ABSTRACT

ANTIGENIC, CHEMICAL, CHROMATOGRAPHIC, AND ELECTROPHORETIC ANALYSES OF UNHEATED CULTURE FILTRATES OF MYCOBACTERIUM BOVIS

by Terry J. Dardas

The antibody response of rabbits inoculated with heat or beta-propriolactone killed Mycobacterium bovis and/or unheated culture filtrates was studied. Antibody was measured by bacterial agglutination and passive hemagglutination (Middlebrook-Dubos test). The sequential production of mercaptoethanol-sensitive (MES) and mercaptoethanol-resistant (MER) antibody was measured. The relative amount and temporal sequence of synthesis of each type of antibody varied among the experimental groups. Increasing the localized dose or concentration of antigen shortened the interval between the detection of MES and MER antibody. When the local antigenic stimulus was decreased (no adjuvant or systemic injection of antigen) or repeated small inoculations were made weekly, MES antibody production was prolonged and the initiation of MER antibody synthesis delayed.

Concentrated unheated filtrates from 2, 3, 4, 5, 6, and 7 month cultures of M. bovis were prepared and analyzed chemically, and by chromatography, electrophoresis, and

immunodiffusion. The culture filtrates were readily separated chromatographically into three or more fractions. The chemical and antigenic composition of the fractions varied with the age of the culture filtrate. The amount of high molecular weight protein in the culture filtrates decreased with longer periods of incubation of the culture.

Disc electrophoresis was a very effective means of separating the culture filtrate components. Between 13 and 30 protein components, and 5 to 9 polysaccharide or glycoprotein components were detected in the culture filtrates. All of the protein components had molecular weights of 5,000 or greater. The number of protein bands increased from 16 in two-month-old culture filtrates to 30 in four-month-old culture filtrates. Thereafter, the number decreased; only 13 bands were detected in filtrates from seven-month-old cultures.

The number of precipitinogens in the culture filtrates decreased with longer periods of incubation. Sixteen immunoprecipitates were detected in immunograms of two-month-old culture filtrates; only six immunoprecipitates were detected in immunograms of filtrates of cultures incubated for seven months. More precipitinogens were detected in the culture filtrates by immunoelectrophoresis. The number of precipitinogens detected by this method varied from 8 to 21.

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INTRODUCTION

Mycobacteria have been studied extensively since the discovery of tubercle bacilli by Robert Koch in 1882. The clinical manifestations and pathological characteristics of tuberculosis have been known for many years. Yet little progress has been made in understanding the pathogenesis of the disease or the immunological responses to the causative agent. Many attempts have been made to find specific antigens, antibodies, or other substances in the blood of diagnostic or prognostic value. The tests are not reliable. Diagnosis depends on a combination of the results from tuberculin tests, X-rays, and the presence of tubercle bacilli in clinical specimens. It must be confirmed by isolation and identification of the causative agent. A basic understanding of the mechanism of antibody production against mycobacterial antigens would greatly supplement our knowledge and understanding of tuberculoimmunity and sensitivity.

Perhaps the greatest challenge lies in the isolation of specific antigens or sensitins from mycobacteria. One of the greatest limitations of immunologic tests in tuberculosis is their inability to reliably differentiate between disease and infection or between tuberculosis and tuberculosis-like disease caused by "atypical" acid-fast organisms. Individual

antigens or haptens must be isolated from various mycobacteria and evaluated for differential specificity in serologic tests and in experimentally infected laboratory animals.

This is a report of the antibody response of rabbits to mycobacterial antigens. The antibody responses to several different antigen preparations and inoculation procedures were compared. Unheated filtrates of cultures of Mycobacterium bovis of different ages were analyzed chemically and by chromatography, disc electrophoresis, and immunodiffusion.

HISTORICAL REVIEW

It is well established that the sera of tuberculous man and animals contains antibodies specific for mycobacterial protein, polysaccharide, and lipid (20;127). The occurrence and relative amounts of these types of antibody are irregular during the course of the disease. Moreover, there is no apparent diagnostic or prognostic correlation between the amount of antibody and the extent, duration, and activity of the disease. Delayed hypersensitivity to tuberculoproteins occurs with few exceptions in tuberculous individuals. However, there appears to be little or no correlation between the degree of hypersensitivity and the activity or extent of disease other than that the reaction may be decreased in the terminal stage. The extent of delayed hypersensitivity is not related to the occurrence of serum antibody (86;164;94). As yet, there is no practical method of demonstrating delayed sensitivity *in vitro*. The tuberculin test has remained the most reliable test for detecting tuberculosis. It does not differentiate between active or closed cases, nor the species of mycobacterium causing the sensitivity.

The significance of the serologic responses in tuberculo-immunity has been the subject of considerable discussion and

controversy for many years (47;127;96). Results from experimental animals and clinical observations in humans have led some investigators to conclude that antibodies play an insignificant role (if any) in tuberculoimmunity (96;50;47). According to this view, resistance is primarily a result of the ability of the hypersensitive state to make the intracellular environment inhibitory to the growth of the tubercle bacilli. Other investigators (149) believe that antibodies play a decisive role in the acquisition of tuberculoimmunity. Several naturally occurring antimycobacterial serum substances, such as lysozyme, react with tuberculopolysaccharide (149). Antipolysaccharide antibody facilitates the removal of excess tuberculopolysaccharide from the extracellular fluids and allows these inhibitors to act. There is a complex interplay and changing balance of the tuberculopolysaccharides, specific antibodies, and nonspecific serum components.

A large portion of the work on the immunology of tuberculosis has been directed toward the development of serologic tests of diagnostic and prognostic value. Sera from individuals immunized or infected with mycobacteria will agglutinate tubercle bacilli (67) and fix complement in the presence of cells or extracts of mycobacteria (132). However, serologic tests based on these reactions are of little diagnostic value because there are many false-negative and flase-positive reactions (20). Both tests have been used to measure the antibody response to the multiple antigens of mycobacterial

cells and culture filtrates following immunization of experimental animals (132;41). Because only low titers of precipitins are produced by tuberculous humans, and domestic and laboratory animals, the tube precipitation test is of little diagnostic or prognostic value (20;29;124).

The passive hemagglutination test was developed by Middlebrook and Dubos and reportedly measures tuberculopolysaccharide-specific antibodies (102;163). Although the test is very sensitive, false-positive reactions occur with sera from normal individuals and patients with other diseases (65;155). Various strains of staphylococci and certain fungi elicited antibody in experimental animals which agglutinated erythrocytes sensitized with tuberculopolysaccharide (79). Hemolytic (103) and anti-globulin (75) modifications have not improved the diagnostic usefulness of the test (68).

A passive hemagglutination test specific for tuberculoprotein was devised by Boyden (17). Tannic acid-treated sheep red blood cells adsorbed protein but not polysaccharide antigens present in old tuberculin (OT). Tuberculoproteins were also attached to phenolyzed red blood cells by tetrazotized benzidine (30). Little correlation exists between the results of these tests and the presence of active tuberculosis (31).

Turcotte and co-workers (172) fractionated sera from healthy tuberculin positive individuals and patients with active tuberculosis with DEAE cellulose. The anti-tuberculoprotein antibody in the chromatographic fractions was

measured using a passive hemagglutination test. Two types of antibody were readily separated chromatographically. Most of the antibody in the sera from healthy individuals had a high anionic affinity and was serologically inactivated by mercaptoethanol (ME). Sera from tuberculous patients contained antibodies of low anionic affinity and were not inactivated by ME. Daniel (39) was unable to confirm these findings.

Other passive agglutination tests have employed carriers other than red blood cells such as latex particles coated with OT or PPD-S (45). Approximately 95% of the sera from patients with active tuberculosis caused agglutination whereas 90% of the sera from healthy individuals sera were negative. When antigen-sensitized bentonite particles were used, two types of agglutinins were found in sera from tuberculous patients; a heat stable (56c, 30 min) antibody and heat labile antibody (32). Heating reduced the titer of sera from tuberculous patients two to six-fold but not of sera from normal individuals or patients with inactive tuberculosis.

Tuberculophosphatide-specific antibodies can be detected in infected or immunized individuals with phosphatide-coated red blood cells or kaolin particles (34;161;162). While anti-protein and anti-polysaccharide antibodies were found in the sera of tuberculous humans and rabbits regardless of the activity or extent of the disease, only anti-phosphatide antibodies were found in individuals with active disease

(164;165). The antibody titer reportedly could be correlated with the extent and activity of the disease. Positive tests were obtained with serum from 95% of 1402 patients with active tuberculosis (166). This test was not a reliable indication of active disease in calves experimentally infected with classical and atypical mycobacteria (128).

In 1948, three investigators independently reported the use of diffusion-in-gel techniques for the analyses of complex antigenic mixtures (113;114;51). Since then, many attempts to develop a meaningful serologic test for tuberculosis have used these techniques. Parlett (118) found precipitins in the sera of 48 of 53 tuberculous patients with a modified immunodiffusion technique. The sera of 38 of 40 tuberculin positive healthy individuals were negative. The number of positive reactions detected varied with the stage of the disease; the percent positive tests were 84.2%, 73.5%, and 57.8% for far advanced, moderately advanced, and minimal tuberculosis, respectively (120). Thurston and Steenken (169) compared the sensitivity of the gel precipitation test and the Middlebrook-Dubos test for the detection of antibody in the sera of tuberculous and nontuberculous patients. Antibody was detected in 80% of the sera from tuberculous patients by both tests. Approximately 17% of the sera from nontuberculous patients were positive by both tests. Further refinements of the gel precipitation test such as readdition of reactants and using several antigen dilutions

has increased somewhat its sensitivity (120;2;105). This test has not been found to be of value in the serodiagnosis of bovine tuberculosis (95). Despite the presence of gross lesions in cattle, very few sera were positive in this test.

The Ouchterlony plate technique (113) was not satisfactory for the detection of precipitins in the sera of patients with active tuberculosis (86). Only 17% of 168 patients with active tuberculosis had detectable precipitins. They occurred most frequently in sera from patients with disease of long duration. Similar results were repeated by Glenchur and Kettel (55) and Burrel et al. (22). Forty-four percent of sera from patients with active tuberculosis had precipitins detectable by the tube gel precipitation test; only 37% of the same sera were positive by the plate technique (89).

No single serologic test is reliable for the diagnosis of tuberculosis and furthermore, a battery of serologic tests is not reliable (54). Sera from 138 tuberculous patients and 41 tuberculin-negative students were tested for antibodies using the tube double diffusion test, the Middlebrook-Dubos HA test, and the Takahashi kaolin agglutination test. Whereas 85% of the patients sera were positive by one or more tests, only 20% of the sera were positive in all of the tests. Seven percent of the sera from the tuberculin-negative students were positive by one or more tests.

Few investigators have studied the antibody response of experimental animals to tubercle bacilli and their many components. A thorough understanding of the antibody response to individual mycobacterial antigens would greatly supplement our knowledge and understanding of tuberculo-immunity. Considerable is known about the antibody response of rabbits and other experimental animals to immunization with a number and variety of soluble, cellular and viral antigens (69;15;170). Variations in the dose (173;158) concentration (52), and physical and chemical nature of the antigen, as well as the route of injection (151;84) and immunization regimen profoundly affect the antibody response.

Recent work has focused on the heterogeneity of the immunoglobulins (123) and the temporal sequence of their production following immunization (173). Stelos (157) demonstrated a change in the electrophoretic mobility and sedimentation velocity of hemolysins produced by rabbits immunized with sheep red blood cells. The primary response consisted of the sequential production of two classes of antibodies. The antibodies produced early had a sedimentation value of 19S and migrated with the $gamma_1$ globulins 1g-M). The antibodies produced later were 7S $gamma_2$ globulins (1g-G). The appearance of 1g-G was accompanied by a decrease or disappearance of 1g-M. Bauer and his associates (8;9) inoculated rabbits with a variety of protein and cellular antigens and confirmed the sequential appearance

of these two classes of antibody. High molecular weight 1g-M lost their precipitating and hemagglutinating activity after reduction with mercaptoethanol (ME); 7S 1g-G retained their serologic activity. Macroglobulins were reductively cleaved to two 7S fragments by sulfhydryl compounds (44). The two main classes of antibody could be separated by DEAE column chromatography (10). Early 1g-M were bound tightly to the adsorbent whereas 1g-G were eluted with the starting buffer.

The antibody response of several animal species has suggested that viral antigens elicit a different antibody response than soluble protein antigens. Svehag and Mandel (158;159;160) made a thorough study of the kinetics of antibody production in rabbits immunized with polio virus. Two classes of ME-sensitive 1g-M appeared in the circulation 8 to 16 hrs after immunization and persisted for 4 to 5 days. After 36 to 48 hours, two types of 1g-G were distinguished by differences in their electrophoretic mobility. The duration of the latent period for the 1g-G could not be shortened by increasing the amount of antigen injected. Small amounts of antigen elicited low levels of 1g-M. No 1g-G were detected. When larger doses were injected, 1g-M production was followed by the production of 1g-G. The minimal dose of antigen required to initiate 1g-M synthesis was approximately one fiftieth of that required to initiate 1g-G synthesis.

Guinea pigs immunized with bacteriophage produced 1g-M which were detectable 20 hours after inoculation (173). Low doses of bacteriophage elicited only 1g-M; larger doses induced the sequential production of both 1g-M and 1g-G. Similar results have been reported by other investigators (21;12).

In contrast to the detection of antibody within 24 hrs after the inoculation with viral antigens, antibody to soluble protein antigens was not detected for several days to two weeks after injection (9). Both 1g-M and 1g-G were elicited by soluble proteins, however, the kinetics of production and the relative amount of each type of immunoglobulin produced varied with the dose of antigen, the route of injection, the chemical and physical properties of the protein and several other factors (171).

The immune response to cell-attached somatic antigens appears to differ in several respects from that elicited by soluble protein and viral antigens (3;173): both elicited 1g-M but less 1g-G was elicited by somatic antigens (175;81; 123). 1g-G were detected several days after 1g-M in rabbits inoculated with soluble and viral antigens (8;9;159) but there was a longer lag period between the detection of 1g-M and 1g-G in rabbits inoculated with somatic antigens (8;81).

The physical state of the antigen as well as the chemical composition can also profoundly influence the nature of the antibody response. Neter and his co-workers (106) studied

the antibody response of rabbit to extracts of heat-killed enteric bacteria. Intravenous injection of the soluble antigen resulted in either a minimal antibody response or none at all. In contrast, when the antigens were attached to homologous red blood cells and injected intravenously, a strong antibody response was elicited.

Although the antibody response to soluble and particulate protein antigens is similar, particulate antigens elicit more 1g-M (10;107;171). Bacterial flagella elicited only 1g-M in rats; flagellin (a soluble monomeric preparation of flagella) failed to do so (107). Rabbits produced 10-20 times more 1g-M when soluble thyroglobulin and human gamma globulin were attached to acrylic resin particles than when the same antigens were injected in soluble form (171).

Many studies of the kinetics of antibody production to protein and somatic antigens have employed the agglutination test which may not be equally sensitive for 1g-M and 1g-G (11). Rabbit 1g-M were 1000 fold more efficient on a molar basis than 1g-G as agglutinins for S. typhumurium (129), and 750 times more efficient in the agglutination and hemolysis of human red blood cells (58). From 160 to 180 times as many moles of purified anti-hapten 1g-G were required to produce the equivalent amount of hemolysis as produced by one mole of 1g-M (110).

Both 1g-M and 1g-G were detected by radioimmunoelectrophoresis (RIE) 5 to 7 days after rabbits were inoculated with

alum-precipitated diphtheria toxoid, human serum albumin, and bovine gamma globulin (53). The authors concluded that the sequential production of these two classes of antibody was in fact due to differences in sensitivity of the hemagglubination test for the two immunoglobulins. Rabbits were inoculated with human serum albumin in adjuvant and the serum tested for antibodies by passive hemagglutination, passive cutaneous anaphylaxis (PCA), antigen binding, precipitation and RIE (174). Both 1g-M and 1g-G were detected six days after immunization; 1g-M by passive hemagglutination and RIE and 1g-G by antigen binding, RIE, and PCA. The 1g-G were not detectable by hemagglutination until eight days after inoculation.

There have been a limited number of studies of the kinetics of production of the major types of immunoglobulins following immunization with mycobacterial antigens. Schoenberg and his associates (132) reported the sequential appearance of complement fixing 1g-M and 1g-G in the sera of rabbits inoculated intravenously with M. butyricum. Parlett and Chu (121) inoculated rabbits intravenously with viable tubercle bacilli or cell extracts and measured the serum antibody by gel double diffusion after chromatography of the antisera on DEAE cellulose. The precipitins were in the 1g-G fraction. Daniel (37;38) has made the most detailed study of antibody responses of rabbits to mycobacteria. Rabbits inoculated with soluble OT or alum-precipitated PPD-S

produced only 1g-M specific for tuberculopolysaccharide. Heat-killed M. tuberculosis (H37Rv) or OT in incomplete Freund's adjuvant elicited 1g-M which were followed after several days by 1g-G. Neither the magnitude nor the duration of the antibody response elicited in rabbits by the inoculation of several dosages of viable and heat-killed cells of BCG could be related to the dose or the viability of the antigen (37;38). Whereas 1g-M were produced by rabbits which received either viable or heat-killed BCG, only 2 of 8 rabbits which received heat-killed BCG produced 1g-G antibody. When M. tuberculosis was injected intravenously a second time five days after an initial injection, only 1g-M were detected five days after the last injection (108).

The chemical and antigenic compositions of mycobacteria and their culture filtrates have been studied extensively, since the heated, concentrated culture filtrate known as Old Tuberculin (OT) was used as a chemotherapeutic agent by Koch in 1891 (49). The untoward local, focal, and systemic reactions caused by OT in tuberculous individuals was recognized subsequently. It became important as a skin test reagent for the detection of tuberculosis and is still used for skin tests and as a test antigen in several serologic tests for tuberculosis.

Siebert and Long (92) extensively investigated the chemical and biological activity of various fractions isolated from OT. The skin-reactive substance in tuberculin was

non-dialyzable, precipitated at pH 4.0 with acetic acid and ammonium sulfate, and was destroyed by hydrolysis with hydrochloric acid or several proteolytic enzymes. They concluded that the active substance(s) was a protein but that OT contained many biologically inactive ingredients in addition to the allergenic proteins. Several attempts were made therefore to isolate the proteins free from other biologically inert contaminants by precipitation with ammonium sulfate (133) and trichloroacetic acid (TCA) (134). One of the first tuberculins prepared in this manner was called synthetic medium old tuberculin (SOTT) (135). The first purified protein derivative (PPD) was made by precipitation of the proteins from heated culture filtrates with TCA (93). The proteins in PPD had molecular weights in the range of 2000 to 4000; somewhat smaller than those in SOTT. Purified protein derivative (PPD) did not elicit precipitins in rabbits or precipitate with antisera (138). Much of the polysaccharide and nucleic acids present in PPD could be removed electrophoretically (137).

In 1941, Seibert and Glenn (139) prepared a PPD by precipitation of the proteins from heated culture filtrates of Mycobacterium tuberculosis with ammonium sulfate. It is called PPD-S and is now the international tuberculin standard of the World Health Organization. Although PPD-S contained some polysaccharide and nucleic acid impurities, it was superior to OT and PPD in that it did not induce tuberculin-type hypersensitivity when used repeatedly in the same

individual. The chemical composition and biological activity of PPD-S was more reproducible than PPD (139;143). Although PPD-S was an impressive advancement, physical, chemical, and biological analyses indicated that it was a complex mixture (141;142). Two groups of molecules with different electrophoretic mobilities and sedimentation velocities were found in PPD-S. Differences in the sedimentation patterns observed during ultracentrifugation of heated culture filtrates and PPD suggested that several monomers had polymerized during precipitation with ammonium sulfate (136). The proteins in unheated culture filtrates had molecular weights in the range of 17,000 to 32,000 (100). Heating reportedly caused the unfolding and elongation of the polypeptide chains without any loss in weight. Prolonged heating resulted in degradation of the proteins to 6,000 molecular weight units with a concurrent loss of antigenicity. More recently, the effect of heat on the physical and biological properties of tuberculoproteins was shown to depend greatly on the initial pH of the solution (73).

Because of the denaturative effects of heat on tuberculoproteins and the loss of antigenic specificity and biological activity that results, unheated culture filtrates are more promising materials for the isolation of antigens and sensitins. Unheated culture filtrates were separated into two fractions by moving boundary electrophoresis (140). One of the fractions was much less antigenic than the other and did

not induce tuberculin sensitivity in rabbits. Fourteen fractions were separated by fractional precipitation of unheated culture filtrates of M. tuberculosis with ammonium sulfate (13;101). Two proteins with different molecular weights were isolated. One of the proteins had a molecular weight of 44,000 and was believed to be identical to a protein isolated earlier by Seibert and co-workers (136). The other protein was not purified. On the basis of differences detected by physicochemical and serologic tests, they concluded that only two antigens were present in culture filtrate.

In 1949, Seibert (142) treated unheated culture filtrates with ethanol and acetic acid and isolated three proteins (A, B, and C) and two polysaccharides (I and II). They are frequently used for comparisons in other studies because their physical, chemical, and biological properties have been studied extensively (143;19). The relative concentrations of the five fractions depended on the strain of M. tuberculosis used, the age of the culture, and the medium on which they were grown. The three proteins differed markedly in sedimentation velocity, electrophoretic mobility, chemical composition, antigenic specificity, and allergenicity in tuberculin-sensitive animals (145;147). Several antigens were found in each protein fraction by immunodiffusion (148;90). Using the passive hemagglutination test, only four different antigens were found in unheated culture filtrates fractionated by Seibert's method (18). Three of these antigens were found in

the corresponding fraction isolated from heated culture filtrates. None of the protein fractions were immunogenic in guinea pigs (34).

Polysaccharides I and II differed markedly in physical, chemical, and serologic properties (19). Both failed to elicit an intradermal reaction in tuberculin-sensitive guinea pigs. Polysaccharide II was antigenic in rabbits; polysaccharide I was a hapten. Recently (14) a heteropolysaccharide was isolated from M. tuberculosis cell walls that was identical to polysaccharide I in chemical, chromatographic, and immunological properties. The authors suggested that polysaccharide I found in culture filtrates is released from lipopolysaccharide in the bacterial cell walls during autolysis.

Unheated culture filtrates were precipitated with alcohol and zinc acetate (116). The materials that were obtained were better sensitins and more specific than OT or PPD-S when tested in BCG-vaccinated individuals. A potent and specific tuberculin was obtained from unheated culture filtrates of M. tuberculosis and M. bovis by precipitation with acid at pH 4.7 (76). The precipitate was dissolved, dialyzed, and reprecipitated several times with ethanol. Sensitins prepared by precipitating culture filtrates from classical and atypical mycobacteria with TCA were tested in guinea pigs infected with various mycobacteria (98;99). Although considerable cross sensitivity occurred, it was possible to

distinguish between infections caused by the two groups on the basis of the degree of sensitivity to the tuberculins. Another group of investigators skin-tested several human populations with fractions obtained by acid-alcohol precipitation of unheated culture filtrates. The supernatant fluid which remained after precipitation of the culture filtrate with acid at pH 4.1 was reprecipitated with several concentrations of ethanol. The supernatant fluid obtained after precipitation with 76% ethanol was much more specific than PPD-S for infections caused by M. tuberculosis.

Prior to the development of diffusion-in-gel techniques little progress had been made in the antigenic analyses of mycobacteria (17). This method of antigenic analyses has largely replaced the older and much less sensitive serologic techniques. Parlett and Youmans (119) detected six antigens in cell suspensions of mycobacteria by immunodiffusion. Only four antigens were found when unheated culture filtrates were tested (117). Inoue (74), Burtin and Kourilsky (23) and Castelnuovo (25) detected 7, 7 and 10 antigens respectively in unheated culture filtrates. Lind (85;87;88;90) found as many as 17 different antigens in culture filtrates of various mycobacteria.

Comparisons of immunodiffusion results are difficult at best. The methods used to obtain mycobacterial antigens and antisera, and the techniques used in immunodiffusion vary markedly. In addition, considerable antigenic differences

exist among different species of mycobacteria (117;119;87; 131). Moreover, the antigenic composition of culture filtrates varied among strains and substrains of the same species (87), and was modified by changes in cultural conditions (25).

Most attempts to obtain satisfactory separation of the culture filtrates by zone electrophoresis on paper have been unsuccessful (125;42). Two to four bands were poorly separated. Starch gel electrophoresis separated four proteins in TCA precipitates from culture filtrate (43). The amino acid composition of the four isolated fractions did not differ significantly. A combination of starch electrophoresis with ammonium sulfate precipitation, and column chromatography yielded two protein antigens from culture filtrates of M. tuberculosis (183;184;185;186). Both antigens were readily extractable from the cell surface and accounted for approximately 90% of the antigenic, protein material in the culture medium (185).

A very potent hemagglutinating substance was isolated from culture filtrates of M. tuberculosis (4). The supernatant fluid from culture filtrate after TCA precipitation was dialyzed and reprecipitated several times with ethanol. After extraction of the precipitate with chloroform and amyl alcohol, it was dissolved and separated into three fractions by starch block electrophoresis. The active material was a low molecular weight lipopolysaccharide-peptide of cell wall

origin. The purified hapten did not elicit antibody production in rabbits.

The best electrophoretic separation of mycobacterial constituents reported has been by disc electrophoresis (1;131;46). Nineteen protein bands were demonstrated in culture filtrates from human tubercle bacilli (H37Ra) (1). Fractional precipitation of the culture filtrates with ammonium sulfate was performed according to the procedure described by Yoneda (183). All five ammonium sulfate-precipitated fractions contained from 10 to 14 bands when analyzed electrophoretically. Concentrated culture filtrates from three month old cultures of M. bovis, M. avium, and two strains of Group III of unclassified mycobacteria of bovine origin contained between 18 and 24 protein components (131). From five to eight polysaccharide components were detected in each culture filtrate. Several protein bands were isolated from each culture filtrate by elution from the acrylamide gels after preparative disc electrophoresis. Analyses of the eluates obtained from each culture filtrate was performed by immunodiffusion with four homologous antisera and eleven other antisera. One or more antigens were detected in each culture filtrate which was not found in any of the other culture filtrates. However, re-electrophoresis of the eluates at a different concentration of acrylamide gel separated some eluates into more than one band. An eluate from a single band was not necessarily a single antigen.

Ion exchange column chromatography has been used with some success to separate the antigens in culture filtrates of mycobacteria. Rhodes (126) separated a fraction obtained by Seibert's method (142) into four components on DEAE-cellulose. No single component was isolated when analyzed by starch gel and paper electrophoresis. Each fraction contained several antigens and elicited approximately the same skin reactivity when tested in tuberculin-sensitive guinea pigs.

As many as 12 fractions were separated by DEAE-cellulose chromatography of unheated culture filtrates of three strains of M. tuberculosis (78). All of the fractions contained several antigens when analyzed by immunodiffusion and although not separated, there did appear to be species-specific and strain-specific antigens.

Five fractions were obtained by chromatography of unheated culture filtrates on carboxymethyl cellulose (90). None were antigenically pure. Rechromatography of the isolated peaks failed to improve significantly the antigenic homogeneity of the fractions.

Two polysaccharide antigens were separated from culture filtrates of M. tuberculosis by chromatography on Dowex-50 and DEAE-cellulose (179). One of the antigens was pure polysaccharide; the other antigen contained alanine, glutamic acid, and diaminopimelic acid. Both antigens were probably derived from cell wall lipopolysaccharides by autolysis.

Unheated culture filtrates were separated into dialyzable and non-dialyzable fractions by Baer and Chaparas (6). The non-dialyzable constituents were separated into acetic acid-soluble and acid-insoluble fractions at pH 4.0. The acid-soluble fraction contained polysaccharides primarily and retained its skin test reactivity after digestion with proteolytic enzymes (7). Several fractions in the non-dialyzable fraction were separated by Sephadex G-50 (26). The concentrated dialyzable fraction contained several precipitinogens and elicited skin reactions in guinea pigs sensitive to tuberculin (26;27). All of the fractions from Sephadex G-25 and G-50 chromatography of the dialysate contained several antigens.

Since the yield of biologically active constituents that can be recovered from culture filtrates is usually relatively small, many attempts have been made to obtain soluble extracts from intact or broken cells. These procedures permit the recovery of native or only slightly denatured proteins.

Mycobacteria contain readily extractable antigens located at or near the cell surface (19;34). Heidelberger and Menzel (64) extracted killed M. tuberculosis with solutions of increasing alkalinity. At least three antigens were detected by serologic analyses of subfractions obtained by sodium sulfate precipitation of the extracts.

A material extracted from broken cells of M. bovis with dilute alkali elicited skin reactions in guinea pigs sensitive

to tuberculin (60). The extract was separated into several components by zone electrophoresis. Extracts from different strains varied markedly in their chemical and biological properties.

Heckly and Watson (62;63) disrupted tubercle bacilli by shaking with glass beads and extracted the debris with phosphate and borate buffers containing ether. The extracts were qualitatively similar. The borate buffer extract contained mostly protein and little nucleic acid or carbohydrates. Nucleic acid impurities were removed with calcium phosphate and the extracts fractionated by precipitation with ethanol. The most active protein had three times the skin test reactivity of an equivalent amount of PPD-S.

Saline and aqueous extracts of three species of mycobacterial cells reportedly contained group-specific and strain-specific antigens that could be differentiated serologically (167;168). The antigens were isolated by successive extraction with saline. Aqueous extracts of intact tubercle bacilli closely resembled culture filtrates when analyzed chemically and by electrophoresis (143). Extracts from different strains differed in chemical composition and biological activity.

Protein, polysaccharide, and lipid antigens were extracted from phenol-killed human tubercle bacilli with dilute buffers (74). Immunodiffusion analyses of the three preparations revealed the presence of 6, 2, and 1 antigen, respectively.

Ide and co-workers (70;71) extracted broken mycobacteria with alkaline phosphate buffers. The extracts closely resembled homologous culture filtrates in physicochemical and biological properties. Four fractions were obtained by chromatography of the extracts on DEAE-cellulose (72). The first peak contained largely carbohydrate; the next three peaks were essentially protein. All three of the protein peaks elicited skin reactions in quinea pigs sensitive to tuberculin. All of the fractions contained several precipitating antigens (187).

Azuma (5) isolated a polysaccharide antigen from an alkaline extract of M. tuberculosis by fractional precipitation with ethanol. The antigen was purified by chromatography on Dowex-50, DEAE cellulose, and Sephadex G-75 and G-200. The active material contained only one precipitating antigen.

Paraffin oil extracts of mycobacteria contained two main antigens; a low molecular weight protein that elicited skin reactions in sensitized animals and an antigenic lipopolysaccharide (28).

Cooper and Clark (33) prepared a potent tuberculin by allowing mycobacterial cells to autolyze under toluene at 37°C. They felt that in this way the denaturative effects of mechanical and physicochemical isolation procedures were avoided.

Solid urea was first used to isolate antigens from mycobacterial cells by Stacey (156). Extracts obtained from

M. tuberculosis contained a complex mixture of lipids, nucleic acids, proteins, and polysaccharides. One main component with the electrophoretic mobility and ultraviolet adsorbency of Seibert's C protein was found in urea extracts of human tubercle bacilli (146). The extract was not immunogenic but elicited skin reactions in tuberculin-sensitive animals and inhibited the migration of leucocytes from tuberculin-sensitive animals in tissue culture. Urea extracts of M. bovis contained five to six antigens (40). Twenty or more antigens were found in sonic extracts of another lot from the same batch of cells.

Yamamura and his co-workers (104;153;109;154) isolated a low molecular weight tuberculin-active peptide (TAP) from heat killed M. tuberculosis by extraction with 0.1 N hydrochloric acid. After neutralization, the extract was precipitated several times with picric acid. The solubilized precipitate was separated into four active fractions by chromatography on DEAE-cellulose. The active fractions did not contain any lipid or carbohydrate. In contrast to PPD-S which evoked sensitivity to tuberculin when injected repeatedly into guinea pigs, repeated injection of TAP did not sensitize guinea pigs to tuberculin. The inability of TAP to elicit delayed hypersensitivity to tuberculoproteins was attributed to the absence of lipid or polysaccharides. Raffel (123) was unable to sensitize guinea pigs to tuberculin by repeated injections of either purified tuberculoprotein

or purified waxes from mycobacteria. When they were injected together, delayed hypersensitivity to OT and PPD-S could be demonstrated several weeks later.

Several investigations have shown that mycobacteria contain several readily extractable immunogenic substances (34; 177). Methanol extracts (176;178) were slightly less immunogenic on a weight basis than intact inactivated bacilli. Crowle (36) isolated a water soluble immunogen from acetone killed tubercle bacilli by digestion with trypsin. Selective degradation or removal of peptides, polysaccharides, lipids, and nucleic acids from the extract indicated that the active substance was a polysaccharide. It was probably derived from the cell wall or cytoplasmic membrane. The purified immunogen was five times as effective as intact bacilli on a weight basis and induced immunity of the same degree, longevity, and specificity. Ribosomes and ribonucleic acid isolated from disrupted cells of M. tuberculosis were immunogenic in mice (181;182). Isolated cell walls from various species of mycobacteria were as immunogenic as BCG in mice (83). Cell walls isolated from cells disrupted in oil were more immunogenic than those prepared by aqueous disruption. The purified cell walls contained little protein but substantial amounts of lipopolysaccharides. Rabbits could be sensitized to tuberculin by injection of cell walls but not by protoplasm from disrupted tubercle bacilli (82).

Extracts of tubercle bacilli broken in a French press were fractionated by gel filtration and chromatography on DEAE-cellulose (55;56;57). All of the fractions contained several antigens and elicited immediate and delayed skin reactions in tuberculin-sensitive guinea pigs.

Dietz (46) used disc electrophoresis to analyze extracts of M. tuberculosis and M. kansasii obtained by repeated freezing and thawing and by disruption with glass beads. Four of the ten bands detected were isolated and tested for allergenicity in tuberculin-sensitive guinea pigs. Two of the eluates elicited skin reactions but lacked specificity. Neither of the eluates elicited antibody production in animals.

Mycobacterial phage lysates have been used as test antigens in immunodiffusion test for tuberculosis but no attempt was made to analyze the chemical or antigenic composition of the lysates.

Extracts of M. bovis obtained by disintegration of two month old cells with ultrasound contained at least twenty antigens when analyzed by immunoelectrophoresis (40). Variable numbers of antigens were detected in urea, guanidine, triton, desoxycholate, and phosphate buffer extracts of viable cells.

In view of the antigenic and chemical complexity of mycobacteria, it is perhaps not surprising that difficulty has been encountered in attempts to isolate immunochemically

pure antigens or sensitins. Evidence exists that specific-sensitins and antigens are present in culture filtrate and cell extracts. Their isolation and evaluation remains a challenge.

MATERIALS AND METHODS

Mycobacterium bovis.

Culture 310-2 was isolated in 1962 from a gross lesion cow and identified as *Mycobacterium bovis* by growth and morphologic characteristics, cytochemical tests, virulence for laboratory animals and cattle, and allergenicity for guinea pigs.

Culture filtrates.

