IMMUNOCHEMICAL INVESTIGATION OF THE SOLUBLE ANTIGENS OF *MYCOCOCCUS* AND ITS RELATIONSHIP TO THE *ACTINOMYCETALES*

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by

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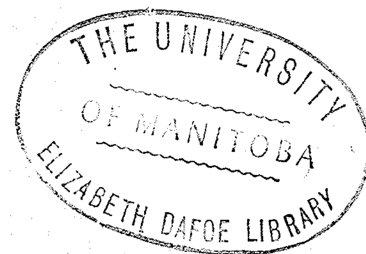

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ABSTRACT

Soluble antigens in the form of cytoplasm, capsule and culture filtrate were obtained from two strains of Mycococcus, identified as M. capsulatus. These preparations were examined by selected physicochemical and immunological methods. The different polymer-categories of the soluble antigens were separated and purified by electrofocusing, preparative polyacrylamide gel electrophoresis and gel filtration. The major component of the cytoplasm consisted of a phospholipid-galactose-nucleoprotein complex. The exoantigen- and capsular antigen preparations were composed mainly of galactoproteins and nucleoproteins, respectively.

The chemical analysis revealed that the cytoplasm consisted of 57% protein, 15% saccharide, 24% nucleic acids, 2% phospholipid, 13 amino acids and galactose. The macromolecular complex purified from the cytoplasm contained 11 amino acids and galactose. The capsular antigen consisted of 42% protein, 22% saccharide, 19% nucleic acids and 15% lipid. The exoantigen was composed of 64% protein, 30% saccharide and 3% nucleic acids.

The cytoplasmic antigen of Mycococcus revealed close immunological relationships to seven cytoplasmic serogroups of nocardiae, four serogroups of scotochromogenic mycobacteria and to one cytoplasmic serogroup of Dermatophilus. The biological position of Mycococcus appears to be in the middle of these Actinomycetales.

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INTRODUCTION

The main objective of this work was the immunochemical characterisation of Mycococcus and its biological relationship to the Actinomycetales, by the investigation of its soluble, macromolecular components. Taxonomically, this genus has been placed in the order Actinomycetales, although this classification had been based mainly on the morphological and physiological characteristics of these bacteria. The immunochemical properties of Mycococcus were not known prior to this work; thus, the review of pertinent literature is dealing mainly with the immunochemical characteristics of soluble antigens occurring in the other genera of the Actinomycetales. This part of review is preceded by a summary of the morphological, cultural and physiological properties of Mycococcus, as published in the microbiological literature.

Mycococcus usually occurs in the form of spherical cells, appearing singly, in short chains, or in clusters. Sometimes, especially in liquid media, rod-shaped cells may be found. The coccal cells are quite variable and pleomorphic in size and shape with V and Y forms, in fact club- and amoeboid-shaped cells are quite common. They are Gram +ve, not acid-fast bacteria. The cultural and physiological characteristics resemble those of Mycobacterium by their production of red, orange or yellow pigments, their lack of mycelium, their oxygen requirement and their reaction to Gram staining. The cultural and physiological characteristics of Mycococcus are summarily presented in Table I.

TABLE I

CULTURAL AND PHYSIOLOGICAL PROPERTIES OF MYCOCOCCUS SPECIES
(KRASSILNIKOV,1938, c.f. BREED, MURRAY AND SMITH, 1957)

	M. albus	*M. ruber*	*M. capsulatus*	*M. luteus*	*M. flavus*	*M. citreus*
Colonies	large, moist and shiny	dough-like consistency	mucoid and spreading	smooth and shiny	smooth and shiny	smooth and waxy
Pigment production	-	red, orange	rose	yellow	yellow	yellow
Milk peptonisation	+	-	-	-	+	+
Gelatin liquefaction	+	-	-	+/-	+	+
Starch hydrolysis	+	-	-	*	-	+
Sucrose inversion	+	-	-	+/-	*	+
Acid from glucose	+	*	*	+	+	+
Acid from fructose	+	*	*	+	*	+
Acid from sucrose	*	*	*	-	*	+
Nitrate reduction	+	-	-	*	*	+
Citrate and Acetate as carbon source	+	+	+	-	+	-
Resistance to high salt concentration	*	+	+	*	*	*

* Not reported
+/- Denotes a weak reaction.

REVIEW OF THE LITERATURE

GENERAL

Mycococcus was first isolated from soil and described and named by Krassilnikov (1938). A series of biochemical tests was done, designed to classify this organism as a member of the family Mycobacteriaceae and to distinguish between different species (Breed, Murray and Smith, 1957). According to Nellis (1955), the germination of the resting cells of Mycococcus was analogous to that of the conidiospores of the actinomycetes. She proposed that these organisms should not be placed in a known group, since their properties had not been fully elucidated.

Waksman (1961) included the genus Mycococcus within the order Actinomycetales, but he regarded them as mutants or variants, not as 'true' actinomycetes. Csillag (1964) postulated that Mycococcus arose from the 'form 2' mycobacteria which had been isolated from 'form 1' of strains of Mycobacterium tuberculosis. She cultivated the 'form 2' mycobacteria and found that Gram +ve granules were liberated into the medium after intermittant aeration. These gradually developed into small cocci which were identified as Mycococcus on the basis of the descriptions of Krassilnikov, who regarded these cocci as a stage in the normal development of Nocardia, rather than as mutants.

In the opinion of Hilson (1965), 'form 2' organisms were not part of a complex mycobacterial life-cycle, but contaminants. Recently Pease (1970) found that Streptococcus MG also passed through a growth phase which possessed the morphological characteristics of Mycococcus. These Gram +ve cocci, in the course of prolonged culture, became variable in size and produced rod-like projections which later developed into small mycobacteria.

CHEMICAL COMPOSITION OF THE SOLUBLE ANTIGENS OF THE ACTINOMYCETALES

Cytoplasmic Antigen

Kwapinski (1966 b) observed that the chief organic constituents of the cytoplasm of the *Actinomycetales* were proteins and nucleic acids. He found that the different cytoplasmic preparations of mycobacteria consisted of roughly 55% protein, 10% nucleic acids and 33% polysaccharides. Most of the proteins appeared to be strongly bound to the nucleic acids and were found as nucleoprotein complexes. The polysaccharides in the cytoplasm occurred either in a free form or were weakly bound to the proteins. These cytoplasmic polysaccharides were found to consist of the following monosaccharides: glucosamine, galactose, mannose and arabinose (Kwapinski and Snyder, 1961). The following amino-acids were discovered in the cytoplasmic proteins by Kwapinski (1966 b): alanine, arginine, glutamic acid, lysine, valine, phenylalanine, glycine, threonine, tyrosine, cysteine and leucine, with the first four occurring as the major components.

The different cytoplasmic antigens of nocardiae were found to consist of 46.3-67.8% protein, 24.8-39.3% polysaccharides, 6.6-9.0% RNA and 1.2-2.8% DNA depending on the species or groups of these microorganisms (Kwapinski, 1966 b). He found that the protein fraction of the cytoplasm contained up to 12 amino acids: alanine, serine, arginine, glutamic acid, lysine, valine, phenylalanine, leucine, glycine, tyrosine, cysteine and threonine. The following monosaccharides occurred in the polysaccharide fraction of the cytoplasm of all nocardiae, except 5 species: glucose, arabinose, galactose and a trace amount of mannose. No arabinose or mannose

was found in the cytoplasms of Nocardia madurae and N. turbata, and the cytoplasms of N. polychromogenes, N. pelletieri and N. rangoonensis contained only glucosamine and ribose. The chief soluble component of the Nocardia cytoplasm had been shown by Kwapinski (1964, 1966 b) to be a polysaccharide-nucleoprotein complex which was relatively heat resistant.

Kwapinski (1966 b) found that the soluble part of the cytoplasm from Actinomyces contained an average of 54.2% protein, 37.3% carbohydrate, 8.9% RNA and 2.2% DNA. The following amino acids were discovered in the protein component: alanine, glutamic acid, lysine, glycine, serine, valine and leucine as the major components; arginine, threonine, tyrosine, cysteine and phenylalanine as the minor ones. The following monosaccharides were found to be present in the polysaccharide fraction of the cytoplasm: arabinose, mannose, galactose, glucosamine and ribose.

The components of the cytoplasms of Waksmania, Streptomyces and Micromonospora were examined by Kwapinski (1966 b). The latter two consisted roughly of 50% protein, 40% carbohydrate, 7% RNA and 2% DNA. The major components of the protein were alanine, glutamic acid, lysine, serine, glycine, valine and leucine. Arginine, tyrosine, cysteine and phenylalanine were minor components. The carbohydrate consisted mainly of glucosamine and mannose. Traces of arabinose were detected in the cytoplasmic fluid of Micromonospora. The cytoplasm of Waksmania contained more protein but less carbohydrate than the two above mentioned microorganisms.

Kwapinski and Simmons (1967) examined the cytoplasmic antigens from 22 different Dermatophilus strains, and recorded between 54.5% protein, 19.0-30.0% carbohydrate, 2.1-6.7% RNA, 1.6-3.9% DNA and 6.0-9.7% lipid.

Protein components contained alanine, glutamic acid, lysine, glycine, serine, valine, phenylalanine and leucine. Glucosamine, galactose, arabinose and ribose occurred as monosaccharides in the carbohydrate components, whereas stearic, palmitic and oleic acids occurred as fatty acids in the lipids of the cytoplasm. The chemical compositions of the cytoplasmic antigens from the Actinomycetales are summarized in Tables II and III.

Exoantigen

Exoantigens are soluble antigens, liberated from bacterial cells into the culture medium. Bevilacque and McCarter (1948) demonstrated the presence of two different proteins with sedimentation factors of 3.4 and 2.0, in the culture filtrates of human tubercle bacilli. Seibert (1949), using alcohol fractionation, observed three different proteins (designated as A, B and C fractions) and two polysaccharide fractions. Seibert et al.(1955) showed that the fractions consisted of 6.9-12.0% protein, 0.3-1.0% nucleic acids and about 2% carbohydrate, although the fraction A possessed 24.0-40.2% of the carbohydrate.

Using electrophoresis, Kara and Keil (1958) isolated from the exoantigen of Mycobacterium tuberculosis a glycoprotein which consisted of alanine, glutamic acid and α,ε-diaminopimelic acid in equimolar quantities. Takeya et al. (1961), on the other hand, obtained a glycoprotein fraction consisting of alanine, glutamic acid, α,ε-diaminopimelic acid, trace amount of glycine and aspartic acid, muramic acid, glucosamine, galactose and arabinose. Kato and Lederer (1961) observed that the chemical composition of this glycoprotein fraction was strikingly similar to the glycoprotein and

glycopeptide extracted by them from the mycobacterial cell walls and whole cells of the tubercle bacilli, respectively. Therefore, the fraction was probably split off from the cell walls by an autolytic enzyme, during prolonged cultivation. Affronti et al. (1965) separated electrophoretically the culture filtrates of mycobacteria into three different, specific macromolecular components: protein, nucleic acids and polysaccharides with varying amounts of lipids. Most of the nucleic acid was found to be combined with protein.

Keeler and Pier (1965) isolated and purified a fraction from the exoantigen of Nocardia asteroides. This fraction contained 50-66% protein, 7-14% carbohydrate in terms of hexose,about 1.5% pentose and trace amounts of nucleic acids. The fraction contained alanine, aspartic acid, serine, glutamic acid, glycine, cysteine, lysine, histidine, arginine, threonine, proline, phenylalanine, isoleucine, valine and methionine as amino acids, and glucose, galactose, mannose, xylose and ribose as monosaccharides.

Kwapinski (1966 a) purified exoantigen preparations from 21 Dermatophilus strains and observed that all the exoantigens contained glycine, glutamic acid and leucine as amino acids in the protein component; xylose, galactose and galactosamine as saccharides in the carbohydrate component, with traces of glucosamine and tyrosine detected too. From these data, he showed that Dermatophilus exoantigens were mucoids of the hexosamine-xylose-galactose-peptide type. The presence of xylose in these chemical complexes distinguished Dermatophilus from the other members of the Actinomycetales which had been shown to contain arabinose as the characteristic pentose. The chemical composition of the exoantigens of the Actinomycetales are presented in Table IV.

TABLE II

CHEMICAL COMPOSITIONS OF ORGANIC MATERIALS OF CYTOPLASMS OF ACTINOMYCETALES

Microorganisms	Range (%) of organic materials in dry mass*					Author
	Protein	Polysaccharide	DNA	RNA	Lipid	
Mycobacterium	45.9-60.4	19.6-42.6	1.5-3.6	8.7-9.7	-	K
Nocardia	46.3-67.8	24.8-39.3	1.2-2.8	6.9-9.0	-	K
Actinomyces	54.1-54.6	36.2-37.5	2.2	8.9	-	K
Streptomyces and Micromonospora	45.3-49.3	36.3-40.9	2.1-3.1	6.7-9.0	-	K
Dermatophilus	54.5-70.0	19.0-30.0	1.6-3.9	2.1-6.7	6.0-9.7	K & S
Thermoactinomyces and Waksmania	66.8-70.3	18.4-19.2	2.0-2.6	7.2-9.2	-	K

* For cytoplasms of various strains.

K = Kwapinski, 1966 b.

K & S = Kwapinski and Simmons, 1967.

TABLE III

AMINO ACIDS AND SACCHARIDES DETECTED CHROMATOGRAPHICALLY IN THE CYTOPLASMS OF THE ACTINOMYCETALES

Amino Acids	*Mycobacterium*	*Nocardia*	*Actinomyces*	*Streptomyces*	*Micromonospora*	*Dermatophilus*
Serine	+	+	+	+	+	+
Alanine	+	+	+	+	+	+
Arginine	+	+	+	+	+	
Glutamic acid	+	+	+	+	+	+
Lysine	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+
Leucine	+	+	+	+	+	+
Glycine	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	
Threonine	+	+	+	+	+	
Cysteine	+	+	+	+	+	
Saccharides						
Glucosamine	+	+	+	+	+	+
Galactose	+	+	+			+
Mannose	+/-	+/-	+	+	+	
Arabinose	+	+	+	+/-	+/-	+
Ribose	+	+	+	+	+	+
Author	Kwapinski (1966b)	Kwapinski (1966b)	Kwapinski (1966b)	Kwapinski (1966b)	Kwapinski (1966b)	Kwapinski and Simmons (1967)

+/- = Trace amount.

TABLE IV

AMINO ACIDS AND SACCHARIDES DETECTED CHROMATOGRAPHICALLY IN THE EXOANTIGENS OF THE ACTINOMYCETALES

Amino Acids	Mycobacterium	Nocardia	Dermatophilus	Micromonospora, Actinomyces and Streptomyces*
Serine		+		
Alanine	+	+		
Arginine		+		
Glutamic acid	+	+	+	
Lysine		+		
Histidine		+		
Aspartic acid	+/-	+		
Valine		+		
Phenylalanine		+		
Proline		+		
Isoleucine		+		
Methionine		+		
Leucine		+	+	
Glycine	+	+	+	
Tyrosine			+	
Threonine		+		
Cysteine		+		
Diaminopimelic acid	+**			
Saccharides				
Galactosamine			+	
Glucosamine	+		+	
Galactose	+	+	+	
Mannose		+		
Arabinose	+	+		
Ribose		+		
Glucose		+		
Muramic acid	+			
Xylose		+	+	
Author	T	K	Kwapinski (1966a)	

* Not reported.

** Glycopeptide isolated electrophoretically from the exoantigens of Mycobacterium tuberculosis (Kara and Keil, 1958).

T = Glycoprotein fraction obtained from the culture filtrates of tubercle bacilli (Takeya et al., 1961).

K = A purified fraction obtained from the exoantigens of Nocardia asteroides (Keeler and Pier, 1965).

SEROLOGICAL CHARACTERISATIONS ON THE SOLUBLE ANTIGENS OF THE ACTINOMYCETALES

Cytoplasmic Antigen

Kwapinski (1964, 1966 b) demonstrated the presence of a polysaccharide-nucleoprotein complex in the cytoplasmic antigen which appeared as serologically homogeneous material in the diffusion precipitation test. The cytoplasmic antigens were found to be relatively thermostable, apparently due to the presence of the polysaccharide component in this macromolecular complex. It was suggested that degraded parts of the complex were also liberated into the cytoplasmic preparation. These would migrate with different velocities in an electric field of a semi-solid medium due to the difference in diffusion coefficients and electric charges.

Kwapinski (1971) advanced a hypothesis that the non-degraded, native macromolecules of the complex possessed several determinant groups, located in different positions on the macromolecule or on its constituents. Therefore antibodies obtained by immunisation with this cytoplasm complex would have diversified reactive sites, corresponding to a specific determinant of the macromolecule, due to a varied distribution and conformation of the different determinants. Since both the native macromolecular complex and products of degradation at different stages of polymerisation were usually present in the cytoplasmic preparations, distinct bands at different locations representing several antigen-antibody complexes would be observed when the cytoplasmic antigen was allowed to react with the corresponding antiserum in diffusion precipitation test. This polysaccharide-nucleoprotein macromolecular complex of the cytoplasmic antigen, possessing

numerous and dissimilar determinants, would react not only with the homologous antiserum, but also with the heterologous ones, provided that the antibody fitted at least one of the determinants. Immunoglobulins, induced by different determinants of the macromolecule, might react with any cytoplasmic molecule carrying at least one of the determinants. Furthermore, by absorption studies, Kwapinski et al. (1971) was able to show that anti-cytoplasmic sera could be absorbed completely with different, although antigenically related cytoplasms. This phenomenon demonstrated that immunoglobulin molecules, which possessed reactive sites of different configuration located closely to each other, could be saturated with any antigen molecule possessing one or more corresponding determinants.

Protein and polysaccharide antigens isolated by Kwapinski and Snyder (1961) from the cytoplasmic fluids of Mycobacterium tuberculosis and M. phlei were shown to possess a serological similarity between themselves and were related to similar fractions obtained from Actinomyces israelii, Streptococcus pyogenes and Diplococcus pneumoniae. Castelnuovo et al. (1964) did a study on the cytoplasms of M. tuberculosis, M. bovis, M. avium, M. kansasii, M. scrofulaceum, M. marinum, M. thamnopheos, Battey strains, M. phlei, M. pellegrino, M. lacticola and M. minetti. They found that these cytoplasmic antigens contained an antigen shared with the cytoplasms of Nocardia asteroides, N. brasiliensis, N. rubra, N. corallina and Nocardia rubropertincta. Kwapinski (1966 b) demonstrated the presence of five serogroups by examining the cytoplasms of 42 mycobacteria by a diffusion precipitation test. It was observed that the cytoplasmic antigens obtained from M. tuberculosis strains were serologically related to 12 types of

nocardiae, and also to Streptomyces and Waksmania. Those of M. smegmatis, M. phlei, M. butyricum and the scotochromogenic strains contained serological factors identical with, or closely related to, 20 to 26 different Nocardia strains and to members of the genera Actinomyces and Thermoactinomyces. The cytoplasm of M. fortuitum was also found to cross-react with some 10 strains of Nocardia and one strain of Waksmania.

Using the cytoplasmic preparations from 62 different strains of scotochromogenic (Runyon's group II) mycobacteria, Kwapinski and Alcasid (1970a)found that the most common antigenic factor was shared by one-third of all the cytoplasmic preparations, and one of three other major factors occurred in about one-fourth of the strains. They also found that six strains of scotochromogenic mycobacteria possessed only a single minor antigenic factor. The cytoplasms of five other strains were proved to be related to the Battey-type mycobacteria and/or to the rapid growers. The scotochromogenic mycobacteria were thus divided into seven different cytoplasmic serogroups (1-7), with most of the strains being placed in group nos. 1, 2 and 3 (Table V).

