ABSTRACT

IMMUNOLOGIC RESPONSES OF RABBITS TO *MYCOBACTERIUM BOVIS*; CHEMICAL, ELECTROPHORETIC AND ANTIGENIC ANALYSES OF BACILLARY EXTRACTS

by Gary F. Dardas

Antibody production was studied in experimental models designed to simulate (a) active tuberculosis, (b) closed tuberculosis with tuberculoimmunity and tuberculin hypersensitivity, (c) no tuberculosis with tuberculoimmunity and tuberculin sensitivity, and (d) no tuberculosis with tuberculoimmunity and reduced tuberculin sensitivity. Passive hemagglutination and bacterial agglutination tests were used for antibody determinations.

Rabbits infected with virulent *M. bovis* (strain 310) (a) produced negligible amounts of antibody. The antibody response of rabbits infected with attenuated *M. bovis* (strain BCG)(b) was comparable to that elicited by killed cells. Antibody elicited by injections of heat or acetone-killed cells was greater than was elicited by inoculation with betapropriolactone-killed cells (c) or when subsequently extracted with methanol and acetone (d).

A sequential production of mercaptoethanol-sensitive followed by mercaptoethanol-resistant antibody was detected

in all rabbits regardless of the antigen preparation. Mercaptoethanol-sensitive and resistant antibody was detected in sera from most rabbits for the duration of the experimental period, up to 23 weeks.

Skin testing with PPD-S 14 weeks post-inoculation stimulated antibody production in approximately 50% of the rabbits tested. Both the absolute and relative amounts of the two types of antibody produced were changed as a result of skin testing. Extraction of betapropiolactone-killed cells of M. bovis with methanol and acetone considerably reduced their ability to sensitize rabbits to tuberculin (d).

The antigenic and chemical composition of ultrasonic extracts of viable cells of M. bovis varied with the length of incubation of the cells, prior to insonation, and the intensity of insonation used for extraction. Disc electrophoresis effectively separated the greatest number (24) of constituents in ultrasonic extracts. Both carbohydrate and protein components were detected using disc electrophoresis. Antigens were detected in ultrasonic extracts by Ouchterlony immunodiffusion and immunoelectrophoresis. Ultrasonic extracts of cells from two and one-half month-old cultures contained 20-22 separate antigen-antibody systems detected by immunoelectrophoresis.

Chemical extraction of viable cells with Triton X-100, sodium desoxycholate, urea, guanidine and phosphate buffer

containing ethyl ether yielded antigen preparations of varying complexity. From 4-13 components were detected in chemical bacillary extracts by disc electrophoresis. The number of antigens detected by Ouchterlony immunodiffusion was usually less than was detected by immunoelectrophoresis.

IMMUNOLOGIC RESPONSES OF RABBITS TO *MYCOBACTERIUM BOVIS*; CHEMICAL, ELECTROPHORETIC AND ANTIGENIC ANALYSES OF BACILLARY EXTRACTS

By

Gary Frank Dardas

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1967

ACKNOWLEDGEMENTS

The author wishes to express his appreciation and thanks to Dr. V. H. Mallmann for her interest and guidance throughout this investigation. Dr. Mallmann's fine example of high ethical and academic standards will always be admired and appreciated. Appreciation is also extended to Dr. W. L. Mallmann for his wise counsel on many aspects of this study.

To the author's brother, Dr. Terry Dardas, goes my special thanks for many fine suggestions and contributions to this research.

A special expression of appreciation goes to the author's wife, Patricia, and family for their great understanding and inspiration without which this work would not have been possible.

The author is also indebted to the many members of the tuberculosis project who have contributed so much directly and indirectly to this investigation.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued Page

LIST OF TABLES

LIST OF TABLES - Continued

LIST OF TABLES - Continued

LIST OF FIGURES

LIST OF FIGURES - Continued

FIGURE

LIST OF FIGURES - Continued

FIGURE Page

LIST OF FIGURES - Continued

LIST OF FIGURES - Continued

INTRODUCTION

Tuberculosis is the single major bacterial disease for which serologic procedures fail to yield reliable information of diagnostic or prognostic significance. Isolation and identification of the causative agent is troublesome and time consuming but remains the only reliable proof of active disease. The tuberculin test fails to differentiate between infection and disease but serves as a major tool in the detection of tuberculosis.

Understanding of the role of atypical mycobacteria in infection and heterologous sensitization to tuberculin necessitates the use of more specific mycobacterial sensitins for skin testing (159,227,112).

Very few investigations have been concerned with the fundamental properties of the antibody response elicited by mycobacterial antigens. Only recently have such studies been undertaken (40,42,136). A better understanding of the antibody response to tuberculosis may eventually lead to the development of meaningful serological procedures useful in diagnosis.

The antigenic composition of different mycobacteria is largely unknown despite much investigation. Mycobacterial preparations currently available for skin tests, including

"purified protein derivatives" (PPD), are crude and antigenically complex mixtures. A need exists for specific antigens and sensitins from the classical and atypical mycobacteria which are of pathogenic and epidemiologic significance.

This is a report of studies of antibody production and induction of hypersensitivity in rabbits to constituents of M. bovis. Passive hemagglutination and bacterial agglutination were used to detect antibodies produced in rabbits inoculation with virulent, attenuated, or killed M. bovis. Disc electrophoresis, Ouchterlony immunodiffusion and immunoelectrophoresis were used to study the chemical and antigenic composition of bacillary extracts of viable cells of M. bovis prepared by ultrasonic disruption or chemical treatment.

HICTORICAL REVIEW

The need for a reliable diagnostic test for tuberculosis still exists. A prime objective of such a test would be to differentiate between infection and disease and between active and closed cases of the disease if it existed. Despite much investigation, no such test or combination of tests have been found. The tuberculin test is the most widely used test for the detection of tuberculosis. This test also fails to distinguish between infection and disease, or between present and past infections. Furthermore, it does not indicate conclusively which species of mycobacteria has induced sensitivity. The tuberculin reaction has been reviewed as to the specific and nonspecific immunologic factors involved (6,137); the general mechanisms (122) and epidemiologic significance (54). It is an area of considerable research activity today but fundamental knowledge is meager.

Sensitization by many of the well-defined mycobacterial species as well as the anonymous (unclassified, atypical) mycobacteria has been well documented. This imposes many complications on diagnosis and epidemiology (54,138,112,159, 227,226). The need exists for skin test preparations (sensitins) with which the causative agent of sensitization, with or without disease, can be determined more reliably.

Antibodies with specificities for various antigenic constituents of mycobacteria may be present in sera from infected individuals. A number of serologic tests for the detection of antibody in serum from tuberculous individuals have been used. These tests include agglutination, passive hemagglutination, passive hemolysis, precipitation and complement fixation reactions and many modifications. Middlebrook and Dubos developed a passive hemagglutination test with which antibodies specific for tuberculopolysaccharide can be detected (117). Many modifications and conflicting results on the reliability of the test have been reported (157,174,98,180,147,195,70,160,88). Treatment of erythrocytes with tannic acid (19) decreases their adsorption of polysaccharides and increases the adsorbtion of protein. It has been used to detect tuberculoprotein-specific antibodies (20,195). Protein antigens can be coupled to formalinized erythrocytes with bis-diazotized benzidine (30) and has been used to detect tuberculoprotein-specific antibodies (28,29). Turcotte and co-workers used the bis-diagotized modification to examine serums from tuberculous individuals (203,204,64). Serum to be tested was fractionated by chromatography on DEAE-cellulose or treated with mercaptoethanol to destroy the activity of macroglobulin antibody, IgM. The results obtained suggested that only the presence of 7 S antibody, IgG, was indicative of active disease.

Tuberculophosphatides have been adsorbed to kaolin particles to detect antibodies specific for the phosphatide fractions (196,193,194,195). The specific agglutination of the tuberculophosphatide-kaolin particles are independent of specific reactions with tuberculoprotein or tuberculo-polysaccharide (196). The kaolin-phosphatide test was of no value in detecting disease or differentiating causative agents in calves inoculated with different mycobacteria, and phosphatides extracted from the different mycobacteria did not improve the test (153).

Takahashi and his associates described the occurrence of antibodies of more specificities in patients with tuberculosis, antiprotein, polysaccharide and phosphatide (192).

The sequence of production and the titers obtained of antiphosphatide antibody most faithfully reflected the degree of development of the infection (192). Anti-protein and anti-polysaccharide antibody was produced for long periods without regard to the virulence of the infecting organism. There was no apparent relationship between the occurrence of antibodies and the presence of delayed type hypersensitivity to mycobacterial constituents (192).

A combination of an immediate-type reaction and precipitins (agar double diffusion test) has been used for the diagnosis of active pulmonary tuberculosis. The antigens used for both of these tests were either crude extracts or chromatographic fractions of extracts of mechanically disrupted mycobacteria (71,72,73).

A double diffusion test was developed (132,133,134,135), which clinical evaluation indicates is a diagnostic aid (2,119,67,200,108).

Our knowledge of many fundamental properties of antibodies and the immune response has been greatly expanded during the past decade. The development of refined techniques for biochemical analyses has facilitated investigations of the physical and chemical properties of immunoglobulins (62). Stelos (184,185,186) detected two molecular sizes of antibody elicited in rabbits by injections of ovine or bovine erythrocytes. The different molecular species of antibody had differences in their serological activity. The production of at least two different molecular species of antibodies by rabbits was confirmed and extended (11,12,13,15). There was a sequential production of the two different antibodies. The high molecular weight antibody with a sedimentation constant of approximately 19 S (macroglobulin, $gamma_1$, IgM) was produced first and subsequently, a lower molecular weight antibody ($gamma_2$-globulin, IgG) with a sedimentation constant of approximately 7 S ($gamma_2$-IgG). Human serum macroglobulins were sensitive to reductive cleavage by mercaptoethanol (50) and the reduction was used to distinguish between the immunoglobulins. Reductive cleavage by mercaptoethanol and fractionation of serum proteins has aided in determining the temporal sequence of the kinds of immunoglobulins elicited by a variety of antigens in different species of animals (23,205, 208,158,206,17,211,121).

When viral antigens were injected into rabbits, the IgM was first detected four-five hours after a single intravenous injection of virus; IgG was detected one-two days after antigen injection (189,190,191). The rate of formation, amount and persistence of antibody were antigen-dose dependent. Prolonged production of IgG and transient responses of only IgM were produced. Approximately 50 times more antigen was required to induce production of IgG than to induce production of IgM. Gamma M and gamma G antibody responses differed in four fundamental properties; (1) the amount of antigen required for induction and maintenance of antibody synthesis, (2) the kinetics of antibody synthesis, (3) specific anamnestic response, and (4) in sensitivity to irradiation.

There have been extensive studies on the effect of route of injection and dosage of antigen upon the gross properties of the antibody response (100,136,207,140,189,190, 191,210); variations in antibody response to soluble or particulate antigens (120,202,155); and variations in the electrophoretic mobility of the immunoglobulins involved (218,123,202,142).

Results of recent investigations using sensitive methods for antibody detection have indicated that IgG and IgM may be synthesized simultaneously and not sequentially as had been previously observed (210,162,3,65). There is evidence that specific agglutination reactions may be more sensitive for the detection of IgM than for IgG (15,76,16,210) and

that differences exist between the binding constants of IgG and IgM (125,75). The IgM is reportedly 750 times more efficient than IgG on a molar basis as hemolysins or hemagglutinins (75); 60-180 times as hemolysins and hemagglutinins using an anti-azobenzenearsonate antibody system (123); 22 times as bacterial agglutinins, 120 times more potent in sensitizing bacteria for complement lysis and 500-1000 times more efficient as an opsonin (152). The production of IgM and IgG may be independent responses and, depending on the nature of the antigens, the amount of antigen and the inoculation schedule of the antigen, the two antibody types may be synthesized simultaneously or sequentially (210).

There have been few studies on the kind of antibodies elicited by the mycobacteria which cause natural or experimental disease. Chronicity and the inconsistency of antibody production of relationship to diagnosis or prognosis is well-established. The mechanism of enhanced resistance to tuberculosis in vaccinated individuals inoculated with BDG (Bacillus of Calmette-Geurin; attenuated, viable M. bovis) has been and continues to be of major academic and practical importance. Theories have been proposed but to date none are universally accepted (52,144,22,172,146,31). The role of antibodies is controversial. Seibert (172) proposed a complex and changing balance in the host of tuberculopolysaccharides, antibodies and lysozyme (171,172). Generally, viable cells are necessary to induce tuberculoimmunity.

Delayed sensitivity is also induced. Its role is unknown. Skin testing has, however, been reputed to stimulate increased antibody production (111,153,174).

The rabbit antibody responses to mycobacteria do not seem to differ significantly from the responses to other particulate antigens (40,41,42,43,136,45).

Results from very recent studies with mycobacterial antigens indicate that the route of inoculation, antigen concentration and use of adjuvants influence the nature of the antibody response (40,45). Both IgM and IgG have elicited in rabbits by viable BCG. The detection of IgG was more indicative of active disease in rabbits than detection of IgM but was not consistent with active disease in humans (43). Parlett and Chu (136) detected antibody production earlier with antigens in cell extracts and culture filtrates than to viable cell suspensions. They suggested that the availability of antigen or the relative dosage received was responsible. The present difficulty experienced in interpreting the results of serological tests may be due to our lack of knowledge of temporal patterns of antibody production in infected tuberculous individuals (136).

Immunogenic and Allergenic Constituents of Mycobacteria

The basis of tuberculoimmunity (enhanced resistance to tuberculosis) is unknown. The relationship between tuberculin hypersensitivity and antibody production in tuberculosis

is controversial. Mycobacterial components can be antigenic (stimulate antibody production), allergenic (induce hypersensitivity), and immunogenic, although these conditions do not necessarily coexist.

Suspensions of particulate material from mycobacteria have been shown to induce delayed type hypersensitivity and induce enhanced resistance in experimental animals to virulent organisms. Ribi and co-workers studied cell wall and cytoplasmic fractions from mechanically disrupted cells of various species of mycobacteria (150,99,151). Cell wall constituents induced delayed type hypersensitivity and caused dermal lesions in normal rabbits (150,99). Protoplasmic fractions failed to cause dermal lesions or to induce delayed hypersensitivity. A delayed hypersensitivity reaction was elicited by protoplasmic fractions when injected intradermally into rabbits which had previously been sensitized with intact cells or a cell wall fraction.

A cell wall constituent other than "wax D" was responsible for eliciting hypersensitivity (99). This factor consisted of a firmly bound lipid and a peptide composed of alanine, glutamic acid and diaminopamilic acid. The specificity of the delayed hypersensitivity reaction provoked in sensitive animals by intradermal injections of cell walls or protoplasm differed significantly. Cell wall preparations from different species of mycobacteria elicited reactions in animals sensitized with heterologous species. In contrast,

delayed reactions using protoplasmic fractions were greater in intensity with animals sensitized with the homologous species. Cell walls from a variety of mycobacterial species contained closely related constituents whereas protoplasmic fractions contained more species or strain-specific constituents.

Mice immunized with a killed vaccine consisting of cells mechanically disrupted in mineral oil were as resistent to virulent organisms as mice which had been vaccinated with the Bacillus of Calmette-Geurin (BCG). The potency of this vaccine was correlated with the presence of cell wall fractions from the disrupted cells. Only oil disruption products from either BCG or M. tuberculosis (H37Ra) were immunogenic. Cell walls from several strains of atypical mycobacteria and BCG had a common factor which was correlated with the protective potency and virulence of the organism.

Cell wall preparations from various mycobacteria have been shown to induce enhanced resistance to infection with other species and genera of bacteria (59,60,61). Mycobacterial cell walls extracted with 20% urea yielded a resistance-enhancing factor which represented 15% of the dry weight of the cell wall (61). The extract was chemically complex and consisted of protein, carbohydrate, lipid and nucleic acid. It was not antigenic and when injected into experimental animals, induced resistence against a variety of viral and bacterial agents (60).

Enhanced resistance was induced in animals inoculated with either whole cells or chemical fractions from various mycobacteria (215). Vaccination of mice with live or inactivated acetone, methanol, NaOH-extracted BCG induced resistance to subsequent infections with virulent staphylococci (51). Methanol extracts from live or killed BCG induced enhanced resistance in mice to subsequent infection with either Staphylococcus aureus or M. fortuitum. Moreover, if methanol extracts of BCG or the residue fraction from methanol extracts were injected simultaneously or shortly after injection of mice with Staphylococcus aureus or M. fortuitum, their mean survival time was shortened (161). Enhanced resistance was attributed to a polysaccharide present in the methanol extract and was described as being broadly specific rather than nonspecific (217). Weiss and co-workers (212,213) found that methanol-soluble fractions from BCG had little protective effect whereas vaccination with phenol killed cells extracted with chloroform, methylchlorobenzene or methanol provided enhanced resistance (213).

A highly immunogenic particulate fraction was isolated from the cytoplasm of mechanically, chemically, or enzymatically disrupted mycobacteria (225). Neither soluble cytoplasmic constituents nor the intracellular particulate fraction from disrupted organisms induced hypersensitivity in normal animals although both provoked delayed hypersensitivity reactions in animals which had been previously sensitized with cell walls. Ribosomes obtained from young cells

by extraction with sodium dodocyl sulfate were immunogenic and resembled the intracellular particulate fractions. The immunogenecity of this preparation was destroyed by treatment with RNAase.

Crowle (31) reviewed the subject of immunizing constituents from mycobacteria. Of the many chemical fractions of tubercle bacilli tested for immunogenecity, the lipid components were thought to be most active. "Antigen methyleque" obtained from acetone washed, autoclaved, air-dried cells, increased resistance in animals to virulent organisms. Bacillary extracts containing "antigen methyleque" are antigenic and contain tuberculophosphatide in relatively pure form although other lipid and nitrogenous components have been detected (31). Tuberculophosphatide is the single, chemically defined mycobacterial constituent which has been shown to be immunogenic. The wax fractions from tubercle bacilli do not appear to be immunogenic although wax B has not been thoroughly investigated (31). Water soluble cell-free extracts from acetone washed, trypsin digested M. tuberculosis were studied for their immunogenicity (33,34,35,36). Immunization with the trypsin extract was as effective as with whole bacilli (35). The level of resistance was greatest when the homologous strain or species was used to challenge the immunity of the animals. Immunization of mice or guinea pigs with the trypsin extract did not induce hypersensitivity to tuberculin, did not elicit antibody formation

and did not precipitate with antiserum (36). Chemical analyses of the extract indicated the active component was carbohydrate and was normally present in the cell wall (37).

A major disadvantage of vaccination with mycobacteria or products derived from mycobacteria is the possibility of inducing delayed type hypersensitivity which eliminates the use of skin tests for the detection of tuberculosis. Protein-lipid complexes may be responsible for both immunogenicity and allergenicity (214). Components responsible for these two possibly related phenomenon have not been chemically separated (175). Extraction of mycobacteria with neutral solvents have been reported on the one hand to increase their immunogenicity, and on the other hand not to increase their immunogenicity (63). Progressive removal of the lipid components from mycobacteria was found to decrease both the immunogenicity and allergenicity of defatted organisms (63). Lipopolysaccharide fractions from paraffin oil extracts of killed cells did not induce a significant level of resistance in mice to virulent organisms (154).

Raffel (145) demonstrated that neither tuberculo-protein nor wax constituents alone were capable of eliciting hypersensitivity but that a combination of the two constituents was necessary. The protein-containing wax fractions would induce hypersensitivity to several materials including ovalbumin and picryl chloride. Tuberculopolysaccharides either alone or with other bacillary components were not immunogenic.

Hypersensitivity was induced in guinea pigs with tuberculoprotein but resistance was not enhanced. Raffel concluded that acquired resistance and delayed sensitivity could not be attributed to any chemically pure substance of the tubercle bacillus.

Guinea pigs inoculated with crude protein-containing fractions from borate buffer extracts of mechanically disrupted cells did not have increased resistance although they developed hypersensitivity to tuberculin and produced antibody specific for tuberculoprotein (31).

Freund and Stone (66) isolated a component from M. tuberculosis which was responsible for the adjuvant effect and designated it wax D. White and Marshall (216) reported that either wax D or a cell wall fraction consisting of firmly bound lipids and a peptide containing glutamic acid, alanine and diaminopamilic acid was necessary for the development of hypersensitivity.

A basic failure of much of the research on immunogenicity and allergenicity of fractions of mycobacteria is that these fractions have not been chemically defined and purified. In order for immunogenicity, antigenicity and allergenicity to be fully understood, more purified components from mycobacteria must be used. Procedures for evaluating immunogenicity must be standardized.

Antigens of Mycobacteria

The chemical composition of mycobacteria has been reviewed by a number of authors (168,5,183). Despite much investigation, the chemical and antigenic nature of mycobacterial constituents are poorly understood. A characteristic of mycobacteria is the high percentage of lipid, up to 40% of the dry weight of the cell (7). Lipids may be responsible for many of the biological and chemical properties of the mycobacteria including immunologic adjuvant activity, induction of antibody formation, induction of delayed hypersensitivity, acid-fastness, their hydrophobic nature and possibly virulence (7). Although the polysaccharide components of mycobacteria are diverse and chemical complex, there is little or no evidence that strain-specific polysaccharides exist (181). Crowle (31) noted that tubercle bacilli contain at least nine different proteins, at least two distinct polysaccharides, three different lipids and a variety of yet undetermined constituents.

Cell walls from certain species of Mycobacterium, Nocardia and Corynebacterium have been found to be similar in chemical composition suggesting that these three genera are closely related taxonomically (38,39). The principle components detected were galactose, arabanose, hexosamine, glucose, alanine, glutamic acid and diaminopamilic acid.

Tuberculopolysaccharide antigens have been found within the cell wall (39). Meynell (116) suggested that the outermost superficial layers consisted of polysaccharide while

those more deeply situated in the cell envelope were protein.

From defatted mechanically disrupted cells, three antigenic components were isolated by salt fractionation from aqueous extracts and two were isolated by extraction of the cell debris with neutral salts (114,115). Three different antigenic proteins were obtained from frozen or vacuum dried mycobacteria by extraction with acetone and alkaline solutions of different pH (114). The components responsible for the specificity of mycobacteria resided in the protein constituents of the cells rather than in the carbohydrates.

A protein fraction was obtained from live tubercle bacilli by extraction with solid urea for several days at 37°C (87). The antigenic fraction was 95% homogenous as determined by electrophoresis, yielded positive complement fixation reactions with serums from tuberculous patients, and elicited hypersensitivity reactions in sensitized individuals. A polysaccharide component was extracted from the moist, steam-killed tubercle bacilli by extraction with solid urea (182). The extract had a single electrophoretic component and reacted *in vitro* with sera from tuberculous patients.
A single antigenic protein fraction was extracted with urea which had an electrophoretic mobility similar to the C protein isolated from culture filtrates (196). It was more potent than C protein in eliciting skin reactions in sensitized animals. The fraction was pyrogenic but not immunogenic. Injections of the urea extract in the normal guinea pigs induced delayed hypersensitivity to tuberculin. An interesting

property of the urea extract was the ability to inhibit the *in vitro* migration of polymorphonuclear neutrophils from tuberculous guinea pigs (196). Analyses of urea extracts by immunodiffusion detected two or more precipitinogens (170).

Serologic relationships among mycobacteria and related genera were studied using chemical fractions from intact or disintegrated cells (95,96,97). Three serologic groups of mycobacteria were distinguished with the chemical extracts from the disrupted cells; Group I--*Mycobacterium bovis*, *M. fortuitum*, *M. avium*; Group II--atypical mycobacteria, and Group III--saprophytic mycobacteria. Seventy-two chemical fractions from several mycobacteria, actinomycetes and streptococci were compared with antisera (95). Antigens obtained from the cytoplasmic fractions of the mechanically disrupted cells of the different groups were related and cross reacted among various members. However, polysaccharide antigens from the cell walls of the different organisms possessed some degree of specificity. Among the mycobacteria there were a large number of cross reactions among the polysaccharides from the cell walls. Two explanations for the lack of serologic specificity among the mycobacteria were offered: (1) the specific components could not be separated from the nonspecific or cross reacting materials, or (2) the specific components, if present, were too low in concentration to stimulate antibody production.

Attempts to isolate pure, undenatured specific antigens from bacillary extracts of tubercle bacilli are not new (77). Bacillary extracts were chosen for antigen preparations in preference to concentrated culture filtrate because of the various stages of degradation in the culture filtrate proteins. Therefore, more homogenous antigen preparations could be obtained from whole extracts. Mechanically disrupted mycobacteria were extracted with borate buffer and phosphate buffer containing ether (80,81). Both removed the same cell constituents but in different proportions. Phosphate buffer extracts contained nucleic acid and less protein and carbohydrate than the borate buffer extract. Extracts of young cells had more tuberculin activity than extracts of cells from old cultures.

Antigens obtained from mycobacteria by aqueous and saline extractions of intact viable cells were more specific than those in O.T. (97,98,99). Mycobacterium tuberculosis and M. bovis were closely related antigenically. There was considerable cross reactivity among the different species of mycobacteria. It was suggested that antigens were stratified in the cell with cross reacting polysaccharide antigens located in the outermost surface of the cell. Antigens with more strain specificity were located deeper within the cell and were only removed by subsequent extractions. Serologic specificity was attributed to the protein moiety of carbohydrate-protein complexes. The aqueous extracts contained precipitinogens.

Glenchur and his associates examined bacillary extracts to find a tuberculospecific antigen for serodiagnostic tests (71,72,73). Chemical and antigenic differences between bacillary extracts and PPD were found using chromatography on ion exchange resins and gel filtration. Bacillary extracts were more complex chemically than PPD and contained several additional components. Stepwise elution of proteins from DEAE-cellulose with phosphate buffers yielded seven separate protein-containing fractions from bacillary extracts and only three fractions from PPD. Fractions from ion exchange chromatography were tested for their ability to elicit delayed hypersensitivity reactions in sensitized animals. A fraction from bacillary extracts was chosen for testing sera from tuberculous humans. A total of seven precipitating antigen-antibody systems were detected in the bacillary extracts. Pepys and co-workers (138) found antigenic differences between bacillary extracts and culture filtrates of different mycobacteria. Antibody elicited in experimental animals by inoculation with products of the tubercle bacilli were specific for polysaccharide but not protein. They suggested that the so-called chemical impurities in tuberculin may actually be responsible for potentiating the tuberculin reaction. Tissues used for the Schultz-Dale test were reciprocally desensitized with culture filtrate antigens or lipopolysaccharide extracts from homologous mycobacterial cells.

Culture filtrates obtained from mycobacteria after different growth periods and bacillary extracts prepared by

mechanical disruption of mycobacterial cells in a pressure cell were compared (25). The antigenic composition of bacillary extracts from a single species remained constant regardless of the age of the culture from which the cells were obtained. More variation in the antigenic content was observed with culture filtrates of different ages. Thus, bacillary extracts were again reportedly superior to culture filtrates for analyses of the antigen constituents of different strains and species of mycobacteria.

Precipitinogens in chemical fractions from mechanically disrupted cells were examined chemically. A polysaccharide preparation contained two distinct precipitinogens, a lipid fraction contained only one. In a similar study, bacillary extracts prepared from mechanically disintegrated cells contained more than six distinct precipitinogens (78). Yamaguchi (219) isolated protein, carbohydrate and lipid fractions from both culture filtrates and bacillary extracts. Polysaccharide antigens were precipitated in two discrete zones in immunodiffusion reactions. Lipid and protein antigens were difficult to detect. The protein fractions from bacillary extracts and the culture filtrates were different.

Bacillary extracts from sonic disruption of tubercle bacilli with glass powder were fractionated with ion exchange chromatography on DEAE-cellulose (84,85,86,228). Five major antigen-containing peaks were obtained. Ion exchange chromatography separated protein components from the nucleic acid

and carbohydrate components of the extract. Three protein-containing fractions elicited skin reactions in sensitized animals equal to protein fractions from homologous culture filtrates. The tuberculin activity was associated with the tuberculoprotein which was retained even if the tuberculoproteins were denatured.

Few attempts have been made to obtain antigen-containing bacillary extracts from mycobacteria using ultrasonic disruption. Serologically active antigens were obtained by this method which were reportedly not chemically or antigenically altered by this extraction procedure (201,187,18). Soluble and particulate fractions from BCG contained 61.1% lipids, 21.2% reducing substances, 3% total nitrogen and 0.36% phosphorus (93,94). The principle chemical components were arabinose, galactose, hexosamine, alanine, glutamic acid and diaminopimilic acid. Cell walls induced delayed hypersensitivity. Cell wall and intracellular particulate fractions were most antigenic but many cross reactions occurred.

Disc electrophoresis of ultrasonic extracts detected 10 or more discrete components of which four were considered to be of major taxonomic importance (48,49). Several disc bands were capable of provoking delayed type hypersensitivity reactions in sensitized animals without eliciting antibody production. The skin activity was associated with polysaccharide-protein complexes. Three disc components were

detected in extracts which were common to a number of representative strains of atypical mycobacteria.

Tuberculin-active peptides have been isolated from mycobacteria by precipitation with picric acid (177,178). The peptide preparation did not induce delayed hypersensitivity but provoked delayed reactions in sensitized guinea pigs. Culture filtrates have been used as a major source of mycobacterial antigens. Seibert and co-workers developed methods for obtaining and standardizing a purified protein derivative (PPD) to be used as tuberculin (163,164,165,167). From these classical studies the international standard for PPD, PPD-S, was developed. Using an alcohol fractionation procedure, Seibert separated PPD into three protein containing fractions, designated A, B, and C and two polysaccharide fractions, I and II. Analyses of these fractions proved that they were chemically complex (107,166,21,1).

Culture filtrates were fractionated by dialysis and molecular exclusion chromatography (8). Thirteen distinct precipitating antigen antibody systems were detected in a nondialyzable fraction from culture filtrates of BCG (8). Both nondialyzable and dialyzable fractions had tuberculin activity in sensitized animals. The different components varied in their chemical composition, sedimentation constants and electrophoretic mobility. Tuberculin activity was associated with precipitating antigens and carbohydrate was also found to play a role in the delayed type reaction (26,27).

Identical or closely related antigenic determinants were shared by molecules of different sizes.

Yoneda and co-workers employed several different procedures to fractionate culture filtrates from a virulent strain of M. tuberculosis (221,222,68,69). Salt fractionation with ammonium sulfate separated tuberculoproteins into three fractions: 0-30% $(NH_4)_2SO_4$-insoluble fraction; 30-50% $(NH_4)_2SO_4$-insoluble fraction and 50-80% $(NH_4)_2SO_4$-insoluble fraction. Starch block electrophoresis of these fractions yielded four apparently homogenous protein components. Ultracentrifugation and antigenic analyses by immunodiffusion indicated that these fractions were not homogenous. Ion exchange chromatography on DEAE-cellulose improved the purity of several fractions and freed them from contaminating nucleic acids and carbohydrates. Two major protein fractions, designated alpha and beta antigens, comprised approximately 70% of the total protein released by cells into the culture medium. Alpha and beta antigens were shown to be type specific, heat labile, protein antigens representing a major portion of the protein complement of the cell. Both antigens could be removed from the cell surface without apparent damage to the cell. Ouchterlony immunodiffusion was used to determine the distribution of the alpha and beta antigens in culture filtrates from 120 strains in mycobacteria (69). Four serologic groups were established on the presence or absence of one or both of the antigens.

Twelve fractions were separated from culture filtrates of four strains of Mycobacterium tuberculosis by ion exchange chromatography on DEAE-cellulose (91,92). Antigenic analyses by Ouchterlony immunodiffusion indicated that no fractions were antigenically pure. As many as eight discrete antigen-antibody systems was detected in a single fraction. Twenty distinct antigen-antibody systems were detected in the fractions from culture filtrates from a single organism. Twenty percent of the antigens were reported to be strain specific and of potential diagnostic significance.

A tuberculoprotein fraction from culture filtrates was separated into four components by column chromatography on DEAE-cellulose and into three fractions by paper electrophoresis (148,149). These fractions differed significantly in their ability to provoke delayed type hypersensitivity reactions in sensitized animals. Ion exchange chromatography on carboxymethyl cellulose did not provide adequate separation of antigens from unheated culture filtrates (101). However, optimal conditions of pH and ionicity of the eluting buffers may not have been used. Various other electrophoretic and chromatographic procedures have been used for the separation and purification of individual components of culture filtrates of M. tuberculosis, M. bovis, and other mycobacteria (148).

Antigens from culture filtrates have been used to study the antigenic relationships among different strains and

species of mycobacteria (131,132,156,222,188,101,102,103, 104). The results of these studies have contributed greatly to our understanding of the antigens of mycobacteria and have supported the idea that different mycobacteria may contain specific antigens. Dardas (44) found that the number of antigenic components in culture filtrates from M. bovis was dependent upon the age of the culture from which the filtrate was obtained.

Disc electrophoresis is a relatively new method which has been used for the separation of mycobacterial components on the basis of electrophoretic mobility and molecular size (128). Affronti (1) found up to 19 components in various fractions. Roszman (156) separated 18 to 24 protein components and 5 to 8 polysaccharides in unheated culture filtrates from M. bovis, M. avium and two Group III mycobacteria. The preparative disc electrophoretic technique was developed by which larger quantities of components from the disc bands could be obtained. A single band after being eluted, and re-electrophoresed on a different concentration of gel in disc electrophoresis, contained more than one component. However, what had appeared to be the original band was still present in the greatest quantity and the reelectrophoresis may provide a method of obtaining relatively pure fractions.

Cell Disruption by Ultrasound

The application of ultrasound in bacteriology, immunology and biochemistry was initiated by the observation that

cellular structures could be disrupted by this method of treatment (79). Grabar and Royer (74), showed that sensitivity to the destructive effects of ultrasound varied among different species of bacteria and among strains of the same species. Mycobacterium tuberculosis was more resistant to ultrasound than any of the other pathogenic bacteria tested. The sensitivity of different species and strains of mycobacteria to ultrasound differed greatly.

Exposure to ultrasound of sufficient intensity and duration to kill all the cells of a BCG culture only inactivated 15% of a strain of M. tuberculosis isolated from a clinical specimen (74). Varying degrees of morphologic disorganization were observed in mycobacterial cells exposed to ultrasound (209). The envelope of some cells was fractured and cytoplasmic material was released into the medium; in other cells the damage was at the end or center of the cell. The effects of ultrasound upon bacteria depends upon the intensity and duration of insonation as well as the density of the suspension of cells used (46). Increasing cell densities (number of cells/unit volume of medium) reduced the bactericidal effect on various strains of mycobacteria (110). The effect of ultrasound on bacteria also depends to a certain extent upon their morphological features and physiological state (57).

The mode of action by ultrasound on microorganisms is controversial. Cavitation undoubtedly is a principal agent

causing the bacteriocidal effect. Altering the conditions of the medium to suppress cavitation decreases the bacteriocidal action. Although the medium absorbs a substantial amount of ultrasound energy as heat, temperature is thought to play a secondary role (58).

Exposure of microorganisms to ultrasound causes damage to the cell wall and membranes. Since the disruptive forces of ultrasound are thought to be caused by cavitation, the distance between a cavitation bubble and its object are important. The intensity of the shock wave varies inversely with the square of the distance and acts over a distance of several microns (55). The nature of the cell wall and membranes are important in this respect. If the cell envelope is hydrophobic the formation of cavitation bubbles at the cell surface-water interface is favored and the organisms should be sensitive to the effects of ultrasound. If the cell surface is hydrophilic the formation of cavitation bubbles at the cell surface-medium interface will be inhibited and the organisms should be more resistant to the effects of cavitation. Alterations in sensitivity to bacteriophage, sensitivity to antibiotics, and disinfectants or ultraviolet light and altered cell morphology are probably due to surface effects (58).

Cavitation is not thought to occur within the cytoplasm of cells due to its high viscosity (57). However, a number of changes are induced within the cytoplasm of cells (56).

Alterations in the physicochemical state of the cytoplasm may lead to various degree of degradation or death.

An important aspect of the application of ultrasound to microbiology is the possibility of extracting biologically or chemically active soluble or particulate constituents from cells. Biomacromolecules are extracted from viable cells by ultrasound with very little alteration in their physical or chemical state (57). Live and co-workers (106) obtained protective antigens in soluble extracts from ultrasonically disrupted cells of Brucella sp. The sedimentable fraction from ultrasonically disrupted Brucella cells conferred protection to mice against subsequent infection with virulent organisms (113).

Ultrasonic extracts from various microorganisms have been used as a source of antigens for serological tests and antigenic analyses (223,118,89). Nine distinct precipitating antigen were detected by immunoelectrophoresis in ultrasonic extracts of Brucella abortus (22). Thirteen antigen were detected in ultrasonic extracts of intact cells of Brucella suis. Ten of the antigens were precipitated with antiserum elicited with intact cells (14). Three additional antigens were detected using spheroplast-specific antiserum. Four antigens were associated with the cell surface. Highly specific and very sensitive allergins were isolated from extracts of Brucella brucei and Pasteurella tularensis prepared by ultrasonic disruption (130). Cells of the yeast stage of Histoplasma capsulatum were disrupted by ultrasound

for the purpose of obtaining a specific antigen for serologic testing (141). The soluble extract contained constituents which were more antigenic than components found in the sedimentable fraction of the disrupted cells. Two antigens were detected in ultrasonic extracts from cells of Corynebacterium hofmanii (10). A study was made of the antigenic changes which accompanied sporulation in Bacillus cereus (9). Immunoelectrophoresis detected seven thermal-resistant and 8-10 thermal sensitive antigens in ultrasonic extracts from intact vegatative cells. Five thermal-resistant antigens present in spore extracts were absent in extracts of the vegetative cells. Changes in the antigenic composition of different developmental stages of slime molds were detected using sonic extracts of cells at different ages (179). Ultrasonic extracts from cells of Mycoplasma pneumoniae were fractionated by gel filtration (176,143). One lipid fraction, three protein fractions and four polysaccharide-containing fractions were obtained. Antigens detected by component-fixation tests were associated with the lipid components of the extract; precipitnogens were associated in polysaccharide-containing fractions (143).

Ultrasonic disruption has been shown to be an effective means of obtaining antigens from a variety of bacterial species. Thus far, however, very few attempts have been made to prepare antigens and sensitins from mycobacteria. This technique has great promise for obtaining undenatured mycobacterial constituents.

MATERIALS AND METHODS

Mycobacterial Inoculums for Rabbits and Inoculation Protocol

Mycobacterium bovis, 310-2, was isolated from a naturally infected, tuberculin-positive, gross lesion cow. It was identified by morphologic, cultural, biochemical and pathogenicity tests.

Mycobacterium bovis (310-2) was grown three weeks at 35°C in 20 ml tubes containing 1 ml of modified Proskauer Beck medium (224). The supernatant fluid was removed after centrifugation (1500 x g, 15 min). The cells were washed with 0.15 M phosphate buffered saline (PBS) solution, pH 7.2. Portions of the cells were resuspended in PBS-solution to contain 0.1 mg wet weight/ml. The remainder of the cells were killed by the procedures indicated below.