Cultures of *M. bovis* were grown on the surface of one liter of a modified Proskauer-Beck synthetic medium (180) in diphtheria toxin bottles. The medium contained the following:

Asparagin	5.0 gm
Monopotassium phosphate	5.0 gm
Potassium sulfate	0.5 gm
Glycerol	20.0 ml
Magnesium citrate	1.5 gm
Distilled water *q.s.ad.*	1000.0 ml

The first four ingredients were dissolved in order in 700 ml of the H_2O, each being added after the previous ingredient had completely dissolved. The pH of the solution was adjusted to 7.0 with 40.0% sodium hydroxide, poured into a diphtheria bottle, and autoclaved for 20 minutes at 121°C. To this was added 300 ml of sterile (20 min, 121°C) 0.5% magnesium citrate solution.

The medium was seeded with fragments of surface growth of M. bovis and incubated 2, 3, 4, 5, 6, and 7 months at 35°C. Bottles with turbid culture fluid beneath the pellicle were discarded. The clear, amber colored culture fluid was drawn off aseptically into 250 ml plastic bottles and centrifuged three hours at 2000 xg. The supernatant fluid from individual cultures of the same age were pooled and filtered (Berkefeld filter, N grade). The sterile filtrates were poured into sterile 4.5 cm (flat diameter) dialysis tubing (Visking Corporation) and dialyzed two days at 4°C against approximately 15 volumes of 0.015 M phosphate buffer pH 7.2. The dialyzed filtrates were concentrated ten fold by pervaporation at room temperature. Concentrated, dialyzed, sterile culture filtrate (CF) of the same age was pooled, filtered (Millipore, 0.45 μ pore size), dispensed into 50.0 ml screw capped tubes, and stored at -80°C.

Chemical analyses.

Protein in the CF and chromatographic fractions was measured by the Folin method (77). One ml each of 4.9% sodium potassium tartrate and 2.0% copper sulfate solutions was added to 100.0 ml of 4.0% sodium carbonate solution. Ten ml of the reagent was mixed with one ml of the samples of CF containing 10 to 250 μg of protein and incubated 45 minutes at room temperature. One ml of Folin phenol reagent diluted 1:3 with distilled water was added, mixed immediately, and incubated 15 minutes at room temperature. The optical

density (OD) of the solutions at 660 mμ was measured with a Beckman D.U. spectrophotometer. The amount of protein in the unknown samples was calculated by comparing the OD of the samples (minus the blank) to those of a standard solution of bovine serum albumin as represented on a standard curve.

Carbohydrates were assayed by use of the thymol-sulfuric acid method (150). Seven ml of cold 77% sulfuric acid was placed into 13 ml glass stoppered centrifuge tubes. One ml samples containing 5 to 100 μg of carbohydrate were layered on the sulfuric acid and the tubes placed in an ice bath for 30 minutes. One-tenth ml of a 10% thymol solution in absolute ethanol was carefully layered over the samples and 0.9 ml of distilled water added. The tubes were stoppered, shaken, and placed in a boiling water bath for twenty minutes. They were then transferred to an ice water bath for five minutes and allowed to stand at room temperature for twenty-five minutes. The OD of the solutions was determined at 500 mμ in a Beckman D.U. spectrophotometer. The amount of carbohydrate in the samples was calculated by comparing the OD of the samples to those of standard solutions of glucose represented on a standard curve.

The amount of nucleic acids present in the CF and chromatographic fractions was measured by ultraviolet absorption spectrophotometry at 280 and 260 mμ using a one cm light path. The nucleic acid concentration was calculated by comparing the OD of the samples to those of standard solutions of enolase and nucleic acid represented on a nomogram.

The absorbency of ultraviolet light in the range of 190 to 320 mμ by culture filtrates and chromatographic fractions was measured in a Beckman D.B. spectrophotometer using a one cm light path.

Production of antisera.

Several preliminary experiments were performed to determine the best way to elicit high titered antisera against mycobacterial antigens.

Eight groups of three Dutch rabbits per group were inoculated according to the schedule shown in Table 1. Cells of M. bovis strain 310 were grown for three weeks in modified Proskauer Beck media and washed with phosphate buffered saline solution (PBS) pH 7.2 prior to inactivation with heat or beta-propiolactone (Testagar and Company).

Cells were heat inactivated at 100°C (moist heat) for thirty minutes. Beta-propiolactone (BPL)-inactivated cells were prepared according to the method of Onyekwere (111). Washed cells were suspended in triple distilled water to a concentration of approximately 10 mg wet weight per ml. The suspension was transferred to a screw capped tube, the pH adjusted to 8.4 with 0.5 M disodiumphosphate and cooled in an ice water bath. A cold solution of BPL was added with constant stirring to a final concentration of 0.4%. The suspension was incubated in a 37°C water bath for two hours with constant agitation. During this period, the pH of the suspension was adjusted to 7.6 at 15 minute intervals by the

Table 1. Inoculation schedule for rabbits which received killed cells and/or Mycobacterium bovis culture filtrate.

Group	Antigen	Cells(mg)	Protein(mg)	Adjuvant[1]	Route	Schedule
I	CF[2]	0	2.0	+	SC[3]	Multiple[4]
II	CF + H[5] cells	9.0	2.0	+	SC	Multiple
III	CF + BPL[6] cells	9.0	2.0	+	SC	Multiple
IV	CF + BPL cells	9.0	2.0	+	SC	Sequential[7]
V	CF + BPL cells	9.0	2.0	-	SC	Multiple
VI	CF + BPL cells	9.0	2.0	-	IV[8]	Sequential
VII	CF + BPL cells	9.0	2.0	+	IV-SC[9]	Multiple
VIII	CF + BPL cells	9.0	2.0	+	IV-SC	Sequential

[1]Adjuvant - Freund's incomplete adjuvant (Difco)

[2]CF - Unheated culture filtrate

[3]SC - Subcutaneous

[4]Multiple - Six simultaneous SC injections in different sites

[5]H cells - Heat killed M. bovis

[6]BPL cells - Betapropiolactone killed M. bovis

[7]Sequential - Six weekly subcutaneous injections

[8]IV - Intravenous

[9]IV-SC - Initial IV injection of CF followed by SC injections on the same day

addition of 0.5 M disodium phosphate. The cells were removed after centrifugation, washed four times with sterile distilled water, and suspended in sterile 0.15 M sodium chloride solution to a concentration of approximately 1.5 mg wet weight per ml.

Rabbits which were inoculated with inactivated cells each received a total of approximately nine mg wet weight. All rabbits received two mg of CF protein. The inocula for all the groups except Groups V and VI were prepared by emulsifying the antigen suspension in an equal volume of incomplete Freund's adjuvant (Difco Laboratories). Rabbits in Groups I, II, III, V and VII were given six subcutaneous injections at different sites on the same day (multiple). Six sequential subcutaneous or intravenous injections were given by one injection per week to the rabbits in Groups IV and VI, respectively (sequential). Rabbits in Groups VII and VIII each received an initial intravenous injection of one ml of CF followed by multiple or sequential subcutaneous injections.

Blood samples were collected from the marginal ear vein of each rabbit prior to inoculation and weekly for eight weeks after inoculation. Sera were decanted, clarified by centrifugation, heat inactivated for thirty minutes at 56^{o}C, and if to be used in hemagglutination tests, absorbed twice with sheep red blood cells, and stored at -80^{o}C. A portion of each serum was mixed with an equal volume of 0.2 M 2-mercaptoethanol (ME) and incubated twelve hours at room temperature.

The treated serums and untreated serums were titrated for antibody immediately after ME treatment.

Polysaccharide-specific antibody was measured by a modification (97) of the passive hemagglutination (HA) test devised by Middlebrook and Dubos (102). Sheep red blood cells were collected aseptically into an equal volume of sterile modified Alsevers solution. The cells were removed after centrifugation and washed three times with PBS. Six ml of a 1:15 dilution of mammalian Old Tuberculin (USDA) were added to 0.1 ml of the washed, packed cells and incubated two hours at 37°C. The sensitized cells were removed after centrifugation and washed three times with PBS. They were resuspended in PBS to a concentration of 0.5% and used within 24 hours.

Serial two-fold dilutions of the serums were made in PBS starting with a 1:10 dilution. Three drops of the sensitized erythrocyte suspension were added to 0.5 ml of the serum dilutions. The tubes were shaken, incubated for two hours in a 37°C water bath, incubated two hours at room temperature, and overnight at 4°C. The reciprocal of the highest serum dilution which caused visible hemagglutination was recorded as the HA titer. The ME-resistant antibody titer was determined by titrating the serums after treatment with ME. The titer of ME-sensitive antibody is the difference between the titers of the serums before and after treatment with ME.

Bacterial agglutinins were measured by a tube agglutination test which employed a uniform suspension of BPL-inactivated M. bovis. Six month old cells were inactivated with BPL as indicated previously. Aggregated cells were dispersed in a tissue grinder. The turbidity of the suspension was adjusted to an OD of 0.2 at 525 mu with PBS. Twenty-five hundredths ml of the cell suspension was added to 0.25 ml of the serially diluted ME-treated and untreated serums. The suspension was incubated twelve hours at 37°C. The presence of antibody was indicated by visible agglutination. The antibody titer was recorded as the highest dilution of the serum which caused visible agglutination. The ME-sensitive and ME-resistant antibody titers were recorded as previously described.

The efficacy of aluminum chloride and aluminum potassium sulfate (alum) to precipitate CF (3 month incubation, 2.5 mg/ml) precipitinogens was compared. Twenty ml of distilled water and 20.0 ml of 10% alum (66) were added to 16.0 ml of CF. The pH of the solution was adjusted to 6.8 with 5 N sodium hydroxide and the resulting precipitate washed twice with PBS containing 0.01% merthiolate. The washed precipitate was resuspended in 40.0 ml of merthiolated PBS.

The aluminum chloride-precipitated inoculum was prepared by adding 2.0 ml of 10.0% aluminum chloride and 2.0 ml of distilled water to a 16.0 ml of CF. The pH of the solution was adjusted to 6.8 with 20.0% sodium hydroxide.

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Two groups of four Dutch Belted rabbits per group were inoculated as shown in Table 2. Each rabbit received 5 ml (10 mg protein) of aluminum hydroxide-precipitated CF and 1.0 ml (2.5 mg protein) of untreated CF. The serums obtained from each rabbit were tested individually for anti-CF precipitins by Ouchterlony immunodiffusion and immunoelectrophoresis.

Inocula were prepared by aluminum chloride precipitation of pooled three and four month incubation CF, and pooled five, six and seven month incubation CF. Two groups of Dutch rabbits, six rabbits per group, were inoculated as given under A in Table 2. The inoculation schedule was repeated after a one month interim. The serums obtained from each rabbit were tested individually by Ouchterlony immunodiffusion for anti-CF precipitins against the homologous CF pool. Satisfactory antisera within each group were pooled, dispensed in small tubes, and stored at -80°C. The antisera are herafter referred to as reference antiserum A (anti-3 and 4 month old CF), and reference antiserum B (anti-5, 6, and 7 month old CF).

Statistical analyses.

The hemagglutination and agglutination titers of the serums collected weekly from each group of rabbits (Table 1) was converted to the corresponding dilution tube number. The mean of the tube dilution numbers in each group was used for statistical analyses. The variance among the means for

Table 2. Inoculation schedule of rabbits which received Mycobacterium bovis precipitated with aluminum hydroxide.

Day	Operation	Amount (ml) A[1]	Amount (ml) B[2]	Route A	Route B
1	Bled	10.0	10.0	C[3]	C
1	Inoculated	5.0	5.0	Im[4]	Im
14	Inoculated	0	5.0	--	Im
32	Inoculated	1.0[5]	1.0	Ip[6]	Ip
36	Bled	25.0	25.0	C	C
40	Bled	25.0	25.0	C	C

[1]A - Rabbits which received aluminum chloride precipitated culture filtrate (10.0 mg protein per rabbit)

[2]B - Rabbits which received aluminum potassium sulfate precipitated culture filtrate (10.0 mg protein per rabbit)

[3]C - Cardiocentesis

[4]Im - Intramuscular

[5]1.0 - Non-precipitated culture filtrate, 2.5 mg protein per ml

[6]Ip - Intraperitoneal

each week was analyzed by the multiple range test developed by Duncan (48). Significance was determined at the 95% level.

Ouchterlony double diffusion.

Clean 3 1/4 x 4 inch glass lantern slides were evenly covered with ten ml of melted 1% agar (Difco) in 0.15 M PBS containing 0.01% merthiolate. The gels were "aged" for at least three hours in a humidified diffusion chamber. Immediately before use, the circular reactant wells were cut and the agar plugs removed. Several different well diameters and interwell diffusion distances were tried. Subsequently, all wells of 6 mm diameter were cut with 6 mm diffusion distance between wells.

The protein concentration of the samples was adjusted to approximately 2.5 mg per ml. After the reactants were added to the wells, the slides were incubated at 28°C in a humidified diffusion chamber and observed daily. Replenishments of the reactants were made daily for three days with undiluted antiserum and 1:3 dilutions of the antigens. After incubation, the unreacted protein was removed by soaking the gel-covered plates three days in six changes of 0.015 M PBS, pH 7.4. Salts were removed by a 12 hr rinse in distiled water.

The plates were overlaid with moist filter paper, dried at 37°C overnight, and stained for ten minutes in a protein triple stain (35). The staining solution contained the following:

Thiazine red R	0.1 gm
Amidoschwarz 10 B	0.1 gm
Light green SF	0.1 gm
Acetic acid	2.0 gm
Mercuric chloride	0.1 gm
Distilled water q.s.ad.	100.0 ml

The plates were differentiated in 2% acetic acid and thoroughly rinsed in tap water.

Immunoelectrophoresis.

Immunoelectrophoresis was done according to the procedure described by Hirschfeld (66) with modifications. A 2% solution of agar (Difco) in distilled water was poured to 1 cm depth in a flat dish, allowed to solidify, and cut into 1 cm square cubes. The cubes were washed for three days in running tap water and for several days with frequent changes of distilled water, and stored in distilled water at 4°C.

A barbital buffer system, discontinuous with respect to ionicity, was used: the internal buffer solution (pH 8.6, ionicity 0.09) was used to prepare the gel; the external buffer solution (pH 8.6, ionicity 0.06) was used in the electrode vessels of the migration chamber. The buffer solutions contained the following:

	External buffer	Internal buffer
Diethylbarbituric acid	1.38 gm	1.66 gm
Sodium diethylbarbiturate	8.76 gm	10.51 gm
Distilled water q.s.ad.	1000 ml	1000 ml

Two parts of the internal buffer solution were mixed with one part of distilled water and heated in flowing steam. An equal volume of melted 2% agar containing 0.02% merthiolate was added. Two and one-half ml of the warm agar solution were spread evenly over the surface of clean 1 x 3 inch microscope slides. After solidification, the slides were "aged" at least three hours before used.

Immediately before electrophoresis, two circular antigen wells and an antiserum trough were cut in the gels with an LKB gel punch (LKB Instruments, Inc.). The antigen wells were enlarged with a blunt 12 gauge needle and removed. Five μl of the concentrated (approximately 10 mg of protein per ml) CF were placed in the antigen wells and electrophoresis begun immediately.

Electrophoresis was carried out in a Shandon migration chamber (Colab Laboratories, Inc.). The glass microscope slides were supported by a plexiglass plate placed across the bridge supports. Eight slides were used at one time; four sample slides and four blank. The latter were placed on the anode side of the plate to prevent electrodecantation of the sample slides. Electrical connection between the slides and the electrode vessels was made with buffer impregnated filter paper strips (Whatman #1). Electrophoresis was carried out at 4°C for one and one-half hours at a current of 1.25 mA per slide (eight volts per cm).

Following electrophoresis, the agar plugs in the antiserum troughs were removed and antiserum added. The slides

were incubated in a humidified diffusion chamber for 12 hours with replenishments of antiserum as needed. Incubation was continued for an additional three days at 4°C with daily addition of antiserum. The slides were washed, dried, and stained as described under Ouchterlony double diffusion.

Disc electrophoresis.

The apparatus and technique employed were similar, but slightly modified, to that described by Ornstein and Davis (112). The composition of the reagents used for preparing the gels is shown in Tables 3 and 4.

The small pore solution was prepared and placed into 0.5 x 6.3 cm glass tubes to a depth of 5.1 cm. It was overlaid carefully with distilled water and allowed to stand 45 minutes. The water was removed and replaced by a layer of large pore solution 1 cm deep. The large pore solution was carefully overlaid with water and photopolymerized for 15 minutes under a fluorescent lamp.

The gels were transferred to the upper buffer reservoir. A sample containing approximately 0.25 mg of protein in not less than 0.05 ml and not more than 0.2 ml, was layered on the spacer gel by displacement. Bromphenol marking dye was added to the upper vessel, and a constant current of 3 mA per tube applied at room temperature until the bromphenol blue marking dye had migrated 4.9 cm. The constant current was applied by a Vokam 500 mA DC power supply (Colab Laboratories, Inc.).

Table 3. Composition of the stock reagents used for disc electrophoresis.

(A)		(B)	
INHCl	48.0 ml	INHCl	48.0 ml
TRIS[1]	36.6 gm	TRIS	5.98 gm
TEMED[2]	0.23 ml	TEMED	0.46 ml
DH_2O g.s.ad.	100.0 ml	DH_2O g.s.ad.	100.0 ml
pH 8.9[3]		pH 6.7[3]	
(C)		**(D)**	
Acrylamide	28.0 gm	Acrylamide	10.0 gm
BIS[4]	0.74 gm	BIS	2.5 gm
DH_2O g.s.ad.	100.0 ml	DH_2O g.s.ad.	100.0 ml
(E)		**(F)**	
Riboflavin	4.0 mg	Ammonium persulfate	0.14 gm
DH_2O g.s.ad.	100.0 ml	DH_2O g.s.ad.	100.0 ml

[1]TRIS - Tris (hydroxymethyl) aminomethane
[2]TEMED - N, N, N', N', - tetramethylenediamine
[3]pH - Adjusted by titrating with INHCl
[4]BIS - N, N' - methylenebisacrylamide

Table 4. Composition of the working solutions used in disc electrophoresis.

Small pore solution A	Small pore solution B	Large pore solution	Stock Buffer solution for reservoirs[1]	
1 part A	4 parts F	1 part B	TRIS[2]	6.0 gm
2 parts C		2 parts D	Glycine	28.8 gm
1 part DH_2O		1 part E	DH_2O g.s.ad.	1000 ml
pH 8.9		4 parts DH_2O pH 6.7	pH 8.3	

[1]Diluted 1:10 with DH_2O before use

[2]TRIS = tris (hydroxymethyl) aminomethane

After electrophoresis, the gels were chilled and removed from the glass tubes. The proteins in the gels were stained by immersing the gels for thirty minutes in a 0.5% solution of amido black in 5% acetic acid. Excess stain was removed electrophoretically in 5% acetic acid.

Polysaccharides in the gels were stained by the periodic acid Schiff method (24). After the gels were removed from the glass tubes, the gels were immersed in 7.5% acetic acid one hour at room temperature. The gels were transferred to a 0.5% periodic acid solution for one hour at 4°C. Excess periodic acid was removed from the gels electrophoretically in 7.5% acetic acid. The polysaccharides were stained by placing the gels in cold Schiff reagent and stored in the reagent at 4°C (24).

Dialysis.

One hundred ml each of CF of three, four, and five month incubation were placed in individual dialysis tubes (Visking Corporation) and dialyzed separately for two days against three changes of 2 L each of distilled water. The retentates were concentrated ten-fold by pervaporation at room temperature and redialyzed eight days against daily changes of 100 ml each of distilled water. The dialysates from the latter were pooled, lyophylized, and reconstituted with ten ml of 0.015 M phosphate buffer pH 7.2. The retentates and reconstituted dialysates were analyzed by immunodiffusion, chromatography on Sephadex G-25, disc electrophoresis, and skin-tests in sensitized rabbits.

Ion exchange column chromatography.

Dry diethylaminoethyl cellulose powder (DEAE, Schleicher and Schuell Co.) type 20 was thoroughly mixed after settling into a 1 N solution of sodium hydroxide. The mixture was transferred to a Buchner funnel fitted with filter paper and washed repeatedly with the alkali. The filter cake was resuspended in a minimal amount of 1 N NaOH. Sufficient 1 N HCl was added to make the mixture strongly acidic. The adsorbent was washed immediately with water on a Buchner funnel, resuspended in alkali, washed thoroughly with water again, and resuspended in starting buffer solution. Excess "fines" were removed by decanting the supernatant fluid after allowing the adsorbent to settle one hour in the starting buffer solution. Concentrated phosphoric acid was added to adjust the pH of the adsorbent to 8.6. The adjusted adsorbent was washed with the starting buffer solution and resuspended to approximately a 2% suspension in the same buffer.

Sephadex laboratory columns (1.5 x 30 cm) were packed with the adsorbent until the bed level was slightly above the desired height. The packed columns were washed for several days with the starting buffer solution.

Culture filtrate from a four month old culture of M. bovis was prepared for chromatography by dialysis for 24 hours against 100 volumes of the starting buffer solution (0.005 M TRIS-phosphate pH 8.6). The sample was placed on the column and allowed to sink into the adsorbent.

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The filter pad and sides of the column were washed with several three ml portions of the starting buffer. A 5 cm layer of the starting buffer was added to the adsorbent and continuous flow (35 ml per hour) of the eluting fluid started.

Proteins were eluted from the adsorbent by continuous gradient elution. A concave salt gradient was produced by a cone-sphere buffer vessel device. This consisted of a 250 ml Erlenmeyer flask which contained 200 ml of the limit buffer solution (0.3 M TRIS phosphate pH 5.0 containing 1.6 M sodium chloride) and a 500 ml round bottomed Florence flask which contained 400 of the starting buffer solution. Five ml eluant fractions were collected, and the adsorbency of the eluant at 280 mμ was continuously measured and recorded.

Molecular exclusion chromatography.

Culture filtrates, dialysates, and retentates were fractionated chromatographically using medium grade Sephadex G-25 (Pharmacia Fine Chemicals, Inc.) and Bio-Gels P-100, P-150, and P-200 (Bio-Rad Laboratories). The dry Sephadex and Bio-Gel beads were slowly added to distilled water with constant stirring, and allowed to stand without stirring at least 48 hours before use. Excess "fines" were removed by decanting the supernatant fluid after allowing the hydrated gels to settle one hour in replenished starting buffer solution.

Columns used for chromatography with Sephadex G-25 and Bio-Gels were 2.5 x 45 cm and 1.5 x 30 cm, respectively. The inside of the columns were coated by pouring in a warm 1% solution of dimethyl-dichlorosilane in benzene. The solution was removed after several minutes and the benzene evaporated. This procedure was repeated. The gels were deaerated in a vacuum flask and gradually poured into the columns until the gel column was slightly above the desired bed level. The gel columns were washed for several days with distilled water.

Prior to sample application, the void volume of each column was determined using Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.), a high molecular weight (MW = 2,000,000) dextran polymer coupled with a blue chromatophore.

Samples were placed in the sample applicator at the top of the column and allowed to settle into the gel. The sample applicator and sides of the column were rinsed with several three ml portions of the eluent (distilled water). A 4 cm layer of eluent was placed on the gel column before continuous flow (30 ml per hour) was started. Chromatography was done at room temperature. Three ml fractions were collected, and the absorbency of the eluant at 280 mμ was measured and recorded continuously.

The contents of selected tubes were pooled, lyophilized (The Virtis Co.) and reconstituted to one-half the original volume. The concentrated fractions obtained from several

fractionations were pooled and rechromatographed. The contents of appropriate tubes were pooled, lyophylized, reconstituted to the original sample volume, and stored at -80°C. Designated fractions were analyzed by immunodiffusion, cellulose acetate membrane electrophoresis, disc electrophoresis, skin tests in sensitized rabbits, and analyzed for protein, carbohydrate, and nucleic acid.

Cellulose acetate membrane electrophoresis.

Culture filtrates and rechromatographed fractions from Sephadex G-25 were analyzed by cellulose acetate membrane electrophoresis. Oxoid 2.5 x 12 cm cellulose acetate membranes were supported in a Shandon migration chamber (Colab Laboratories, Inc.). Current was supplied by a Vokam constant current 500 mA DC power supply. The barbital acetate buffer described by Owen (115) with calcium lactate omitted was employed. The buffer solution, pH 8.6, ionicity 0.07, contained the following:

Sodium diethylbarbiturate	5.0 gm
Sodium acetate (anhydrous)	3.25 gm
1 N Hydrochloric acid approximately	34 ml[1]
Distilled water q.s.ad.	1000 ml

Five µl samples were applied directly over the cathode on each buffer impregnated strip and electrophoresed at a current of 1 mA per strip for two hours at 4°C. The strips were stained overnight in 0.001% nigrosin in 2% acetic acid.

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[1]pH adjusted by titrating with 1 N HCl.

RESULTS

Antibody responses of rabbits to mycobacterial cells and culture filtrates.

The antibody titers of sera from rabbits inoculated with CF in Freund's incomplete adjuvant or with CF+ killed cells of *M. bovis* are shown in Tables 5 and 6. Mercaptoethanol-sensitive (MES) and mercaptoethanol-resistant (MER) passive-hemagglutinin and agglutinin titers are given in Tables 7 and 8. Many of the rabbits produced antibodies detectable by both methods one week after inoculation and all but three rabbits by two weeks post-inoculation (Figs. 1 through 16). All of the detectable antibody produced during the first two weeks post-inoculation was MES. Some sera obtained three weeks post-inoculation contained MES antibody; all sera except from Group V rabbits contained MER antibody at four weeks post-inoculation.

Group I rabbits produced significantly more MES bacterial agglutinins for the first two weeks than rabbits in Group II and III (Figs. 2, 4, and 6); there were no significant differences among MES hemagglutinin titers. Detectable bacterial agglutinins and hemagglutinins were not produced by rabbits in Group II until two and three weeks post-inoculation, respectively, and there were not significant differences between the bacterial agglutinin and hemagglutinin titers at any week.

Table 5. Mean titers[1] of polysaccharide-specific antibody produced by rabbits inoculated with killed cells and/or *Mycobacterium bovis* culture filtrate.

Table 5. Mean titers[1] of polysaccharide-specific antibody produced by rabbits inoculated with killed cells and/or Mycobacterium bovis culture filtrate.

Group[2]	WEEKS AFTER INOCULATION 1	2	3	4	5	6	7	8
I	3.3	4.0	6.7	6.7	7.0	6.7	7.0	5.7
II	0	4.3	6.0	7.0	7.3	7.0	6.7	7.7
III	3.0	5.3	7.0	7.7	7.7	8.0	7.7	7.7
IV	0.7	4.3	5.3	6.0	6.7	7.3	7.7	7.7
V	2.0	3.3	4.0	4.0	4.0	3.3	3.0	3.7
VI	4.0	8.0	8.0	8.0	8.0	7.0	6.0	4.5
VII	2.0	3.7	6.0	7.3	7.3	6.3	6.0	6.0
VIII	4.3	4.7	5.7	6.3	7.7	8.0	8.3	7.0

[1]Titers 0, 10, 20, 40, etc., to 5120 are coded as the serum dilution number 0, 1, 2, 3 etc., to 10, respectively.

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

Table 6. Mean titers[1] of bacterial agglutinins produced by rabbits inoculated with killed cells and/or *Mycobacterium bovis* culture filtrate.

Group[2]	WEEKS AFTER INOCULATION 1	2	3	4	5	6	7	8
I	2.0	2.7	3.3	5.0	5.7	6.0	6.3	7.0
II	0	0	1.0	3.7	5.3	6.7	7.0	7.0
III	0.7	1.3	3.7	5.0	5.7	6.3	7.0	7.3
IV	0.7	0.7	2.0	2.7	4.7	5.3	6.3	7.0
V	0.3	0.7	1.7	2.3	3.3	3.7	5.0	5.0
VI	3.0	3.0	4.5	4.5	5.5	5.5	5.0	4.5
VII	0	0.7	3.7	5.3	5.3	6.3	7.3	6.3
VIII	2.0	3.0	3.7	4.0	5.0	6.0	6.3	6.7

[1]Titers 0, 10, 20, 40 etc., to 5120 are coded as the serum dilution number 0, 1, 2, 3, etc., to 10 respectively.

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

Table 7. Mean titers[1] of mercaptoethanol-sensitive and mercaptoethanol-resistant polysaccharide-specific antibody produced by rabbits inoculated with killed cells and/or Mycobacterium bovis culture filtrate.

	WEEKS AFTER INOCULATION															
	1		2		3		4		5		6		7		8	
Group[2]	S[3]	R[4]	S	R	S	R	S	R	S	R	S	R	S	R	S	R
I	3.3	0	3.3	0.7	6.7	0	2.3	4.3	1.7	5.3	0.3	6.3	0.3	6.7	0	5.7
II	0	0	4.0	0	2.3	3.7	0	7.0	0.7	6.3	0	7.0	0	6.7	0	7.7
III	3.0	0	5.3	0	3.3	3.7	1.0	6.3	1.0	6.7	1.0	7.0	0	7.3	0.7	7.0
IV	0.7	0	4.3	0	4.6	0.7	1.7	4.3	1.3	5.3	0.7	6.7	1.0	6.7	0.3	7.3
V	2.0	0	3.3	0	4.0	0	4.0	0	4.0	0	2.7	0.7	1.3	1.7	1.7	2.0
VI	4.0	0	8.0	0	5.0	3.0	3.5	4.5	5.5	2.5	5.5	1.5	3.0	3.0	2.5	2.0
VII	2.0	0	3.7	0	5.0	1.0	1.3	6.0	2.3	5.0	0.3	6.0	0	6.0	0.3	5.7
VIII	4.3	0	4.7	0	4.0	1.7	2.7	3.7	3.0	4.7	1.7	6.3	1.3	7.0	0	7.0

[1]Titers 0, 10, 20, 40, etc., to 5120 are coded as the serum dilution number 0, 1, 2, 3, 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 8. Mean titers[1] of mercaptoethanol-sensitive and mercaptoethanol-resistant bacterial agglutinins produced by rabbits inoculated with killed cells and/or Mycobacterium bovis culture filtrate.

	WEEKS AFTER INOCULATION															
	1		2		3		4		5		6		7		8	
Group[2]	S[3]	R[4]	S	R	S	R	S	R	S	R	S	R	S	R	S	R
I	2.0	0	2.3	0.3	1.3	2.0	0.7	4.3	1.0	4.7	0.3	5.7	0.7	5.7	0.3	6.7
II	0	0	0	0	0.7	0.3	0.3	3.3	0.7	4.7	0.3	6.3	0	7.0	0	7.0
III	0.7	0	0.7	0.7	1.7	2.0	0	5.0	0.3	5.3	0	6.3	0	7.0	0	7.3
IV	0.7	0	0.7	0	1.3	0.7	0.3	2.3	0.3	4.3	0.3	5.0	0	6.3	0	7.0
V	0.3	0	0.7	0	1.7	0	1.7	0.7	1.3	2.0	0.3	3.3	0.3	4.7	0	5.0
VI	3.0	0	3.0	0	4.5	0	0.5	4.0	1.0	4.5	0.5	5.0	0	5.0	0	4.5
VII	0	0	0.7	0	3.3	0.3	1.3	4.0	1.0	4.3	0.3	6.0	1.0	6.3	0.3	6.0
VIII	2.0	0	3.0	0	3.3	0.3	0.7	3.3	0.3	4.7	0	6.0	0	6.3	0	6.7

[1]Titers 0, 10, 20, 40, etc., to 5120 are coded as the serum dilution number 0, 1, 2, 3, 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

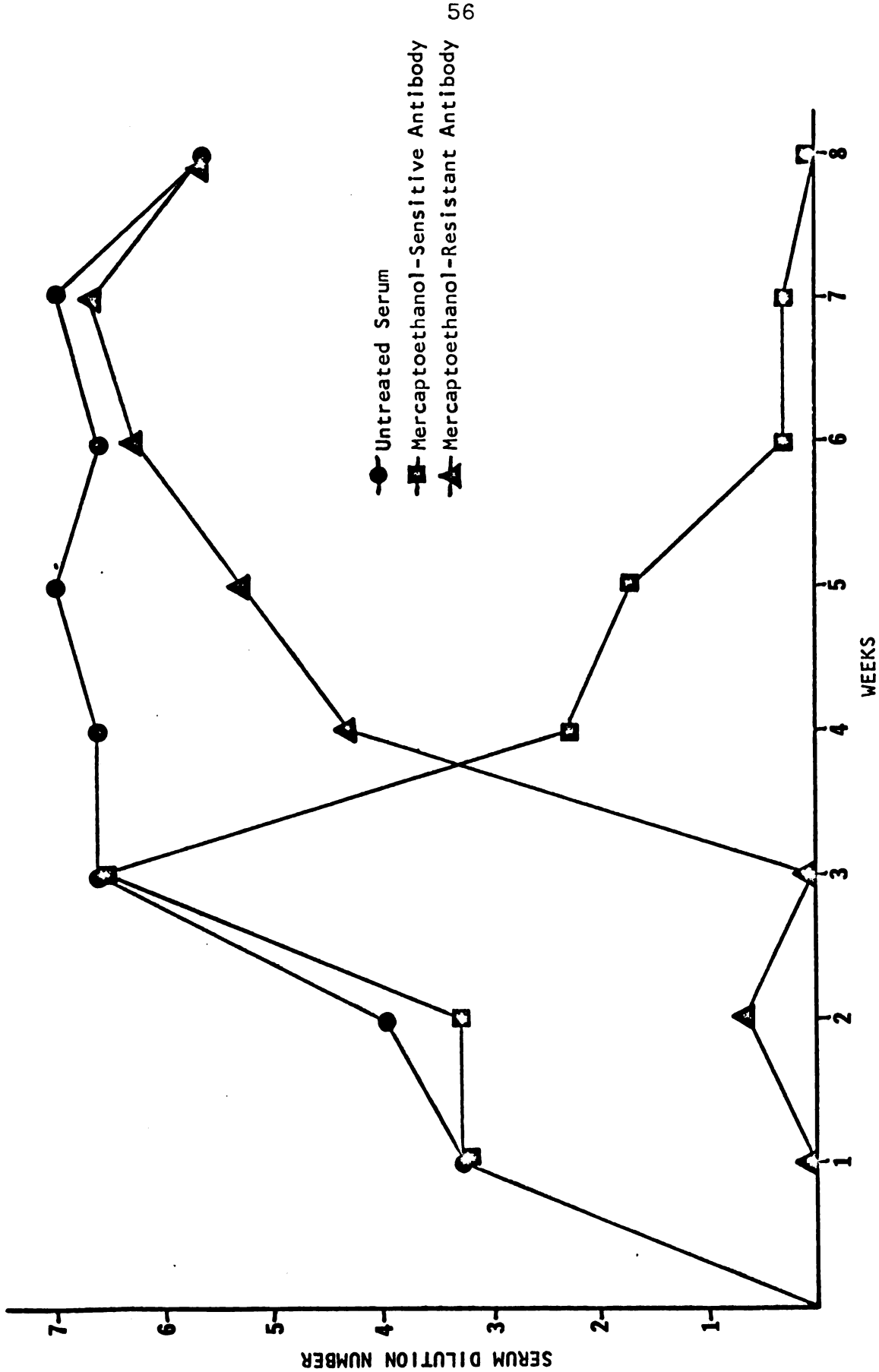

Figure 1. Hemagglutinins produced by rabbits in Group I (Table 1).