Castelnuovo et al. (1964) showed that Nocardia rubra, Mycobacterium rhodochrous and M. pellegrino were serologically identical with each other, and that N. corallina shared cytoplasmic antigens with M. rhodochrous. An extensive serological study on the cytoplasmic antigens of Nocardia strains (Kwapinski, 1966 b), revealed four main serogroups:

I. N. asteroides and N. lutea - cross-reacting with Thermoactinomyces, Corynebacterium and Mycobacterium.

II. N. caviae and N. sebivorans - serologically related to Actinomyces,

Thermoactinomyces and Mycobacterium.

IIIa. N. brasiliensis, N. erythropolis, N. madurae, N. corallina and some 'atypical' nocardiae - serologically related to various parasitic and saprophytic mycobacteria, but not to Thermoactinomyces, Actinomyces and Corynebacterium.

b. N. caprae, N. farcinica and N. turbata - cross-reacting only with the saprophytic, but not with the parasitic mycobacteria.

IV. N. rangoonensis and N. leishmanii - serologically not related to Thermoactinomyces, Actinomyces, Mycobacterium and Corynebacterium.

Cytoplasmic preparations from 125 strains of Nocardia, were found by Kwapinski et al. (1971) to react in one to four locations with one to fourteen different anti-cytoplasm sera. They were antigenically related by more than two antigenic factors or determinants. By this investigation, 22 cytoplasm serogroups (1-22) of the nocardiae had been distinguished, with each group having a specific antigenic pattern, represented by capital letter A-R (Table VI). By comparison with the serological activities revealed among the saprophytic mycobacteria, the author postulated that the relatively wide serological cross-reactions of the cytoplasmic antigens of the nocardiae were contributed by the strong affinities of the cytoplasms to the anti-cytoplasm immunoglobulins.

Kwapinski and Snyder (1961) found that cytoplasmic constituents of the mycobacteria reacted with the antisera of Actinomyces. Using the diffusion precipitation test, Kwapinski (1964) was able to show that the cytoplasms of Actinomyces, M. tuberculosis, M. bovis, Corynebacterium diphtheriae and C. hoffmannii were also serologically interrelated. Cytoplasms of the

different strains of Actinomyces were found to be serologically related to those of Thermoactinomyces, and partly to those of M. phlei, M. rhodochrous, scotochromogenic mycobacteria, N. sebivorans and N. caviae (Kwapinski, 1966b).

The cytoplasm of streptomycetes, as reported by Kwapinski (1968), consisted of seven serogroups (1-7). It seemed to possess a relatively simple antigenic composition, as compared to the other members of the Actinomycetales. Thus, the cytoplasmic preparations of streptomycetes were found to reveal only one to three, and seldom four, different antigenic factors. By doing cross-reaction studies, Kwapinski (1966 b) had shown that the cytoplasmic preparations of Streptomyces had no serological interrelationship to the actinomycetes, but they were closely related to two to six Nocardia strains. Cytoplasmic antigens of Micropolyspora, Micromonospora and partly Streptomyces listeri were found to have very little in common with the mycobacteria. In contrast, S. griseus, Thermoactinomyces and some strains of Waksmania rosea, were quite closely related to the mycobacteria, especially to the saprophytic and scotochromogenic ones.

No serological relationship was observed between the cytoplasmic antigens of Dermatophilus and those of the other representative strains of the Actinomycetales (Gordon, 1964 and Kwapinski, 1966 b). Kwapinski and Simmons (1967) distinguished five cytoplasm serogroups for Dermatophilus (A-E). By using complement-fixation test, they demonstrated that three strains possessing the least 'typical' morphological characteristics of Dermatophilus showed an affinity to the antisera against N. turbata, N. caviae and N. rangoonensis.

TABLE V

CYTOPLASMIC-ANTIGEN RELATIONSHIPS OF SCOTOCHROMOGENIC MYCOBACTERIA (KWAPINSKI AND ALCASID, 1970 a)

CYTOPLASM ANTI-SERUM / CYTOPLASM OBTAINED FROM MYCOBACTERIUM STRAIN NUMBER:

Number	Factor	19981	19275	4243	14473	14470	23409	15975	15079	15983	15080	23429	23427
12670	a	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒
14474	b												
19981	c	X	X										
19277	d												
15080	e		X								X	X	X
23283	f									X			
23401	g												
23414	h		X							X			
23424	i												
23408													
23416													
23396													
23419													
P-19													
19275				X									
19073													
15983										X			
23285													
Serogroup No.		1											
Main antigenic factor(s)		a											

Number	Factor	14474	23395	15982	23410	23424	15081	19277	12670	19073	23033	23414	23035	23411
12670	a	▒	▒	▒	▒	▒	▒	▒	▒	▒				
14474	b	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒
19981	c							▒	▒	▒	▒	▒	▒	
19277	d							X	X					
15080	e							X						
23283	f							X						
23401	g							X				X	X	
23414	h							X			X	X		
23424	i	X	X					X	X					
23408								X				X		
23416								X						
23396							X							
23419														
P-19										X				
19275										X				
19073										X				
15983														
23285														
Serogroup No.		2												
Main antigenic factor(s)		ab(c)												

Number	Factor	15978	23401	23428	23399	23408	23398	23283	23423	23404	23413
12670	a										
14474	b	▒	▒	▒							
19981	c										
19277	d	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒
15080	e								▒	▒	▒
23283	f						X	X			
23401	g							X			
23414	h						X				
23424	i										
23408							X				
23416											
23396											X
23419						X					X
P-19											
19275						X		X			
19073								X			
15983								X			
23285											
Serogroup No.		3									
Main antigenic factor(s)		d									

Number	Factor	23421	23422	23432	23425	23405	23426	23418	23419	23417
12670	a									
14474	b									
19981	c									
19277	d									
15080	e	▒	▒	▒	▒	▒	▒	▒	▒	▒
23283	f									
23401	g									
23414	h									
23424	i									
23408										
23416										
23396										
23419									X	
P-19										
19275										
19073										
15983										
23285										X
Serogroup No.		4								
Main antigenic factor(s)		e								

Number	Factor	23415	23400	23402	23416	23420	23397	23009	23286	23406	23430	23396	P-19	23285	23431	23284	23407	23412	15078
12670	a																		
14474	b																		
19981	c																		
19277	d																		
15080	e	▒																	
23283	f	▒	▒	▒	▒														
23401	g								X										
23414	h							X		X									
23424	i					▒	▒	▒											
23408											X								
23416					X														
23396												X							
23419					X			X											
P-19													X						
19275									X					X					
19073									X						·	·	·		
15983																	:	:	:
23285																			
Serogroup No.		5				6			7										
Main antigenic factor(s)		f				i			other than abcdefi						non-scoto group				

LEGEND: Shaded and X areas denote antigen–antibody reaction.
· Reactive with anti-Battey 15 serum.
: Reactive with *M. borstelense* No. 23030 serum.

TABLE VI

SEROLOGICAL REACTIONS BETWEEN CYTOPLASMS OF NOCARDIAE AND ANTI-CYTOPLASM SERA, DETECTED BY IMMUNODIFFUSION TEST (KWAPINSKI *ET AL.*, 1971)

ANTI-ENDOPLASM SERUM		CYTOPLASM OBTAINED FROM *NOCARDIA* STRAIN NUMBER:-																																																											
NUMBER	SYMBOL	10011	10051	12007	10022	10075	10084	8595	11024	10016	10017	11013	10085	10072	11019	11021	12005	10008	10012	10081	10001	10092	10003	11033	10082	11020	15904	14008	12013	659	12008	10010	10091	11028	B-685	13723	14011	283	10071	12009	4277	10000	11031	14007	13000	14009	10005	14010	6860	10086	4524	4273	20002	11001	13003	10078	13005	11018	11004	10077	10089	14629
4324	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10017	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																									
11016	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																												+	+	+	+	+	+	+	+	+	+	+
12013	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								+	+	+	+	+	+	+														+	+	+	+	+	+	+											
10056	E	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+							+	+	+							+	+	+							+					+	+	+	+	+	+	+
13000	F	+	+	+	+	+	+	+	+	+	+							+	+	+	+				+						+	+							+	+	+		+	+	+	+					+									+	+	+
10067	G	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+		+		+	+	+		+	+		+	+	+		+	+	+	+	+	+			+	+	+	+	+	+		+			+	+	+	+		+		+		+	+	+	+
152	H	+	+	+	+	+	+	+	+			+	+	+	+	+		+	+				+			+	+						+						+				+	+												+	+		+	+	+	
4273	I	+	+	+	+	+									+					+	+	+	+		+	+	+	+		+		+				+	+		+	+	+		+			+	+	+	+			+				+			+	+		+
3409	J	+	+	+										+	+		+	+				+					+			+			+	+					+				+		+										+	+						
1070	K	+		+	+			+				+			+						+		+	+	+		+		+	+	+	+				+	+							+	+		+		+								+					+
20004	L	+																		+			+					+								+					+							+	+								+			+	+	+
9999	M	+						+						+				+	+								+		+										+	+											+											
91	N										+			+									+					+								+	+		+							+	+						+							+		
104	O		+	+				+				+	+													+													+								+										+	+				
B-685	P					+		+						+															+						+									+				+								+						+
6761	Q					+		+					+		+										+									+								+	+					+		+		+					+					
14009	R										+									+			+			+											+										+														+	
ANTIGENIC PATTERN		ABCDEF(GHI)										ABCD(E)						ABC(E)							ABD							AB						A(F)							AD							AC				ACE						
SEROGROUP NUMBER		1										2						3							4							5						6							7							8				9						

(continued)

TABLE VI (continued)

ANTI-ENDOPLASM SERUM / CYTOPLASM OBTAINED FROM NOCARDIA STRAIN NUMBER:-

NUMBER	SYMBOL	17895	11008	10083	11039	10004	11022	10021	6761	6846	10079	11016	152	10029	12002	12010	12004	10067
4524	A	+	+	+	+	+	+	+	+	+	+	+	+					
10017	B													+	+	+	+	+
11016	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12013	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10056	E					+	+	+	+	+	+	+	+	+				
13000	F										+	+	+	+	+	+		+
10067	G	+	+	+		+				+	+	+			+	+		+
152	H				+	+		+		+	+	+	+					+
4273	I	+	+	+	+			+		+	+			+			+	
3409	J								+	+	+					+		
1070	K	+	+					+		+								+
20004	L																	
9999	M																	
91	N													+	+		+	
104	O																	
B-685	P								+									
6761	Q				+		+									+		
14009	R																+	
ANTIGENIC PATTERN		ACD				ACDE								BCD				
SEROGROUP NUMBER		10				11								12				

NUMBER	SYMBOL	11027	11030	12011	10087	11002	11029	11032	10065	13002	12001	10039	10070	10076	13781	12012	12006	11014
4524	A																	
10017	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11016	C	+	+	+	+	+												
12013	D						+	+	+	+	+							
10056	E	+						+	+			+	+	+				
13000	F		+	+					+	+		+			+	+		
10067	G	+	+		+		+	+		+		+	+	+		+		
152	H		+				+			+								+
4273	I		+	+				+	+	+				+	+			+
3409	J		+			+				+		+	+	+	+			
1070	K			+			+		+	+			+	+			+	
20004	L													+	+		+	
9999	M					+			+							+		
91	N												+	+				
104	O		+	+	+							+	+					
B-685	P											+						
6761	Q	+			+							+	+			+		
14009	R		+											+				
ANTIGENIC PATTERN		BC					BD					BEG			B(F)			
SEROGROUP NUMBER		13					14					15			16			

NUMBER	SYMBOL	14816	11034	13258	11041	11036	10015	3409	10020	10007	10090	12000	17039	14630	9999
4524	A														
10017	B														
11016	C	+	+	+	+	+	+	+	+	+					
12013	D		+	+				+	+	+	+	+	+	+	+
10056	E		+		+	+	+	+	+	+	+	+	+		+
13000	F			+				+				+	+	+	
10067	G	+				+	+				+			+	+
152	H	+	+		+			+	+					+	
4273	I	+	+	+	+	+	+	+			+	+	+	+	+
3409	J							+			+				
1070	K														
20004	L				+									+	
9999	M	+					+							+	+
91	N				+		+	+							+
104	O				+		+		+		+	+			
B-685	P														
6761	Q	+		+					+	+	+	+	+		
14009	R												+		
ANTIGENIC PATTERN		C(DEHI)									DI(E)				
SEROGROUP NUMBER		17									18				

NUMBER	SYMBOL	10073	14004	12014	10034	5654	13259	13004	13006	91	11037	10073	17896	20004	20003	1070	13724
4524	A																
10017	B																
11016	C																
12013	D	+	+	+	+	+	+	+									
10056	E								+	+							
13000	F		+								+	+	+				
10067	G	+	+		+			+	+		+	+	+	+			
152	H	+		+									+				
4273	I					+								+			
3409	J				+	+											
1070	K						+						+			+	
20004	L		+											+	+		
9999	M																+
91	N	+	+	+					+				+				
104	O		+					+	+								
B-685	P																
6761	Q				+												
14009	R												+				
ANTIGENIC PATTERN		D(G)							E		FG			L		K	M
SEROGROUP NUMBER		19							20		21			22			

The exoantigens of Nocardia were found to be serologically closely related to, and largely dependent on the cytoplasmic antigen complex. Therefore the serological spectra of the exoantigens of Nocardia were similar to, but not identical with the cytoplasm spectra. Kwapinski (1967 b) differentiate between five major exoantigen serogroups (A-E), with one or two types in each group.

Kwapinski (1966 c) reported four exoantigen serogroups A, B, C and D among the 21 strains of Dermatophilus culture filtrate preparations he used. In contrast to the close relationships between the exoantigens among the other members of the Actinomycetales, he found that the exoantigens of Dermatophilus were not serologically related to those of the Actinomycetales.

Using 112 different strains of the Actinomycetales, Kwapinski (1966a) showed that there was a close serological relationship between the exoantigens of Actinomyces, Thermoactinomyces and M. tuberculosis. One group of Nocardia revealed a marked affinity to Mycobacterium and Actinomyces, whereas cross-reactivities of another group were mostly confined to various Nocardia strains. He also observed that the exoantigens of S. griseus and Waksmania were serologically closely related to each other, and to Thermoactinomyces and N. sebivorans. Furthermore, the exoantigens of Micromonospora and Micropolyspora were found mutually related , but the serological activities were different from the other members of the Actinomycetales. In the same paper, he proposed that the exoantigens of the Actinomycetales possessed a single major antigen , which consisted of multiple determinants for various species or types of these microorganisms.

MATERIALS AND METHODS

MICROORGANISMS

Strains of Mycococcus Nos. 13556 and 13557 were obtained from American Type Culture Collection, Washington, D. C.

CULTURE MEDIA

Kwapinski's semi-synthetic liquid and solid culture media (1969) were used to prepare materials for cytoplasmic antigens and exoantigens. The composition of liquid medium is given below:

Solution A.	Casamino acids	5.0 gm
	Sodium pyruvate	1.0 gm
	Magnesium citrate	0.1 gm
	K_2HPO_4	1.5 gm
Solution B.	L(+) cysteine hydrochloride	0.7 gm
	Maltose	1.0 gm
	Galactose	1.0 gm
	Dextrose	5.0 gm

Solution A was autoclaved at 121°C for 15 minutes and cooled. Solution B was sterilized by filtration and added to solutionA.The mixture was made up to 1 litre with distilled water, and the pH adjusted to 6.8-7.1.

Solid medium was prepared the same way except that 4% of Bacto-agar was added to solution A before autoclaving. Aliquots of 500 ml were dispensed into 2 litre Pavitsky's tissue culture flasks.

Gaudy and Wolfe's glucose-peptone medium (1962) was used for the production of capsules and/or slime layers. Constitution of this medium is as follows:

Glucose	0.5%
Peptone	1.0%
Magnesium sulphate (7 H_2O)	0.02%
Calcium chloride	0.005%

Ferric chloride (6 H_2O)	0.001%

with a slight modification of adding

Glycerol	1.0%
Bacto-agar	4.0%

After autoclaving, the pH of the solution was adjusted to 7.1 and 500 ml aliquots were dispensed into 2 litre Pavitsky's tissue culture flasks.

Basal Medium was used for testing the ability of the microorganisms to utilize carbohydrates and organic acids as sole carbon sources (Pridham and Gottlieb, 1948).

Solution A.	$(NH_4)_2 SO_4$	7.92 gm
	KH_2PO_4	7.14 gm
	K_2HPO_4	16.95 gm
Solution B.	$MgSO_4 \cdot 7 H_2O$	3.00 gm
	$CuSO_4 \cdot 5 H_2O$	0.0192 gm
	$FeSO_4 \cdot 7 H_2O$	0.0033 gm
	$MnCl_2 \cdot 4 H_2O$	0.0237 gm
	$ZnSO_4 \cdot 7 H_2O$	0.0045 gm
	Bacto-agar	45.00 gm

Solution A was dissolved in 1 litre of distilled water, autoclaved, and the pH adjusted to 7.0. Solution B was dissolved in 2 litres of distilled water, autoclaved, and the pH adjusted to 7.0. Different carbohydrates, polyhydric alcohols and organic acids, sterilized by filtration, were added aseptically to the basal medium to make final concentrations of 1%, 1% and 0.2%, respectively.

Yeast Extract-Glycerol medium was used for testing the sensitivity of microorganisms to various dyes (Jones Jr. and Kubica, 1963). Composition of the medium is given below:

Yeast extract	10 gm
Dextrose	10 gm
Glycerol	70 ml
Bacto-agar	15 gm
Distilled water	930 ml

The respective dye was added to make a 0.01% final concentration (W/V). The pH of the medium was adjusted to 6.8 and the medium was sterilized by autoclaving before being poured into plastic Petri dishes.

The above medium was also used for testing salt tolerance of the microorganisms after a 10% solution of sodium chloride or sodium sulphate was added.

All the other media were prepared according to Difco Manual, 9 th Ed. (Difco Laboratories, 1953).

The different cytoplasms and anti-cytoplasmic sera of the Actinomycetales used in the cellulose acetate immunodiffusion experiments are shown in Table VII.

