit sera were tested for antibacterial activity and all were shown to inhibit respiration significantly. There was some fluctuation in the activity of various preparations but this fell to within reasonable limits for a biologically active macromolecule prepared by the fractionation of serum. Activity

studies would have been more meaningful had the specific activity of each preparation been calculated. The effectiveness of ABA, however, in reducing the respiration of staphylococci is beyond dispute. This is not to say that the amount of ABA in rat serum is equivalent to the amount of ABA in rabbit serum or human serum, but that both animal species possessed a bactericidal serum protein in sufficient quantity for it to be isolated in relatively large amounts.

Coagulase reversal of ABA indicated that possible receptor sites on the cell surface were no longer available for ABA attachment. Because of the limited information available on the nature of such receptor sites, it would be of interest to uncover the manner in which coagulase causes the cell surface to antagonize the ABA. In this connection, Table 23 presents data which suggest that coagulase-producing organisms are more resistant to ABA than are non coagulase-producing ones. Even in the presence of exogenous coagulase at a concentration of 0.5 μg/ml the coagulase-producing virulent S. aureus E33 was 29% and 55% more resistant than S. aureus E33V, a noncoagulase-producing virulent mutant, and S. epidermidis, respectively.

Exogenous coagulase increased resistance to ABA by 20-42% at 0.5 μg/ml and 9-54% at 1.0 μg/ml, respectively. The decrease in susceptibility to ABA of S. aureus E33, and E33V and of S. epidermidis indicates that inherent coagulase production plays a role in the breakdown of the host defense mechanism.

In this connection, it was of interest that neither osmotically fragile spheroplasts nor L-forms of staphylococci were protected by the

addition of exogenous coagulase. The concentration of coagulase (500 μg/ml) was more than sufficient to neutralize the most potent ABA when used to protect intact cells. However, in the absence of cell wall, there was no absorption of coagulase to the surface of the bacteria and hence no protection. (Tables 26, 27). This work confirms Yotis' original postulation that coagulase protected the staphylococcal cell by binding the cell surface thereby preventing ABA access to the susceptible organisms. These studies also indicated that coagulase did not bind to the exposed cytoplasmic membrane of either spheroplasts or L-forms; nor was coagulase able to directly neutralize ABA in the absence of cell wall. We can conclude from these results that coagulase had prevented ABA activity by masking surface absorption sites, thus precluding ABA absorption which in the intact cell preceded the bactericidal effect of membrane disruption. In the absence of such surface receptors ABA was unaffected by the exogenous coagulase and damaged the cytoplasmic membrane. In a recent review, Kaback (57) discussed the release of pericytoplasmic proteins from the cell following spheroplast formation with E. coli and the effect these proteins exerted on transport across the spheroplast membrane.

At the time Myrvik characterized the serum bactericidin, he established that calcium ions reversed the inactivation which followed the addition of sodium salts of citrate, oxalate, and phosphate, and that sodium citrate inactivated the serum bactericidin by increasing the ionic strength. These finding confirmed the studies of Yotis (113) who demonstrated that ABA was inactivated by buffer at greater than 10^{-4} M. The role of various ions on

the activity of ABA had been previously investigated by Yotis and Ortiz (115). The authors had reported that the antibacterial activity of rat serum was decreased in the presence of excessive levels of exogenous ferric iron. These findings confirmed the earlier report of Bornside et al. (9) on the reversal of serum inhibition of B. subtilis by ferric iron. On the other hand, the presence of calcium ions did not significantly increase rat serum bactericidal activity in the hands of Yotis and Ortiz, contrary to the report by Jacox (50) that calcium enhanced human serum activity against B. subtilis. Myrvik et al.(71) had also demonstrated human serum bactericidin to be more active against B. subtilis following the addition of both calcium and bicarbonate ions, while both rat and rabbit serum bactericidin were dependent only on bicarbonate ions.

Our studies have indicated that ABA activity was significantly reversed upon the addition of bicarbonate ions at the same concentration used by Myrvik and his co-workers. In addition to the negative effect of bicarbonate, calcium ions were also found to decrease the effectiveness of ABA to a lesser degree (Tables 28, 29). The lack of an activating effect of calcium ions on human serum ABA correlates with the earlier work of Yotis and Ortiz with rat serum ABA. The use of human serum ABA for these studies was of importance in order to more directly contrast our findings with those of Jacox as well as with the results of Myrvik and co-workers. Jacox measured the effect of calcium on the bactericidal activity of serum by viability counts as did Donaldson and his co-workers. In order to more readily compare our results, studies with ions were done by plate count.

To determine that ABA was the factor in serum responsible for the antibacterial activity demonstrated, the specific activities of the substance was calculated at each of the steps in the ammonium sulfate fractionation. The original data reported the respiration inhibition as a percentage relative to the control. For an average experiment the absolute amount of oxygen consumed was 100 μl O_2 consumed/hr. Using this value then as our control we have calculated the specific activity, units of activity, and purification for each step in the fractionation procedure. These values are presented in Table 36.

Prior to investigating the chemical composition of ABA, the heterogeneous nature of the molecule was established by a variety of electrophoretic techniques. Few techniques are more sensitive to small differences of ionic change in a molecule than isoelectric focusing.

Density gradient isoelectric focusing can be attributed to the work of Svensson (99a) and Vesterberg and Svensson (102a). It was Svensson who first developed the idea of using a non-ionic substance such as sucrose to create a density gradient in which zones of proteins would be stabilized after focusing by zonal electrophoresis. Density gradients are prepared by mixture of heavy sucrose solution with a light solution containing no sucrose. For our experiments the density gradients were prepared manually. During isoelectric focusing, the electrolysis is continued until the ohmic resistance of the column no longer changes. All proteins are characterized by very sharp isoelectric points. It is consequently possible to get extremely thin and highly concentrated isoelectric zones of proteins if only the density

gradient is able to carry them. At increased voltages the zone of protein condenses into a smaller volume, with the result that the zone density ex-exceeds that of the zone below it; the protein zone then starts to sediment in small droplets. This is known as "droplet sedimentation" (99a). Zone stability is greater therefore at lower voltage, however resolution is decreased. In order to circumvent this problem one must use very narrow pH gradients. If one does set up a pH gradient in the approximate range of the protein isoelectric point, the whole space of the column becomes available for protein zones and electrolysis can be carried out at higher voltages without the risk of "droplet sedimentation". To this end it was largely the discoveries of Vesterberg and Svensson (102a) which uncovered the procedure for the synthetic production of suitable carrier ampholytes to form narrow pH gradients on electrolysis. The product obtained by this procedure is a mixture of low-molecular aliphatic poly-amino-poly-carboxylic acids. Such ampholytes are extremely heterogeneous and it is the combination of various mixtures which allows a wide range of pH gradients to be established. Using these ampholytes a resolving power of 0.01 pH unit was attained. This resolving power can be influenced only by way of the pH gradient and the field strength. Theoretically there is no limit to the pH gradient shallowness; field strength can be increased by more efficient cooling or different design of apparatus. The limitation of isoelectric focusing does not lie in its resolving power but in other areas. The solubility of proteins and the rate of protein diffusion among them. Density pH gradient isoelectric focusing as well as both polyacrylamide gel disc-electrophoresis and sodium dodecyl

sulfate (SDS) polyacrylamide gel electrophoresis were used to assay ABA. The results presented in Figs. 2, 3, 4 indicated that ABA had an isoelectric point of between 4.73 to 4.80 depending on the range of the pH gradient used in the determination. While the ABA elution pattern as measured by ultraviolet absorption at 280 nm appeared as a homogeneous protein profile, an analysis of the volume occupied by ABA suggested that the major protein component was contaminated with minor fractions of other serum proteins. Those proteins which were highly suspected were orosomucoid (α_1 acid-glycoprotein) and transferrin. The results of Ouchterlony double diffusion two-dimensional analysis of the relationship of these proteins and their respective antisera to ABA and its antisera are discussed below. In the Ouchterlony diffusion of transferrin (Fig. 9), the precipitin reaction shown between anti ABA and transferrin was due to the presence of ABA in the commercial enriched transferrin preparation. This conclusion is valid because we have also shown that anti-transferrin did not react with ABA and therefore ABA was not contaminated with transferrin. Fig. 10 illustrates the same point with orosomucoid. Antisera to whole human serum reacted to yield precipitin lines with orosomucoid, however antisera to ABA did not react with orosomucoid. These findings helped to differentiate both of these major serum fractions from ABA. However, these findings do not indicate that ABA was homogeneous, rather that neither orosomucoid nor transferrin were contaminants of ABA.