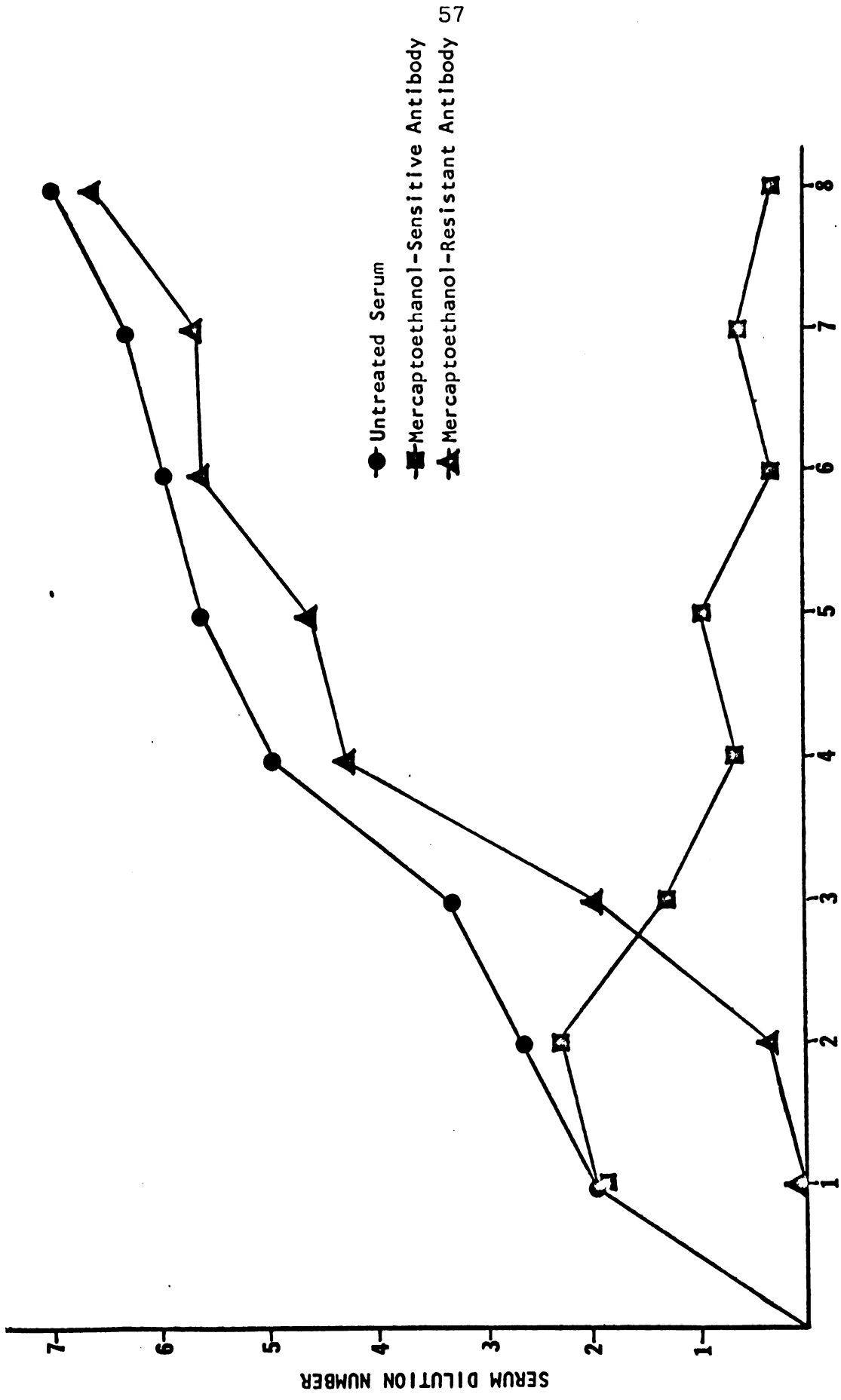

Figure 2. Bacterial agglutinins produced by rabbits in Group I (Table 1).

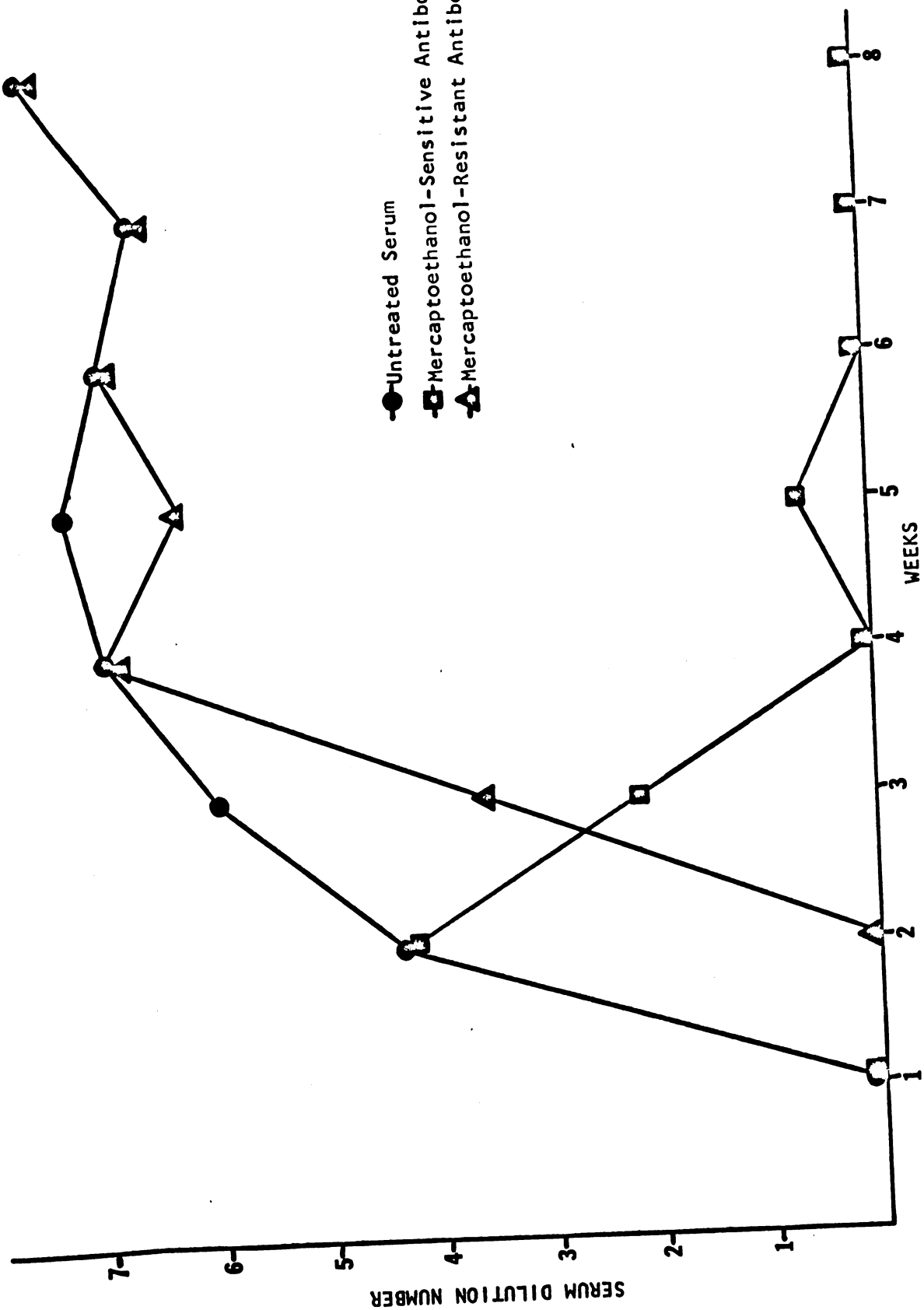

Figure 3. Hemagglutinins produced by rabbits in Group II (Table 1).

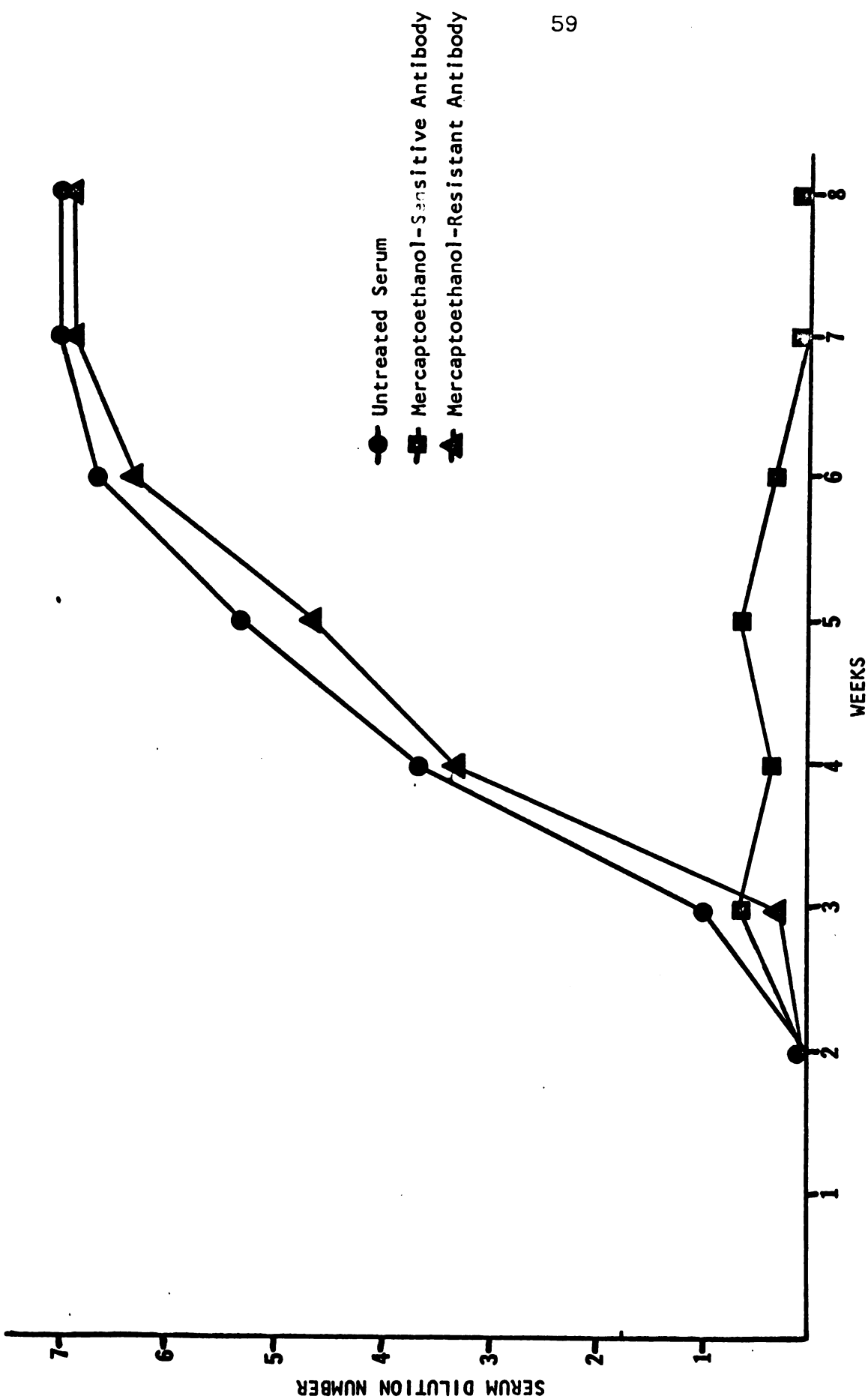

Figure 4. Bacterial agglutinins produced by rabbits in Group II (Table I).

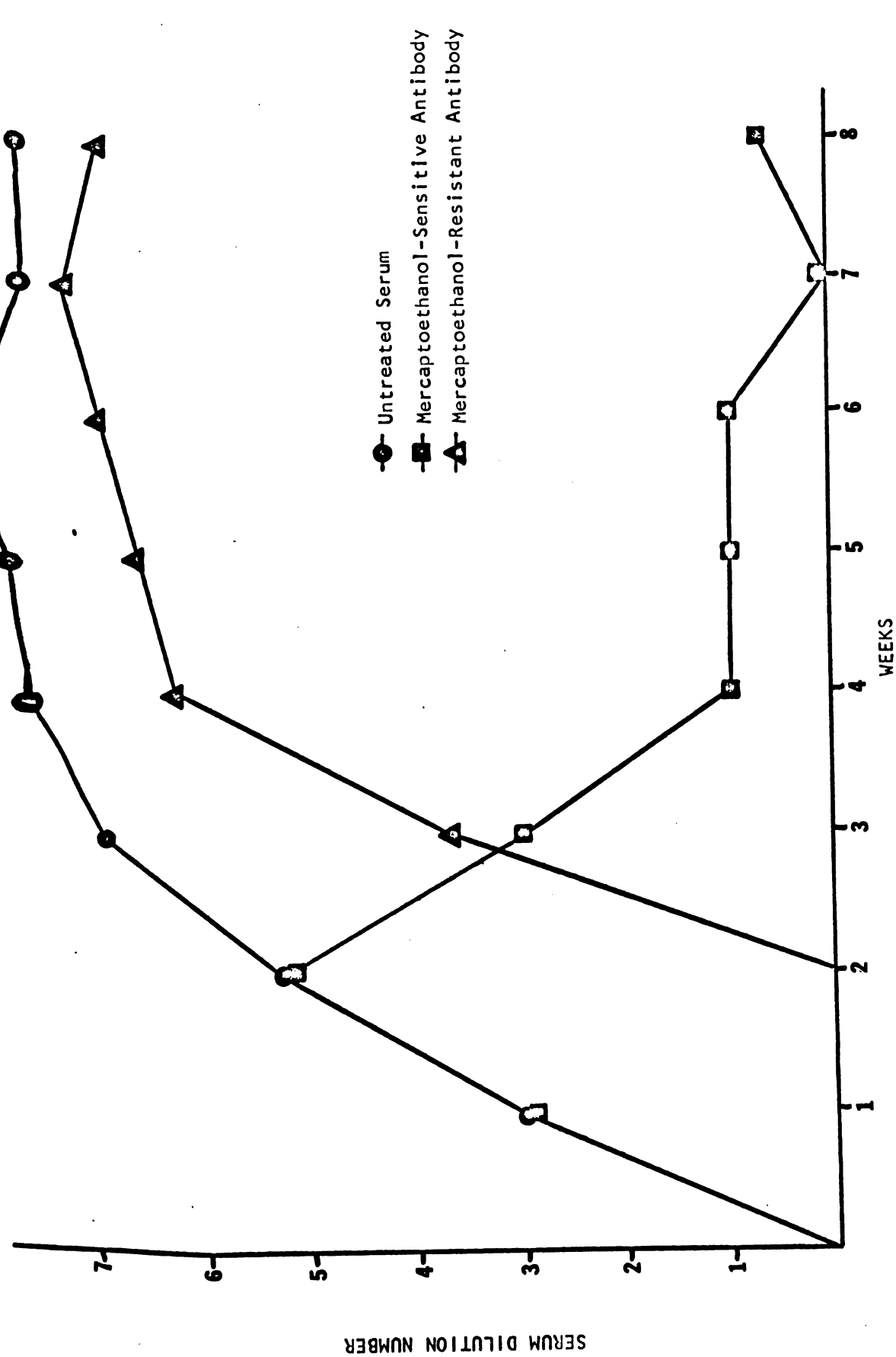

Figure 5. Hemagglutinins produced by rabbits in Group III (Table I).

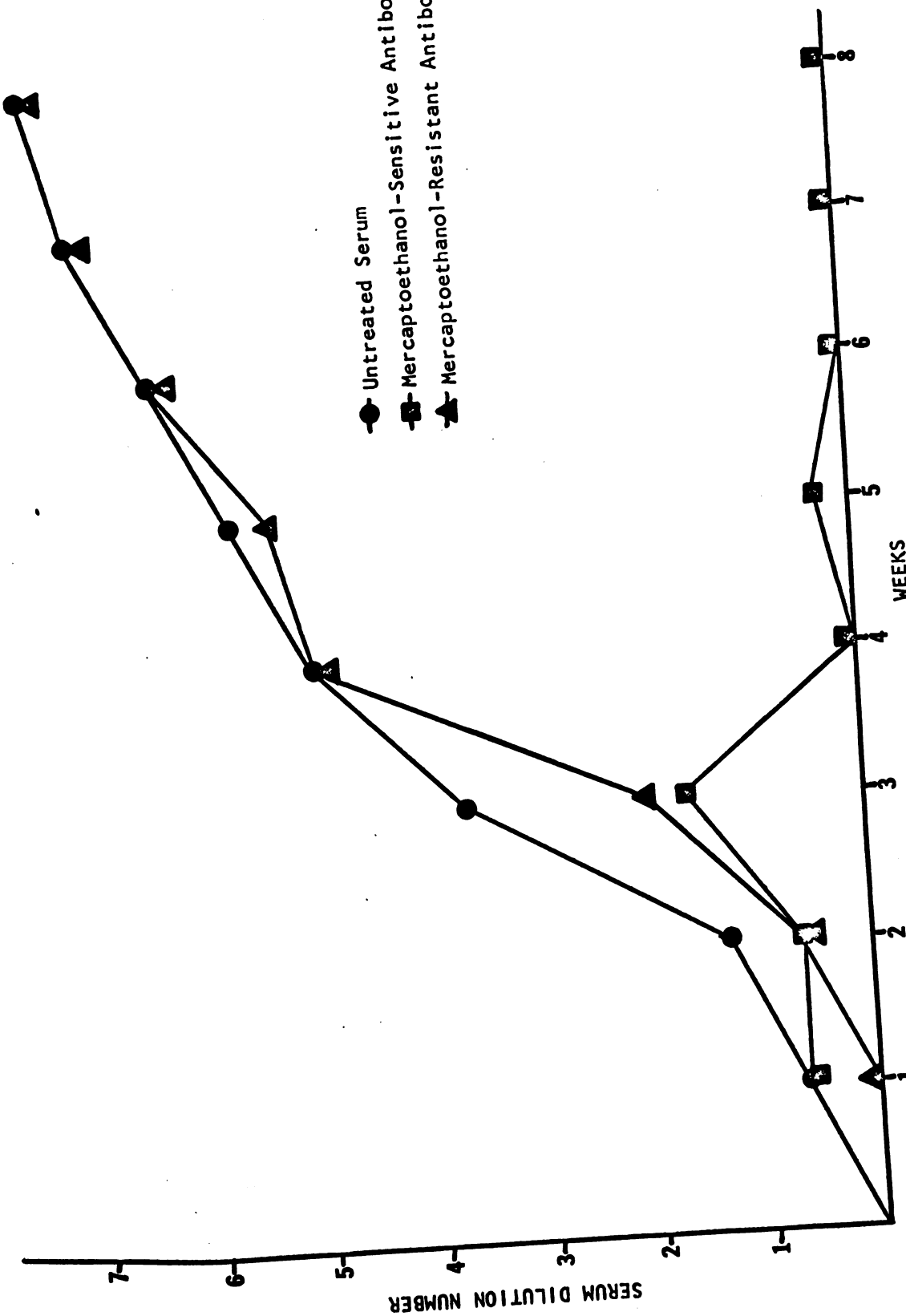

Figure 6. Bacterial agglutinins produced by rabbits in Group III (Table 1).

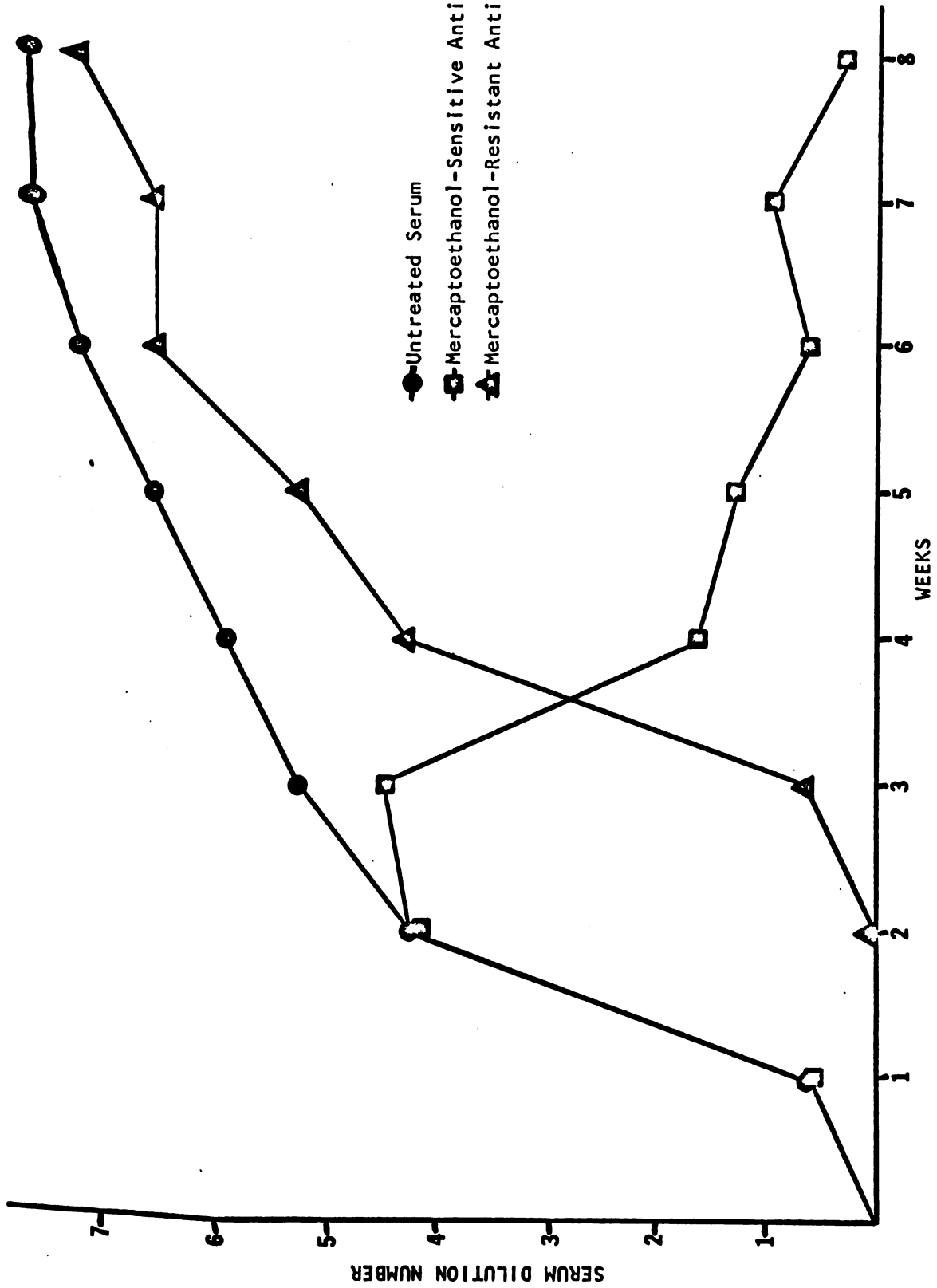

Figure 7. Hemagglutinins produced by rabbits in Group IV (Table 1).

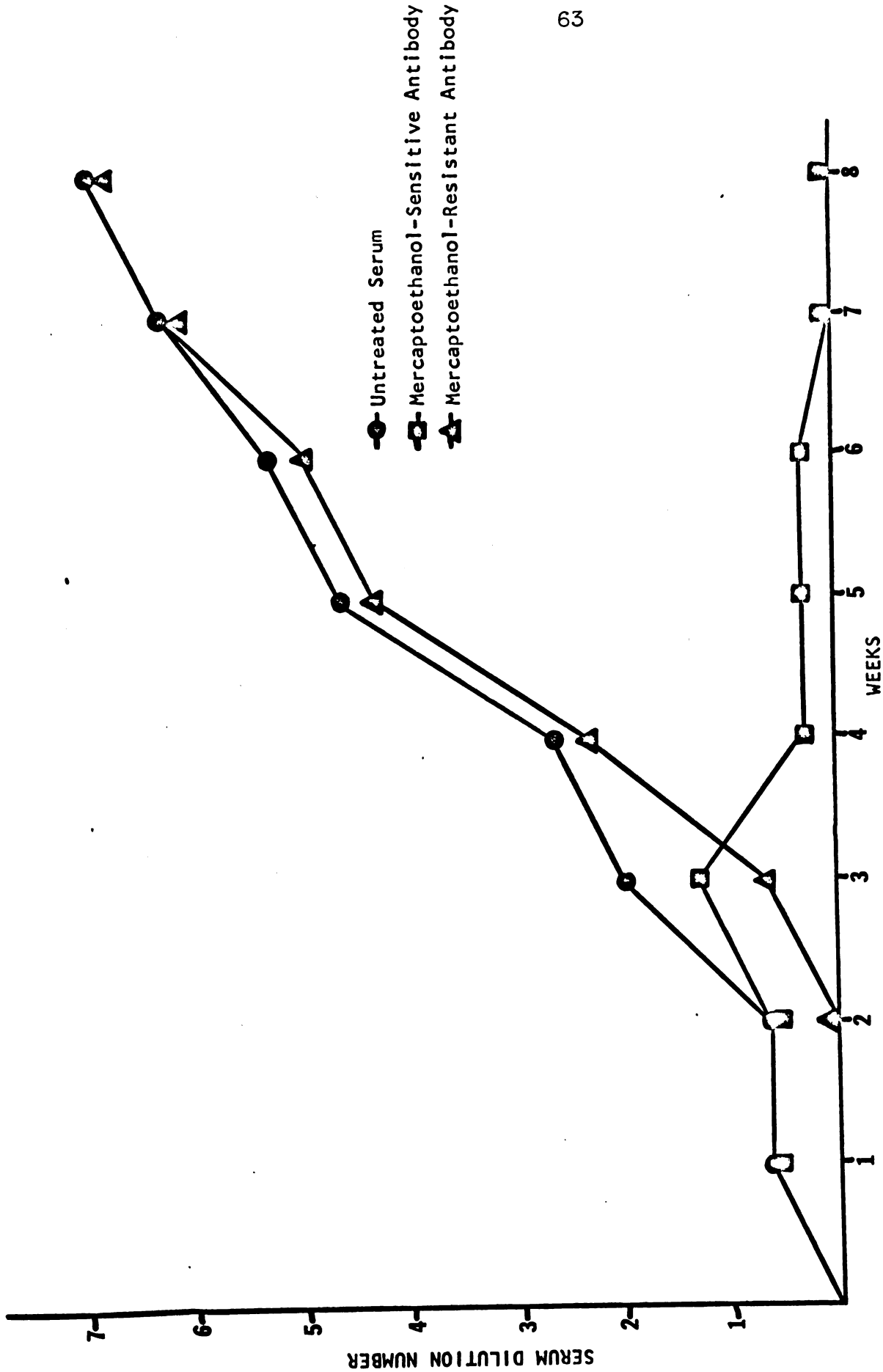

Figure 8. Bacterial agglutinins produced by rabbits in Group IV (Table 1).

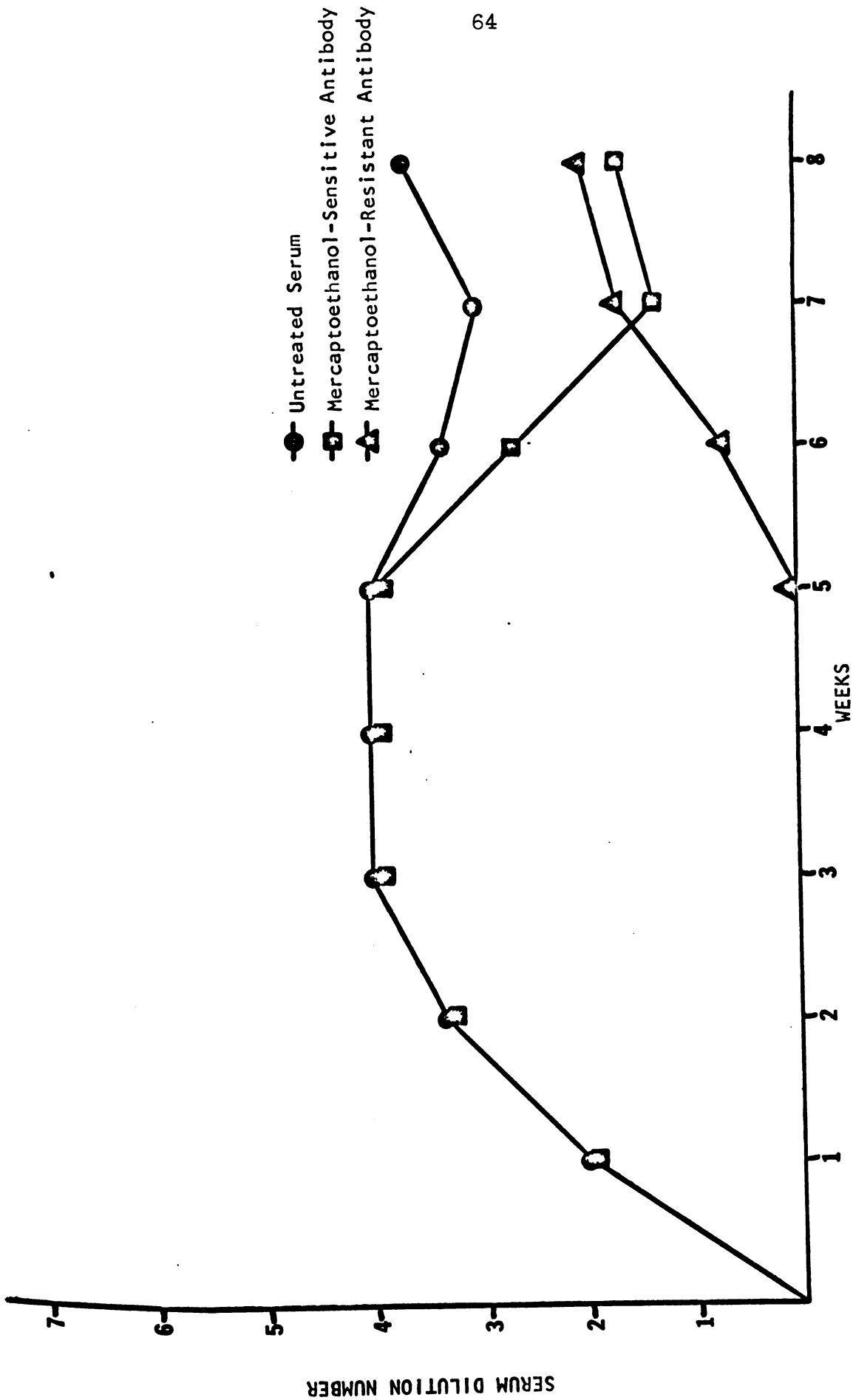

Figure 9. Hemagglutinins produced by rabbits in Group V (Table 1).

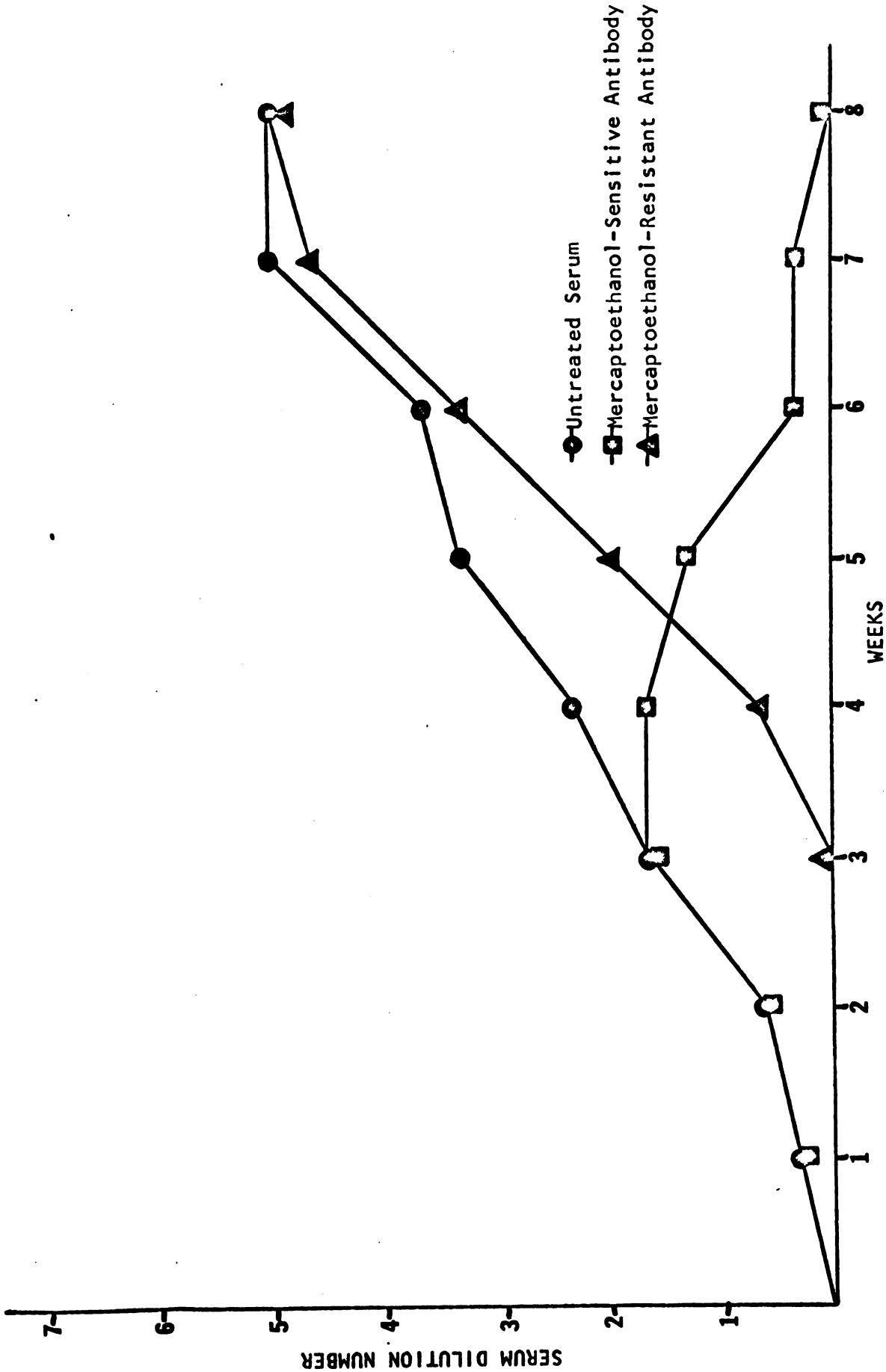

Figure 10. Bacterial agglutinins produced by rabbits in Group V (Table 1).