TABLE VII

CYTOPLASMS AND ANTI-CYTOPLASMIC SERA OF THE ACTINOMYCETALES USED FOR THE DETECTION OF CROSS-REACTIVITIES OF MYCOCOCCUS

Organism	Strain No.
M. tuberculosis	102, 202, $H_{37}Ra$
M. bovis	601, 11166, BCG
M. avium	701, 801
M. paratuberculosis	12227
M. kansasii	927, 12478, P-24
M. murium	1601
M. balnei	11564
M. berolinense	356
Battey mycobacteria	15, 2922, 2929
M. fortuitum	6841, 9820, 19542, 23043, 23048
M. phlei	354, 19249
M. butyricum	357
M. smegmatis	14468, 23011, 23019, 23037
M. peregrinum	23001, 23015, 23022, 23023
M. flavescens	14474, 23416, 23033, 23395
M. scrofulaceum	15079, 15081, 15983, 19073, 19881, 23245, 23414, 23419, 23425, 23431
M. scotochromogenes	15080, 23429, P-19, 23426
M. borstelense	19235, 19237, 23030
M. vaccae	15483
M. marinum	19275

(continued)

TABLE VII (continued)

Organism	Strain No.
M. aquae	19277, 23283, 23397, 23401, 23404 23422, 23408
M. thermoresistibile	19527
M. abscessus	19977, 23044
M. gordonae, M. acapulcensis	14470, 14473
M. parafortuitum	19686
N. rangoonensis	77
N. caviarum	91
N. asteroides	93, 10017, 10067, 10077
N. turbata	152
N. lutea	192
N. caprae	659
N. farcinica	1361, 4524
N. pelletieri	160, 9999
N. madurae	1070, 20004
N. brasiliensis	283, 11016, 11032
N. rubra	12007, 12013, B-685
N. paraffinica	14009
N. polychromogenes	3409, 14011
N. corallina	13000, 13005, 13006
N. sebivorans	8595
A. israelii	A36, A48, A56, A58

(continued)

TABLE VII (continued)

Organism	Strain No.
Dermatophilus	499, 494, 694, 1758, 2688, 2778
S. cinereoruber	5012
S. achromogenes	5028
S. parasinopilosus	5098
S. cellostaticus	5189
S. griseus	8781

MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL EXAMINATIONS

The microorganisms were maintained by subculturing on the semi-synthetic liquid culture medium. A three-day old culture was used as the inoculum. Cultural tests on solid media were performed on agar plates with the usual streaking technique; whereas a 0.1 ml bacterial suspension was used as an inoculum for liquid media. The method described by Pridham and Gottlieb (1948) was used for testing the microorganisms' ability to utilize carbohydrates and organic acids as sole carbon sources. The sensitivity of Mycococcus to various dyes was performed according to Jones Jr. and Kubica (1963). All the other biochemical tests were done according to Gordon and Smith (1955) and Gordon and Mihm (1957).

PREPARATION OF SOLUBLE ANTIGENS

Exoantigen

The strains of Mycococcus were grown in Kwapinski's semi-synthetic liquid culture medium at 37°C for 3 days. Each bottle, containing 200 ml of the medium, was inoculated with 5 ml of an aqueous suspension of cells from a 24 hour-culture on Brain Heart Infusion broth (Difco Laboratories, Michigan). The purity of the cultures was checked by microscopic examination and by cultural test on the semi-synthetic medium containing 2% Bacto-agar.

Pure cultures were treated with formaldehyde solution to make 0.5% final concentration. They were left at room temperature for 16 hours, and then centrifuged at 23,500xg for 15 minutes in an International Refrigerated Centrifuge Model B-20 (International Equipment Co., Mass., U. S. A.). The

supernatant fluid was collected and passed through a 0.22 μ membrane filter (Millipore, Gelman Co.). It was then dialysed against frequent changes of distilled water at 4°C for 3 days until the dialysate was free from amino acids and carbohydrate, as determined by Ninhydrin (Schiffman, 1958) and Molisch (Dische, 1955) reactions. The crude culture filtrate was then concentrated by lyophilisation.

In order to remove any material adhering to the chemical complexes present in the dialysed and lyophilised culture filtrate, the lyophilisate obtained from the semi-synthetic medium was dissolved in distilled water, pH 6.0. It was then heated for 5 minutes at 60°C, and any residue was removed by centrifugation at 23,500 x g for 5 minutes. The supernatant was collected, dialysed against distilled water at 4°C , and again lyophilised. The lyophilisate was further purified by repeating the same procedure as above. A control using only the semi-synthetic medium was treated in the same manner.

Cytoplasmic Antigen

The strains of *Mycococcus* were grown in Kwapinski's semi-synthetic solid culture medium at 37°C for 3 days. Each Pavitsky's flask, containing 500 ml of the medium, was inoculated with 20 ml of an aqueous suspension of cells from a 24 hour-culture on Brain Heart Infusion broth. The flask was then rotated gently in order to obtain an even distribution of the bacterial suspension on the agar. The purity of the cultures was checked as before.

Pure cultures were washed out with 0.85% phosphate buffered saline.

Whole cells, obtained as sediment after the centrifugation of the pure cultures obtained, were washed repeatedly with distilled water, followed by centrifugation until no trace of amino acids and carbohydrate were revealed in the washings. To the final washing, 5 times volume of 10^{-5} M EDTA (ethylenediaminetetraacetic acid), pH 7.5 was added and the suspension was sonicated by a Raytheon Sonic Oscillator (Raytheon Manufacturing Co., Mass., U.S.A.) for 20 minutes at 9,000 Kc until most of the cells were disrupted. After centrifugation at 20,000 x g for 15 minutes to remove the non-disrupted cells, cell wall debris and cytoplasmic membranes, the supernatant fluid was filtered through a 0.22 μ membrane filter. A drop of the fluid was checked for particulate and possibly virus materials using an electron microscope.

Capsular Antigen

The microorganisms were grown in the modified Gaudy and Wolfe's medium for 3 days at 37°C. The capsular material was extracted by Kwapinski's (1965) technique for capsular antigen. The procedure was as follows:

After purity of the cultures had been established, the organisms were washed several times with double glass distilled water and centrifuged at 1,500 x g for 5 minutes without shaking to remove most of the contaminations from the medium. The cells were then suspended in 0.15 M, pH 6.5 phosphate buffer containing 0.9% sodium chloride. The mixture was agitated for 6 hours on an electric shaker at 2°C and centrifuged for 10 minutes at 10,000 x g. The sediment was washed with a small volume of phosphate buffer, centrifuged, and the washings were combined with the original supernatant.

The combined supernatant fluids, containing materials released from the surface of cells, were filtered through a 0.22 μ membrane filter, dialysed exhaustively against distilled water and lyophilised. Controls consisting of the medium alone and the inoculated semi-synthetic solid culture medium were treated in the same way.

CHEMICAL EXAMINATION

Qualitative Determination of Polymer-Categories by Disc-Electrophoresis

Different fractions of the individual soluble antigen were identified preliminary using the comprehensive disc-electrophoresis technique (Kwapinski, 1971a)using the basic acrylamide-gel electrophoresis of Ornstein and Davis (1964). Polyacrylamide gels of 5% total solids were used with low conductivity TRIS (hydroxymethyl aminomethane) - glycine HCl buffer of pH 8.91. Samples of about 500 μg were applied to the top of the gel columns and a trace of 0.001% Bromophenol Blue was added as a tracking dye. Anionic system with anode at bottom was used with a current of 1.25 mA per tube in the beginning and increased to 2.50 mA after the dye had reached the upper gel. At the end of the run, the gels were rimmed out and the dye bands cut off to facilitate the measurements of the R_s values. A control was also done with the dye band in the middle of the gel and without cutting to check if there was any component present in the antigen which could have migrated faster than the tracking dye. Different components of the individual soluble antigen were then identified by the following staining methods:

Protein (Clark, 1964) - Gel slabs were stained for 30 minutes in a 0.1% Naphthol Blueblack solution (Amido-black) in 7% glacial acetic acid. The residual dye was then removed by electrophoretic destaining in 3% glacial acetic acid. The R_s values were calculated as the ratio:

$$\frac{\text{Distance of band moved from origin}}{\text{Distance of tracking dye moved from origin}}$$

Nucleic Acids (Kwapinski, 1971) - Gel slabs were stained for 4 hours in a mixture containing 2% acridine orange, 1% lanthanum acetate and 15% glacial acetic acid. The residual dye was removed by destaining electrophoretically in 15% glacial acetic acid employing 50 V D.C. and 0.5 A. DNA fractions were distinguished from RNA by viewing the gels over an ultraviolet light source (UVS. 11, Ultra-violet Products Inc., California). DNA bands appeared brown while those of RNA showed green color.

Saccharides (Fairbairn, 1953) - A modified anthrone reagent consisting of 1% purified anthrone in 72% sulphuric acid was used to stain the gels. The acrylamide gel slabs were immersed in this reagent for 30-40 minutes and were washed in distilled water. The presence of greenish-blue bands indicated the saccharide fractions.

Phosphatides (Rouser et al., 1956) - Gel slabs were stained for 3 hours in a solution of 0.05% Rhodamine B in alcohol and destained by leaching. Phosphatides were shown by the appearance of red violet bands that fluoresced red on UV light against a pale pink background.

The electrophoresis technique together with the different staining methods were also used to confirm the homogeneity of the purified macromolecular complex.

Determination of Chemical Components occurring in the Antigenic Preparations

Dry Weight Determination - A 5 ml solution or suspension of the individual soluble antigen was introduced to a pre-weighed weighing bottle. It was then dried to constant weight over P_2O_5 in a vacuum oven (Thelco Model 10 Precision Scientific Co., Chicago, Illinois) at 70°C for 4-6 hours. The weight was determined in a Sartorius Microbalance three times during the period of 3 days. The content of the dry material per ml of the original solution or suspension was estimated.

Ash Value Determination (Steyermark, 1961) - Ash value was determined by the difference in weight between a pre-weighed combustion glass tubing and the complete combustion of the sample at 700°C. It was considered to be a correction factor for metals and non-volatile salts which might be present in the sample.

A series of chemical tests was done on the cytoplasms, exoantigens and capsular preparations. Their chemical compositions were determined quantitatively using the following methods, after the lipid fractions were extracted out quantitatively.

Lipid Extraction - Lipids were extracted by mixing 1 part sample with 5 parts organic solvent consisting of chloroform and acetone (4:1). After vigorous shaking in a separatory funnel for 10 minutes and allowed to stand for 1 hour at 4°C, the organic phase was collected and the aqueous phase extracted twice again. All the organic phases were pooled together, poured to a large flask and dried under nitrogen gas. The residue was redissolved in a minimun amount of chloroform-acetone mixture and poured to

a pre-weighed weighing bottle. The flask was rinsed with the organic solvent and combined with the above fraction. It was then dried in a vacuum oven over P_2O_5 at 45°C for 3 days until constant weight was obtained.

Phosphorus - Total phosphorus was determined by Fiske and Subbarow (1926) method on the individual soluble antigen and also on the extracted lipid fractions. Pure mono-potassium phosphate was used as standard. The blue color produced was read at 830 nm using the Unicam SP500 Series 2 spectrophotometer (Unicam Instruments Ltd., Cambridge, England).

Phospholipid - The percentage of phospholipid in the sample was obtained by multiplying the percentage of phosphorus in lipid by a factor of 25 (A conversion factor derived from the composition of lecithin).

Phosphorus in Nucleic Acids - The percentage of phosphorus in nucleic acids was calculated by subtracting the percentage of phosphorus in lipid from the total percentage of phosphorus in the sample.

Nucleic Acids - The total amount of nucleic acids in terms of total dry mass was assayed by multiplying the amount of phosphorus in nucleic acids by a conversion factor of 9.89.

Deoxyribonucleic Acid (DNA) - Total DNA was determined after hydrolysis of the sample, by Dische's (1930) method modified by Burton (1956). Sodium salt of deoxyribonucleic acid obtained from calf thymus gland was used as standard (B.D.H. Lab. Chemicals). Hydrolysis was done in 5N HCl in sealed ampules for 12 hours in an oven at 110°C.

Ribonucleic Acid (RNA) - Total RNA was obtained by taking the difference between the total nucleic acids and the total DNA.

Ribose in RNA - The percentage of ribose in RNA was calculated by dividing the percentage of RNA by a conversion factor of 2.3.

Pentose - Total pentose was determined by the dichromatic scanning using the Bial orcinol reaction (Brown, 1946). Glucose and xylose were used as standards and the wavelengths used were those of 670 and 520 nm.

Pentose in Carbohydrate - The percentage of pentose in the carbohydrate portion was calculated by taking the difference between the total pentose and the amount of ribose in RNA.

Carbohydrate - Total carbohydrate in terms of hexose was determined by using Fairbairn's (1953) modification of Dreywood's (1946) anthrone method. Galactose and glucose were used as standards and the blue color produced was read at 625 nm.

Protein - Total protein was assayed by using Lowry's *et al*. (1951) method and checked by Weichselbaum's biuret (1946) method modified by Dittebrandt (1948). Bovine serum albumin (B.S.A. Sigma Chemical Co.) was used as standard for protein estimation.

Nitrogen - The nitrogen content was determined by the micro-Kjeldahl method (Markham, 1942) using a Steam Distillation Apparatus.

Chemical analyses on the purified macromolecular complex were also carried out to obtain the ratio of protein, DNA, RNA, carbohydrate and phosphatides to check if it fitted closely with that of the non-purified one.

Preparative Separation and Purification of the different Polymer-Categories

The polymers were separated and purified by the following procedures: isoelectric focusing, polyacrylamide gel electrophoresis and gel filtration.

The Isoelectric Focusing Technique

The procedure of Vesterberg *et al*. (1967) was followed. The experiments were performed on LKB 8101 and 8102 columns (LKB-Produkter AB, Stockholm) utilizing different ampholytes with different pH ranges.

A pH range of 7-10 was used for the initial separation of the cytoplasmic antigen, whereas pH ranges of 6-8 and 3-6 were used for the separation of the capsular antigen and exoantigen respectively. The pH value of each fraction was measured at 22°C immediately after the elution of the column. After exhaustive dialysis against distilled water, the fractions were then concentrated by pervaporation and made to constant volumes. The amounts of protein, DNA, RNA and carbohydrate in terms of hexose were measured by chemical tests using Lowry's *et al*. (1951) method; Dische's (1930) diphenylamine reaction modified by Burton (1956); Mejbaum's (1939) method, and Fairbairn's (1953) modification of Dreywood's (1946) anthrone method respectively.

All the fractions were then extracted several times for lipids using a mixture of chloroform and acetone (4:1). The extracts of different fractions were then tested for phosphorus by the method of Fiske and Subbarow (1926).

Preparative Polyacrylamide Gel Electrophoresis

The procedure designed by Duesberg and Rueckert (1965) was followed using Shandon's Preparative Polyacrylamide Gel Electrophoresis Apparatus (Shandon Scientific Co. Ltd., London). The major components obtained from five isoelectric focusing runs (pH 7-10) were pooled. After dialysis and concentration, they were applied on top of the acrylamide gel for further purification. A 7.5% acrylamide gel was prepared at a pH of 9.5 in 6M urea. The power supply was adjusted to 30 mA at constant current with anode at bottom and increased to 80 mA after 1 hour of the run. The different fractions were carried by the elution buffer and the absorbance was read at 280 nm and 254 nm using an ISCO Dual Beam Ultraviolet Analyzer Model UA-2 with a built-in recorder (Instrumentation Specialties Co., Lincoln, Nebraska). The materials collected from the peak zone of the macromolecular complex (Fraction I) were further purified by isoelectric focusing using an ampholyte with a different pH range (pH 6-8).

Gel Filtration

A Sephadex K50/100 column (Pharmacia Canada Ltd., Montreal) was used. About 60 gm of Sephadex G-200, purchased from the same company, was slowly added to distilled water with continuous stirring and was heated at 60-80°C for 5 hours. The fines were decanted off five or six times with 0.1M TRIS-buffer, pH 7.1 added to the swollen gel. The dilute slurry of the Sephadex gel in TRIS-buffer was then poured into a R 50 type reservoir (Pharmacia Fine Chemicals, Uppsala, Sweden) for even packing of the gel. The column was stabilized by flushing the buffer through overnight, and at

the same time a 0.1% sodium azide was allowed to run through the column to act as a preservative.

A sample size of 20 ml of 0.5% Blue Dextran 2000 (Pharmacia Canada Ltd.) in distilled water was passed through the column using upward flow at a flow rate of 35 ml/hr to determine the void volume (V_o). Twenty milliliters of the material were applied to the column and the effluents were collected and scanned at 280nm and 254 nm using the ISCO UV Analyzer. Anthrone test for carbohydrate in terms of hexose was then performed on all the fractions obtained, and they were measured at an absorbance of 625nm. The experiment was performed at 4^oC using the Buchler Fractomat fraction collector with built-in refrigeration system (Buchler Instruments Inc., Fort Lee, New Jersey).

Determination of the Qualitative Composition of the different Polymers

A two-dimensional thin layer chromatography technique was used to determine the compositions of amino acids and monosaccharides present in the individual soluble antigen and also the purified macromolecular complex.

Thin Layer Chromatography of Amino Acids

About 5 mg sample of a material was hydrolysed in 5N HCl in a sealed ampule for 20 hours in an oven regulated at 110^oC. The hydrolysate was decolorized with active decolorizing charcoal, centrifuged, evaporated and neutralized in a vacuum oven over P_2O_5 and NaOH pellets placed in separate beakers. About 5 µl of the hydrolysate was spotted at the left hand top corner, one inch from each side of a glass plate (8x8 inches) coated

with a thoroughly blended mixture of MN 300 Cellulose (Brinkmann Instruments, Westbury, New York), alcohol and water (4:1:24). The plates were coated by spraying 30 times with a spray gun (Sprayit No. 525 Paint Sprayer, Thomas Industries Inc., Johnson City, Tennessee) held one foot vertically above the plates. The air pressure applied was 10 lb/sq. in., and the coated plates were dried at 70°C and stored in a dessicator. The chromatogram was first run in an ascending manner for 3 hours in a solvent consisting of propylene glycol, acetone, 95% ethyl alcohol and distilled water,in the ratio 6.5:50:23.5:20,respectively (Kwapinski and Snyder, 1961). The ascending chromatography was carried out in a rectangular tank (Desaga, Germany) previously saturated with the solvent, and at a temperature of no more than 23°C to prevent spot distortion. The plates were then dried at 100°C, turned by 90 degrees, and developed for 5 hours in a basic solvent consisting of the upper phase of n-butanol and 1% aqueous ammonia (1:1). The plates were again dried and run in the same direction for 2 hours in the first solvent. The chromatoplates were dried at 100°C, sprayed with 0.5% ninhydrin solution in 95% ethyl alcohol and heated at 100°C until color spots had appeared. The positions of the spots produced by different amino acids were compared to the two-dimensional map obtained by the examination of 22 standard amino-acids.

Thin Layer Chromatography of Saccharides

The chromatographic analysis of saccharides was conducted on MN 300 Cellulose coated plates applying for 4 hours Kwapinski's (1965) solvent consisting of a 10:5:2:1 mixture of n-butanol, 95% ethyl alcohol, glacial acetic acid and distilled water. For the two-dimensional chromatography, the above solvent was used for 4 hours, followed by Kwapinski and Snyder's (1961) solvent in the second direction for 2 hours. The sugar spots were detected with a 1:1 mixture of 0.1% aniline in ethanol and 2.5% oxalic acid at 100°C. Acetylhexoses were detected by spraying with Salton's (1959) reagent consisting of 10 ml of a 2% w/v solution of p-dimethylamino benzaldehyde (DMAB) in glacial acetic acid, 30 ml n-butanol and 0.4 ml concentrated hydrochloric acid. Hydrolysis was done in 1.5N sulfuric acid in a sealed ampule for 5 hours at 100°C. The hydrolysate was adjusted to pH 4.5, using barium carbonate. The resulting precipitate was discarded by centrifugation, and about 10 μl of the hydrolysate was used for the chromatography.

SEROLOGICAL EXAMINATION

Antiserum Production

Albino rabbits weighing from 2.5 to 3.0 kilograms were injected with the soluble antigens according to Kwapinski's (1969) immunization schedule:

Subcutaneously	0.3 ml
Intramuscularly	0.4 ml
Foot pad	0.4 ml
Another foot pad	0.4 ml
Intravenously	0.4 ml
Subcutaneously	0.5 ml

The antigenic preparations were adjusted to give an optical density of 0.5 at 280 and 254 nm absorbance using a 1 cm-wide cuvette. Injections were spaced by 3-5 days and were continued until strongly reactive antisera were obtained. Active sera were collected one week after the last injection, and were preserved in the frozen state.