Sodium dodecyl sulfate gel electrophoresis was developed as a technique for the determination of the molecular weight of polypeptide subunits of proteins by Shapiro and co-workers (91a). By incubating ABA in the presence

of 1% β-mercaptoethanol, 6 M urea, and 1% SDS, the disulfide and hydrogen bonds which impart the secondary, tertiary and quaternary structure to the molecule were destroyed. Sodium dodecyl sulfate is important in such studies more so because it negates the electrical charges on the polypeptides than for its detergent properties. By neutralizing ionized amino acids, the electrophoretic mobility of all polypeptides is equalized and all separations are based on the sieving effect of the 10% polyacrylamide gel on the polypeptides of various molecular weights. On SDS gel electrophoresis ABA was resolved into a two-component system when a sample of 100 μg/ml was applied to the gel; at concentrations up to 60 μg/ml, ABA appeared as a single band (Fig. 18). On the basis of these studies we can conclude: 1) that either ABA consisted of two separate and distinct proteins each composed of a single polypeptide species, or 2) that ABA was formed by the junction of two polypeptide chains of different molecular weight muchlike the immunoglobulins. Disc-electrophoresis was developed in its present day form by Ornstein and Davis (16). While Ornstein was responsible for the development of the theory of disc-electrophoresis it was Davis who developed the technique in the laboratory, as Ornstein himself admits: "The first attempt, designed and executed by B. J. Davis, was so successful that it encouraged Davis and me through almost a year of successive failures until an understanding of mechanism and a reasonable degree of reproducibility began to be achieved" (75a).

The mechanism which Ornstein theorized to be responsible for the success of disc-electrophoresis in increasing the resolution of serum pro-

teins is based on two primary mechanisms: 1) The viscous properties of gels and solutions of very long chain polymers are unique. 2) The thinner the starting zone in the direction of the electric field, the higher will be the resolution.

The pore size of an average 7.5% polyacrylamide gel is about 50 Angstroms. Because the average pore size of a gel depends on the concentration of polymer, the pore size can be tailored to the dimensions of the molecule to be separated. The thin starting zones are based on the premise of Kohlraush's regulating function (61a) which stated that if two solutions of ions were layered over one another, such that the faster ion were placed below the slower of the same sign of charge, then the boundary between the two ions would be maintained as they migrated in an applied electric field.

Practically speaking, in an anionic buffer system (pH 8.5) the serum proteins will concentrate into very thin discs, one stacked on top of the other in order of decreasing mobility, with the last followed by glycine and all are preceeded by chloride. If the chloride boundary (and the following stack of protein discs) is permitted to pass into a region of smaller pore size (the separating gel) such that the mobility of the fastest protein drops below that of glycine, the glycine will overrun all the protein discs and run directly behind the chloride, and the proteins will now be in a uniform linear voltage gradient. Alternatively, if the boundary (and the following stack of discs) is permitted to pass into a region of higher pH (e.g., the pKa of glycine), the glycine will now overrun all the proteins discs and will run directly behind the chloride, and the proteins will now be in a uniform

linear voltage gradient, each effectively in an extremely thin starting zone and will migrate as in free electrophoresis.

A cationic buffer system has been perfected by Reisfeld et al. (83a) in which potassium serves as the leading (chloride) ion and β alanine acts as the trailing (glycine) ion with migration directed towards the cathode. ABA was subjected to disc-electrophoresis under both anionic and cationic buffer conditions, and under anionic conditions was resolved into a two component system comprised of one major and one minor band (Fig. 7). In the cationic gel system ABA was resolved into three protein bands: 2 major and 1 minor components (Fig. 8).

In conjunction with the analysis of ABA, the 50% ethanol soluble fraction of Cohn's fraction IV-1 (known as CE-ABA) was subjected to disc-electrophoresis and isoelectric focusing to determine the degree of purity of the molecule. Under anionic disc electrophoresis conditons, CE-ABA was resolved into 4 protein bands (Fig. 8) while under cationic conditions the same protein resolved into 7 distinct protein bands (Fig. 8). Clearly the heterogeneous nature of the CE-ABA preparation was indicated. No further purification of CE-ABA was carried out primarily because ABA prepared by ammonium sulfate fractionation was a much less heterogeneous mixture with equal antibacterial activity. The isoelectric focusing of CE-ABA resolved the protein mixture into two protein components. These pools were subjected to disc electrophoresis under cationic buffer (pH 4.5) conditions and are presented in Fig. 8 .

The nature of the major band in the ABA preparation was hinted at in the immunoelectrophoretic analyses presented in Table 2 and Fig. 1. The use

of sodium barbital (veronal) buffer pH 8.6 and 0.1 ionic strength for these studies insured that the optimal conditions for immunoelectrophoresis was taken into consideration. At pH 8.6 most proteins carry an important negative charge and therefore migrate toward the anode. Above pH 8.6 denaturation has been found to occur. The ionic strength is set at 0.1 because at higher electrolyte concentrations the conductivity of the buffer would cause the development of an undesirable amount of heat and a slow migration rate of proteins. At lower concentrations, the buffering capacity would become too low. Barbiturate anions are desirable because they are univalent, therefore they contribute little to ionic strength, and interact minimally with proteins. In addition they are voluminous, and therefore they migrate slowly in an electrical field so that conductivity and Joule-effect heat for which they are responsible can be kept reasonably low. Just as the buffer strength and pH is important to the electrophoretic separation of the antigen, the concentration of antigen is equally important to the formation of the immune precipitate arc. It was significant therefore that transferrin only reacted with anti ABA antisera at a concentration of 24 mg/ml, orosomucoid did not react with anti ABA antisera even at a concentration of 10 mg/ml. On the other hand, the concentration of ABA which reacted with anti human α_1-antitrypsin was only 1 mg/ml. Consequently, Ouchterlony double diffusion analyses were performed to determine the extent to which ABA and α_1-antitrypsin are related.

For the interpretation of comparitive patterns, it is essential to remember the basic factors which influence the formation of precipitates with

the double diffusion arrangement. The site of a precipitate depends on the initial concentration of the reactants and their diffusion coefficients. Balanced systems produce stable precipitin lines, unbalanced systems may produce "migrating" precipitates and eventually duplication of lines. Established precipitates may dissolve in an excess of antigen and for certain immunosystems in an excess of antibody as well. A precipitin line acts as an immunospecific barrier for the reactants to a certain degree. Bi- and multi-specific antigens as well as bi-specific antibodies should be taken into consideration (76a).

The results in Fig. 11 clearly indicate that α_1 antitrypsin and ABA contain a common antigenic site. Both antigens were tested against homologous and heterologous antisera and in all instances a definite line of identity developed. It is quite possible therefore to conclude from these studies the following: 1) ABA and α_1 antitrypsin are one and the same protein and antisera to either protein will react monospecifically with both proteins; 2) ABA is composed mainly of α_1 antitrypsin and some other minor contaminants such that the ABA molecule contains more than 1 antigenic determinant. Antisera to ABA reacts with α_1 antitrypsin to yield two precipitin lines and with ABA to yield one precipitin line. The development of two precipitin lines in the α_1 antitrypsin: anti-ABA antiserum system caused us to wonder if our original conclusion that a line of identity developed was correct. Upon further examination it was determined that the α_1 antitrypsin molecule was composed of what appeared to be at least two components which reacted to form precipitin lines with ABA. One of these components

matched the ABA:anti-ABA precipitin line exactly; the other appeared to migrate more closely to the antiserum well and to form a very small spur across the ABA:anti-ABAline. The question of line duplication in simple immunosystems is important for the analysis of comparative patterns. The possibility that duplication of lines is caused by the existence of different determinants attached to the antigen molecule has been suggested by Jennings et al. (52a). The immunosystem may have been unbalanced with the antigen (α_1 antitrypsin) in excess. This would have caused the dissolution of the precipitin line barrier and allowed the migration of the antigen closer to the antiserum well. To resolve this potential source of error, Ouchterlony analyses was performed on dilutions of the antigen α_1-antitrypsin down to 2 mg/100 ml or 20 µg/ml. At this concentration it can be shown that only one precipitin line developed between anti-ABA and α_1-antitrypsin.

It can be concluded therefore that ABA does contain α_1 antitrypsin most probably as the major component seen on disc-electrophoresis. The identity of the minor component in ABA is still unknown, but may be found in the commercial preparation of transferrin as a contaminant. This conclusion is based on the amount of transferrin (24 mg/ml) to react with anti-ABA on immunoelectrophoresis and the results of SDS gel electrophoresis. The mobility of the minor ABA component matches that of one of the very minor polypeptides found in transferrin on SDS gel electrophoresis. Of the α_2 globulins, there is one protein which could be considered a strong possibility as the minor ABA component. That protein is α_2-Neuramino-glycoprotein. This glycoprotein contains 42.6% carbohydrate and migrates behind transferrin in

starch gels, and in the α_2 region on paper electrophoresis. Because of its high carbohydrate content, it could easily be a very minor contaminant of ABA and still significantly raise the level of carbohydrate residue in ABA. The fact that α_2-Neuramno-glycoprotein migrates near transferrin is of interest and enhances the probability of it being the minor ABA component. However, the identity of the minor component of ABA is purely speculative at this time.