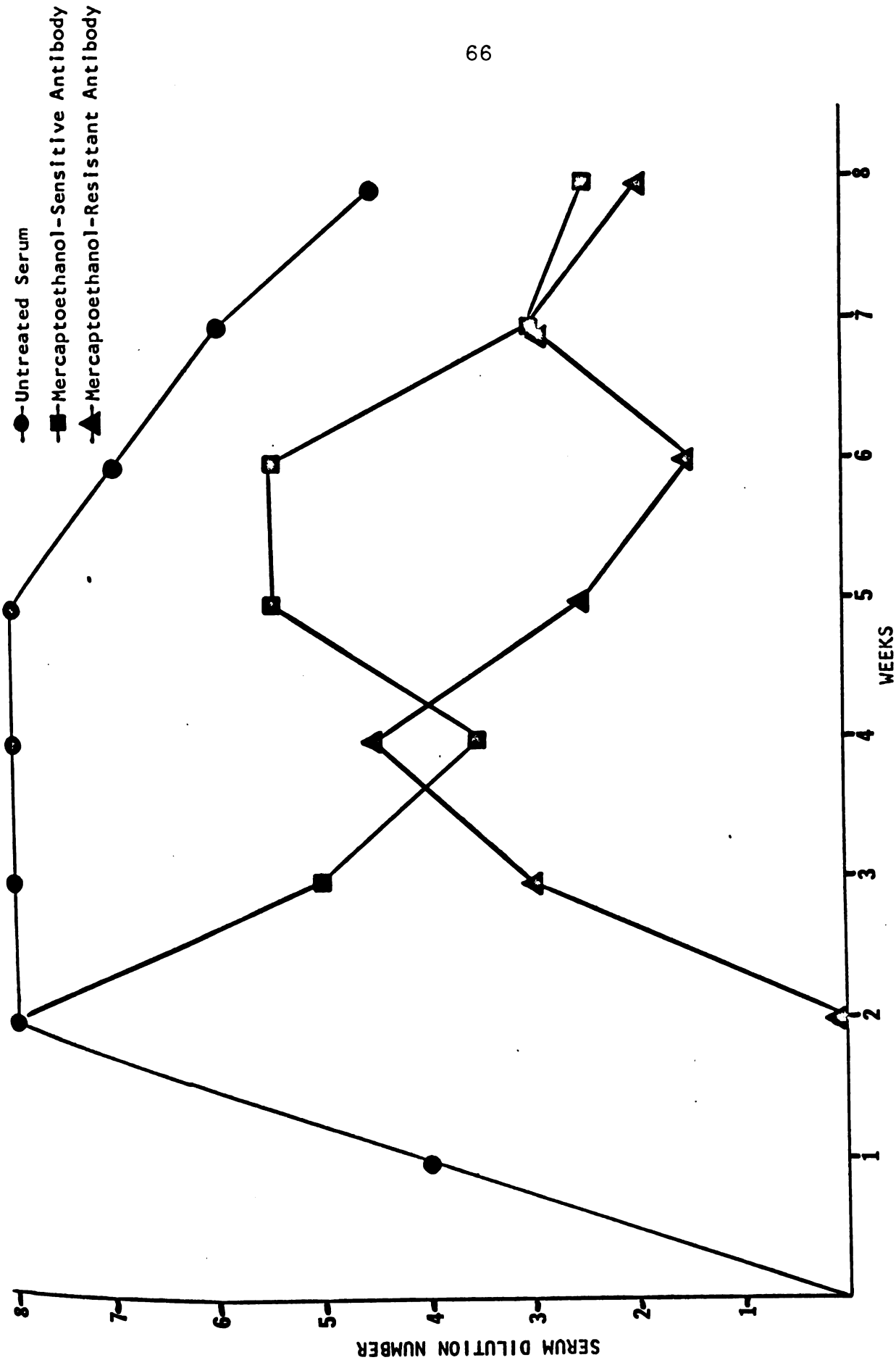

Figure 11. Hemagglutinins produced by rabbits in Group VI (Table 1).

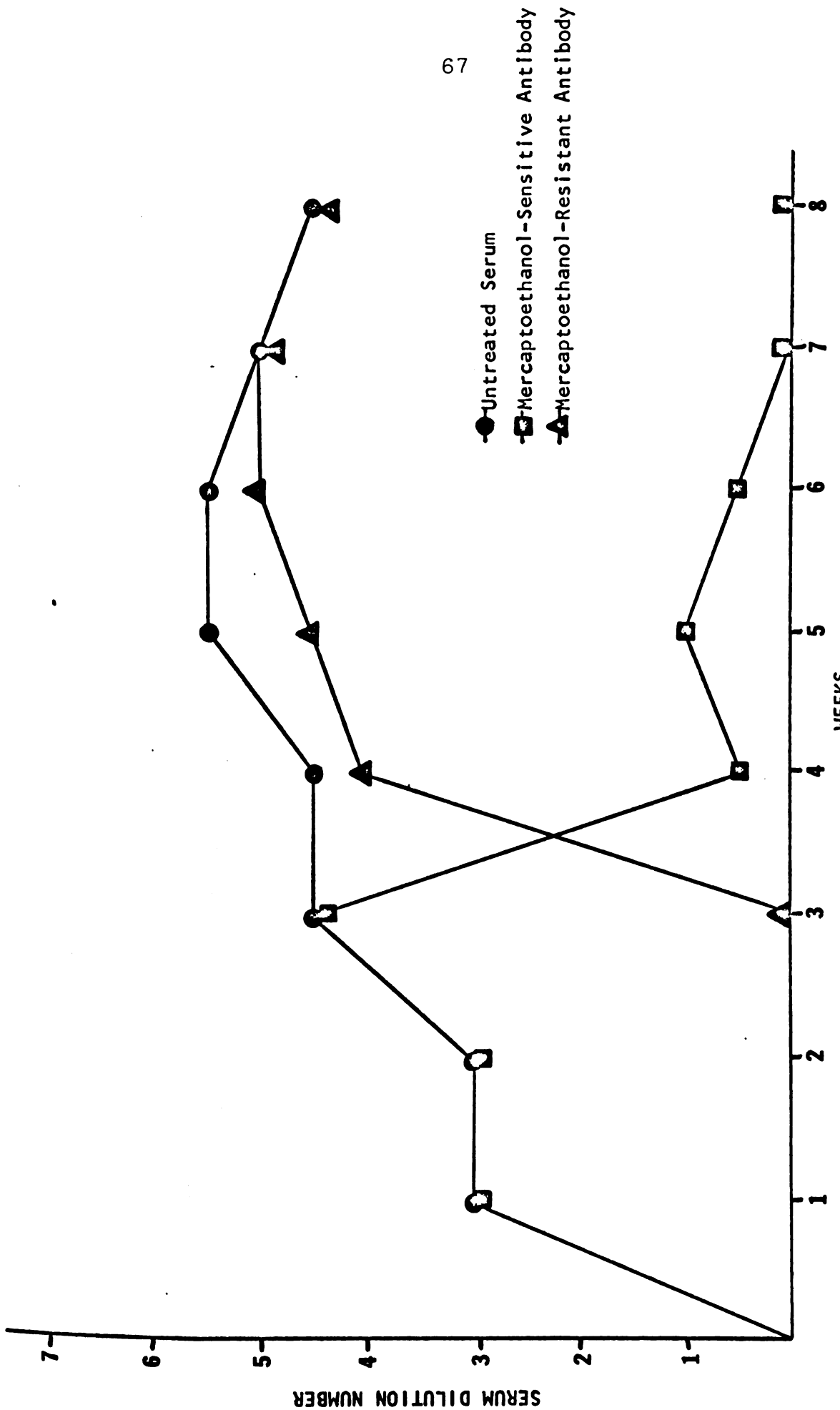

Figure 12. Bacterial agglutinins produced by rabbits in Group VI (Table 1).

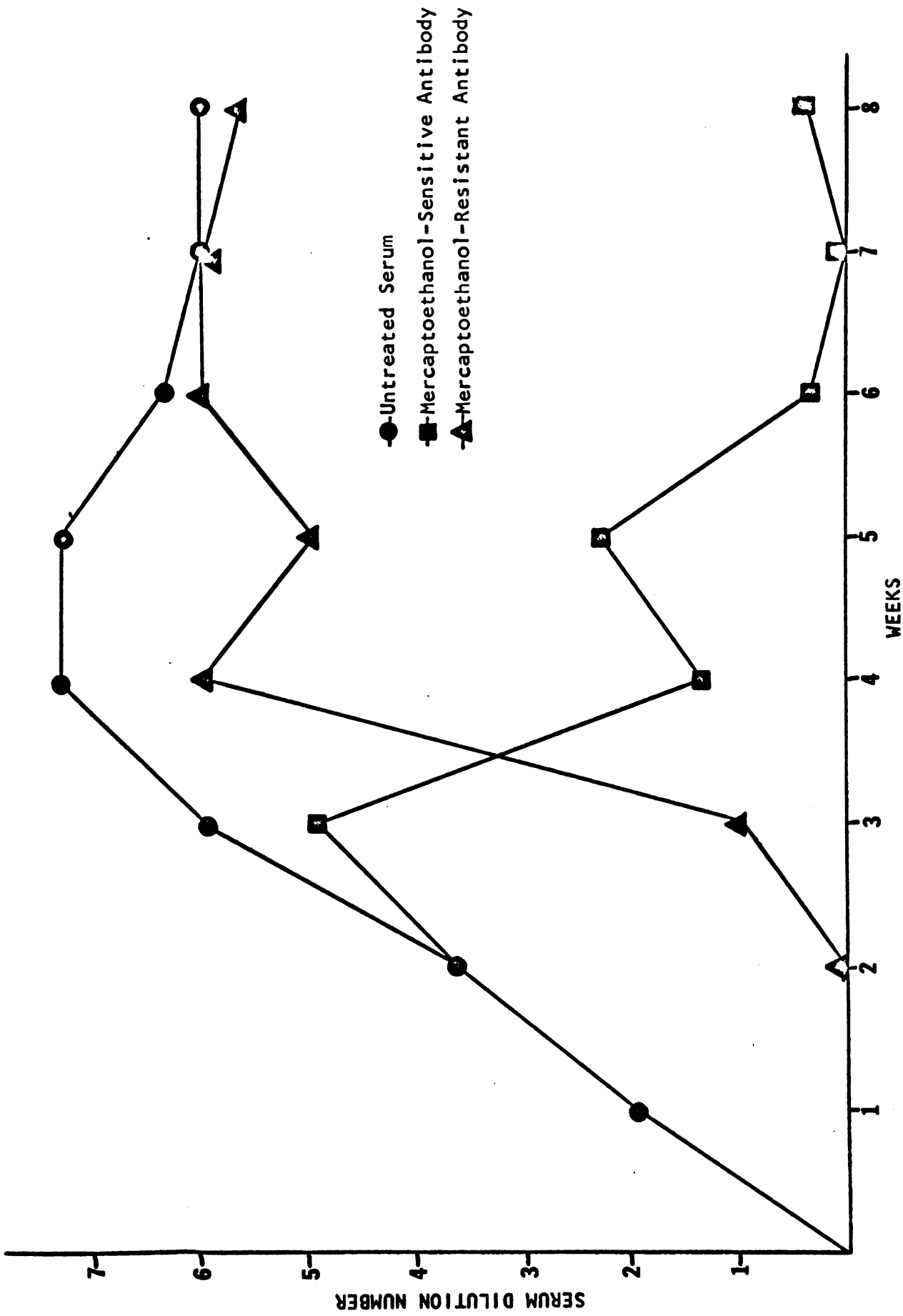

Figure 13. Hemagglutinins produced by rabbits in Group VII (Table 1).

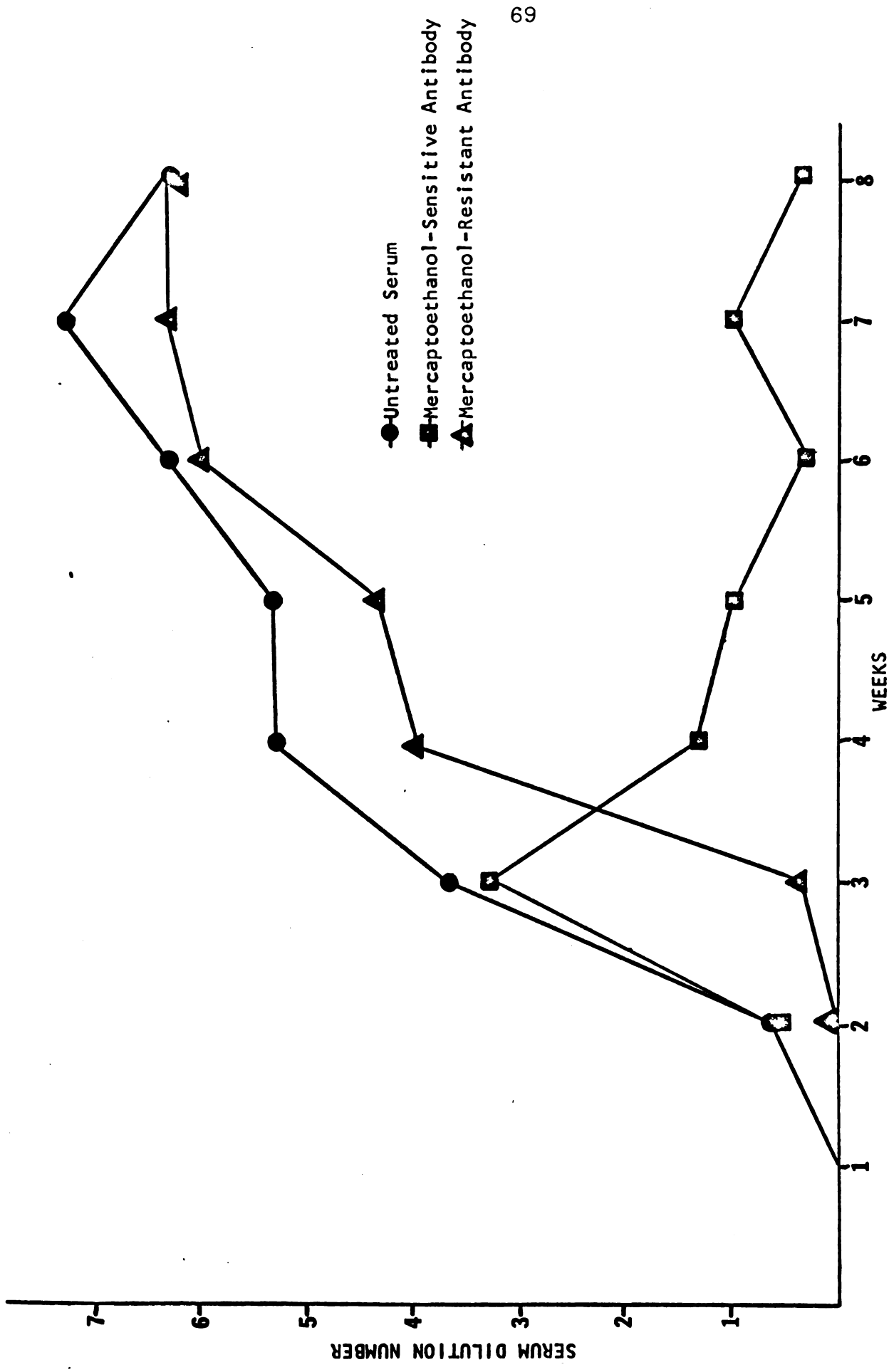

Figure 14. Bacterial agglutinins produced by rabbits in Group VII (Table 1).

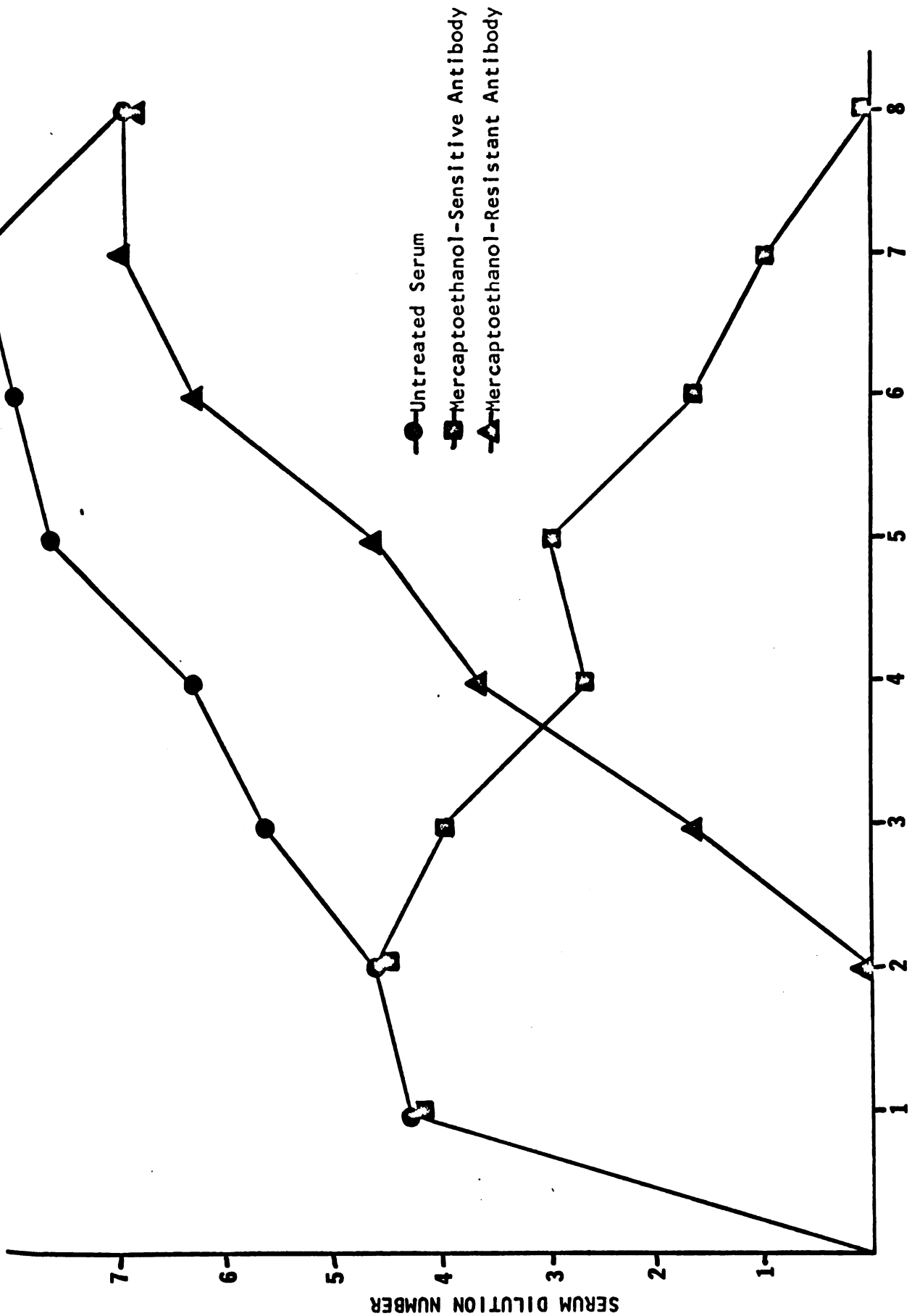

Figure 15. Hemagglutinins produced by rabbits in Group VIII (Table I).

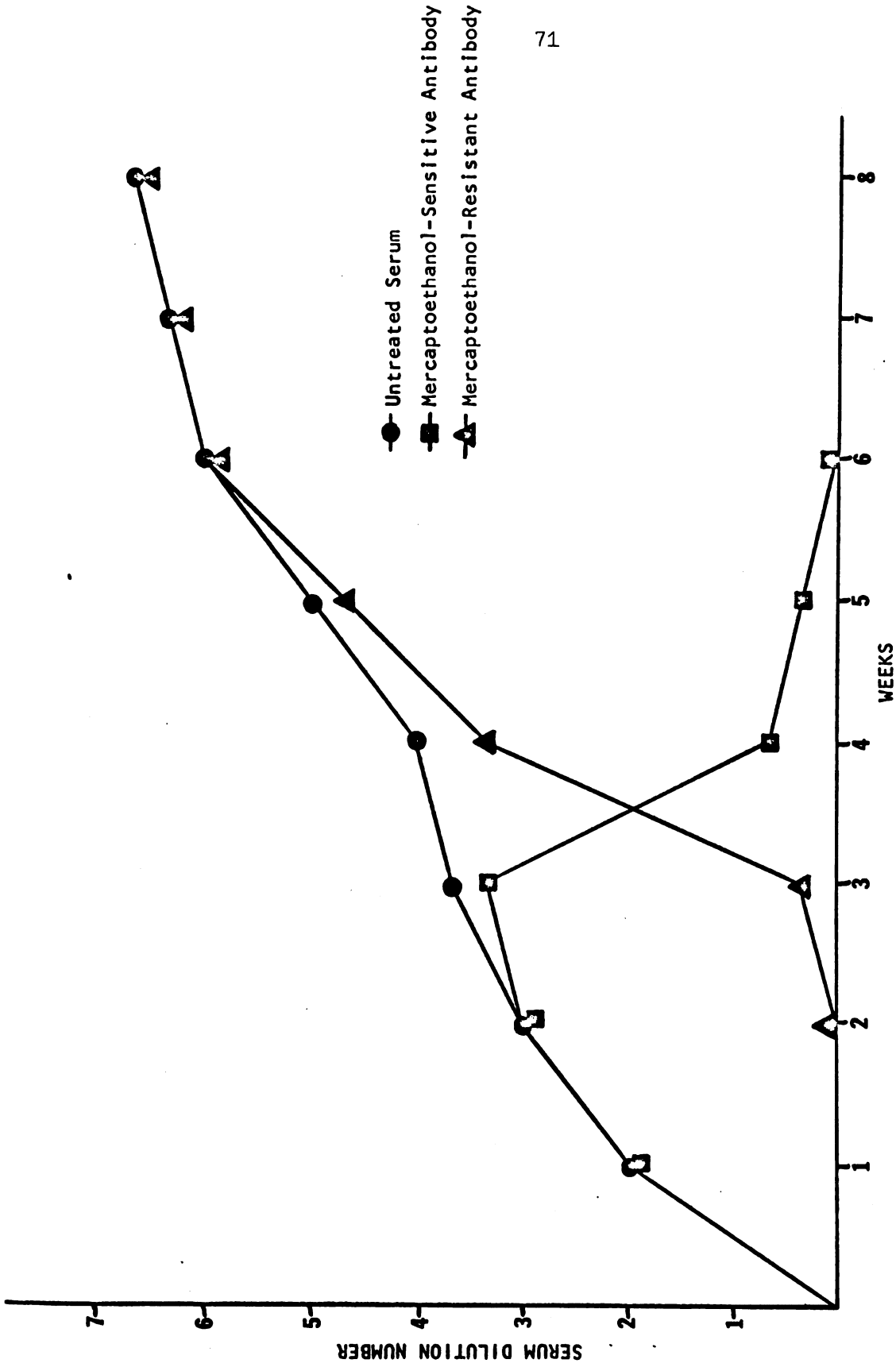

Figure 16. Bacterial agglutinins produced by rabbits in Group VIII (Table 1).

An early and sustained production of MES hemagglutinins was elicited with CF in incomplete Freund's adjuvant (Group 1). After three weeks an increasing amount of MER hemagglutinins were produced (Fig. 1). In contrast, rabbits which received CF+ killed cells (Groups II and III) produced less MES hemagglutinins and for a shorter time (Figs. 3 and 5), and MER antibody was detected one week earlier in these groups and in significantly higher titer. The sequential production of MES and MER bacterial agglutinins was similar in all three groups (Figs. 2, 4, and 6).

Hemagglutinins produced by rabbits in Group V were predominately MES (Fig. 9); mercaptoethanol-resistant hemagglutinins were not detected until six weeks post-inoculation and only in one serum. The temporal sequence of the production of bacterial agglutinins was similar to that of hemagglutinins. However, a stronger and earlier MER antibody response occurred (Fig. 10). Sera from all of the rabbits in this group contained MES antibody throughout the experiment.

There were no significant differences in the temporal sequence or the amount of MES or MER antibodies elicited by CF with heat-killed (Group II) or BPL-killed (Group III) M. bovis (Tables 7 and 8). Antibody was detected one week earlier in the sera from rabbits in Group III. Multiple injections of CF+ BPL-killed M. bovis (Group III) elicited MES and MER hemagglutinins one week earlier than sequential injections of the same antigen in Group IV (Figs. 5 and 7).

The amount of MES and MER antibody produced by both groups was not significantly different.

Repeated intravenous injections of CF with BPL-killed M. bovis elicited the highest titers of MES antibody (Tables 7 and 8). Mercaptoethanol-resistant bacterial agglutinins and hemagglutins were not detected until three and four weeks respectively, post-inoculation (Figs. 11 and 12). Mercaptoethanol-sensitive hemagglutinins were produced throughout the duration of the experiment; MES agglutinins were not detected after the six week period.

The antibody response of rabbits which received sequential or multiple injections of antigens without an intravenous injection of CF, Groups III and IV, differed from those which received an initial injection (Groups VII and VII)(Figs. 13 and 15). The peak hemagglutinin titer was produced one week later, and less MER hemagglutinins were produced by Group III rabbits than by Group VII. There were no significant differences between titers of bacterial agglutinins. Rabbits in Group VIII produced more MES hemagglutinins for the first six weeks post-inoculation, less MER hemagglutinins at 4, 5, and 6 weeks post-inoculation, and more bacterial agglutinins for the first four weeks than rabbits in Group IV (Tables 7 and 8).

Rabbits in all groups except Group V produced precipitins. Two to four immunoprecipitates were formed with CF.

Precipitins elicited by alum-precipitated and aluminum chloride-precipitated culture filtrate.

From 12 to 15 immunoprecipitates were visible in immunoelectrophorograms of CF with both antisera. No consistent differences were observed in the number or displacement of the lines formed with the antisera obtained from rabbits inoculated with alum-precipitated CF or aluminum chloride-precipitated CF.

Chemical analyses of culture filtrates.

The concentration of protein, polysaccharide, and nucleic acids, and the ultraviolet absorbancy ratio of the culture filtrates at 280 and 260 mμ are shown in Table 9. Culture filtrates B and C contained the greatest amounts of protein, polysaccharide and nucleic acid. The 280/260 absorbancy ratio was approximately the same for all of the culture filtrates. A representative ultraviolet absorption spectrum of one of the culture filtrates (CF-C) is shown in Fig. 17. The peak absorption occurred at approximately 210 and 270 mμ. Similar absorption spectra were obtained for all of the culture filtrates.

Chromatography of culture filtrates.

The culture filtrates were separated into three or four major 280 mμ-absorbing fractions by molecular exclusion chromatography on Sephadex G-25 (Figs. 18, 19, 20, 21, 22, and 23). The chemical composition of the major fractions from the individual culture filtrates are shown in Tables 10,11,12,13,14, and 15. The chromatogram of CF-A contained

Table 9. Chemical composition of the six unheated, ten fold concentrated Mycobacterium bovis culture filtrates.

CF[1]	Age[2] (months)	280/260[3]	FRM[4] (mg/ml)	TRM[5] (mg/ml)	NA[6] (mg/ml)
A	2	0.61	2.9	0.63	0.81
B	3	0.68	8.8	1.26	1.16
C	4	0.72	10.7	1.03	0.62
D	5	0.66	3.3	0.48	0.37
E	6	0.67	6.5	0.55	0.31
F	7	0.66	6.5	0.37	0.42

[1]CF - Culture filtrate

[2]Age - Length of the incubation period

[3]280/260 - Ratio of the ultraviolet absorbancy at 280 mu and 260 mu

[4]FRM - Folin-reacting material

[5]TRM - Thymol-reacting material

[6]NA - Nucleic acid

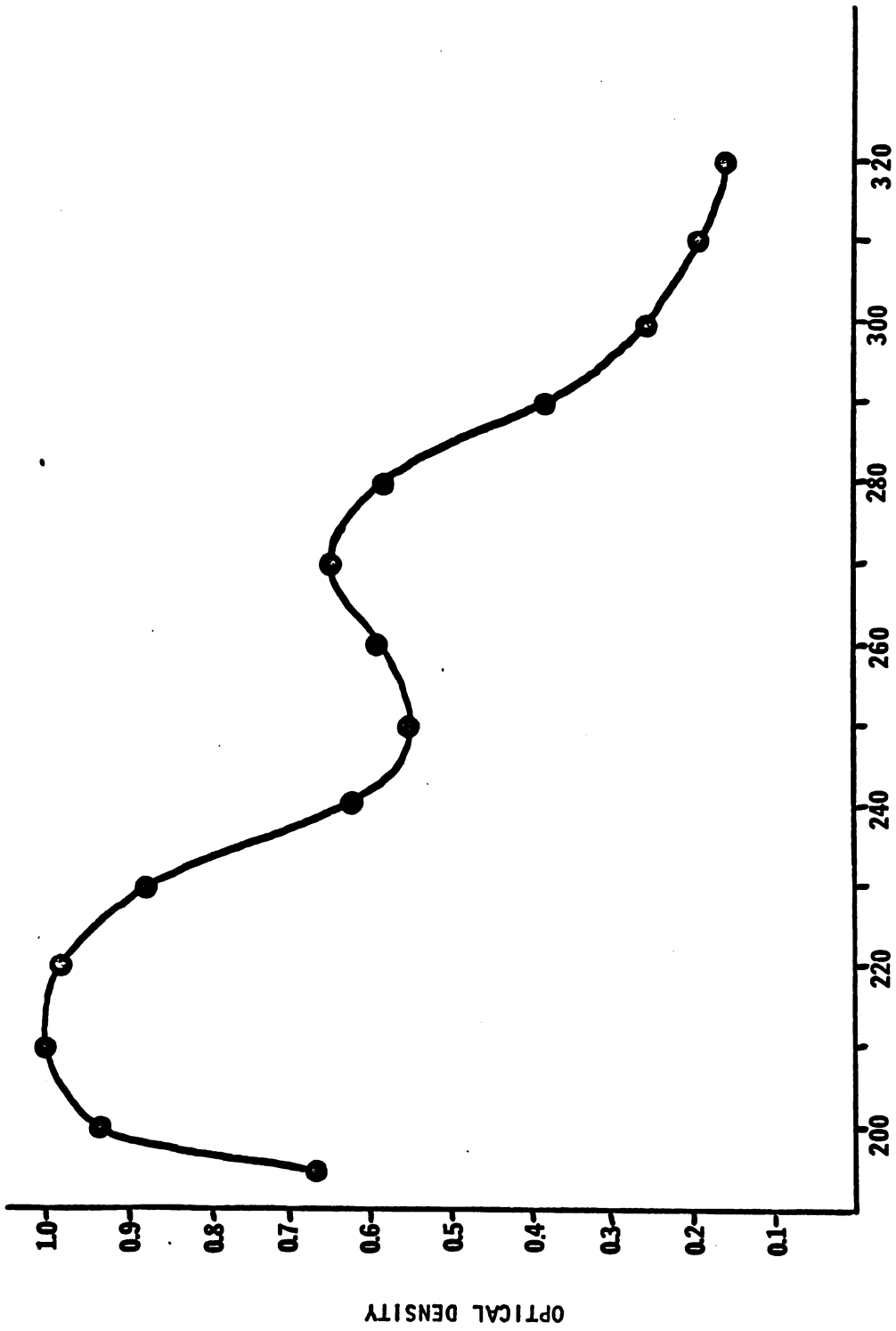

Figure 17. Ultraviolet absorption spectrum of Mycobacterium bovis culture filtrate C (Table 9).

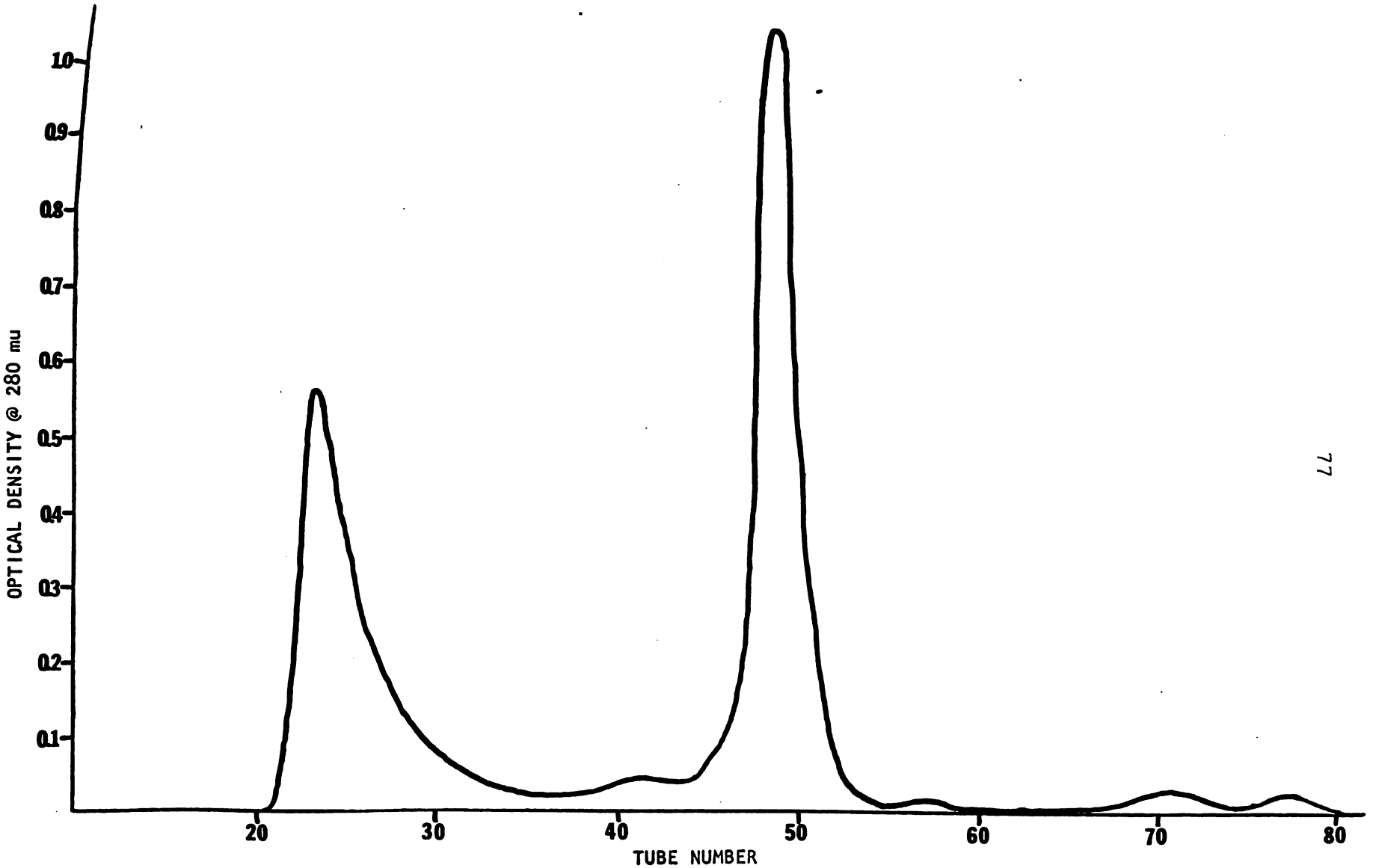

Figure 18. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate A (Table 9) on Sephadex G-25.