Agar-Gel Immunodiffusion

The prepared antisera were tested against the soluble antigenic preparations obtained from *Mycococcus*. The method designed by Ouchterlony (1962) was followed. Agar gel was prepared in 0.85% sodium chloride and Ionagar No. 2 (Oxoid) with the addition of a few drops of 1:10,000 Merthiolate to make a 0.5% final concentration of agar. Wells were cut with a Feinberg agar-gel cutter. The diameters of the center well and the outer wells were 14 mm and 4 mm respectively. The distance between the center and the outside wells was that of 10 mm apart. About 0.1 ml of the optimum concentration of the antigen was applied to the outside well whereas the center well received 0.5 ml of the antiserum. The plates were incubated in a moist chamber at 37°C for 40 hours. Precipitate bands were recorded as lines of identity, non-identity and partial identity of the different soluble antigens with the antiserum.

Disc-Immunoelectrophoresis (Finkelstein *et al*., 1966)

Gel slabs obtained from disc-electrophoresis were rinsed once in distilled water and imbedded in 15 ml of molten 0.5% Ionagar No. 2 (Oxoid) in 0.85% sodium chloride with a few drops of 1:10,000 Merthiolate in a Petri

plate. Agar was allowed to solidify and a 2 mm trough was cut at the center of the plate with a Feinberg agar-gel cutter parallel and approximately 5 mm distance from each gel. Mycococcus cytoplasm antiserum was placed into the trough and the resulting precipitation between the gel and the antiserum trough was observed after 40 hour incubation at 37°C. Prestained gels were placed at exactly the same position for comparison of areas of protein staining and antigen distribution.

Cellulose Acetate Immunodiffusion

Buffered isotonic saturated cellulose acetate membrane (Millipore Co., Bedford, Mass.) was used and the test was run in a Saravis (1965) immunocell consisting of a template, support and base. The template consisted of two double rows of 10 wells situated on both sides, each having a diameter of 1.5 mm, and two 1.5 mm wide troughs, 5 mm from the wells. The troughs were filled with the prepared antisera and 0.1 ml of the optimum concentration of the cytoplasmic preparations obtained from selected strains of Mycobacterium, Nocardia, Actinomyces, Streptomyces and Dermatophilus were introduced into wells opposite to each other. The template was fastened to the base with nylon bolts at 5 inch-pounds pressure. The plates were incubated in a moist chamber for 48 hours at 37°C, washed with saline, and then stained with 0.2% Ponceau-S dye made in 3% trichloroacetic acid for 5-7 minutes. The membrane was then decolorised with 6% glacial acetic acid, and examined in a transmitted light viewer, equipped with a 10X magnifying glass (Picker X-ray Engineering Ltd., Montreal). Each test was set in duplicate and was repeated three times.

RESULTS

I. Morphological, cultural and biochemical characteristics of Mycococcus spp.

The results obtained from different tests are presented summarily in TablesVIIIa, b and c. It is apparent that both strains of *Mycococcus* were morphologically, culturally and biochemically similar to each other. It was also shown in TableVIIIb that the growth in Kwapinski's semi-synthetic medium occurred in the form of a slimy, greyish deposit, but a turbidity and slimy, greyish deposit were formed in the glucose broth, whereas a viscous, greyish layer, near the top, was produced in the thioglycollate broth. Colonies on the solid semi-synthetic medium were tiny, smooth, slimy and opaque. Colonies on blood agar were slightly larger, greyish-yellow, slightly confluent, smooth and slimy. As shown in TableVIIIc, both strains were able to grow most abundantly at 37° C and slightly less abundantly at 28° C, but some growth still occurred at 15° and 45° C. The cultures exposed to 60° C for 4 hours lost their viability. The growth of both strains in the presence of 10% sodium chloride indicated that they were resistant to high concentration of salts. Neither strain was able to decompose tyrosine, urea, starch or gelatin, peptonize milk and reduce nitrate, but they produced catalase. The following saccharides were utilized readily as sole source of carbon: galactose, arabinose, glucose, maltose and sucrose, and less readily fructose, mannose, raffinose, xylose and erythritol. Succinate, pyruvate, propionate, acetate and citrate were also utilized as sole source of carbon, but not benzoate, paraffin, lactate and tartrate. The color of the pigment produced, the presence of capsule and/or slime layer and the ability to utilize citrate, acetate and succinate as sole carbon source strongly suggested that they belonged to *Mycococcus capsulatus* or *M. ruber*.

TABLE VIII a

MORPHOLOGICAL CHARACTERISTICS OF *MYCOCOCCUS* SPP.

Media	Strain No. of *Mycococcus* 13556	13557
Blood agar	Gram +ve, very pleomorphic; mainly coccobacillary or spherical forms to short rods (3:1 length:width) with round ends. About 0.2-0.7 μ in diameter or 0.3x 1.0-1.2 μ. Some cells amoeboid in shape; sometimes club-shaped cells arranged singly, in pairs, in chains or clusters also found.	Gram +ve, less pleomorphic than strain no. 13556; mainly coccal shape. Some oval rods and irregular-shaped cells discovered too. Cells in clusters, singles, pairs and chains surrounded by slime layer. Chains of cocci usually in V or Y forms. About 0.2-0.7 μ in diameter. Club-shaped cells rarely found.
Kwapinski's semi-synthetic solid and liquid culture media	Gram +ve, very pleomorphic; mainly cylindrical form with round ends. About 0.3 μ in width and 1.0-1.4 μ in length. Some cells amoeboid in shape or club-shaped. Sometimes cells found arranged in excentric clusters, short chains or singly.	Gram +ve, less pleomorphic than strain no. 13556; mainly round and oval shapes in singles, clusters or chains. Cells 0.5-0.7 μ in diameter. Older cultures slowly develop into large, lemon-shaped cells.
Modified Gaudy and Wolfe's medium	Gram +ve, very pleomorphic; the cellular morphology resembles those grown in the semi-synthetic liquid and solid media, except that cells are found surrounded by a slime layer.	Same as strain no. 13556.

TABLE VIII b

CULTURAL CHARACTERISTICS OF MYCOCOCCUS SPP.

	Strain No. of Mycococcus	
	13556	13557
From solid media*		
Blood agar	Greyish-yellow, slimy, slightly confluent, smooth round and soft colonies, 1-3 mm. in diameter.	Colonies irregular in shape, mucoid and spreading, with rosy-pink pigment produced.
Kwapinski's semi-synthetic medium	Tiny, slimy, greyish colonies.	Same as strain no. 13556.
Gaudy and Wolfe's medium	Greyish-yellow, slimy, smooth and soft colonies.	Same as strain no. 13556.
Nutrient agar	Greyish-white, smooth and shiny colonies.	Same as strain no. 13556.
Soil Extract agar	Abundant growth; colonies soft, shiny, more entire and with deep orange-yellow pigment produced.	Colonies smooth, shiny and spreading, with pink pigment produced.
Maltose Yeast Extract agar	Creamy yellow, soft, shiny and irregular colonies.	Same as strain no. 13556.
From liquid media*		
Glucose broth	Turbidity and greyish, slimy deposit.	Same as strain no. 13556.
Brain Heart Infusion broth	Turbidity and yellowish, slimy deposit.	Same as strain no. 13556.
Thioglycollate broth	Viscous growth, mainly in the top layer.	Same as strain no. 13556.
Semi-synthetic medium	Scanty growth, with slimy greyish deposit.	Same as strain no. 13556.

* All the cultures were incubated at 37° C for 3 days.

TABLE VIII c

BIOCHEMICAL CHARACTERISTICS OF MYCOCOCCUS SPP.

	Strain No. of Mycococcus	
	13556	13557
Catalase production	+*	+
Motility	+	+
Litmus milk peptonisation	-	-
Gelatin liquefaction	-	-
Starch Hydrolysis	-	-
Tyrosine decomposition	-	-
Nitrate reduction	-	-
Growth at:		
4° C	-	-
15° C	+	+
28° C	++	++
37° C	+++	+++
45° C	+	+
55° C	-	-
Resistance to 60° C for 4 hours	-	-

(continued)

TABLE VIII c (continued)

	Strain No. of *Mycococcus*	
	13556	13557
<u>Growth on</u>:		
10% NaCl	+/-**	+/-
10% Na_2SO_4	+	+
<u>Growth on dyes</u>:		
Congo Red	+	+
MacConkey's	+	+
Methyl Blue	+	+
Brilliant Green	-	-
Eosin Y	-	-
Malachite Green	-	-
<u>Utilisation of</u>:		
<u>Hexose Monosaccharides</u>		
Fructose	+/-	+/-
Galactose	+	+
Glucose	+	+
Mannose	+/-	+/-
<u>Disaccharides</u>		
Maltose	+	+
Sucrose	+	+
Lactose	-	-

(continued)

TABLE VIII c (continued)

	Strain No. of *Mycococcus*	
	13556	13557
Trisaccharide		
Raffinose	+/-	+/-
Pentose Monosaccharides		
Arabinose	+	+
Xylose	+/-	+/-
Rhamnose	-	-
Polyhydric Alcohols		
Dulcitol	-	-
Glycerol	-	-
Erythritol	+/-	+/-
Inositol	-	-
Mannitol	-	-
Organic Acids		
Succinate	+	+
Pyruvate	+	+
Propionate	+	+
Acetate	+	+
Citrate	+/-	+
Benzoate	-	-

(continued)

TABLE VIII c (continued)

	Strain No. of *Mycococcus*	
	13556	13557
Paraffin	-	-
Lactate	-	-
Tartrate	-	-

* + denotes a reaction or growth.

**+/- denotes a weak reaction or scanty growth.

II. Preparation of antigens

Exoantigen - Exoantigen preparations were obtained in the form of a brownish yellow liquid, soluble at a pH below 6.5, and precipitable at higher pH values. The control sample consisting of the semi-synthetic liquid medium had a negligible amount of organic material. This indicates that the exoantigen preparations possessed no contaminating material from the semi-synthetic medium.

Cytoplasm Antigen - The cytoplasm preparations were obtained in the form of an opalescent liquid which remained in solution at alkaline pH values, but precipitated at a pH below 5.1.

Capsular Antigen - The capsular antigen preparations were in the form of an opalescent liquid. Acidic or alkaline pH values did not cause precipitation of the antigen. The control sample consisting of the modified Gaudy and Wolfe's medium possessed a negligible amount of organic material. *Mycococcus* cells grown in Kwapinski's semi-synthetic liquid or solid medium produced neither capsule nor slime layer (Figure 1), whereas the bacteria grown in the modified Gaudy and Wolfe's medium formed capsules and/or slime layers (Figure 2). After the extraction procedure following Kwapinski's (1965) technique, it was found that almost all the capsules or slime layers were removed from the cells (Figure 3).

Figure 1

Electron micrograph of whole cells of _Mycococcus_ (strain 13556) grown in Kwapinski's semi-synthetic medium. Note the coccobacillary form and the cylindrical shape of the cells. Capsular material was not found when cells were grown in this medium.

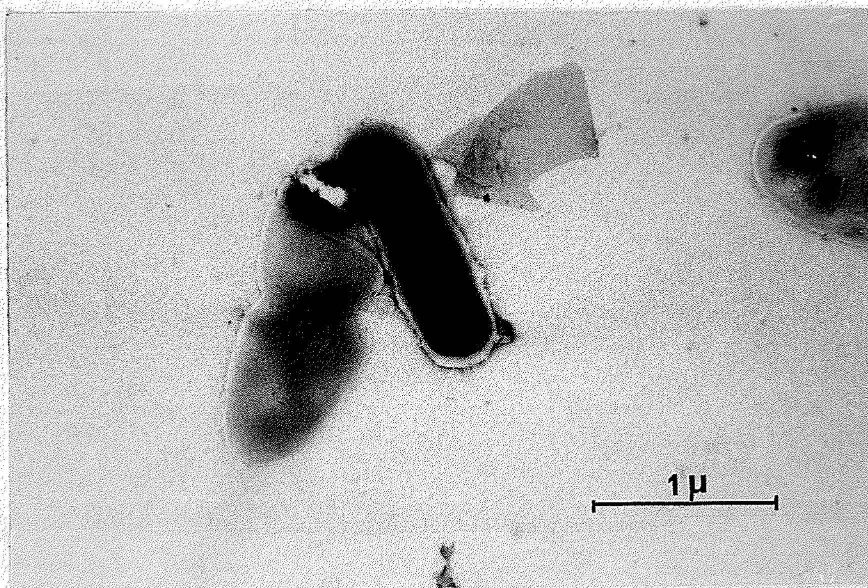
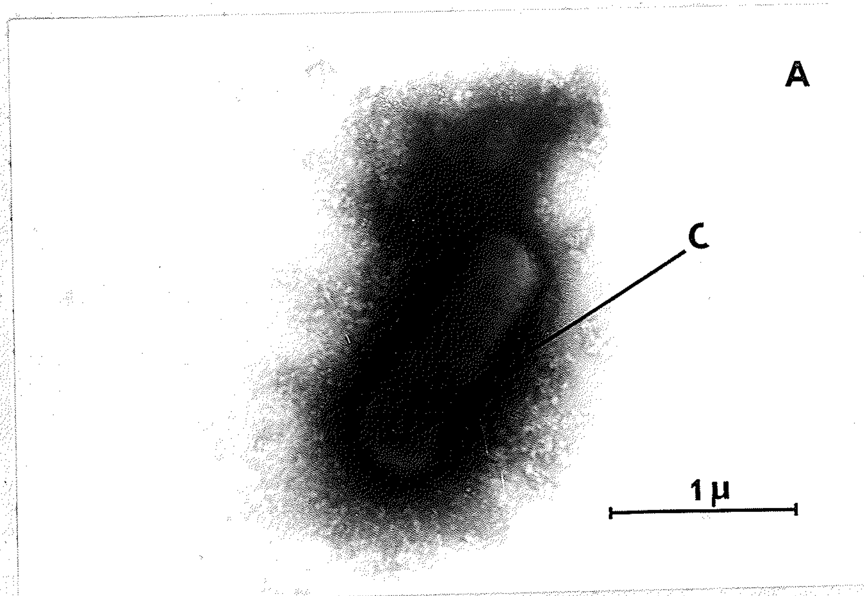

1 µ

Figure 2

Electron micrographs of *Mycococcus* cells grown in the modified Gaudy and Wolfe's glucose-peptone medium. Note the presence of capsule or slime layer (C).

A: *Mycococcus* (strain 13556).

B: *Mycococcus* (strain 13557).


A
C
1 µ

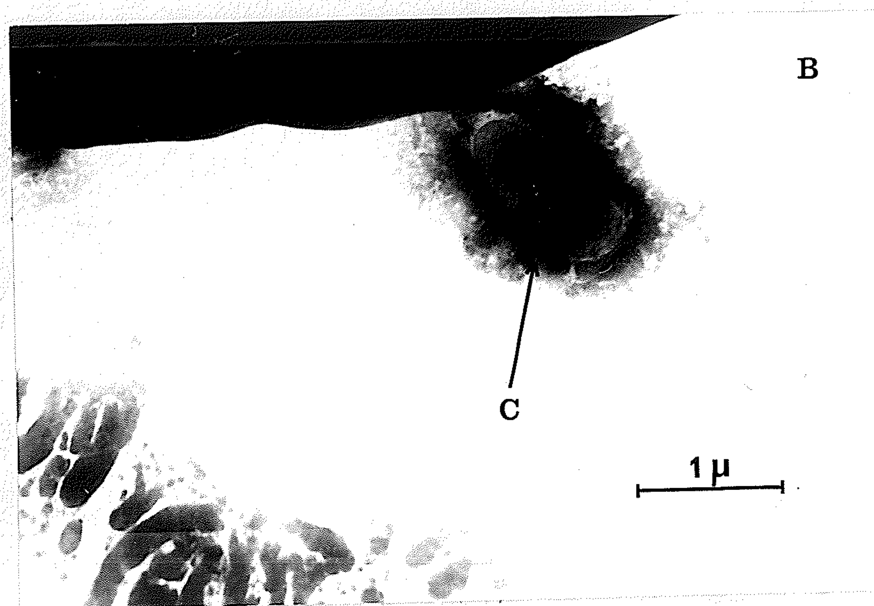
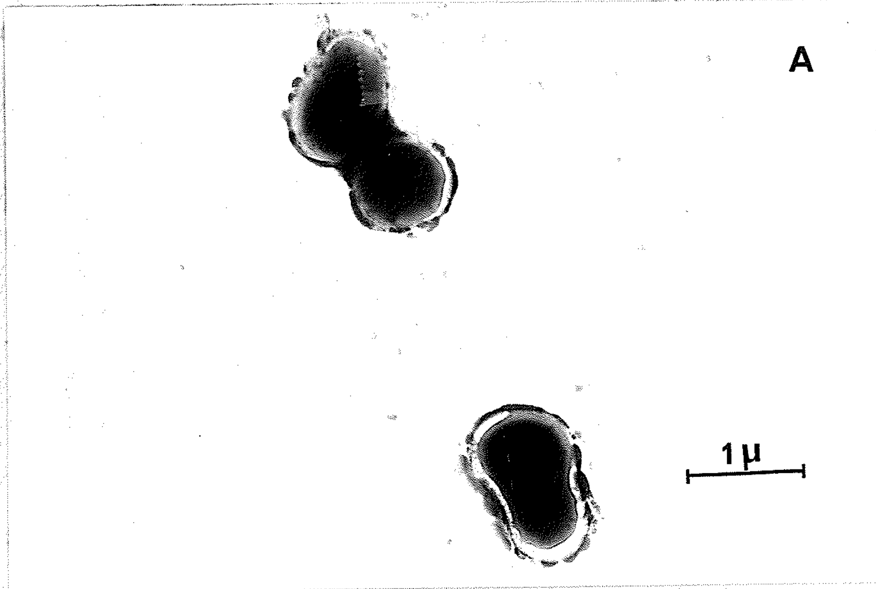

B
C
1 µ

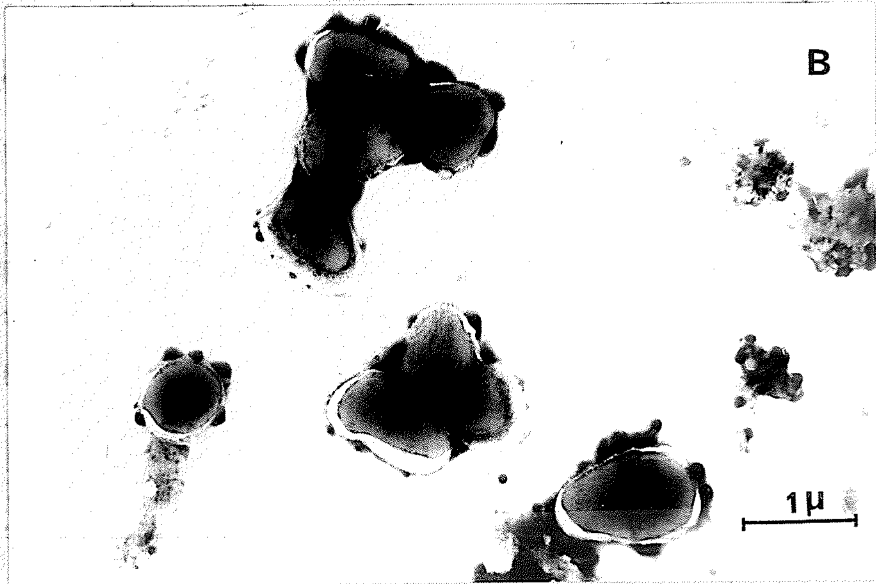
Figure 3

Electron micrographs of _Mycococcus_ cells after decapsulation with 0.15 M phosphate buffer, pH 6.5, with 0.9% sodium chloride. Note the disappearance of the capsule or slime layer.