Yotis and Lentino (114) have reported that human ABA when reacted with rabbit antihuman ABA antiserum yielded two precipitin arcs by immunoelectrophoresis. The presence of a second arc can be due to the presence of a contaminant normally found in large amounts in serum. Because the contaminating protein would be precipitated along with ABA during the fractionation procedure, only a minute amount would be required to react with anti-human ABA antisera resulting in the formation of two precipitin arcs; or the ABA molecule may have been presented to the non-immune animal in a variety of molecular configurations, which could stimulate antibody production.

Such contaminants are unlikely however to account for the biological activity of ABA. Orosomucoid was tested in preliminary experiments for antibacterial activity by manometric technique. There was no decrease in oxygen consumption by cells exposed to orosomucoid (2 mg/ml) for 1 hr at 4°C compared with either unexposed cells or cells exposed to heat-inactivated orosomucoid.

Assuming ABA to be a heterogeneous substance under the experimental conditions, composed of one major polypeptide species and a minor trace of a second contaminating polypeptide species, we investigated the chemical

composition of the molecule. The results of qualitative carbohydrate analysis by paper chromatography indicated both galactose and mannose to be present in ABA'. Dische et al. (18) had previously reported the presence of both of these hexoses in a biologically active protein isolated from salivary secretions. While some confusion over the identification of the galactose spot did exist, the results of the glucose oxidase test indicated that glucose was not present in ABA. This test is based on the production of one mole of D-glucono-1, 5, lactone and one mole of hydrogen peroxide for each mole of glucose oxidized. The hydrogen peroxide is measured with peroxidase and dianisidine (3, 3' -dimethoxybenzidine). Peroxidase acts on H_2O_2 to transfer oxygen to the reduced form of the chromogen and this oxygen oxidizes the dianisidine reagent in proportion to the amount of glucose which was originally oxidized by the glucose oxidase. The test is measured at 420 nm in a Klett colorimeter after 90 min. at 37°C in a shaking water bath. The only other hexose which migrated a similar distance and to which the unknown could be compared was galactose. Both mannose and fucose were identified from the chromatograms. The hexosamine was not specifically identified as either glucosamine or galactosamine on the chromatograph of ABA (Fig. 12).

Quantitative analysis of ABA revealed the molecule was composed of 22.56% carbohydrate (Table 31), which may explain the increased thermal stability of ABA as reported by Yotis and Ekstedt (111). ABA was shown to be active when isolated from a 25% ammonium sulfate fractionation of serum after heating up to 70°C for 30 minutes. Previous investigators have also

indicated other serum glycoproteins to possess increased heat stability which they explained as a function of high carbohydrate content (8). Quantitative carbohydrate analyses were based on the results of various colorimetric tests. Hexoses were determined primarily by the anthrone test in which the protein bound hexoses are heated in the presence of sulfuric acid to 5 hydroxymethyl furfural. This product reacts with the anthrone reagent to yield at least 2 different chromogens (48a, 87a) which are read at 620 nm against a reagent blank. The absorption curve for hexoses is altered in the presence of protein primarily because of the effect of tryptophan , because of a three way reaction between tryptophan , anthrone reagent and carbohydrate. The product of this interaction absorbs best at 530 nm. Different levels of tryptophan, added to carbohydrate solutions increase the absorption at 530 nm and excess levels of tryptophan decrease absorption at 620 nm by competing with anthrone for the furfural derivative. Care must be taken also to exclude any extraneous water from the samples since this produces turbidity. Fucose will contribute to the color development and should be determined separately. Hexosamines do not give any color in this reaction, sialic acid gives a yellow-green color equivalent to approximately 8% of the color given by galactose.

Hexosamines were determined by Rimington's modification of the Elson-Morgan reaction. The Elson-Morgan reaction depends on 1) reaction with 2,4-pentanedione (acetyl acetone), 2) conditions of treatment with Ehrlich reagent, 3) wavelength at which the red color obtained is measured.

The reaction between hexosamine and acetyl acetone takes place in hot mildly alkaline conditions to yield mainly 2-methylpyrrole through a condensation reaction. This chromogen is responsible for 70% of the red color which is obtained when Ehrlich's reagent (p-dimethylaminobenzaldehyde) is added. The presence of nitrogenous amino acids, especially lysine in combination with hexoses in protein hydrolysates can interfere with color development. Our hexosamine values were determined with a glucosamine standard at 530 nm against a water blank.

Fucose was determined by the sulfuric acid-cysteine reaction which results in the formation of 5-methyl furfural imparting a brown color to the sample solution. The addition of cysteine accentuates the color development by forming a complex substance the structure of which is unknown at this time. However the proposed mechanism through which these chromogens are formed is thought to be the Maillard reaction. The ten minute boiling time employed is necessary to lessen the interference by hexoses and makes the test specific for 5 methylpentoses. In addition the hexoses present in the sugar sample form chromogens which absorb light maximally at 390 nm. The amount of fucose is calculated from the difference in light absorbance at 396 nm and 430 nm. This difference will increase with 5 methyl pentoses but will decrease with pentoses. Both fucose and hexoses absorb at 396 nm but not at 430 nm. Therefore the latter wavelength is most important in measuring actual fucose values. The stability of the chromogen formed is dependent on the water content of the reaction. Without the addition of cysteine the chromogen 5 methyl-furfural absorbs maximally at 330 nm.

Dische is the one investigator who is most responsible for the development of the above method (17a,17b).

Sialic acid determined by the periodate-thiobarbituric acid method of Warren in which the resultant chromogen is read at 532 nm and 549 nm. The test depends on the production of β-formyl pyruvic acid by periodate oxidation and the subsequent reaction of this compound with thiobarbituric acid (TBA) to yield a chromophore. The most common interfering substance is 2-deoxy-ribose which yields malonaldehyde upon periodate oxidation. The chromophore of malonaldehyde and TBA absorbs best at 532 nm while the β-formylpyruvic acid and TBA chromophore absorbs at 549 nm. Other compounds containing 2-keto-3-deoxyonic acid groups will react as sialic acids. The sialic acid must be released by acid or enzymatic hydrolysis prior to application of the TBA reaction. Only sialic acids with an unsubstituted glycosidic hydroxyl group are reactive in the TBA test. L-fucose decreases the optical density given by sialic acids in the Warren method by 35%. This effect is probably due to the acetaldehyde which is produced upon periodate oxidation of fucose.

The loss of most of the sialic acid and a significant proportion of L-fucose from the heat-inactivated ABA does not come as a surprise. Sialic acid is characteristically located at the ends of covalently bound carbo-hydrate chains. It seems only reasonable therefore that the sialic acid residue be susceptible to removal during the denaturation of the ABA molecule by heat. Fucose is also known to exist in less secure terminal positions; and under mildly acidic conditions can be readily split from the more stable sugar residues of galactose and mannose.

This extensive carbohydrate content may very well have a functional role in the biological activity of ABA. At the moment however, direct evidence for the involvement of the carbohydrate in the active site of glycoproteins is scanty. Most likely the carbohydrate moiety exerts its effect through structural changes rather than enzymatic activity (63a). Indeed it has been shown that desialylated hormones have little or no activity left following neuraminidase treatment. In terms of structure, the carbohydrate may be present in very small amounts or as a major portion of the molecule. It may occur in simple or complex units either closely spaced or attached at only a few locations along the peptide chain. The mode of linkage between carbohydrate and peptide vary. It seems most realistic to consider the carbohydrate along with the amino acids as an integral part of the moelcule and to assume together with the peptide portion it equips the molecule to perform its biological function (98a). While carbohydrates have been implicated in facilitating the transport of plasma protein molecules (29a), it does not seem that this property is responsible for the effectiveness of ABA. Rather it appears that the interaction between ABA and the staphylococcal membrane is a result of either structural, enzymatic or ionic properties conferred on the molecule by sialic acid. The distribution of acid and basic phospholipids in biological membranes is largely unknown at this time. However the majority of minor phospholipids carry a net negative charge at physiological pH (7a). It is concievable that the ABA molecule could be attached to the negative portion of the membrane surface by the NH_3^+ groups it contains, simultaneously sialic acid could be responsible for increasing the solubility of ABA and

consequently enhancing the permeability of the membrane. This phenomenon has been reported most recently by Bretscher (11a), who indicated that glycoproteins can penetrate the lipid bilayer and consequently present hydrophilic groups at external and cytoplasmic interfaces. It has been suggested by Gingell (35a) that any factors which overcome repulsive forces between mobile subunits will lead to their aggregation and that aggregation causes a local increase in membrane permeability. Each mobile unit must bear an electrostatic charge in order to avoid immediate aggregation with others. The mobile subunit charge is almost certainly negative if the cellular surface charge is negative. Perhaps the addition of the sialic acid containing ABA to the exposed membrane surface of the staphylococci activates the membrane subunits such that sufficient reduction of the electrostatic repulsion between subunits occurs to permit adhesian of the molecules among themselves. This is possible by virtue of attractive electrodynamic (Van der Waals) forces which act between like bodies. Therefore the net result of ABA activity would be a structural alteration of the staphylococcal membrane due to the introduction of similar ionic charges leading to mutual adhesive properties among the membrane molecules.