Cells were killed with betapropriolactone (BPL)[1] (126). Washed cells were suspended in triple distilled water, 1.0 mg wet weight/ml. The pH was adjusted to 8.4 with 0.5 M Na_2HPO_4. A 250 ml centrifuge bottle containing the suspension was placed in an ice bath and cold BPL added slowly with constant agitation to a concentration of 0.4%. The mixture was incubated in a constant-shaker water bath at 37°C for two hours. The pH was adjusted to 7.6 at 15 minute intervals

---

[1]Betaprone, Testagar and Co. Inc.

with 0.5 M Na_2HPO_4. After incubation, the mixture was centrifuged and the supernatant fluid removed. The cells were washed four times with sterile distilled water and suspended in isotonic saline solution to a concentration of 1.5 mg wet weight/ml.

Acetone-killed cells were prepared by adding acetone to washed, packed cells, one mg wet weight cells/ml acetone, and incubating at 4°C for seven days with daily changes of acetone. The cells were washed and resuspended in 0.15 M PBS-solution pH 7.2.

Cells were killed by moist heat, 100°C for 30 minutes. The heat-killed cells were resuspended in 0.15 M PBS-solution pH 7.2.

A portion of the BPL-killed cells were extracted with methanol and acetone. Ten mg of BPL-killed cells were suspended in 10 ml of acetone and shaken for three hours at 37°C. The mixture was centrifuged and the supernatant fluid removed. The cells were suspended in methanol overnight. This procedure was repeated three times, after which the cells were washed and resuspended in 0.15 M PBS-solution pH 7.2.

The attenuated strain of M. bovis, BCG, was prepared as described for M. bovis, 310.

Six groups of adult Dutch rabbits, three rabbits per group, were inoculated with preparations of mycobacterial cells according to the protocol in Table 1.

Table 1. Protocol for rabbit inoculations of live and killed *Mycobacterium bovis*.[1]

Rabbit Group	Preparation of M. bovis	Cells mg/ml	Adjuvant[2]	Schedule
I	Live *M. bovis*	0.01	-	Single[3]
II	Live BCG	0.6	+	Multiple[4]
III	Δ-killed *M. bovis*[5]	9.0	+	Multiple
IV	BPL-killed *M. bovis*[6]	9.0	+	Multiple
V	BPL-killed Ma[7] extracted *M. bovis*	9.0	+	Multiple
VI	Acetone killed *M. bovis*			

[1]Virulent strain of *Mycobacterium bovis* 310-2 except Group III which received BCG, attenuated *M. bovis*.

[2]Adjuvant--incomplete Freunds adjuvant (Difco)

[3]Single--a single subcutaneous injection.

[4]Multiple--six sub cutaneous injections given at separate sites on the first day.

[5]Moist heat--100 C, 30 min.

[6]BPL--betapropriolactone (Betaprone, Testagar Co.)

[7]Ma-extracted--BPL-killed cells extracted with methanol and acetone.

Each rabbit in Group I received a single subcutaneous injection of 0.01 mg wet weight of live M. bovis (310-2) without adjuvant. Each rabbit in Group II received multiple injections containing a total of 0.6 mg (wet weight) of attenuated, viable M. bovis (BCG) emulsified in adjuvant.

On the same day, each rabbit in Groups III-VI received six subcutaneous injections of killed cell preparations, 9.0 mg (wet weight) of cells in adjuvant. The inocula were prepared by emulsifying equal parts of killed cells in 0.15 M PBS-solution pH 7.2 with incomplete Freunds adjuvant.

Antibody Titrations

Blood was collected from the marginal ear vein from each rabbit in Groups I-VI prior to and weekly for at least eight weeks post-inoculation. Sera were decanted from the clotted blood, centrifuged, and decanted. Sera from rabbits in Group I were filtered[1] before being centrifuged. Approximately three ml of each serum were dispensed into sterile brucella tubes, merthiolate was added (1:10,000), and the sera were stored at -70°C. Sera to be tested by passive hemagglutination were inactivated and adsorbed twice for 30 min each at 37°C with sheep red blood cells prior to storage.

Polysaccharide-specific antibody was measured by a modification of the Middlebrook-Dubos passive hemagglutination

---

[1]Seita pad supported on a Swinney filter.

test (HA) (111). Sheep blood was collected aseptically in an equal volume of modified Alsevers solution and stored at 4°C for not more than ten weeks. Erythrocytes were washed three times in PBS-solution before being used. One tenth ml of packed, washed erythrocytes were mixed with 6.0 ml of Old Tuberculin,[1] diluted 1:15 with buffered saline-solution pH 7.2 and incubated in a water bath at 37°C for two hours. The suspension was centrifuged at 550 x g for four minutes and the supernatant fluid discarded. The sensitized cells were washed three times and resuspended in 0.5% in PBS-solution. The cell suspension was used within 24 hours.

Serial dilutions of serum were made in 0.15 M PBS-solution pH 7.2 beginning with 1:10 dilution. Three drops of the 0.5% sensitized erythrocyte suspension were added to one ml of the serum dilutions in 12 x 75 mm tubes and shaken. The tubes were incubated in a water bath at 37°C for two hours, at room temperature for two hours and at 4°C overnight. Tubes were observed for settling patterns and visible clumping of erythrocytes when the tubes were shaken gently. The titer was recorded as the reciprocal of the highest dilution of serum which caused hemagglutination.

Bacterial agglutinins were measured with BPL-killed M. bovis. A uniform suspension of the cells in PBS-solution pH 7.2 was prepared by dispensing cells in a tissue grinder,

---

[1]Agriculture Research Service, U. S. Dept. Agriculture. U. S. Vet Licence No. 107.

and centrifuging at approximately 250 x g for four minutes. The supernatant fluid was removed and diluted to an absorbency of 0.3 at 525 mμ.[1] Twenty-five hundredths ml of the antigen suspension was mixed with an equal volume of twofold serial dilutions of antiserum. The mixtures were incubated 12 hours at 37°C. The titer was recorded as the reciprocal of the highest dilution of serum which caused agglutination.

Bacterial agglutinin and hemagglutinin titers were determined before and after treatment of the sera with 2-mercaptoethanol (ME). Equal volumes of 0.2 M ME and serum were mixed and incubated for 8-12 hours at room temperature. The difference between titers obtained before and after treatment of the serum with ME were recorded as the titer of ME-sensitive antibody.

Statistical Analyses

The results of antibody titrations were analyzed using the multiple range test developed by Duncan (53). The analyses were made with the number of the dilution tube of the titer as follows:

1:10 = 1	1:80 = 4	1:640 = 7
1:20 = 2	1:160 = 5	1:1280 = 8
1:40 = 3	1:320 = 6	1:5120 = 9

The significance of the variance between treatment means was determined at the 95% level.

---

[1]Bausch and Lamb "Spectronic 20" Spectrophotometer.

Tuberculin Tests

Rabbits in Group I, IV and V were tested intradermally 14 week post-inoculation with 0.1 ml of second strength PPD-S.[1] The diameters of palpable induration at the site of injection were observed and recorded at 30 minutes, 5, 24, 48 and 72 hours post-inoculation.

Cultures for Extraction of Cellular Components

Mycobacterium bovis, 310-2, was grown in diphtheria toxin bottles on the surface of 500 ml or 1 liter of modified Proskauer Beck (PB) medium (224). The medium was prepared in 26 liter lots as follows:

Reagent	Amount	Final Concentration
Lasparagine	125.0 gm	0.5%
KH_2PO_4	125.0 gm	0.5%
K_2SO_4	12.5 gm	0.05%
Glycerol	500.0 ml	0.15%

Dissolve each ingredient sequentially and completely in the order listed in 17.5 l of distilled H_2O. Adjust the pH to 7.0 with 40% of NaOH. Dispense 700 ml in each diphtheria bottle. Sterilize (121°C, 30 min). For each bottle of 700 ml, dissolve 1.5 gm Mg titrate (final concentration--0.15%) in 300 ml of water, and sterilize (121°C, 30 min). Aseptically, add the contents of one flask to each of the bottles.

The medium was incubated at room temperature several days before seeding with M. bovis. To seed, portions of confluent, surface growth was transferred with a small wire

---

[1]Parke Davis and Company.

screen on an inoculating wire. The protions were deposited on the surface of the medium in the horizontal bottles. The bottles were carefully transferred to the 35°C incubator and incubated for the periods of time indicated in the appropriate sections.

After incubation, the contents of the bottles were removed by suction into 250 ml centrifuge bottles, centrifuged at 1500 x g for 30 minutes, and the supernatant fluid and cells removed separately.

Extraction of Cells with Ultrasound

Four ultrasonic extracts (USE) were made differing in the age of the culture from which cells were derived, cell suspension density and duration of insonation. The different ultrasonic extracts are described in Table 2.

Table 2. Ultrasonic extracts obtained from viable cells of Mycobacterium bovis.

Designation	Amount of Cells (mg wet wt)	Age of Culture	Suspension Density Percent W/V	Insonation Duration (min)
USE-A	51	6 mo.	10	20
USE-B	45	6 mo.	7	35
USE-C	47	6 mo.	7	60
USE-D	25	2½ mo.	7	20

A Biosonik[1] 20 kilocycle ultrasonic apparatus was used for cell disruption. The apparatus consisted of an air cooled 400 watt generator, transducer housing and a solid magnetostrictive type transducer. The transducer was attached to a stainless steel insonation chamber which could be detached and sterilized separately. The system was "tuned" before use by placing approximately 30 ml of water in the chamber and adjusting the electronic circuit to maximum power output. Insonation was performed in a ventilated bacteriological hood fitted with an ultraviolet light.

Sixty ml of a pre-cooled cell suspension in 0.002 M phosphate buffer (PB) pH 7.2 were placed in the insonation chamber. The gasketed top was made secure with four wing bolts and the entire chamber was covered with cheesecloth saturated with 5% phenol solution. The transducer housing, coolant reservoir and circulating pump were covered with plastic bags to prevent chance contamination of internal components. The chamber was cooled by an oscillating pump circulating ice water from an insulated reservoir through a cooling coil within the chamber. Preliminary experiments were performed to determine the maximum temperature reached during different intervals of insonation. The cell suspension was allowed to reach thermal equilibrium in the chamber before insonation was begun.

---

[1]Bronwill Scientific Division, Will Corporation, Rochester 1, N. Y.

Following insonation, the chamber was unopened for approximately twenty minutes. The cell suspension was transferred aseptically to 250 ml centrifuge bottles and centrifuged at 2°C for 30 minutes at 1,500 x g. The supernatant fluid was decanted and the cell debris washed once with approximately 70 ml of sterile 0.002 M PB at 4°C. The original extract and the supernatant fluid from the cell washing were combined and stored in 250 ml centrifuge bottles at 4°C. The cell debris was washed again following the same procedure and saved for further extraction.

Ultrasonic extracts were sterilized by filtration (size 6, S-1 Seitz filter sheets and Millipore filter membranes, pore size 0.45 μ). The extracts were concentrated approximately ten-fold by pervaporation and stored at -70°C.

Chemical Extraction of Mycobacterial Cells

Cells were harvested from six-month-old cultures and washed three times with either 0.002 M tris-HCl buffer pH 8.6 or 0.002 M PB, pH 7.4. Ten percent suspensions of washed cells were placed in 250 ml plastic centrifuge bottles containing the appropriate concentration of extractant. The extractants were as follows: 1.5% sodium desoxycholate in the tris-HCl buffer; 1:320 dilution of Triton-X-100 in tris-HCl buffer; 4.5 M urea in PB; and 4.5 M quanidine-HCl in PB. The sodium desoxycholate (SDE) and triton extracts (TE) were prepared by continuous agitation of the cell suspension in extractant for 18 hours at 2-6°C. The urea (UE) and

guanidine-HCl extracts (GE) were prepared by continuous agitation of cell suspensions in extractant for 18 hours at room temperature. All extractions were performed in a bacteriological hood.

Following extraction, the cell suspensions were centrifuged at 1500 x g for 45 minutes at 4°C and the supernatant fluids were decanted. The cell debris were washed once with 50 ml portions of the suspending buffer and supernatant fluids from these washings were combined with the original extracts. Extracts were sterilized by filtration through Seitz and Millipore filter pads and dialyzed for 72 hours at 4°C against daily changes of the suspending buffers. Extracts were concentrated approximately ten-fold by pervaporation and frozen.

Cellular debris from the ultrasonic disruption of bacterial cells from six-month-old cultures were extracted with PB containing ethyl ether (80). Approximately 14 gm wet weight of cell debris contained in 150 ml of 0.002 M PB pH 7.2 were placed in a 250 ml plastic centrifuge bottle. Fifty ml of ethyl ether at -70°C were added and the mixture was shaken vigorously to achieve a stable emulsion. The mixture was frozen at -70°C and remained frozen for approximately six hours. It was thawed and centrifuged at 4°C to separate the organic and aqueous phases. The aqueous phase was decanted and sterilized, dialyzed, concentrated and stored as described for the other extracts.

Acetone, Ethanol and Trichloracetic Acid Extractions of Ultrasonic Extract C

Carbohydrates from USE-C were precipitated by slowly adding 40 ml of cold acetone with constant stirring to 20 ml of the extract (90). The mixture was allowed to stand overnight at 4°C and the precipitate was collected by centrifugation at 4°C for 30 minutes at 2,000 x g. The precipitate was washed three times with a cold solution containing one part of distilled water and two parts of acetone. After washing, the precipitate was dissolved in five ml of 0.002 M PB pH 7.2 and dialyzed for 72 hours at 4°C against several changes of the same buffer. Twenty ml of USE-C were treated with 60 ml of 95% ethanol by the method described for acetone.

A trichloroacetic acid precipitate of USE-C was prepared following a procedure previously described (4). Ten ml of the extract was slowly mixed with an equal volume of 0.5 M trichloroacetic acid at 0°C. After standing for three hours at 4°C, a slight precipitate formed which was removed by centrifugation at 2,000 x g for 30 minutes at 4°C. The supernatant fluid was decanted and dialyzed for 48 hours at 4°C against 0.002 M phosphate buffer pH 7.2. The extract was concentrated approximately two-fold by pervaporation and stored at -70°C.

Production of Ultrasonic Extract-Specific Antisera

One-half ml of a 10% solution of $AlCl_3$ was slowly added with continuous stirring to each ten ml portion of USE-A.

The pH of the mixture was adjusted to 6.5 with 20% NaOH. The inoculum was stored at 4°C and was used within 14 days of preparation.

Four adult rabbits were each inoculated on days one and 14 with six ml of the inoculum containing approximately six mg of protein. Each rabbit was inoculated intraperitoneally on day 14 with one ml (4.0 mg protein) of USE-A without adjuvant. The rabbits were bled by carbiocentesis on days 49 and 51. Serums were decanted from the clotted blood and centrifuged for 30 min at 1000 x g. Each antiserum was tested for precipitins by Ouchterlony gel diffusion and satisfactory antisera were pooled. Merthiolate was added (1:10,000) and three ml portions of serum stored in brucella tubes at -70°C.

Zone Electrophoresis in Cellulose Acetate Membranes

Zone electrophoresis was performed in a Shandon migration chamber with a Vokam DC power supply. Oxoid 12 x 2 1/2 cm cellulose acetate membrane strips were used as the supporting medium. A 0.007 M barbital buffer pH 8.6 was used (129). The composition of this buffer was as follows:

Ingredient		Quantity
Sodium diethylbarbiturate		5.0 gm
Sodium acetate (anhydrous		3.25 gm
Hydrochloric acid (0.1N)		34.20 ml
Calcium lactate*		0.38 gm
Distilled water	to	1,000.00 ml

*Omitted for electrophoresis of bacillary extracts.

Five microliter samples were dispensed from a lambda pipette onto a buffer impregnated cellulose acetate membrane strip. Sample application was facilitated by placing a ruler across the cathode reservoir. The sample was applied as an even line using the ruler edge as a guide. Serum samples were not concentrated for electrophoresis. Bacillary extracts were concentrated 20-30 fold. A constant current of one ma per strip was applied for 1 1/2-2 hours at 4°C. Following electrophoresis, the protein was stained with 0.2% Ponceau S in 5.0% aqueous trichloracetic acid. Serum protein distribution was measured by a double beam recording and integrating microdensitometer[1] and the serum gamma-globulin concentrations computed.

Disc Electrophoresis

Disc electrophoresis was performed using the procedures and apparatus described by Ornstein and Davis (127), and later modified by Davis (47). The composition of stock and working solution are given in Table 3.

Samples to be electrophoresed were applied to the top of the spacer gel by displacement. No sample gel was used. Bacillary extracts were concentrated approximately 20-30 fold to contain approximately 280 micrograms of protein in 0.1-0.2 ml. Electrophoresis was performed at room temperature using a Vokkam DC power supply. A constant current of three ma

---

[1]N and L--Joyce Chromoscan.

Table 3. Stock and working solutions used for disc electrophoresis.

STOCK SOLUTIONS

Reagent A		Reagent B	
IN HCl	48 ml	IN HCl	approximately 48.0 ml[1]
TRIS	86.6 gm	TRIS	5.98 gm
TEMED	0.23 ml	TEMED	0.46 ml
DHOH to pH 8.9	100.0 ml	DHOH to pH 6.7	100.0 ml

Reagent C		Reagent D	
Acrylamide	28.0 gm	Acrylamide	10.0 gm
BIS	0.735 gm	BIS	2.5 gm
DHOH to	100.0 ml	DHOH to	100.0 ml

Reagent E		Reagent F	
Riboflavin	4.0 mg	Sucrose	40.0 gm
DHOH to	100.0 mg	DHOH to	100.0 ml

[1]pH adjusted by titrating with IN HCl
TRIS--Tris (hydroxymethyl)aminomethane
TEMED--N,N,N^1,N^1-tetramethylethylenediamine
BIS--N,N'-methylenebis acrylamide
DHOH--distilled water

WORKING SOLUTIONS

Small pore Solution #1	Small pore Solution #2	Large pore solution	Stock buffer solution for electrode reservoir
1 part A	Ammonium	1 part B	TRIS 6.0 gm
2 parts C	persulfate	2 parts D	Glycine 28.8 gm
1 part DHOH	0.14 gm	1 part E	DHOH to 1 liter
pH 8.9		4 parts F	pH 8.3
(8.8-9.0)		pH 6.7	
		(6.6-6.8)	

per tube was applied for a length of time sufficient to allow the migration of bromphenol blue to within 5 mm from the anodic end of the gel column.

Gel columns were stained for protein by immersion in a 0.5% solution of Amido Swarz 10 B in 5% acetic acid for 30 minutes at room temperature. The unbound stain was removed by electrophoresis in 5% acetic acid. Polysaccharides and glycoproteins were stained by the periodic acid-Schiff (PAS) procedure described by Canalco (24). Following electrophoresis, the gel columns were immersed in 7.5% acetic acid for one hour at room temperature. The gel columns were removed and placed in a 0.5% solution of periodic acid and allowed to stand for one hour at $4^{o}C$. Excess periodic acid was removed electrophoretically for one hour in 7.5% acetic acid. Following treatment with periodic acid, the gel columns were immersed and stored in Schiff reagent at $4^{o}C$.

Schiff reagent was prepared by the method described by Crowle (32).

The stained disc columns were observed under indirect fluorescent light and diagrammatic representation of line patterns were drawn as composited from several columns run simultaneously. Rf valves were compiled as the ratio of the migration distance of individual lines to the most anodic component detected.

Immunoelectrophoresis

Immunoelectrophoresis was performed using a modification of the Hirschfeld procedure (83). Semi-purified agar was prepared by washing solidified one inch square cubes of 2% Difco agar in distilled water for several months. A barbital buffer system was employed for immunoelectrophoresis:

Constituents	Electrode vessel buffer	Agar buffer
Diethylbarbituric acid	1.38 gm	1.66 gm
Sodium barbital	8.76 gm	10.51 gm
Distilled water to	1,000.00 ml	1,000.00 ml

A solution of buffered agar was prepared by mixing two parts of agar buffer with one part of distilled water and heating to approximately 70°C. Three parts of melted two percent agar were then added to the heated buffer solution and two and one-half ml of this mixture containing merthiolate (1:10,000) were layered on each 1 x 3 in thoroughly cleaned microscope slide. The agar slides were incubated three-five hours in a humidified chamber at room temperature before use. An LKB[1] gel punch was used to cut two, 2 1/2 mm diameter sample basins in the agar approximately 44 mm from the anodic end of the slide and equidistant between the edges of the slide and a centrally located 1 1/2 mm wide antiserum trough.

A Shandon migration chamber was adapted to accommodate slides by inserting a 6 x 8 1/2 inch plexiglass casette across

---

[1]LKB Instruments, Inc., Rockville, Maryland

the chamber bridge. Electrical connections were established with filter paper wicks impregnated with electrode vessel buffer. Two rows of 4-8 slides each were positioned on the casette connected by a filter paper wick. Slides on the anode side of the casetts served as "blanks." Antigens were added to the sample basins using 26 gauge needles on one-half ml syringes. Bacillary extracts were concentrated 20-fold or greater for immunoelectrophoresis. Serum samples were used without concentration.

A constant current of 1.25 ma per slide was applied for 90 minutes at 4°C using a Volkam DC power supply. Following electrophoresis, the antiserum was added. Slides were incubated in a humidified chamber at 28 C for 72 hours with one replenishment of a 1:3 dilution of antiserum, 12 hours after the start of incubation. Following incubation, the slides were washed in 0.015 M PB-solution pH 7.2 for 72 hours and in distilled water for 12 hours. The slides were then dried and the protein was stained with triple stain (32).

Ingredient		Amount
Thiazine Red R		0.1 gm
Amidoswarz 10 B		0.1 gm
Light Green SF		0.1 gm
Acetic acid		2.0 gm
Mercuric chloride		0.1 gm
Distilled water	to	100.0 ml

Ouchterlony Immunodiffusion

Ten ml of a melted, buffered agar solution were layered on thoroughly cleaned 3 1/4 x 4 inch glass microscope slides. The composition of the buffered agar solution is given below:

Ingredient	Amount
Difco Agar	0.5 gm
0.15 M KH_2PO_4	7.5 ml
0.15 M Na_2HPO_4	17.5 ml
0.15 M NaCl	25.0 ml
Merthiolate	0.02 %
pH	7.2

After solidification of the agar, the slides were "aged" for three to five hours in a humidified chamber. Six mm sample basins were made in the agar using a template placed beneath the slide as a guide. Several patterns were employed using inter-basin diffusion distances of six-seven mm.

Reactants were added to the sample basins using disposable pipettes and the slides were incubated at 28°C in humidified, six in diameter plastic Petri dishes. Preliminary experiments were performed to determine optimum antigen and antiserum concentrations for immunodiffusion. The protein concentration of bacillary extracts and their fractions were adjusted to 1.8-2.0 mg/ml for immunodiffusion analyses. Plates were incubated for four days with daily readditions of antigen and antiserum diluted 1:3 with sterile saline solution. Immunoprecipitate patterns were observed and recorded

daily. When incubation was completed, the slides were immersed for three days in daily changes of 0.15 M PBS solution, pH 7.2. They were rinsed in distilled water, dried to a thin agar film by evaporation through Whatman filter paper and stained with Crowle's triple stain (32). Immunoprecipitates were observed under indirect fluorescent light against a black background.

Chromatography

Molecular exclusion chromatography was performed using superfine dextran beads (Sephadex)[1] and acrylamide beads (BioGel).[2] Dry beads were swollen in distilled water for varying lengths of time depending upon the degree of cross linkage of the beads.

Unjacketed Sephadex laboratory columns[3] 1.5 x 30 cm and 2.5 x 45 cm were coated with a 1% solution of dimethyldichlorosilane prior to packing. The coating solution was heated to approximately 60°C and poured into a clean, dry column and allowed to stand for several minutes. The column was emptied and dried in a hot air oven. This procedure was repeated. Swollen beads were gradually added to a column bed height of approximately 32 and 37 cm in the two column sizes, respectively. The gel beds were washed under low hydrostatic pressure with ten or more void volumes of eluent.

---

[1]Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

[2]Calbiochem, Los Angeles, California

[3]Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

The gel bed was stabilized and 0.2% Blue Dextran 2000[1] was used to determine the void volume of each column and the homogeneity of packing.

The volume of material to be chromatographed varied with the size of the column and the protein concentration of the material. Samples were carefully added to the top of the gel and allowed to "soak" into the gel bed. The sides of the column and the sample pad were washed with several two-four ml portions of eluent before the eluent reservoir was connected to the column for continuous operation.

Chromatography was performed at room temperature with flow rates adjusted to 15-18 ml per hour. Three-five ml portions of eluate were collected on an automatic, rotating drum fraction collector fitted with a time drop unit.[2] The eluate was analyzed for adsorption at 280 or 260 mμ by a ultraviolet analyzer.[3] The corresponding elution diagram was automatically recorded with a recording unit integrated with the analyzer. Tubes containing fractions corresponding to discrete areas of the effluent diagram were identified by an event marker attachment on the recorder. Tubes containing fractions registered under the same peak in the effluent diagram were pooled, concentrated by lyophilization and re-dissolved to the original sample volume. Rf values were

---

[1]Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

[2]Vanguard.

[3]Ibid.

determined for individual fractions by computing the ratio of the void volume (Vo) to the eluant volume of the respective fraction (Ve).

Ion exchange chromatography was performed using selected Type 20 DEAE cellulose[1] capacity 0.62 meq/gm as the adsorbent. The adsorbent was prepared for use following procedures described by Peterson and Sober (139). Dry powder was allowed to sink into 1 N NaOH and become thoroughly saturated. After mixing, the suspension was filtered through a Buchner funnel using Whatman No. 1 filter paper. The adsorbent was washed with 1 N NaOH until the filtrate was clear. It was suspended in a small volume of 1 N NaOH, acidified with 1 N HCl, immediately washed with distilled water, resuspended in 1 N NaOH, and washed with distilled water. The adsorbent was suspended in starting buffer and "fines" were removed by decanting the supernatant fluid after the adsorbent had settled. The pH of the adsorbent was adjusted to that of the starting buffer and washed thoroughly before the columns were packed.

A suspension of adsorbent in starting buffer was slowly poured into dry, "coated" 1.5 x 30 cm Sephadex laboratory columns to a bed height of approximately 23 cm. The adsorbent bed was washed for several days under low hydrostatic pressure with the starting buffer.

Ultrasonic extracts were concentrated approximately 20-fold for ion exchange chromatography. Seven ml of ultrasonic

---

[1]Carl Schleicher and Schuell Co., Keene, N. H.

extract B were dialyzed against the starting buffer for 24 hours at 4°C before application to a column. A continuous concave gradient of decreasing pH and increasing molarity was used to elute proteins from the adsorbent. The gradient was prepared using a modification of the cone sphere method (139). A 500 ml round-bottomed flask containing 450 ml of the starting buffer was connected by thin rubber tubing to a 250 ml Erlenmeyer flask containing 225 ml of the limit buffer. The composition of the buffers were as follows:

Buffer	Composition
Starting	0.005 M TRIS-phosphate pH 8.6
Limit	0.3 M TRIS-phosphate in 1.7 N NaCl pH 5.0

The flask of starting buffer contained a magnetic stirrer. The sample was applied to the top of the adsorbent bed, washed into the column with small portions of starting buffer and buffer reservoirs were connected to the column for continuous elution. Five ml portions of eluant were collected, analyzed and recorded. Fractions were pooled, dialyzed for 24 hours at 4°C against 0.015 M PBS solution pH 7.2 and concentrated to the original sample volume.

Chemical Analyses

Protein determinations were made using the Lowry modification of the Folin-Ciocalteu method (109). The reagents were: 4.9% sodium potassium tartrate, 4.0% sodium carbonate and 2.0% copper sulfate $5H_2O$. Reagent A was prepared by mixing one ml of the copper sulfate solution, one ml of the

tartrate solution and 100 ml of the carbonate solution. Reagent B was prepared by the addition of one part of Folin reagent to two parts of distilled water. One ml of the protein-containing sample was added to 10 ml of Reagent A with thorough mixing and the mixture incubated 45 minutes at room temperature. After incubation, one ml of Reagent B was added, mixed immediately and incubated 15 minutes at room temperature. Absorbancy was determined in a one ml cuvette at 660 mμ using a Beckman DU Spectrophotometer. A standard protein curve was made with bovine serum albumin.

Carbohydrate determinations were made using the thymol sulfuric acid reaction described by Shetlar (173). The following reagents were used.

Thymol reagent: 10 gms dissolved in 100 ml of absolute ethanol.

Sulfuric acid reagent: 77% by volume, specific gravity 1.84.

Add 770 ml of sulfuric acid at 15°C to 230 ml distilled water.

Seven ml of the sulfuric acid reagent were dispensed into fifteen ml stoppered glass centrifuge tubes and cooled to 4°C. One ml of the test solution containing between five and 100 micrograms of carbohydrate/ml was layered on the chilled sulfuric acid and incubated for 30 minutes at 4°C. The tubes were stoppered and shaken after adding 0.1 ml of the Thymol reagent and 0.9 ml of distilled water, and

placed in a boiling water bath for 20 minutes. They were cooled at $4^{o}C$ for five minutes and at room temperature 25 minutes. Absorbancy was determined in a one ml cuvette at 500 mμ using a Beckman DU spectrophotometer.

Nucleic acid concentrations were estimated from absorbancy measurements at 280 and 260 mμ using a one cm light path in a DU spectrophotometer. The nucleic acid concentration was read from a standard nomograph based on the ratios of absorbancy at the two wave lengths. Ultraviolet absorption spectra of ultrasonic extracts and fractions were obtained by absorbancy measurements at wave lengths of 200-320 mμ using a one cm light path in a Beckman DB spectrophotometer.

Dialysis

Fifty ml portions of ultrasonic extract A were dispensed into wetted dialysis tubing (Visking Co.) and dialyzed six days against 0.01 M PBS solution pH 7.2. The dialysate and retentate were collected and lyophilized.

RESULTS

Antibody Responses Elicited by Cells of *Mycobacterium bovis*

Antibody titers were low and inconsistent in sera from rabbits inoculated subcutaneously with 0.01 mg wet weight of virulent *M. bovis* cells, strain 310, without adjuvant (Group I). Tuberculous lesions were present in the rabbits at necropsy 8-12 weeks post-inoculation.

Antibodies produced by rabbits in Groups II-III during the first two weeks post-inoculation were exclusively ME-sensitive (Figs. 1-10, Tables 4-7). Thereafter, increasing amounts of ME-resistant antibody were detected which decreased the ratios of ME-sensitive/Me-resistant antibody (Tables 8 and 9). Mercaptoethanol-resistant bacterial agglutinins were detected in sera from most rabbits by three weeks post-inoculation; ME-resistant hemagglutinins were detected by four weeks post-inoculation. Most of the rabbits produced ME-sensitive hemagglutinins and bacterial agglutinins until the sixth week post-inoculation.

At the first and second weeks post-inoculation, mercaptoethanol-sensitive hemagglutinin titers were significantly higher in sera from rabbits in Groups II, III and IV than in sera from rabbits in Groups V and VI. Thereafter, rabbits in Groups II, III and VI produced significantly more

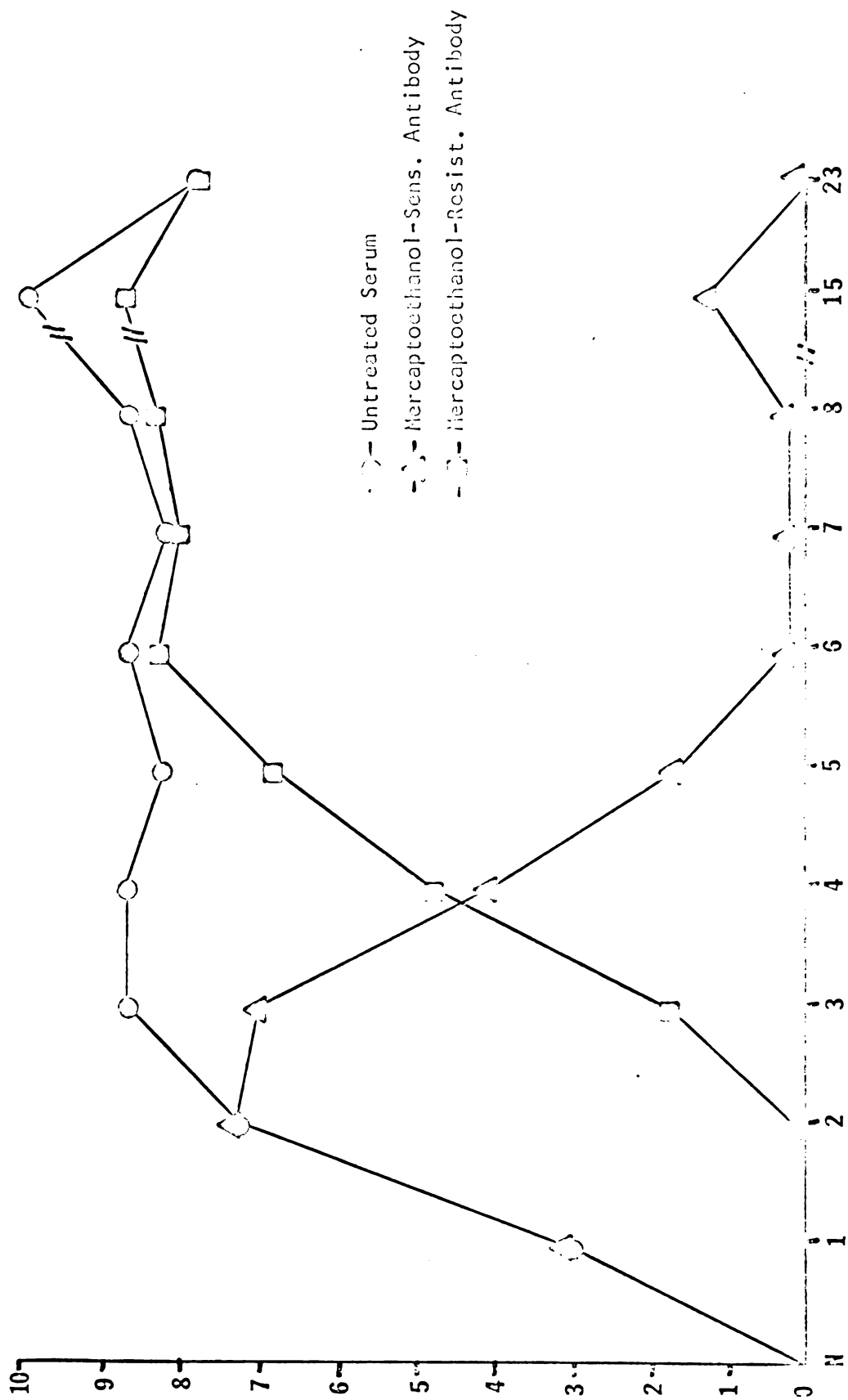


Fig. 1. Hemagglutinins produced by rabbits in Group IV (Table I).

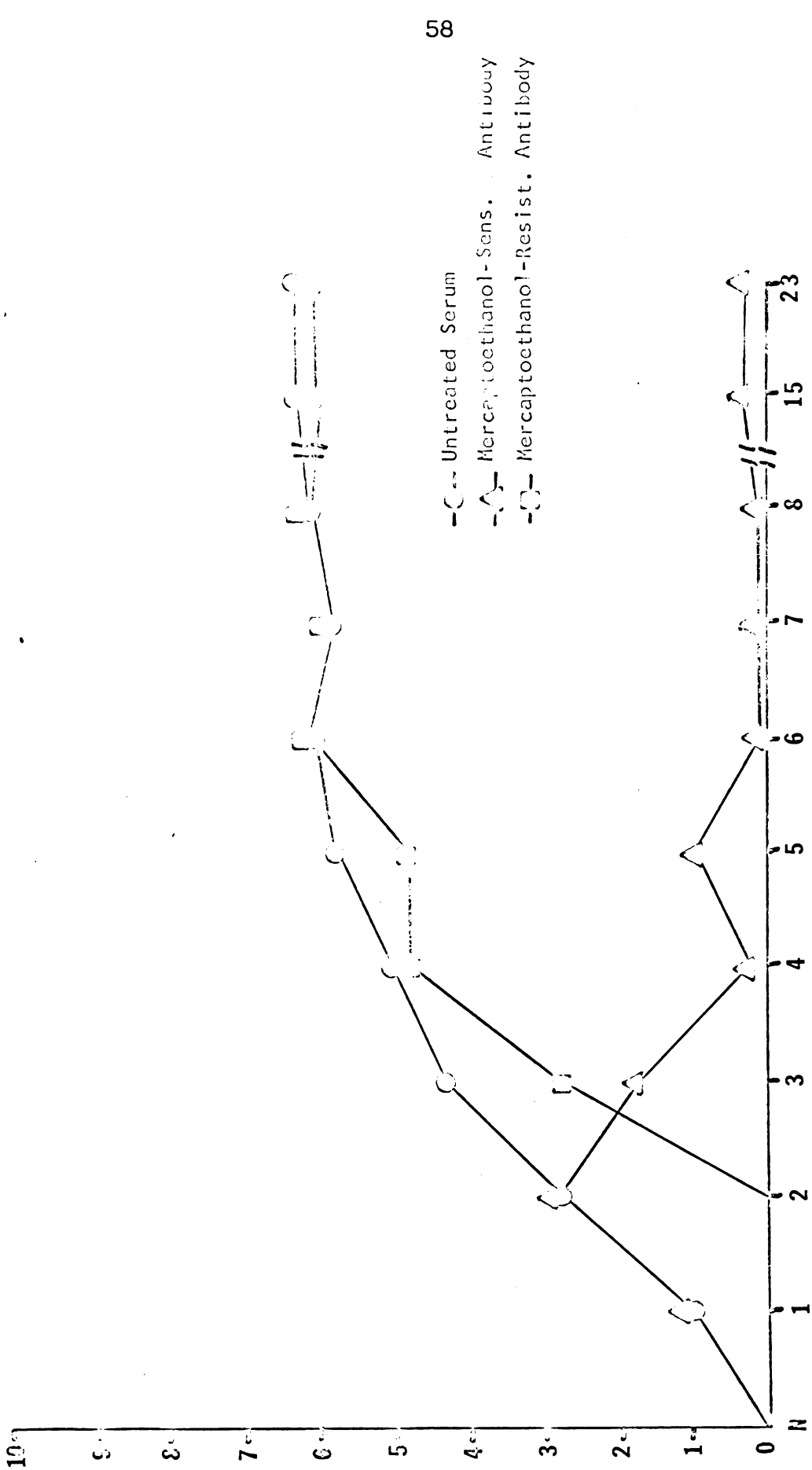


Fig. 2. Bacterial agglutinins produced by rabbits in Group II (Table 1).