A: _Mycococcus_ (strain 13556).

B: _Mycococcus_ (strain 13557).


A
1μ


B
1μ

III. Physicochemical properties of the soluble antigens of *Mycococcus*

Comprehensive disc-electrophoresis

By using the different staining techniques described in Materials and Methods, the R_s values of the different components of the cytoplasmic antigen are presented in Table IX . It was shown that the cytoplasm of *Mycococcus* tested contained eleven polymer fractions, as follows: 2 protein-, 3 nucleoprotein-, 3 galactoprotein-, 1 phospholipid-galactonucleoprotein-, and 2 galactonucleoprotein fractions. The respective R_s values of the different fractions were: 0.09 and 0.20; 0.43, 0.58 and 0.73; 0.50, 0.90 and 0.98; 0.83; and 0.77 and 0.66.

The capsular antigen of *Mycococcus* was shown to consist of a simpler pattern than that of the cytoplasmic antigen. Only seven polymer fractions were detected. They were: 1 protein- (R_s 0.59), 1 nucleic acid- (R_s 0.67), 2 nucleoprotein- (R_s 0.35 and 0.86), and 3 polysaccharide fractions (R_s 0.33, 0.42 and 0.92). The polysaccharide components, as shown in Table X , were not closely associated with either the protein or the nucleic acid moieties. The disc-electrophoretic patterns of the cytoplasmic antigen of *Mycococcus* are shown in Figures 4 and 5.

Chemical Composition

Chemical composition of the different soluble antigens are summarily presented in Table XI. It was found that the cytoplasm consisted of roughly 57% protein, 15% saccharide, 9% DNA, 15% RNA and 2% phospholipid. The capsular antigen comprised approximately 42% protein, 22% saccharide, 14% DNA, 5% RNA and 15% lipid. The organic matter of the exoantigen was found to contain roughly 70% nucleoprotein and 30% carbohydrate.

Figure 4

Disc-electrophoresis pattern of Mycococcus (strain 13556) cytoplasm on 5% acrylamide gel with anode at bottom. The gel slab was stained with 0.1% Amido-black for protein components.

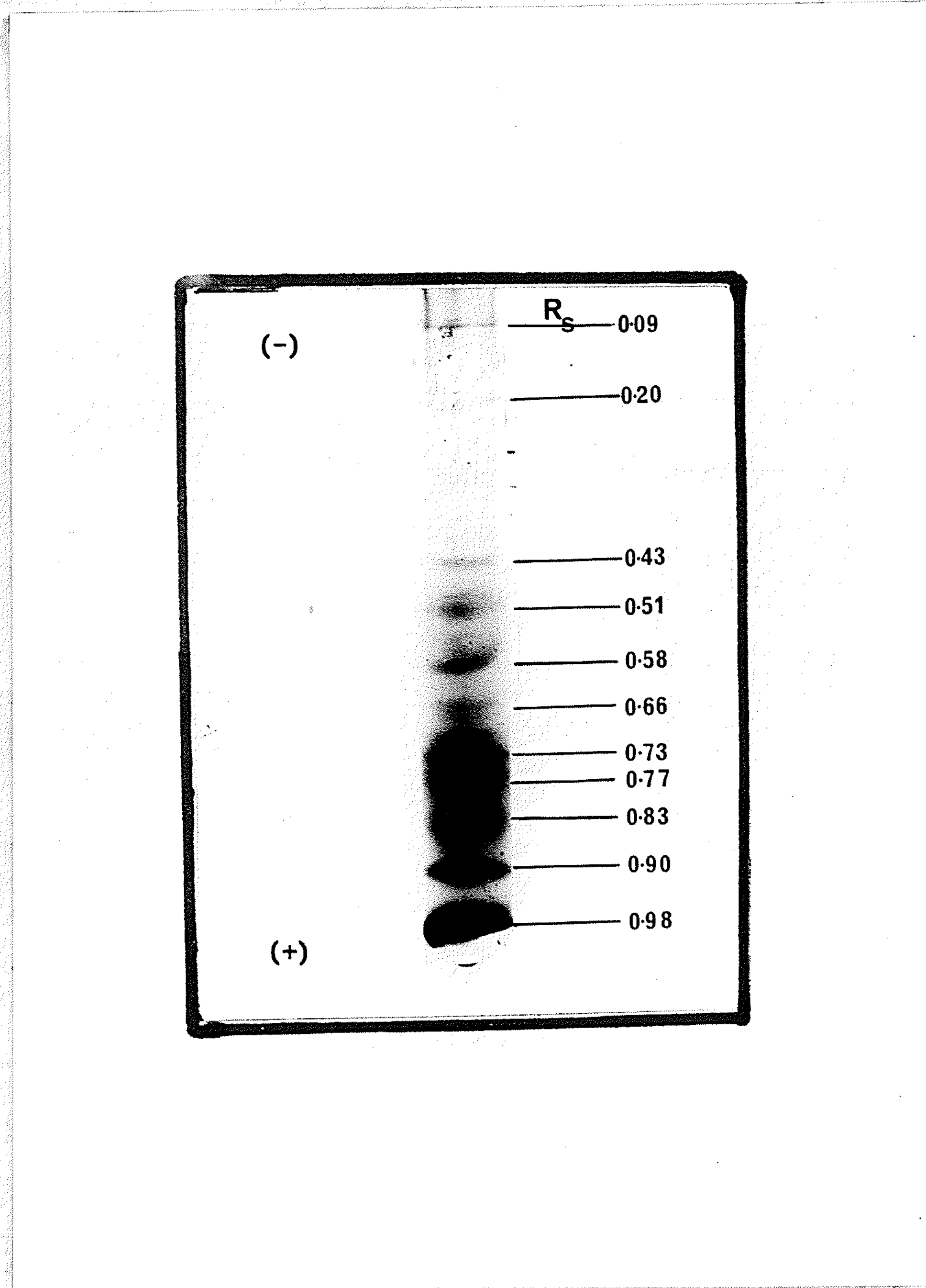

(-)
(+)
Rs
0·09
0·20
0·43
0·51
0·58
0·66
0·73
0·77
0·83
0·90
0·98

Figure 5

Disc-electrophoresis pattern of *Mycococcus* (strain 13556) cytoplasm on 5% acrylamide gel with anode at bottom. The gel slab was stained with the modified anthrone reagent for saccharide components.

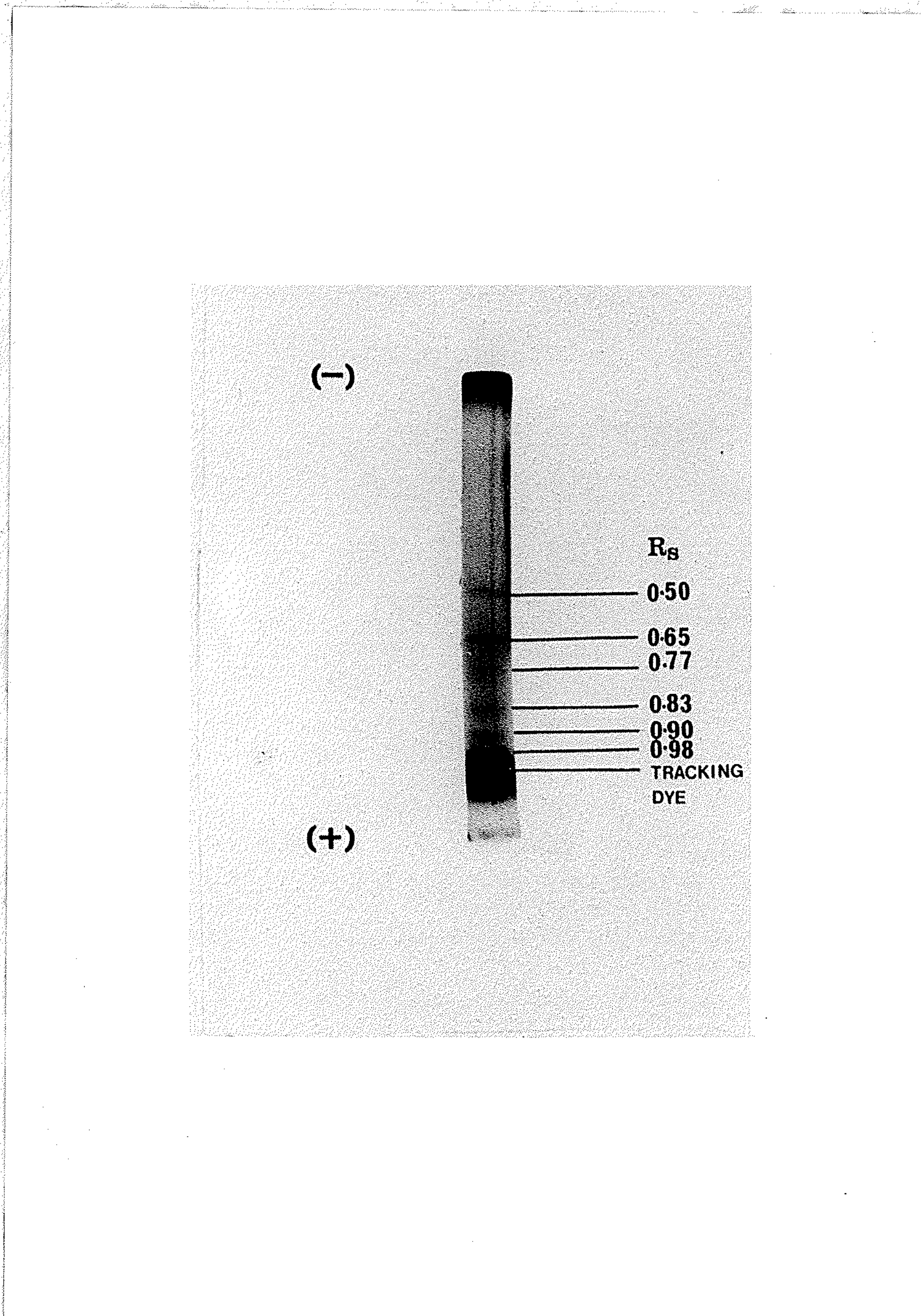

(−)
Rs
0·50
0·65
0·77
0·83
0·90
0·98
TRACKING
DYE
(+)

TABLE IX

POLYMERS SEPARATED BY DISC-ELECTROPHORESIS OF THE CYTOPLASMIC ANTIGEN OF MYCOCOCCUS, AS REVEALED BY DIFFERENT DYES

Band No.	R_s^* values of			
	Protein	Carbohydrate	Nucleic Acid	Phospholipid
1	0.09			
2	0.20			
3	0.43		0.43	
4	0.50	0.50		
5	0.58		0.58	
6	0.66	0.66	0.66	
7	0.73		0.73	
8	0.77	0.77	0.77	
9	0.83	0.83	0.83	0.83
10	0.90	0.90		
11	0.98	0.98		

* R_s values were calculated from the ratio:

$$\frac{\text{Distance of band moved from origin}}{\text{Distance of tracking dye moved from origin}}$$

TABLE X .

POLYMERS SEPARATED BY DISC-ELECTROPHORESIS OF THE CAPSULAR ANTIGEN OF *MYCOCOCCUS*, AS REVEALED BY DIFFERENT DYES

Band No.	R_s^* values of		
	Protein	Carbohydrate	Nucleic Acid
1		0.33	
2	0.35		0.35
3		0.42	
4	0.59		
5			0.67
6	0.86		0.86
7		0.92	

* R_s values were calculated from the ratio:

$$\frac{\text{Distance of band moved from origin}}{\text{Distance of tracking dye moved from origin}}$$

TABLE XI

CHEMICAL COMPOSITIONS OF THE SOLUBLE ANTIGENS OF <u>MYCOCOCCUS</u>

Element or Compound	Percentage of dry mass of:					
	Cytoplasmic Antigen*		Capsular Antigen		Exoantigen	
Lipid	2.0	3.9	14.0	16.0	0	0
Lipid-phosphorus	0.076	0.082	0	0	0	0
Total phosphorus	2.56	2.59	1.8	2.0	0.38	0.39
Phospholipid	1.90	2.05	0	0	0	0
Nucleic acid-phosphorus	2.48	2.51	1.8	2.0	0.38	0.39
Total nucleic acids	24.50	26.50	17.7	20.1	3.5	3.9
DNA	8.40	10.80	13.2	14.9	2.4	2.6
RNA	15.60	16.20	4.5	5.2	1.1	1.3
RNA-pentose	6.80	7.00	2.0	2.2	0.48	0.56
Total pentose	6.80	7.00	1.9	2.3	1.98	2.86
Carbohydrate-pentose	0	0	0	0	1.5	2.3
Carbohydrate-galactose	16.00	14.00	20.8	21.8	30.9	31.1
Nitrogen	5.22	5.32	5.81	6.0	9.8	11.4
Protein	56.4	58.7	41.8	42.6	63.5	65.5
Ash	0	0	4.3	4.5	2.0	2.8

* The first of two vertical columns of each soluble antigen refers to strain no. 13556, and the second column refers to strain no. 13557.

Quantitative separation of different polymer-categories by isoelectric focusing

The patterns of the different polymer-categories of the cytoplasmic antigen, capsular antigen and exoantigen obtained by isoelectric focusing are presented in Tables XII, XIV and XVI, and Figures 6, 7 and 8 respectively.

The cytoplasm was found by this method to consist of twelve polymer fractions, as follows: five galactonucleoprotein-, three nucleoprotein-, two nucleic acid-, one protein- and one galactoprotein fractions. The two major constituents of the cytoplasmic antigen were denoted as Fractions I and II, with isoelectric points of pH 6.40 and 2.10, respectively. These two fractions accounted for approximately 80% of the total materials present in the cytoplasm (Fraction I counting from tube nos. 39-41; Fraction II counting from tube nos. 46-48). With tube no. 40 alone, the total amount of organic materials was found to be 23%, whereas tube no. 47 alone consisted of 20% of the total dry mass. Fraction I appears to be a macromolecular complex of the cytoplasmic antigen because the peak positions of the different components (protein, carbohydrate, DNA and RNA) all were situated at tube no. 40. The chemical composition of fractions obtained are shown in Table XIII. The pattern of distributions of the various components coincided quite well with that revealed by the disc-electrophoresis experiment. Also as shown in Table XIII, the ratio of the percentage of protein: RNA: carbohydrate: DNA present in Fraction I was calculated to be approximately 67: 24: 6 :3.

TABLE XII.

COMPOSITION OF PEAKS SEPARATED BY ISOELECTRIC FOCUSING OF MYCOCOCCUS (STRAIN 13556)CYTOPLASM USING AMPHOLYTE pH 7-10 RANGE

Tube no.*	pH**	Protein	Carbohydrate	DNA	RNA
5	11.9	+	+	+	+
8	9.9	+	+	+	
11	9.7	+	+		
13	9.4	+		+	
18	9.2	+			
20	9.0	+		+	
24	8.8	+	+	+	
30	8.5	+		+	
40	6.4	+	+	+	+
46	2.8				+
47	2.1	+	+	+	
49	2.0				+

* Tube number with 8 ml. elution volume, made up to 10 ml. solution after dialysis and concentration.

**The pH of each fraction was measured at 22° C immediately after elution of column.

TABLE XIII.

CHEMICAL COMPOSITION OF FRACTIONS OBTAINED BY ISOELECTRIC FOCUSING OF THE CYTOPLASM OF MYCOCOCCUS STRAIN NO. 13556 USING THE AMPHOLYTE RANGE OF pH 7-10

Tube no.	pH	Protein*	%	CHO*	%	DNA*	%	RNA*	%
1**	12.0	210	0.55	0	0	0	0	0	0
2	12.0	240	0.63	18	0.44	0	0	0	0
3	12.0	280	0.73	44	1.06	0	0	6.9	0.10
4	11.9	380	0.99	74	1.80	12	0.32	11.5	0.16
5	11.9	462	1.20	107	2.60	96	2.53	41.4	0.58
6	11.3	315	0.82	15	0.37	23	0.60	0	0
7	10.5	300	0.78	0	0	0	0	0	0
8	9.9	306	0.80	135	3.28	162	4.26	0	0
9	9.8	120	0.31	108	2.62	22	0.58	0	0
10	9.7	30	0.21	14	0.33	40	1.05	0	0
11	9.6	246	0.64	138	3.35	61	1.60	0	0
12	9.4	120	0.31	74	1.81	70	1.84	0	0
13	9.4	175	0.45	47	1.15	95	2.50	0	0
14	9.3	100	0.26	32	0.79	20	0.53	0	0
15	9.3	40	0.10	26	0.63	0	0	0	0
16	9.3	20	0.05	0	0	0	0	0	0
17	9.3	40	0.10	0	0	0	0	0	0
18	9.20	135	0.35	0	0	41	1.08	0	0
19	9.1	62	0.16	0	0	67	1.76	0	0
20	9.0	132	0.34	0	0	108	2.84	0	0
21	8.9	80	0.21	0	0	24	0.63	0	0
22	8.9	60	0.15	0	0	0	0	0	0
23	8.9	80	0.21	0	0	15	0.39	0	0
24	8.8	156	0.41	121	2.93	83	2.18	0	0
25	8.8	110	0.28	0	0	0	0	0	0
26	8.7	82	0.21	0	0	0	0	0	0
27	8.6	65	0.17	0	0	0	0	0	0
28	8.5	70	0.18	0	0	0	0	0	0
29	8.5	100	0.26	0	0	14	0.37	0	0
30	8.5	144	0.37	0	0	72	1.89	0	0
31	8.4	122	0.32	0	0	0	0	0	0
32	8.4	122	0.32	0	0	0	0	0	0
33	8.2	140	0.36	0	0	0	0	0	0
34	8.1	200	0.52	0	0	0	0	0	0
35	7.8	300	0.78	0	0	0	0	4.0	0.06
36	7.7	535	1.39	0	0	0	0	18.4	0.26
37	7.6	1050	2.74	0	0	0	0	55.2	0.78
38	7.5	1600	4.17	20	0.48	75	1.97	197.8	2.79

(continued)

TABLE XIII (continued)

Tube no.	pH	Protein*	%	CHO*	%	DNA*	%	RNA*	%
39	7.1	2700	7.04	455	11.04	270	7.10	811.9	11.46
40	6.4	6222	16.22	576	14.01	312	8.21	2217.2	31.30
41	6.3	2900	7.56	34	0.82	80	2.10	423.2	5.97
42	5.9	1700	4.43	0	0	25	0.66	186.3	2.63
43	5.5	1200	3.13	0	0	0	0	82.8	1.17
44	4.6	1060	2.76	12	0.30	0	0	73.6	1.04
45	3.9	1425	3.72	45	1.09	124	3.26	66.7	0.94
46	2.8	2400	6.26	627	15.23	380	10.00	386.4	5.45
47	2.1	6672	17.53	930	22.59	684	18.00	218.5	3.08
48	2.0	2350	6.13	414	10.06	210	5.53	52.9	0.75
49	2.0	1525	3.98	50	1.20	64	1.68	241.5	3.41
50	2.0	1145	2.99	0	0	0	0	108.1	1.53
51	2.0	410	1.20	0	0	0	0	25.3	0.36
52	1.9	0	0	0	0	0	0	13.8	0.19
53	1.9	0	0	0	0	0	0	9.2	0.13
54	1.9	0	0	0	0	0	0	0	0
TOTAL		39559μg	105.8%	4116 μg	99.9%	3249μg	85%	5253μg	74%
AMOUNT APPLIED		38332μg		4126 μg		3800μg		7084μg	