The fact that desialylated ABA is inactive strengthens this argument. However, it must be kept in mind that such an argument as presented above is largely speculation and remains to be verified in future work.

In addition to a variety of carbohydrates involved in the activity of glycoproteins, the amino acid composition as determined by automated amino acid analysis invites speculation as to which amino acids are involved in

the biological activity of ABA. Others have reported a number of basic polypeptides such as leukin (94), plakin (2) and β-lysin (21) to have enhanced biological activity against microorganisms and have attributed such activity to the basic nature of these polypeptides. ABA has been shown to contain increased levels of basic amino acids such as lysine and arginine as well as high levels of aspartic acid and glutamic acid (Table 33). The values of these amino acids indicated the protein moeity of ABA carried a net negative charge. The levels of valine and leucine were increased over that of the other neutral amino acids. Because of the difficulty of hydrolysing such branched chain amino acids under most conditions, the increased values of these hydrophobic amino acids was significant. Their presence indicated the ABA molecule could have a sufficient number of hydrophobic residues to make an attachment to lipid laden membranes a distinct possibility. While the hydrophobic amino acids may account for the mechanism of action of ABA, the hydrophilic amino acids present in such large amounts, e.g., the NH_3^+ groups of lysine and arginine and the COO^-groups of glutamic and aspartic acids account for the solubility of ABA in water. The amino acid analyses indicated the protein content of ABA to be 77.26% and confirms the more fundamental colorimetric tests. While the biuret test assays for the presence of peptide bonds by the formation of a violet reddish complex between copper ions (supplied by $CuSO_4$ in dilute alkatine solution) and the amide linkages of proteins; the Lowry test measures the tyrosine equivalent of proteins. In the Lowry reaction the phenolic groups of tyrosine reacts with the Folin-Ciocalteau reagent. This Folin reagent is composed of phosphotungstate and phosphomolybdate and the

reaction is dependent on the reduction of Folin reagent by the copper-alkali treated protein. The optimum pH of the reaction is pH 10. The reaction occurs very quickly and must be well buffered. The chemistry of the Folin-Ciocalteau reaction is poorly understood. The biuret test is more dependable in terms of reproducibility than is the Lowry test (91).

Molecular weight studies were performed by the gel filtration method of Andrews (5a). Porath and Flodin (82a) discovered a means of obtaining a molecular-seive effect by passing a mixture of solutes of different molecular sizes through a column of grains of water-soaked cross-linked dextran. In addition it was Porath (82b) and Flodin (33a) who described a method in which the chains of dextran monomers were cross-linked by means of epichlorhydrin. It was left to Andrews however to elucidate the behavior of many proteins on gel filtration. According to Porath the experimental estimation of the distribution coefficient is facilitated by the fact that the total distribution volume of a solute is equal to its elution volume V_e. Therefore it may be written as:

$$K_d = \frac{V_e - V_o}{V_t}$$

Porath (82b) had suggested that the effective radius of a protein is proportional to the square root of its molecular weight. This assumption leads to a formula which postulates a linear relationship between $K_d^{1/3}$ and $M^{1/2}$:

$$K_d = k \quad 1 - k_1 \frac{M^{1/2}}{(Sr - \alpha)^{1/3}} \quad 3$$

where K_d = the distribution coefficient; M = the molecular weight; S_r = the solvent regain; k, k_1 and α = constants.

The theoretical suggestions of Porath are based on the assumption that the volume Vi consists of a collection of cones, cylinders and crevices in the de tran gel which can be occupied by the solute molecules. Absorption phenomena are neglected.

Laurent and Killander (61b) have described an alternative model to Porath's. According to these authors the gel suspension of Sephadex may be considered as a system of randomly distributed straight fibers. The space accessible to solutes can then be expressed as an enponential function which depends on the concentration and radius of the fibers on one hand, and the molecular radius of the solute on the other hand. As in Porath's model the available space can be calculated from the volume required for the elution of the solute (V_e) and the void volume of the column (V_o):

$$K_{av} = \frac{V_e - V_o}{V_x} = \frac{V_e - V_o}{V_t - V_o}$$

This formula was the one used to calculate the molecular weight of ABA. By plotting the K_{av} value against the molecular weight of standard proteins and glycoproteins on semi-logarithmic paper we obtained an approximation of the ABA molecular weight. The molecular-seiving properties of gel filtration are the basis for determination of molecular weight of polypeptides by SDS gel electrophoresis. Originally proposed by Shapiro et al. in 1967, SDS gel electrophoresis has proven to be an accurate and rapid method for molecular weight determination. Despite the range of isoelectric points of a group of proteins, the molecular seive effect of the polyacrylamide gel is not affected by electrophoretic migration. The SDS minimizes the native charge differences

and all proteins migrate as anions as the result of complex formation with SDS. The extensive disruption of hydrogen, hydrophobic and disulfide linkages by SDS, urea and β-mercaptoethanol results in the solubilization of many relatively insoluble proteins. Since Shapiro and co-workers published their work, other investigators have described modifications of the technique (22a,105). We used the method of Weber and Osborn (105) to calculate ABA molecular weight. The value obtained indicated this method was more sensitive than that of Sephadex gel filtration, having resolved ABA into two components of different molecular weights. The main component of ABA was shown to have a molecular weight of approximately 62000, this compares favorably with the results of the gel filtration method and has increased the probability that this value is the true molecular weight of ABA. The standard protein preparations used were by no means all homogeneous, however they were all known glycoproteins, the molecular weights of which had been calculated by other means. We measured electrophoretic mobility by the distance traveled by the most dense protein band. In those instances where the band was more than 5 mm wide, the center of the protein band served as the protein migration point. In this way we hoped to negate any spurious results which may have been the result of molecular diffusion.

There have been few α-globulin serum glycoproteins described which contain such a large carbohydrate moiety. ABA has been distinguished from the majority of α_2 globulins. Both haptoglobin and ceruloplasmin were immunologically distinct molecules (Table 2), while pseudocholinesterase failed to demonstrate antibacterial activity (Table 1). Molecular weight

determinations have indicated ABA to be between 55,000-74,000 daltons by Sephadex gel filtration and 62,000 daltons by SDS gel electrophoresis, a more sensitive and reproducible technique. The α_2 lipoproteins and α_2 macroglobulins therefore can be readily distinguished from ABA on the basis of their increased molecular weights. The carbohydrate content of α_2-neuraminoglycoprotein was double that of ABA and therefore does not appear to be the major ABA component. However as was mentioned earlier, it may be a contaminant of ABA responsible for the increased carbohydrate content of ABA over that of α_1 antitrypsin. Concievably ABA may be related to α_2-HS glycoprotein or Zn α_2 glycoprotein, however all the α_2 globulins, reported to date, have isoelectric points distinct from that of ABA. ABA however has the electrophoretic mobility characteristic of an α_1 globulin. It can not be conclusively argued however that ABA is an α_1 globulin by the electrophoretic evidence alone. On the other hand, no known α_2 globulin corresponds to ABA in all properties.

Heimburger and Haupt (43) had recently characterized α_1 X-glycoprotein as a heat labile serum factor containing 22.8% carbohydrate while the total carbohydrate content matched that of ABA, the composition was dissimilar. However, the molecular weights of the two molecules closely approximated each other. Electrophoretically identified as α_1 antichymotrypsin, α_1 X-glycoprotein most probably is a distinct entity from ABA. The α_1-glycoprotein described by Schultze et al. (90) as an easily precipitable α_1 globulin was shown to be related to α_1-antitrypsin immunologically. Both of these glycoproteins contain less than 15% carbohydrate while α_1 acid glycoprotein

on the other hand contains more than 40% carbohydrate (40). These α_1 globulins may be considered distinct from ABA on the basis of their carbohydrate content and composition. On the other hand, immunological data indicated that α_1 antitrypsin and ABA were related (Figs. 1, 11, 19). The increased carbohydrate may be due to trace contamination of ABA by another serum glycoprotein. Other α_1 globulins such as transcortin, 4.65-postalbumin, and thyroxin-binding globulin can be distinguished from ABA primarily because of their low plasma concentration and their lower carbohydrate content (91).