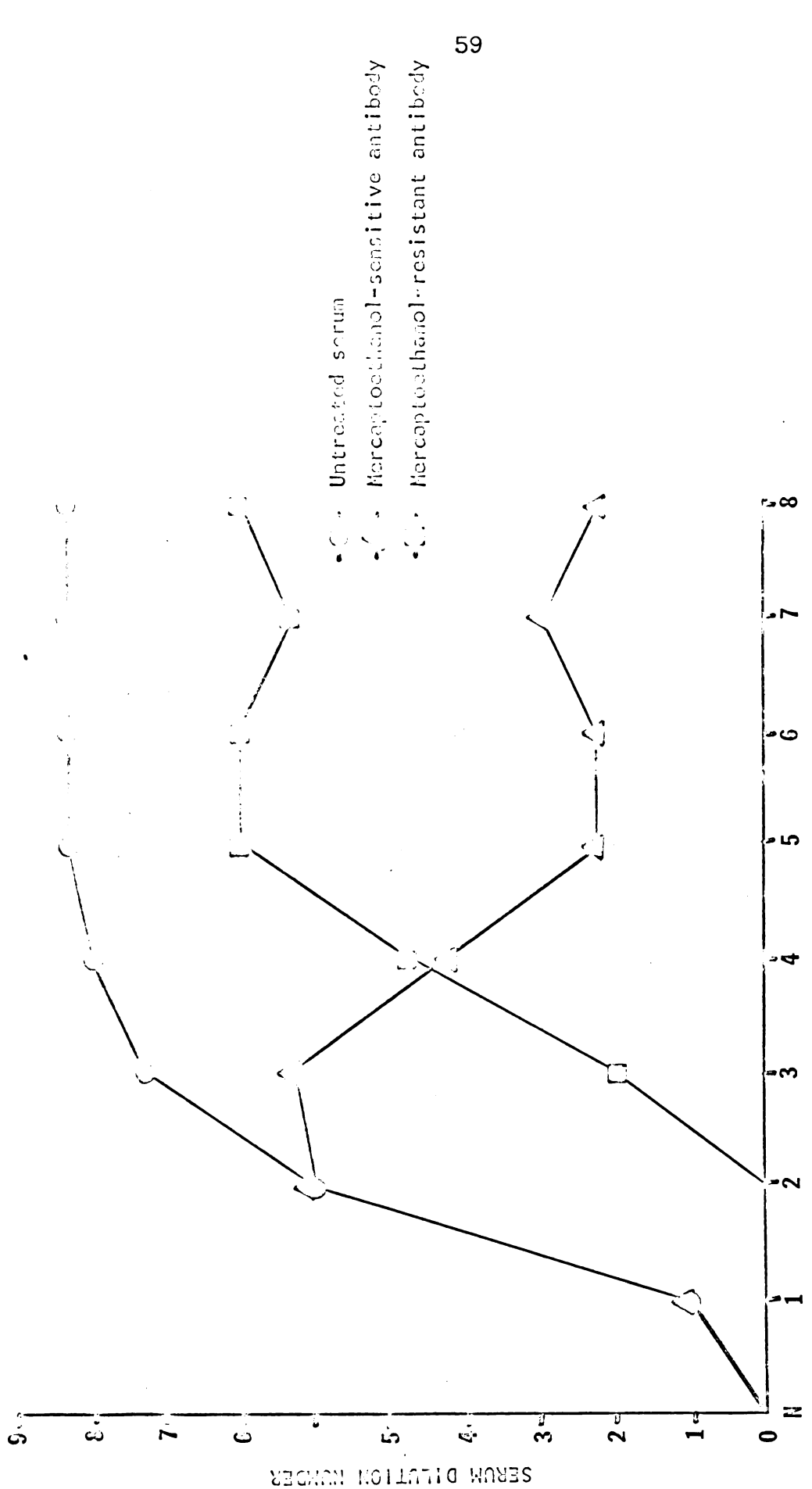


Fig. 3. Hemagglutinins produced by rabbits in Group III (Table 1).

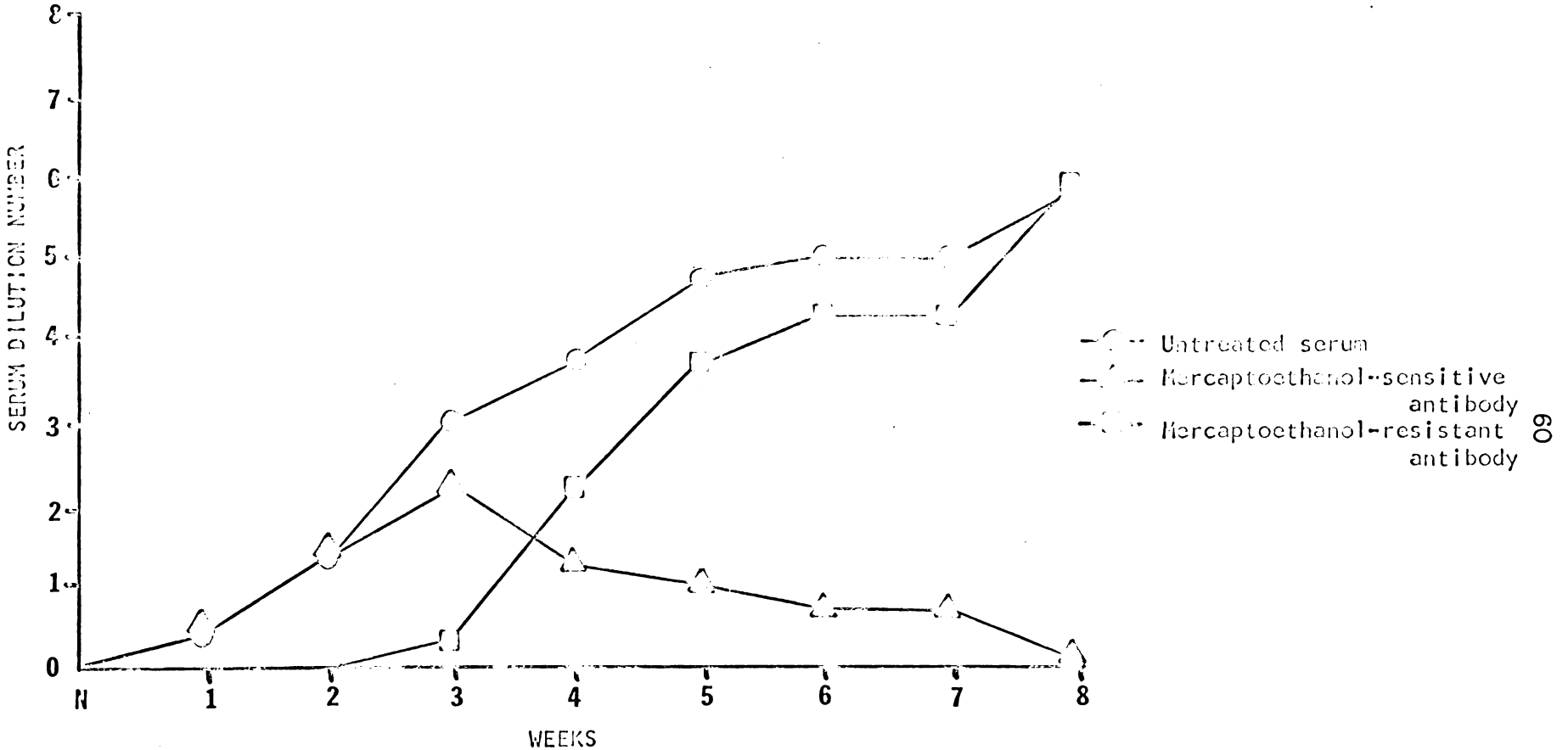


Fig. 4. Bacterial agglutinins produced by rabbits in Group III (Table 1).

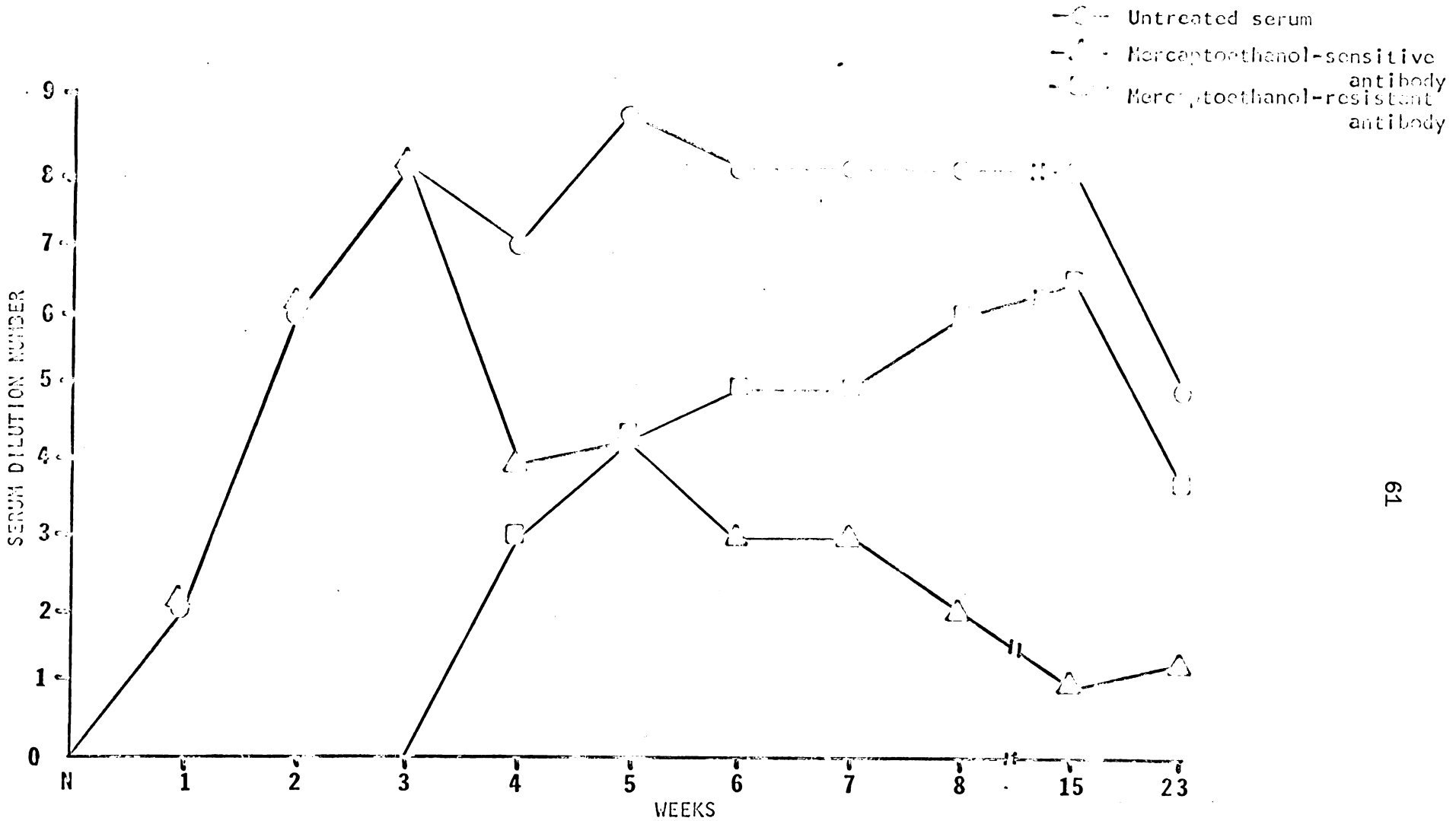


Fig. 5. Hemagglutinins produced by rabbits in Group IV (Table 1).

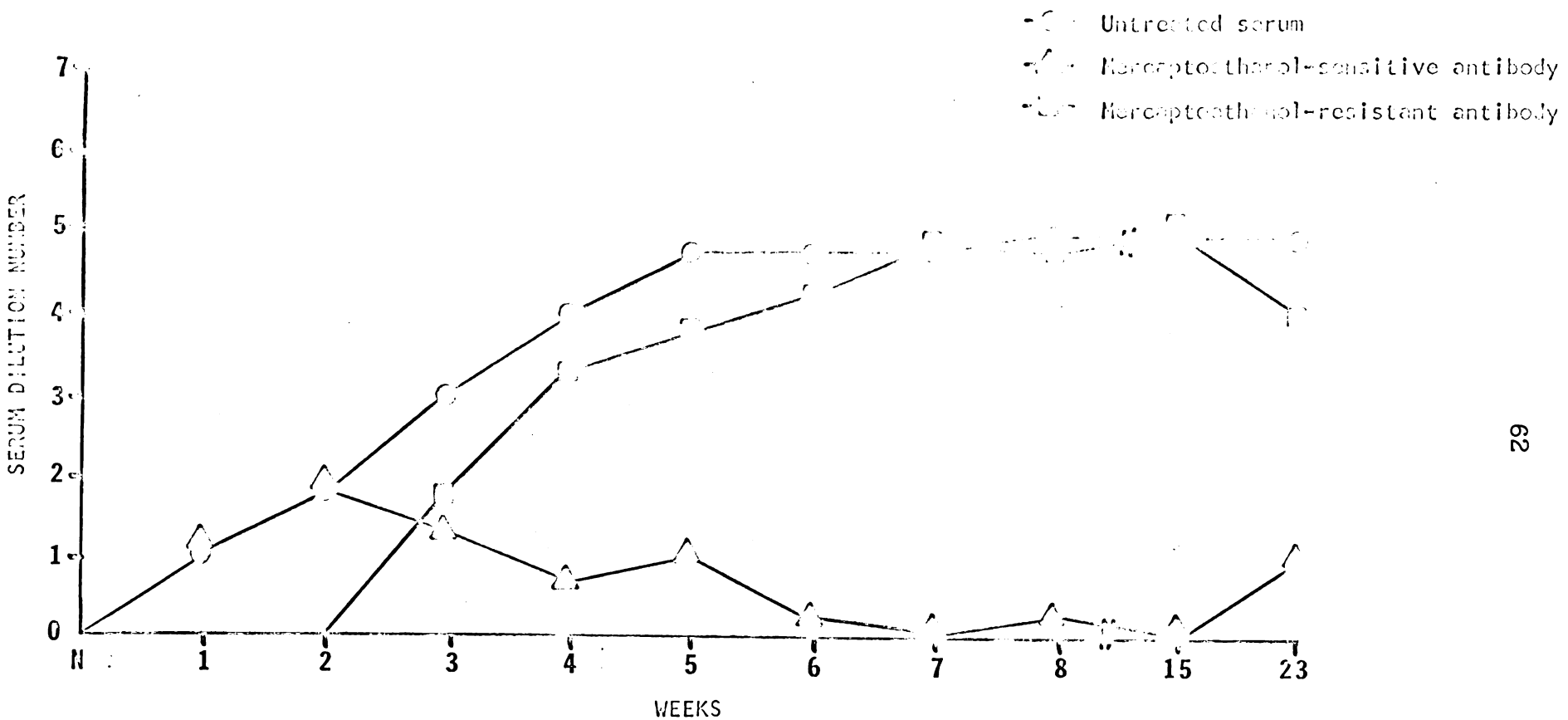


Fig. 6. Bacterial agglutinins produced by rabbits in Group IV (Table 1).

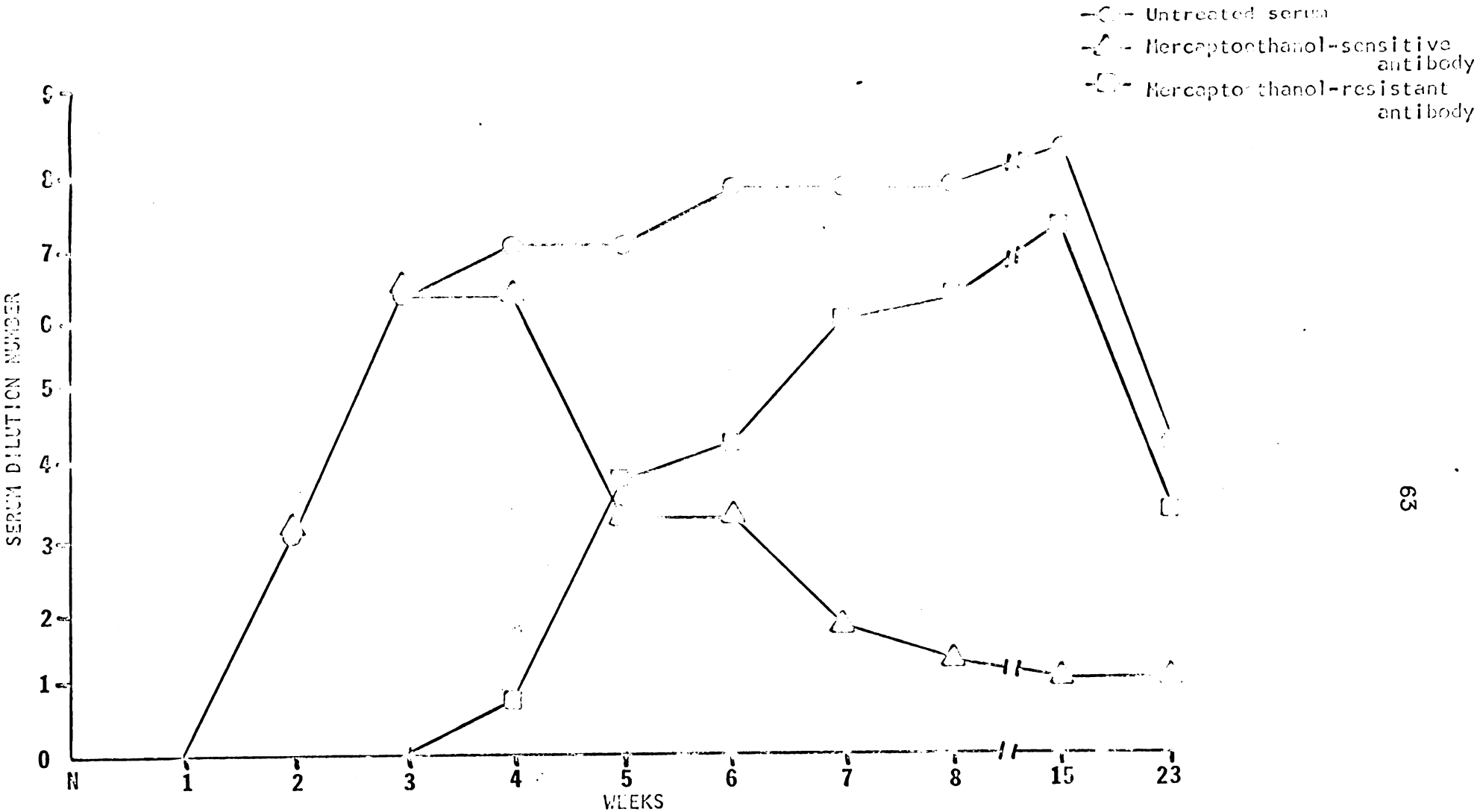


Fig. 7. Hemagglutinins produced by rabbits in Group V (Table 1).

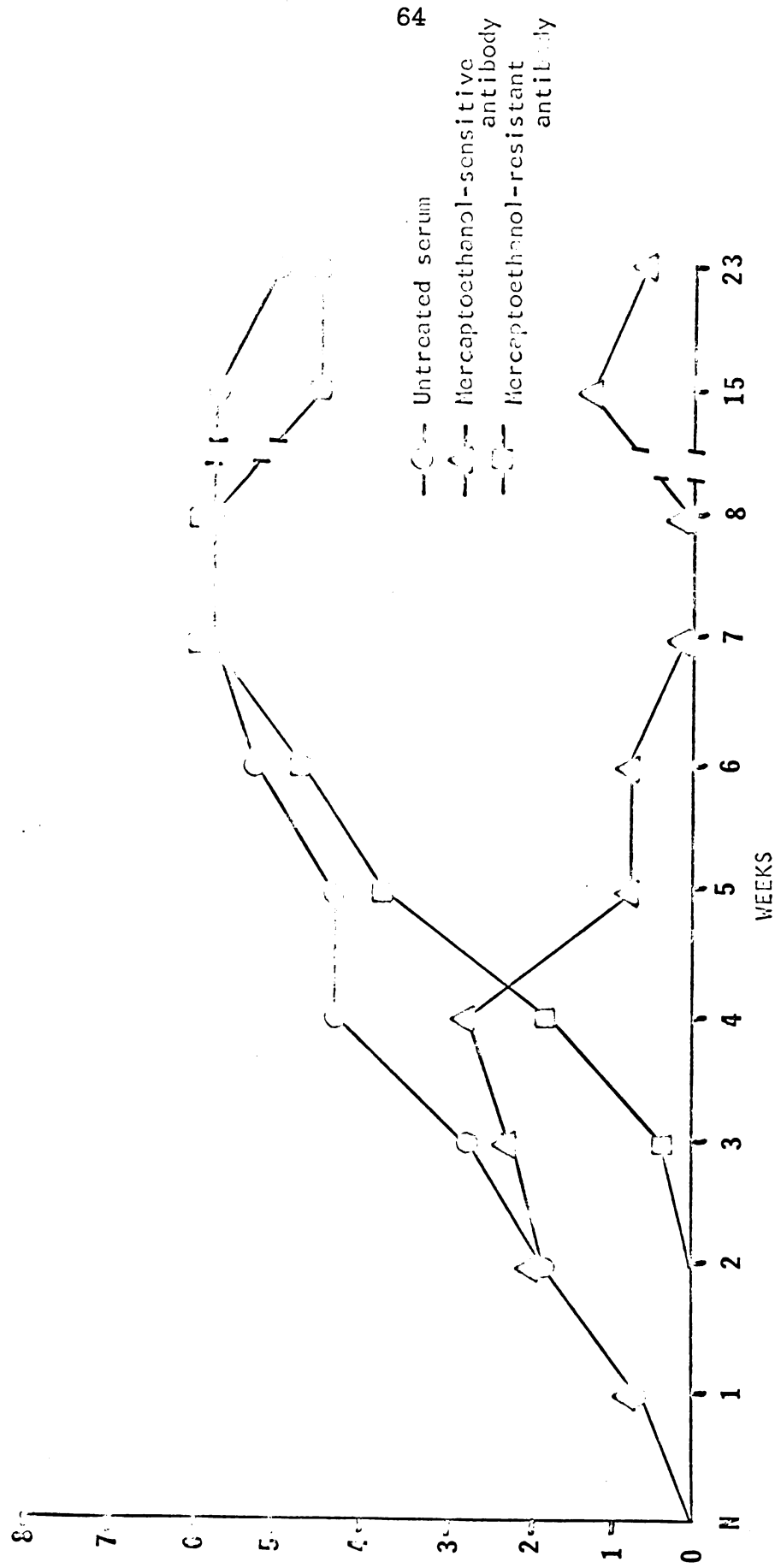


Fig. 8. Bacterial agglutinins produced by rabbits in Group V (Table 1).

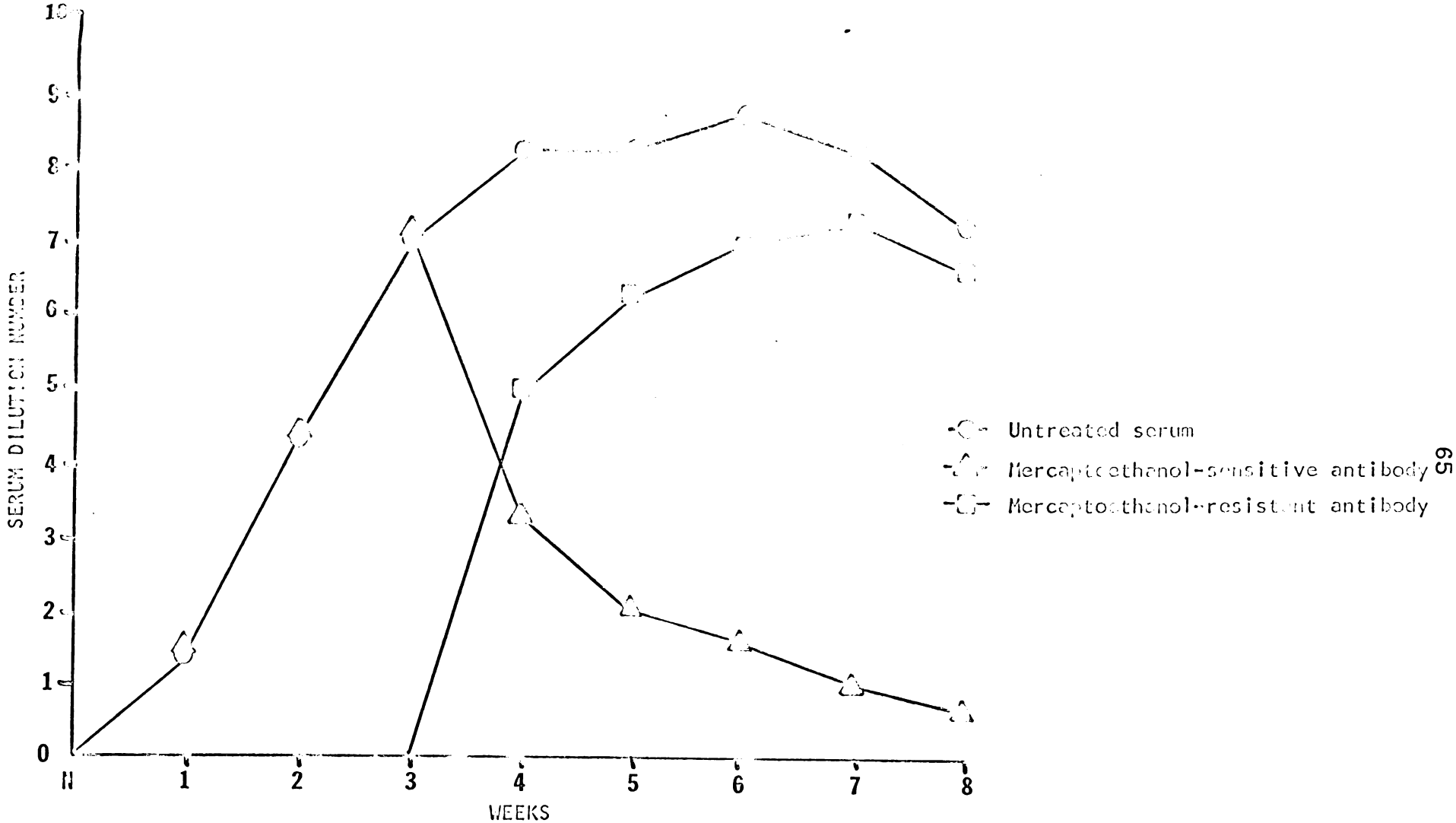


Fig. 9. Hemagglutinins produced by rabbits in Group VI (Table 1).

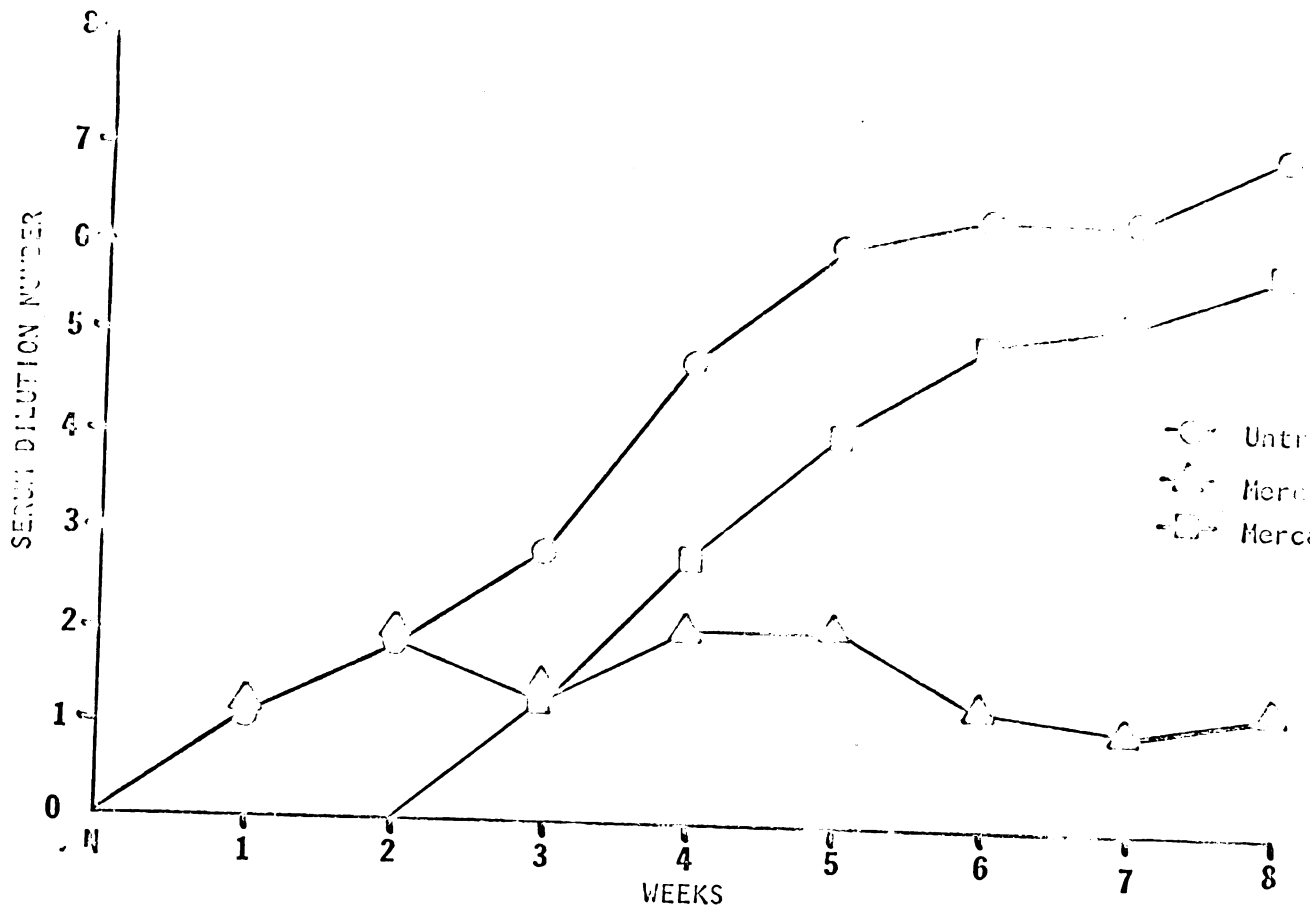


Fig. 10. Bacterial agglutinins produced by rabbits in Group VI(Table I).

Table 4. Mean hemagglutinin titers[1] in sera from rabbits inoculated with viable or killed cells of Mycobacterium bovis.

Group[2]	WEEKS AFTER INOCULATION									
	1	2	3	4	5	6	7	8	9	10
II	3.0	7.3	8.7	8.7	8.3	8.7	8.3	8.7	10	7.7
III	1.0	6.0	8.3	9.0	9.3	9.3	9.3	9.3	--	--
IV	2.0	6.0	8.0	7.0	8.7	8.0	8.0	8.0	8.0	5.0
V	0	3.0	6.3	7.0	7.0	7.7	7.7	7.7	8.3	4.3
VI	1.3	4.3	7.0	8.3	8.3	8.7	8.3	7.3	--	--

[1] Titers 0, 10, 20 etc., to 5120 coded as serum dilution numbers 0, 1, 2 etc., to 10 respectively.

[2] Group - Three rabbits per group, inoculated as shown in Table 1.

Table 5. Mean bacterial agglutinin titers[1] in sera from rabbits inoculated with viable or killed cells of *Mycobacterium bovis*.

Group[2]	WEEKS AFTER INJECTION									
	1	2	3	4	5	6	7	8	19	23
II	1.0	2.7	4.3	5.0	5.7	6.0	5.7	6.0	6.3	6.3
III	0.3	1.3	3.0	3.7	4.7	5.0	5.0	5.7	--	--
IV	1.0	1.7	3.0	4.0	4.7	4.7	4.7	5.0	5.0	5.0
V	0.6	1.7	2.7	4.3	4.3	5.3	5.7	5.7	5.7	5.0
VI	1.0	1.7	2.7	4.7	6.0	6.3	6.3	7.0	--	--

[1]Titers 0, 10, 20 etc., to 1280 coded as serum dilution numbers 0, 1, 2, etc., to 8 respectively.

[2]Group - Three rabbits per group, inoculated as shown in Table 1.

Table 6. Titers[1] of mercaptoethanol-sensitive and mercaptoethanol-resistant hemagglutinins in sera from rabbits inoculated with viable or killed cells of *Mycobacterium bovis*.

	WEEKS AFTER INJECTION																			
	1		2		3		4		5		6		7		8		15		23	
Group[2]	S[3]	R[4]	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
II	3.0	0	7.3	0	7.0	1.7	4.0	4.7	1.7	6.7	0.3	8.3	0.3	8.0	0.3	8.3	1.3	8.6	0	7.7
III	1.0	0	6.0	0	6.3	2.0	4.3	4.7	2.3	7.0	2.3	7.0	3.0	6.3	2.3	7.0	--	--	--	--
IV	2.0	0	6.0	0	8.0	0	4.0	3.0	4.3	4.3	3.0	5.0	3.0	5.0	2.0	6.0	1.0	7.0	1.3	3.7
V	0	0	3.0	0	6.0	0	6.3	0.7	3.3	3.7	3.3	4.3	1.7	6.0	1.3	6.3	1.0	7.3	1.0	3.3
VI	1.3	0	4.3	0	7.0	0	3.3	5.0	2.0	6.3	1.6	7.0	1.0	7.3	0.7	6.6	--	--	--	--

[1]Titers 0, 10, 20 etc., to 5120 coded as serum dilution number 0, 1, 2 etc., to 10 respectively.

[2]Group - Three rabbits per group, inoculated as shown in Table 1.

[3]S = Mercaptoethanol-sensitive antibody

[4]R = Mercaptoethanol-resistant antibody

Table 7. Titers[1] of mercaptoethanol-sensitive and mercaptoethanol-resistant bacterial agglutinins in sera from rabbits inoculated with viable and killed cells of *Mycobacterium lewis*.

	WEEKS AFTER INJECTION																			
	1		2		3		4		5		6		7		8		15		23	
Group[2]	S[3]	R[4]	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
II	1.0	0	2.7	0	1.7	2.7	0.3	4.7	1.0	4.7	0	6.0	0	5.7	0	6.0	0.3	6.0	0.3	6.0
III	0.3	0	1.3	0	2.3	0.7	1.3	2.3	1.0	3.7	0.7	4.3	0.7	4.3	0	5.7	--	--	--	--
IV	1.0	0	1.7	0	1.3	1.7	0.7	3.3	1.0	3.7	0.3	4.3	0	4.7	0.3	4.7	0	5.0	1.0	4.0
V	0.7	0	1.7	0	2.3	0.3	2.7	1.7	0.7	3.7	0.7	4.7	0	5.7	0	5.7	1.2	4.5	.5	4.5
VI	1.0	0	1.7	0	1.3	1.3	2.0	2.7	2.0	4.0	1.3	5.0	1.0	5.3	1.3	5.7	--	--	--	--

[1]Titers 0, 10, 20 etc., to 1280 coded as serum dilution numbers 0, 1, 2, etc., to 8 respectively.

[2]Group - Three rabbits per group, inoculated as shown in Table 1.

[3]S = Mercaptoethanol-sensitive antibody

[4]R = Mercaptoethanol-resistant antibody

Table 8. Ratios of mercaptoethanol-sensitive/mercaptoethanol-resistant hemagglutinins in sera from rabbits inoculated with viable or killed cells of *Mycobacterium bovis*.

Group[1]	ME-S/ME-R[2] Hemagglutinins									
	Rabbit No.									
	1	2	3	4	5	6	7	8	9	10
II	3.0	7.3	4.1	0.8	0.3	0.04	0.04	0.04	0.2	0
III	1.0	6.0	3.15	0.9	0.3	0.3	0.5	0.3	--	--
IV	2.0	6.0	8.0	1.3	1.0	0.6	0.6	0.3	0.1	0.3
V	0	3.0	6.3	0.9	0.9	0.8	0.3	0.2	0.3	0.1
VI	1.3	4.3	7.0	0.7	0.3	0.2	0.1	0.1	--	--

[1]Group - Three rabbits per group, inoculated as shown in Table 1.

[2]ME-S/ME-R - Ratios of mercaptoethanol-sensitive/mercaptoethanol-resistant hemagglutinins.

Table 9. Ratios of mercaptoethanol-sensitive/mercaptoethanol-resistant bacterial agglutinins in sera from rabbits inoculated with viable or killed cells of *Mycobacterium bovis*.

	ME-S/ME-R[2] Agglutinins WEEKS AFTER INJECTION									
Group[1]	1	2	3	4	5	6	7	8	15	23
II	1.0	2.7	0.6	0.1	0.2	0	0	0	0.05	0.05
III	0.3	1.3	3.3	0.6	0.3	0.2	0.2	0	--	--
IV	1.0	1.7	0.8	0.2	0.3	0.1	0	0.1	0	0
V	0.7	1.7	7.6	1.2	0.2	0.2	0	0	0.3	0.1
VI	1.0	1.7	1.0	0.7	0.5	0.3	0.2	0.2	--	--

[1]Group - Three rabbits per group, inoculated as shown in Table 1.

[2]ME-S/ME-R - Ratio of mercaptoethanol-sensitive/mercaptoethanol-resistant agglutinins.

ME-resistant hemagglutinins than rabbits in Groups IV and V. Rabbits in Group II produced significantly more ME-resistant antibody by the third week than rabbits in Groups II and VI. Hemagglutinins were not detected in sera from any of the rabbits in Group V at one week post-inoculation.

The ratio of ME-sensitive/ME-resistant hemagglutinins decreased to less than one by four weeks post-inoculation in all groups except Group III. The ME-sensitive/ME-resistant bacterial agglutinin ratio decreased to less than one in all Groups by the fifth week. Mercaptoethanol-sensitive hemagglutinins were detected in sera from some rabbits in all Groups for at least eight weeks post-inoculation and in sera from rabbits in Groups IV and V until the 23rd week. Sera from rabbits in Groups III and VI were not tested past the eighth week. Mercaptoethanol-resistant hemagglutinins were detected in sera from rabbits in all Groups tested at 23 weeks post-inoculation.

ME-sensitive bacterial agglutinins were detected in serum from rabbits in all Groups five weeks post-inoculation but only in sera from rabbits in Groups IV and VI at the eighth week. Mercaptoethanol-resistant bacterial agglutinins were detected in sera from rabbits in all Groups tested at 23 weeks post-inoculation.

Skin testing of rabbits in Groups II, IV and V with 0.1 ml of second strength PPD-S 14 weeks post-inoculation simulated antibody production (Figs. 1-2 and 5-8). Mean hemagglutinin titers were elevated at the fifteenth week in

Groups II and V but remained unchanged in Group IV. Mean bacterial agglutinin titers were higher at the fifteenth week in Group II but remained unchanged in Groups IV and V. Ratios of ME-sensitive/ME-resistant hemagglutinins were correspondingly increased at the fifteenth week in Groups II and V but decreased in Group IV. The ME-sensitive/ME-resistant bacterial agglutinin ratio was higher at the fifteenth week than at the eighth week in Groups II and V.

No reactions were detectable in rabbits in Groups II, IV and V, five hours after skin tests. Delayed reactions occurred which reached their maximum diameters 24 hours post-inoculation. The mean diameters of palpable induration at the skin test sites were greatest in rabbits of Group II and smallest in rabbits of Group V (Fig. 11).

Precipitins specific for culture filtrate antigens were detected in sera from most of the rabbits in Groups II-VI by five weeks post-inoculation. One immunoprecipitate was usually detected in the Ouchterlony double diffusion test, occasionally, two lines occurred.

Serum from normal and immunized rabbits was separated into six to eight fractions by zone electrophoresis in cellulose acetate membranes (Fig. 12).

Gamma-globulin concentrations increased above normal levels one week post-inoculation (Table 10). A decrease frequently occurred at the second week, and subsequently, a progressive increase.