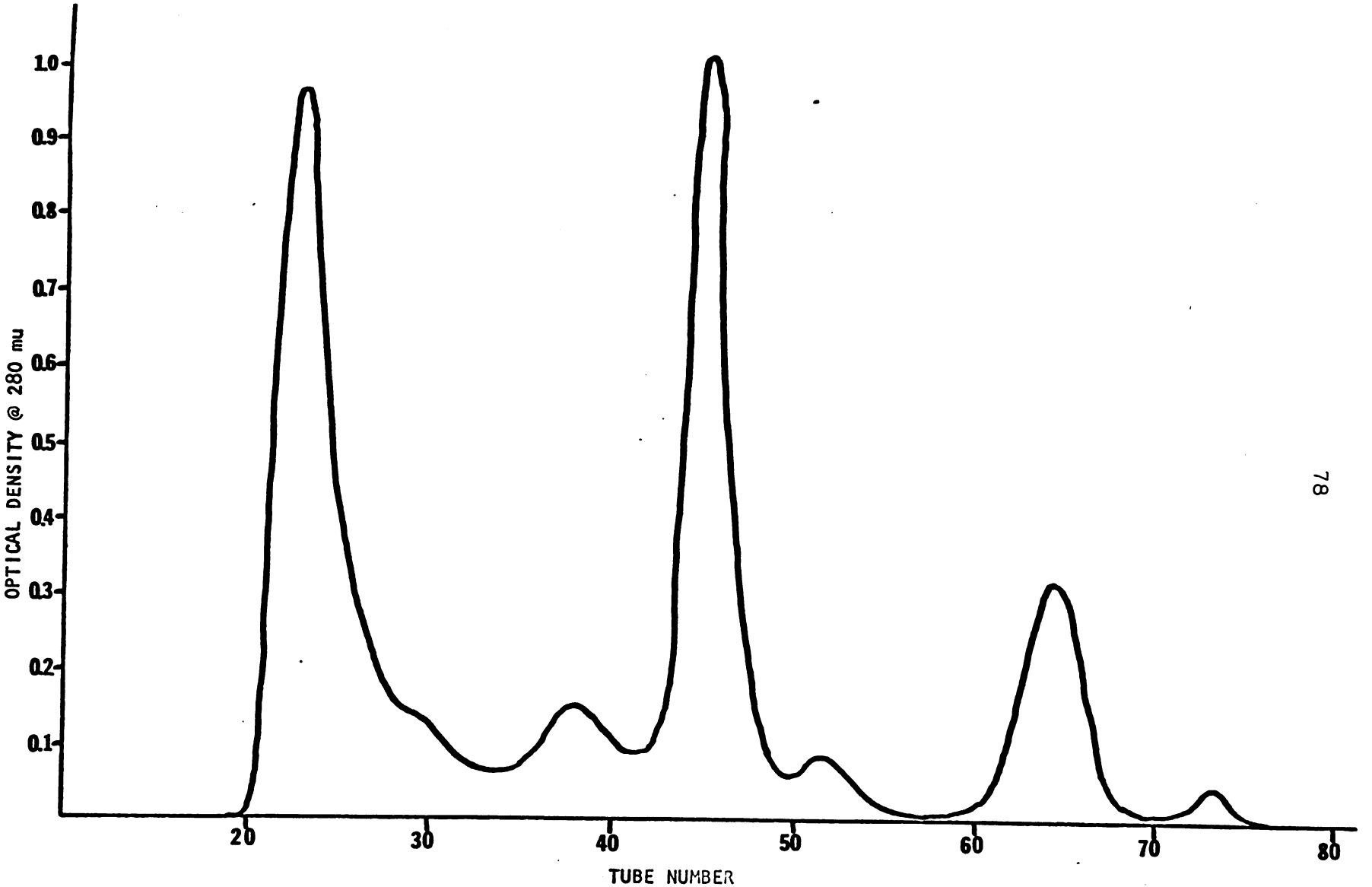

Figure 19. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate B (Table 9) on Sephadex G-25.

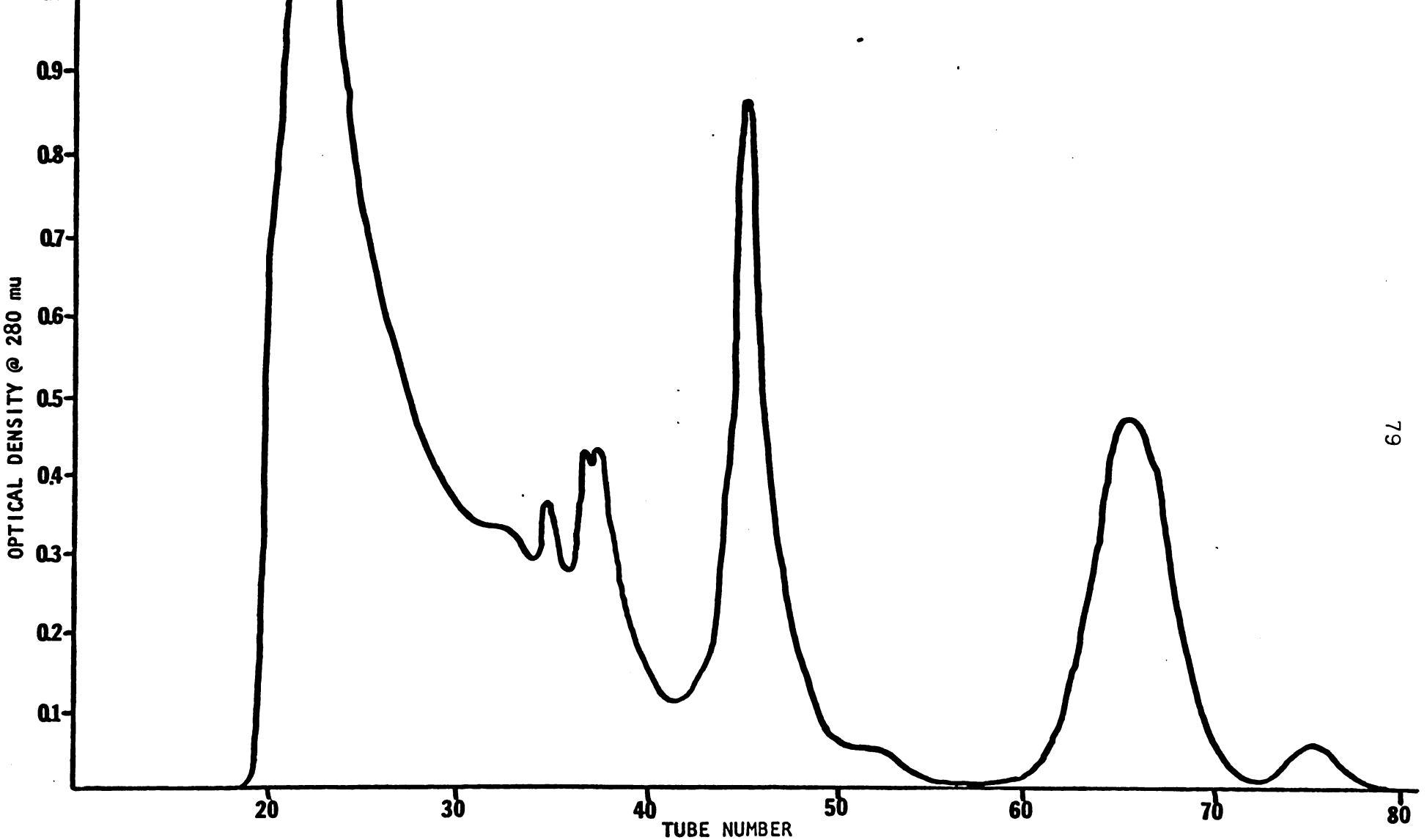

Figure 20. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

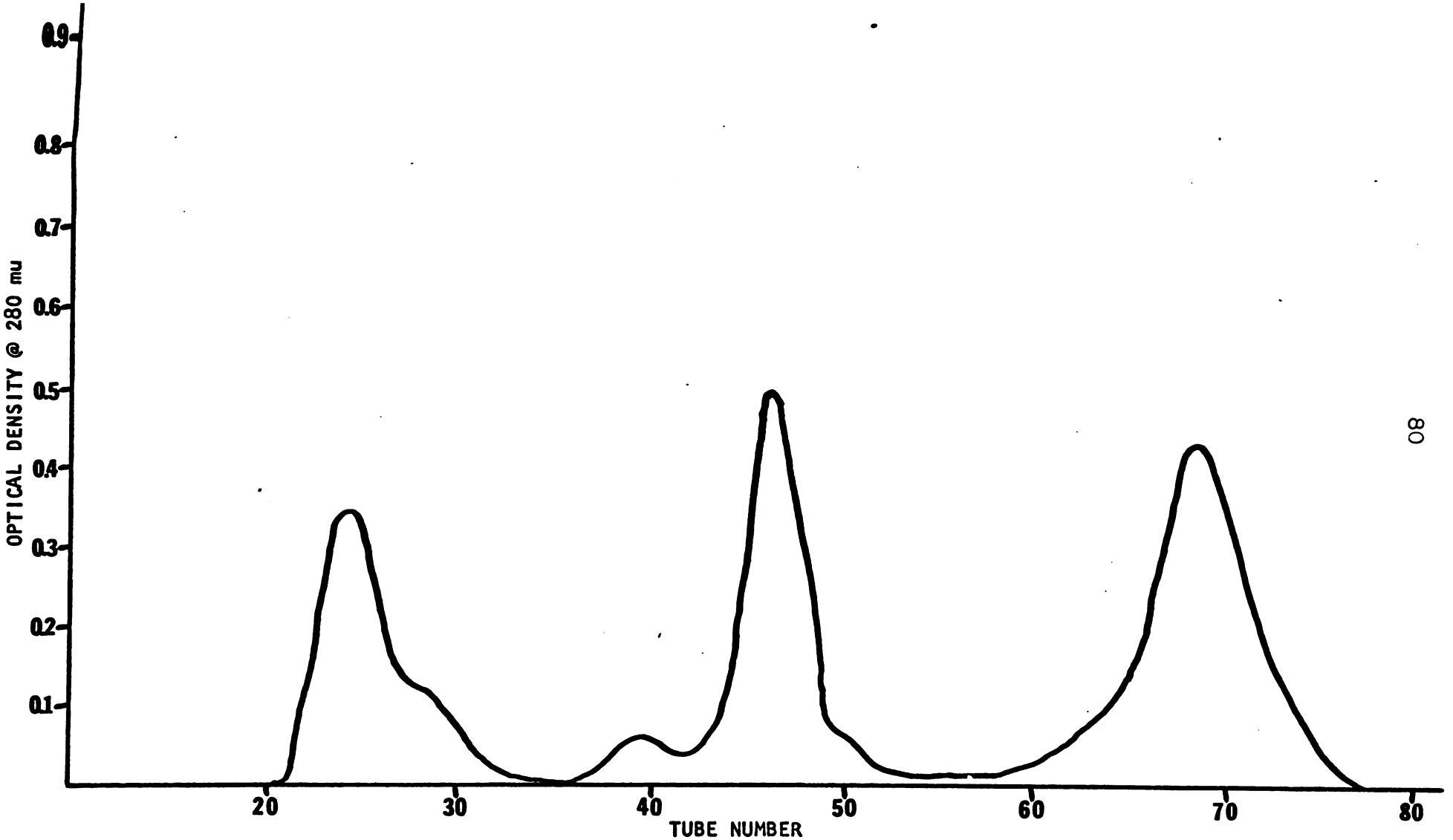

Figure 21. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate D (Table 9) on Sephadex G-25.

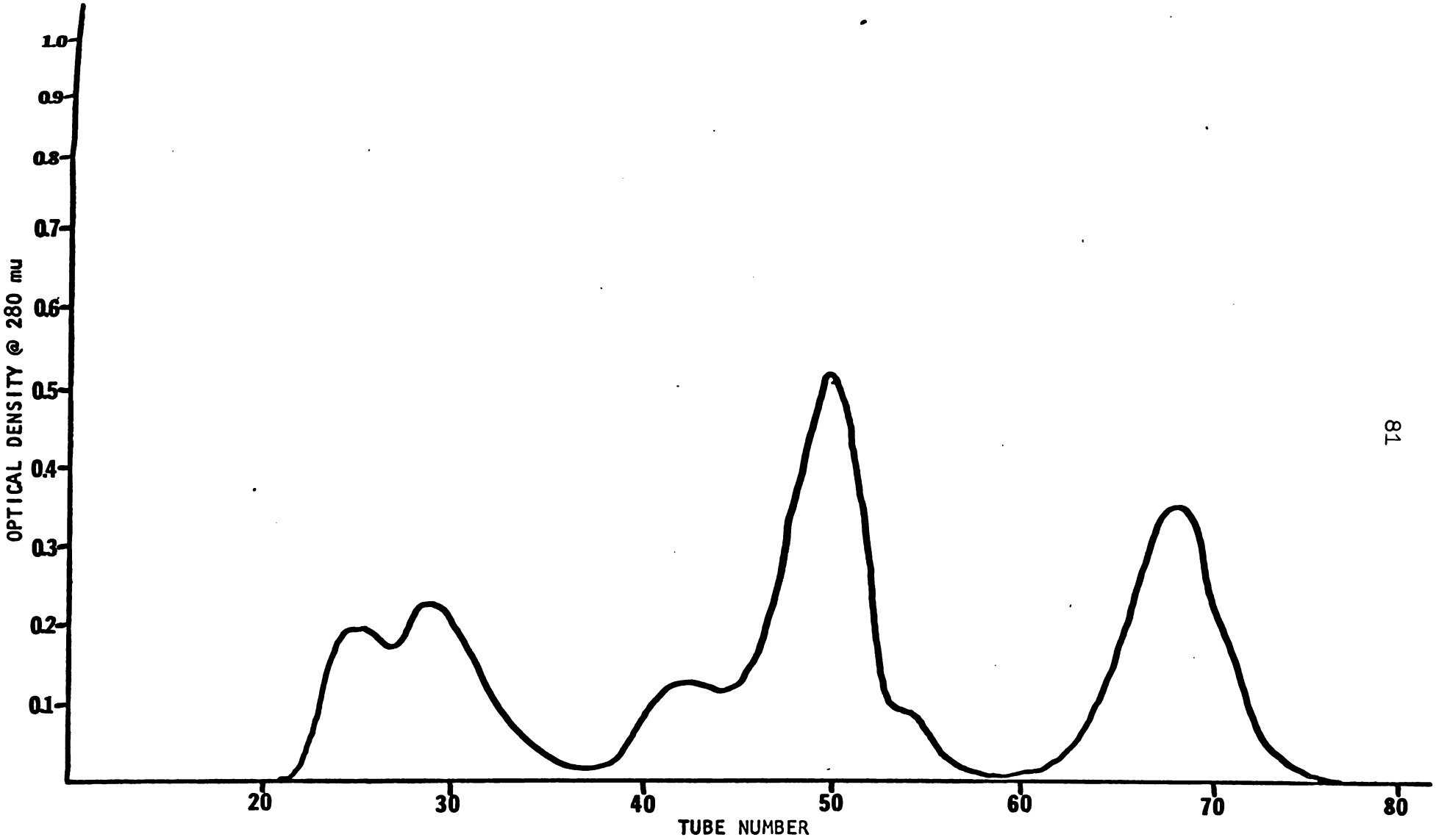

Figure 22. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate E (Table 9) on Sephadex G-25.

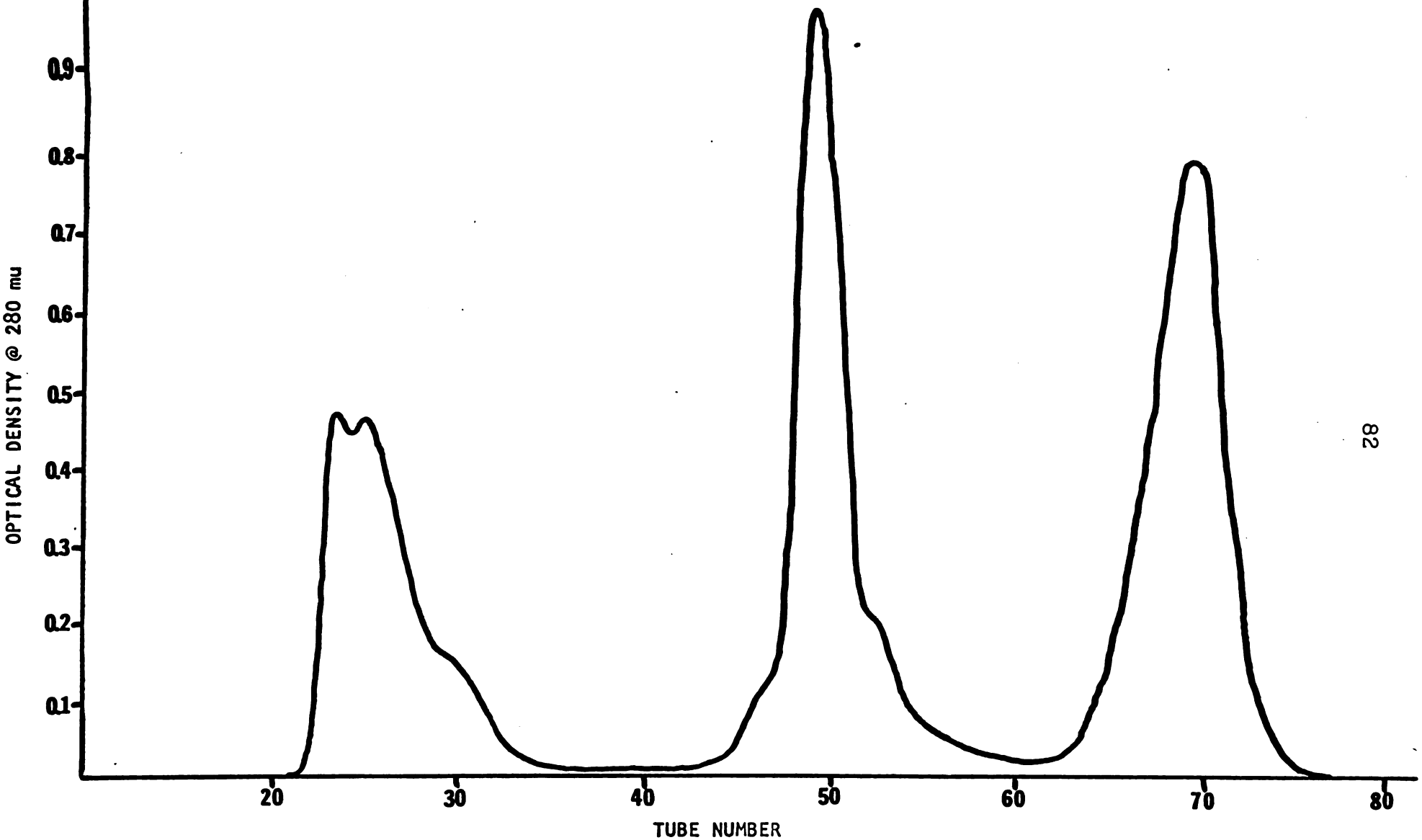

Figure 23. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate F (Table 9) on Sephadex G-25.

Table 10. Chemical composition of the fractions obtained by chromatography of Mycobacterium bovis culture filtrate A[1] on Sephadex G-25.

Fraction[2]	% 280-AM[3]	% FRM[4]	% TRM[5]	% NA[6]	280/260[7]	Rf[8]
I	44.3	82.0	93.9	15.4	0.88	1.0
II	53.6	18.0	6.1	83.3	0.53	0.38
III	1.5	0	0	1.3	0.48	0.25
IV	0.6	NM[9]	NM	NM	NM	0.21

[1] Obtained from cultures incubated for two months

[2] Fraction - Major eluant fraction

[3] % 280-AM - Percent of the total 280 mu-absorbing material in the individual fractions

[4] % FRM - Percent of the total Folin-reacting material in the individual fractions

[5] % TRM - Percent of the total thymol-reacting material in the individual fractions

[6] % NA - Percent of the total nucleic acids in the individual fractions

[7] 280/260 - Ratio of the ultraviolet absorbancy at 280 mu and 260 mu

[8] Rf - Ratio of the column void volume and the eluant volume in which the fraction was detected

[9] NM - Not measured

Table II. Chemical composition of the fractions obtained by chromatography of Mycobacterium bovis culture filtrate B[1] on Sephadex G-25.

Fraction[2]	% 280-AM[3]	% FRM[4]	% TRM[5]	% NA[6]	280/260[7]	Rf[8]
I	49.1	81.2	96.4	13.6	0.94	1.0
II	36.0	17.2	3.6	85.9	0.56	0.40
III	12.9	1.5	0	0.4	0.64	0.27
IV	1.8	NM[9]	NM	NM	NM	0.23

[1]Obtained from cultures incubated for three months
[2]Fraction = major eluant fraction
[3]% 280-AM = percent of the total 280 mu absorbing material in the individual fractions
[4]% FRM = percent of the total Folin-reacting material in the individual fractions
[5]% TRM = percent of the total thymol-reacting material in the individual fractions
[6]% NA = percent of the total nucleic acids in the individual fractions
[7]280/260 = ratio of the ultraviolet absorbancy at 280 mu and 260 mu
[8]Rf = ratio of the column void volume and the eluant volume in which the fraction was detected
[9]NM = not measured

Table 12. Chemical composition of the fractions obtained by chromatography of Mycobacterium bovis culture filtrate C[1] on Sephadex G-25.

Fraction[2]	% 280-AM[3]	% FRM[4]	% TRM[5]	% NA[6]	280/260[7]	Rf[8]
I	52.3	90.4	94.8	28.1	0.89	1.0
II	35.1	9.6	5.2	70.5	0.70	0.36
III	10.3	0	0	1.3	0.72	0.24
IV	2.2	NM[9]	NM	NM	NM	0.22

[1] Obtained from cultures incubated for four months
[2] Fraction = major eluant fraction
[3] %280-AM = percent of the total 280 mu absorbing material in the individual fractions
[4] % FRM = percent of the total Folin-reacting material in the individual fractions
[5] % TRM = percent of the total thymol-reacting material in the individual fractions
[6] % NA = percent of the total nucleic acids in the individual fractions
[7] % 280/260 = ratio of the ultraviolet absorbancy at 280 mu and 260 mu
[8] Rf = ratio of the column void volume and the eluant volume in which the fraction was detected
[9] NM = not measured

Table 13. Chemical composition of the fractions obtained by chromatography of Mycobacterium bovis culture filtrate D[1] on Sephadex G-25.

Fraction[2]	% 280-AM[3]	% FRM[4]	% TRM[5]	% NA[6]	280/260[7]	Rf[8]
I	39.1	82.6	92.8	20.9	0.90	1.0
II	28.3	6.8	7.2	75.4	0.65	0.37
III	32.5	6.8	0	3.7	0.60	0.25

[1] Obtained from cultures incubated for five months
[2] Fraction = major eluant fraction
[3] % 280-AM = percent of the total 280 mu-absorbing material in the individual fractions
[4] % FRM = percent of the total Folin-reacting material in the individual fractions
[5] % TRM = percent of the total thymol-reacting material in the individual fractions
[6] % NA = percent of the total nucleic acids in the individual fractions
[7] 280/260 = ratio of the ultraviolet absorbancy at 280 mu and 260 mu
[8] Rf = ratio of the column void volume and the eluant volume in which the fraction was detected

Table 14. Chemical composition of the fractions obtained by chromatography of Mycobacterium bovis culture filtrate F^{1} on Sephadex G-25.

Fraction[2]	% 280-AM[3]	% FRM[4]	% TRM[5]	% NA[6]	280/260[7]	Rf[8]
I	28.3	61.2	91.2	11.9	0.96	1.0
II	38.8	26.3	8.8	78.8	0.90	0.39
III	32.9	13.5	0	9.2	0.53	0.27

[1]Obtained from cultures incubated for six months
[2]Fraction = major eluant fraction
[3]% 280-AM = percent of the total 280 mu absorbing material in the individual fractions
[4]% FRM = percent of the total Folin-reacting material in the individual fractions
[5]% TRM = percent of the total thymol-reacting material in the individual fractions
[6]% NA = percent of the total nucleic acids in the individual fractions
[7]280/260 = ratio of the ultraviolet absorbancy at 280 mu and 260 mu
[8]Rf = ratio of the column void volume and the eluant volume in which the fraction was detected

Table 15. Chemical composition of the fractions obtained by chromatography of Mycobacterium bovis culture filtrate[1] on Sephadex G-25.

Fraction[2]	% 280-AM[3]	% FRM[4]	%TRM[5]	% NA[6]	280/260[7]	Rf[8]
I	27.6	78.0	86.5	15.2	0.96	1.0
II	40.6	14.6	13.5	78.9	0.91	0.37
III	31.7	7.4	0	6.9	0.54	0.26

[1]Obtained from cultures incubated for seven months

[2]Fraction - Major eluant fraction

[3]% 280-AM - Percent of the total 280 mu-absorbing material in the individual fractions

[4]% FRM - Percent of the total Folin-reacting material in the individual fractions

[5]% TRM - Percent of the total thymol-reacting material in the individual fractions

[6]% NA - Percent of the total nucleic acids in the individual fractions

[7]280/260 - Ratio of the ultraviolet absorbancy at 280 mu and 260 mu

[8]Rf - Ratio of the column void volume and the eluant volume in which the fraction was detected

two major 280 mμ-absorbing fractions and two minor fractions (Fig. 18). Fraction I was eluted in the column void volume (Rf = 1.0) and contained 82.0%, 93.9% and 15.9% respectively, of the protein, polysaccharide and nucleic acid in the sample (Table 10). Fraction II had a small shoulder on both sides of the major peak and was composed of molecules with molecular weights less than 5,000 (Rf = 0.38). The yellow pigment seen in all of the culture filtrates was eluted in this peak. Most of the nucleic acids in the sample (83.3%) were found in fraction II. Only 1.5% of the total 280 mμ-absorbing material in the sample was found in fraction III. This fraction contained no polypeptides or oligosaccharides and was almost all low molecular weight polynucleotides. Fraction IV contained 0.5% of the 280 mμ-absorbing materials in the sample. Fractions I and II elicited immediate-type skin reactions in sensitized rabbits.

Four main fractions were seen in the chromatogram of CF-B (Fig. 19). They contained 49.1%, 36.0%, 12.9%, and 1.8% respectively, of the total 280 mμ-absorbing material in the sample (Table 11). The first fraction was eluted in the column void volume (Rf = 1.0) and had a small shoulder on the trailing front. The chemical composition of this fraction was similar to fraction I from CF-A (Table 10). Fraction II had a well-defined shoulder on the leading and trailing front of the major peak. The chemical composition and Rf were similar to fraction II in CF-A (Table 10). Fraction III in

CF-B was much larger than the same fraction in CF-A. Fraction III contained no oligosaccharides, very few polynucleotides, and small amounts of low molecular weight polypeptides (Rf = 0.27). The first two peaks elicited immediate-type skin reactions in sensitized rabbits.

The chromatogram of CF-C had four fractions (Fig. 20). Fraction I contained 52.3% of the total 280 mμ-absorbing material in the sample and 94.4%, 94.8%, and 28.1% of the protein, polysaccharide and nucleic acids, respectively (Table 12). The leading front was symmetrical although a shoulder and several small peaks were seen on the trailing front. Fraction I was divided into three peaks. Lower concentrations of polypeptides and polynucleotides were found in this fraction than in the corresponding fraction from CF-B (Table 11). The first three peaks elicited immediate-type skin reactions in sensitized rabbits. Fraction III contained no polypeptides, or oligosaccharides. Fraction III and IV accounted for only 12.5% of the total 280 mμ-absorbing material in the sample. The ultraviolet absorption spectra of rechromatographed fractions I, II, and III from CF-C are shown in Figs. 24, 25, and 26. Similar spectra were obtained with the corresponding fractions from all of the culture filtrates. Fraction I had a greater 280/260 mμ absorbancy ratio than fractions II or III (Table 12). The absorbancy spectra of fractions II and III were very similar.

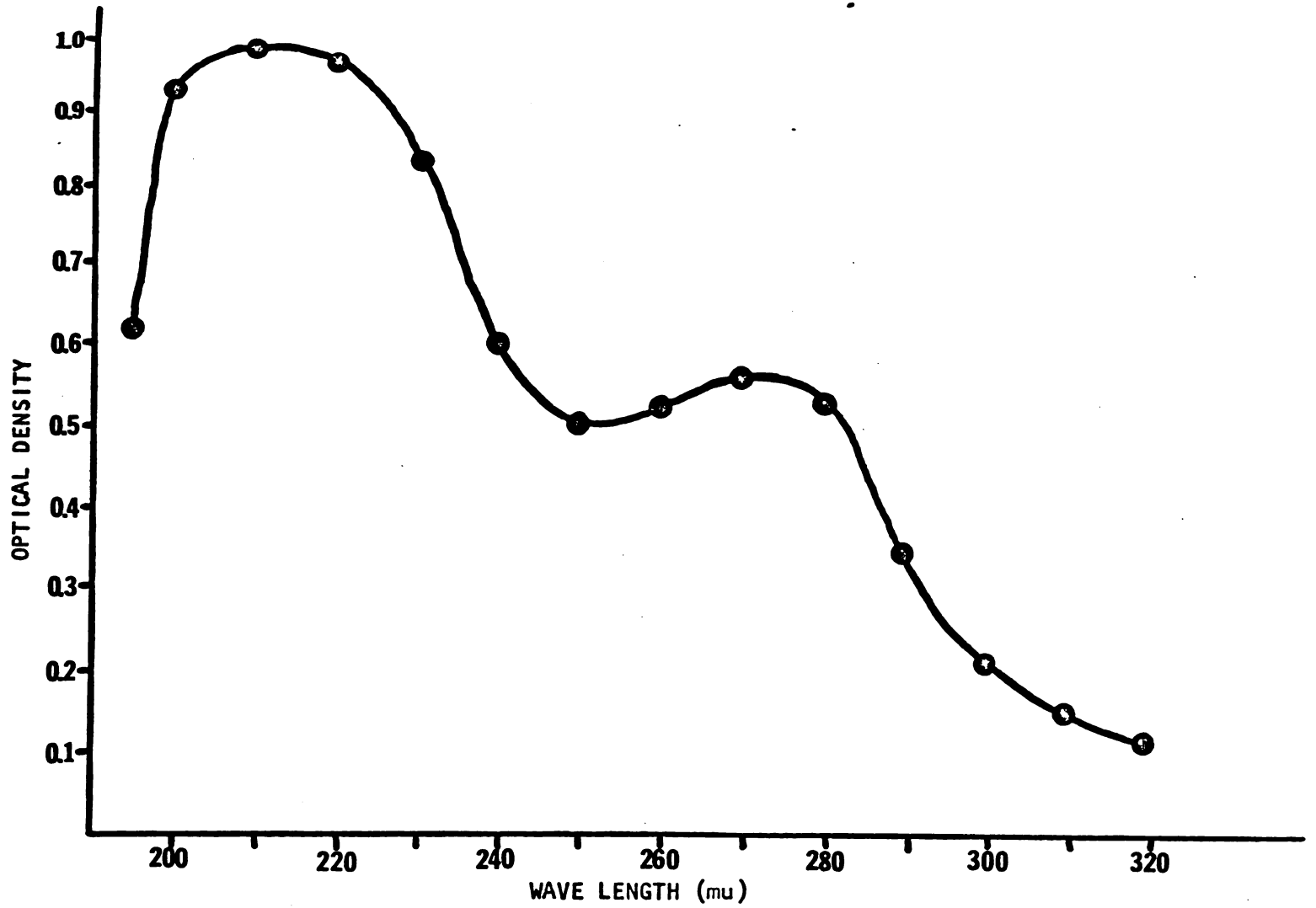

Figure 24. Ultraviolet absorption spectrum of Fraction I (Table 12) obtained by chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

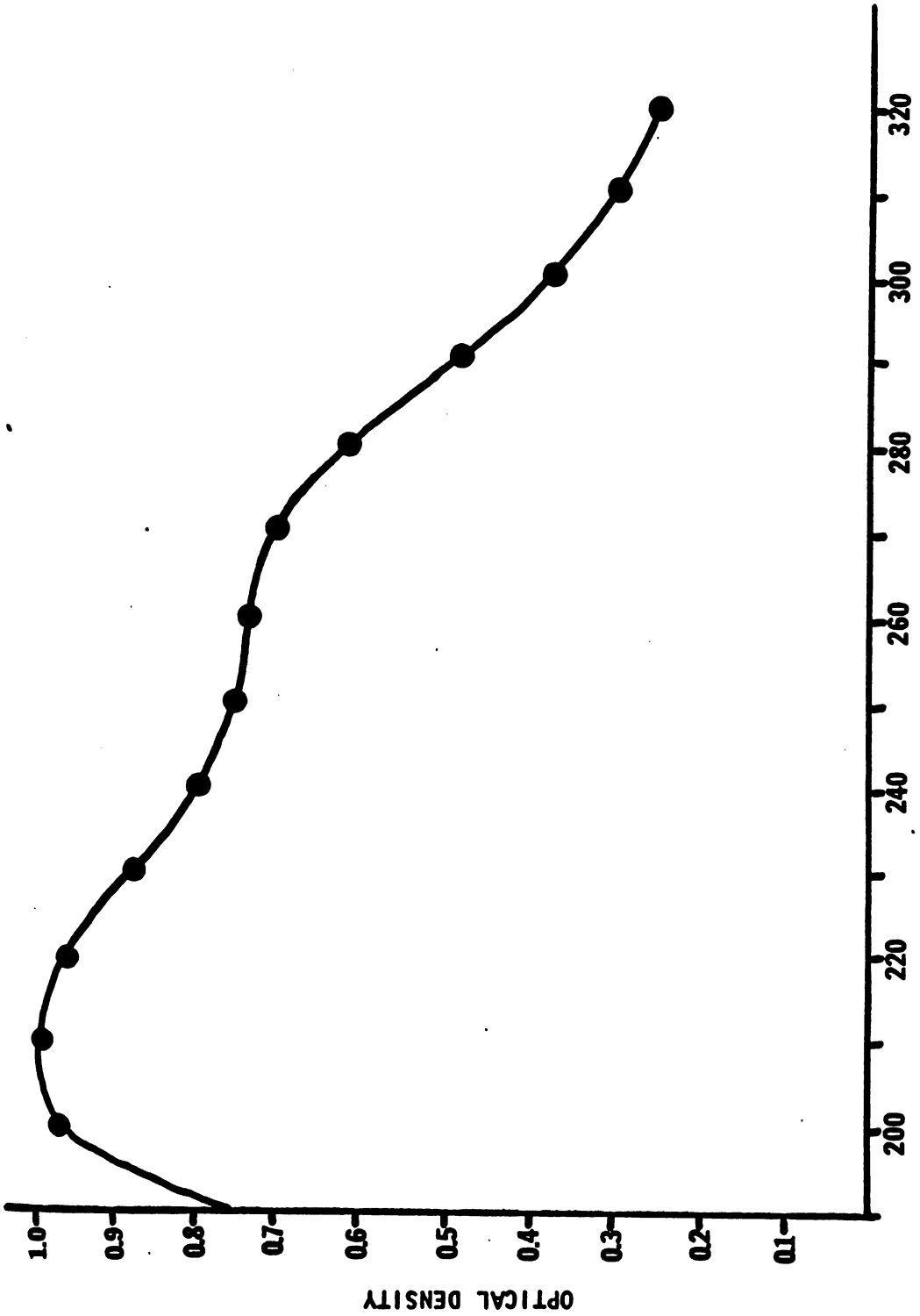

Figure 25. Ultraviolet absorption spectrum of Fraction II (Table 12) obtained by chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

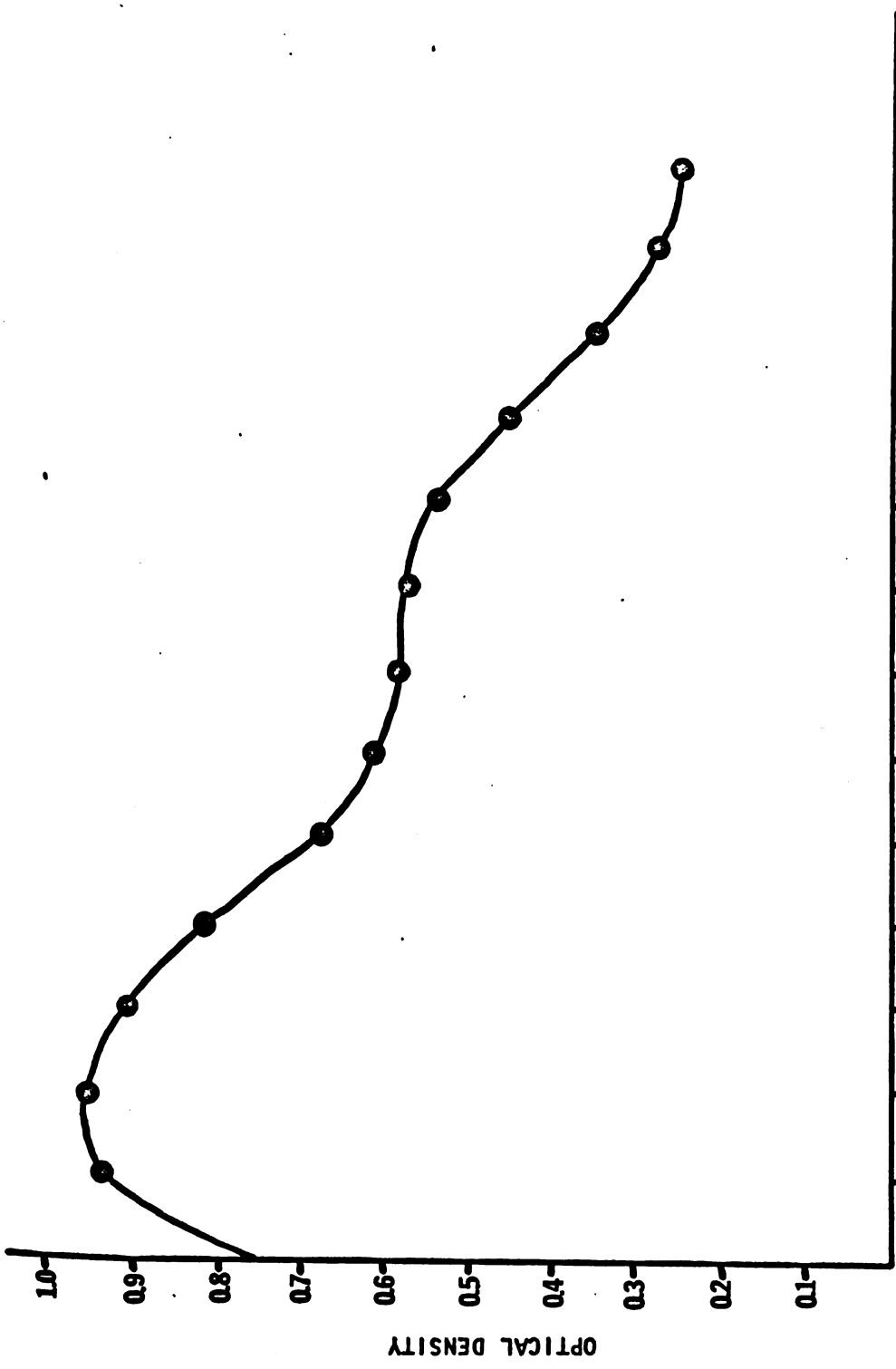

Figure 26. Ultraviolet absorption spectrum of Fraction III (Table 12) obtained by chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

Culture filtrate D was separated into three major fractions (Fig. 21). Fraction I was eluted in the column void volume (Rf = 1.0((Table 13). It contained approximately one-third of the total 280 mμ-absorbing material and 82.6%, 92.8%, and 20.9% of the protein, polysaccharide, and nucleic acids, respectively. Fraction II was fairly symmetrical but contained two other small peaks. The chemical composition of this fraction was similar to that from CF-C. Fraction III contained low molecular weight polypeptides (Rf = 0.25), no oligosaccharides, and a small amount of polynucleotides.