Both α_1 and α_2 "acute phase" globulins have been shown to increase in response to nonspecific host injury (51, 52, 87). It is of interest therefore to note that infectious processes most often are followed by a rise in the α_1 globulin level (65, 67, 108). Many of these reports are concerned with the α_1 acid glycoprotein (orosomucoid) which we have shown to be distinct from ABA on the basis of Oucherlony double diffusion tests. Others have dealt with α_1 antitrypsin (66a, 99b, 108). The rapid rise of the α_1 antitrypsin level in serum following infection has been shown to be characteristic of this protein. Orosomucoid however is thought to rise two days after the initial infectious process. A most interesting report by Moskowitz and Heinrich (66a) concerns the inactivation of α_1 antitrypsin by cultures or culture supernatants of Ps. aeruginosa. The authors postulate that the antitrypsin inhibition of these bacteria may account for an inflammatory mechanism involving α_1 antitrypsin. S. aureus was not shown to produce the antitrypsin inhibitor. The investigators findings supported an enzymatic rather than binding action of antitrypsin inhibitor in antitrypsin inactivation.

It is most interesting to speculate on the mechanism by which ABA works should it prove to be α_1 antitrypsin. By its very nature we know α_1 antitrypsin must serve to inactivate various protease enzymes in the blood, however there remains the possibility that ABA or α_1 antitrypsin may contain either a lipase or esterase action responsible for the membrane damaging effects against staphylococci. To this end, Yotis (113a) has suggested the ABA can and does hydrolyze a variety of synthetic esters. However, this is not to say that the esterase and antibacterial activity of the ABA glycoprotein are one and the same. However, evidence on hand favors the assumption that the bactericidal properties are possessed by and a direct result of the same protein moiety which possessed esterase activity.

While not complete the list of plasma proteins, which qualify as both α globulin and glycoprotein, is representative of the major known α globulin glycoprotein substances. No known glycoprotein corresponds to ABA in all physiocochemical properties (91). This suggests that ABA therefore may well be a previously unknown molecule completely autonomous from any described glycoprotein. Until the ABA molecule is more completely characterized we shall classify it as an α_1 globulin, most likely α_1 antitrypsin.

SUMMARY

An α globulin glycoprotein has been isolated from the sera of humans as well as from selected animal species. This glycoprotein demonstrated antibacterial activity against the staphylococci and was differentiated from the antibacterial substances β-lysin, described by Donaldson and coworkers, and bactericidin, reported by Myrvik and coworkers.

A variety of known serum proteins have been indicated to be immunologically distinct from ABA and include: ceruloplasmin, haptoglobin, β-lipoprotein, C-reactive protein, fibrinogen, orosomucoid, transferrin, many of the α globulins such as α_2 HS-glycoprotein, ZN α_2-glycoprotein, α_1 T-glycoprotein, α_1 B-glycoprotein, α_1 antichymotrypsin, inter α trypsin inhibitor and GC globulin, as well as the immunoglobulins IgA, IgM and IgG. In addition only the α_1 antitrypsin fraction reacted with antisera to ABA on immunoelectrophoresis. The α-globulin fraction of serum has been shown to inhibit the viability and respiration of staphylococci as did the antibacterial agent (ABA). This activity was neutralized by prior exposure of staphylococci to appropriate concentrations of exogenous coagulase. The relationship of coagulase production to reversal of ABA was investigated by a comparative study with S. aureus E33, a coagulase producing organism and S. aureus E33V, a non-coagulase producing mutant. The results indicated that exogenous coagulase is less effective in neutralizing ABA activity against strain E33 than against strain E33V. The relative percent reversal of ABA due to the addition of coagulase was 10%, 36%, and 49.5% for S. aureus E33, E33V and S. epidermidis respectively. These data indicated that S. aureus E33 was afforded less

protection due to exogenous coagulase and suggested that inherent coagulase production may play a greater role in the breakdown of the host defense mechanism. In contrast to Myrvik and coworkers, significant reversal of animal sera ABA was effected by the addition of increased concentrations of coagulase. It has been suggested that coagulase forms a chemical union with unknown receptor sites on the cell surface thereby precluding ABA access to these cellular receptors. We have confirmed this hypothesis in our studies with cell wall deficient forms of staphylococci. While ABA reduced the respiration of intact cells by 55%, the anti-respiratory effect was shown to be increased to 67% and 85% for spheroplasts and L-forms respectively. In addition, respiratory inhibition levels, comparable to those obtained with whole cell suspensions in 1 hr, could be attained against the staphylococcal L-forms in 10 min. At no time could we demonstrate coagulase reversal of ABA activity against the cell wall deficient forms. The data suggested that coagulase protected intact cells by masking cellular receptor sites thus precluding ABA access to the susceptible organisms. These studies also indicated that coagulase did not bind to the exposed cytoplasmic membrane of either spheroplasts or L-forms; nor was coagulase able to directly neutralize ABA in the absence of cell wall. In the absence of such surface receptors ABA was unaffected by the exogenous coagulase and damaged the cytoplasmic membrane.

Additional studies indicated that neither cell wall nor peptidoglycan absorbed ABA quickly enough to be the main sites of attachment. Although a ribitol teichoic acid free mutant S. aureus H52A5 was not susceptible to ABA, the lack of ribitol teichoic acid in the cell wall may have so structurally

altered the cell wall that access to the membrane by ABA was precluded. In contrast to other bactericidal systems, ABA was inhibited by low concentrations of both bicarbonate and calcium ions. These ions were shown to have a stimulatory effect at equivalent concentrations in antibacterial systems described by both Jacox and Myrvik and coworkers.

An analysis of the chemical composition of ABA indicated the α globulin to be a glycoprotein composed of 22.5% carbohydrate and 77.2% protein (as determined by amino acid analysis). The total carbohydrates included 11.08% protein-bound hexoses, 0.86% hexosamines, 4.3% sialic acid and 6.3% fucose. The mean isoelectric point, determined by isoelectric focusing in the range pH 3-6, was 4.73, while this value was 4.80 when determined in the range pH 4-6. Fluctuation of the isoelectric point was a reflection of the high carbohydrate content. Molecular weight studies on Sephadex G-200 dextran gel columns calibrated with proteins and glycoproteins indicated the molecular weight of ABA to be 55,000 - 74,000 daltons respectively. SDS-gel electrophoresis indicated the molecular weight of ABA was 62,000; this is in close agreement with the molecular weight of α_1 antitrypsin as reported by Schultze and coworkers. In addition to similarities in molecular weight, ABA and α_1 antitrypsin share common electrophoretic mobility patterns as seen on disc electrophoresis (Fig. 19) and cross react to yield a line of identity on Ouchterlony gel diffusion analysis (Fig. 11).

Because of the immunochemical, electrophoretic and physical similarities between ABA and α_1-antitrypsin, an effort was made to uncover antitryptic activity in ABA or antistaphylococcal activity in α_1-antitrypsin. While slight antitryptic activity was found in the ABA preparations, no antirespiratory activity could be discovered in α_1-antitrypsin. However these findings do not eliminate the possibility that ABA and α_1-antitrypsin are related molecules, as the presence of biological activity could be greatly dependent on the method of preparation of the substance.

LITERATURE CITED

1. Adinolfi, M., A.A. Glynn, M. Lindsay, and C.M. Milne. 1966. Serological properties of γ A antibodies present in human colostrum. Immunology, 10: 517-526.

2. Amano, T., K. Kato, and R. Shimizu. 1952. Studies on the role of plakin. Med. J. Osaka Univ. 3: 293-311.

3. Amano, T., M. Shima, and K. Kato. 1953. Studies on the role of plakin. III. The damage of semipermeability of bacteria caused by plakin. Med. J. Osaka Univ. 4: 277-284.

4. Amano, T., K. Kato, T. Morishima, and K. Okada. 1953. Studies on the role of plakin. IV. The effect of plakin on the respiratory enzyme system of Bacillus megatherium. Med. J. Osaka Univ. 4: 285-290.

5. Amano, T., K. Kato, and K. Okada. 1953. Studies on the role of plakin. V. On the correlationship of phosphate metabolism to the maintenance of the organization of bacterial cells. Med. J. Osaka Univ. 4: 291-298.

5a. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91: 222-233.

6. Archibald, A.R., J. Baddiley, and N.L. Blumson. 1968. The teichoic acids. Advan. Enzymol. 30: 223-253.

6a. Armstrong, J.J., J. Baddiley, J.G. Buchanan, B. Carss, and G.R. Greenberg. 1958. Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial cell walls. J. Chem. Soc. Pt. 4: 4344-4345.