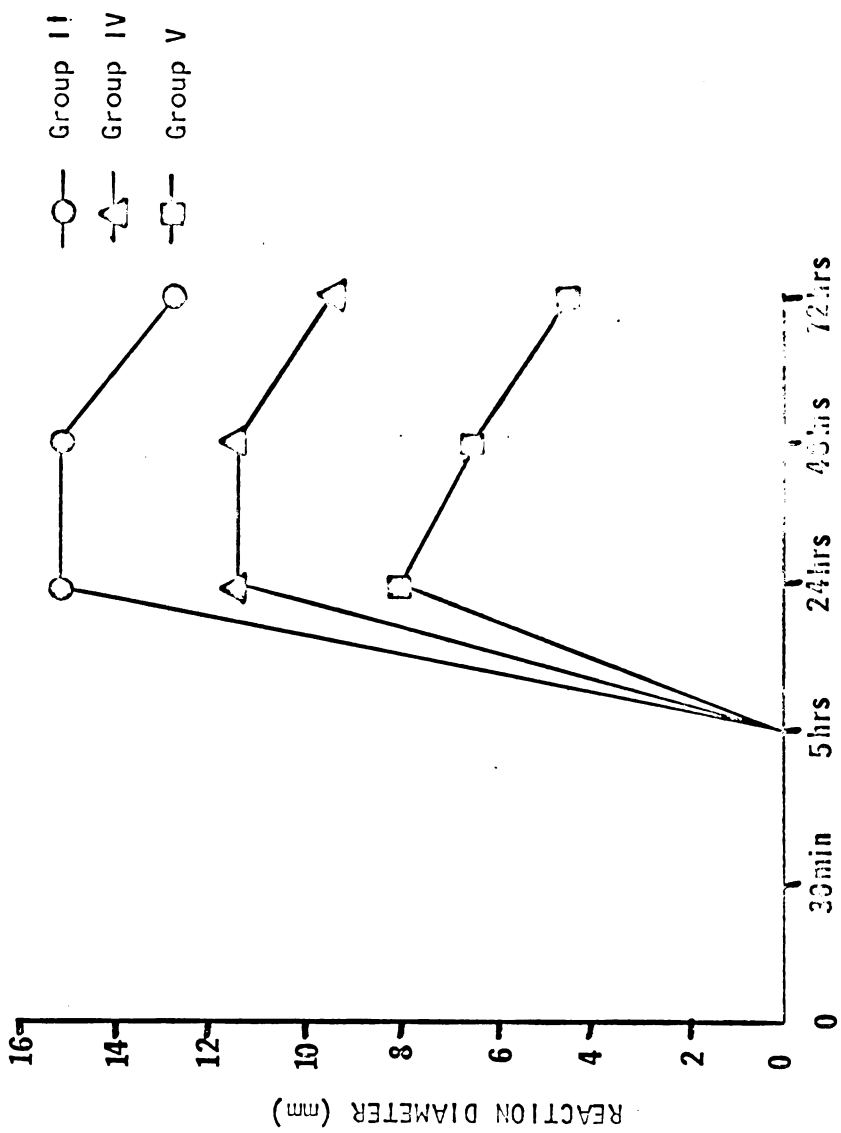


Fig. 11. Skin test reactions elicited in rabbits of Groups II, IV and V (Table 1) by an intradermal injection of PFD-S 14 weeks post-inoculation of antigen.

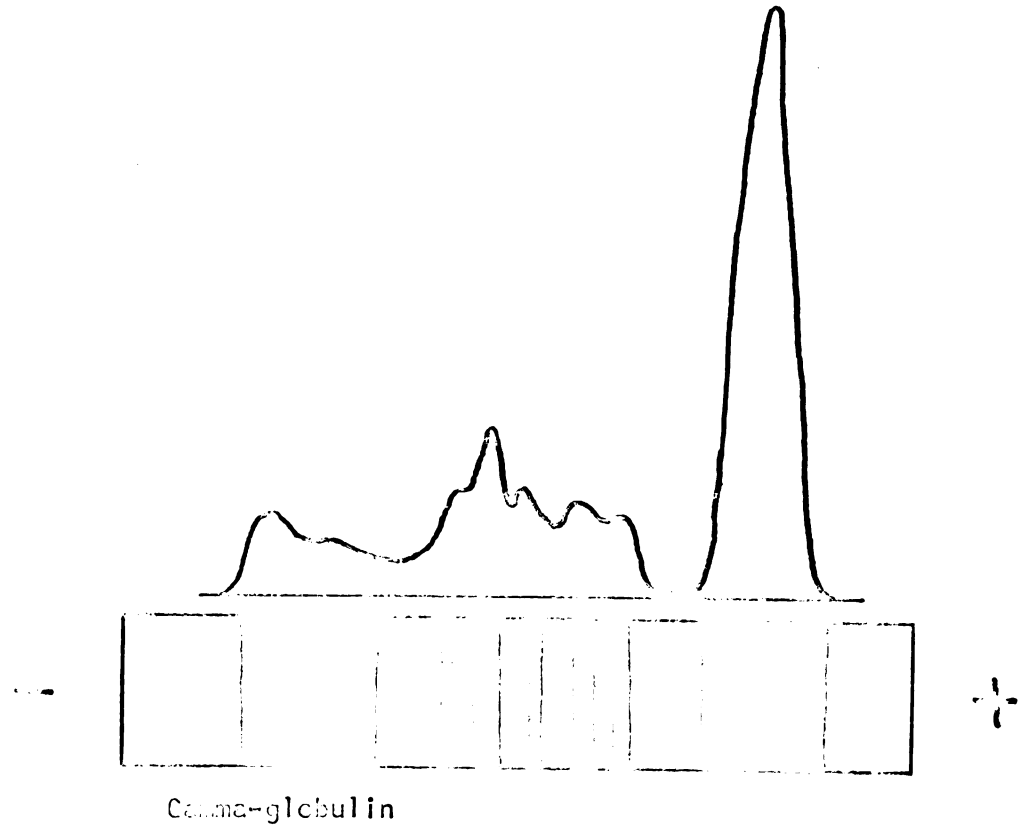


Fig. 12. Representative densitometric recording of cellulose acetate electrophoresis of normal rabbit serum.

Table 10. Relative percentage of gamma-globulin in sera from rabbits inoculated with viable or killed cells of *Mycobacterium bovis*.

Group[1]	Per cent Gamma-Globulin										
		WEEKS AFTER INJECTION									
	N[2]	1	2	3	4	5	6	7	8	15	23
II	9.9	11.5	10.0	10.6	15.0	15.8	16.1	16.3	19.0	21.8	17.0
III	12.0	17.5	17.3	17.2	17.2	21.0	23.6	24.0	24.6	--	--
IV	9.0	11.3	8.2	9.0	12.8	14.6	15.8	19.1	19.3	18.8	15.3
V	6.5	14.1	15.7	13.5	11.4	15.6	17.0	19.7	21.3	20.8	10.0

[1]Group - Three rabbits per group, inoculated as shown in Table 1.

[2]N - Serum from normal rabbits obtained prior to antigen injection.

Chemical Analyses of Bacillary Extracts Prepared by Ultrasound

Ratios of absorbancy at 280 and 260 mμ and the amounts of protein, carbohydrate and nucleic acid in ultrasonic extracts (USE) are listed in Table 11. Ultrasonic extracts A and B were very similar in chemical composition. There was approximately 4 mg of Folin-reactive material (protein), 0.18 mg of carbohydrate and 0.42 mg of nucleic acid per ml of concentrated extracts. Ratios of absorbancy at 280 and 260 mμ were between 0.55 and 0.6. Ultrasonic extract C contained significantly more Folin-reactive material, carbohydrate and nucleic acid than USE A or B; 0.5 mg of carbohydrate, 7.8 mg Folin-reactive material and 0.68 mg of nucleic acid were detected/ml. Ultrasonic extract D contained more protein, approximately the same amount of carbohydrate and a somewhat higher concentration of nucleic acid than USE-A or B. Ultraviolet absorbancy ratios at 280 and 260 mμ of USE-C and D were similar, and higher than USE-A or B.

Bacillary extracts were clear and slightly yellow in color. Some precipitation occurred when frozen extracts were thawed.

Chromatography of Ultrasonic Extracts

Molecular exclusion chromatography of ultrasonic extracts in Sephadex G-25 separated three major fractions on the basis of absorption at 280 mμ or 260 mμ (Figs. 13-16). Fraction I was eluted in the external volume of the column and consisted

Table 11. Amounts of protein, carbohydrate and nucleic acid in ultrasonic extracts obtained from viable cells of Mycobacterium bovis.

Ultrasonic Extract	FRM[1] (mg/ml)	CHO[2] (mg/ml)	NA[3] (mg/ml)	280/260[4]
A	4.2	0.16	0.4	0.59
B	4.0	0.2	0.4	0.56
C	7.8	0.5	0.63	0.68
D	5.0	0.22	0.53	0.67

[1]FRM = Folin-reactive material (protein)

[2]CHO = Carbohydrate

[3]NA = Nucleic acid

[4]280/260 - Ratio of absorbancy at 280 and 260 mu.

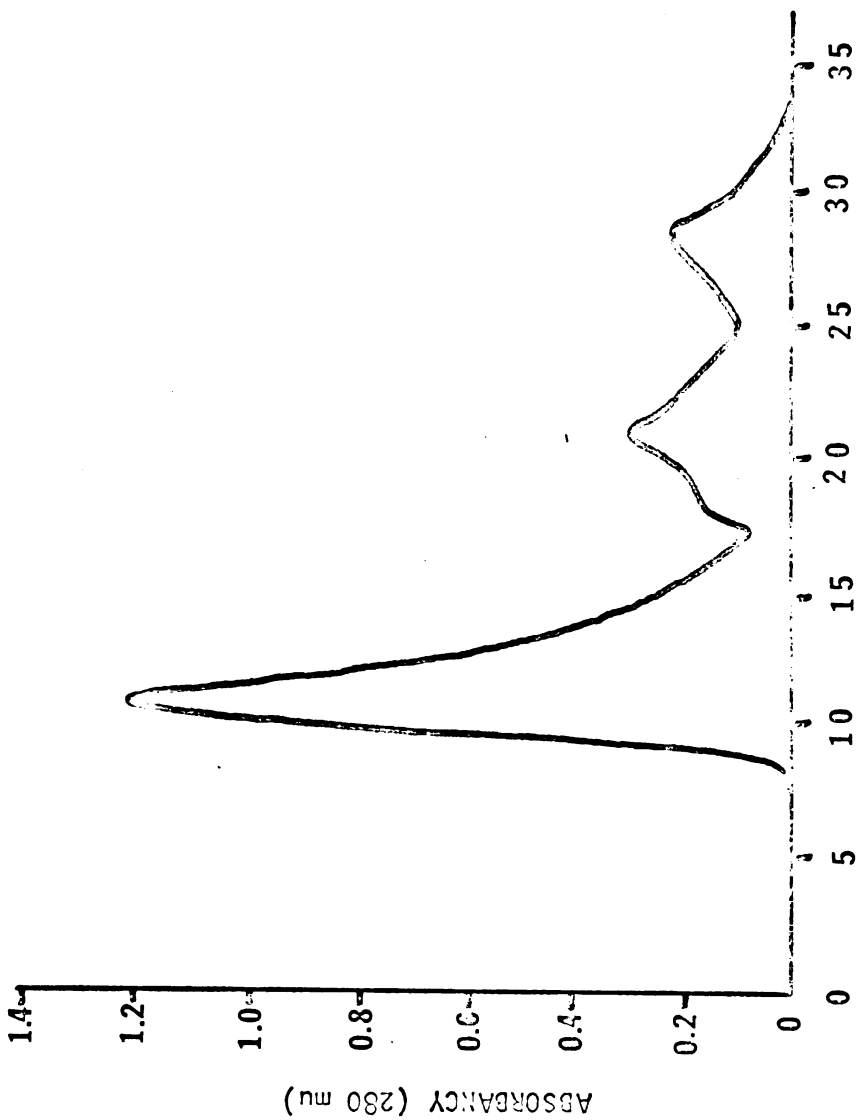


Fig. 13. Molecular exclusion chromatography of ultrasonic extract A (Table 2) in Sephadex G-25.

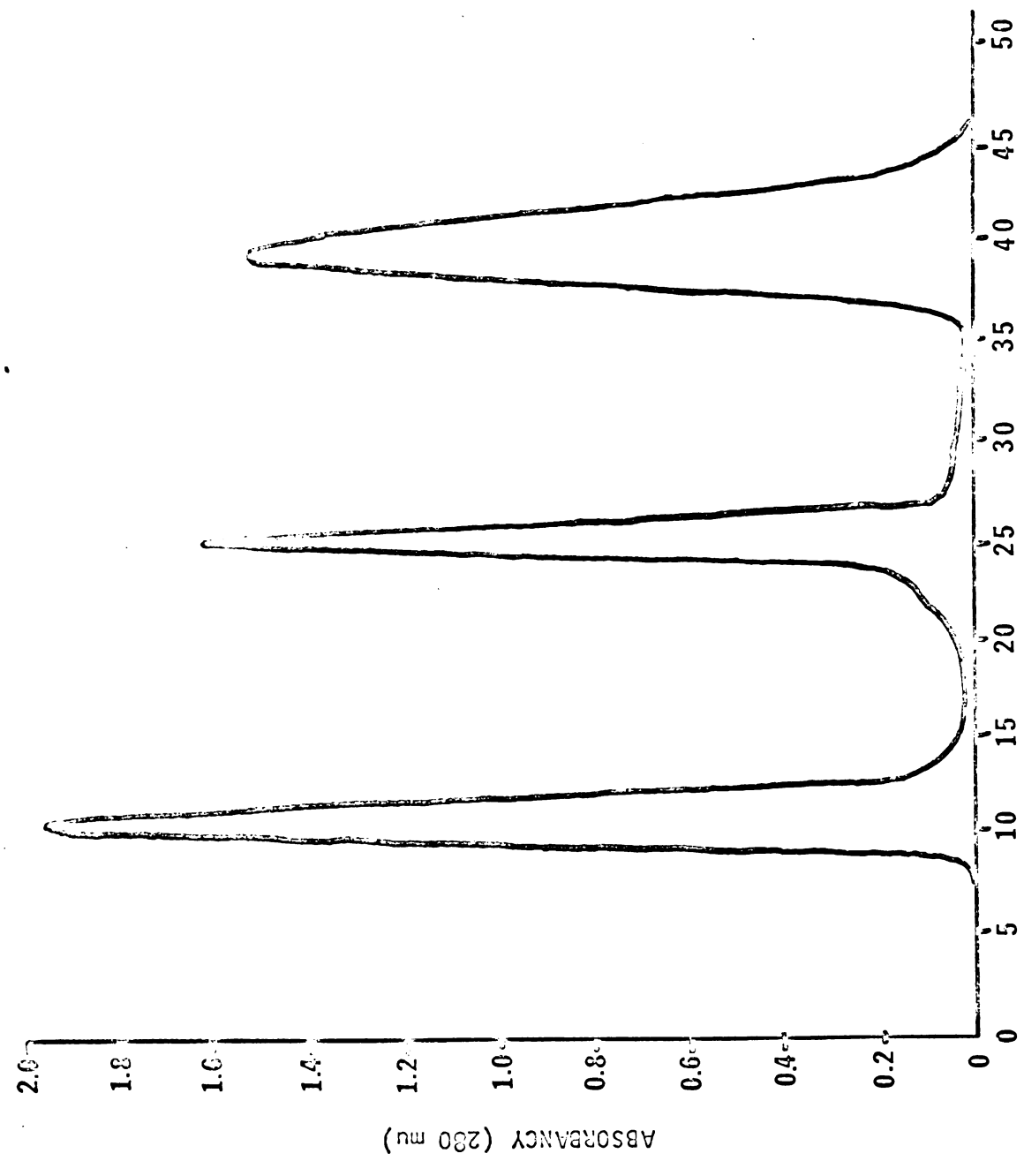


Fig. 14. Molecular exclusion chromatography of ultrasonic extract B (Table 2) in Sephadex G-25.

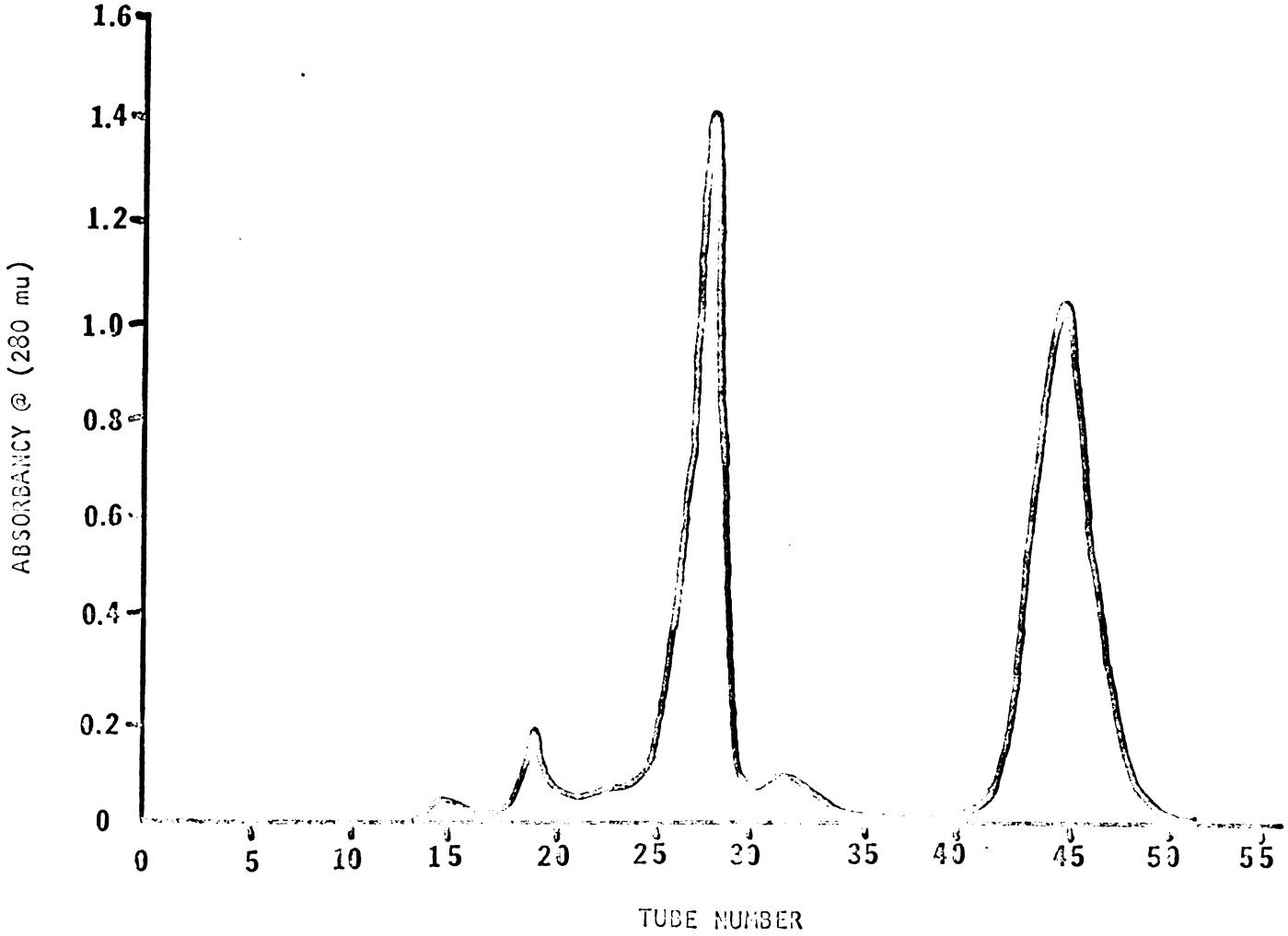


Fig. 15. Molecular exclusion chromatography of ultrasonic extract C (Table 2) in Sephadex G-25.

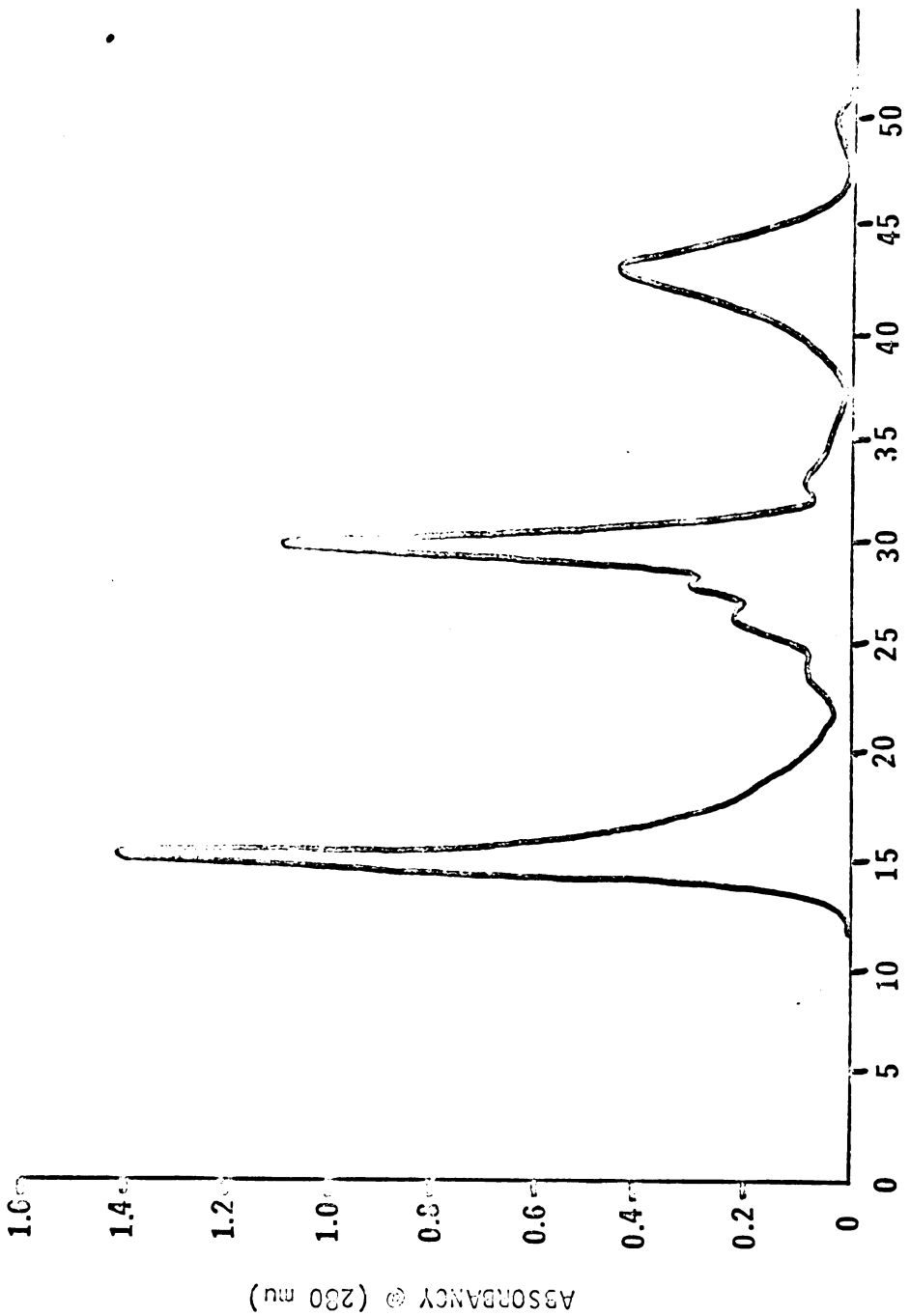


Fig. 16. Molecular exclusion chromatography of ultrasonic extract D (Table 2) in Sephadex G-25.

of a single peak except in USE-C, where fraction one consisted of at least two subfractions contained in three discrete peaks (Fig. 15). Fractions II and III were eluted within the internal volumes of the columns. In USE-B, fraction II consisted of a single peak. Fraction II from USE-A and C was composed of at least two subfractions (Figs. 13 and 15). Fraction II from USE-D consisted of at least five subfractions consisting of one major peak with four closely associated sholders (Fig. 16). Fraction III usually consisted of a single, rather broad 280 mμ-absorbing peak. In USE-D, fraction III had two subfractions with a very small peak associated with the major fraction. Fractions I and III were white and readily dissolved. Fraction II was yellow-brown, granular and difficult to dissolve.

The distribution of carbohydrate, nucleic acid, Folin-reactive material and 280 mμ-absorbing material in chromatographic fractions from different ultrasonic extracts are listed in Tables 12-15. Ultrasonic extracts differed in the relative distribution of 280 mμ-absorbing material in the three major chromatographic fractions from Sephadex G-25. Fractions I, II and III from USE-A contained 63.4, 22.4 and 14.2% of the 280 mμ-absorbing material respectively (Table 12). Although similar in chemical composition, the distribution of 280 mμ absorbing material in USE-A and B differed. Ultrasonic extract B contained less fraction I and II and more of fraction III than USE-C (Table 13). Ultrasonic

Table 12. Distribution of protein, carbohydrate, and nucleic acid in the major chromatographic fractions of ultrasonic extract A eluted from Sephadex G-25.

Fraction number[1]	% FRM[2]	% CHO[3]	% NA[4]	% 280-AM[5]	Rf
I	62.5	100	31	63.4	1.0
II	35.7	0	59	22.4	0.4
III	1.8	0	10	14.2	0.3

[1]Fraction number - Major chromatographic fractions eluted from Sephadex G-25.

[2]FRM = Folin-reactive material (protein)

[3]CHO = Carbohydrate

[4]NA = Nucleic acid

[5]280-AM = 280 mu-absorbing material

Table 13. Distribution of protein, carbohydrate, and nucleic acid on the major chromatographic fractions of ultrasonic extract B eluted from Sephadex G-25.

Fraction[1] Number	% FRM[2]	% CHO[3]	% NA[4]	% 280-AM[5]	PE
I	48.8	100	31.5	48.8	1.0
II	48.8	0	60.3	17.0	0.46
III	3.8	0	8.2	34.1	0.32

[1]Fraction number - Major chromatographic fractions eluted from Sephadex G-25

[2]FRM = Folin-reactive material (protein)

[3]CHO = Carbohydrate

[4]NA = Nucleic acid

[5]280-AM = 280 mu-absorbing material

Table 14. Distribution of protein, carbohydrate and nucleic acid in the major chromatographic fractions of ultrasonic extract C eluted from Sephadex G-25.

Fraction Number[1]	% FRM[2]	% CHO[3]	% NA[4]	% 280-AM[5]	RF
I	43.8	98.5	10.4	10.1	1.0
II	56.2	1.5	59.3	30.3	0.42
III	0	0	30.2	59.6	0.30

[1]Fraction number - Major chromatographic fractions eluted from Sephadex G-25.

[2]FRM = Folin-reactive material (protein)

[3]CHO = Carbohydrate

[4]NA = Nucleic acid

[5]280-AM = 280 mu-absorbing material

Table 15. Distribution of protein, carbohydrate and nucleic acid in the major chromatographic fractions of ultrasonic extract D eluted from Sephadex G-25.

Fraction Number[1]	% FRM[2]	% CHO[3]	% NA[4]	% 280-AM[5]	Rf
I	41	99	39	42	1.0
II	56.1	1	57	36.4	0.5
III	2.9	0	4.0	22.1	0.3

[1]Fraction number - Major chromatographic fractions eluted from Sephadex G-25

[2]FRM = Folin-reactive material (protein)

[3]CHO = Carbohydrate

[4]NA = Nucleic acid

[5]280-AM = 280 mu-absorbing material

extract C contained substantially less fraction I and more of fraction II and fraction III than USE-A or B. Only approximately 10% of the total amount of 280 mμ-absorbing material detected in USE-C was in fraction I. Fraction I, II and III from USE-D contained 42.0, 36.4 and 22.1% respectively of the total amount of 280 mμ-absorbing material detected.

Ultrasonic extracts differed in the distribution of nucleic acid in the three major chromatographic fractions eluted from Sephadex G-25. In general, elution profiles based upon absorbancy at 260 mμ overlayed corresponding profiles based upon absorbancy at 280 mμ although some differences in peak amplitude and width were observed. Nucleic acid concentrations in fraction I from USE-A and B were similar and somewhat lower than in USE-D (Tables 12, 13 and 15). Considerably less nucleic acid (10.1%) was detected in fraction I from USE-C (Table 14). The percent of nucleic acid detected in fraction II was similar in different ultrasonic extracts and varied between 57 and 60.3%. More variation was observed in the relative distribution of nucleic acid in fraction III from different ultrasonic extracts. Fraction III from USE-D contained 4% of the total nucleic acid. Eight and 10% of the total nucleic acid were detected in fraction III from USE-B and A respectively. Almost one-third of the nucleic acid detected in USE-C was found in fraction III.

Carbohydrate was detected in both fractions I and II from USE-C and D and only in fraction I from USE-A and B. The majority of the carbohydrate detected in ultrasonic extracts was present in chromatographic fraction I.

The distribution of protein (Folin-reactive material) in the three major chromatographic fractions from Sephadex G-25 varied among the different ultrasonic extracts. Fraction I from USE-A contained 62.5% of the total amount of Folin-reactive material detected in the sample (Table 12). Somewhat lower values were detected in fraction I from USE-B, C and D; 48.8, 43.8 and 41.0% respectively were detected. Fraction II contained the majority of the remaining Folin-reactive material. From zero to 4% of the Folin-reactive material was detected in fraction III. Good correlation was observed between the relative percentage of 280-absorbing material and Folin-reactive material in fraction I from USE-A, B and D. No correlation existed between these values in fractions II and III.

Rf values were computed for each of the three major 280 mμ-absorbing fractions separated from ultrasonic extracts by chromatography in Sephadex G-25 (Tables 12-15). Fraction I had an arbitrary Rf value of 1.0. Fraction II from different ultrasonic extracts had Rf values ranging from 0.42-0.50. Rf values for fraction III varied from 0.29-0.32.

Representative ultraviolet absorption spectra from unfractionated ultrasonic extract (USE-B) and major chromatographic fractions I, II and III eluted from Sephadex G-25

are shown in Figures 17 and 18. Absorption spectra of the unfractionated extract and fraction I were virtually identical (Fig. 17). Two broad absorption peaks were observed, one between 280 and 260 mμ and a second between 240 mμ and 280 mμ. Chromatographic fraction II had a single continuous peak with maximum absorption beginning at approximately 260 mμ (Fig. 18). Fraction III had two absorption maxima, approximately 250 and 200 mμ (Fig. 18).

Representative chromatograms of fraction I from ultrasonic extract B obtained by chromatography in Sephadex G-25 and rechromatographed in BioGels P-100, P-150 and P-200 are shown in Fig. 19. Fraction I (G-25) from USE-A was separated into two subfractions in BioGel media on the basis of absorption at 280 mμ. Each subfraction consisted of a single peak. In each case, the first subfraction was eluted within the external volume of the column and the second was eluted within the internal volume of the column. The percent distribution of 280 mμ-absorbing material in each of these subfractions from the different BioGels is given in Table 16. Eighty-four percent of fraction I G-25 from USE-B was eluted in the first subfraction from BioGel P-100. The remaining 16% was eluted in the second subfraction. Approximately 82% and 18% of fraction I G-25 from this extract was eluted in the first and second subfractions from BioGel P-150 respectively. Fifty-nine percent of the 280 mμ-absorbing material contained in fraction I G-25 was eluted within the

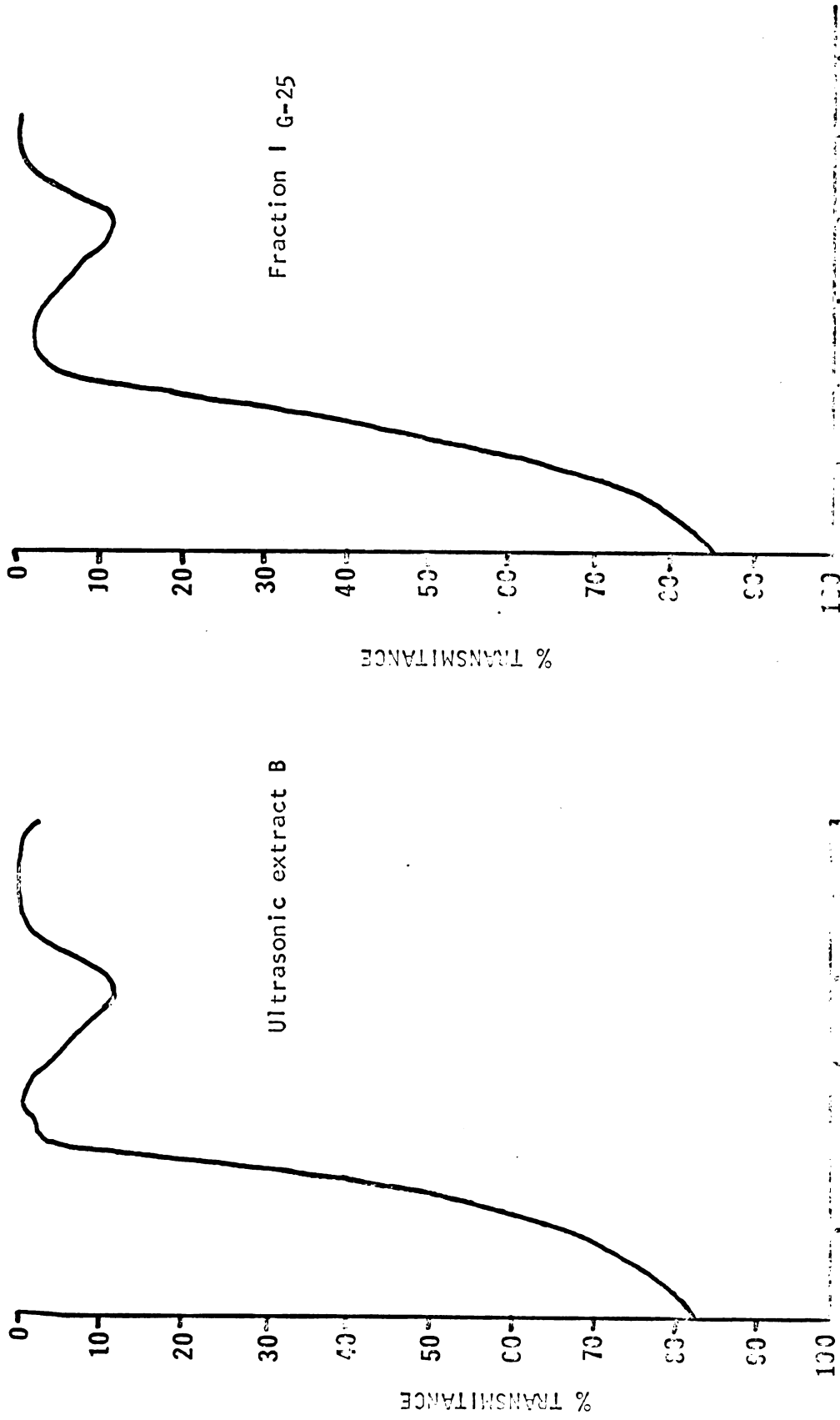


Fig. 17. Ultraviolet absorption spectra of ultrasonic extract B (Table 2) and chromatographic fraction I obtained from Sephadex G-25.

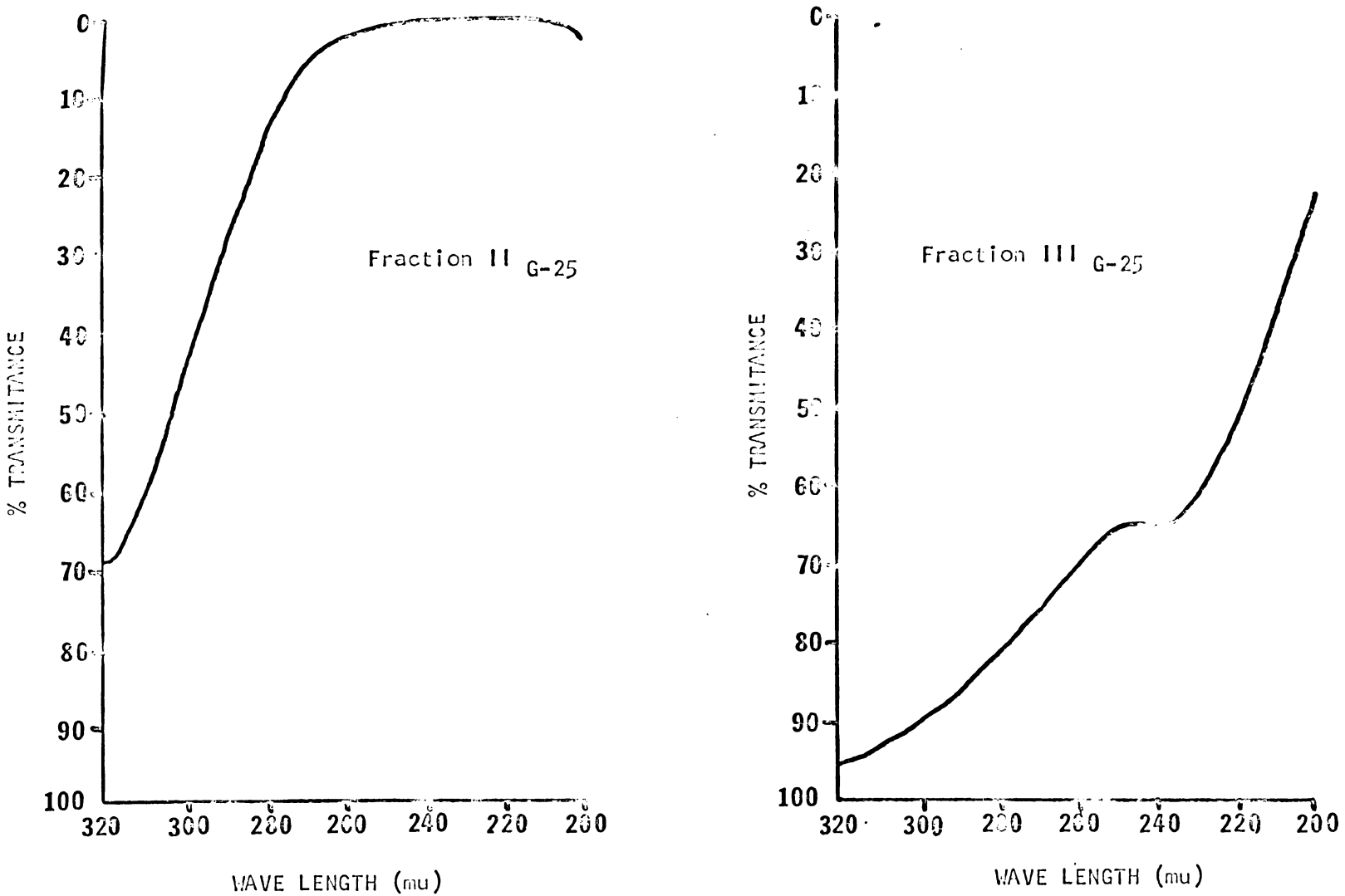


Fig. 18. Ultraviolet absorption spectra of chromatographic fraction II and III from ultrasonic extract B (Table 2) obtaincd from Sephadex G-25.