The chromatogram of CF-E had three fractions (Fig. 22). Fraction I was separated into two subfractions and contained less than one-third of the 280 mμ-absorbing material in the sample (Table 14). Fraction II contained more 280 mμ-absorbing material, polypeptides, oligosaccharides, and polynucleotides than fraction II from CF-D. The chemical composition of fraction III from both culture filtrates was similar.

Culture filtrate F was separated into three major fractions (Fig. 23). The first fraction was eluted in the column void volume and contained two subfractions. Less protein, polysaccharide, and nucleic acid was found in this fraction than in fraction I from Cf-E (Table 15). More polypeptides and polynucleotides were found in fractions II and III than in the corresponding fractions from CF-E.

Chromatograms of the six culture filtrates on Bio-Gels P-100, P-150, and P-200 are shown in Figs. 27, 28, 29, 30, 31, and 32. The distribution of protein and 280 mμ-absorbing material in the chromatographic eluants are shown in Table 16. All of the culture filtrates except CF-F were separated into two fractions on all three Bio-Gels. Only one fraction was obtained by chromatography of CF-F on Bio-Gel P-200 (Fig. 32). From 53.1% to 61.9% of the proteins in culture filtrate A-E were eluted in the void volume of P-100 columns (Table 16). Fraction I from Cf-F contained 23.2% of the protein in the sample. The first fraction eluted from P-200 columns contained from 33.3% to 44.8% of the protein in the culture filtrates A-D (Table 16). Twenty-six percent of the protein in CF-E was eluted in the column void volume. All of the protein in CF-F had an Rf value of less than 1.0 when chromatographed on Bio-Gel P-200. The percent distribution of the culture filtrate proteins in fraction I from Sephadex G-25 and Bio-Gels P-100 and P-200 are shown in Table 17.

The nondialyzable proteins in fraction I from CF-C obtained by rechromatography on Sephadex G-25 were separated into five fractions by gradient elution from DEAE-cellulose (Fig. 33). Approximately 50% of the proteins in the sample were recovered in the chromatographic eluants. Fractions I, II, III, and V were composed of several subfractions with different affinities for the adsorbent.

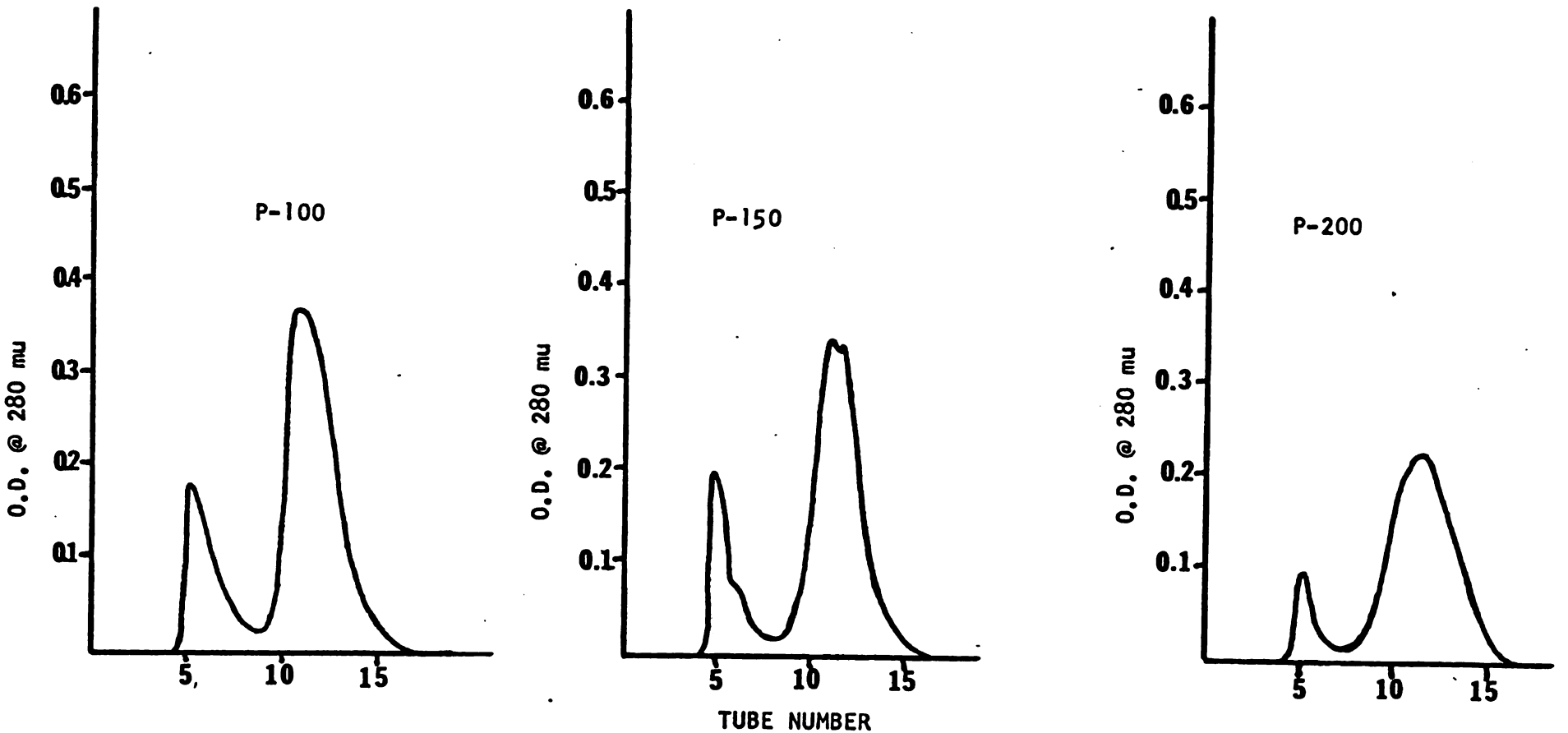

Figure 27. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate A (Table 9) on Bio-Gels P-100, P-150, and P-200.

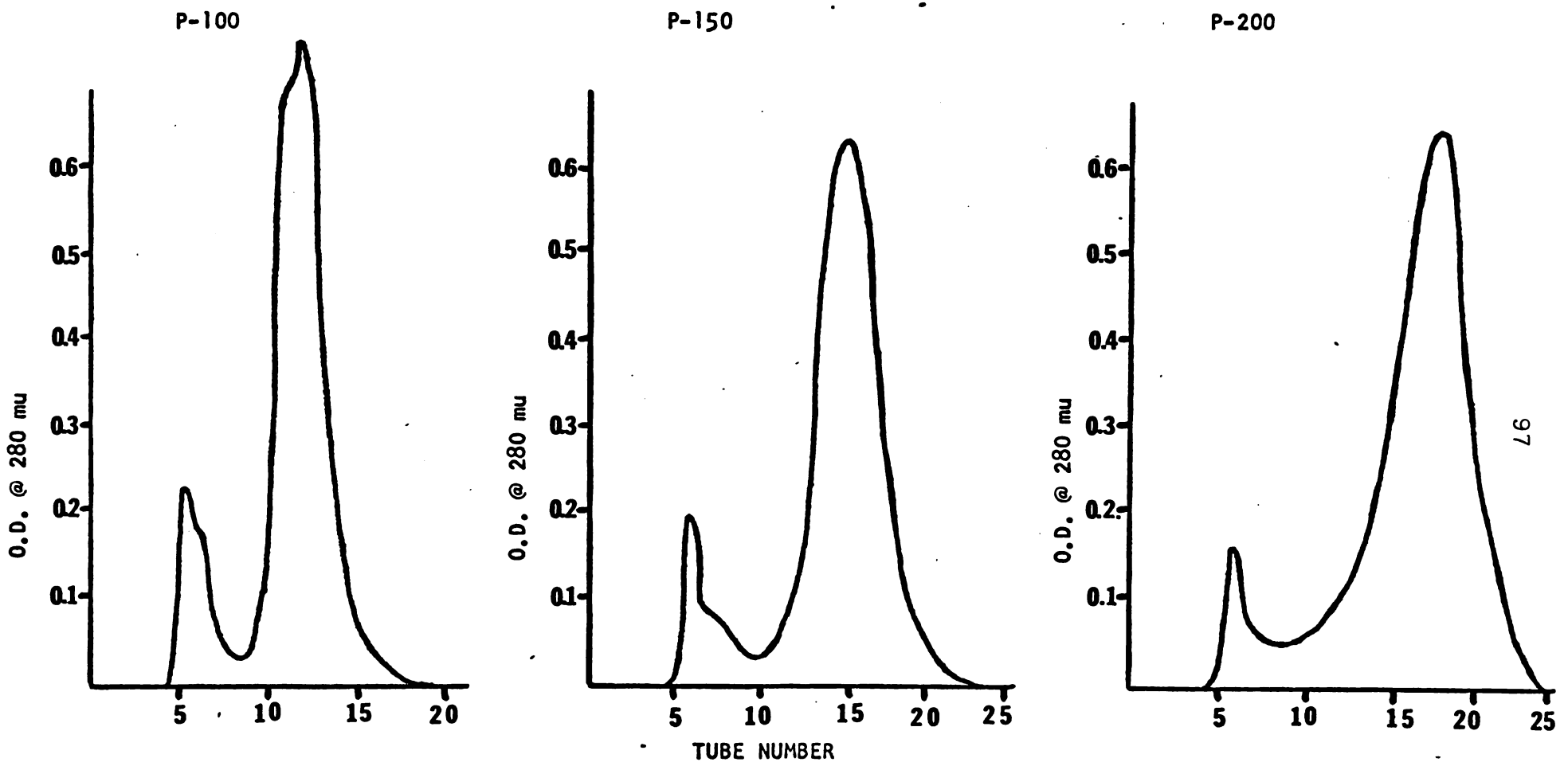

Figure 28. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate B (Table 9) on Bio-Gels P-100, P-150, and P-200.

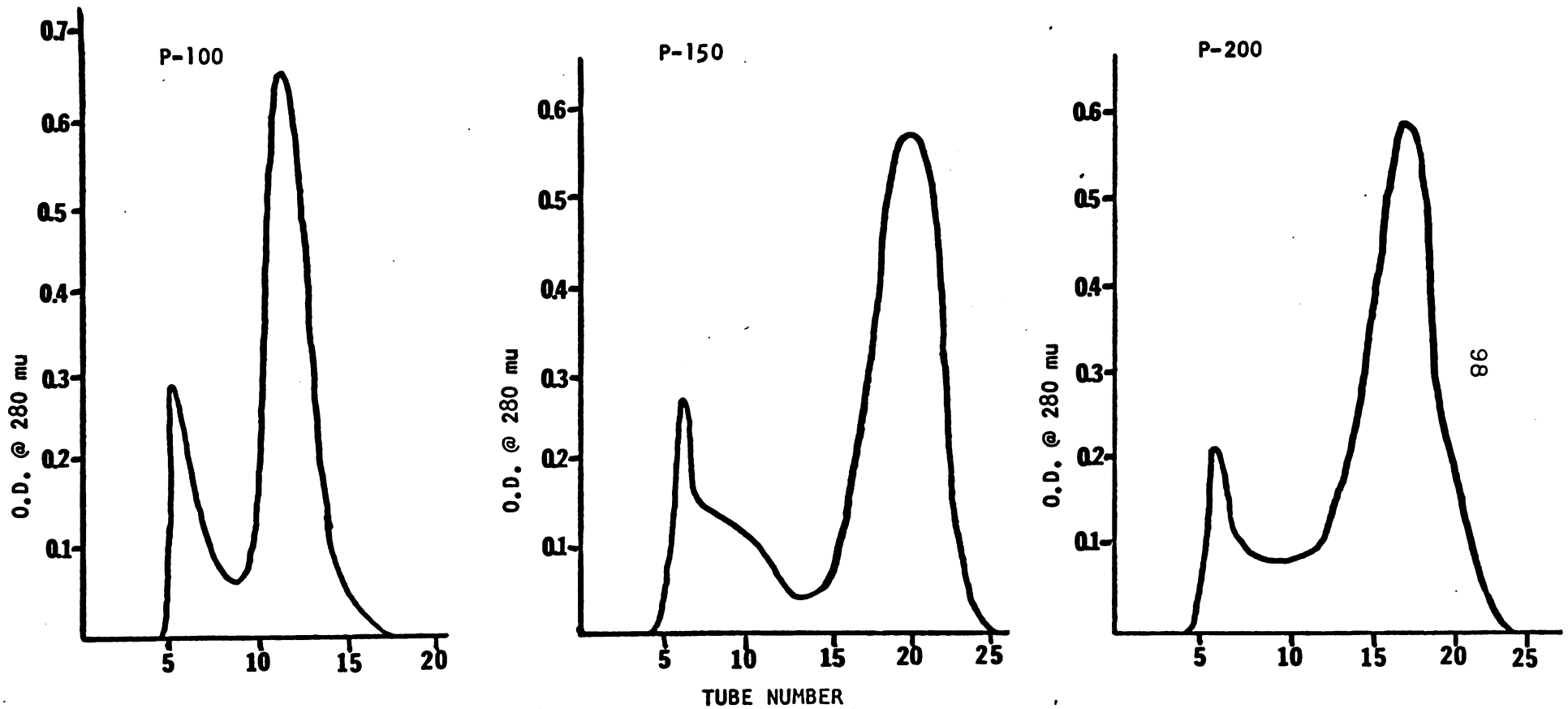

Figure 29. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Bio-Gels P-100, P-150, and P-200.

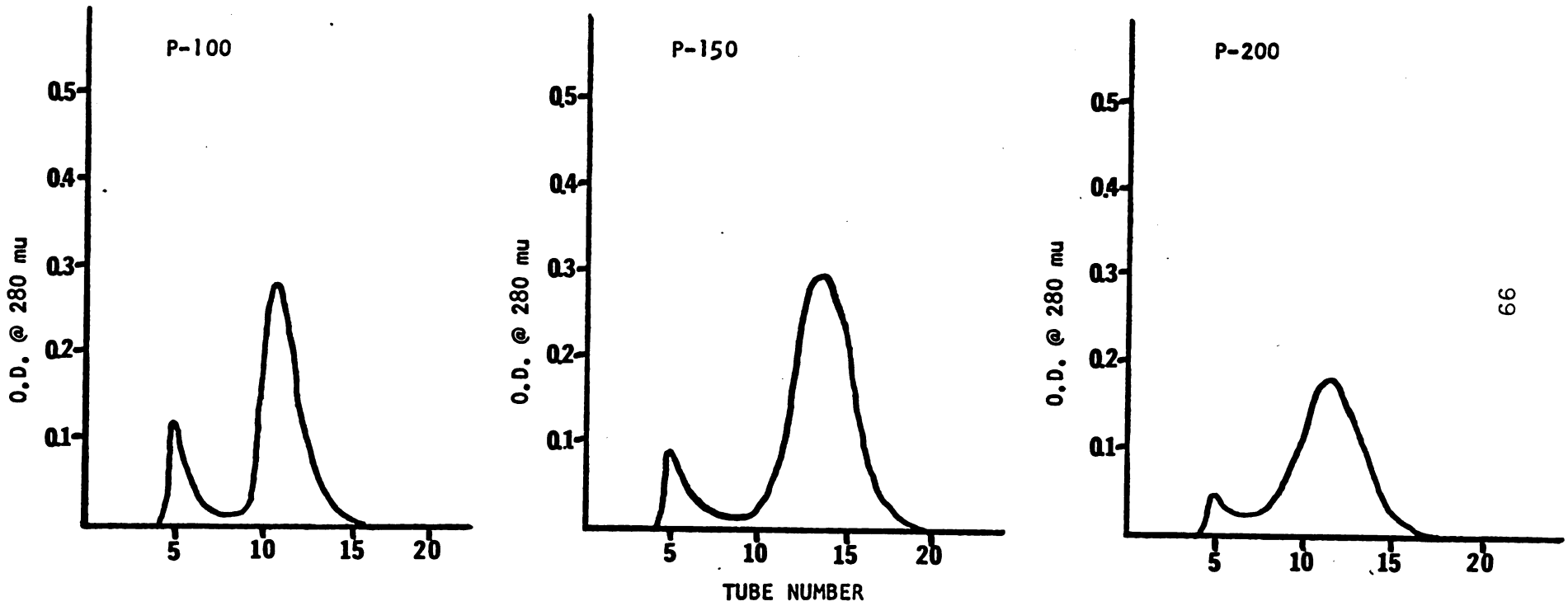

Figure 30. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate D (Table 9) on Bio-Gels P-100, P-150, and P-200.

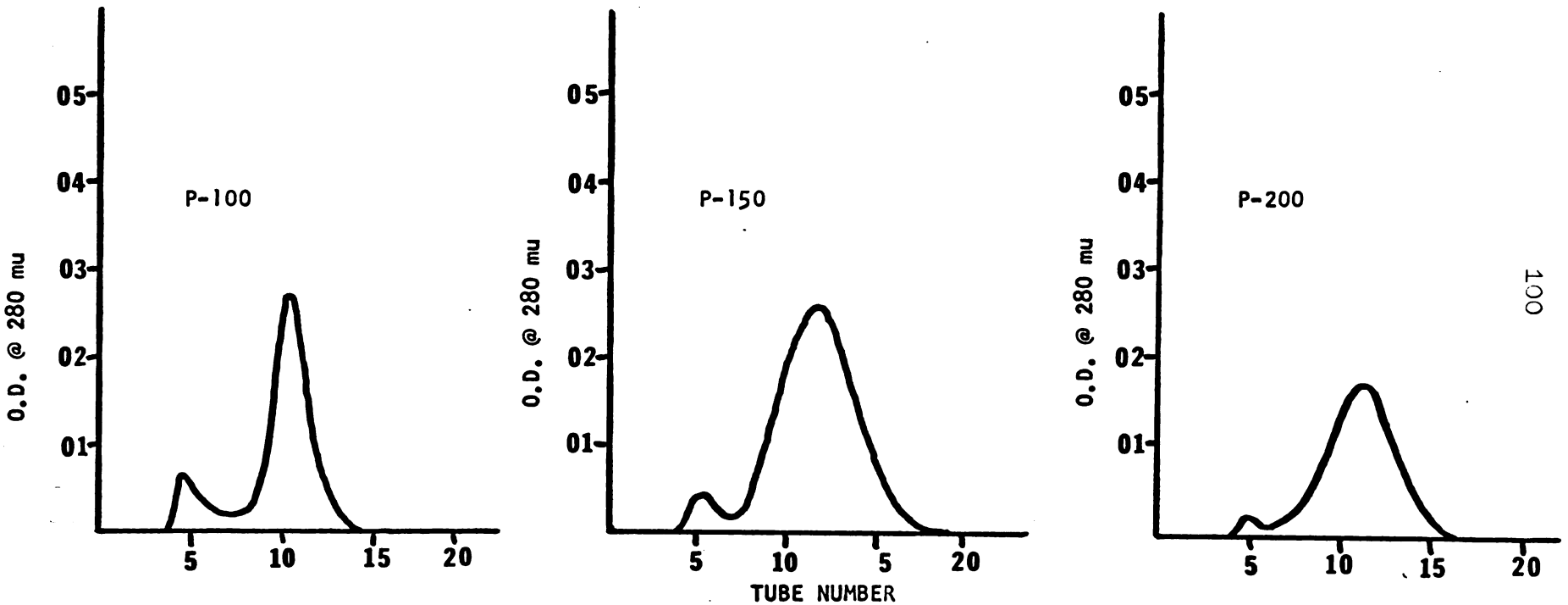

Figure 31. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate E (Table 9) on Bio-Gels P-100, P-150, and P-200.

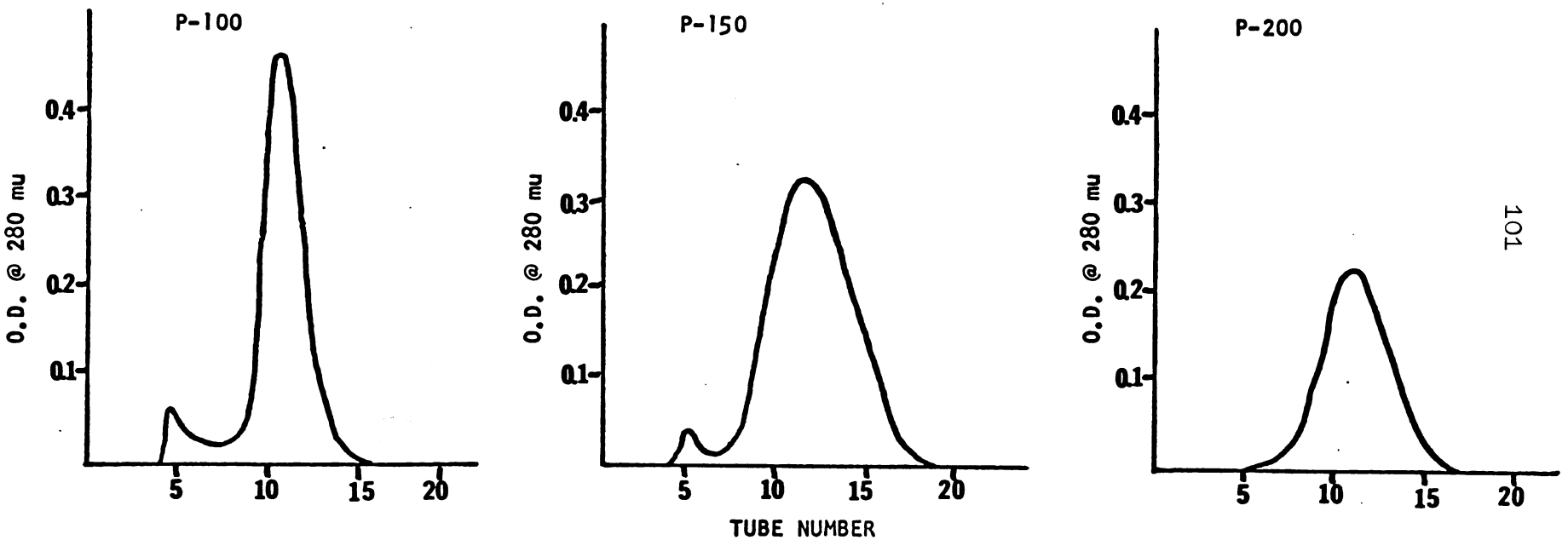

Figure 32. Molecular exclusion chromatography of Mycobacterium bovis culture filtrate F (Table 9) on Bio-Gels P-100, P-150, and P-200.

Table 16. Distribution of 280 mu-absorbing and Folin-reacting material in fractions obtained by chromatography of the six Mycobacterium bovis culture filtrates on Bio-Gels P-100, P-150, and P-200.

	P-100[1]				P-150[2]		P-200[3]			
	I[4]		II[5]		I	II	I		II	
CF[6]	%280-AM[7]	%FRM[8]	%280-AM	%FRM	%280-AM	%280-AM	%280-AM	%FRM	%280-AM	%FRM
A[9]	22.1	61.9	77.9	38.1	19.7	80.3	13.5	42.0	86.5	58.0
B	20.6	56.4	79.4	43.6	16.1	83.9	12.4	33.3	87.6	66.7
C	21.5	63.0	78.5	37.4	17.9	82.1	16.3	41.7	83.7	58.3
D	15.8	53.1	84.2	46.9	11.4	88.6	9.0	44.8	91.0	55.2
E	12.3	57.7	87.7	42.3	5.7	94.3	1.7	26.2	98.3	73.8
F	6.0	23.2	94.0	76.8	4.6	95.4	0	0	100.0	100.0

[1] P-100 = Bio-Gel P-100
[2] P-150 = Bio-Gel P-150
[3] P-200 = Bio-Gel P-200
[4] I = major eluant Fraction I
[5] II = major eluant Fraction II
[6] CF = culture filtrate
[7] % 280-AM = percent of the 280 mu-absorbing material in the individual fractions
[8] % FRM = percent of the total Folin-reacting material in the individual fractions
[9] A = culture filtrate A described in Table 9

Table 17. Distribution of Folin-reacting material in Fraction I obtained by chromatography of the six Mycobacterium bovis culture filtrates on Sephadex G-25, Bio-Gel P-100 and Bio-Gel P-200.

	G-25	P-100	P-200
CF[1]	% FRM[2]	% FRM	% FRM
A[3]	82.0	61.9	42.0
B	81.2	56.4	33.6
C	90.4	63.0	41.7
D	82.6	53.1	44.8
E	78.0	57.7	26.2
F	61.2	23.2	0.0

[1] CF - Culture filtrate

[2] % FRM - Percent of the total Folin-reacting material in Fraction-I

[3] A - Culture filtrate A described in Table 9.

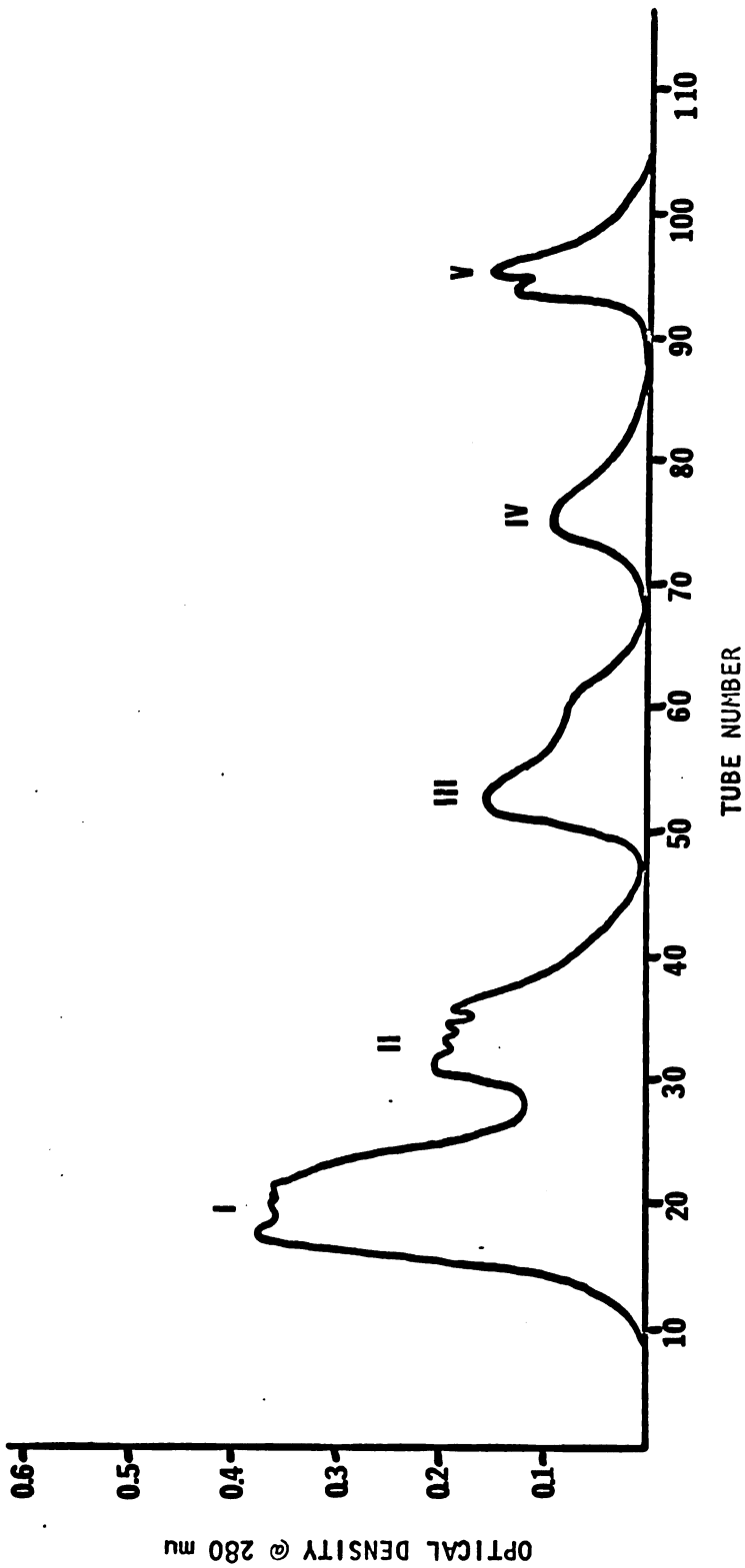

Figure 33. DEAE-cellulose chromatography of Fraction I obtained by molecular exclusion chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

Dialysis of culture filtrates.

The dialyzable material (dialysate) from all of the culture filtrates was amber-colored and had an unpleasant odor. The nondialyzable material was colorless and almost odorless. The dialysate contained trace amounts of fraction I, almost all of fraction II, and most of fraction III when rechromatographed on Sephadex G-25 (Fig. 34). No precipitinogens were detected in the dialysates from any of the culture filtrates (Fig. 35). Both the dialysates and the nondialyzable fractions elicited immediate-type skin reactions in sensitized rabbits. The nondialyzable fraction contained essentially all of the 280 mμ-adsorbing material found in fraction I and some of the material found in fraction III of CF (Fig. 34). All of the precipitinogens found in CF were nondialyzable (Fig. 35). The nondialyzable fraction contained all of the protein component found in the homologous CF by disc electrophoresis. Trace amounts of two or three of these components were found in disc electrophorograms of the dialysates.

Cellulose acetate membrane electrophoresis of culture filtrates.

Rechromatographed proteins from fraction I from culture filtrates A, B, and C were separated into five to six fractions by cellulose acetate membrane electrophoresis (Fig. 36). Three to four poorly separated bands were observed when unfractionated CF was electrophoresed in the same manner.

Figure 34. Molecular exclusion chromatography of dialyzable (broken line) and non-dialyzable (solid line) constituents of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

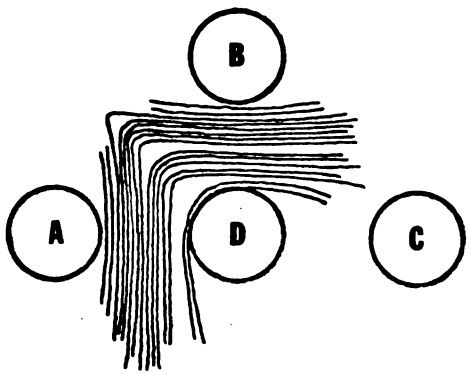

Figure 35. Immunogram of dialyzable (C) and non-dialyzable (B) constituents from Mycobacterium bovis culture filtrate C (Table 9) (A). The antiserum was placed in well D.

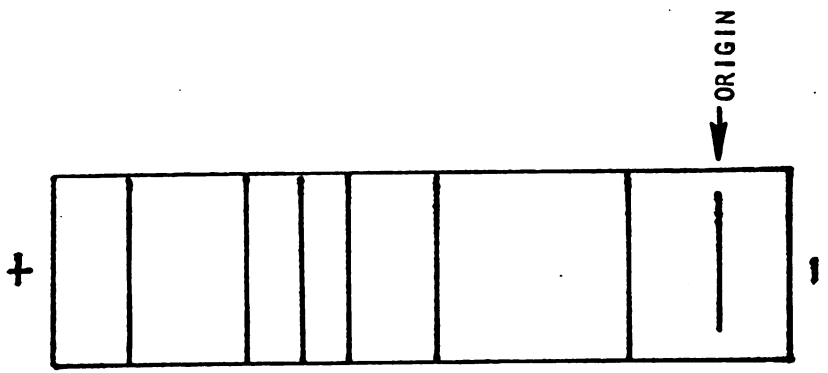

Figure 36. Cellulose acetate electrophorogram of Fraction I obtained by molecular exclusion chromatography of Mycobacterium bovis culture filtrate C (Table 9) on Sephadex G-25.