6b. Baddiley, J., J.G. Buchanan, B. Carss and A.P. Mathias. 1956. Cytidine diphosphate ribitol from Lactobacillus arabinosis. J. Chem. Soc. Pt 4: 4583-4588.

6c. Baddiley, J., J.G. Buchanan, U.L. Rajbhandary and A.R. Sanderson. 1962. Teichoic acid from the walls of Staphylococcus aureus H. Structure of the N-acetyl glucoaminylribitol residues. Biochem. J. 82: 439-448.

7. Baird-Parker, A.C. 1963. A classification of micrococci and staphylococci based on physiological and biochemical tests. J. gen. Microbiol. 30: 409-427.

7a. Bangham, A.D. 1972. Lipid bilayers and biomembranes. Ann. Rev. Biochem. 41: 753-776.

8. Bettelheim-Jevons, F.R. 1958. Protein - carbohydrate complexes. Advan. Prot. Chem. 13: 35-105.

9. Bornside, G.H., C.B. Merritt, and A.C. Weil. 1964. Reversal by ferric iron of serum inhibition of respiration and growth of Bacillus subtilis. J. Bacteriol. 87: 1443-1451.

10. Bourrillon, R., and J. Michon. 1959. Resolution et determination quantitative par chromatographie sur papier des glucides lies aux proteins. Bull. Soc. Chim. Biol. 41: 267-276.

11. Boyden, S.V. 1966. Natural antibodies and the immune response. Advan. Immunol. 5: 1-28.

11a. Bretscher, M.S. 1972. Major human erythrocyte glycoprotein spans the cell membrane. Nature New Biology 231: 229-232.

11b. Buchanan, J.G., C.A. Dekker, and A.G. Long. 1950. The detection of glycosides and non-reducing carbohydrate derivatives in paper partition chromatography. J. Chem. Soc. Pt. 4: 3162-3167.

12. Buchner, H. 1891. Zur Nomenklatur der schützenden Eiweisskorper. Centr. Bakteriol. Parasitenk. 10: 699-701.

12a. Bundy, H.F. and J.W. Mehl. 1959. Trypsin inhibitors of human serum. II. Isolation of the α_1-inhibitor and its partial characterization. J. Biol. Chem. 234: 1124-1128.

12b. Catsimpoulas, N. 1973. Analytical scanning isoelectrofocusing. Ann. N.Y. Acad. Sci. 209: 65-79.

13. Chattejee, A.N., D. Mirelman, H.J. Singer, and J.T. Park. 1969. Properties of a novel pleiotropic bacteriophage resistant mutant of Staphylococcus aureus H. J. Bacteriol. 100: 846-853.

14. Cohn, E.J., L.E. Strong, W.L. Hughes, Jr., D.J. Mulford, J.N. Ashworth, M. Melin, and H.L. Taylor. 1946. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein component, of biological tissues and fluids. J. Amer. Chem. Soc. 68: 459-475.

15. Cybulska, J., and J. Jeljaszewicz. 1966. Bacteriostatic activity of serum against staphylococci. J. Bacteriol. 91: 953-962.

16. Davis, J. 1964. Disc Electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404-427.

17. Dische, Z., and L.B. Shettles. 1948. A specific color reaction of methyl pentoses and a spectrophotometric micro method for their determination. J. Biol. Chem. 175: 595-603.

17a. Dische, Z. 1955. Color reactions for determination of sugars in polysaccharides. Methods of Biochem Anal. 2: 313-358.

17b. Dische, Z. 1962. Color reactions of carbohydrates. p. 477. In R.L. Whistler and M.L. Wolfrom (ed.), Methods in carbohydrate chemistry. Vol. 1. Acad. Press, New York.

18. Dische, Z., C.M. Burgher, A. Danilchenko, and C. Roghschild. 1969. Variations in the composition of glycoproteins of the submaxillary and parotid saliva of the dog in relation to intensity of secretory stimulus. Arch. Biochem. Biophys. 135: 1-9.

19. Dettlebrandt, M. 1948. Application of the Weichselbaum buiret reagent to the determination of spinal fluid protein. Amer. J. Clin. Path. 18: 439-441.

20. Donaldson, D.M., and S. Marcus. 1958. Studies on serum bactericidal activity. Inter relationships of heparin, citrate, protamine and x-irradiation on serum and plasma bactericidal activity against Bacillus subtilis. J. Immunol. 81: 292-296.

21. Donaldson, D.M., B. Ellsworth, and A. Matheson. 1964. Separation and purification of β-lysin from normal serum. J. Immunol. 92: 896-901.

22. Donaldson, D.M., R.S. Jensen, B.M. Jensen, and A. Matheson. 1964. Serological relationships among β-lysin, plakin, and leukin. J. Bacteriol. 88: 1049-1955.

22a. Dunker, A.K. and R.R. Rueckert. 1969. Observations on molecular weight determinations on polyacrylamide gel. J. Biol. Chem. 244: 5074-5080.

23. Ehrenkranz, N.J., D.F. Elliott, and R. Zarco. 1971. Serum bacteriostasis of Staphylococcus aureus. Infection and Immunity. 3: 664-670.

24. Ehrlich, P. 1906. Studies on immunity. Chap. 2. (Translated by C. Bolduan). John Wiley & Sons, New York.

25. Ekstedt, R.D., and W.J. Nungester. 1955. Coagulase in reversing antibacterial activity of normal human serum on Micrococcus pyogenes. Proc. Soc. Expt. Biol. Med. 89: 90-94.

26. Ekstedt, R.D. 1956a. Further studies on the antibacterial activity of human serum on Micrococcus pyogenes and its inhibition by coagulase. J. Bacteriol. 72: 157-161.

27. Ekstedt, R.D. 1956b. The effect of coagulase on the antibacterial activity of normal human serum against selected strains of Micrococcus pyogenes. Ann. N.Y. Acad. Sci. 65: 119-131.

28. Ekstedt, R.D. 1965. Mechanisms of resistance to staphylococcal infection: Natural and acquired. Ann. N.Y. Acad. Sci. 126: 301-334.

29. Elson, L.A., and W.T.J. Morgan. 1933. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. 27: 1824-1828.

29a. Eylar, E.H. 1965. On the biological role of glycoproteins. J. Theoret. Biol. 10: 89-113.

29b. Feinberg, J.G. 1959. Feinberg agar gel cutters. Data sheet - Shandon Scientific Co., London.

30. Feingold, D.S., J.N. Goldman, and H.M. Kuritz. 1968. Locus of the action of serum and the role of lysozyme in the serum bactericidal reaction. J. Bacteriol. 96: 2118-2126.

31. Feingold, D.S., J.N. Goldman, and H.M. Kuritz. 1968. Locus of the lethal event in the serum bactericidal reaction. J. Bacteriol. 96: 2127-2131.

32. Fleming, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. London, Series B. 93: 306-317.

33. Fletcher, S. 1962. The growth of staphylococci in human serum. J. Path. Bact. 84: 327-335.

33a. Flodin, P. 1962. Dextran gels and their application in gel filtration University of Uppsala, Uppsala.

34. Ghuysen, J.M., J.L. Stominger, and D.J. Tipper. 1968. Bacterial cell walls, p. 53-104. In M. Florkin and E.H. Stotz (ed.). Comprehensive biochemistry, vol. 26A. Elsevier Publishing Co., Amsterdam.

35. Giblett, E.R. 1962. The plasma transferrins, p. 34-63. In A.G. Steinberg and A.G. Bearn (ed.), Progress in Medical Genetics, vol. 2. Grune & Stratton, New York.

35a. Gingell, D. 1973. Membrane permeability change by aggregation of mobile glycoprotein units. J. Theoret. Biol. 38: 677-679.

36. Gladstone, G.P., and H.H. Johnston. 1955. The effect of cultural conditions on the susceptibility of Bacillus anthracis to lysozyme. Brit. J. Expt. Path. 36: 363-372.

37. Glynn, A.A., and C.M. Milne. 1965. Lysozyme and immune bacteriolysis. Nature (London). 207: 1309-1310.

38. Glynn, A.A., and C.M. Milne. 1967. A kinetic study of the bacteriolytic and bactericidal action of human serum. Immunology. 12: 639-653.

39. Glynn, A.A. 1969. The complement lysozyme sequence in immune bacteriolysis. Immunology 16: 463-471.

40. Gottschalk, A. 1972. Glycoproteins: their composition, structure and function. 2nd ed. Elsevier Publishing Co., Amsterdam.

40a. Guenther, C.A., M.H. Welch, and J.F. Hammarsten. 1971. Alpha$_1$ antitrypsin deficiency and pulmonary emphysema. Ann. Rev. Med. 22: 283-292.

41. Haglund, H. 1967. Isoelectric focusing in natural pH gradients - a technique of growing importance for fractionation and characterization of proteins. Science Tools. 14: 17-23.