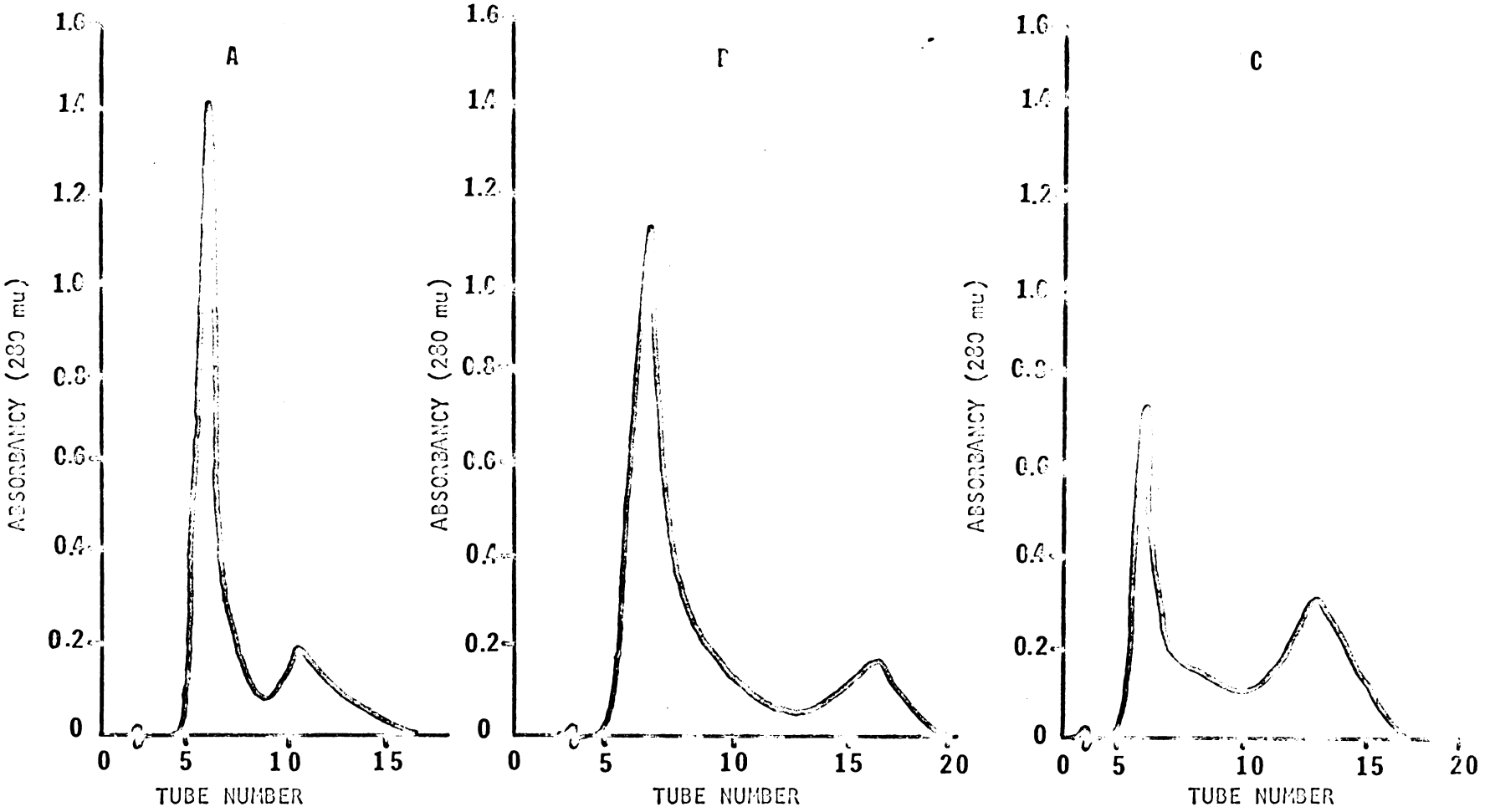


Fig. 19. Rechromatography of fraction I $_{G-25}$ from ultrasonic extract B (Table 2) in Bio Gels P-100 (A), P-150 (B) and P-200 (C).

Table 16. Distribution of 280 mu-absorbing material in chromatographic fractions from Bio Gel media following rechromatography of fraction $I_{G\ 25}$ from ultrasonic extracts.

FI_{G-25}[2] from Ultrasonic Extract	Chromatography Medium[1]					
	P-100		P-150		P-200	
	I[3]	II	I	II	I	II
B	84	16	81.7	18.3	59	41
C	61	39	59	41	22	78
D	82	18	77	23	20	80

[1]Chromatography medium - P-100, P-150 and P-200 are Bio Gels with different molecular exclusion limits

[2]FI_{G-25} - Chromatographic fraction I from ultrasonic extracts eluted from Sephadex G-25

[3]I and II refer to fraction numbers eluted from different Bio Gels

external volume of columns of BioGel P-200. The remaining (41%) was eluted as the second subfraction. Twenty-two percent of fraction I G-25 from USE-C was eluted in the external volume of BioGel P-200 columns. Twenty percent of the 280 mμ-absorbing material in fraction I G-25 from USE-D was eluted within the external volume of BioGel P-200 columns.

Representative chromatograms of USE-B, C and D in BioGels P-100, P-150 and P-200 are presented in Figs. 20-22. Chromatography in BioGel yielded two major fractions. Both fractions consisted of single 280 mμ-absorbing peaks. Fraction I was eluted within the external volume of the columns in every case. Fraction II from BioGel columns was excluded within the columns internal volume. The percent distribution of 280 mμ-absorbing material in chromatographic fractions from ultrasonic extracts is listed in Table 17. The percent distribution of fraction I from chromatography of USE-A, C and D on Sephadex G-25 and BioGel P-100 were considerably different. No significant differences were noted in concentrations of fraction I from BioGel P-100 and BioGel P-150. Differences were noted in the concentration of fraction I from BioGel P-150 and BioGel P-200.

Ultrasonic extract A was separated into dialyzable and nondialyzable components. Representative elution profiles of these two components from Sephadex G-25 columns are shown in Fig. 23. No dialyzable components were found in fraction I

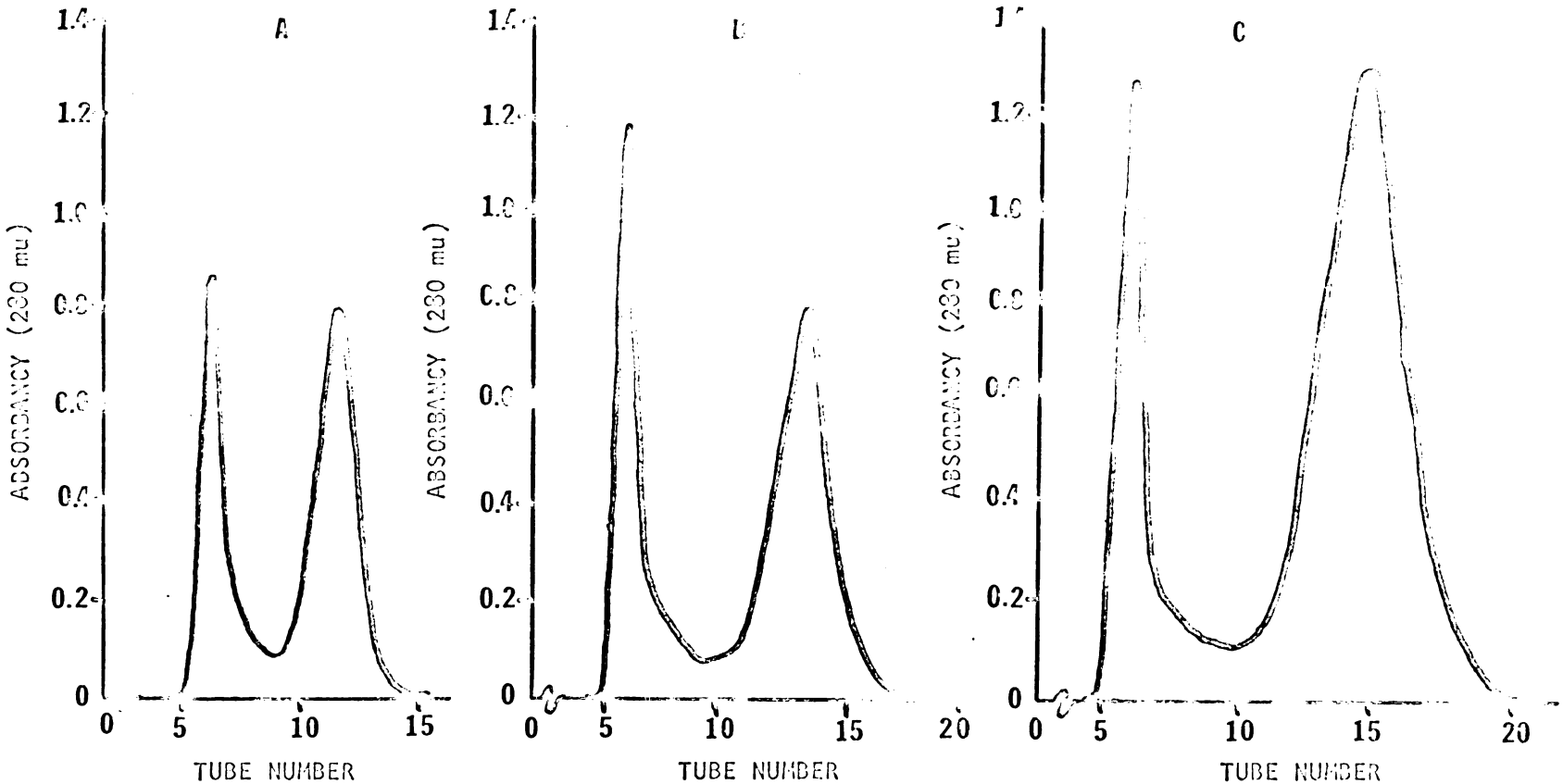


Fig. 20. Molecular exclusion chromatography of ultrasonic extract B (Table 2) in Bio Gels P-100 (A), P-150 (B) and P-200 (C).

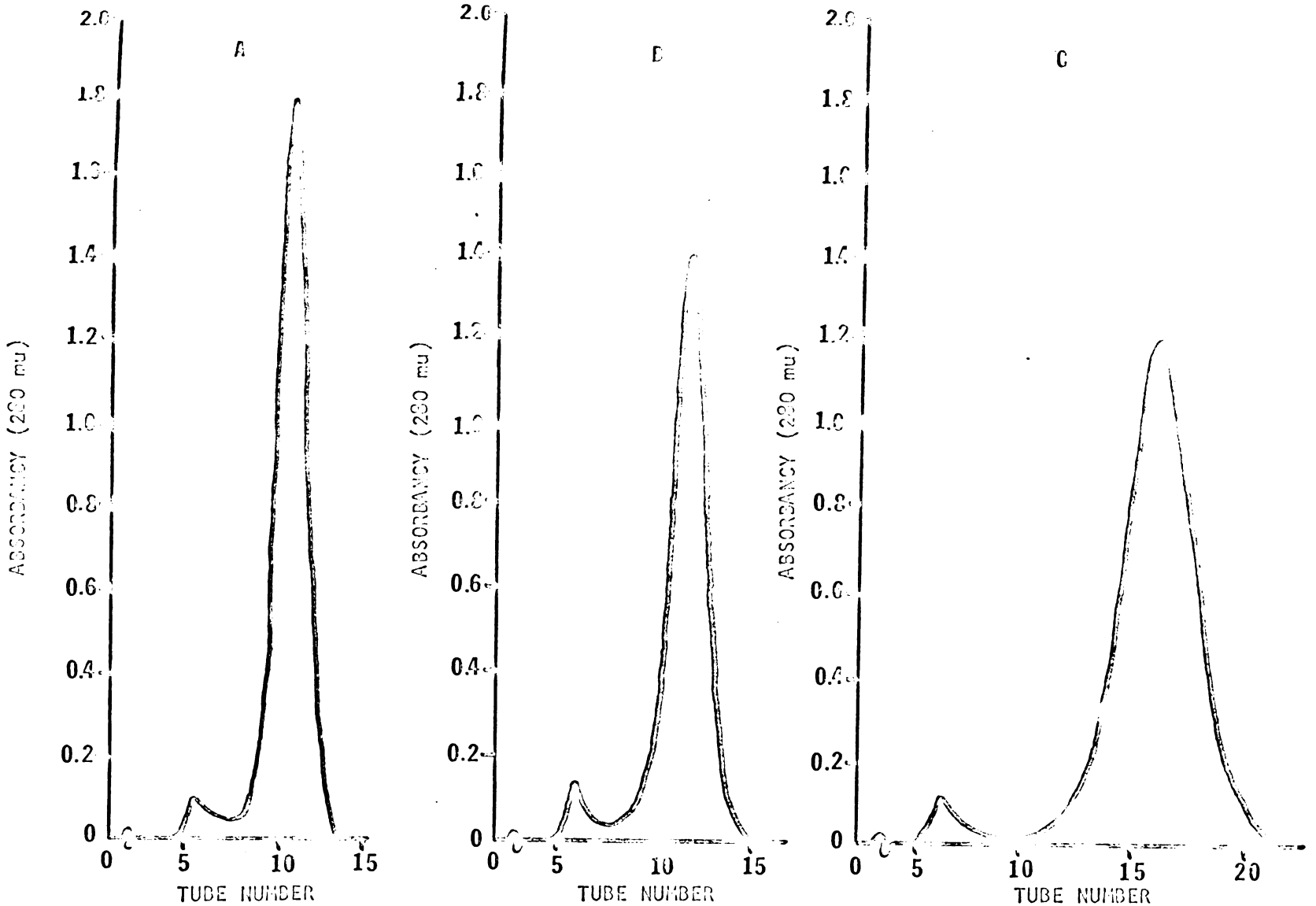


Fig. 21. Molecular exclusion chromatography of ultrasonic extract C (Table 2) in Bio Gels P-100 (A), P-150 (B) and P-200 (C).

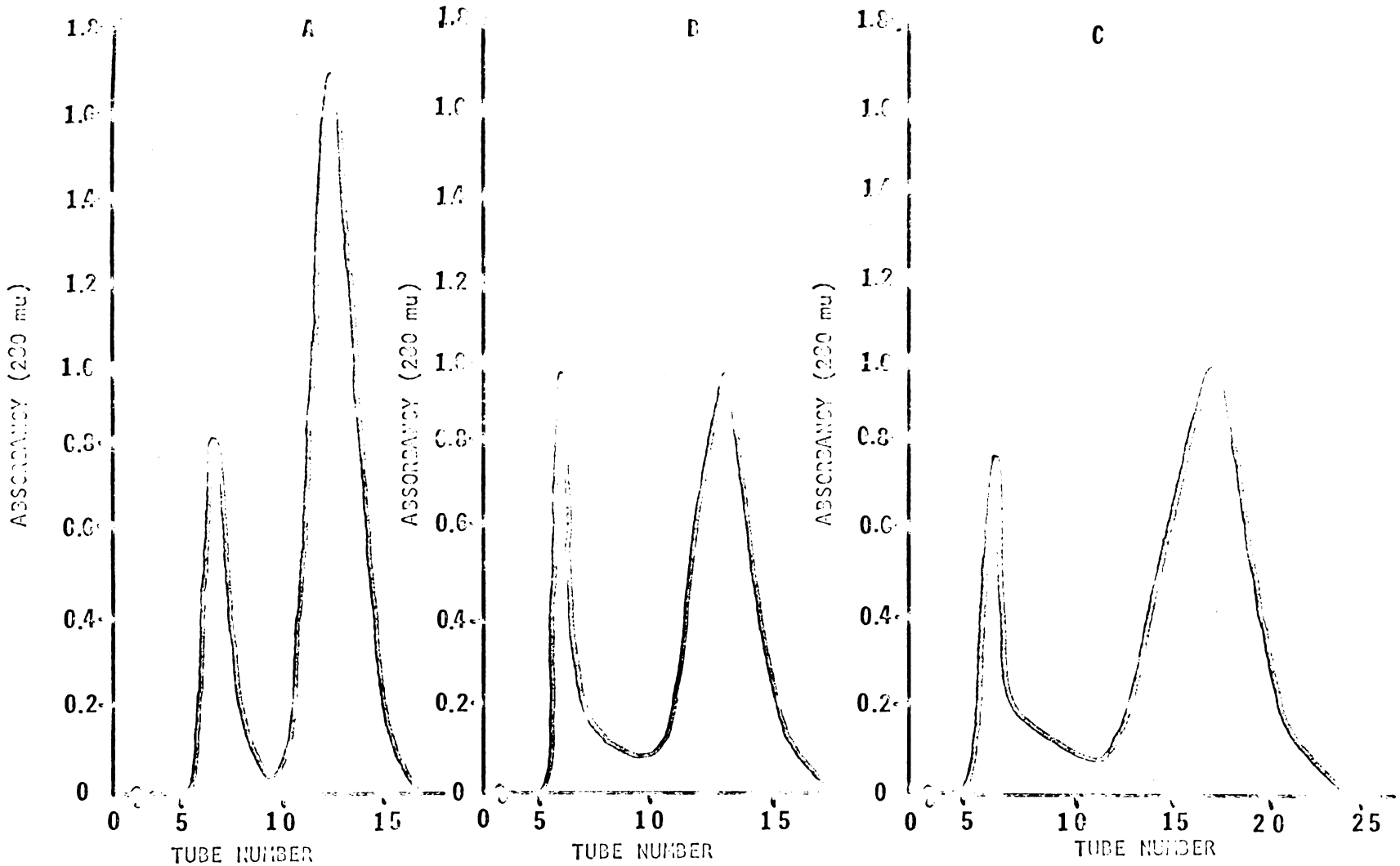


Fig. 22. Molecular exclusion chromatography of ultrasonic extract D (Table 2) in Bio Gels P-100 (A), P-150 (B) and P-200 (C).

Table 17. Distribution of 280 mu-absorbing material in fractions obtained by molecular exclusion chromatography of ultrasonic extracts obtained from viable cells of Mycobacterium bovis.

	Chromatography Medium[1]							
	G-25		P-100		P-150		P-200	
Ultrasonic Extract	I[2]	II & III	I	II	I	II	I	II
A	63.4	36.6	44	56	43	57	26	74
B	48.8	51.3	46	54	45.4	54.6	29	71
C	10.1	89.9	4.0	96	4.5	95.5	4.3	95.7
D	42	58	26	74	26	74	23	77

[1]Chromatography medium - G-25 refers to Sephadex G-25; P-100, P-150 and P-200 are Bio Gels having different molecular exclusion limits

[2]I, II and III refer to different fractions eluted from chromatography media

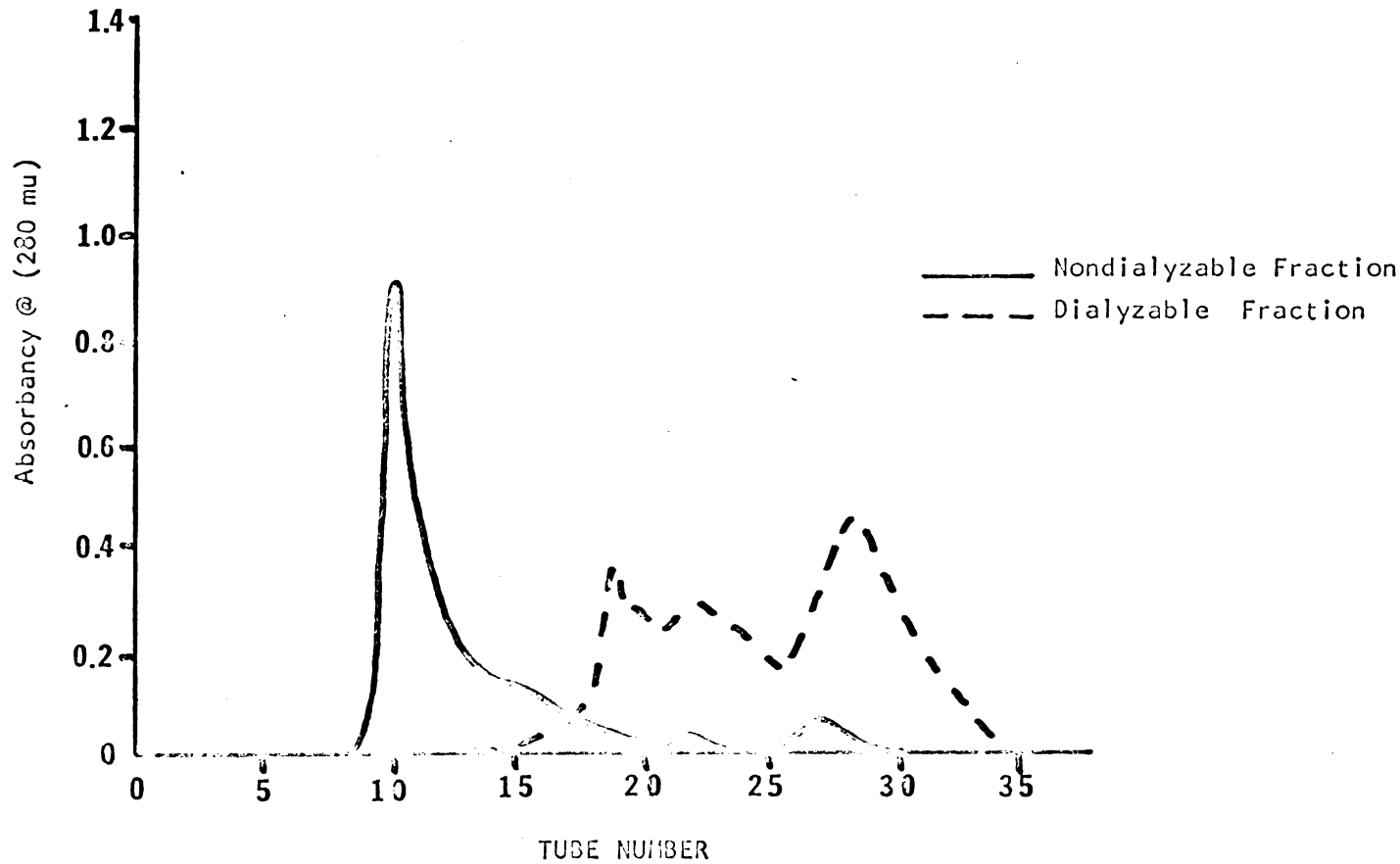


Fig. 23. Molecular exclusion chromatography of dialyzable and nondialyzable fractions from ultrasonic extract A (Table 2) in Sephadex G-25.

but considerable amounts of fractions II and III were detected. Small amounts of fractions II and III were detected in the nondialyzable component but it consisted primarily of fraction I.

A representative elution profile from ion exchange chromatography of USE-B on DEAE-cellulose is presented in Fig. 24. Seven protein-containing peaks were eluted from the adsorbent using a continuous concave gradient with a phosphate-NaCl buffer system.

Immunodiffusion Analyses of Ultrasonic Extracts

Between 1.8 and 2.0 mg of protein/ml was optimal for the development of immunoprecipitates using ultrasonic extracts and reference antisera in Ouchterlony immunodiffusion. One percent agar in phosphate buffered 0.15 M NaCl solutions pH 7.2 was a suitable medium for immunodiffusion analyses of mycobacterial antigens. Immunoprecipitate formation was not enhanced by incubation at 4 or 37°C, or by the use of polyvalent sheep anti-rabbit serum. Incubation for four days at 28°C with daily replenishments of antigen and antiserum diluted 1:3 produced the maximum number of immuno-precipitates in the reference system.

The number of precipitinogens detected by Ouchterlony immunodiffusion in the different ultrasonic extracts is listed in Table 18. Diagrammatic immunograms showing comparative analyses of ultrasonic extracts and chromatographic

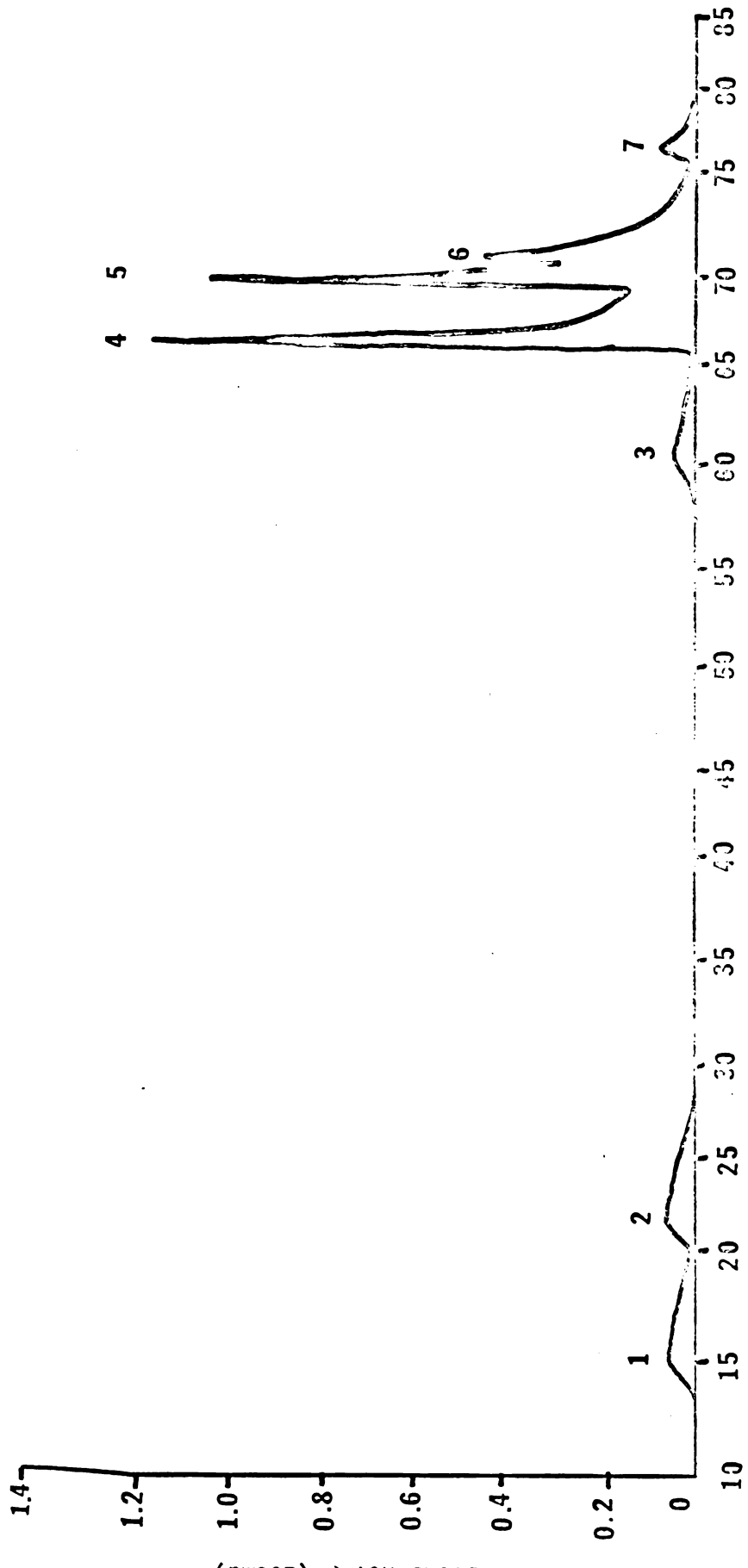


Fig. 24. Ion exchange chromatography of ultrasonic extract 3 (Table 2) in DEAE-cellulose.

Table 13. Number of mycobacterial components detected in ultrasonic extracts by disc electrophoresis, Ouchterlony immunodiffusion and immunoelectrophoresis.

Ultrasonic Extract	DE[1]		Number of Components Detected	
	AB[2]	PAS[3]	OI[4]	IE[5]
A	18	15	15	18
B	18	15	15	18
C	16	17	13	9+
D	24	18	16-17	20-22

[1]DE = Disc electrophoresis
[2]AB = Amido black-positive components
[3]PAS = Periodic acid Schiff-positive components
[4]OI = Ouchterlony immunodiffusion
[5]IE = Immunoelectrophoresis

fractions from Sephadex G-25 are presented in Figs. 25-28. The number of antigen-antibody systems detected in the ultrasonic extracts were as follows: 15 in USE-A and B (Figs. 25 and 26); 13 in USE-C (Fig. 27); and 16-17 in USE-D (Fig. 28).

Several of the immunoprecipitates were faint. Immunoprecipitates frequently occurred in clusters and individual lines could only be reliably distinguished as the different immunoprecipitates developed. No immunoprecipitation occurred when either normal rabbit serum or saline solution was substituted for antiserum. No antigens were detected in any of the unfractionated extracts which were absent in fraction I from chromatography in Sephadex G-25 although the same line in different preparations frequently varied in density and displacement. Immunoprecipitates were not detected in either chromatographic fractions II or III from ultrasonic extracts.

The number of immunoprecipitates detected in chromatographic fractions from ultrasonic extracts in common identity with the original extract is listed in Table 19. Fewer immunoprecipitates were detected in fraction I from different BioGels as the molecular exclusion limit of the chromatography medium increased.

Unheated filtrate from two-month-old cultures of Mycobacterium bovis and USE-D were antigenically undistinguishable using anti-USE serum (Fig. 29). However, using anti-filtrate serum prepared against antigens in filtrate

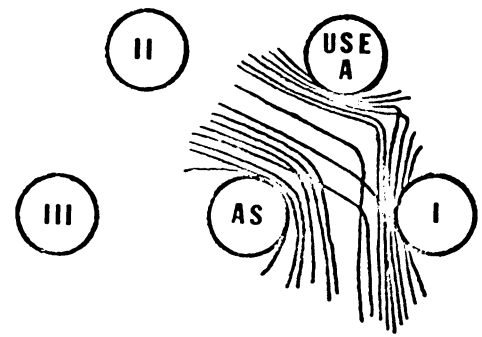


Fig. 25. Schematic immunogram of ultrasonic extract A (USE-A) (Table 2) and chromatographic fractions I (I), II (II), and III (III) from Sephadex G-25.

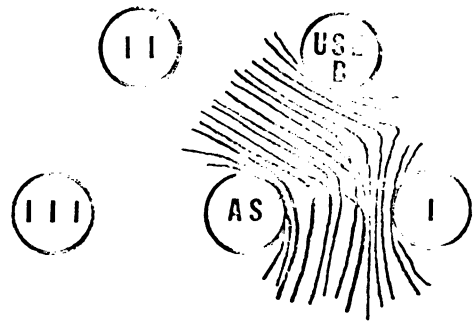


Fig. 26. Schematic immunogram of ultrasonic extract B(USE-B) (Table 2) and chromatographic fractions I (I), II (II), and III (III) from Sephadex G-25.

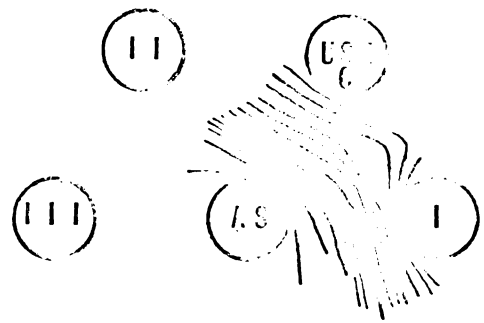


Fig. 27. Schematic immunogram of ultrasonic extract C (USE-C) (Table 2) and chromatographic fractions I (I), II (II), and III (III) from Sephadex G-25.

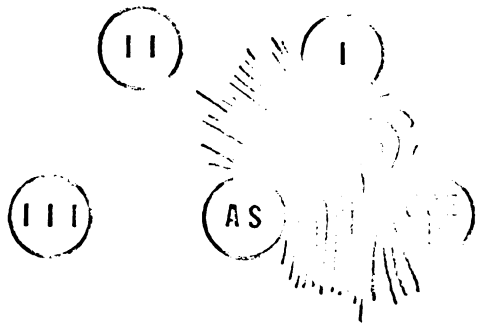


Fig. 23. Schematic immunogram of ultrasonic extract D (USE-D) (Table 2) and chromatographic fractions I (I), II (II), and III (III) from Sephadex G-25.

Table 19. Number of mycobacterial components detected in chromatographic fractions of ultrasonic extracts C and D by Ouchterlony immunodiffusion analysis.

Ultrasonic Extract	Number of Components Detected			
	FI_{G-25}[1]	FI_{P-100}[2]	FI_{P-150}[3]	FI_{P-200}[4]
C	13	10	8-9	5-6
D	16-17	12-14	11-12	9-11

[1] FI_{G-25} - Chromatographic fraction I eluted from Sephadex G-25

[2] FI_{P-100} - Chromatographic fraction I eluted from Bio Gel P-100

[3] FI_{P-150} - Chromatographic fraction I eluted from Bio Gel P-150

[4] FI_{P-200} - Chromatographic fraction I eluted from Bio Gel P-200

Fig. 29. Schematic immunograms of ultrasonic extract D (USE-D) (Table 2) and culture filtrates from two and one-half (CF_2) and six-month-old (CF_6) cultures of *Mycobacterium bovis*.

from six-month-old cultures, only ten antigens were detected in USE-D (Fig. 29).

Eleven immunoprecipitates were detected in immunograms of ethanol or acetone precipitates of USE-C (Fig. 30). These preparations were antigenically indistinguishable. Three immunoprecipitates were detected in immunograms of an antigen preparation obtained by TCA precipitation of USE-C (Fig. 31).

Electrophoretic Analyses of Ultrasonic Extracts

Optimal protein concentration for disc electrophoresis of ultrasonic extracts was between 250 and 300 μg in a sample volume of 0.1-0.2 ml. Schematic disc electrophorograms of different extract preparations are presented in Figs. 32-35. The number of protein-containing components and carbohydrate-containing components in the different ultrasonic extracts are listed in Table 18.

Disc electrophorograms of USE-A and B both contained 18 amido black-positive components (Figs. 32 and 33). They differed in only one component having slightly different Rf values. Sixteen and 24 protein components were detected in disc electrophorograms of USE-C and D respectively (Figs. 33 and 34). Sixteen components were common in the four ultrasonic extracts. A nonstained, yellow component in ultrasonic extracts migrated with or preceded the most anodic component. No protein bands were detected in the spacer gel. Rf values for amido-black positive components in disc electrophorograms of ultrasonic extracts are listed in Table 20.

Fig. 30. Comparative antigenic analyses of ultrasonic extract C (USE-C) (Table 2) and acetone (AP) and ethanol precipitates (EP) of ultrasonic extract C.

Fig. 31. Comparative antigenic analysis on ultrasonic extract C (USE-C) (Table 2) and trichloroacetic acid precipitate (TCA-P) obtained from ultrasonic extract C.

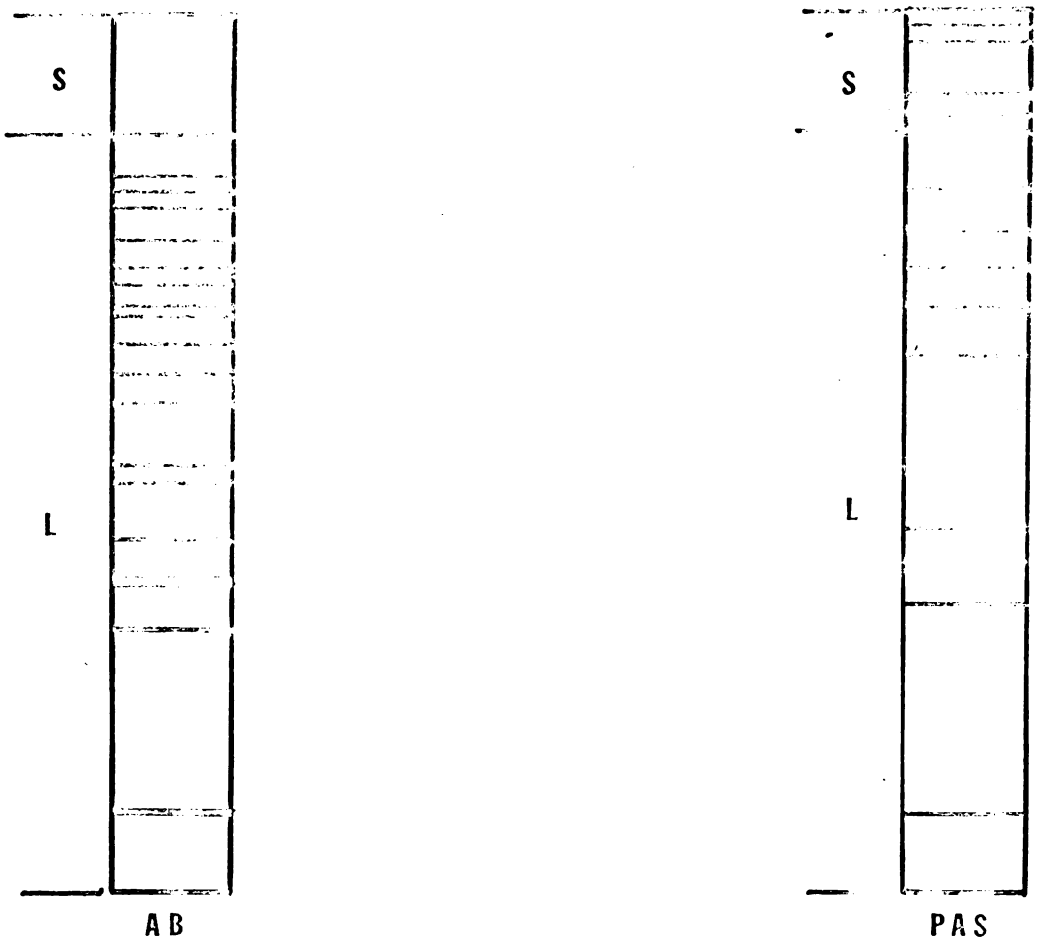


Fig. 32. Schematic disc electrophorograms of ultrasonic extract A (Table 2)
AB = Amido black stain for protein; PAS = Periodic acid Schiff stain for carbohydrate.