No stainable protein bands were detected in fractions II or III in any of the culture filtrates when analyzed electrophoretically.

Disc electrophoresis of culture filtrates.

The number of amido black-stained bands in the six culture filtrates were as follows: 16 in CF-A; 23 in CF-B; 30 in CF-C; 23 in CF-D; 19 in CF-E; and 15 in CF-F (Table 18); Figs. 37, 38, 39, 40, 41, and 42. All of the culture filtrates contained five bands with similar or identical Rf values (Table 19). Twelve other bands with the same mobility were found in three or more of the culture filtrates. No protein bands were found in the spacer gel of any of the disc electrophorograms. There is no satisfactory method for determining the relative amount of proteins in each of the bands.

The number of PAS-stained bands in the culture filtrates were as follows: 9 in CF-A; 8 each in CF-B and CF-C; 7 in CF-D; and 5 each in CF-E and CF-F (Table 18). All of the culture filtrates had PAS-stained bands in the spacer gel, at the spacer gel-lower gel interface, and at the lower end of the lower gel associated with the most anodic protein component (Figs. 37, 38, 39, 40, 41, and 42). Most of the PAS-stained bands in the lower gel could be correlated with protein components with identical Rf values.

Fraction I isolated and rechromatographed on Sephadex G-25 from all of the culture filtrates contained all of the

Table 18. Number of components found in the six Mycobacterium bovis culture filtrates by disc electrophoresis, Ouchterlony double diffusion and immunoelectrophoresis.

CF[1]	DE[2] AB[3]	PAS[4]	ODD[5]	IE[6]
A[7]	16	9	16	19
B	23	8	15	19
C	30	8	15	21
D	23	7	13	16
E	19	5	8	10
F	15	5	6	8

[1]CF - Culture filtrate

[2]DE - Disc electrophoresis

[3]AB - Amido black-stained components

[4]PAS - Periodic acid Schiff-stained components

[5]ODD - Ouchterlony double diffusion

[6]IE - Immunoelectrophoresis

[7]A - Culture filtrate A, described in Table 9.

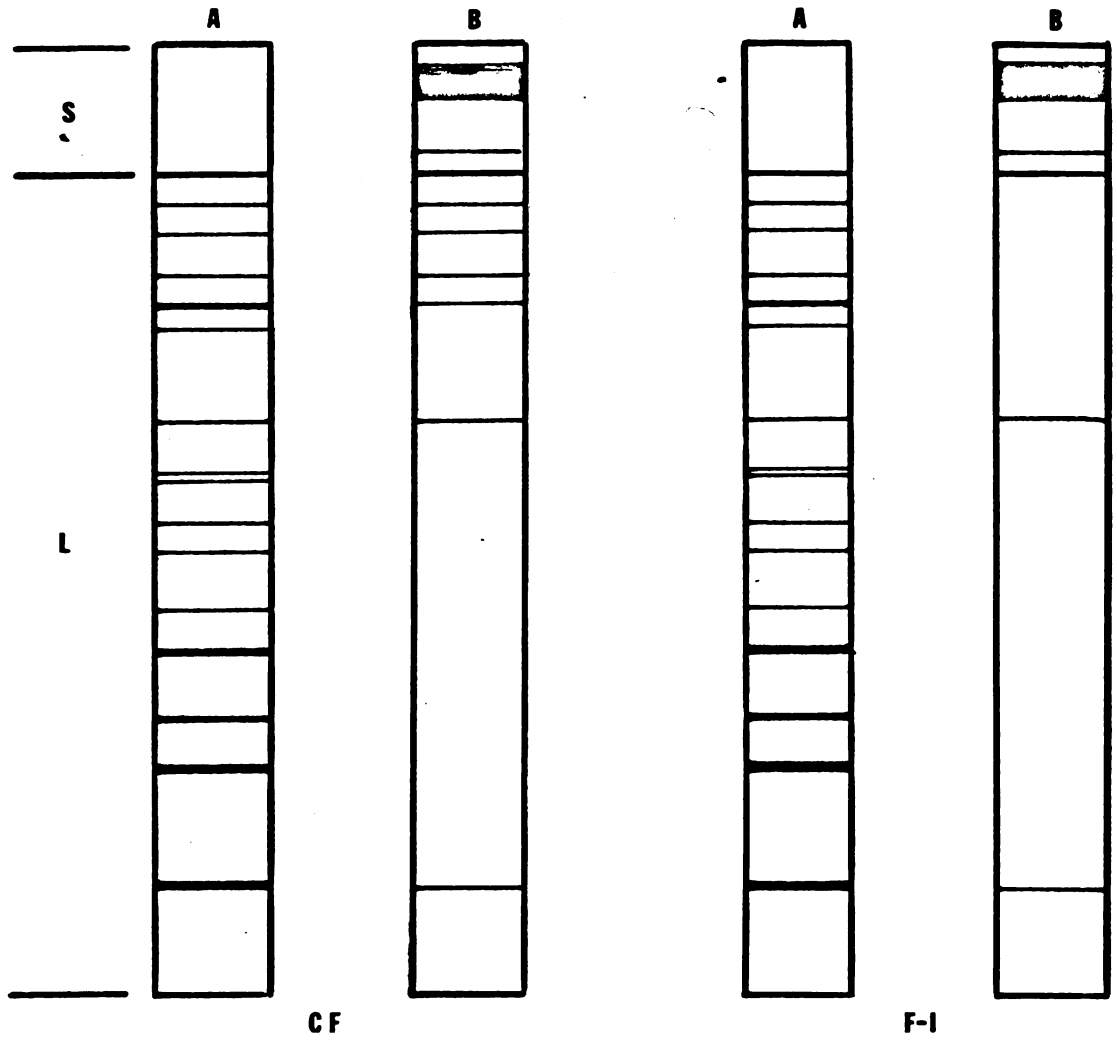

Figure 37. Schematic representation of the protein (A) and PAS-positive (B) components in disc electrophorograms of Mycobacterium bovis culture filtrate A (Table 9) (CF) and Fraction 1 (F-1) obtained by chromatography on Sephadex G-25. The letters S and L refer to the spacer and lower gel areas, respectively.

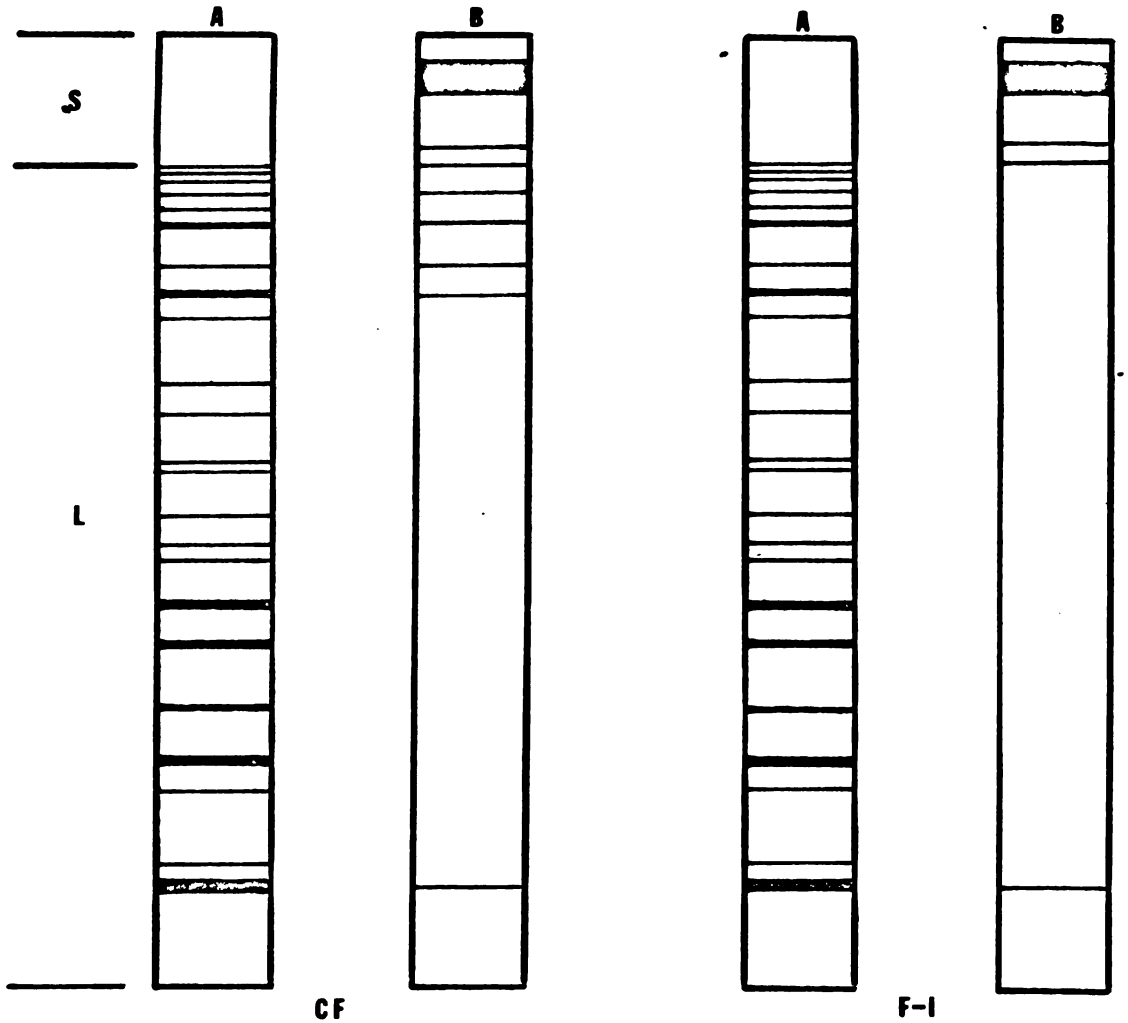

Figure 38. Schematic representation of the protein (A) and PAS-positive (B) components in disc electrophorograms of Mycobacterium bovis culture filtrate B (Table 9) (CF) and Fraction I (F-I) obtained by chromatography on Sephadex G-25. The letters S and L refer to the spacer and lower gel areas, respectively.

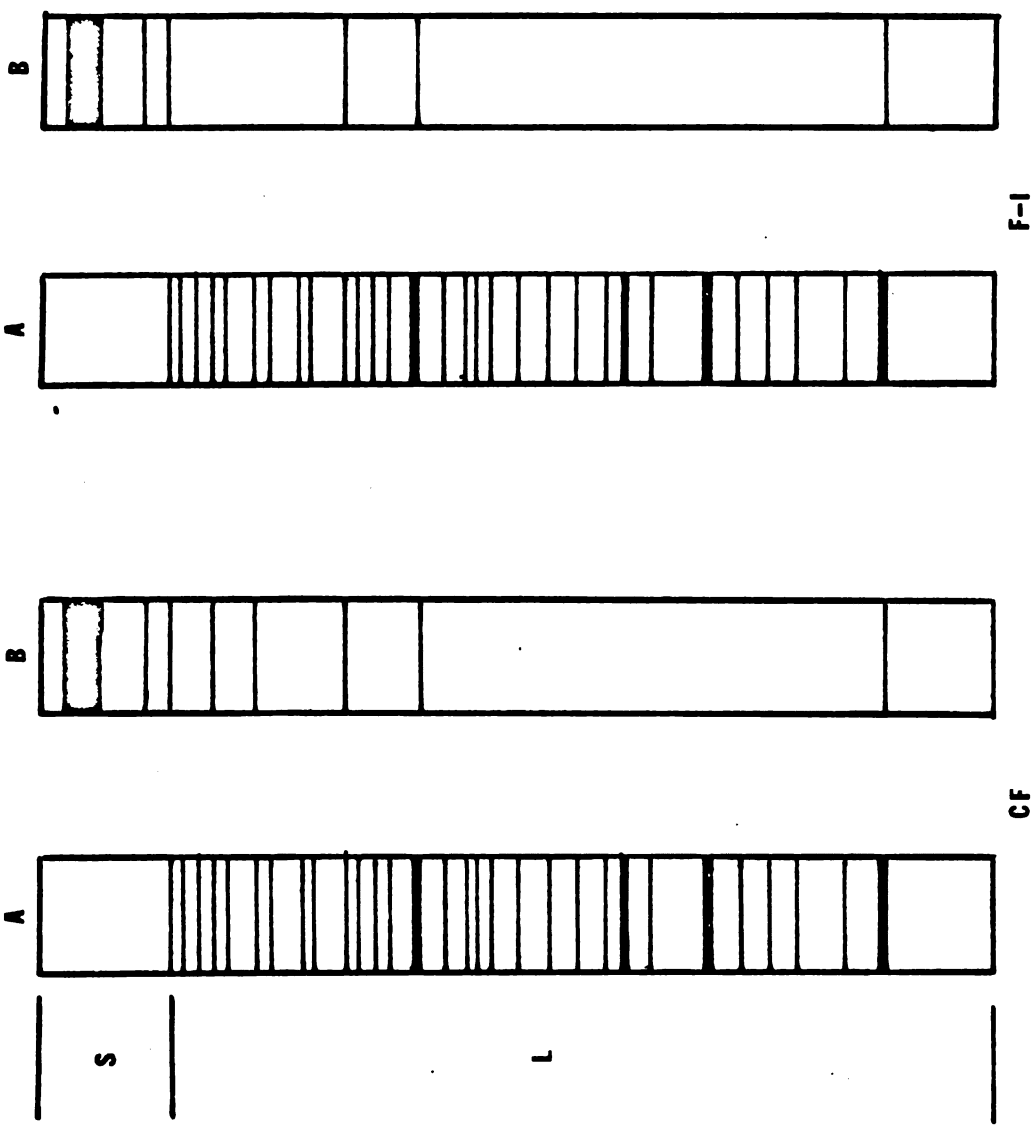

Figure 39. Schematic representation of the protein (A) and PAS-positive (B) components in disc electrophorograms of Mycobacterium bovis culture filtrate C (Table 9) (CF) and Fraction I (F-I) obtained by chromatography on Sephadex G-25. The letters S and L refer to the spacer and lower gel areas, respectively.

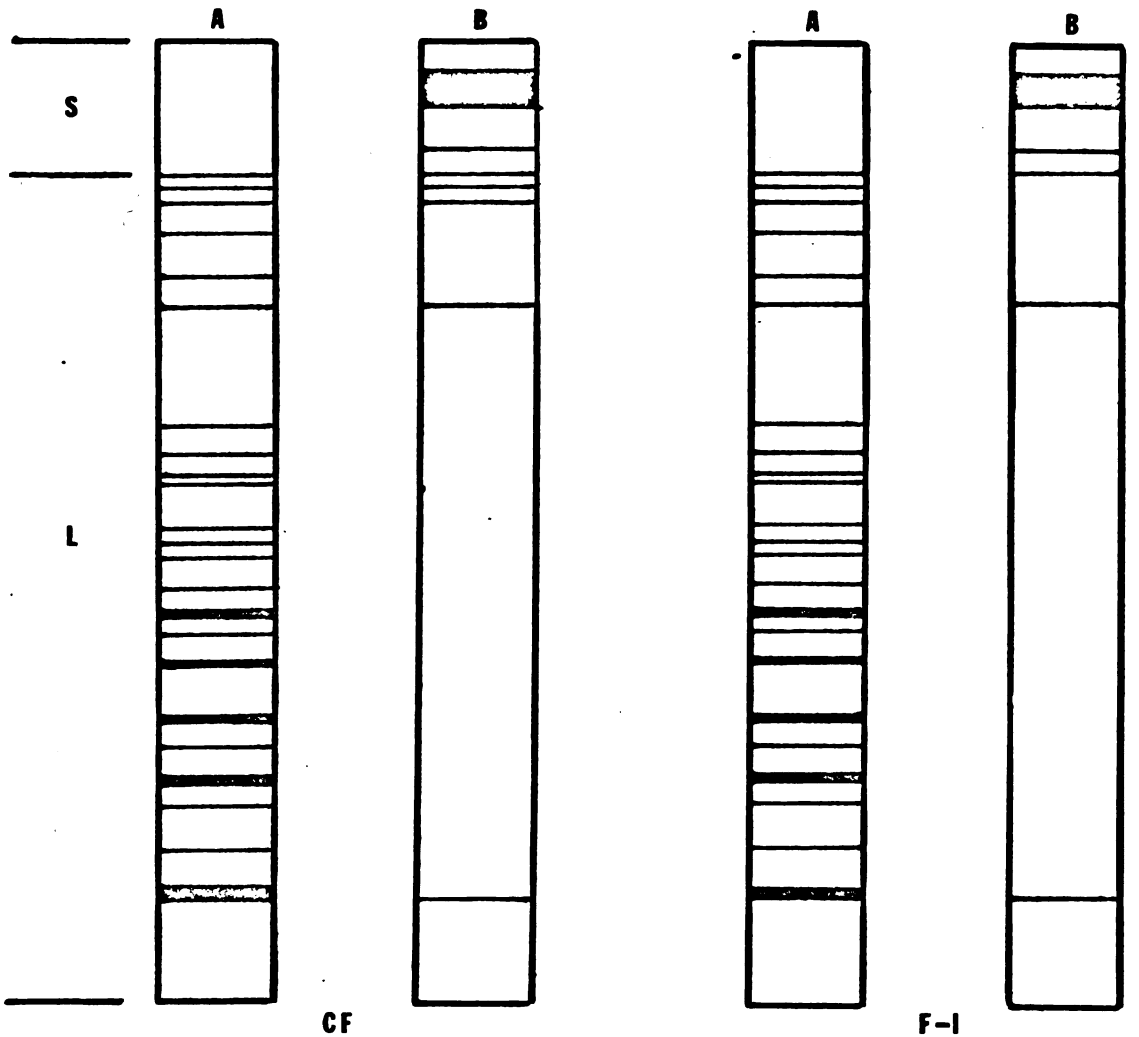

Figure 40. Schematic representation of the protein (A) and PAS-positive (B) components in disc electrophorograms of Mycobacterium bovis culture filtrate D (Table 9) (CF) and Fraction I (F-1) obtained by chromatography of Sephadex G-25. The letters S and L refer to the spacer and lower gel areas, respectively.

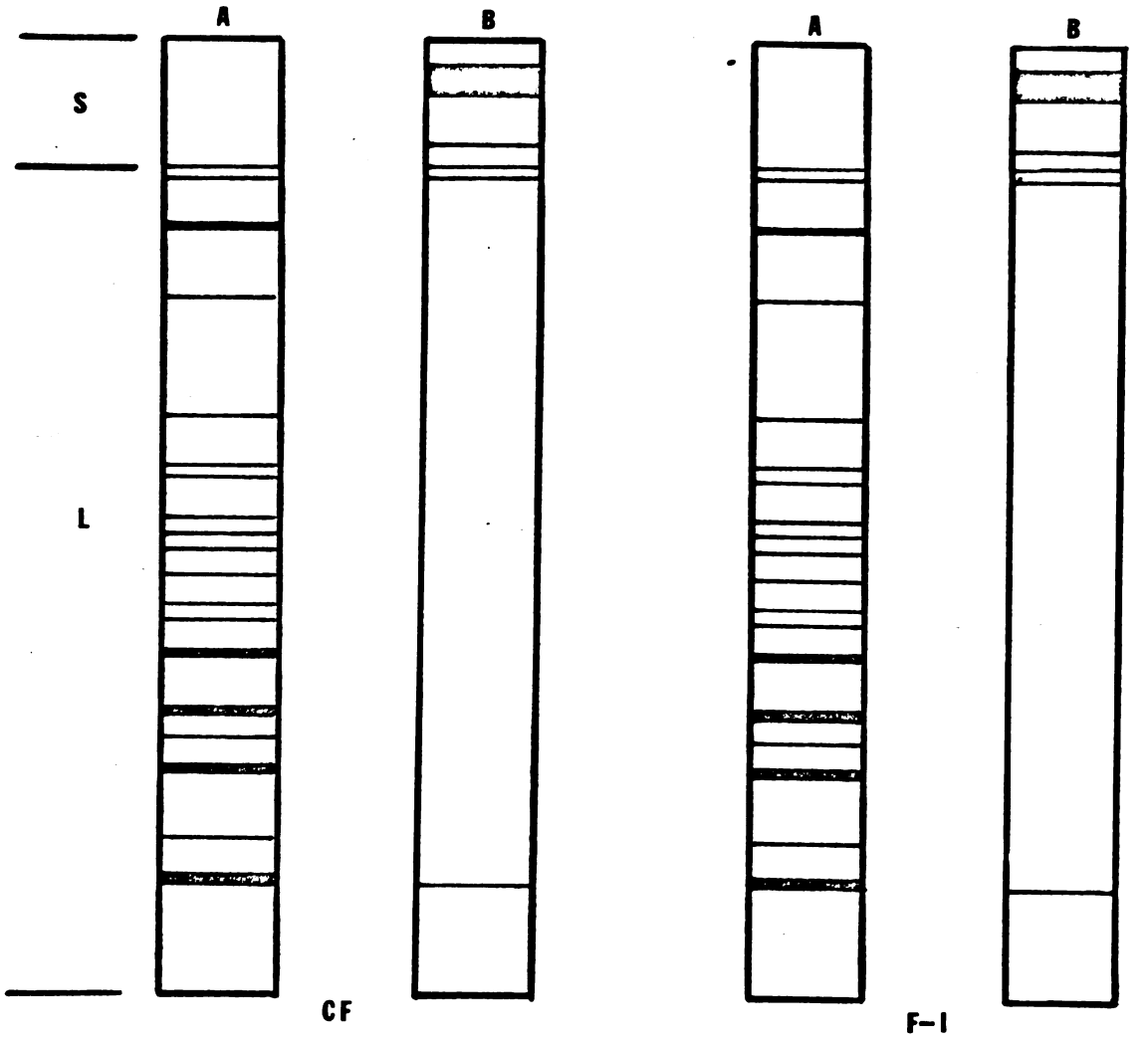

Figure 41. Schematic representation of the protein (A) and PAS-positive (B) components in disc electrophorograms of Mycobacterium bovis culture filtrate E (Table 9) (CF) and Fraction I (F-1) obtained by chromatography of Sephadex G-25. The letters S and L refer to the spacer and lower gel areas, respectively.

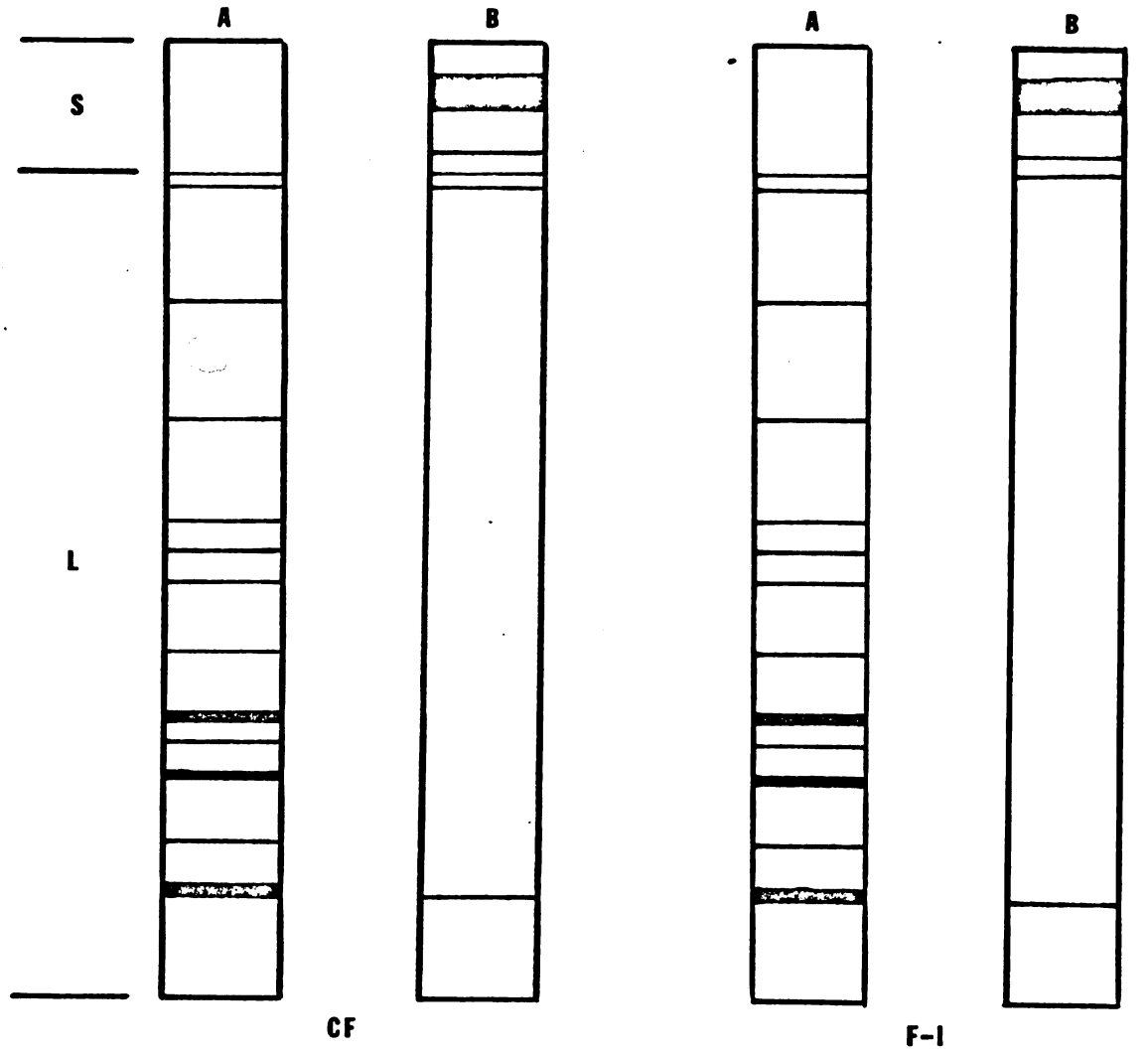

Figure 42. Schematic representation of the protein (A) and PAS-positive (B) components in disc electrophorograms of Mycobacterium bovis culture filtrate F (Table 9) (CF) and Fraction I (F-1) obtained by chromatography on Sephadex G-25. The letters S and L refer to the spacer and lower gel areas, respectively.

Table 19. Rf values of the protein components in the six Mycobacterium bovis culture filtrates.

		CF[1]					
d(cm)[2]	Rf[3]	A[4]	B	C	D	E	F
0.00	0.00	+	+	+	+	+	+
0.05	0.01		+				
0.10	0.02		+	+	+	+	+
0.20	0.04	+	+	+	+		
0.30	0.06		+	+			
0.40	0.08		+	+	+	+	
0.60	0.12			+			
0.70	0.14	+	+	+	+		
0.90	0.18	+	+	+	+	+	+
1.10	0.22	+	+	+			
1.20	0.24			+			
1.30	0.26			+			
1.40	0.28			+			
1.50	0.31		+	+			
1.70	0.35	+	+	+	+	+	+
1.90	0.39			+	+		
2.05	0.42	+	+	+	+	+	
2.10	0.43	+	+	+	+	+	
2.20	0.45			+			
2.40	0.49	+	+	+	+	+	+
2.50	0.51				+	+	
2.60	0.53	+	+	+	+	+	+
2.70	0.55		+				
2.80	0.57			+	+	+	+
3.00	0.61	+	+	+	+	+	
3.10	0.63			+	+	+	
3.30	0.67	+	+	+	+	+	+
3.70	0.76	+	+	+	+	+	+
3.90	0.80			+	+	+	+
4.10	0.84	+	+	+	+	+	+
4.30	0.88		+	+	+		
4.60	0.94		+	+	+	+	+
4.90	1.00	+	+	+	+	+	+

[1]CF = culture filtrate
[2]d(cm) = distance in centimeters
[3]Rf = ratio of the distance moved by the most anodic component and the distance moved by the individual component
[4]A = culture filtrate A described in Table 9

protein bands detected in the unfractionated culture filtrates (Figs. 37, 38, 39, 40, 41, and 42). In only one case (CF-E) did purified fraction I contain all of the PAS-stained components found in the unfractionated CF.

No protein or PAS-stained components were detected in isolated and rechromatographed fractions II or III from the culture filtrates. The yellow pigment in fraction II migrated in close association with the bromphenol blue tracking dye.

Analyses of the culture filtrates and chromatographic eluants by immunodiffusion.

None of the sera from rabbits used for the production of antibodies specific for CF contained precipitins prior to immunization. The number of immunoprecipitates detected in immunograms of the six culture filtrates with antiserum A plus B was as follows: 16 in CF-A; 15 each in CF-B and CF-C; 13 in CF-D; 8 in CF-E; and 6 in CF-F (Table 18). Fraction I isolated from all the culture filtrates and rechromatographed on Sephadex G-25 columns contained the same number of precipitinogens as found in the unfractionated culture filtrates (Table 20). Some displacement of several individual lines was observed (Figs. 43, 44, 45, 46, 47, and 48). All but two or three of the antigens in each CF-fraction I system formed lines of identity. The lines that did not join were near the antigen well. No precipitinogens were detected in fractions II and III from any of the culture

Table 20. Precipitinogens demonstrated in the six Mycobacterium bovis culture filtrates and fractions obtained by chromatography on Sephadex G-25 and Bio-Gels P-100, P-150, and P-200.

Preparation	CULTURE FILTRATE A[1]	B	C	D	E	F
CF[2]	16	15	15	13	8	6
F-I (G-25)[3]	16	15	15	13	8	6
F-II (G-25)	0	0	0	0	0	0
F-III (G-25)	0	0	0	0	0	0
F-I (P-100)	13	12	13	9	8	6
F-I (P-150)	12	11	12	8	7	--
F-I (P-200)	6	5	4	5	2	--

[1]A - Culture filtrate A described in Table 9.

[2]CF - Culture filtrate

[3]F-I (G-25) - Fraction I obtained by chromatography of CF-A on Sephadex G-25

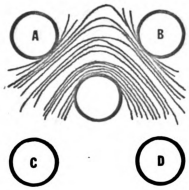

Figure 43. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate A (Table 9) (A) and Fractions I (B), II (C), and III (D) obtained by chromatography on Sephadex G-25. The antiserum was placed in the center well.

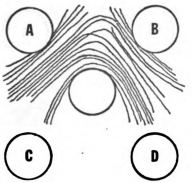

Figure 44. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate B (Table 9) (A) and Fractions I (B), II (C), and III (D) obtained by chromatography on Sephadex G-25. The antiserum was placed in the center well.

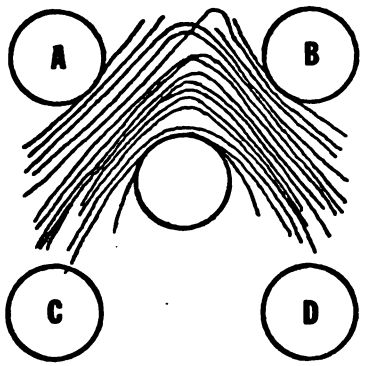

Figure 45. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate C (Table 9) (A) and Fractions I (B), II (C), and III (D) obtained by chromatography on Sephadex G-25. The antiserum was placed in the center well.

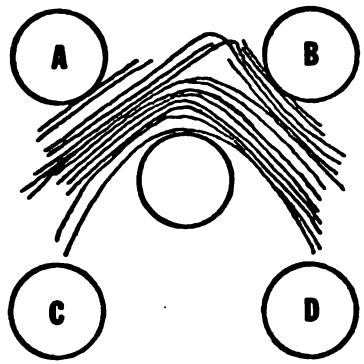

Figure 46. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate D (Table 9) (A) and Fractions I (B), II (C), and III (D) obtained by chromatography on Sephadex G-25. The antiserum was placed in the center well.

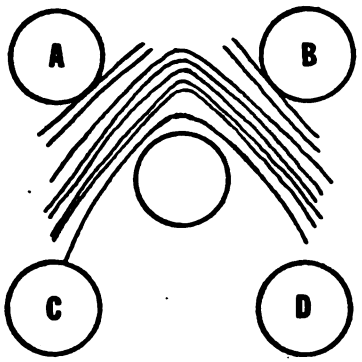

Figure 47. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate E (Table 9) (A) and Fractions I (B), II (C), and III (D) obtained by chromatography on Sephadex G-25. The antiserum was placed in the center well.

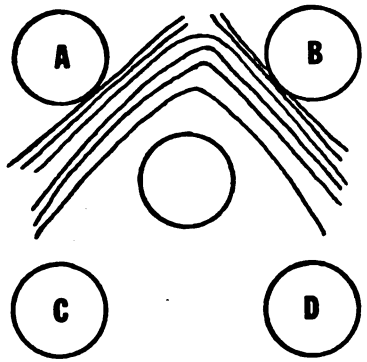

Figure 48. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate F (Table 9) (A) and Fractions I (B), II (C), and III (D) obtained by chromatography on Sephadex G-25. The antiserum was placed in the center well.

filtrates (Table 20). In most cases (CF-A, CF-B, CF-C and CF-D), two to four of the precipitinogens found in the culture filtrates were not found in the corresponding fraction I from Bio-Gel P-100 (Figs. 49, 50, 51, and 52). Fraction I from CF-E and CF-F contained all the precipitinogens in the corresponding CF (Figs. 53 and 54). Fraction I obtained by chromatography of culture filtrates A-E on Bio-Gel P-150 contained all but one of the precipitinogens found in the corresponding fraction from Bio-Gel P-100 (Figs. 49, 50, 51, 52, and 53). From one-quarter to less than one-half of the precipitinogens in the unfractionated culture filtrates were detected in the corresponding fraction I from Bio-Gel P-200 (Table 20).

Culture filtrate A and ultrasonic extracts (USE) from two-month-old cells of M. bovis were antigenically indistinguishable when analyzed with antiserum specific for CF or USE. Several other filtrates from three month old cultures of M. bovis reacted poorly or not at all with antiserum A. Antiserum against any of these culture filtrates formed detectable lines with only two or three antigens in CF-B. The number of precipitinogens detected in the six culture filtrates with antiserum B was as follows: 10 each in CF-A, CF-B, CF-C and CF-D; and 6 each in CF-E and CF-F (Table 21).

Immunoelectrophoresis of culture filtrates.

The number of antigens found in the six culture filtrates by immunoelectrophoresis was as follows: 19 each in CF-A and

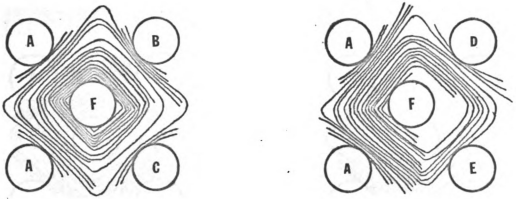

Figure 49. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate A (Table 9) (A) and Fraction I obtained by chromatography on Sephadex G-25 (B) and Bio-Gels P-100 (C), P-150 (D), and P-200 (E). The antisera were placed in the center wells (F).