% RECOVERY

Protein $= \frac{39559}{38332} \times 100\% = 103.2\%$

CHO $= \frac{4116}{4126} \times 100\% = 99.76\%$

DNA $= \frac{3249}{3800} \times 100\% = 85.5\%$

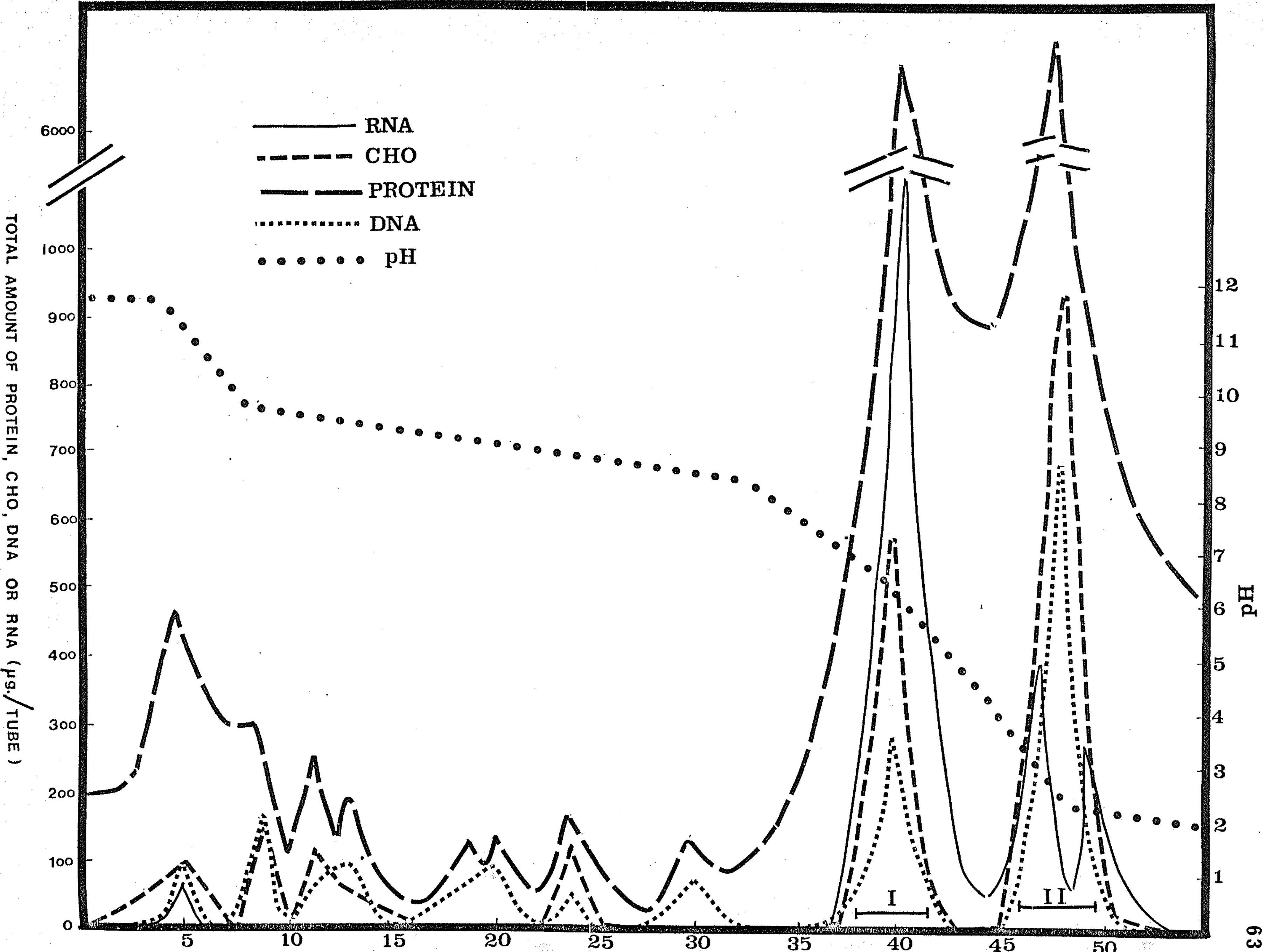
RNA $= \frac{5253}{7084} \times 100\% = 74.2\%$

* Total amount of individual material (μg.) calculated per tube.

**The fractions (8 ml) collected were dialysed against distilled water, concentrated and made up to 10 ml.

Figure 6

Pattern of polymers detected by isoelectric focusing of the cytoplasm of *Mycococcus* strain no. 13556 using the ampholyte range of pH 7-10 with cathode at bottom. A sample size of 3 ml of the initial cytoplasmic antigen consisting of a dry mass of 65 mg of the total materials was applied to LKB 8102 electrofocusing column.


RNA
CHO
PROTEIN
DNA
pH
TOTAL AMOUNT OF PROTEIN, CHO, DNA OR RNA (µg./TUBE)
pH
TUBE NO. (10 ml./TUBE)
I
II
6000
1000
900
800
700
600
500
400
300
200
100
0
5
10
15
20
25
30
35
40
45
50
12
11
10
9
8
7
6
5
4
3
2
1

The capsular antigen possessed a simpler pattern than that of the cytoplasmic antigen (Figure 7), since only three saccharide-, two nucleic acid-, one nucleoprotein- and two protein fractions were resolved by the same method. The components were found to occur quite freely and were not in the form of complexes, except one nucleoprotein fraction (tube no. 13, isoelectric point of pH 3.15). This nucleoprotein component represented the major constituent of the capsular antigen of Mycococcus and accounted for roughly one third of the total dry mass. The main polysaccharide fraction (tube no. 14, isoelectric point of pH 2.25) consisted of approximately two third of the total polysaccharide present in the capsular antigen (Table XV). The pattern of distributions of the different polymer fractions is shown in Table XIV.

As the exoantigen was found to be precipitated at a pH above 6.5, an ampholyte range of pH 3-6 was used in the electrofocusing experiment. The composition of peaks obtained is presented in Table XVI. The main component of the exoantigen of Mycococcus existed in the form of a saccharide-protein moiety with an isoelectric point of pH 7.0. This fraction accounted for roughly 30% of the total dry mass (Table XVII). Another complex present in the exoantigen occurred in the form of a nucleoprotein fraction with an isoelectric point of pH 3.30, whereas all the other components were freely distributed. The pattern of distributions of the different polymer fractions is shown in Figure 8.

TABLE XIV

COMPOSITION OF PEAKS SEPARATED BY ISOELECTRIC FOCUSING OF MYCOCOCCUS (STRAIN 13557) CAPSULAR ANTIGEN USING AMPHOLYTE pH 6-8 RANGE

Tube no.*	pH**	Protein	Carbohydrate	DNA	RNA
2	12.2		+		+
3	11.7	+			
5	7.7		+		
6	7.5			+	
7	7.2	+			
8	7.0		+		
10	6.7			+	
13	3.2	+		+	+
14	2.3		+		

* Tube number with 6 ml. elution volume, made up to 10 ml. solution after dialysis and concentration.

**The pH of each fraction was measured at 22°C immediately after the elution of column.

TABLE XV

CHEMICAL COMPOSITION OF FRACTIONS OBTAINED BY ISOELECTRIC FOCUSING OF THE CAPSULAR ANTIGEN OF MYCOCOCCUS STRAIN NO. 13557 USING THE AMPHOLYTE RANGE OF pH 6-8

Tube no.	pH	Protein*	%	CHO*	%	DNA*	%	RNA*	%
1**	12.5	10	0.2	13	0.4	24	1.4	0	0.0
2	12.2	70	1.2	40	1.4	24	1.4	14	2.3
3	11.7	105	1.9	20	0.7	24	1.4	0	0.0
4	8.8	30	0.5	25	0.8	31	1.7	0	0.0
5	7.7	80	1.4	30	1.0	36	2.0	0	0.0
6	7.5	189	3.4	20	0.7	166	9.4	0	0.0
7	7.2	390	7.0	55	1.9	52	2.9	0	0.0
8	7.0	145	2.6	70	2.4	48	2.7	0	0.0
9	6.8	45	0.8	67	2.3	40	2.3	9	1.4
10	6.7	0	0.0	67	2.3	177	10.0	10	1.7
11	6.5	180	3.2	87	3.0	57	3.2	16	2.6
12	5.5	835	14.9	162	5.5	52	2.9	41	6.8
13	3.1	1785	31.9	552	18.8	267	15.1	329	54.4
14	2.2	1185	21.1	1050	35.9	52	2.9	185	30.6
15	2.2	0	0.0	380	12.9	48	2.7	94	15.6
16	2.2	0	0.0	0	0.0	0	0.0	0	0.0
17	2.2	0	0.0	0	0.0	0	0.0	0	0.0
18	2.2	0	0.0	0	0.0	0	0.0	0	0.0
19	2.2	0	0.0	0	0.0	0	0.0	0	0.0
TOTAL		5049μg	90.1%	2638μg	90.0%	1098μg	62.0%	698μg	115.4%
AMOUNT APPLIED		5600μg		2925μg		1768μg		604μg	

% RECOVERY

Protein $= \frac{5049}{5600} \times 100\% = 90.0\%$

CHO $= \frac{2638}{2925} \times 100\% = 90.2\%$

DNA $= \frac{1098}{1768} \times 100\% = 62.1\%$

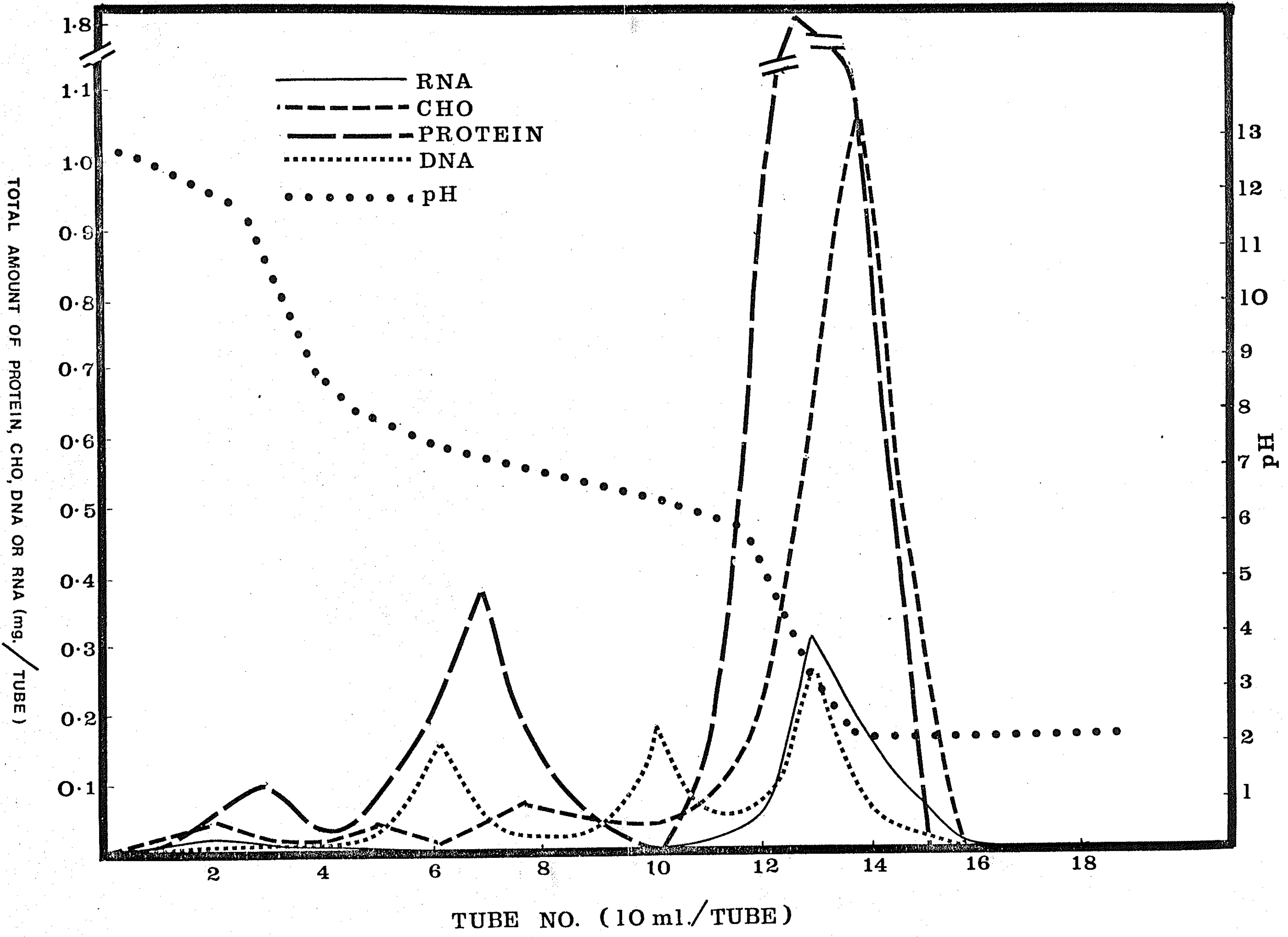
RNA $= \frac{698}{604} \times 100\% = 115.5\%$

* Total amount of individual material (μg.) calculated per tube.

**The fractions (6 ml) collected were dialysed against distilled water, concentrated and made up to 10 ml.

Figure 7

Pattern of polymers detected by isoelectric focusing of the capsular antigen of Mycococcus strain no. 13557 using the ampholyte range of pH 6-8 with cathode at bottom. A sample size of 4 ml of the initial antigen consisting of a dry mass of 15 mg of the total materials was applied to LKB 8101 electrofocusing column.



RNA
CHO
PROTEIN
DNA
pH
TOTAL AMOUNT OF PROTEIN, CHO, DNA OR RNA (mg./TUBE)
TUBE NO. (10 ml./TUBE)
pH

TABLE XVI

COMPOSITION OF PEAKS SEPARATED BY ISOELECTRIC FOCUSING OF MYCOCOCCUS (STRAIN 13557) EXOANTIGEN USING AMPHOLYTE pH 3-6 RANGE.

Tube no.*	pH**	Protein	Carbohydrate	DNA	RNA
2	8.2			+	+
3	7.0	+	+		
4	4.8				+
5	4.1			+	
6	3.8	+			
8	3.6	+			
9	3.5		+		
10	3.3	+		+	+
12	2.4			+	
13	2.3	+			
14	2.2			+	

* Tube number with 6 ml. elution volume, made up to 10 ml. solution after dialysis and concentration.

**The pH of each fraction was measured at 22°C immediately after the elution of column.

TABLE XVII

CHEMICAL COMPOSITION OF FRACTIONS OBTAINED BY ISOELECTRIC FOCUSING OF THE EXOANTIGEN OF MYCOCOCCUS STRAIN NO. 13557 USING THE AMPHOLYTE RANGE OF pH 3-6

Tube no.	pH	Protein*	%	CHO*	%	DNA*	%	RNA*	%
1**	8.5	0.4	0.79	1.2	5.42	0.02	1.12	0.01	1.07
2	8.2	1.5	2.95	2.4	10.66	0.20	11.23	0.62	66.67
3	7.0	16.0	31.49	4.2	18.97	0.06	3.37	0.01	1.07
4	4.85	7.3	14.37	1.4	6.10	0.06	3.37	0.07	7.53
5	4.1	2.0	3.94	1.1	5.15	0.18	10.11	0.02	2.15
6	3.8	8.1	15.94	1.0	4.42	0.05	2.81	0	0
7	3.7	2.5	4.92	0.4	1.99	0.03	1.68	0	0
8	3.6	3.6	7.09	0.3	1.17	0.04	2.24	0	0
9	3.5	1.3	2.55	0.7	3.16	0.06	3.37	0	0
10	3.3	1.5	2.95	0.1	0.54	0.42	23.60	0.05	5.37
11	2.5	0.3	0.59	0.0	0	0.02	1.12	0	0
12	2.4	0	0	0	0	0.14	7.86	0	0
13	2.3	0.3	0.59	0	0	0.03	1.68	0	0
14	2.2	0	0	0	0	0.15	8.43	0	0
15	2.0	0	0	0	0	0.04	2.24	0	0
16	1.9	0	0	0	0	0.02	1.12	0	0
17	1.9	0	0	0	0	0	0	0	0
18	1.9	0	0	0	0	0	0	0	0
TOTAL		44.8 mg	87.2%	12.8mg	57.6%	1.5mg	85.3%	0.78mg	83.9%
AMOUNT APPLIED		50.8 mg		22.1mg		1.8mg		0.93mg	

% RECOVERY

Protein $= \frac{44.8}{50.8} \times 100\% =$ 88.2%

CHO $= \frac{12.8}{22.1} \times 100\% =$ 57.6%

DNA $= \frac{1.5}{1.8} \times 100\% =$ 84.3%

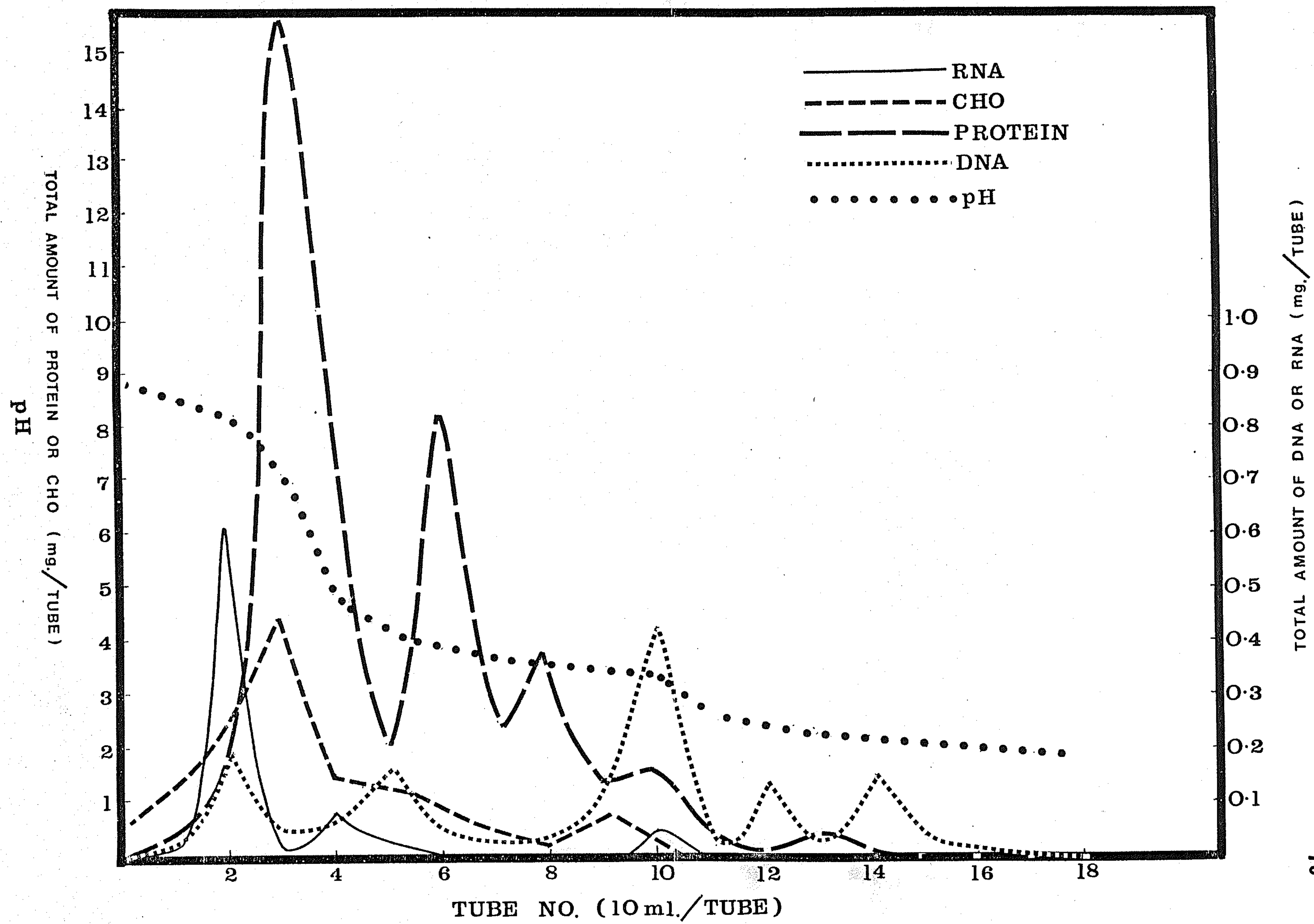
RNA $= \frac{0.78}{0.93} \times 100\% =$ 83.9%

* Total amount of individual material (mg.) calculated per tube.