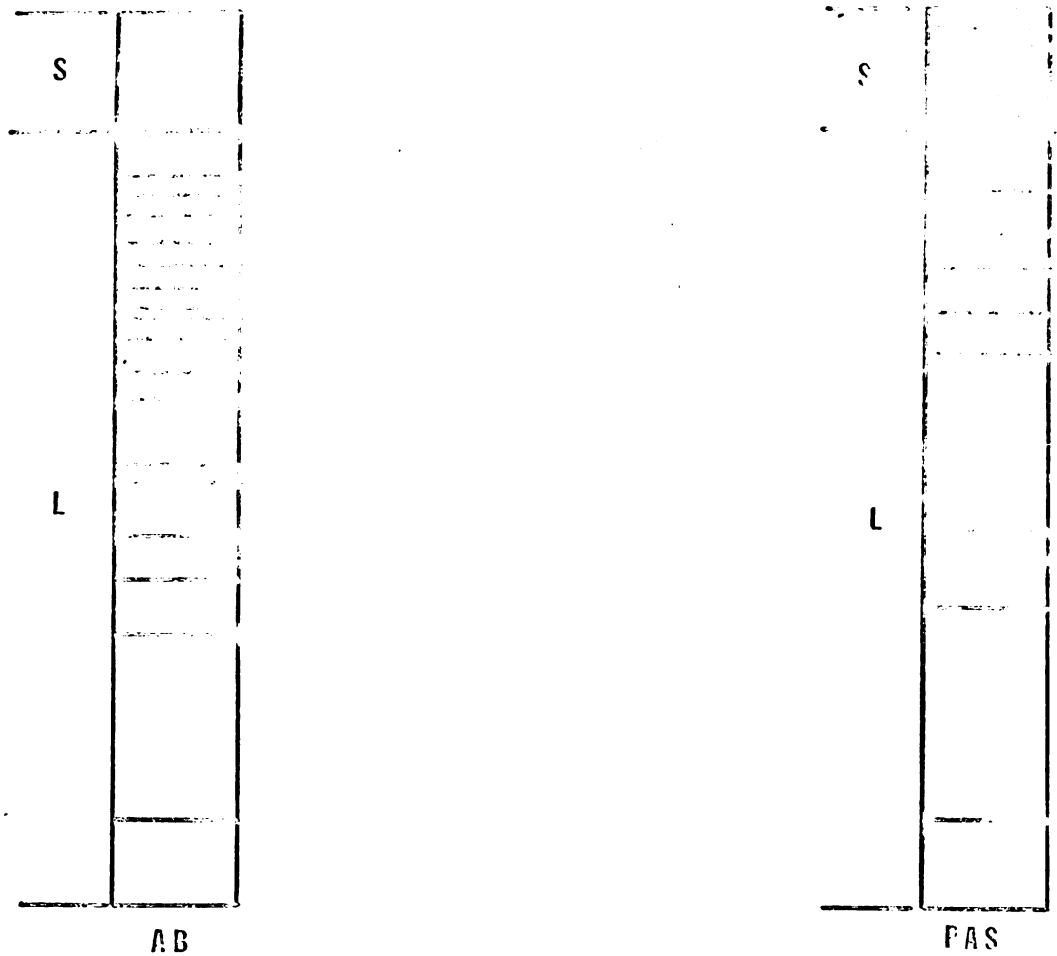


Fig. 33. Schematic disc electrophorogram of ultrasonic extract B (Table 2).
AB = Amido black stain for protein
PAS = Periodic acid Schiff stain for carbohydrate

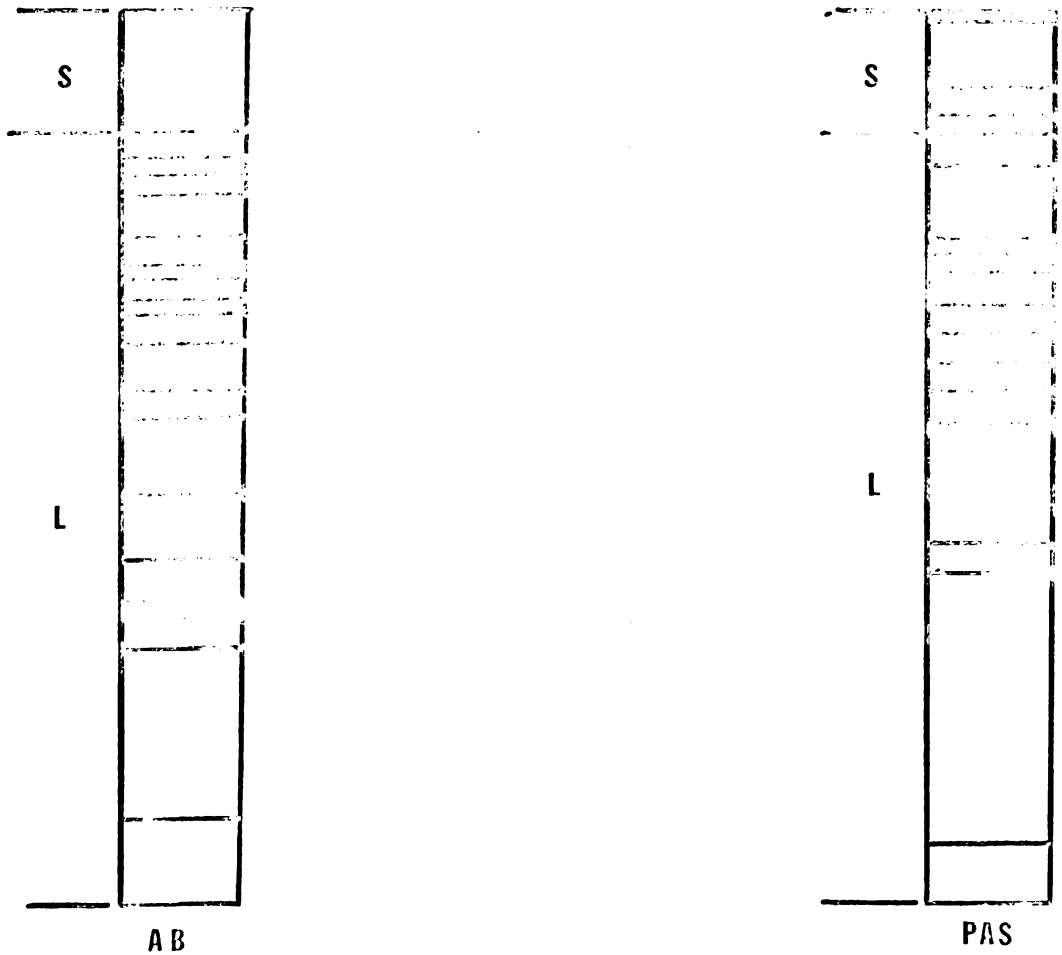


Fig. 34. Schematic disc electrophorogram of ultrasonic extract C (Table 2).
AB = Amido black stain for protein
PAS = Periodic acid Schiff stain for carbohydrate

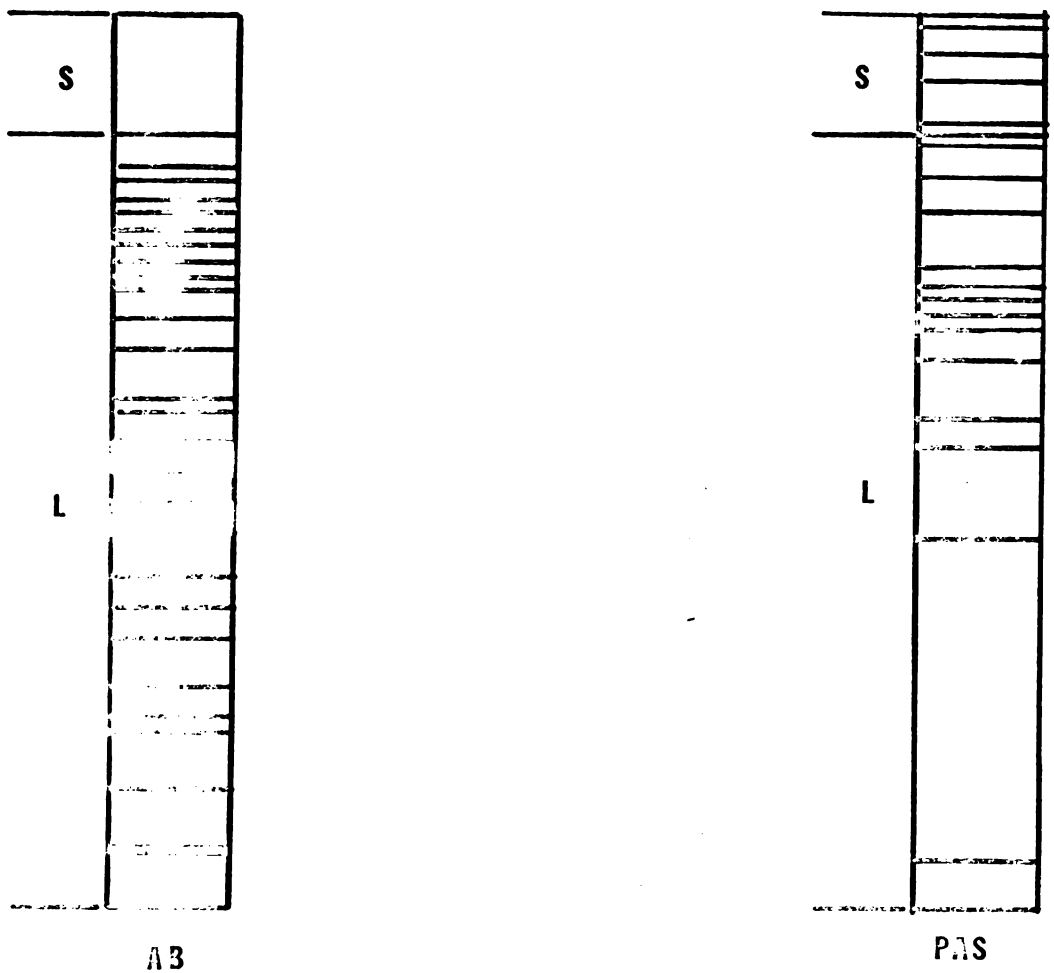


Fig. 35. Schematic disc electrophorogram of ultrasonic extract D (Table 2).
AB = Amido black stain for protein
PAS = Periodic acid Schiff stain for carbohydrate

Table 20. Distribution of amido black-positive components in disc electrophorograms of ultrasonic extracts obtained from viable cells of *Mycobacterium bovis*.

	Ultrasonic Extract			
	A	B	C	D
0.16	×	×	×	×
0.19	×	×	×	×
0.21	×	×		×
0.23	×	×		×
0.25		×		×
0.27	×		×	×
0.30				×
0.32	×	×	×	×
0.34	×	×	×	×
0.36	×	×	×	×
0.38	×	×		
0.41	×	×	×	×
0.46	×	×	×	×
0.48	×	×	×	×
0.52-0.55	×	×	×	×
0.59-0.63	×	×	×	×
0.66	×	×		×
0.71			×	×
0.73			×	×
0.75	×	×	×	×
0.80				×
0.84				×
0.88				×
0.93				×
1.0	×	×	×	×

Carbohydrate or glycoprotein components were detected in all of the ultrasonic extracts. Some PAS-positive components were very faintly stained. Many of the PAS-positive components were correlated with amido black-positive components. Both the spacer gel and the lower gel contained PAS-positive components from ultrasonic extracts. Schematic disc electrophorograms stained with PAS-strain are presented in Figs. 32-35. The number of PAS-positive components and their Rf values in the different ultrasonic extracts is given in Table 21. PAS-positive components were detected in disc electrophorograms of USE-A and B. Seventeen and eighteen components were detected in USE-C and D respectively.

Disc electrophorograms of unfractionated ultrasonic extracts and fraction I from chromatography in Sephadex G-25 were identical although the density of individual lines in the different preparations sometimes differed. Fraction II from chromatography in Sephadex G-25 contained no amido black-positive components. Two very faintly stained PAS-positive components may have been present in very low concentration in several preparations of peak II. What may have been the PAS-positive bands were located at the top and bottom of the lower gel. A non-stained, yellow component frequently observed in unfractionated ultrasonic extracts was also detected near the anodic end of the lower gel.

Disc electrophorograms frequently contained sharply defined opalescent lines which did not stain with either carbohydrate of protein stains.

Table 21. Distribution of PAS-positive components in disc electrophorograms of ultrasonic extracts obtained from viable cells of Mycobacterium bovis.

Rf	Ultrasonic Extract			
	A	B	C	D
0	×	×	×	×
0.04	×	×	×	×
0.05	×	×		×
0.09	×	×	×	×
0.11	×	×	×	×
0.16	×	×	×	×
0.21			×	×
0.23	×	×		×
0.27	×	×	×	
0.30			×	×
0.32	×	×	×	×
0.34			×	×
0.36	×	×		×
0.38			×	×
0.41	×	×	×	×
0.45			×	
0.48			×	×
0.52				×
0.56	×	×	×	×
0.67	×	×	×	
0.73	×	×		
1.0	×	×	×	×

Zone electrophoresis of ultrasonic extracts in cellulose acetate membranes separated four-five components (Fig. 36). Five protein-stained components were detected in USE-D, four each were detected in USE-A, B and C. The density and mobility of the same line in different extracts varied somewhat.

Immunoelectrophoretic Analyses of Ultrasonic Extracts

Immunoelectrophorograms of USE-A and B were very similar; eighteen immunoprecipitates were detected (Table 18). Differences were noted in the density and displacement of the same immunoprecipitates in the two extracts (Figs. 37 and 38). Nine immunoprecipitates were detected in immunoelectrophorograms of USE-C (Fig. 39). Between 20 and 22 immunoprecipitates were observed in immunoelectrophorograms of USE-D (Fig. 40). Some immunoprecipitates were very lightly stained. No differences were detected in immunoelectrophorograms of unfractionated ultrasonic extracts and fraction I from chromatography in Sephadex G-25. Immunoprecipitates did not stain using carbohydrate-specific or lipid-specific stains.

Immunoprecipitates were formed on both sides of the sample basin in the axis of migration although more precipitin arcs were detected in the anodic region of the slide. A diffuse immunoprecipitate was frequently observed approximately one cm anodically and at the same level as the sample basin. Individual immunoprecipitates were difficult to distinguish in this area. The use of sheep-anti-rabbit antiserum

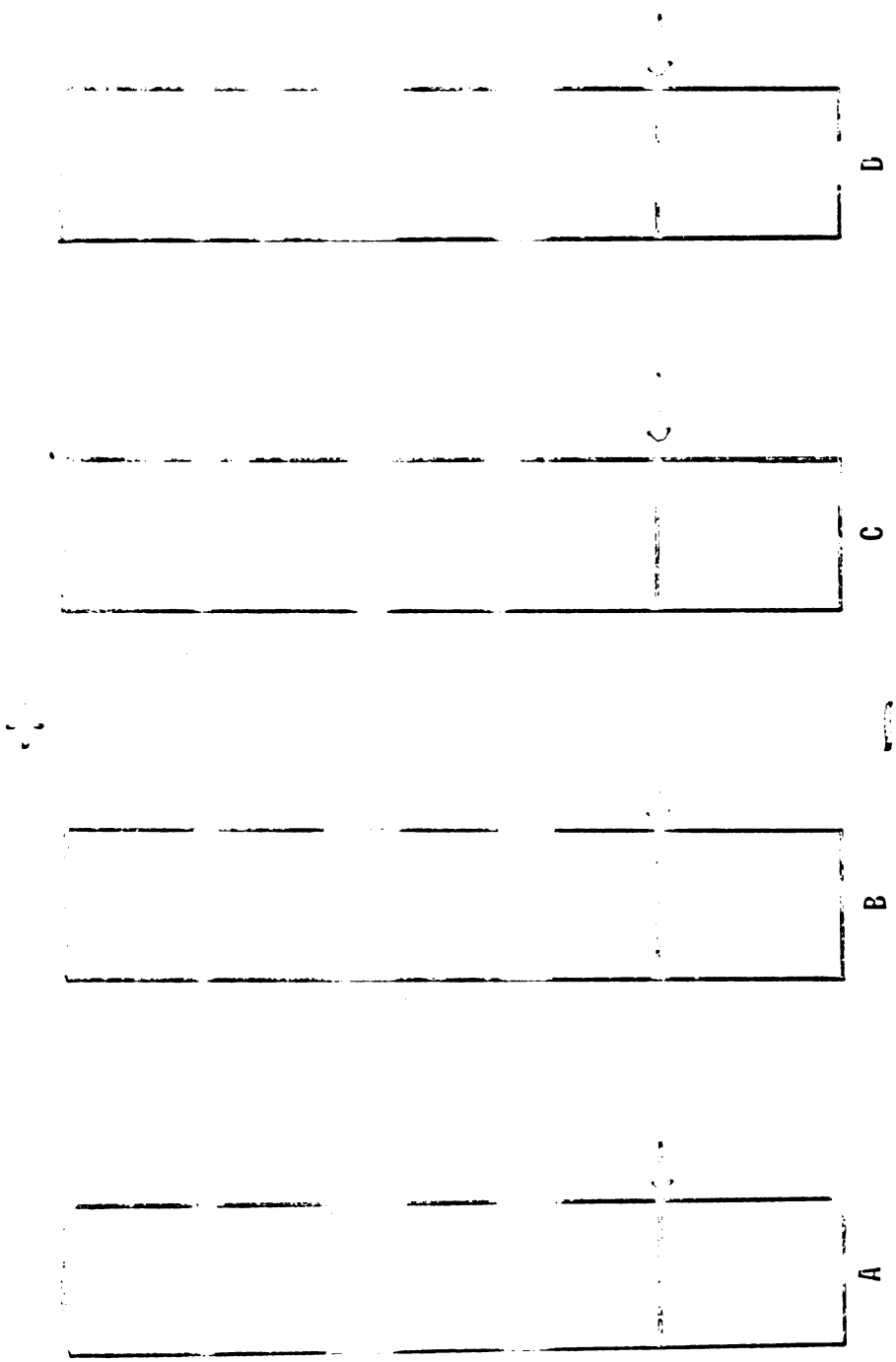


Fig. 36. Cellulose acetate electrophorograms of ultrasonic extracts A, B, C, and D (Table 2).

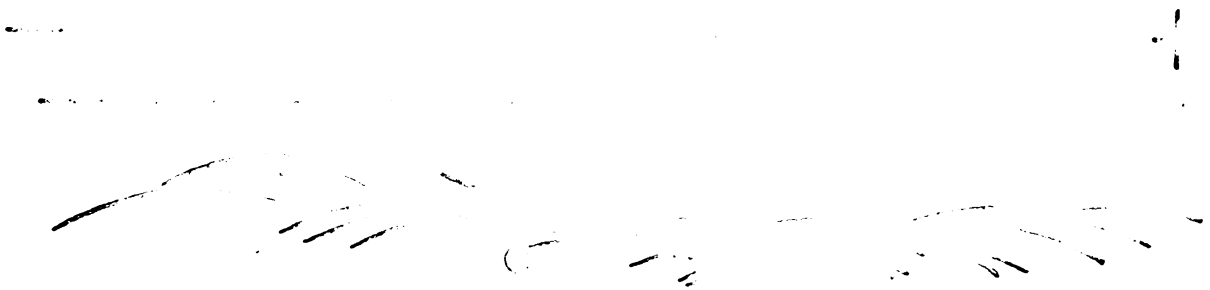


Fig. 37. Diagrammatic immunoelectrophorogram of ultrasonic extract A (Table 2).

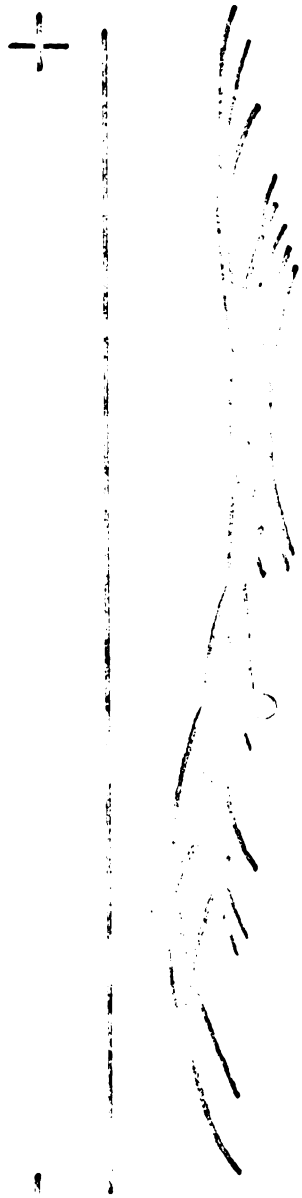


Fig. 33. Diagrematic immunoelectrophorogram of ultrasonic extract B (Table 2).

Fig. 39. Diagramatic immunoelectrophorogram of ultrasonic extract C (Table 2).

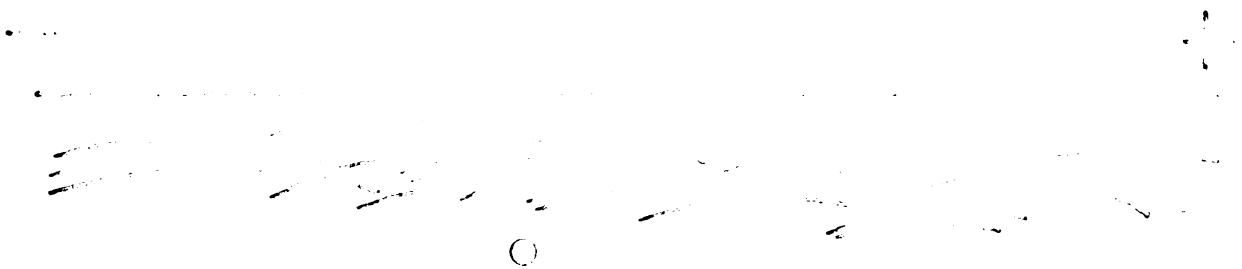


Fig. 40. Diagramatic immunoelectrophorogram of ultrasonic extract D (Table 2).

following development of the slides did not increase the number of detectable immunoprecipitates or increase the clarity of those which were originally present. Immunoprecipitation did not occur when normal rabbit serum was used in place of anti-ultrasonic extract antiserum.

Immunodiffusion, Electrophoretic and Immunoelectrophoretic Analyses of Chemical Bacillary Extracts

The number of components detected in chemical bacillary extracts by immunodiffusion, disc electrophoresis and immunoelectrophoresis is listed in Table 22. Schematic immunograms of the different chemical bacillary extracts with anti-ultrasonic extract antisera are presented in Figs. 41-45. The number of immunoprecipitates detected were: 11 in desoxycholate extract (SDE) (Fig. 43); 11 in Triton extract (TE) (Fig. 41); 5-7 in guanidine extract (GE) and urea extract (UE) (Figs. 44 and 45); and three in ether extract (EE) (Fig. 42). Immunograms of TE and UE from heat-killed cells contained three-four and two components respectively (Fig. 46).

Schematic immunoelectrophorograms of the different chemical bacillary extracts are presented in Figures 47-50. The number of components detected using anti-ultrasonic extract serum was as follows: 11 in SDE (Fig. 48); 15 in TE (Fig. 47); three^{+} in GE (Fig. 50); and at least eight in UE (Fig. 49). The protein concentration in EE was insufficient for immunoelectrophoretic analyses.

Table 22. Number of mycobacterial components detected in chemical bacillary extracts by disc electrophoresis, Ouchterlony immunodiffusion and immunoelectrophoresis.

	DE[1]			
Chemical Extract	AB[2]	PAS[3]	OI[4]	IE[5]
SDE[6]	13	6	11	11
TE[7]	6	4	11	15
UE[8]	4	3	5	9
GE[9]	6	3	5-7	34

[1] DE = Disc electrophoresis
[2] AB = Amido black-positive components
[3] PAS = Periodic acid Schiff-positive components
[4] OI = Ouchterlony immunodiffusion
[5] IE = Immunoelectrophoresis
[6] SDE = Sodium desoxycholate extract
[7] TE = Triton extract
[8] UE = Urea extract
[9] GE = Guanidine extract

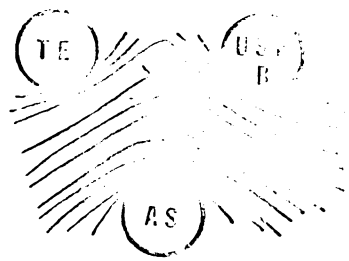


Fig. 41. Comparative antigenic analysis of triton extract (TE) and ultrasonic extract B (USE-B) (Table 2).

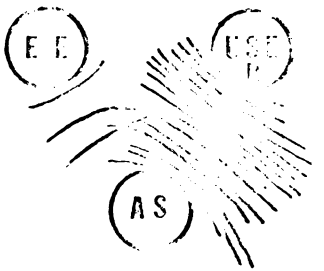


Fig. 42. Comparative antigenic analysis of ether extract (EE) and ultrasonic extract B (USE-B) (Table 2).

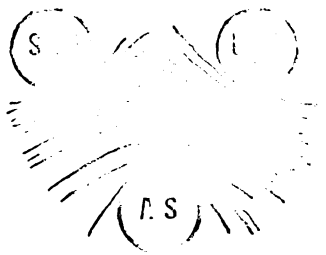


Fig. 43. Comparative antigenic analysis of sodium desoxycholate extract (SDE) and ultrasonic extract B (USE-B) (Table 2).

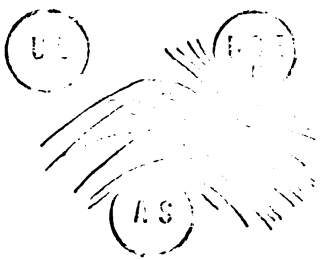


Fig. 44. Comparative antigenic analysis of urea extract (UE) and ultrasonic extract B (USE-B) (Table 2).

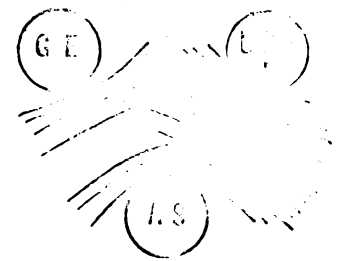


Fig. 45. Comparative antigenic analysis of guanidine extract (GE) and ultrasonic extract B (USE-B) (Table 2).

Fig. 46. Comparative antigenic analyses of urea (UE) and triton extract (TE) obtained from viable(v) and killed (h) cells of *Mycobacterium bovis*.

Fig. 47. Diagrammatic immunoelectrophorogram of triton extract of viable cells of *Mycobacterium bovis*.

Fig. 4. Diagrammatic immunoelectrophorogram of sodium desoxycholate extract of viable cells of *Mycobacterium bovis*.

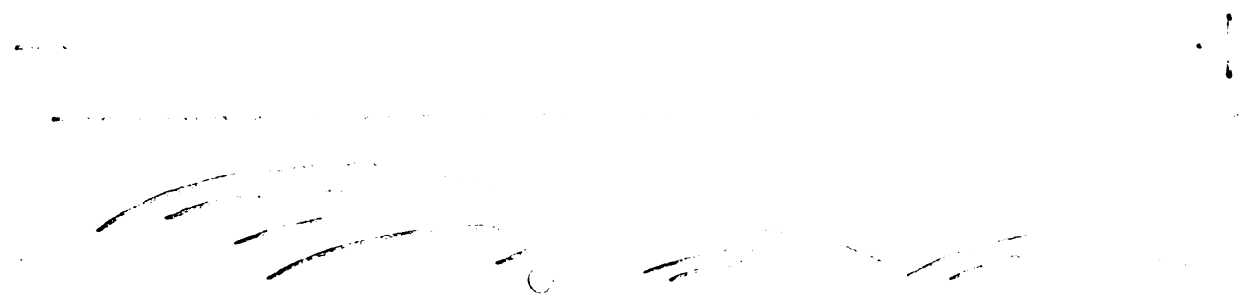


Fig. 49. Digramatic immunoelectrophorogram of urea extract of viable cells of *Mycobacterium bovis*.

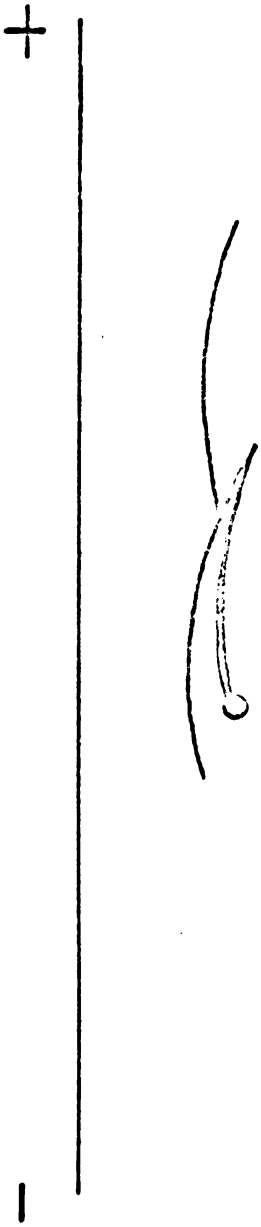


Fig. 50. Diagramatic immunoelectrophorogram of guanidine extract of viable cells of Mycobacterium bovis.

Schematic disc electrophorograms of the different chemical bacillary extracts are presented in Figs. 51-54. The number of detectable amido black-positive and PAS-positive components in chemical bacillary extracts were six amido-black and four PAS-positive components in TE (Fig. 51); 13 amido black and six PAS-positive components in SDE (Fig. 52); four amido black and three PAS-positive components in UE (Fig. 53); and six amido black and three PAS-positive components in GE (Fig. 54). Rf values for the disc components detected in chemical extracts are listed in Tables 23 and 24.

Zone electrophoresis in cellulose acetate membrane separated each of the chemical bacillary extracts into at least three protein-stained zones (Fig. 55). The relative amounts in each of these fractions differed among different extracts.

Schematic immunograms showing comparative analyses of chemical bacillary extracts are presented in Figs. 56-59, common antigens were detected among many of the chemical extracts. Comparative immunodiffusion analyses of chemical bacillary extracts and filtrate from six-month-old cultures demonstrated the presence of common antigens: six in SDE (Fig. 60) and seven in TE (Fig. 60).

Skin Testing with Ultrasonic and Chemical Bacillary Extracts

Unfractionated ultrasonic extracts and chromatographic fraction I G-25 elicited dermal reactions in normal rabbits

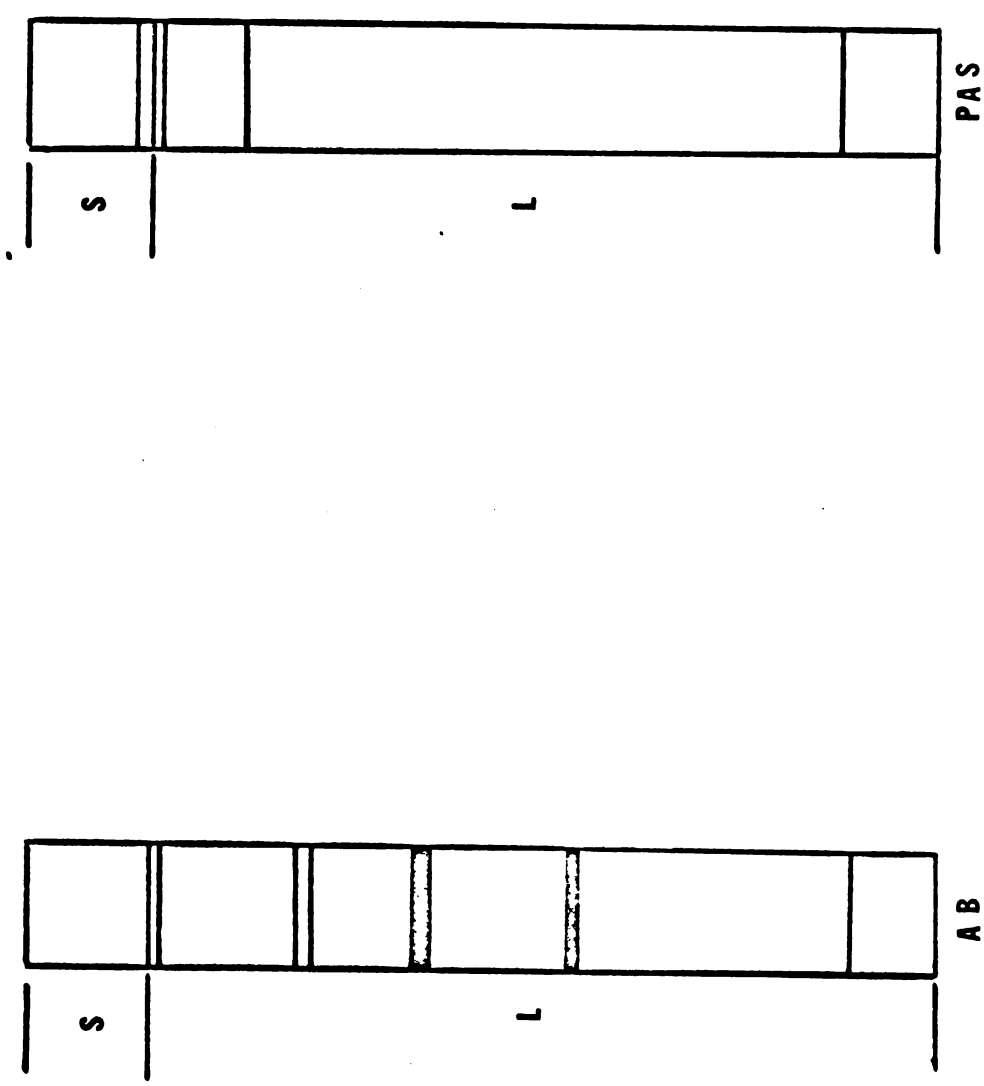


Fig. 51. Diagramatic disc electrophorogram of triton extract of viable cells of Mycobacterium bovis.

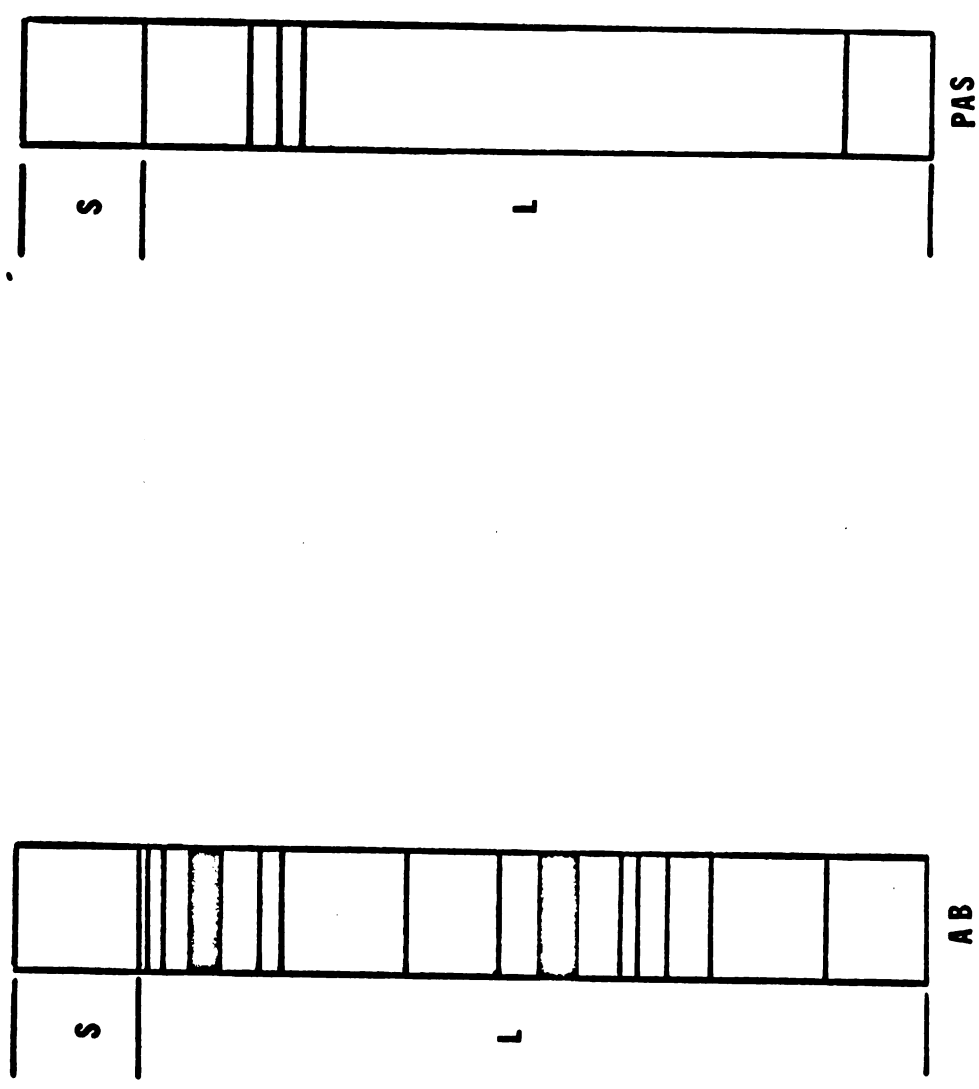


Fig. 52. Diagramatic disc electrophorogram of sodium desoxycholate extract of viable cells of Mycobacterium bovis.

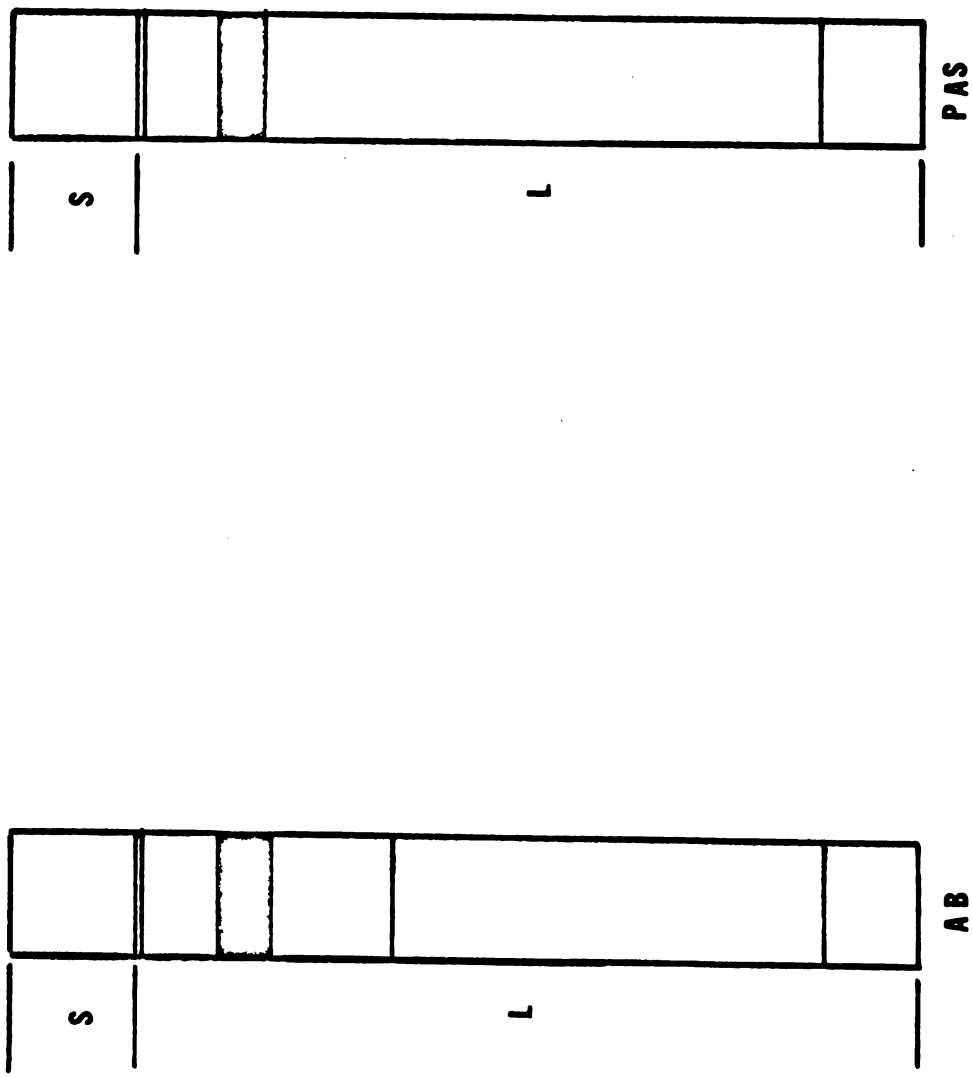


Fig. 53. Diagramatic disc electrophorogram of urea extract of viable cells of Mycobacterium bovis.