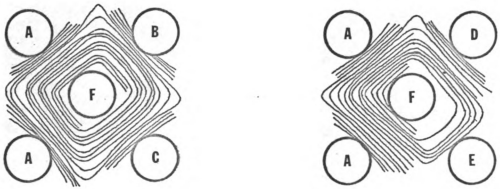

Figure 50. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate B (Table 9) and Fraction I obtained by chromatography on Sephadex G-25 (B), and Bio-Gels P-100 (C), P-150 (D), and P-200 (E). The antisera were placed in the center wells (F).

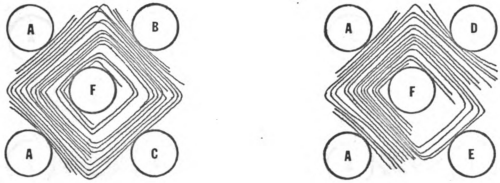

Figure 51. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate C (Table 9) and Fraction I obtained by chromatography on Sephadex G-25 (B) and Bio-Gels P-100 (C), P-150 (D), and P-200 (E). The antisera were placed in the center wells (F).

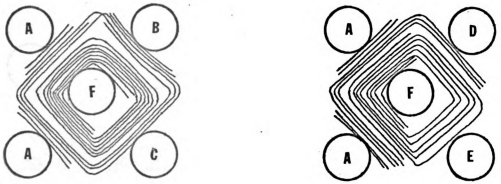

Figure 52. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate D (Table 9) and Fraction I obtained by chromatography on Sephadex G-25 (B) and Bio-Gels P-100 (C), P-150(D), and P-200 (E). The antisera were placed in the center wells (F).

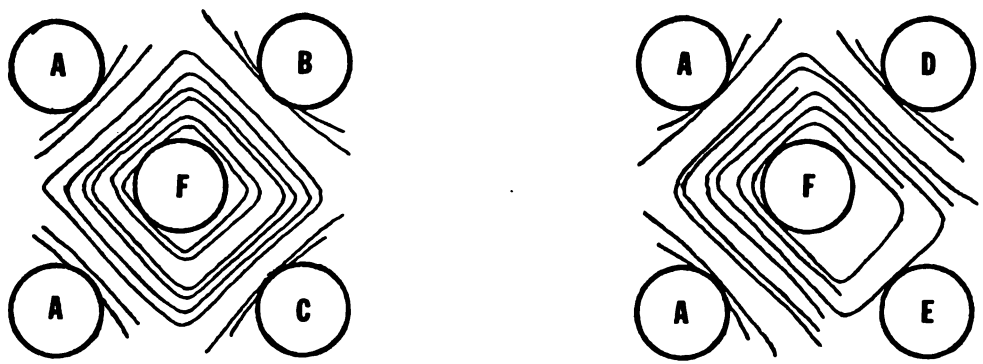

Figure 53. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate E (Table 9) (A) and Fraction I obtained by chromatography on Sephadex G-25 (B) and Bio-Gels P-100 (C), P-150 (D), and P-200 (E). The antisera were placed in the center wells (F).

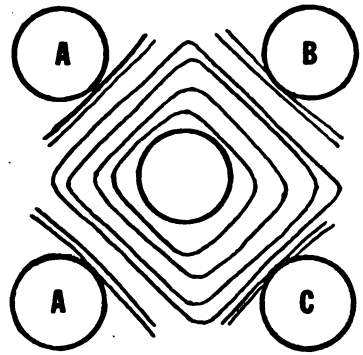

Figure 54. Ouchterlony double diffusion of Mycobacterium bovis culture filtrate F (Table 9) (A), and Fraction I obtained by chromatography on Sephadex G-25 (B) and Bio-Gel P-100 (C). The antiserum was placed in the center well.

Table 21. Precipitinogens demonstrated in the six Mycobacterium bovis culture filtrates by Ouchterlony double diffusion with antiserum B[1].

CF[2]	A[3]	B[4]									
A	10	a	b	c	d	e	f	g	h	i	j
B	10	a	b	c	d	e	f	g	h	i	j
C	10	a	b	c	d	e	f	g	h	i	j
D	10	a	b	c	d	e	f	g	h	i	j
E	6			c	d	e	f	g			j
F	6			c	d	e	f	g			j

[1]Antiserum B elicited in rabbits by culture filtrates D, E, and F (Table 9)

[2]CF - Culture filtrate

[3]A - Number of immunoprecipitates detected in immunograms of the individual culture filtrates

[4]B - Precipitinogens demonstrated

CF-B; 21 in CF-C; 16 in CF-D; 10 in CF-E; and 8 in CF-F (Table 18). Anodic and cathodic antigens were found in all of the immunoelectrophorograms (Figs. 55, 56, 57, 58, 59, and 60). Many of the lines were very lightly stained with the protein-specific stain and were difficult to see. None of the lines were stained by lipid or polysaccharide-specific stains. The individual lines in each immunoelectrophorogram differed slightly in their lateral and migrational displacement. No attempt was made to identify or locate individual antigens by this method.

Figure 55. Immunoelectrophorogram of Mycobacterium bovis culture filtrate A (Table 9).

Figure 56. Immunoelectrophorogram of Mycobacterium bovis culture filtrate B (Table 9).

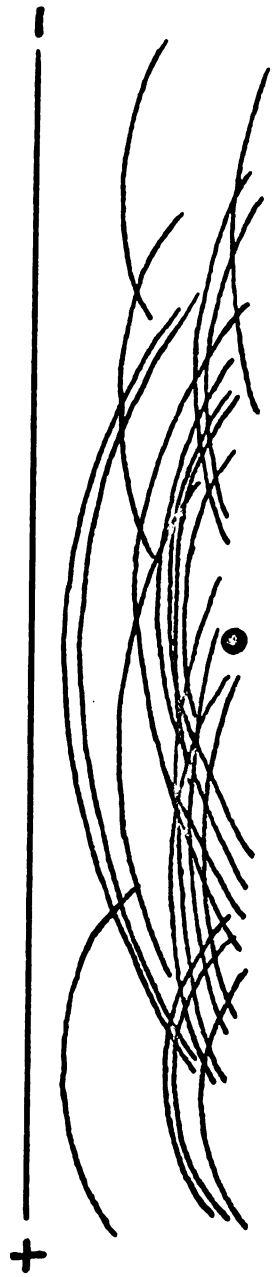

Figure 57. Immunoelectrophorogram of Mycobacterium bovis culture filtrate C (Table 9).

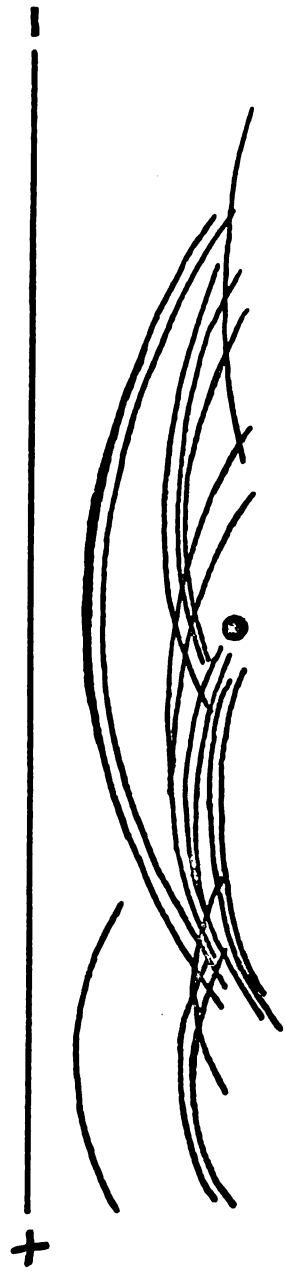

Figure 58. Immunoelectrophorogram of Mycobacterium bovis culture filtrate D (Table 9).

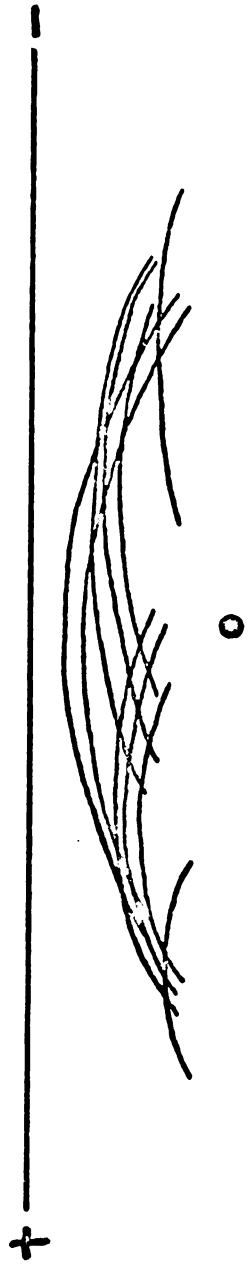

Figure 59. Immunoelectrophorogram of Mycobacterium bovis culture filtrate E (Table 9).

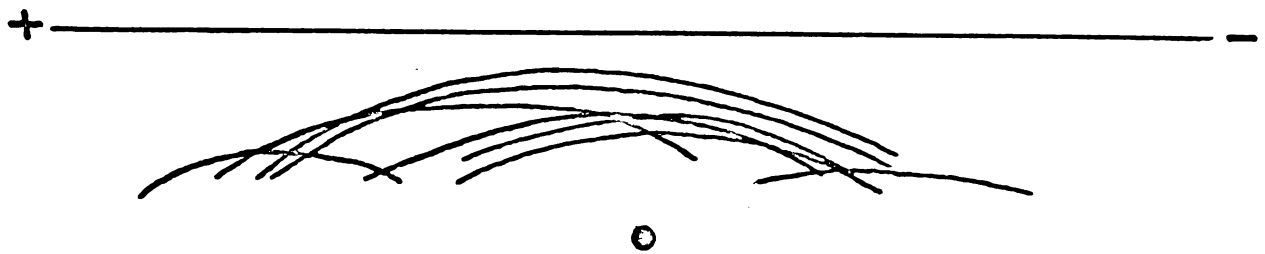

Figure 60. Immunoelectrophorogram of Mycobacterium bovis culture filtrate F (Table 9).

DISCUSSION

Considerable advancements have been made in the past decade in understanding the nature of specific antibodies. Rabbits produce at least two classes of immunoglobulins when inoculated with a variety of antigens (8;9;173). The primary response consists of the sequential production of these two immunoglobulins. The first antibodies to be produced (1g-M) are 19S gamma$_1$-globulins which are sensitive to reductive cleavage by 2-mercaptoethanol (ME). This is followed, after several days, by the production of lower molecular weight, 7S gamma$_2$-globulins (1g-G) which retain their serologic activity after treatment with ME. Following secondary antigenic stimulation, much higher titers of 1g-G are produced shortly after inoculation (9). The temporal sequence and extent of 1g-M production is similar to the primary response.

The sequential production of MES and MER hemagglutinins and bacterial agglutinins was observed in rabbits inoculated with soluble and cell-bound antigens of M. bovis. The relative amount and temporal sequence of production of each type of antibody varied among the experimental groups. Although none of the pre-inoculation sera were positive when tested by both serologic tests, it is possible that some of the

rabbits used in this investigation had previously been sensitized to mycobacterial antigens. Primary sensitization can result from repeated exposure to ubiqutous saprophytic mycobacteria. Certain antigens are shared by many, if not all mycobacteria and cross reacting antigens can be obtained from apparently diverse sources.

Depending on the dosage and frequency of antigenic stimulation, antibody may or may not be elicited. Low doses of antigen elicit only a small amount of 1g-M and no lasting immunologic memory results (173). When larger doses of antigen are injected, both 1g-M and 1g-G are produced and a typical anamnestic response can be demonstrated upon reinjection of the antigen. Thus, some of the sera may have contained undetectable amounts of 1g-M or 1g-G prior to inoculation.

It is also possible that detectable amounts of antibody were present in some of the sera but that neither OT-sensitized red blood cells nor the intact bacterial cells contained homologous antigen(s). This is unlikely, however, because of the antigenic complexity of OT and the frequency of cross agglutination of different mycobacteria (67).

It is common practice to tuberculin test experimental animals prior to immunization or collection of sera or cells for <u>in vitro</u> studies. Tuberculin tests cause an anamnestic antibody response in tuberculous humans (152), cattle and cattle and swine (97). Antibody can also be elicited in normal

tuberculin-negative animals by tuberculin testing. There was a sharp increase in the production of MES hemagglutinins elicited by tuberculin in rabbits sensitized with M. bovis (40). To avoid the possibility of sensitization or the elicitation of an anamestic response in previous sensitized rabbits, the rabbits used in the study were not tuberculin tested prior to use. They were obtained from a colony in which other rabbits are and remain tuberculin-negative.

Antibody in the sera of immunized rabbits was detected by bacterial agglutination and the Middlebrook-Dubos passive hemagglutation test. The sequential production of MES and MER antibody was detected by both serologic tests. The mean hemagglutinin titers of all of the groups were consistently higher at each week than the mean bacterial agglutinin titers. These differences can be anticipated due to the greater sensitivity of the passive hemagglutination test, but the differences do not infer that the same antigens are measured. It is most likely that the tests measure different antigen-antibody systems. Culture filtrates contain polysaccharides believed to originate from cell wall lipopolysaccharides during autolysis (14), and normal sheep red blood cells selectively absorb tuberculopolysaccharides. To absorb proteins cells must first be treated, as for example with tannic acid. It is also possible that tuberculopolysaccharides in the medium in which the cells grew absorb to the surface of the mycobacterial cells prior to BPL-inactivation. This is not

likely since the cells were treated with various solutions, temperatures and mechanical treatment during inactivation, dispersing and washing. The suspension contains some cell debris but many intact cells were observed when stained with fluorscein-conjugated antibody.

The apparent sequential production of MES and MER antibody may be due to the different sensitivity of the agglutinin test for the two types of immunoglobulins (174). If this is true, much more MER antibody than MES antibody may have to be produced in order to reach detectable concentrations. Further studies will have to be conducted using other serologic tests to determine whether the production of 1g-M and 1g-G is simultaneous or sequential.

Multiple injections of CF in incomplete Freund's adjuvant (Group I) elicited higher titers of MES bacterial agglutinins for the first two weeks post-inoculation than culture filtrate and heat-killed M. bovis in incomplete adjuvant. This may be due to the differences in the relative amounts of effective agglutinogens in cells and CF. The agglutinogens in CF are soluble and readily accessible to antibody producing cells. The same antigens in cells must be liberated prior to processing by antibody producing cells. It is also possible that bacterial agglutinins were specifically sequestered by intact cells in the inocula in vivo. This does not seem probable.

The rabbits which received CF with BPL-killed M. bovis (Group III) produced antibody from two to three weeks sooner

than rabbits which received CF and heat-killed M. bovis (Group II). It is not known whether these differences are due to variations in the antigen dosage. Although the cells were repeatedly shaken prior to preparation of the inocula, many of the cells were aggregated. It is likely that all of the rabbits did not receive exactly the same amount of cells. It is unlikely that the small difference in the concentrations would account for the differences. These results may also be attributed to differences in the nature or extent of antigen inactivation by heat and BPL. Perhaps heat-killed M. bovis contains less effective antigen than BPL-killed M. bovis or this may reflect only at BPL-inactivated cells used to test agglutinins. It is important to realize however, that the serologic tests used probably measure several different antigen-antibody systems. Consequently, neither test is likely to reveal meaningful information about the alteration of antigenic specificity in complex mixtures of antigens.

The magnitude and duration of MES hemagglutinin production was lower in rabbits inoculated with inactivated cells plus CF (Groups II and III) than in rabbits which received only CF (Group I) or inactivated M. bovis. Moreover, earlier and higher titers of MER hemagglutinins were produced by the rabbits in Groups II and III. Sequential injection of CF plus BPL-killed cells (Group IV) prolonged the production of MES and delayed the onset of MER hemagglutinin production when compared to multiple injections of the same inoculum

(Group III). Rabbits given six simultaneous subcutaneous injections of CF without adjuvant (Group V) produced only MES hemagglutinins until six weeks post-inoculation. A similar pattern of bacterial agglutinins production was observed. An initial intravenous injection of CF followed by multiple injections of CF with BPL-killed M.bovis (Group VIII) extended the duration of MES hemagglutinin production longer than rabbits which did not receive initial intravenous injection (Group III). More and earlier MER hemagglutinins were produced by rabbits in Group III. These results may be best understood by relating the mode and route of injection to the localized dose or concentration of the antigen.

The interval between the appearance of MES antibody and the first appearance of MER antibody could be shortened by concentration of the antigen or localizing the dose, that is, injecting in an area and/or with a substance so that the antigen is retained longer at the site of injection. On the other hand, when the localized antigenic stimulus was decreased by not using adjuvant, systemic injections, or repeated small injections at weekly intervals, MES antibody production was prolonged and the initiation of MER antibody synthesis delayed. How can this be rationalized with the theory that 1g-G acts as an inhibitor of 1g-M? Is the prolonged production of 1g-M only an indirect effect and could it be more correctly stated that intravenous injection suppressed 1g-G production, or at least, increase the time

required for localized antigen to elicit 1g-G antibodies?

The addition of heat or BPL-killed M. bovis (Groups II and III) increased both the localized dose and concentration of antigen in contrast to the inoculation of CF or cells alone. Similarly, rabbits which received multiple inoculations of CF plus killed M. bovis received a greater initial antigenic stimulus. Although rabbits in Groups III and VII received the same amount of antigen, the localized dose of antigen was less in these rabbits which received an intravenous inoculation (Group VII). The longest interval between the appearance of MES and MER antibody (7 weeks) occurred in rabbits given CF with inactivated M. bovis without adjuvant. None of the rabbits in the group had palpable granulomae or produced detectable precipitins. Importance of adjuvant most likely lies in the retention of antigen in the tissue and the associated inflammatory response which thereby potentiates the production of antibody.

The preferential evocation of MES antibody by the intravenous inoculation of particulate antigens is well-known (171). Repeated intravenous injections of CF plus BPL-killed M. bovis elicited high titers of MES antibody that persisted throughout the duration of the experiment. The production of MES antibodies was also potentiated by an initial intravenous injection of CF (Group VII and VIII).

It was of considerable importance to determine the best way of eliciting large amounts of antibodies specific for

CF antigens. Aluminum hydroxide-precipitated CF elicited a much better precipitin response than either killed M. bovis or inactivated cells plus CF incorporated in Freund's adjuvant. These results are not comparable, however, due to the differences in the antigen dose and route of inoculation. Washing the alum hydroxide-precipitate did not noticeably reduce the efficacy of the precipitate to elicit precipitins.

The biopolymers found in mycobacterial culture filtrates are derived from bacterial cells primarily as a result of autolysis. Materials may also be liberated into the culture fluid during cell growth or may be extracted from the cell envelopes. Autolysis is a complex chemical process involving a state of dysequilibrium between cell wall biosynthesis and catabolism. Autolysis of some cells occurs during every phase of the growth cycle of most bacteria (80). However, more occurs at the end of the stationary phase when nutrient exhaustion or the accumulation of toxic substances halts the growth of the culture. During autolysis, cytoplasmic materials are released from cells as the result of enzymatic attack on cell walls. This process occurs gradually so that in the case of mycobacteria cultures, considerable time may be required before the culture media contains suitable concentrations of antigens. Thus, there is ample time for considerable denaturation and degradation of the macromolecules by protolytic enzymes, peptidases, carbohydrases, and nucleases. The moderate temperature of incubation (35C)

and aqueous environment facilitate enzymatic hydrolysis of macromolecules (61).

All of the culture filtrates of M. bovis contained proteins, polysaccharides, and nucleic acids in various states of polymerization and denaturation. The relative concentrations of these macromolecules varied considerably in the culture filtrates. The chemical and antigenic composition of the filtrates varied with the length of incubation of the culture. There was no correlation between the age of the culture and the protein, polysaccharide or nucleic acid concentration. However, CF-B and CF-C contained the highest concentrations of these constituents. It is possible that this is in part due to variations in the amount of growth that occurred in the cultures. No actual measurements of the number of cells in the individual cultures were made, so it cannot be stated with certainty that this is not the case. However, a thin confluent layer of growth formed on the surface of the culture media within one month after inoculation and large lots of filtrates were pooled. Moreover, the filtrates were all prepared in the same manner.

The constituents in the culture filtrates were readily separated into three or more fractions by molecular exclusion column chromatography on Sephadex G-25. In this technique, molecules are separated solely on the basis of molecular size.

The approximate molecular weight range of a solute can be estimated by relating the volume of eluent required to

elute the solute to the void volume of the column. Molecules that have Rf values of 1.0 are eluted in the column void volume and have molecular weights equal to or greater than the exclusion value of the gel. Thus, molecular exclusion chromatography is a rapid and convenient means of detecting changes in the state of polymerization of the three major classes of biomacromolecules in culture filtrates of different ages.

The amount of protein in the chromatographic eluants was measured chemically by the Folin method and spectrophotometrically at 280 mμ. Although the results of both tests were in fairly good agreement for fraction I of culture filtrates A through D, they were considerably different for fraction I of culture filtrates E and F. The reasons for these discrepancies is unknown. Both test measure the amino acids tyrosine and tryptophane. Although nucleic acids have a maximal absorbancy at 260 mμ, they also absorb 280 mμ ultraviolet light. The partial absorption of 280 and 260 mμ ultraviolet light by nucleic acids and proteins accounts for the maximal absorbancy at 270 mμ in the ultraviolet absorption spectra of the culture filtrates. This may also account for the discrepancies in the spectrophotometric determinations of protein in the chromatographic eluants.

The percent distribution of proteins of molecular weight 5,000, 100,000 and 200,000 or greater varied with the age of the culture filtrate. From 80% to 90% of the proteins in

culture filtrates A through E had molecular weights equal to or greater than 5,000. Only 50% to 60% of the proteins in the same culture filtrates were greater than 100,000 molecular weight. Sixty percent of the proteins in CF-F had molecular weights greater than 5,000; 23.2% had molecular weights greater than 100,000. From 33% to 45% of the proteins in culture filtrates A, B, and C had molecular weights greater than 200,000. Only 26% of the proteins in CF-E were greater than 200,000 molecular weight. None of the proteins in CF-F had molecular weights in this range. Thus, considerable degradation of the large mycobacterial proteins occurs in the culture fluid during incubation.

Most, if not all, bacteria contain peptidases that are released into the culture medium during autolysis (130). These enzymes hydrolyze small proteins to low molecular weight polypeptides and amino acids. The Folin reacting materials in fraction II obtained by chromatography of the culture filtrates on Sephadex G-25 had molecular weights considerably less than 5,000 (Rf = 0.36 to 0.40). They are probably polypeptides. It is difficult to determine whether these polypeptides represent products of degradation of larger proteins or arise from polypeptide pools within viable cells. Twice as much polypeptide was found in fraction II from CF-F as in the same fraction from CF-A. It is most likely that the majority of these polypeptides were derived from larger proteins by enzymatic hydrolysis.

The presence of low molecular weight polypeptides in fraction III is further indication of degradation of larger proteins. Polypeptides were not detected in fraction III from culture filtrates A, B, or C. However, increasing amounts were found in this fraction obtained from culture filtrates D, E, and F.

Most of the chemical tests for the measurement of protein are based on the determination of aromatic amino acids, nitrogen, the peptide bond, or absorption of 280 mμ ultraviolet light. None of these test, however, give any indication of the size or molecular weight of the proteins being measured. The dividing line between the size requirement for proteins and polypeptides has been arbitrarily set at 10,000. This is also near the lower limit for antigenicity of proteins, although some lower molecular weight "proteins" are antigenic (16). These factors must be considered when mixtures such as CF are used to elicit antibody. This is exemplified by the fact that only 60% of the Folin-reacting material in CF-F had molecular weights of 5,000 or greater.

The presence of 280 mμ and 260 mμ-absorbing material in the culture filtrates is indicative of autolysis. The percent distribution of these materials in chromatograms of the six culture filtrates varied depending on the age of the culture. The chromatogram of CF-A contained very little fraction III. However, approximately one-third or more of the total 280 mμ-absorbing materials in culture filtrates

D, E, and F were found in this fraction. Increasing amounts of 280 mμ-absorbing material were found in fraction I of culture filtrates A, B, and C. Thereafter, the relative amount of 280 mμ-absorbing material in fraction I of culture filtrates D, E, and F decreased. Moreover, fraction I in the older culture filtrates (D, E, and F) was split into several subfractions. These changes reflect the greater amount of autolysis and degradation of macromolecules that occurred in the older cultures.

Further indication of the extent of autolysis and degradation that occurred in the cultures was provided by the distribution of nucleic acids in the three fractions. Most of the nucleic acids in the culture filtrates were found in fraction II. Fraction I of CF-C and CF-D contained greater amounts of nucleic acid than the same peak from any of the other filtrates. This suggests that considerable autolysis occurred at this time. Culture filtrate C also contained high concentration of proteins and polysaccharides.

The culture filtrates contained variable amounts of dialyzable and nondialyzable materials. The dialysate contained only trace amounts of fraction I but most of fractions II and III found in the culture filtrates. This provides further evidence for the low molecular weight of these materials. The dialysate elicited immediate-type skin reactions in sensitized rabbits but no precipitinogens were detected by Ouchterlony double diffusion or immunoelectrophoresis.

This result differs from that reported by Chaparas and Baer (27). They found several precipitinogens in the dialyzable fraction from BCG culture filtrates. Only trace amounts of several protein components were seen in disc electrophorograms of the dialysates. The nondialyzable fractions contained all of the precipitinogens and protein components found in the homologous culture filtrate. These results indicate that dialysis does not appreciably reduce the number of antigens in CF. This is a good way of removing low molecular weight materials from CF that interfere with protein determinations. Moreover, the dialysate contains very little protein but may be a good source of sensitin materials.

The proteins in culture filtrates A, B, and C were not well separated by cellulose acetate membrane electrophoresis. Considerable trailing occurred and only three to four bands were discernable. However, from five to six bands were discernable in electrophorograms of purified fraction I from the same culture filtrates. The bands were fairly well separated. There were no qualitative differences in the protein patterns obtained. The improved separation and resolution obtained with these fractions is most likely due to the absence of low molecular weight materials. This may explain the failure of other investigators to obtain satisfactory separation of the proteins in culture filtrates by this method (125). Elution of these bands from large cellulose

acetate strips may be useful as a primary step in the isolation of mycobacterial antigens.

Disc electrophoresis was a very effective means of separating the components in the six culture filtrates. Moreover, the technique is simple, rapid, and the patterns obtained are highly reproducible. The number of amido black-stained (protein) and PAS-stained (polysaccharide or glycoprotein) components detected in disc electrophorograms varied with the length of the incubation of the culture. The number of protein bands increased from 16 in CF-A to 30 in CF-C. Thereafter the number decreased; only 13 protein bands were detected in CF-F. All of the filtrates contained five protein bands with similar or identical electrophoretic mobility. Seven other bands with the same Rf value were found in three or more of the culture filtrates. Culture filtrate C contained all but two of the protein bands found in the other filtrates. It appears, therefore, that new components did not appear in the culture medium after the fourth month of incubation. Rather as the length of incubation continued, there was a progressive degradation of the proteins already present.

The number of polysaccharide or glycoprotein components in the culture filtrates decreased with longer periods of incubation. The disc electrophorograms of all the filtrates had two polysaccharide bands in the spacer gel. There was no consistent pattern in the presence or absence of these glycoproteins in culture filtrates of different ages.

All of the protein and polysaccharide components detected in the disc electrophorograms had molecular weights of 5,000 or greater. This is indicated by the fact that the disc electrophorograms of fraction I from Sephadex G-25 contained all the bands seen in the corresponding CF. No bands were seen in disc electrophorograms of fractions II or III. This affords further evidence for the non-protein nature (polypeptide) of the Folin-reacting material in these fractions.

Immunodiffusion is a very useful means of analyzing the precipitinogenic content of the culture filtrates. However, the results obtained by this technique vary greatly depending on the way the test is performed and the quality of the antiserum employed. Repeated inoculation of CF in incomplete Freund's adjuvant was not an effective means of eliciting anti-CF precipitins in rabbits. Prolonged immunization in this manner may be more effective, however, the specificity of the antisera would probably be decreased. Only six antigens were found in concentrated culture filtrates when analyzed with antisera elicited by this method. The addition of killed M. bovis to culture filtrates from the same species of mycobacterium did not improve the precipitinogenicity of the inocula. Immunograms of filtrates from three month cultures of M. bovis had only three lines when analyzed with antisera elicited in this manner. In the present investigation, only four lines were detected in

immunograms of filtrates of three month old cultures of M. bovis.

Preliminary studies showed that multiple intramuscular injections of aluminum hydroxide-precipitated CF elicited good precipitating antisera in rabbits. From six to eight lines were detected in immunograms of culture filtrates of M. bovis with these antisera. However, by carefully selecting the optimal conditions for analyses, as many as 16 lines could be detected. Results of disc electrophoresis and molecular exclusion chromatography indicate that CF may contain as many as 30 different protein components with molecular weight of 5,000 or greater. Most of the precipitinogens in CF have molecular weights greater than 150,000. It is likely that most if not all, of these proteins may elicit precipitins under suitable conditions. From 19 to 21 lines were detected in immunoelectrophorograms of culture filtrates A, B, and C. These results point out the antigenic and chemical complexity of mycobacterial culture filtrates. It is clear that earlier investigations have detected relatively few of the antigens in CF. The methods on which reports of fewer numbers of antigen have been based should be seriously questioned.

Immunodiffusion was an effective means of enumerating the precipitinogens in culture filtrates of different ages. There was a progressive reduction in the number of precipitinogens in culture filtrates of increasing age. Sixteen

lines were detected in immunograms of CF-A; immunograms of CF-F had only six lines. These results were confirmed by immunoelectrophoresis.

Fewer precipitinogens were detected in all but one of the filtrates (CF-F) when analyzed with antiserum B than with antisera A. This is most likely due to the fact that younger filtrates contain antigens that are absent or present only in low concentrations in older filtrates. The detection of more antigens in culture filtrates D and E with antiserum A than with the homologous antiserum B is not so readily explained. Many (perhaps most) of the proteins present in the older culture filtrates (D, E, and F) have undergone some degree of denaturation due to the prolonged exposure to the relatively warm temperatures in an aqueous environment. Denaturation occurs by unfolding and/or refolding of the polypeptide chains resulting in a new tertiary structure. The extent of denaturation and alteration of antigenic specificity that results depends on the extent of chain rearrangement that occurs. It is also probable that smaller proteins are eliminated from the rabbit more readily which reduces the relative or effective concentration of the protein.

Denatured proteins elicit precipitins that may or may not react with the native protein. Therefore, it is possible that antiserum against the native proteins (antiserum A) will react with the denatured form whereas the converse will not occur. Some support for this interpretation is provided by results

of immunodiffusion analyses of different batches of M. bovis culture filtrates of the same age with homologous and heterologous antisera. Whereas strong reactions occurred with homologous systems (8 to 10 lines) only two or three lines were detected with heterologous antisera. One would expect that if the proteins were in the native undenatured state (no change in antigenic specificity) stronger cross reactions would occur. Antisera specific for ultrasonic extracts of M. bovis (40) reacted equally well with CF-A. The sonic extract presumably contained native antigens. Moreover CF-A and ultrasonic extracts from two month old M. bovis were indistinguishable when analyzed with antiserum A. These results indicate that filtrates from two month old cultures contain antigens in a native or only slightly denatured state. Continued incubation of the culture may lead to considerable denaturation of the proteins and the acquisition of new antigenic specificity.

Immunoelectrophoresis was superior to Ouchterlony double diffusion for the enumeration of the precipitinogens in the culture filtrates. In every case, more precipitinogens were detected by immunoelectrophoresis than by the Ouchterlony method. The number of lines detected in immunoelectrophorograms were from 21 in CF-C to 8 CF-F. The possibility of superimposition (masking) of precipitates after immunoelectrophoresis is considerably less than after Ouchterlony double diffusion since the antigens are first separated by electrophoresis. Two or more antigens can form superimposed

precipitates only if their electrophoretic mobility, diffusion coefficient, and reactant ratios are nearly identical. The probability of the simultaneous occurrence of all of these factors is very low. Despite the demonstrated sensitivity of this technique, several antigens in each CF were undoubtedly not detected because of masking of immunoprecipitates.

A great need exists for purified specific antigens and sensitins from mycobacteria that could be used for the diagnosis of tuberculosis and identification of the causative agent. Although much progress has been made, most investigations have failed to obtain immunochemically pure preparations. Moreover, almost nothing is known about the role of individual mycobacterial antigens in tuberculoimmunity or hypersensitivity. It is obvious that purified antigens must be used in order to draw meaningful conclusions regarding their respective role in tuberculosis.

Disc electrophoresis hold much promise as a means of isolating individual antigens (131). However, the application of this technique is limited to proteins that have dissimilar electrophoretic mobility. Unfortunately, due to the great number of CF proteins with similar mobilities, the usefulness of this technique is limited to only a few proteins. A preliminary procedure is needed to separate the proteins in CF based on properties other than electric charge. Proteins with dissimilar mobility could then be readily isolated by

preparative disc electrophoresis and studied in experimentally infected animals. The results of molecular exclusion chromatography on Bio Gel P-200 suggest that this technique may be useful in this regard. Fraction I from CF-A contained only 6 of the 16 precipitinogens detected in the unfractionated CF. Moreover all of these antigens have molecular weights of 200,000 or greater and are free of lower molecular weight constituents. The technique is rapid, easily performed, and can be used on a preparative scale.

SUMMARY

Antibodies elicited in rabbits by concentrated unheated culture filtrates and heat or betaprone-killed cells of M. bovis were measured. Bacterial agglutinins and passive hemagglutinins (tuberculopolysaccharide specific) were titrated before and after treatment with 2-mercaptoethanol. The variance among the mean weekly antibody titers were analyzed statistically. Mercaptoethanol sensitive (MES) and mercaptoethanol resistant (MER) antibodies were produced sequentially but relative amounts and temporal sequences were altered by the state of the antigen and the inoculation schedule. Injecting the total amount of antigen initially shortened the interval after the production of MES antibody and the production of MER antibody. Repeated small inoculations at weekly intervals, no adjuvant, or intravenous inoculations of antigens prolonged MES antibody production and delayed production of MER antibody.

The constituents in unheated concentrated culture filtrates from 2, 3, 4, 5, 6, and 7 month-old-cultures of M. bovis were readily separated into 3 or more fractions by molecular exclusion chromatography on Sephadex G-25. The first fraction contained all of the protein and precipitinogens, and most of the polysaccharides. Fractions II and III contained predominately polynucleotides, polypeptides, and

oligosaccharides. The percent distribution of proteins of molecular weight of 5,000, 100,000, and 200,000 or greater varied with the length of incubation of the culture. The older culture filtrates contained less high molecular weight protein than the younger culture filtrates.

None of the precipitinogens in culture filtrate were dialyzable. The components in the dialysate were largely materials from fractions II and III, only trace amounts of fraction I were found. By disc electrophoresis, between 13 and 30 amido black-staining components and five to nine PAS-staining components in the culture filtrates were separated. All of the amido black-staining components had molecular weights greater than 5,000. The number of components detected varied with the length of the incubation of the culture. There were 16 protein bands in CF-A, 30 in CF-C, and 13 bands in CF-F.

Sixteen immunoprecipitates were detected in immunograms of CF-A; six immunoprecipitates in CF-F. In every case, more precipitinogens were detected in the culture filtrates by immunoelectrophoresis than by the Ouchterlony method. The number varied from 21 in CF-C to 8 in CF-F by immunoelectrophoresis.

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