**The fractions (6 ml) collected were dialysed against distilled water, concentrated and made up to 10 ml.

Figure 8

Pattern of polymers detected by isoelectric focusing of the exoantigen of Mycococcus strain no. 13557 using the ampholyte range of pH 3-6 with cathode at top. A 2 ml sample containing 71.2 mg of the initial exoantigen was applied to LKB 8101 electrofocusing column.


RNA
CHO
PROTEIN
DNA
pH
TOTAL AMOUNT OF PROTEIN OR CHO (mg./TUBE)
pH
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
TOTAL AMOUNT OF DNA OR RNA (mg./TUBE)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2
4
6
8
10
12
14
16
18
TUBE NO. (10 ml./TUBE)

Purification of the macromolecular complex (Fraction I) from the cytoplasm of Mycococcus

Preparative polyacrylamide gel electrophoresis

Fraction I consisting of tubes no. 40 from the previous five runs of electrofocusing at a pH range of 7-10 was pooled. After dialysis and concentration, a sample containing about 50 mg of the material was applied to Shandon's Polyacrylamide Gel Electrophoresis Apparatus for further purification. As shown in Figure 9, Fraction II, which appeared very close to Fraction I in the initial separation, was almost completely separated from the latter. Fraction I containing an effluent volume from 330-370 ml was collected. It was then dialysed and concentrated.

Isoelectric focusing

A pH range of 6-8 was used for the further purification of the complex. As shown in Figure 10, Fraction I was the only peak detected in the sucrose gradient, whereas no Fraction II was present. The isoelectric point of the homogeneous fraction was found to have a pH of 6.40, which was exactly the same as that of the initial electrofocusing at the range of pH 7-10. It should be noted that the different fractions eluted from the column were measured at an absorbance of 280 and 254 nm before dialysis, therefore the first peak (elution volume from 0-10 ml) was only an artifact due to the presence of sucrose and sodium hydroxide. This artifact was absent after the dialysis of the different fractions.

Sephadex G-200 gel filtration

The fraction was finally purified by Sephadex G-200 gel filtration after the dialysis of the preparation of Fraction I. The elution pattern is shown in Figure 11. The macromolecular complex was found to come out almost immediately after the void volume (V_o). Besides measuring the absorbance at 280 and 254 nm, the different fractions were also measured at 625 nm after anthrone test for carbohydrate was performed. The pattern showed that after the different steps of purification, Fraction I still consisted of protein, carbohydrate and nucleic acids. Therefore, it was assumed that these substances were combined quite firmly together in the form of a complex.

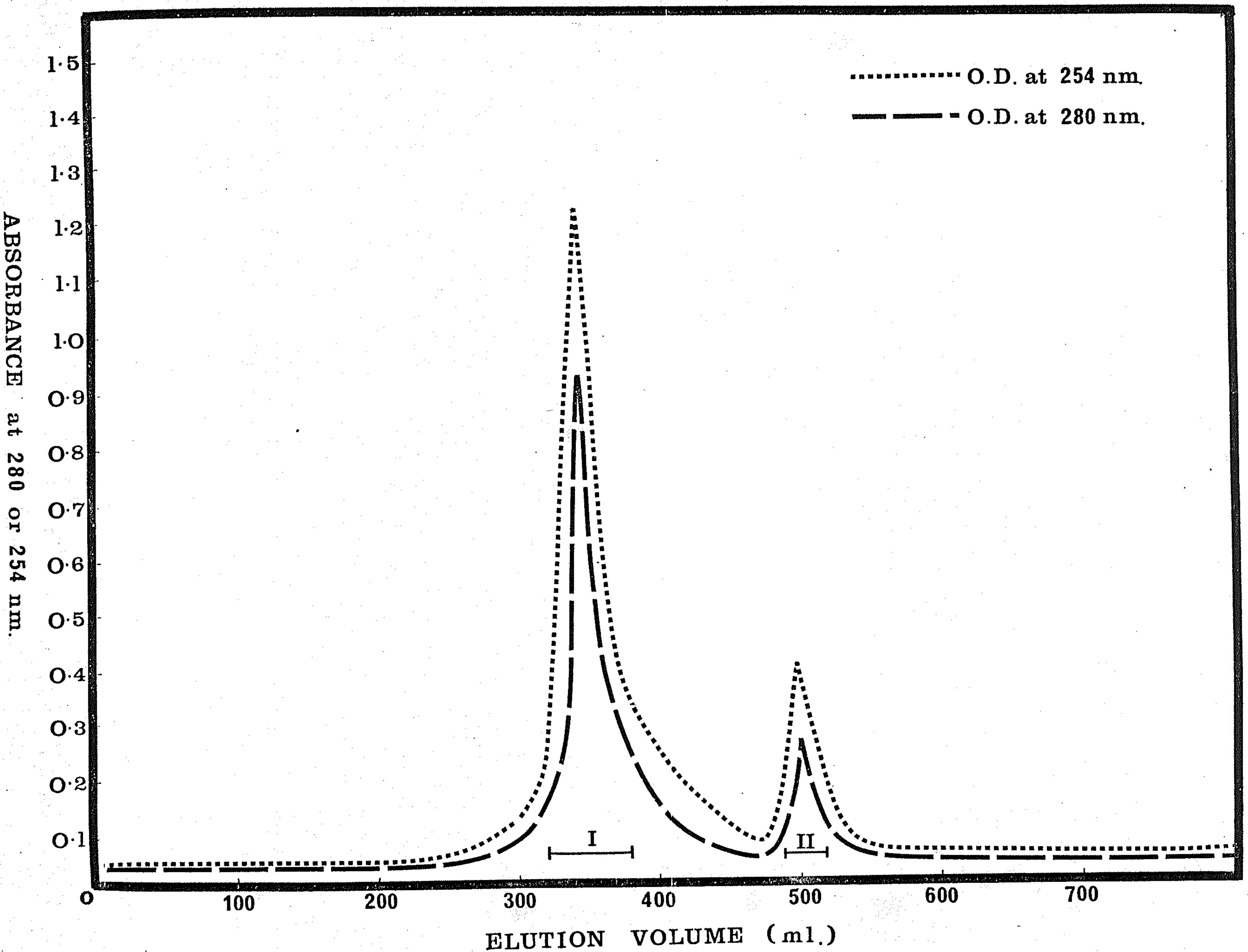
Confirmation of the homogeneity of the complex

A portion of the homogeneous fraction was tested by disc-electrophoresis described under Materials and Methods. The gel slabs were stained by the different staining techniques and the R_s values calculated. Only one band was observed by each staining method, and the R_s value of the individual protein-, DNA-, RNA-, carbohydrate- and phospholipid band was calculated to be 0.83 (Figures 12 and 13).

The ratio of the percentage of protein: RNA: carbohydrate: DNA present in the purified complex was found to be 66: 24: 7: 3, respectively, by different chemical analyses. This ratio was found to coincide quite well with that of the complex before purification, which was determined previously to be 67: 24: 6: 3, respectively. This strongly suggested that the different composing homo-polymers present in the macromolecular complex were bound quite firmly together, and were not separated by the different purification procedures.

Figure 9

Purification of Fraction I of Mycococcus (strain 13556) cytoplasm by Polyacrylamide Gel Electrophoresis using a 7.5% acrylamide gel at pH 9.5 in 6 M urea.



O.D. at 254 nm.
O.D. at 280 nm.
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCE at 280 or 254 nm.
I
II
0
100
200
300
400
500
600
700
ELUTION VOLUME (ml.)

Figure 10

Purification of Fraction I of *Mycococcus* (strain 13556) cytoplasm by the isoelectric focusing technique in ampholyte range pH 6-8 with cathode at bottom.

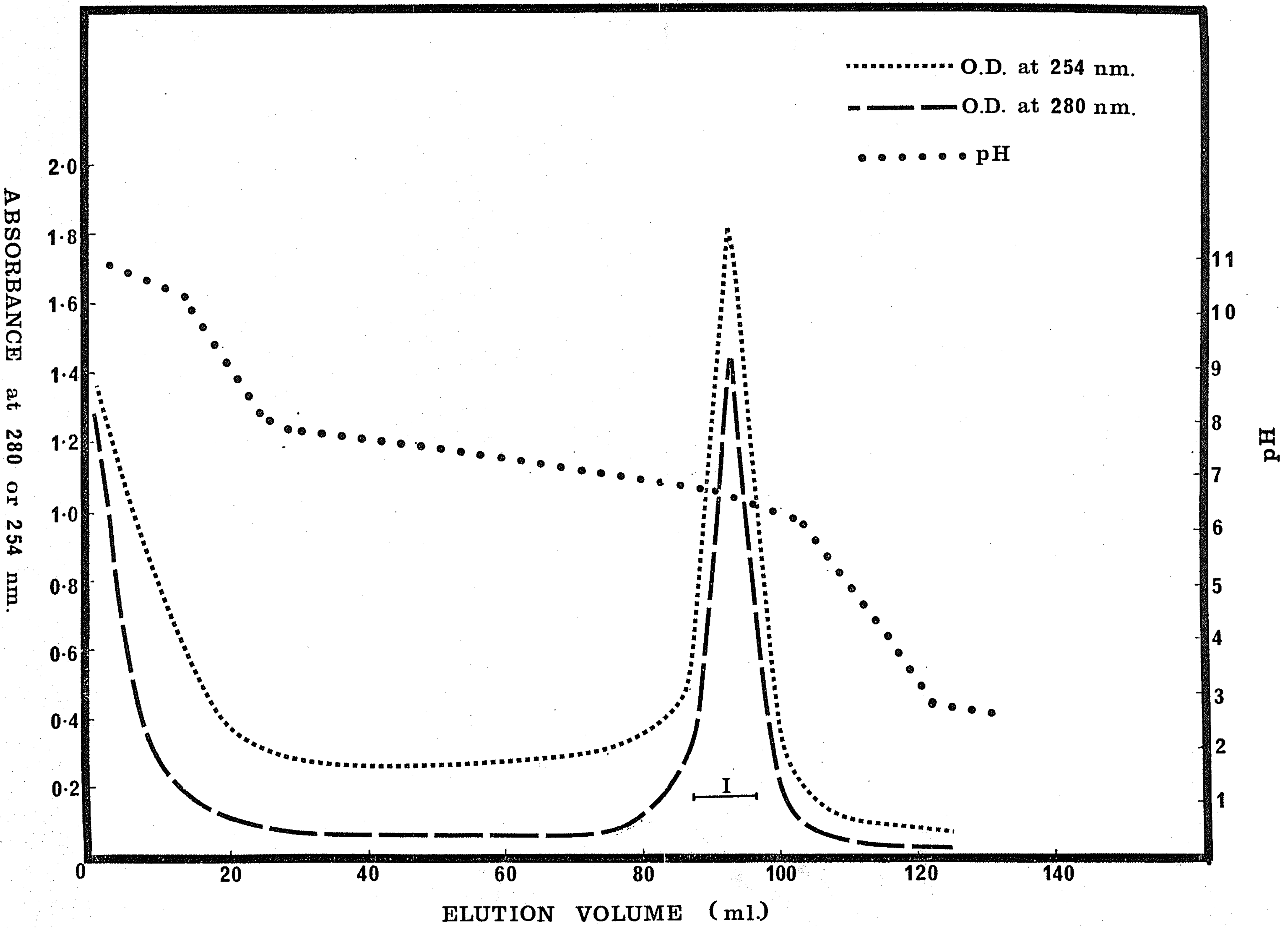
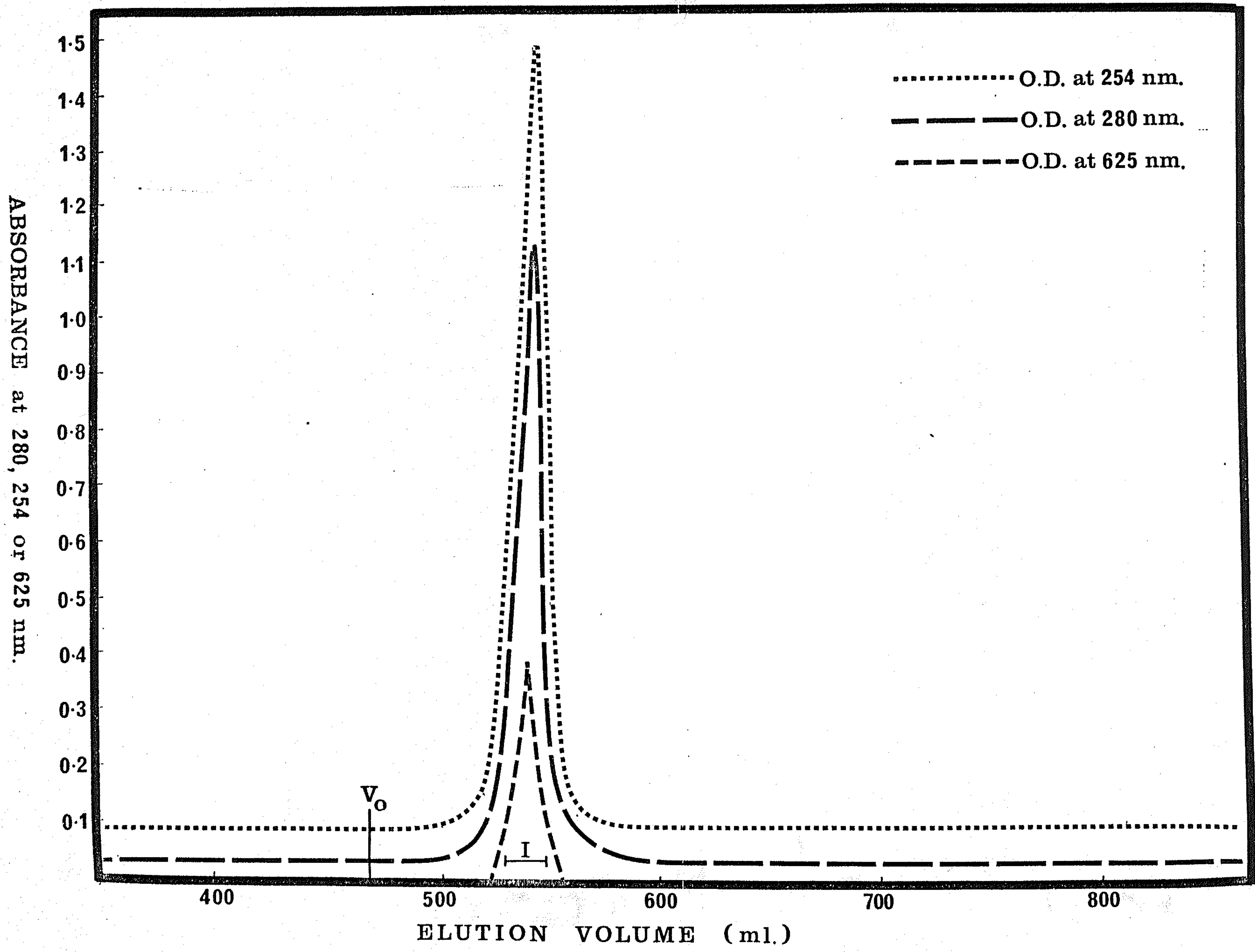

O.D. at 254 nm.
O.D. at 280 nm.
pH
ABSORBANCE at 280 or 254 nm.
ELUTION VOLUME (ml.)
pH
I
0
20
40
60
80
100
120
140
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
1
2
3
4
5
6
7
8
9
10
11

Figure 11

Purification of Fraction I of Mycococcus (strain 13556) cytoplasm by Sephadex G-200 gel filtration. The fractions were scanned at 280 and 254 nm. absorbance; and also at 625 nm. after testing for carbohydrate by the anthrone test.



O.D. at 254 nm.
O.D. at 280 nm.
O.D. at 625 nm.
ABSORBANCE at 280, 254 or 625 nm.
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Vo
I
400
500
600
700
800
ELUTION VOLUME (ml.)

Figure 12

Disc-electrophoresis of the purified macromolecular complex of <u>Mycococcus</u> (strain 13556) cytoplasm on 5% acrylamide gel with anode at bottom. Protein staining revealed only one band with an R_s value of 0.83.