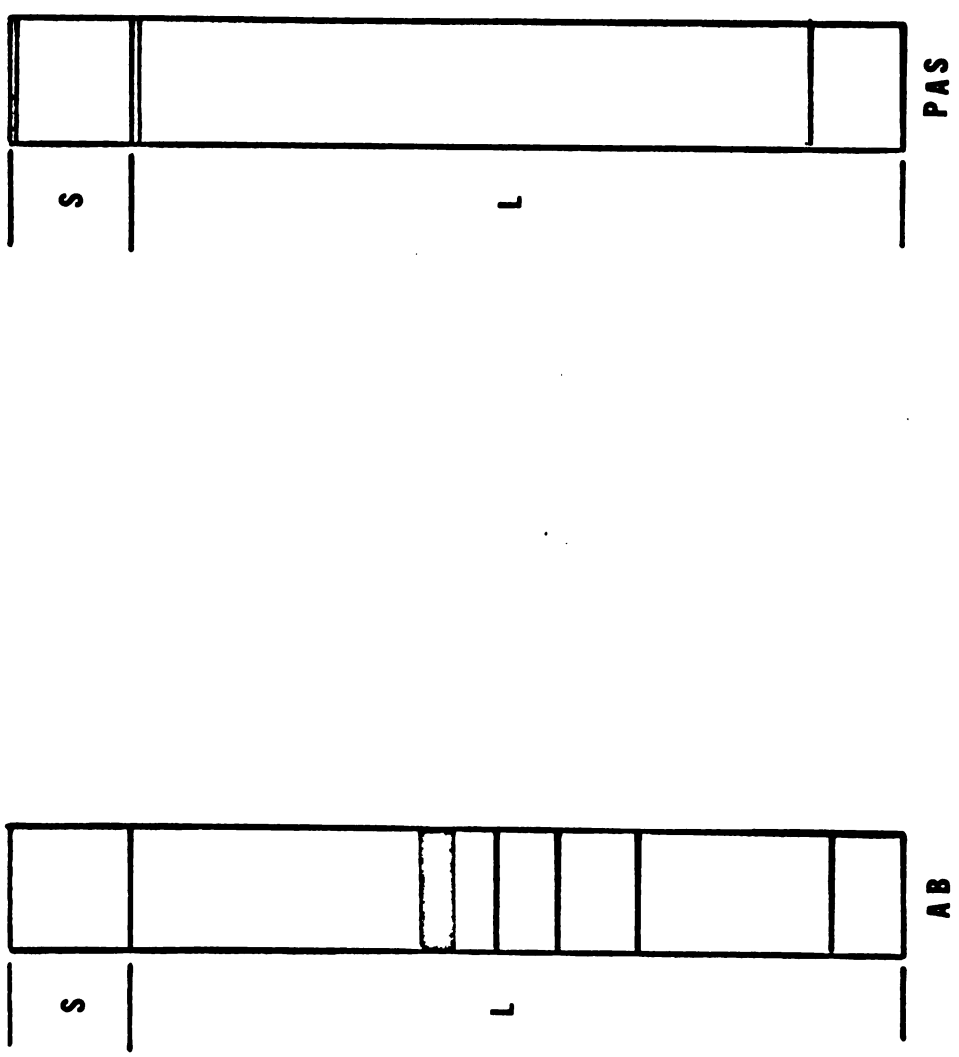


Fig. 54. Digramatic disc electrophorogram of guanidine extract of viable cells of Mycobacterium bovis.

Table 23. Distribution of amido black-positive components in disc electrophorograms of chemical bacillary extracts obtained from viable cells of Mycobacterium bovis.

	Chemical Bacillary Extract			
Rf	TE[1]	SDE[2]	UE[3]	GE[4]
0.15	x	x		x
0.17		x	x	
0.21		x		
0.31	x	x		
0.33	x	x	x	x
0.48	x	x	x	
0.60		x		x
0.65	x	x		x
0.75		x		x
0.77		x		
0.81		x		
0.87		x		
1.0	x	x	x	x

[1] TE = Triton extract

[2] SDE = Sodium desoxycholate extract

[3] UE = Urea extract

[4] GE = Guanidine extract

Table 24. Distribution of PAS-positive components in disc electrophorograms of chemical bacillary extracts obtained from viable cells of Mycobacterium bovis.

	Chemical Bacillary Extract			
Rf	TE[1]	SDE[2]	UE[3]	GE[4]
0		x		x
0.15	x			
0.17	x	x	x	x
0.28	x	x		
0.31		x		
0.33		x	x	
1.0	x	x	x	x

[1] TE = Triton extract

[2] SDE = Sodium desoxycholate extract

[3] UE = Urea extract

[4] GE = Guanidine extract

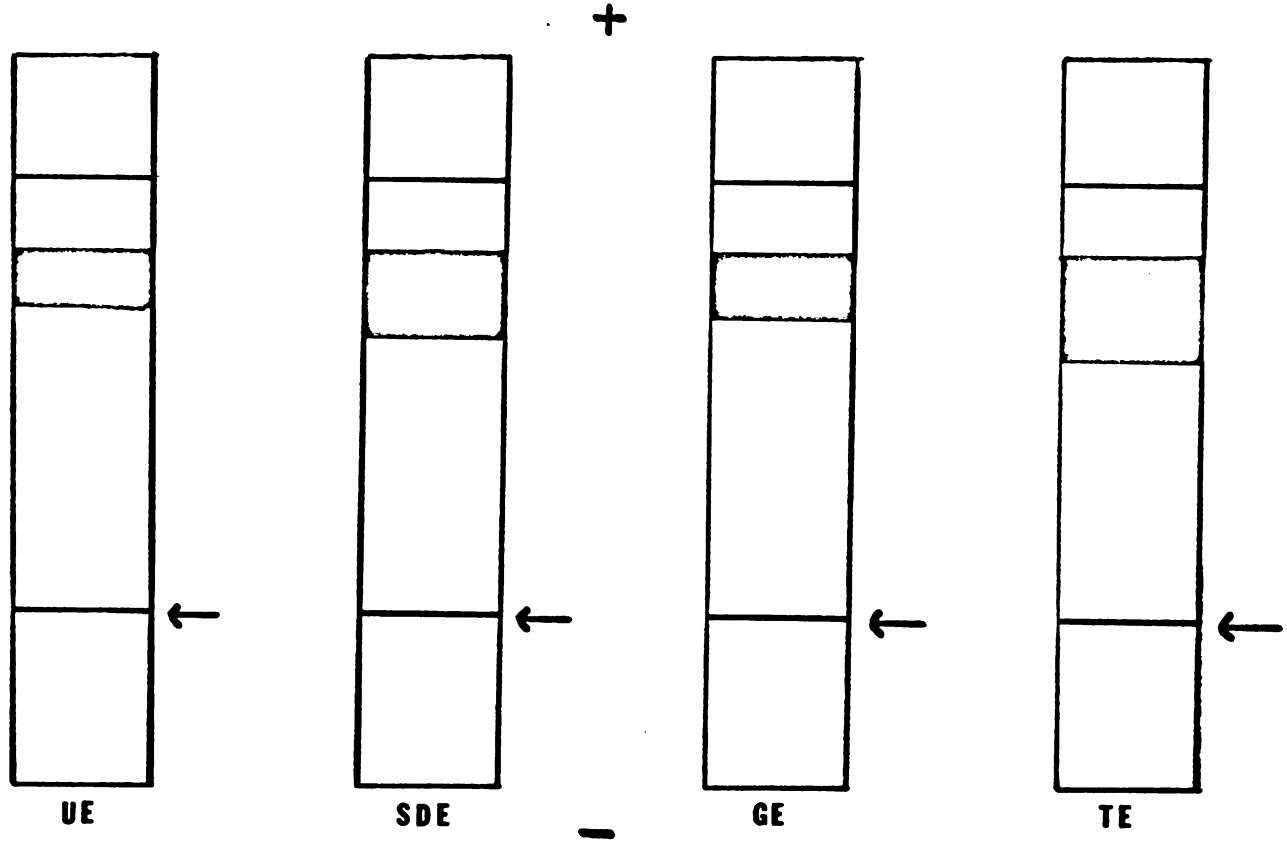
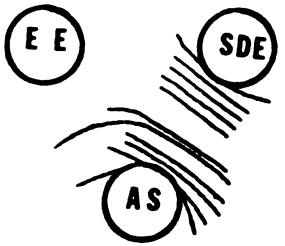


Fig. 55. Cellulose acetate electrophorograms of chemical bacillary extracts of viable cells of Mycobacterium bovis. UE = urea extract; SDE = sodium desoxycholate extract; GE = guanidine extract; TE = triton extract.

Fig. 56. Comparative antigenic analyses of chemical bacillary extracts.
TE = Triton extract; SDE = Sodium desoxycholate extract;
UE = Urea extract; AS = antiserum.

Fig. 57. Comparative antigenic analyses of chemical bacillary extracts.
EE = Ether extract; GE = Guanidine extract; TE = Triton extract; AS = Antiserum.



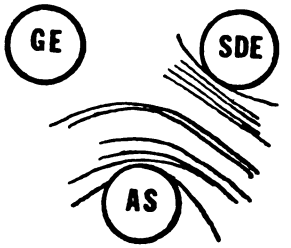
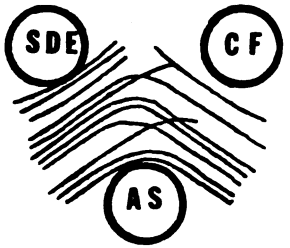


Fig. 58. Comparative antigenic analyses of chemical bacillary extracts.
SDE = Sodium desoxycholate extract; GE = Guanidine extract; EE = Ether extract;
AS = Antiserum.

Fig. 59. Comparative antigenic analysis of chemical bacillary extracts. SDE = Sodium desoxycholate extract; UE = Urea extract; TE = Triton extract; EE = Ether extract; AS = Antiserum.



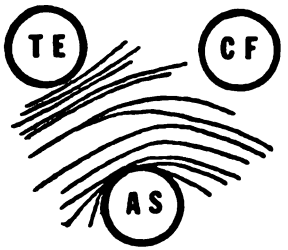


Fig. 60. Comparative antigenic analyses of triton (TE and sodium desoxycholate extracts (SDE) and filtrate obtained from six-month-old cultures of Mycobacterium bovis (CF).

detectable three hours post-inoculation. The erythematous reactions usually subsided within 24-48 hours. Unfractionated ultrasonic extracts and chromatographic fractions I and II G-25 elicited immediate reactions in rabbits sensitized with heat-killed M. bovis, strain 310. Slight reactions were detected with chromatographic fraction III in sensitized but not in normal rabbits. Reactions elicited in sensitized rabbits were greater and persisted longer than in normal rabbits.

Slight reactions were detected in normal rabbits skin tested with the chemical bacillary extracts. All of the chemical bacillary extracts elicited immediate reactions in sensitized rabbits which were greater than in normal rabbits.

DISCUSSION

Antibody production was studied in experimental models analogous to (a) active tuberculosis, (b) limited (closed) tuberculosis with tuberculoimmunity and tuberculin sensitivity, (c) no tuberculosis with tuberculoimmunity and tuberculin sensitivity, and (d) no tuberculosis with tuberculoimmunity and reduced sensitivity. Mycobacterium bovis of different strains or prepared by different methods were injected into rabbits to simulate these conditions as follows:

(a) active tuberculosis: Group I; viable, virulent M. bovis (strain 310)

(b) limited tuberculosis with tuberculoimmunity and tuberculin sensitivity: Group II--attenuated M. bovis, BCG

(c) no tuberculosis with tuberculoimmunity and tuberculin sensitivity:
Group III heat inactivated M. bovis (strain 310)
Group IV-BPL inactivated M. bovis (strain 310)
Group VI-acetone inactivated M. bovis (strain 310)

(d) no tuberculosis with tuberculoimmunity and reduced sensitivity: Group V-betaprone inactivated M. bovis (strain 10) treated repeatedly with acetone and methanol

Antibody production in infected individuals is generally considered to be of little diagnostic or prognostic significance in tuberculosis or tuberculoimmunity. The role of delayed hypersensitivity in either is not known.

Virulent M. bovis (strain 310) infects and produces tuberculosis in rabbits. Therefore, a small inoculum

administered subcutaneously was used to avoid a rapidly progressive, fulminating and fatal disease in rabbits of Group I. It was hoped that the antibody response would vary during the prolonged period of disease. Antibody production by these rabbits was negligible. Antibody was detected in very low amounts and was irregular in occurrence. The irregular occurrence of antibody in diseased individuals is a general characteristic of tuberculosis (159).

Sufficient experimental precedent has established the use ME-sensitivity to differentiate IgM from IgG. Mycobacterial cells elicited first IgM and then IgG in the experimental rabbits. This general pattern existed in all the groups tested, although variations occurred in the temporal and quantitative relationships of hemagglutinins and bacterial agglutinins. Antibody produced during the first two weeks was exclusively IgM. Thereafter IgG was produced in increasing amounts. The IgM persisted throughout the duration of the experimental period (8-23 weeks) in all groups tested except Group I. This is consistent with other reports of prolonged IgM synthesis in rabbits immunized with inactivated mycobacteria. This is undoubtedly associated with the relatively slow degradation of mycobacteria in the host thus providing a prolonged exposure to the antigen. This is in contrast to the reaction elicited by a more rapidly metabolized antigen in that the IgM is produced for a relatively short period of time.

The antibody response to infection with BCG (Group II) was comparable to that induced by any of the killed cell preparations. Mercaptoethanol-sensitive hemagglutinin titers were significantly higher at the first and second week post-inoculation in sera from rabbits in Groups II, III and IV than in sera from rabbits in Groups V and VI. Thereafter, rabbits in Groups II, IV, and VI produced significantly more ME-resistant hemagglutinins than rabbits in Groups IV and V. Bacterial agglutinins were produced by rabbits in Group II in significantly greater amounts than by rabbits which received inactivated cells. Because there was multiplication of BCG in the rabbits in Group II, the actual number of cells to which the rabbits were exposed is not known. It would probably be less than the number of virulent M. bovis cells (Group I) and more than the number of killed cells (Groups III-VI). The significantly better antibody response to BCG than to inactivated cells may be due to differences in antigen dose or to the differences in processing antigens in viable, multiplying cells and killed cells. It is interesting that sera from rabbits in Group II, inoculated with BCG, consistently yielded higher titers of bacterial agglutinins using BPL-killed cells than sera from rabbits in Groups IV and V which were inoculated with BPL-killed cells. This indicates the close antigenic relatedness of somatic surface antigens between M. bovis (310) and BCG.

A more important point can be suggested from the relatedness of BCG and BPL-killed cells. Betapropriolactone

effectively inactivates M. bovis and BPL-killed cells have been shown to elicit tuberculoimmunity in mice and guinea pigs and tuberculin sensitivity in the latter. Betaproprio-lactone reportedly does not denature proteins and therefore provides a means of inactivation without loss of antigenicity. The reaction of the BPL-killed cells with the sera from rabbits inoculated with BCG may indicate promise of the use of inactivated cells in agglutination tests.

Rabbits in Group IV and V which received BPL-killed cells produced significantly less ME-resistant antibody than rabbits in the other groups. Moreover, rabbits in Group V failed to produde detectable hemagglutinins during the first week post-inoculation. These results contraindicate the use of BPL-killed mycobacteria as antigens for antibody production in rabbits.

Results from recent investigations have indicated that simultaneous rather than sequential production of IgM and IgG may occur with different antigens, and that the two antibody responses are independent of each other (210). An explanation of the earlier detection of IgM antibody is thought to be the greater sensitivity of agglutination reactions for the detection of IgM than for IgG (16). Our results of antibody titrations using agglutination reactions should be interpreted with the understanding that IgM is more efficient in agglutination on a molar basis than IgG and that both immunoglobulin species may be produced simultaneously. To conclusively determine the temporal sequence

of the IgM and IgG, a serologic test is needed which is equally sensitive for the detection of both.

Hemagglutination is more sensitive than bacterial agglutination for antibody determinations. Hemagglutinin titers were consistently higher than agglutinin titers in the same sera with few exceptions. However, the temporal pattern of synthesis of ME-sensitive and ME-resistant antibodies was similar. It is not known whether the two tests measured the same or different antigen-antibodies systems. If the two test measured the same antigen-antibody systems, hemagglutinins would be detected earlier and in greater amounts. Hemagglutinins are reportedly specific for tuberculopolysaccharides. The nature of the agglutinogens are not known other than they are apparently on the surface of the cell. They may also be carbohydrate since the surface antigens of tubercle bacilli are reportedly carbohydrate (116).

Because M. bovis cords, that is, forms chains of cells which tightly adhere to other chains of cells, it is difficult to disperse them evenly to standardize inocula. Therefore, there were undoubtedly some differences in the antigen concentration given to each rabbit which could influence antibody responses. However, it is not believed that the slight differences in the cell concentrations would cause significant differences in the serum titers.

Due to widespread distribution of mycobacteria in the environment, it is possible that some degree of heterologous antigenic stimulation occurred prior to inoculations.

The rabbits were not skin tested prior to inoculation but were obtained from a colony in which the rabbits are and remain tuberculin negative. None of the serum collected prior to inoculation contained detectable antibodies.

Skin testing with PPD-S 14 weeks post-inoculation stimulated antibody production in approximately 50% of the rabbits tested. An anamestic-like response following skin testing has been reported to occur in cattle and swine (111). Mean hemagglutinin titers were elevated in Groups II and V but not in Group IV, following skin tests. Bacterial agglutinin titers were elevated only in the serum from rabbits in Group II. Corresponding elevations of the ratios of ME-sensitive/ME-resistant antibodies indicate that skin testing may have preferentially stimulated the production of Ig-M. Whether this is a specific, homologous anamestic response is not known. It is significant, however, that tuberculin testing apparently changed the absolute and relative amounts of Ig-M and Ig-G synthesized. Perhaps more significant changes in the absolute and relative amounts of ME-sensitive and Me-resistant antibody may have been detected if the rabbits were bled at various intervals after skin tests. It is probable that tuberculin testing alters other immunologic processes in still undetermined ways. For this reason, the effect of tuberculin testing should be carefully evaluated when conducting <u>in vivo</u>, <u>in vitro</u> or serologic tests.

Inoculation of rabbits with viable or killed M. bovis induced sensitivity to tuberculin. Rabbits injected with BCG developed greater tuberculin sensitivity than rabbits which were inoculated with BPL-killed M. bovis or BPL-killed methanol and acetone extracted M. bovis. The ability to induce delayed sensitivity was reduced considerably by the treatment by BPL-killed cells with methanol and acetone.

The kinetics of the antibody response to individual mycobacterial antigens cannot be adequately studied until purified antigens are obtained. It is significant that mycobacterial antigen preparations currently employed for serodiagnosis of tuberculosis are chemically and antigenically heterologous. It is only logical that more reliable results can more probably be obtained with a less heterogenous mixture. If antigens can be isolated there is a greater probability of obtaining one which is specific.

Disruption of viable cells by ultrasound is a safe, effective means of obtaining mycobacterial antigens. The relative amounts of protein, carbohydrate and nucleic acid in extracts differs with the age of the cultures from which the cells are obtained and the duration of insonation to which the cells are exposed.

After centrifugation and prior to filtration, ultrasonic extracts were opalescent and probably contained relatively large amounts of lipoprotein in the form of particulate micells. Membrane filtration clarified the

extracts and probably removed some of the soluble protein as well as lipoprotein material. The qualitative and quantitative loss of chemical and antigenic constituents of ultrasonic extracts due to filtration was not determined. It was considered to be the method of sterilization which would most probably reduce the constituents the least. Because of the nature of the organism some method of sterilization was necessary.

The amount of nucleic acid in ultrasonic extracts was indicative of the extent of cell disruption and was correlated with the intensity of insonation. Ultrasonic extract-C which received the longest period of sonication, contained substantially more nucleic acid than USE-A or B, all three extracts were obtained from cells from six-month-old cultures. When the duration of insonation was the same but of cells from two-month-old cultures (USE-D), more nucleic acid was present than in either the USE-A or B. Cells from younger cultures appear to be more fragile than those from older cultures and shorter periods of insonation should be used with the younger cells to achieve the same extent of cell disruption. This may be due to the fact that cells from older cultures are undergoing extensive autolysis and that a considerable percentage of cells are nonviable.

Another indication of the extent of cell disruption is found in the carbohydrate and protein concentrations in ultrasonic extracts. Again, USE-C which was irradiated for

the longer period, contained more than twice the amount of carbohydrate and significantly more protein than was present in other extracts of cells of the same age but which received a shorter period of insonation.

Ultrasound causes varying degrees of depolymerization of biomacromolecules. The distribution of nucleic acid and protein in the three major chromatographic fractions from Sephadex G-25 are illustrative. Molecular exclusion chromatography is an effective method of comparing the relative sizes of molecules. Major fraction I from Sephadex G-25 contains molecules whose average molecular weight is greater than 5000 since they were eluted within the external volume of the columns. Fraction II and III were eluted within the internal volume of the column and contained molecules whose average molecular weights are less than 5000. Nucleic acids as they occur in viable cells exist as high molecular weight polymerized biomacromolecules. Approximately 30% of the nucleic acid detected in USE-A and B was present in fraction I whereas only 10% was detected in fraction I from USE-C. This indicates that rather extensive depolymerization of the nucleic acid had occurred in USE-C presumably as a result of insonation.

Protein was estimated in ultrasonic extracts using two methods; a chemical determination (Folin-phenol reaction) and absorption at 280 mμ. Values obtained by these two methods for the relative protein concentrations in chromatographic fraction I_{g-25} from USE-A, B and D were in close

agreement. This was not true of fraction I from USE-C. The reason for this discrepancy is not known. No correlation was observed between values obtained for proteins in fractions II and III using these two methods.

Protein concentration, at least in bacillary extracts, cannot always be accurately determined using the Folin-phenol reaction. This is illustrated by the observation that of 7.8 mg of protein detected by the Folin reaction per ml of USE-C only 44% or approximately 3.4 mg/ml actually represented material of molecular weight greater than 5000. Ten percent, or 10.34 mg/ml, was detected by absorption at 280 mμ. Recognition of this limitation of the Folin-reaction for protein determination is important in standardizing antigen-containing solutions for serologic and analytical purposes. A correction factor must be introduced when protein solutions contain low molecular weight Folin-reactive material.

Protein was apparently "denatured" by prolonged exposure to ultrasonic vibrations. This can be seen by the rather high relative concentrations of 280 mμ-absorbing material in fraction II of the chromatogram of USE-C as compared to the relative concentration in the other ultrasonic extracts.

Chemical analyses of chromatographic fractions I, II and III from Sephadex G-25 indicated that fraction I contained proteins of molecular weight greater than 5000,

the majority of carbohydrate and a considerable amount of highly polymerized nucleic acid. The presence of nucleic acid in fraction II was detected by the strong 280 mμ absorption both in chromatograms and ultraviolet spectra. Fraction II contained a substantial amount of Folin-reactive, 280 mμ-absorbing material. It was presumably peptide. Very little carbohydrate and a considerable amount of 260 mμ-absorbing material, presumably oligoneucleotides were detected. This fraction was free of detectable antigens but provoked skin reactions in sensitized rabbits. It may be a promising source of sensitins for detecting tuberculosis. Fraction III consisted of 280 mμ-absorbing material, presumably small peptides, no detectable carbohydrate and some 260 mμ-absorbing material, presumably neucleotides. Fraction III from USE-C contained substantial amounts of the low molecular weight nucleotides and peptides, presumably as the result of depolymerization of the respective biomacromolecules.

Disc electrophoresis effectively separated many of the constituents of ultrasonic extracts of mycobacteria. The separation achieved by zone electrophoresis in cellulose acetate membranes was less satisfactory. Disc electrophoresis is a means for effective reproducible separation and is economical from the standpoint of time, equipment and sample size required for analyses.

The chemical heterogenity of ultrasonic extracts is evident in disc electrophorograms. Between 16 and 24 amido black staining components were detected in different extracts. It should be noted that although disc electrophoresis is reproducible, variations do occur in gel concentrations, the degree of polymerization and in the relative concentrations of individual components in different samples. Thus, variations in line intensity and displacements do occur and not all electrophorograms contain each component consistently each and every time. Optimal results are obtained by running samples in duplicate, preferably in triplicate, and comparing the disc electrophorograms following completion of such sets. It may be possible to increase the resolution of the technique by the alteration of gel concentration since disc bands which appear homogenous may contain more than one antigenic component when separated on a different pore size gel (156).

As many as 18 PAS-stained components were detected in disc electrophorograms of ultrasonic extracts. The PAS reaction results in staining of both carbohydrates and glycoproteins. No amido-black-positive components were detected in the spacer gels indicating that the PAS stained components in this area are carbohydrate. Five to six distinct PAS-stained bands were frequently detected in the spacer gels of electrophorograms of the ultrasonic extracts. PAS-stained components in the lower gel could be either

carbohydrate or glycoprotein. Many of the PAS-positive bands had Rf values identical with amido-black-positive bands indicating that they are probably glycoproteins. The presence in electrophorograms of numerous PAS-positive components from ultrasonic extracts suggests the native state of these molecules since carbohydrate and protein probably exist in the cell wall as complexes containing both carbohydrate and protein.

The most anodic amido black and PAS-positive band was quite prominent in disc electrophorograms of all of the ultrasonic extracts. A yellow, nonstaining component was frequently associated with this band and was also seen in disc electrophorograms of chromatographic fraction II from Sephadex G-25. Mycobacterium bovis is generally described as producing no pigment, however, this is in comparison to other strains of mycobacteria and the component detected is undoubtedly pigment. Disc electrophorograms regularly contained discrete, nonstaining opalescent bands. The significance or chemical nature of these are not known but it is probable that they are of a lipid nature.

Analyses by Ouchterlony immunodiffusion tests was the first method used to approximate the number of antigens in ultrasonic extracts of M. bovis. It had been determined in preliminary tests that the maximum number of immunoprecipitates with the reference system was obtained when the protein concentration was approximately 2 mg/ml. Since

multiple antigen-antibody systems are involved, however, optimal ratios of all of the reactants could not simultaneously exist. Thus, it is practically impossible to achieve maximum resolution of the antigenic composition of complex mixtures using this method. Between 13 and 17 separate immunoprecipitates were detected in immunograms of various ultrasonic extracts. While antigenic and chemical analyses of purified fractions obtained from ultrasonic extracts may eventually yield some classification of mycobacterial antigens, the complexity of the mixtures at this point would make such a classification premature. Maximum resolution was achieved by making daily readditions of dilutions of both reactants. This procedure may, if not properly performed and analyzed, cause splitting of lines and lead to erroneous assumptions. The number of separate antigen-antibody systems enumerated were less than were detected using immunoelectrophoresis. The number of antigens detected are undoubtedly less than the number which actually exist.

Many of the immunoprecipitates detected in immunograms of ultrasonic extracts were lightly stained and difficult to detect consistently unless individual plates were carefully analyzed under optimal conditions. The reference system (USE-A with anti-USE-A) yielded 15 immunoprecipitates. This represents the minimum number of antigens in the extract. Seven distinct antigen-antibody systems have been

reported in extracts of mycobacteria obtained by mechanical disruption in a pressure cell. Culture filtrate, the same culture filtrate from which cells were used for the preparation of USE-D, and ultrasonic extract D were antigenically indistinguishable using anti-USE-A serum. These results are in general agreement with an earlier report (25) and indicate that bacillary extracts and culture filtrates contain the same antigens if filtrates of the appropriate ages are used. The antigenic composition of bacillary extracts is consistent although variations may occur in the relative concentrations of the components.

Antigens with similar diffusion constants but different electrokinetic properties were separated by immunoelectrophoresis. As many as 20 to 22 immunoprecipitates were detected in USE-D, three to five more than detected by Ouchterlony diffusion. It is perhaps significant that a maximum of 18 antigen-antibody systems were detected in immunoelectrophorograms with the reference system. Apparently USE-A contained at least 22 distinct antigens in a concentration sufficient to stimulate antibody production but only 18 of these antigens existed in sufficient concentration to be detected in immunoelectrophoresis. A region of diffuse immunoprecipitation was frequently observed in immunoelectrophorograms of ultrasonic extracts. This was perhaps due to the presence of several antigens which do not differ significantly in electrokinetic properties or diffusion

constants and which precipitate together. A comparison of the number of components detected indicates that disc electrophoresis, immunoelectrophoresis and Ouchterlony immunodiffusion demonstrated decreasing numbers of mycobacterial constituents.

Fractionation of ultrasonic extracts by molecular exclusion chromatography in Sephadex or Bio Gels having different molecular exclusion limits did not yield pure antigen preparations. Fraction I from Sephadex G-25 and unfractionated ultrasonic extract were virtually indistinguishable by Ouchterlony immunodiffusion, immunoelectrophoresis and disc electrophoresis. Thus, fraction I_{G-25} seems to be an ideal preparation to be used for subsequent fractionation for antigen purification since it is free of much of the low molecular weight material present in the crude ultrasonic extract. Dialysis for extended periods with repeated changes of buffer also yields a preparation rich in chromatographic fraction I_{G-25} and contains only small amounts of fractions II and III.

Chromatography in Sephadex G-25 indicated that approximately 49% of the 280 mμ-absorbing material in USE-B had an average molecular weight of greater than 5000. Of this, 84%, 82% and 59%, had molecular weights of 100,000, 150,000 and 200,000 or greater, respectively. It is apparent that a substantial percentage of the antigenic material in ultrasonic extracts is present in a high

molecular weight fraction, greater than 200,000. Ouchterlony immunodiffusion analyses of the fractions eluted from different Bio Gels confirmed that although some degree of purification had been achieved, each fraction remained antigenically complex. Molecular exclusion chromatography alone is not sufficient to yield purified mycobacterial antigens. Hopefully, molecular exclusion chromatography can be allied with other fractionation procedures such as ion exchange chromatography and preparative disc electrophoresis to obtain purified antigens from mycobacteria. Ultrasonic extracts provide a rich source of undenatured antigens from which these purified antigens can be obtained.

Antigens were obtained by chemical extractions of viable mycobacterial cells but no rigorous chemical or antigenic analyses were made of these extracts. The amount of cells of M. bovis by its nature were limited and ultrasonic extraction appeared more promising in preliminary experiments. The chemical extracts were made by procedures which have been employed for other bacterial species and therefore, optimal conditions for extraction of mycobacterial cells may not have been used. The analyses of these extracts may not represent the maximum number of antigens obtainable by the various methods. Many of the chemical bacillary extracts had antigens in common which indicate that different procedures can remove the same antigens. However, the chemical or antigenic nature of these extracts have not been

analyzed sufficiently to classify or identify individual components. They were examined with antiserum specific for ultrasonic extracts, thus, antigens unique to the chemical extracts if they exist would not be detected.

Urea and guanidine denature proteins by the disruption of secondary bonds which disorganizes the tertiary protein structure. Five antigen-antibody systems were detected by immunodiffusion analyses of the urea and guanidine extracts. Differences were detected by immunoelectrophoresis and disc electrophoresis. Because of the denaturative properties of urea and guanidine, the significance of these differences is uncertain.

The mechanisms of action by which tritron X-100 and sodium desoxycholate release components from bacterial cells is speculative. These compounds probably interfere with hydrophobic bonding and thereby disorganize the three dimensional structure of lipoprotein complexes. Ouchterlony immunodiffusion analyses demonstrated 11 antigen-antibody systems in each of these extracts. Immunoelectrophoresis detected 15 and 11 antigens in the triton X- and desoxycholate extracts, respectively. Both compounds appear to be effective chemical extractants for preparing mycobacterial antigens. Disc electrophorograms of the triton extract, however, yielded only six amido-black staining bands. Extensive areas of opalescence were present in the gel columns after electrophoresis of the triton extract which

probably either prevented adequate electrophoretic migration of individual components or interfered with proper staining. It is possible that trace amounts of the triton remain firmly bound to some constituents of the extract and interfere with migration.

When the cellular debris remaining after ultrasonic disruption were extracted with phosphate buffer containing ethyl ether, the extract contained only three detectable antigens.

There is no doubt that the need exists for specific mycobacterial antigens to be used for the detection of tuberculosis. The need exists equally, if not more so, to study fundamental differences among the mycobacteria and to study the reactions of hosts to separate antigens and various combinations of antigens. Mycobacterial antigen preparations available today are nonspecific because of their complexity. These preparations cannot be used to differentiate between infection and disease, infection and sensitization, or the species or strain of Mycobacterium causing the infection, disease and/or sensitization.

Little is known of the antigenic composition of mycobacteria despite years of investigation. Neither is the nature of antibody responses to different mycobacterial antigens understood nor the complex interplay of the various constituents. Crucial areas of ignorance exist regarding the relationship between delayed hypersensitivity, susceptibility

and tuberculoimmunity. In order for these questions to be satisfactorily answered, more detailed information must be obtained regarding the chemical and immunologic properties of the mycobacteria. Purified antigen preparations must be made available and used to investigate fundamental properties of antibody production and delayed hypersensitivity in tuberculosis. Recently developed *in vivo* and *in vitro* procedures are amenable to this type of study if more purified mycobacterial constituents are available.

Extracts obtained by mechanical disruption of *M. bovis* (310) by ultrasound contain a minimum of 22 antigens detectable by specific antibodies. This undoubtedly represents the minimum number present in these extracts. Moreover, each of these antigens may well contain multiple antigenic determinant sites. Which of these antigens is of potential significance in tuberculoimmunity or tuberculin hypersensitivity remains to be determined. Whether any of these antigens are specific is not yet known.

SUMMARY

Antibody production was studied in rabbits inoculated with different preparations of M. bovis. Experimental models were designed to simulate (a) active tuberculosis, (b) limited (closed) tuberculosis with tuberculoimmunity and tuberculin sensitivity, (c) no tuberculosis with tuberculoimmunity and tuberculin sensitivity, and (d) no tuberculosis with tuberculoimmunity and no tuberculin sensitivity. Passive-hemagglutination and bacterial agglutination tests were used for antibody determinations. Rabbits (a) injected with virulent M. bovis had gross lesions and produced negligible amounts of antibody and rabbits, (b) injected with attenuated M. bovis (strain BCG) produced significant amounts of antibody comparable to antibody produced by rabbits, (c) inoculated with killed preparations of M. bovis. Antibody elicited by heat or acetone-killed cells was somewhat greater than elicited by cells which were killed with betapropriolactone, with or without subsequent extraction with methanol and acetone.

A sequential production of first mercaptoethanol-sensitive followed by mercaptoethanol-resistant antibody was observed in all rabbits regardless of the antigen preparation or the serological test used to detect antibody. Antibody detected during the first two weeks post-inoculation was exclusively

mercaptoethanol-sensitive. Thereafter, mercaptoethanol-resistant antibody was produced in increasing amounts. In most cases, mercaptoethanol-sensitive and resistant antibody was detected for the duration of the experimental period, up to 23 weeks post-inoculation.

Skin testing with purified protein derivative 14 weeks post-inoculation stimulated antibody production in approximately 50% of the rabbits tested. Both the absolute and relative amounts of the different types of antibody produced was altered by skin testing. All rabbits inoculated with viable or killed M. bovis preparations became hypersensitive to tuberculin although extraction of betapropiolactone-killed cells with methanol and acetone diminished their ability to sensitize rabbits to tuberculin.

The antigenic and chemical composition of ultrasonic extracts of M. bovis varied with the age of the culture from which the cells were obtained and the intensity of insonation. Ultrasonic extracts of cells from two-month-old cultures contained more antigens than were detected in extracts from six-month-old cultures. The number of antigens in ultrasonic extracts was lessened by exposure of cells to long durations of insonation.

Disc electrophoresis detected the highest number of chemical constituents in ultrasonic extracts. Between 16 and 24 amido black-stained components and 15-18 PAS-stained components were detected in disc electrophorograms of different ultrasonic extracts.

Ouchterlony immunodiffusion detected 13-17 distinct antigen-antibody systems in different ultrasonic extracts. As many as 20-22 antigens were detected in ultrasonic extracts by immunoelectrophoresis.

A substantial porportion of the antigens in ultrasonic extracts have average molecular weights of greater than 200,000. Molecular exclusion chromatography and ion exchange chromatography provide a method of preparative fractionation for antigen purification.

Viable cells of M. bovis were extracted with Triton X-100, sodium desoxycholate, urea, and guanidine, and cellular debris was extracted with phosphate buffer containing ethyl ether. Disc electrophorograms of desoxycholate, Triton, guanidine and urea extracts contained 13, 6, 6, and 4 amido black-stained components respectively, and 11, 15, 3 and 9 antigens respectively, by immunoelectrophoresis.

LITERATURE CITED

1. Affronti, L. F., R. Parlett, and R. Cornesky. 1965. Electrophoresis on polyacrylamide gel of protein and polysaccharide fractions from Mycobacterium tuberculosis. Am. Rev. Resp. Dis. 91:1-5.

2. Alshabkhoun, A., P. Chapman, M. White, and A. DeGroat. 1960. A study of the double dyfusion gel precipitation test in tuberculous patients with special reference to technical problems. Am. Rev. Resp. Dis. 81:704-708.

3. Altemeier, W., J. Robbins, and R. Smith. 1966. Quantitative studies of the immunoglobulin sequence in the response of the rabbit to a somatic antigen. J. Exp. Med. 124:443-460.

4. Anacker, R. L., R. A. Finkelstein, W. T. Haskins, M. Lundy, K. C. Milner, E. Ribi, and P. W. Stasbak. 1964. Origin and properties of naturally occurring hapten from Escherichia coli. J. Bacteriol. 88:1705-1720.

5. Anderson, R. J. 1941. Structural pecularities of acid-fast bacterial lipids. Chem. Rev. 29:45-55.

6. Arnason, B. and B. Waksman. 1964. Tuberculin sensitivity Immunologic considerations. Adv. in Tuberc. Res. 13: 2-97.

7. Asselineau, J. 1952. Lipides du bacille tuberculeus. Adv. in Tuberc. Res. 2-55.

8. Baer, M., and S. Chaparas. 1963. The immunology and chemistry of tuberculin. I. Isolation of dialyzable and non-dialyzable tuberculin-active components from unheated BCG culture filtrates. Amer. Rev. Resp. Dis. 88:28-32.

9. Baillie, A. and V. Norris. 1964. Antigen changes during spore formation in Bacillus cereus. J. Bacteriol. 87:1221-1226.

10. Banach, T. M., and R. Hawirko. 1966. Isolation and characterization of two antigens of Corynebacterium hofmanni. J. Bacteriol. 92:1304-1310.

11. Bauer, D. C. and A. Stavitsky. 1961. On the different molecular forms of antibody synthesized by rabbits during the early response to a simple injection of protein and cellular antigens. Proc. Nat. Acad. Sci. 47:1667-1673.

12. Bauer, D. C., M. Mathies and A. Stavitsky. 1963. Sequence of synthesis of macroglobulin and globulin antibodies during primary and secondary responses to proteins, Salmonella antigens and phage. J. Exp. Med. 117:889-907.

13. Bauer, D. C. 1963. The molecular properties of antibodies synthesized in response to simple haptens. J. Immunol. 90:323-330.

14. Baughn, R. E. and B. Freeman. 1966. Antigenic structure of Brucella suis spheroplasts. J. Bacteriol. 92: 1298-1303.

15. Benedict, A., R. Brown, and R. Ayengar. 1962. Physical properties of antibodies to bovine serum albumin as demonstrated by hemagglutination. J. Exp. Med. 115: 195-208.

16. Benedict, A. 1965. Sensitivity of passive hemagglutination for assay of 7S and 19S antibodies in primary anti-bovine serum albumin sera. Nature 206:1368-1369.

17. Bellanti, J., D. Eitzman, J. Robbins and R. T. Smith. 1963. The development of the immune response. Studies on the agglutinin response to Salmonella flagellar antigen in the newborn rabbit. J. Exp. Med. 117:479-496.

18. Blagovesheenskii, V., and G. Stepanchenok, and L. Zhulins. 1961. Study of the chemical composition of aqueous extracts of BCG cultures treated with ultrasound, and isolation from them of soluble antigens. J. Microbiol. Epidem. and Immunobiol. 32:17-22.