41a. Hanes, C.S. and F.A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature: 164: 1107-1112.

42. Hankin, E.H. 1891. A bacteria killing globulin. Proc. Roy. Soc. London. 48: 93.

43. Heimburger, N., and H. Haupt. 1965. Charaktorisierung von α_1 X-glycoprotein als chymotrypsin-inhibitor des human plasmas. Clin. Chim. Acta. 12: 116-118.

44. Higashi, Y., T. Kurimura, O. Juwahara, M. Ozaki, and T. Amano. 1963. Studies on the role of plakin. (From blood platelets of rat, rabbit and horse). X. Effect on membrane phospholipids. Biken. J. 6: 111-126.

45. Higashi, Y., H. Saito, Y. Yanagase, K. Yonemasu, and T. Amano. 1966. Studies on the role of plakin. XI. Demonstration of phospholipase A in plakin. Biken. J. 9: 249-262.

46. Hirsch, J.G. 1956. Phagocytin: A bactericidal substance from polymorphonuclear keukocytes. J. Expt. Med. 103: 589-611.

47. Hirsch, J.G. 1960. Further studies on preparation and properties of phagocytin. J. Expt. Med. 111: 323-337.

48. Hirsch, J.G. and Z.A. Cohn. 1960. Degranulation of polymorphonuclear leucocytes following phagocytosis of microorganisms. J. Expt. Med. 112: 1005-1014.

48a. Hormann, H. and T.L. Nagabhushen. 1964. Farbreaktion von kohlenhydraten II. Konstitution des Farbstuffs aus glucose und anthron. Hoppe Seyler's Z. Physico. Chem. 335 - 336: 280-287.

48b. Ihrig, J., J. Kleinerman, and D.J. Rynbrandt. 1971. Serum antitrypsins in animals. Studies of species variation, components, and the influence of certain mutants. Amer. Rev. Resp. Dis. 103: 377-389.

49. Inman, J.K., F.C. Coryell, K.B. McCall, J.T. Sgouris, and H.D. Anderson. 1961. A large-scale method for the purification of human transferrin. Vox. Sang. 6: 34-52.

50. Jacox, R.F. 1950. The activating effect of calcium on a bactericidal substance for Bacillus subtilis in human serum. J. Expt. Med. 92: 101-110.

51. Jamieson, J.C., A.D. Friesen, F.E. Ashton, and B. Chou. 1972. Studies on acute phase proteins of rat serum. I. Isolation and partial characterization of an α_1 acid glycoprotein and an α_2 macroglobulin. Can. J. Biochem. 50: 856-870

52. Jamieson, J.C., F.E. Ashton, A.D. Frieson, and B. Chou. 1972. Studies on acute phase proteins of rat serum. II. Determination of the contents of α_1 acid glycoprotein, α_2 macroglobulin, and albumin in serum from rats suffering from induced inflammation. Can. J. Biochem. 50: 871-880.

52a. Jennings, R.K., E. Arditi, and M.A. Kaplan. 1960. Gel diffusion studies of serologically related antigens. I. Zone displacement in complex precipitating systems. Int. Arch. Allergy 17: 257-266.

53. Jensen, R.S., J.G. Tew, and D.M. Donaldson. 1967. Extracellular β-lysin and muramidase in body fluid and inflammatory exudates. Proc. Soc. Expt. Biol. Med. 124: 545-549.

54. Jeppsson, J.O. 1967. Isolation and partial characterization of three human transferrin variants. Biochem. Biophys. Acta. 140: 468-476.

55. Johnson, F.B., and D.M. Donaldson. 1968. Purification of staphylocidal β-lysin from rabbit serum. J. Bacteriol. 96: 589-595.

56. Joos, R.W., and W.H. Hall. 1968. Fate of staphylococci in fresh rabbit blood and serum. Proc. Soc. Expt. Biol. & Med. 128: 49-50.

57. Kaback, H.R. 1970. Transport. Ann. Rev. Biochem. 39: 561-598.

58. Kabat, E.A., and M.M. Mayer. 1967. Kjeldahl nitrogen determination, p. 476-478. In E.A. Kabat and M.M. Mayer (ed.), Experimental Immunochemistry, 2nd Ed. Thomas Publishing Co., Springfield.

59. Keller, W., and R.B. Pennell. 1959. Sterilization of plasma components by heat. I. β_1 metal-combining protein. J. Lab. Clin. Med. 53: 638-645.

59a. Kendall, J. 1926. Smith's inorganic chemistry. The Century Co., New York. p. 476.

60. Kern, R.A., M.J. Kingkade, S.F. Kern, and O.K. Behrens. 1951. Characterization of the action of lysozyme on Staphylococcus aureus and on Micrococcus lysodeikticus. J. Bacteriol. 61: 171-178.

61. Kirkpatrick, C.H., T. Green, R.R. Rich, and A.L. Schade. 1971. Inhibition of Growth of Candida albicans by iron-unsaturated bactoferrin: relation to host-defense mechanisms in chronic mucocutaneous candidiasis. J. Infect. Dis. 124: 539-544.

61a. Kohlraush, F. 1897. Ueber concentrations-verschiebungen durch electrolyse im innern von lösungen und lösungsgemischen. Ann. Physik. 62: 209.

61b. Laurent, T.C., and J. Killander. 1964. A theory of gel filtration and its verification. J. Chromatog. 14: 317-330.

62. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.T. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193: 265-275.

63. Matheson, A. and D.M. Donaldson. 1970. Effect of β-lysin on isolated cell walls and protoplasts of Bacillus subtilis. J. Bacteriol. 101: 314-317.

63a. Marshall, R.D. 1972. Glycoproteins. Ann. Rev. Biochem. 41: 673-702.

64. Michael, J.G. 1969. Natural antibodies. Current Topics in Micro. and Immunol. 48: 43-62.

65. Mitruka, B.M. 1971. Biochemical aspects of Diplococcus pneumoniae infections in laboratory rats. Yale J. Biol. Med. 44: 253-264.

66. Morse, S.I. 1962. Studies on the chemistry and immunochemistry of cell walls of Staphylococcus aureus. J. Expt. Med. 116: 229-245.

66a. Moskowitz, R.W. and G. Heinrick. 1971. Bacterial inactivation of human serum alpha-1 antitrypsin. J. Lab. Clin. Med. 77: 777-785.

67. Murphy, S.G., A.S. Klainer, and D.F. Clyde. 1972. Characterization and patho-physiology of serum glycoprotein alterations in malaria. J. Lab. Clin. Med. 79: 55-61.

68. Mudd, S., B. Lucke, M. McCutcheon, and M. Strumia. 1930. On the mechanism of opsonin and bacteriotrophin. VI. Agglutination and tropin action by precipitin sera. Characterization of the sensitized surface. J. Expt. Med. 52: 313-329.

68a. Myerowitz, R.L., Z.T. Handzel, and J.B. Robbins. 1972. Human serum α_1 antitrypsin: isolation and demonstration of electrophoretic and immunologic heterogeneity. Clin. Chim. Acta. 39: 307-317.

69. Myrvik, Q.N., and R.S. Weiser. 1955. Studies on antibacterial factors in mammalian tissues and fluids. I. A serum bactericidin for Bacillus subtilis. J. Immunol. 74: 9-16.

70. Myrvik, Q.N. 1956-57. Serum bactericidins active against gram-positive bacteria. Ann. N.Y. Acad. Sci. 66: 391-400.

71. Myrvik, Q.N., E. Soto-Figueroa, G. Naff, and E. Henelt. 1958. Studies on antibacterial factors in mammalian tissues and fluids. II. Cofactor requirements of a serum bactericidin for Bacillus subtilis. J. Immunol. 81: 118-122.

72. Myrvik, Q.N., and E.S. Leake. 1960. Studies on antibacterial factors in mammalian tissues and fluids. IV. Demonstration of two non-dialyzable components in the serum bactericidin system for Bacillus subtilis. J. Immunol. 84: 247-250.

73. Naff, G., Q.N. Myrvik, E. Henelt, and E. Soto-Figueroa. 1958. Studies on antibacterial factors in mammalian tissues and fluids. III. Serum bactericidin levels for Bacillus subtilis in myocardial infarction, pneumonia and other diseases of man. J. Immunol. 81: 123-125.

74. Neu, H.C., J. Breyfus, and R.E. Canfield. 1968. Effect of human lysozyme on gram-positive and gram-negative bacteria. Antimicrobial agents and chemotherapy. 8: 442-444.

75. Nuttall, G. 1888. Experiments über die bacterienfeindlicken einflüsse des thierischen körpers. Hyg. Infektionskrankh. 4: 353-394.

75a. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. N.Y. Acad. Sci. 121: 321-349.