19. Boyden, S. V. 1951. The adsorption of protein on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exp. Med. 93:107-120.

20. Boyden, S. V., and E. Sorkin. 1955. A study of antigens active in the tannic acid HA test in filtrates of cultures of Mycobacterium tuberculosis. J. Immunol. 75: 15-27.

21. Boyden, S. V., 1956. Antigens of Mycobacterium tuberculosis. Adv. Tubercu. Res. 1:17-51.

22. Boyden, S. V. 1958. The immunological response to antigens of the tubercle bacillus. Progress in Allergy. V:149-214.

23. Brown, F., B. Cartwright, and J. Newman. 1964. Further studies of the early antibody in the sera of cattle and guinea pigs infected with foot-and-mouth disease virus. J. Immunol. 92:397-402.

24. Canalco. 1963. Canal Industrial Corporation, Bethesda 14, Maryland.

25. Castelnuovo, G., M. E. Duncan, and G. Bellezza. 1964. Mycobacterial antigens. A study of antigens in bacterial extracts and in culture filtrates of different ages. Tubercle 45:246-254.

26. Chaparas, S., and H. Baer. 1964. The immunology and chemistry of tuberculin II. Chromatography with Sephadex of the nondialyzable tuberculin-active constituents of BCG culture filtrate. Amer. Rev. Resp. Dis. 89:41-48.

27. Chaparas, S., and H. Baer. 1964. The immunology and chemistry of tuberculin III. Dialyzable BCG culture filtrate components obtained by Sephadex chromatography and fractional dialysis. Amer. Rev. Resp. Dis. 90:87-96.

28. Cole, L. R., J. Matloff and V. Farrell. 1955. A method for coupling protein antigens to erythrocytes II. Use of the method in the diagnosis of tuberculosis. J. Exp. Med. 102:647-653.

29. Cole, L. R., and C. B. Favour. 1955. Correlations between plasma protein fractions, antibody titers and the passive transfer of delayed and immediate cutaneous reactivity to tuberculin PPD and tuberculopolysaccharides. J. Exp. Med. 101:391-420.

30. Cole, L. R., and V. Farrell. 1955. A method for coupling protein antigens to erythrocytes I. Description of method. J. Exp. Med. 102:631-645.

31. Crowle, A. J. 1958. Immunizing constituents of the tubercle bacillus. Bact. Rev. 22:183-203.

32. Crowle, A. J. 1961. Immunodiffusion. Academic Press, New York.

33. Crowle, A. J. 1962. Tubercle bacillary extracts immunogenic for mice 3. Chemical degredation studies on the immunogen extracted from tubercle bacilli by trypsin digestion. Tubercle. 43:178-184.

34. Crowle, A. J. 1962. Tubercle bacillary extracts immunogenic for mice. 4. Lipids. Proc. Soc. Exptl. Biol. and Med. 109:969-971.

35. Crowle, A. J. 1963. Tubercle bacillary extracts immunogenic for mice. 5. Specificity of tuberculo-immunity induced by trypsin extracts of tubercle bacilli. Tubercle. 44:241-246.

36. Crowle, A. J., and F. Teramura. 1964. Tubercle bacillary extracts immunogenic for mice. 6. Comparative immunogenicity of trypsin extracts of tubercle bacilli in mice and guinea pigs. Tubercle 45:40-46.

37. Crowle, A. J., and C. Ho. 1965. Tubercle bacillary extracts immunogenic for mice. 7. Electrophoretic analyses. Tubercle 46:214-223.

38. Cummins, C. S., and H. Harris. 1958. Studies on the cell wall composition and toxonomy of actinomycetales and related groups. J. Gen. Microbiol. 18:173-188.

39. Cummins, C. S. 1962. Chemical composition and antigenic structure of cell walls of Corynebacterium, Mycobacterium, Nocardia, Actinomyces and Arthrobacter. J. Gen. Microbiol. 28:35-50.

40. Daniel, T. A. 1965. Observations on the antibody response of rabbits to mycobacterial antigens. J. Immunol. 95:100-108.

41. Daniel, T. A. 1966. Characteristics of the antibody response of rabbits to BCG. Abst. Ann. Meeting of the National Tuberculosis Association. San Francisco. P. 67.

42. Daniel, T. A. 1967. Molecular characterization of the antibody response in rabbits to intravenously injected BCG. Am. Rev. Resp. Dis. 95:262-269.

43. Daniel, T. A., and J. Baum. 1967. Immunoglobulin response to tuberculopolysaccharide in patients with tuberculosis. Abst. Annual Meeting of the National Tuberculosis Assocation. Pittsburg. P. 21.

44. Dardas, T. J. 1967. Antigenic, chemical, chromatographic and electrophoretic analyses of unheated culture filtrate of Mycobacterium bovis. Thesis, Michigan State University.

45. Dardas, G. F., T. J. Dardas, and V. H. Mallmann. 1967. Immunologic responses evoked by mycobacterial components. Antibody responses of rabbits to cells and culture filtrates of Mycobacterium bovis. In Press.

46. Davies, R. 1959. Observations on the use of ultrasound waves for the disruption of microorganisms. Biochim. et Biophys. Acta. 33:481-485.

47. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. New York Acad. Sci. 121:404-427.

48. Deitz, T. M., and E. Runyon. 1967. Specificity of mycobacterial sensitins isolated by aceylamide electrophoresis. Bact. Proc. 67th Ann. Meeting. P. 101.

49. Deitz, T. M., M. Smith, and E. Runyon. 1967. Mycobacterial species. Characterization by electrophoretic protein pattern. Abstract, Ann. Meeting National Tuberculosis Assn. Chicago. Pp. 13-14.

50. Deutsch, H. F., and J. Morton. 1957. Dissociation of human serum macroglobulins. Science. 125:600.

51. Dubos, R. J. and R. Schaedler. 1957. Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infection. I. Proteolytic effects. J. Exp. Med. 106:703-717.

52. Dubos, R. J. 1964. Acquired immunity to tuberculosis. Amer. Rev. Resp. Dis. 90:505-515.

53. Duncan, D. B. 1955. Multiple range and multiple F-tests. Biometrics. 11:1-12.

54. Edwards, D. Q., and L. B. Edwards. 1960. Story of the tuberculin test from an epidemiologic viewpoint. Am. Rev. Resp. Dis. 81:1-17.

55. El Piner, I. E. 1952. Ultrasonics in microbiology. Microbiol. 21:228-235.

56. El Piner, I. E. 1952. Ultrasonics and its application in biology. Nature 11:109-111.

57. El Piner, I. E. 1955. Mechanisms of ultrasonic action on microorganisms. Microbiol. 24:371-380.

58. El Piner, I. E. 1964. Ultrasound, Its Physical, Chemical and Biological Effects. Consultants Bureau, New York.

59. Fox, A., G. L. Evans, F. Turner, A. Blavskin, and B. Schwartz. 1964. Stimulation of nonspecific resistance to infection with an enzyme-treated cell wall preparation from Mycobacterium phlei. J. Reticulloendohdial Soc. 1:359-362.

60. Fox, A., G. Evans, F. Turner, B. Schwartz and A. Blaustein. 1966. Stimulation of nonspecific resistance to infection by a cell wall preparation from Mycobacterium phlei. J. Bacteriol. 92:1-5.

61. Fox, A., J. Anschel, G. Evans, R. Monan, and B. Schwartz. 1966. Isolation of a soluble resistance-enhancing factor from Mycobacterium phlei. J. Bacteriol. 92:285-290.

62. Franklin, E. C. 1964. The immune globulins, their structure and function and some techniques for their isolation. Prog. in Allergy. 8:1-148.

63. Frappier, A., V. Portelance, and J. St. Pierre. 1959. Some biological properties of mycobacteria following the extraction of lipids. The power to initiate acquired resistance. Amer. Rev. Resp. Dis. 79:296-306.

64. Freedman, S., R. Turcotte, and A. Sehon. 1964. A serologic test for active tuberculosis. Clin. Res. 12:243.

65. Freeman, S., and A. Stavitsky. 1965. Radio immunoelectrophoretic study of rabbit anti-protein antibody during the primary response. J. Immunol. 95:981-990.

66. Freund, J., and S. Stone. 1959. The effectiveness of tuberculoglycolipid as an adjuvant in eliciting allergic encephalomyelitis and aspermatogenesis. J. Immunol. 82:560-567.

67. Froman, S., T. Edington, D. Will, L. Scammon, D. Faber, and B. Eckmann. 1964. Gel double diffusion testing for tuberculosis. Am. J. Clin. Path. 42:340-345.

68. Fukui, Y., and M. Yoneda. 1961. Extracellular proteins of tubercle bacilli. III. Column chromatographical purification of the proteins of a virulent strain (H37Rv) of Mycobacterium tuberculosis. Biken J. 4: 187-196.

69. Fukui, Y., T. Hirai, T. Uchida, and M. Yoneda. 1965. Extracellular proteins of tubercle bacilli. IV. Alpha and beta antigens as major extracellular protein products and as cellular components of a strain (H37Rv) of Mycobacterium tuberculosis. Biken J. 8:189-199.

70. Gaby, W. L., J. Black, and A. Bondi Jr. 1953. The specificity of the hemagglutination adsorption technique in the serologic study of tuberculosis. Am. Rev. Tuberc. 67:657-664.

71. Glenchur, H., and L. Kettel. 1965. A study of the agar double diffusion test in human tuberculosis. Amer. Rev. Resp. Dis. 91:86-95.

72. Glenchur, H., B. E. Fossieck, and M. Silverman. 1965. An immediate skin test for the diagnosis of active pulmonary tuberculosis. Amer. Rev. Resp. Dis. 92: 741-748.

73. Glenchur, H., B. E. Fossieck, and M. Silverman. 1966. A comparison of purified protein derivative and some fractions of Mycobacterium tuberculosis. Amer. Rev. Resp. Dis. 93:70-77.

74. Grabar, P. and M. Royer. 1945. La disintegration des microbes par les ultrasons. Ann. Inst. Pasteur. 71:154.

75. Greenbury, C., D. Moore, and L. Nunn. 1963. Reactions of 7S and 19S components of immune rabbit antisera with human Group A and AB red cells. Immunol. 6:421-433.

76. Grey, H. 1964. Studies on changes in the quality of rabbit bovine serum albumin antibody following immunization. Immunology. 7:82-89.

77. Gronwall, A. 1947. Physico-chemical analysis of protein antigens isolated from the tubercle bacillus. Upsalla Lekarej. Forsch. 227-234.

78. Gussoni, C. 1962. Antigenic analysis of old tuberculin and purified protein derivative by gel diffusion. Amer. Rev. Resp. Dis. 85:248-257.

79. Harvey, E. N., and A. Loomis. 1932. High speed photomicrography on living cells subjected to supersonic vibrations. J. Gen. Physiol. 15:147-152.

80. Heckly, R. J., and D. Watson. 1950. Extraction of proteins and other constituents from the tubercle bacillus with some observations on their chemical and biological properties. Amer. Rev. Resp. Dis. 61:778-808.

81. Heckly, R., and D. Watson. 1951. Extraction and fractionation of water soluble components from tubercle bacilli. Amer. Rev. Resp. Dis. 64:602-619.

82. Hinsdill, R. D., and D. Berman. 1967. Antigens of Brucella abortus. I. Chemical and immunoelectrophoretic characterization. J. Bacteriol. 93:544-549.

83. Hirschfeld, J. 1960. Immunoelectrophoresis procedure and application to the study of group-specific variations in sera. Science Tools. 7:18-25.

84. Ide, Y., M. Takanami, H. Nemato, H. Yugi, and H. Hatakeyama. 1961. Studies on tuberculin-active substance. I. Preparation of tuberculin active substance from tubercle bacilli bodies. Bull. Nat. Inst. Animal Health. 42:91-97.

85. Ide, Y., H. Yugi, and M. Takanami. 1961. Studies on tuberculin-active substance. III. Fractionation of phosphate buffer extract from tubercle bacilli. Tokyo Nat. Inst. of Animal Health. Quarterly. 1:135-141.

86. Ide, Y., M. Takanami, H. Nemoto, H. Yugi, and H. Hatakeyama. 1961. Biological studies on fractions extracted from tubercle bacilli bodies. Bull. Nat. Inst. Animal Health. 42:90-94.

87. Iland, C. N., A. Jones, and R. Baldwin. 1950. New antigenic fractions from Mycobacterium tuberculosis. Fifth Internat. Congress for Microbiol. Rio de Janeiro. P. 201.

88. Jones, A., and K. Hinson. 1953. Observations on the serology of pulmonary tuberculosis. Amer. Rev. Tuberc. 68:737-745.

89. Kawakami, M., N. Osawa, and S. Mitsuhashi. 1966. Experimental Salmonellosis. VII. Comparison of the immunizing effect of live vaccine and materials extracted from Salmonella enteritidis. J. Bacteriol. 92:1585-1587.

90. King, S., and E. Meyer. 1963. Gel diffusion technique in antigen-antibody reactions of Actinomyces species and "anaerobic diphtheroids." J. Bacteriol. 85:186-190.

91. Knicker, W. T., and D. C. Heiner. 1960. Separation of the antigens of Mycobacterium tuberculosis by ion exchange chromatography and gel diffusion. Am. J. Dis. Child. 100:604-605.

92. Knicker, W. T., and J. B. LaBorde. 1964. The separation of mycobacterial antigens by ion exchange chromatography. I. Details of chromatographic and immunologic proceedures and results with four strains of Mycobacterium tuberculosis. Amer. Rev. Resp. Dis. 89:29-40.

93. Kotani, S., T. Kitaura, T. Hirano, and A. Tanaka. 1959. Isolation and the chemical composition of the cell walls of BCG. Biken J. 2:121-141.

94. Kotani, S., T. Kitaura, M. Migashigawa, M. Kato, Y. Mori, T. Matsukara, T. Tsujimoto. 1960. Studies on the immunologic properties of the cell wall, particulate and soluble fractions of sonicated BCG cells. Biken J. 3:159-172.

95. Kwapinski, J., and M. Snyder. 1961. Antigenic structure and serologic relationships of Mycobacterium, Actinomyces, Streptococcus and Diplococcus. J. Bacteriol. 82:632-639.

96. Kwapinski, J. 1963. Antigenic structure of Actinomycetales. VI. Serological relationships between antigenic fractions of Actinomyces and Nocardia. J. Bacteriol. 86:179-186.

97. Kwapinski, J. 1964. Antigenic structure of the Actinomycetales. VII. Chemical and serological similarities of cell walls from 100 Actinomycetales strains. J. Bacteriol. 88:1211-1219.

98. Landy, M. 1954. On hemagglutination proceedures utilizing isolated polysaccharide and protein antigens. Amer. J. Pub. Health. 44:1059-1064.

99. Larson, C. L., E. Ribi, W. Wicht. and R. List. 1961. Skin reaction produced in rabbits by cell walls and protoplasm of Mycobacterium tuberculosis and Mycobacterium butyricum. Amer. Rev. Resp. Dis. 83:184-193.

100. Leskowitz, S., and B. Waksman. 1960. Studies in immunization. I. The effect of route of injection of bovine serum albumin in Freunds adjuvant on the production of circulating antibody and delayed hypersensitivity. J. Immunol. 84:58-72.

101. Lind, A. 1959. Serological studies of mycobacteria by means of the diffusion in gel technique. I. Preliminary investigations. Int. Arch. Allerg. 14;264-278.

102. Lind, A. 1960. Serological studies of mycobacteria by means of the diffusion-in-gel technique. II. Investigations of the precipitins formed after infection with M. tuberculosis and their relation to the antibodies demonstrated by the Middlebrook-Dubos hemagglutination test. Int. Arch. Allergy. 16:336-351.

103. Lind, A. 1960. Serological studies of mycobacteria by means of the diffusion-in-gel technique. III. A difference in precipitinogenic content found in substrain of BCG. Int. Arch. Allergy. 17:1-9.

104. Lind, A. 1960. Serological studies of mycobacteria by means of the diffusion-in-gel technique. IV. The precipitinogenic relationships between different species of mycobacteria with special reference to M. tuberculosis, M. phlei, M. smegmatus and M. avium. Int. Arch. Allergy. 17:300-322.

105. Lind, A. 1961. Serological studies of mycobacteria by means of the diffusion-in-gel technique. VII. Investigations of the precipitinogenic demonstrated in various preparations of mycobacterial origin. Int. Arch. Allergy. 19:112-126.

106. Live, I., F. Sperling, and E. Stubbs. 1945. Studies on the immunization of guinea pigs against brucella infections. J. Infect. Dis. 77:16-24.

107. Long, E. 1958. The Chemistry and Chemotherepy of Tuberculosis. 3rd Ed. Williams and Wilkins Co. Baltimore.

108. Long, K. R., and J. Top. 1964. The detection of antibody against tubercle bacilli in the serum of tuberculin-positive cattle by an agar double-diffusion precipitation technique. Amer. Rev. Resp. Dis. 89:49-54.

109. Lowry, O., N. Rosebrough, A. L. Farr and R. J. Randall. 1951. The protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

110. Mackeprang, B. 1957. The dispersing and bacterial effect of ultrasonics on BCG. Acta. Tuberc. Scand. 34:297-312.

111. Mallman, V. H., P. Robinson, and M. D. McGavin. 1964. An anamnestic-like reaction elicited by tuberculin. Am. J. Vet. Med. Res. 25:693-698.

112. Mallmann, W. L., V. H. Mallmann, and J. Ray. 1964. Bovine tuberculosis: What we know, do not know, and need to know in order to eradicate. Proc. U. S. Livestock Jan. Assoc. 68th Ann. Mtg. Pp. 327-332.

113. Markenson, J., D. Sulitzeanu, and A. Olitzki. 1959. Immunizing properties of insoluble cell materials derived from Brucella. Nature 183:1693.

114. Menzel, A., and M. Heidelberger. 1938. Cell protein fractions of bovine and avian tubercle bacillus strains and of the thimothy grass bacillus. J. Biol. Chem. 124:301-307.

115. Menzel, A. and M. Heidelberger. 1938. Protein fractions of the human strain (M-37) of tubercle bacillus. J. Biol. Chem. 124:89-101.

116. Meynell, B. 1954. The antigenic structure of Mycobacterium tuberculosis var. hominic. J. Path. and Bacteriol. 67:137-150.

117. Middlebrook, G., and R. J. Dubos. 1948. The specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. J. Exp. Med. 88:521-528.

118. Miller, J., J. DeBruyn, and J. Bekker. 1966. Immunity in experimental syphilis. IV. Serologic reactivity of antigens extracted from Treponems pallidum and Treponema reiteri. J. Bacteriol. 91:583-587.

119. Morton, W., and M. Dodge. 1963. Modifications of the agar double-diffusion precipitation test for tuberculosis. Amer. Rev. Resp. Dis. 88:264-266.

120. Neter, E., H. Wang, T. Suzuki, and E. Gorzynski. 1964. Differences in antibody response to intravenously infected soluble and cell-attached enterobacterial antigen. Immunology. 7:657-664.

121. Nossal, G., A. Szenberg, G. Ada, and C. Austin. 1964. Single cell studies on 19S antibody production. J. Exp. Med. 119:485-502.

122. O'Grady, F. 1967. Tuberculin reactions in tuberculosis. Brit. Med. Bull. Delayed Hypersnesitivity: Specific Cell-Mediated Immunity. 23:76-80.

123. Onove, K., Y. Yaqi, and D. Pressman. 1964. Multiplicity of antibody proteins in rabbit anti-p-aminobenzene-arsonate sera. J. Immunol. 92:173-174.

124. Onoue, K., Y. Yaqi, A. Grossberg, and D. Pressman. 1965. Number of binding sites of rabbit macroglobulin antibody and its subunits. Immunochem. 2:401-415.

125. Onoue, K., N. Tanigaki, Y. Yaqi, and D. Pressman. 1965. IgM and IgG anti-hapten antibody: hemolytic, hemagglutinating and precipitating activity. Proc. Soc. Exptl. Biol. and Med. 120:340-348.

126. Onyekwere, O. O. 1967. The tuberculoimmunogenicity for mice and guinea pigs of Mycobacterium bovis inactivated with betapropiolactone compared to that of BCG, phenol inactivated cells and Youmans extract. Thesis, Michigan State University.

127. Ornstein, L., and B. J. Davis. 1961. Disc electrophoresis. Distillation Products Industries, Rochester, New York. P. 63.

128. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. New York Acad. Sci. 121: 321-349.

129. Owen, J. A. 1956. Determination of serum protein fractions by zone electrophoresis. Analysist. 81:26.

130. Parnas, Y., and K. Lazugi. 1957. The new allergins brucillin RD and tularemin M. J. Microbiol. Epidemiol. and Immunobiol. 2:249-250.

131. Parlett, R., and G. Youmans. 1956. Antigenic relationships among mycobacteria as determined by agar diffusion precipitation technique. Am. Rev. Tuberc. 73:637-649.

132. Parlett, R., and G. Youmans. 1958. Antigenic relationships between 9S strains of mycobacteria using gel-diffusion precipitation technique. Amer. Rev. Tuberc. 77:450-461.

133. Parlett, R., and C. Rehr. 1959. Further studies on gel diffusion tests in tuberculosis. I. Methods for standardization of the antigen and detection of small amounts of antibody. Amer. Rev. Resp. Dis. 80:886-894.

134. Parlett, R., and G. Youmans. 1959. An evaluation of the specificity and sensitivity of a gel double diffusion test for tuberculosis: A double blind study. Amer. Rev. Resp. Dis. 80:153-166.

135. Parlett, R. 1961. A proposed revision of the gel double diffusion test for the detection of mycobacterial antibody. Amer. Rev. Resp. Dis. 84:589-591.

136. Parlett, R., and Y. M. Chu. 1966. Observation on circulating antibody responses to mycobacterial stimuli. Am. J. Clin. Pathol. 45:552-561.

137. Pepes, J. 1955. The relationship of nonspecific and specific factors in the tuberculin reaction. A Review. Amer. Rev. Tuberc. 71:49-73.

138. Pepes, J., R. Augustin, and A. Patterson. 1959. Common antigenic components of mycobacterial extracts. Tubercle. XL. 163-172.

139. Peterson, E. A., and M. Sober. 1962. Column chromatography of proteins: substituted celluloses. Adv. in Enzymol. 5:3-27.

140. Pike, R., and M. Schultz. 1964. Production of 7S and 19S antibodies to the somatic antigens of Salmonella typhosa in rabbits. Proc. Soc. Exptl. Biol. and Med. 115:829-833.

141. Pine, L., C. Boone, and D. McLaughlin. 1966. Antigenic properties of the cell wall and other fractions of the yeast form of Histoplasma capsulatum. J. Bacteriol. 91:2158-2168.

142. Prager, M., and J. Bearden. 1964. Blood group antibody activity among $gamm_1$-A-globulins. J. Immunol. 93: 481-488.

143. Prescott, B., O. Sobeslavsky, G. Caldes, and R. Chanock. 1966. Isolation and characterization of fractions of Mycoplasma pneumoniae. I. Chemical and chromatographic separation. J. Bacteriol. 91:2117-2125.

144. Raffel, S. 1943. Recent advances in the immunology of tuberculosis. Stanford Med. Bull. 1:209-214.

145. Raffel, S. 1948. The components of the tubercle bacillus responsible for the delayed type of "infectious" allergy. J. Infect. Dis. 82:267-273.

146. Raffel, S. 1955. The mechanism involved in acquired immunity to tuberculosis. Ciba Found. Symp. on Exp. Tuberc. pp. 261-282. J. A. Churchill Ltd., London.

147. Rheins, M., and J. Thurston. 1955. Additional tuberculous antibodies demonstrated with erythrocytes sensitized by successive adsorptions of old tuberculin and further studies on the adsorptive capacity of tanned cells. Amer. Rev. Tuberc. 72:210-217.

148. Rhodes, J. M., and E. Sorkin 1954. Paper electrophoretic separation of tuberculin constituents. Experimentia. 10:427.

149. Rhodes, J. M. 1961. Chromatography of tuberculoproteins on DEAE-cellulose. Int. Arch. Allergy. 19:257-270.

150. Ribi, E., C. L. Larson, R. List, and W. Widt. 1958. Immunologic significance of the cell walls of mycobacteria. Proc. Soc. Exp. Biol. and Med. 98:263-265.

151. Ribi, E., C. Larson, W. Widt, R. List, and G. Goode. 1966. Effective nonliving vaccine against experimental tuberculosis in mice. J. Bacteriol. 91:975-983.

152. Robbins, J., K. Kenny, and E. Suter. 1965. The isolation and biological activities of rabbit gamma M and gamma G anti-Salmonella typhimucium antibodies. J. Exp. Med. 122:385-402.

153. Robinson, P. 1964. Tuberculopolysaccharide and tuberculophosphatide passive agglutination with bovine and porcine serums. Thesis. Michigan State University.

154. Robson, J. M., and J. J. Smith. 1961. Immunizing effects of a lipopolysaccharide in mice. Amer. Rev. Resp. Dis. 84:100-102.

155. Rosen, F., and J. Michael. 1963. Association of "natural" antibodies to gram-negative bacteria with gamma$_1$-macroglobulins. J. Exp. Med. 118: 619-626.

156. Roszman, T. 1966. Disc electrophoretic and immunodiffusion studies of mycobacterial culture filtrates. Thesis. Michigan State University.

157. Rothhard, S., A. Dooneief, and K. Hite, 1950. Practical application of a hemagllutination reaction in tuberculosis. Proc. Soc. Exp. Biol. and Med. 74:72-75.

158. Rowley, D., and K. Turner. 1964. Increase in mcaroglobulin antibody in mice and guinea pigs following injection of bacterial lipopolysaccharide. Immunol. 7:394-402.

159. Runyon, S. H. 1965. Pathogenic mycobacteria. Adv. Tuberc. Res. 14:235-287.

160. Salemeron, S., and J. Sheris. 1957. A comparison of erythrocyte adsorbable antigens derived from different strains of human type tubercle bacilli. J. Path. and Bacteriol. 73:519-525.

161. Schaedler, R., and R. J. Dubos. 1957. Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous injections. II. Enhancement of protection. J. Exp. Med. 106:719-726.

162. Schoenberg, M., A. Stavitsky, A. Moore, and R. Freeman. 1965. Cellular sites of synthesis of rabbit immunoglobulins during primary response to diphtheria toxoid Freund's adjuvant. J. Exp. Med. 121:557-590.

163. Seibert, T. B. 1928. The chemical composition of the active principle of tuberculin. X. The isolation in crystalline form and identification of the active principle of tuberculin. Amer. Rev. Tuberc. 17:402-421.

164. Seibert, F. B., and B. Munday. 1932. The chemical composition of the active principle of tuberculin. XV. A precipitated purified tuberculin protein suitable for the preparation of a standard tuberculin. Amer. Rev. Tuberc. 25:724-737.

165. Seibert, F. B. 1941. The chemistry of the proteins of the acid-fast bacilli. Bact. Rev. 6:69-95.

166. Seibert, F. B. 1949. The isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractionation. Their chemical and biological properties. Amer. Rev. Tuberc. 59:86-101.

167. Seibert, F. B. 1950. Comparative immunizing capacity of BCG and polysaccharide in tubercle bacilli culture filtrates. J. Immunol. 65:297-310.

168. Seibert, F. B. 1950. Constituents of mycobacteria. Ann. Rev. of Microbiol. 4:35-52.

169. Seibert, F. B., and A. Fablizio. 1952. The isolation and chemistry of a protein fraction of M. tuberculosis and its ability to sensitize cells. Amer. Rev. Tuberc. 66:314-334.

170. Seibert, F. B., and E. Soto-Figueroa. 1957. Study of tuberculin protein, and polysaccharide antigens by gel-diffusion technique. Amer. Rev. of Tuberc. and Pulmonary Dis. 75:601-607.

171. Seibert, F. B., and M. Seibert. 1957. Relationship between immunity and circulating antibody, complement and tuberculo polysaccharide in tuberculosis. J. Infect. Dis. 101:109-118.

172. Seibert, F. B. 1960. A theory of immunity in tuberculosis perspectives. Biol. and Med. 3:264-281.

173. Shetlor, M., and Y. Masters. 1957. Use of thymol sulfuric acid reaction for determination of carbohydrates in biological material. Analytical Chem. 29:402.

174. Smith, D. T., and N. B. Scott. 1950. Clinical interpretation of the Middlebrook-Dubos hemagglutination test. Amer. Rev. Tuberc. 62:121-127.

175. Smith, D. T., G. Fregnan, L. DeLaguerriere and E. Valdivia. 1964. Induction of acquired resistance in guinea pigs with defatted Mycobacterium tuberculosis vaccines. J. Bacteriol. 88:87-95.

176. Sobeslavsky, O., B. Prescott, W. James, and R. Chanock. 1966. Isolation and characterization of fractions of Mycoplasma pneumoniae. II. Antigenicity and immunogenicity. J. Bacteriol. 91:2126-2138.

177. Someya, S., O. Hayashi, and Y. Yamamura. 1962. Studies on the antigenicity of tuberculin-active peptide. Amer. Rev. Resp. Dis. 86:542-552.

178. Someya, S., K. Koyama, N. Asmi, T. Katoaka, and Y. Yamamura. 1966. Studies on the tuberculin reactivity of tuberculin-active peptide. Japan. J. Microbiol. 10:221-227.

179. Sonneborn, D., M. Sussman, and L. Levine. 1964. Serological analysis of cellular slime mold development. I. Changes in antigenic activity during cell aggragation. J. Bacteriol. 87:1321-1329.

180. Sorkin, E., and S. Boyden. 1955. A study of antigens active in the Middlebrook&Dubos hemagglutination test present in filtrates of cultures of Mycobacterium tuberculosis. J. Immunol. 75:22-27.

181. Stacey, M., and P. Kent. 1938. The polysaccharides of Mycobacterium tuberculosis. Adv. in Carbohydrate Chemistry. 3:311-351.

182. Stacey, M., and P. Kent, and E. Nassau. 1951. Polysaccharide complexes isolated from Mycobacterium tuberculosis (human strain). Biochim. Biophys. Acta. 7:146-152.

183. Stacey, M. 1955. Mycobacterium tuberculosis polysaccharides. Adv. in Tuberc. Res. 6:7-17.

184. Stelos, P. 1956. Electrophoretic and ultracentrifugal studies of rabbit hemolysins. J. Immunol. 77:396-404.

185. Stelos, P. 1958. Comparative study of rabbit hemalysins to various antigens. I. Hemolysins to beef red blood cells. J. Enfect. Dis. 102:103-113.

186. Stelos, P., L. Taliaferro, and P. D'Alesandro. 1961. Comparative study of rabbit hemolysins to various antigens. III. Chromatographic analyses of Forssman hemolysins induced by various antigens. J. Infect. Dis. 108:113-119.

187. Stepanchenok, G., and V. Blagovechchenskii. 1960. The use of ultrasound microbiology and microbial biochemistry. A review of the literature. J. Microbiol., Epidemiol., and Immunobiol. 31:435-439.

188. Sushida, Y., and N. Hirano. 1963. Serological relationships between mycobacteria as determined by agar diffusion precipitation test. II. Experiments with atypical mycobacteria isolated chiefly in America. Japan. J. Microbiol. 7:9-22.

189. Svehag, S. E., and B. Mandel. 1964. The formation and properties of polio virus-neutralizing antibody. I. 19S and 7S antibody formation: difference in kinetics and antigen dose requirement for induction. J. Exp. Med. 119:1-19.

190. Svehag, S. E., and B. Mandel. 1964. The formation and properties of polio virus-neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, anamnesis and sensitivity to X-radiation. J. Exp. Med. 119:21-39.

191. Svehag, S. E. 1964. The formation and properties of polio virus-neutralizing antibody. III. Sequential changes in electrophoretic mobility of 19S and 7S antibodies synthesized by rabbits after a single virus injection. J. Exp. Med. 119:225-240.

192. Takahashi, Y., K. Mochizuki, and Y. Nagayama. 1961. The behavior of three different kinds of antibodies in tuberculosis: antiprotein, anticarbohydrate and antiphosphatide. II. Human tuberculosis. J. Exp. Med. 114:569-579.

193. Takahashi, Y., and K. Ono. 1961. Study on the passive hemagglutination reaction by the phosphatide of M. tuberculosis. I. The reaction and its specificity.

194. Takahashi, Y., and K. Ono. 1961. Study on the passive hemagglutination reaction by the phosphatide of M. tuberculosis. II. Conditions of the reaction. Amer. Rev. Resp. Dis. 83:386-393.

195. Takahashi, Y., S. Fujita, and A. Sasaki. 1961. The specificity of the passive hemagglutination methods used in serology of tuberculosis. J. Exp. Med. 113: 1141-1154.

196. Takahashi, Y. 1962. Specific serum agglutination of kaolin particles sensitized with tubercle phosphatide and its clinical evaluation as a serodiagnostic test for tuberculosis. Amer. Rev. Resp. Dis. 85:708-719.

197. Thurston, J. R., M. Rheins, and T. Huziwara. 1956. Serologic investigations of mycobacterial antigens. I. The specificity of water and saline extracts of BCG., M. phlei and H37Rv. Amer. Rev. Tuberc. 73:563-568.

198. Thurston, J. R., and M. Rheins. 1956. Serologic investigations of mycobacterial antigens. II. Specificities of antigens obtained by successive aqueous extractions of BCG and M. phlei. Amer. Rev. of Tuberc. 73:571-575.

199. Thurston, J. R., and M. Rheins. 1956. Serologic investigations of mycobacterial antigens. III. Direct detection of homologous antibody by BCG extract antigens in serum of rabbits injected with BCG and challenged with M. bovis. Amer. Rev. of Tuberc. 74:756-763.

200. Thurston, J. R., and W. Steenken, Jr. 1960. Comparison of gel precipitation and sensitized erythrocyte technique for the detection of antibody in the serum of tuberculous and nontuberculous patients. Amer. Rev. Resp. Dis. 81:695-703.

201. Togunova, A., A. Karsonova, and G. Stepanchenok. 1959. The antigenic properties of suspensions of Mycobacterium tuberculosis exposed to ultrasound. J. Microbiol., Epidemiol., and Immunobiol. 30:95-98.

202. Torrigiani, G., and I, Roitt. 1965. The enhancement of 19S production by particulate antigen. J. Exp. Med. 122:181-193.

203. Turcotte, R., S. O. Freedman, and A. Sohon. 1963. A new hemagglutination proceedure for the diagnosis of active tuberculosis. Amer. Rev. Resp. Dis. 88: 725-728.

204. Turcotte, R., S. Freedman, and A. Sehon. 1964. Characterization of isohemagglutinins to tuberculin PPD in human serum. Fed. Proc. 23:347b.

205. Turner, M., and D. Rowe. 1964. Characterization of human antibodies to Salmonella typhi by gel filtration and antigenic analysis. Immunol. 7:639-656.

206. Uhr, J. W., M. Finkelstein, and J. Baumann. 1962. Antibody formation. III. The primary and secondary antibody response to bacteriophage ØX 174 in guinea pigs. J. Exp. Med. 115:655-670.

207. Uhr, J. W., and M. Finkelstein. 1963. Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ØX 174. J. Exp. Med. 117:457-477.

208. Uhr, J. W. 1964. The heterogenity of the immune response. Science. 145:457-464.

209. Veltman, G., and K. Woeber. 1949. Beitrag zur bakteriziden wirkung des ultrashalls. Strahlentherapie. 79:587.

210. Wei, M. N. and A. B. Stavitsky. 1967. Molecular forms of rabbit antibody synthesized during the primary response to human albumin. Immunology. 12:431-444.

211. Weidanz, W., A. Jackson, and M. Landy. 1964. Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens. Proc. Soc. Exp. Biol. and Med. 116:832-837.

212. Weiss, D., and R. J. Dubos. 1955. Antituberculosis immunity induced in mice by vaccination with killed tubercle bacilli or with a soluble bacillary extract. J. Exp. Med. 101:313-330.

213. Weiss, D. W., and R. S. Dubos. 1956. Antituberculous immunity induced by methanol extracts of tubercle bacilli--its enhancement by adjuvants. J. Exp. Med. 103:73-85.

214. Weiss, D. W., and A. Q. Wells. 1960. Vaccination against tuberculosis with nonliving vaccines. III. Vaccination of guinea pigs with bacillary fractions. Amer. Rev. Resp. Dis. 82:339-357.

215. Weiss, D. W., R. Bonhag, and J. Parks. 1964. Studies on the heterologous immunogenicity of a methanol-insoluble fraction of attenuated tubercle bacilli, BCG. I. Antimicrobial protection. J. Exp. Med. 119: 53-70.

216. White, R. G., and A. Marshall. 1958. The role of various chemical fractions of Mycobacterium tuberculosis and other mycobacteria in the production of allergic encephalomyelitis. Immunol. 1:111-121.

217. Williams, C. A., and R. J. Dubos. 1959. Studies on fractions of methanol extracts of tubercle bacilli. J. Exp. Med. 110:981-1004.

218. Yagi, Y., P. Maier, D. Pressman, C. Arbesman, and R. Reisman. 1963. The presence of the ragweed-binding antibodies in the B_2-A, B_2-M and gamma-globulins of the sensitive individuals. J. Immunol.. 91:83-89.

219. Yamaguchi, K. 1955. Precipitin reaction of experimental tuberculosis by the agar diffusing technique. Ann. g. Tuberc. 6:56-63.

220. Yoneda, M., and Y. Fukui. 1961. Extracellular proteins of tubercle bacilli. I. Zone electrophoretic purification of the proteins of a virulent strain (H37Rv) of M. tuberculosis. Biken J. 4:25-40.

221. Yoneda, M., and Y. Fukui. 1961. Extracellular proteins of tubercle bacilli. II. Some serological properties of purified extracellular proteins of a virulent strain (H37Rv) of M. tuberculosis. Biken J. 4:121-130.

222. Yoneda, M., Y. Fukui, and T. Yamanouchi. 1965. Extracellular proteins of tubercle bacilli. V. Distribution of alpha and beta antigens in various mycobacteria. Biken J. 8:201-223.

223. Yoshida, K., S. Mizunari, and H. Halleri. 1964. Immunological studies on "Surface antigen" extracted from Stephylococcus aureus. I. Isolation of the antigen and properties of the immuno rabbit serum. Japan J. Microbiol. 8:67-74.

224. Youmans, G., and A. Karlson. 1947. Streptomycin sensitivity of tubercle bacilli. Studies on recently isolated tubercle bacilli and the development of resistance to streptomycin in vivo. Amer. Rev. Tuberc. 55:529-535.

225. Youmans, G., R. Millman, and A. Youmans. 1955. The immunizing activity against tuberculosis injection in mice of enzymatically active particles isolated from extracts of Mycobacterium tuberculosis. J. Bacteriol. 70:557-562.

226. Youmans, G., R. Parlett, and A. Youmans. 1961. The significance of the response of mice to immunization with viable unclassified mycobacteria. Amer. Rev. Resp. Dis. 83:903-905.

227. Youmans, G. 1963. The pathogenic "atypical" mycobacteria. Ann. Rev. Microbiol. 17:473-494.

228. Yugi, H., H. Hatakeyama, H. Nemato, and Y. Ide. 1962. Studies on tuberculin-active substance. IV. Biological studies on phosphate buffer extract from tubercle bacilli. Tokyo. Nat. Inst. of Animal. Health Quarterly. 2:140-147.