76. Osborn, M.J. 1969. Structure and biosynthesis of the bacterial cell wall. Ann. Rev. Biochem. 38: 501-538.

76a. Ouchterlony, O. 1968. Diffusion-in-gel methods for immunological analysis. In Handbook of immuno-diffusion and immunoelectrophoresis. Ann Arbor Sci. Publ. Inc., Ann Arbor.

77. Park, J.T. and R. Hancock. 1960. A fractionation procedure for studies on the synthesis of cell-wall mucopeptide and of other polymers in cells of Staphylococcus aureus. J. gen. Microbiol. 22: 249-258.

78. Partridge, S.M. 1950. Partition chromatography and its application to carbohydrate studies. Biochem. Soc. Symposia. 3: 52-61.

79. Pensky, J., C.F. Hinz, E.W. Todd, R.J. Wedgwood, J.T. Boyer, and I.H. Lepaw. 1968. Properties of highly purified human properdin. J. Immunol. 100: 142-158.

80. Pettersson, A. 1924. Ueber die thermostabilen bakteriolytischen substanzen des normalserums. Z. Immunitätsforsch. 40: 43.

81. Philips, D.C. 1968. The three-dimensional structure of an enzyme molecule, p. 52-64. In R.H. Haynes and P.C. Hanawalt (ed.), The molecular basis of life. W.H. Freeman & Co., San Francisco.

82. Pillemer, L., L. Blum, I.H. Lepaw, O.A. Ross, E.W. Todd, and A.C. Wardlaw. 1954. The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immunephenomena. Science 120: 279-285.

82a. Porath, J. and P. Flodin. 1959. Gel filtration: a method for desalting and group separation. Nature. 183: 1657-1659.

82b. Porath, J. 1962. Cross-linked dextrans as molecular seives. Advan. Prot. Chem. 17: 209-226.

83. Putnam, E.W. 1957. Paper chromatography of sugars, p. 62-72. In S.P. Colowick and N.O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.

83a. Reisfeld, R.A., U.J. Lewis, and D.E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 281-283.

84. Rimington, C. 1940. Seromucoid and the bound carbohydrates of the serum proteins. Biochem. J. 34: 931-940.

85. Rowley, D. 1960. The role of opsonins in non-specific immunity. J. Expt. Med. 111: 137-144.

86. Salton, M.R.J., and R.W. Horne. 1951. Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. Biochim. Biophys. Acta. 7: 177-197.

87. Sarcione, E.J., and M. Bohne. 1969. Synthesis of alpha$_2$ (acute phase) globulin by fetal and neonatal rat liver in vitro. Proc. Soc. Expt. Biol. & Med. 131: 1454-1456.

87a. Sawamura, R., and T. Kayuma. 1964. Color reactions of pentoses with anthrone. II. Color substance produced by the reaction of furfural with anthrone. Chem. Abst. 61: 1818.

88. Schade, A.L. 1963. Significance of serum iron for the growth, biological characteristics, and metabolism of Staphylococcus aureus. Biochem. Z. 338: 140-148.

89. Scheidegger, J.J. 1955. Une micro-methode de l' immuno-electrophorese. Int. Arch. Allergy Appl. Immunol. 7: 103-110.

89a. Schultze, H.E., K. Heide, and H. Haupt. 1962. α_1 Antitrypsin aus human serum. Klin. Wochen. 40: 427-429.

90. Schultze, H.E., K. Heide, and H. Haupt. 1963. Isolation of an easily precipitable α_1-glycoprotein of human serum. Nature (London) 200: 1103.

91. Schultze, H.E., and J.F. Heremans. 1966. Molecular biology of human proteins. Vol. 1., p. 173-235. Elsevier Publishing Co., Amsterdam.

91a. Shapiro, A.L., E. Vinuela, and J.V. Maizel, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Comm. 28: 815-820.

92. Shetlar, M.R. 1952. Use of anthrone reaction for determination of carbohydrates in the presence of serum protein. Analyt. Chem. 24: 1844-1846.

93. Shetlar, M.R., J.V. Foster, and M.R. Everett. 1948. Determination of serum polysaccharides by the tryphophane reactions. Proc. Soc. Expt. Biol. & Med. 67: 125-130.

94. Skarnes, R.C., and D.W. Watson. 1956. Characterization of leukin: an antibacterial factor from leucocytes active against gram-positive pathogens. J. Expt. Med. 104: 829-845.

95. Skarnes, R.C., and D.W. Watson. 1957. Antimicrobial factors of normal tissues and fluids. Bacteriol Rev. 21: 273-294.

96. Skarnes, R.C. 1967. Leukin, a bactericidal agent from rabbit polymorphonuclear leucocytes. Nature (London) 216: 806-808.

97. Smith, I. 1960. Chromatographic and electrophoretic techniques. London.

98. Spackman, D.H., W.H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Analyt. Chem. 30: 1190-1206.

98a. Spiro, R.G. 1970. Glycoproteins. Ann. Rev. Biochem. 39: 599-638.

99. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta. 24: 604-611.

99a. Svensson, H. 1962. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. III. Description of apparatus for electrolysis in columns stabilized by density gradients and direct determination of isoelectric points. Arch. Biochem. Biophys. Suppl. 1: 132-138.

99b. Snyder, S. and G. Ashwell. 1971. Quantitation of specific serum glycoproteins in malignancy. Clin. Chim. Acta. 34: 449-455.

100. Tager, M. 1947-1948. Concentration, partial purification. properties, and nature of staphylocoagulase. Yale, J. Biol. & Med. 20: 487-501.

101. Tew, J.G., W.M. Hess, and D.M. Donaldson. 1969. Lysozyme and β-lysin release stimulated by antigen-antibody complexes and bacteria. J. Immunol. 102: 743-750.

102. Vesterberg, O. 1971. Staining of protein zones after isoelectric focusing in polyacrylamide gels. Biochim. Biophys. Acta. 243: 345-348.

102a. Vesterberg, O. and H. Svensson. 1966. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. Acta. Chem. Scand. 20: 820-834.

103. Wardlaw, A.C., and L. Pillemer. 1956. The properdin system and immunity. V. The bactericidal activity of the properdin system. J. Expt. Med. 103: 553-575.

104. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234: 1971-1975.

105. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4405-4412.

106. Weichselbaum. T.E. 1946. An accurate and rapid method for the determinations of proteins in small amounts of blood serum and plasma. Amer. J. Clin. Path. Tech. Bull. 10: 40.

107. Welshimer, H.J., and C.F. Robinow. 1949. The lysis of Bacillus megatherium by lysozyme. J. Bacteriol. 57: 489-499.

108. Wiedermann, D., D. Wiedermannova, J.P. Vaerman, and J.F. Heremans. 1970. A longitudinal study of serum α_1 antitrypsin, α_2 macroglobulin, transferrin, immunoglobulins IgG, IgA, and IgM, and H- and O- agglutnin titers in children following infections with Salmonella enteritidis. J. Infect. Dis. 121: 74-77.

109. Winzler, R.J. 1960. Glycoproteins, p. 309-347. In F.W. Putnam (ed.), The plasma proteins, vol. 1 Academic Press, Inc., New York.

109a. Worthington Enzyme Manual. 1972. Trypsin p. 125-127. Worthington Biochemical Corporation, Freehold, New Jersey.

110. Wright, A.E., and S.R. Douglas. 1904. Further observations on the role of blood fluids in connection with phagocytosis. Proc. Roy. Soc. London Series B 73: 128-142.

111. Yotis, W.W., and R.D. Ekstedt. 1960. Studies on staphylococci. III. Further studies on purification and mechanism of action of an antibacterial human serum factor. J. Bacteriol. 80: 719-725.

112. Yotis, W.W. 1962. Effect of the antibacterial serum factor on staphylococcal infections. J. Bacteriol. 83: 137-143.

113. Yotis, W.W. 1963. Absorption of the antibacterial serum factor by staphylococci. J. Bacteriol. 84: 911-917.

113a. Yotis, W.W. 1966. Hydrolysis of synthetic esters by the antibacterial agent in serum. J. Bacteriol. 91: 488-493.

114. Yotis, W.W., and J.R. Lentino. 1973. Further studies on a serum nonantibody α globulin with antistaphylococcal activity. J. Appl. Bacteriol. 36: 147-155.

115. Yotis, W.W., and J.S. Ortiz. 1967. An assessment of the inhibitory activity of rat serum on staphylococci. Ant. van Leeuwenhoek. 33: 87-96.

116. Zeldow, B.J. 1963. Studies on the antibacterial action of human saliva. III. The cofactor requirements of a lactobacillus bactericidin. J. Immunol. 90: 12-16.

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APPROVAL SHEET

The dissertation submitted by Joseph Richard Lentino has been read and approved by the members of the advisory committee listed below.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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