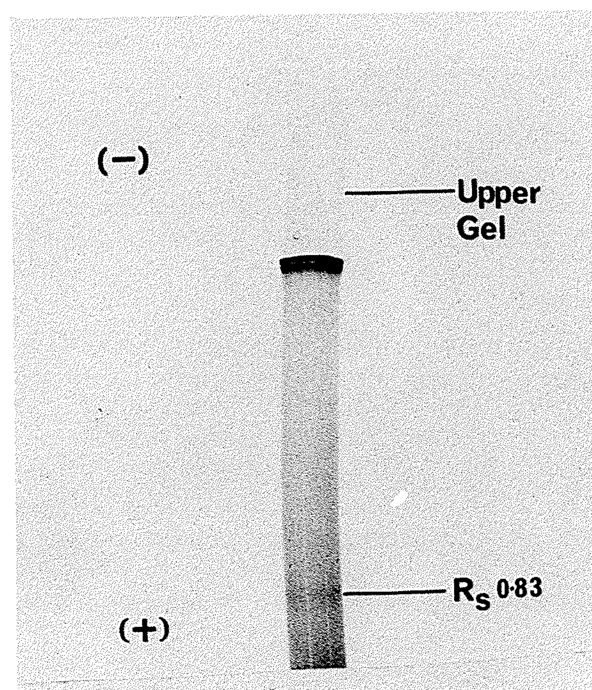
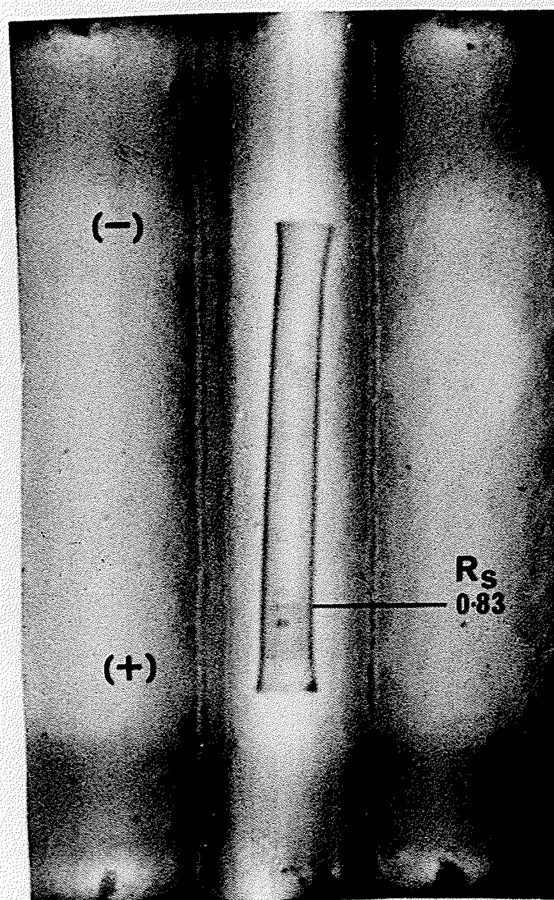

(−)
Upper
Gel
R_S 0·83
(+)

Figure 13

Disc-electrophoresis of the purified macro-molecular complex of Mycococcus (strain 13556) cytoplasm on 5% acrylamide gel with anode at bottom. Staining with alcoholic Rhodamine B revealed only one phospholipid band with an R_s value of 0.83.


(−)
(+)
R_S
0·83

Thin layer chromatography of amino acids

Two-dimensional thin layer chromatography was performed on the individual soluble antigen and also on the purified macromolecular complex obtained from the cytoplasm, after hydrolysis. The two-dimensional chromatograms of the cytoplasmic antigen and the purified complex are shown in Figures 14 and 15, respectively. Leucine and isoleucine could not be separated by this system. There was also a slight overlapping between valine and phenylalanine, but they could be differentiated by the difference in color after spraying with Ninhydrin reagent. Valine showed a pink color, whereas a purple spot was produced by phenylalanine. The amino acids detected in the different soluble antigens and the purified macromolecular complex are presented in Table XVIII.

Thin layer chromatography of saccharides

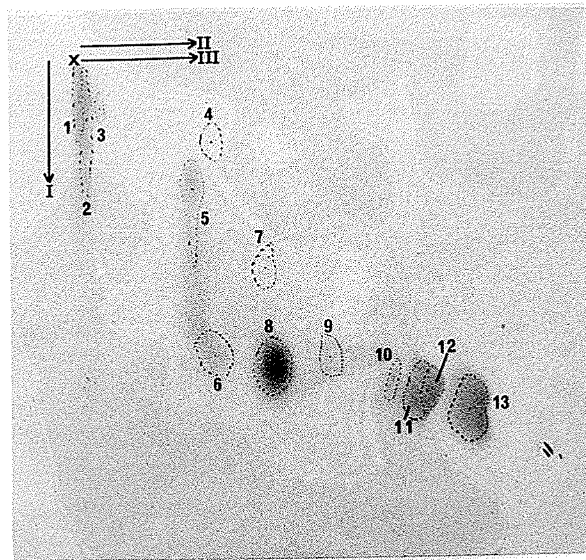
Chromatographic analysis of the cytoplasmic antigen and the main cytoplasmic heteropolymer revealed the presence of galactose. Acetylhexosamine was detected in the capsular antigen, whereas the exoantigen consisted of galactose and arabinose in its polysaccharide component.

Figure 14

Two-dimensional thin layer chromatogram of amino acids of _Mycococcus_ (strain 13556) cytoplasm.

Legend:

1. Arginine 2. Lysine 3. Histidine 4. Serine
5. Glycine 6. Aspartic Acid 7. Glutamic Acid
8. Threonine 9. Proline 10. Alanine 11. Valine
12. Phenylalanine 13. Leucine and/or Isoleucine



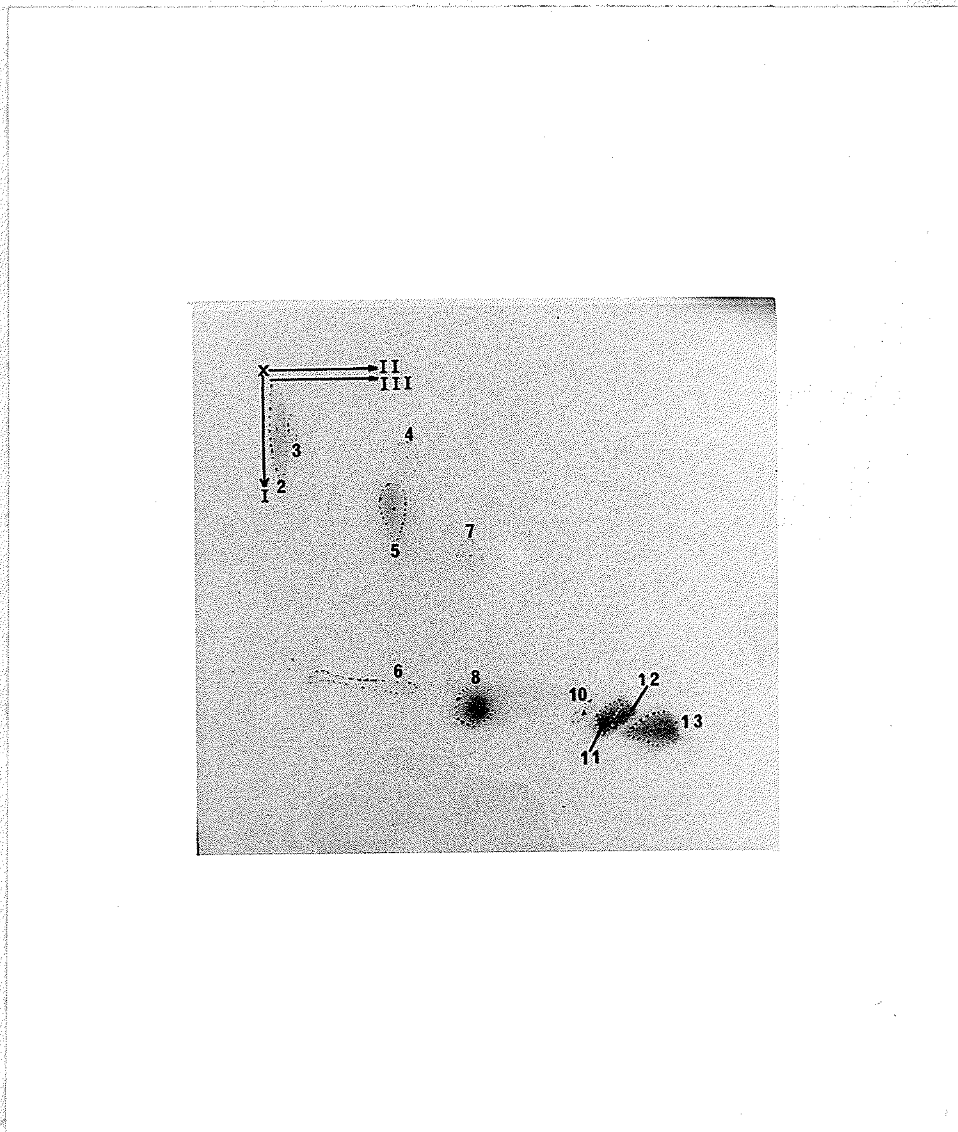
II
III
X
I
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 15

Two-dimensional thin layer chromatogram of amino acids of Mycococcus (strain 13556) macromolecular complex.

Legend:

2. Lysine 3. Histidine 4. Serine 5. Glycine
6. Aspartic Acid 7. Glutamic Acid 8. Threonine
10. Alanine 11. Valine 12. Phenylalanine
13. Leucine and/or Isoleucine



II
III
I
2
3
4
5
6
7
8
10
11
12
13

TABLE XVIII

AMINO ACIDS DETECTED IN THE SOLUBLE ANTIGENS AND IN THE PURIFIED CYTOPLASMIC HETEROPOLYMER OF MYCOCOCCUS BY TWO-DIMENSIONAL THIN LAYER CHROMATOGRAPHY

Amino acid	Cytoplasmic Antigen*		Heteropolymer		Capsular Antigen		Exoantigen	
Arginine	+	+	+	+	-	-	-	-
Lysine	+	+	-	-	+	+	+	+
Histidine	+	+	+	+	-	-	-	-
Glycine	+	+	+	+	+	+	+	+
Serine	+	-	+	-	+	+	+	+
Aspartic acid	+/-	+/-	+/-	+/-	+	+	-	-
Glutamic acid	+	+	+	+	-	-	+	+
Proline	+	+	-	-	+	+	+	+
Alanine	+	+	+	+	-	-	-	-
Cysteine	-	-	-	-	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+
Leucine and/or Isoleucine	+	+	+	+	+	+	+	+
Threonine	+	+	+	+	+	+	+	+

+/- Trace amount

* The first of the two vertical columns of each antigen refers to strain no. 13556, and the second column refers to strain no. 13557.

IV. Serological results

Agar-gel immunodiffusion

Testing of the cytoplasmic antigens against homologous and heterologous antisera revealed that the cytoplasmic preparations contained at least three immunologically active components. After prolonged incubation (5-7 days), a fourth band (closest to the antiserum well) was found separating from the third one. It was shown from Figure 16 that one of the components cross-reacted with the heterologous *Mycococcus* antisera; one component showed a partial immunological identity with the component of the second *Mycococcus* strain, whereas one component was specific for the cytoplasm of a single strain.

Disc-immunoelectrophoresis

This test was performed in order to reveal the antigenicity of the different polymer fractions separated by the disc-electrophoresis experiment. Three components were shown to have immunological activity, as revealed by the precipitation bands observed (Figure 17). By comparison with the pre-stained gel, it was also found that the first band (closest to the bottom) had an R_s value of 0.98, whereas the R_s values of the second and third precipitation bands were 0.83 and 0.77, respectively. The R_s 0.83 antigen was the main macromolecular complex of the cytoplasm, the R_s 0.77 antigen was a galactonucleoprotein, and the R_s 0.98 component was a galactoprotein (Table IX).

Figure 16

Agar-gel immunodiffusion of the cytoplasmic antigens of Mycococcus tested against the anti-Mycococcus cytoplasm serum.

Well I : Cytoplasm of Mycococcus strain 13557.

Well II: Cytoplasm of Mycococcus strain 13556.

Well X : Antiserum against the cytoplasm of Mycococcus strain 13556.

I
II

Figure 17

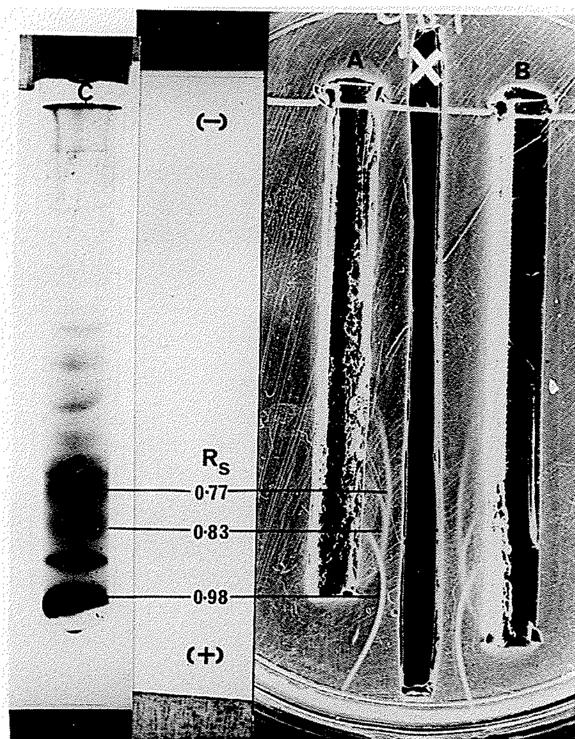
Disc-immunoelectrophoresis of the cytoplasms of *Mycococcus* on 5% acrylamide gel with anode at bottom. Immunodiffusion was carried out after disc-electrophoresis.

A: Unstained gel of the cytoplasm of *Mycococcus* strain 13556.

B: Unstained gel of the cytoplasm of *Mycococcus* strain 13557.

C: Prestained gel of the cytoplasm of *Mycococcus* with 0.1% Amido-black solution.

X: The trough containing antiserum against the cytoplasm of *Mycococcus* strain 13556.


C
(–)
A
B
R_S
0·77
0·83
0·98
(+)

Cellulose acetate immunodiffusion

The cytoplasms of *Mycococcus* were allowed to react with the anti-cytoplasmic sera obtained from selected strains of *Mycobacterium*, *Nocardia*, *Streptomyces*, *Actinomyces* and *Dermatophilus*. It was found that the *Mycococcus* cytoplasms reacted with the anti-cytoplasmic sera of nocardiae belonging to the cytoplasmic serogroup nos. 2, 4, 7, 8, 12, 14 and 20, and with the antiserum as the untypable cytoplasm of strain no. 77 (*Nocardia rangoonensis*).

The cytoplasms of *Mycococcus* were found cross-reactive with most or all of the following *Mycobacterium* anti-cytoplasmic sera: *Mycobacterium fortuitum* (nos. 6841, 9820, 19542, 23043, 23048), *M. borstelense* (nos. 19235, 19237, 23030), *M. peregrinum* (nos. 12670, 23022), *M. scrofulaceum* (nos. 15879, 15081, 23419, 23245, 23431), *M. flavescens* (nos. 14474, 23033, 23395), *M. aquae* (nos. 23283, 23397, 23401, 23422) and with single anti-cytoplasmic sera against nine other scotochromogenic mycobacteria.

The cytoplasms of *Mycococcus*, on the whole, did not react with the anti-*Actinomyces*-, anti-*Streptomyces*- and anti-*Dermatophilus* sera, with the exception of *Dermatophilus* no. 694 and 1758 anti-cytoplasmic sera, both classified in the cytoplasmic serogroup A (Kwapinski and Simmons, 1967).

DISCUSSION

The cultural and biochemical studies on the strains of Mycococcus have revealed that this organism has a similarity to Mycobacterium and Nocardia in respect to the pigment production and the utilization of acetate, succinate, pyruvate and propionate as a sole carbon source.

Chemical composition of the cytoplasm of Mycococcus shows a close similarity to the composition of the cytoplasms of mycobacteria and nocardiae, although the latter two genera contain more polysaccharides and less nucleic acids, whereas the cytoplasm of Mycococcus possesses more nucleic acids than saccharide.

Although a number of polymer fractions had been separated by isoelectric focusing and acrylamide gel electrophoresis, the chief components were mainly in the form of heteropolymers. The relative ratios of protein: RNA: carbohydrate : DNA before and after the purification of the complex had been found to be identical, thus it has been concluded that the macromolecules were firmly bound within the large hetero-complex. The physicochemical analysis of different fractions obtained by isoelectric focusing failed to detect the position of phospholipids because of relatively small amounts of these compounds, but the material separated by disc-electrophoresis from the initial cytoplasmic preparation and the macromolecular complex, stained with alcoholic Rhodamine B, revealed a single phospholipid band. The identical R_s values of these bands thus determined suggest that the phospholipid is one of the components of the main heteropolymer of the cytoplasm. This confirms Kwapinski's et al. (1971) view that the main antigenic complex in the cytoplasm of the Actinomycetales appears in the form of a polysaccharide-nucleoprotein or a phospholipid-polysaccharide-nucleoprotein macro-

molecule, possessing multiple and different determinants.

The two strains of Mycococcus were found identical culturally, morphologically and biochemically. The cytoplasms of the two strains of Mycococcus tested by electrofocusing also revealed identical physicochemical patterns. The only difference found between the two cytoplasmic preparations was the presence of serine in the cytoplasm of strain 13556, and its absence in strain 13557. The amino acids identified in the cytoplasms of Mycococcus showed nine identical amino acids as compared to the cytoplasms of the other members of the Actinomycetales (Kwapinski, 1966 b): alanine, arginine, glycine, glutamic acid, lysine, valine, phenylalanine, leucine and threonine. The cytoplasms of the other Actinomycetales contained two additional amino acids, tyrosine and cysteine, whereas those of Mycococcus contained histidine and proline. The saccharide composition of the cytoplasmic preparations of Mycococcus proved to be different from the saccharide components of the other Actinomycetales. The cytoplasmic antigens of Mycococcus contained only one sugar, identified as galactose. The fact that only Mycobacterium, Nocardia and Actinomyces possess galactose in their cytoplasms but not Dermatophilus, Waksmania, Streptomyces and Thermoactinomyces suggests again that Mycococcus is more closely related to Mycobacterium and Nocardia than to the other genera of the Actinomycetales.

The cytoplasm of Mycococcus was found to possess at least three immunologically active components. One of them was a common antigen among the strains, one showed partial identity, whereas the other one was specific for a given strain. This was confirmed by the appearance of only three bands obtained in the disc-immunoelectrophoresis experiment.

The cross-reactions of the cytoplasm of *Mycococcus* with the anti-cytoplasmic sera obtained from selected strains of *Mycobacterium* and *Nocardia* but not with those of the other genera of the *Actinomycetales* (with the exception of one serogroup of *Dermatophilus*) strongly suggest that *Mycococcus* is closely related to the latter two organisms. The antigenic pattern for the cytoplasm of *Mycococcus*, in terms of the classification scheme of *Nocardia* (Kwapinski *et al*., 1971), has been established as ADGEJN. This is an unusual antigenic pattern, not listed in the original scheme for cytoplasmic groups and types of nocardiae, and it would place *Mycococcus* between the cytoplasmic serogroup 7 and serogroup 8 of the original scheme.

The cytoplasm of *Mycococcus* was found to share the major antigenic components *a* and *b* with the cytoplasmic serogroup no. 2 of scotochromogens, and to contain the antigenic components *e* and *f* occurring in the other cytoplasmic serogroups of scotochromogens distinguished by Kwapinski and Alcasid (1970a).The antigenic component *b*, regarded as one of the main antigenic cytoplasm constituents of the scotochromogens, was also found in some saprophytic mycobacteria of Runyon's group IV (Kwapinski *et al*., 1970b).It appears that through this antigenic component and through another major antigen, detectable with the standard anti-cytoplasmic serum no. 23022, *Mycococcus* is immunologically related to *Mycobacterium fortuitum* and *M. borstelense*.

The cross-reactivity of the cytoplasm of *Mycococcus* with the cytoplasm-antisera of *Dermatophilus* no. 694 and 1758 suggests that the link between *Mycococcus* and *Dermatophilus* is provided by an antigenic component shared by these organisms and by *Nocardia rangoonensis* which is also related to *Dermatophilus* (Kwapinski and Simmons, 1967).

The immunological analysis of the cytoplasmic antigens indicates that the biological position of *Mycococcus* is in the middle between: the "atypical" or higher, slow-growing nocardiae (*N. rangoonensis*, *N. corallina*, *N. blackwellii*, *N. brasiliensis*, *N. caprae*, *N. rubra*), the higher saprophytic mycobacteria (*M. fortuitum*, *M. berolinense*), the lower scotochromogens (*M. flavescens*, *M. scrofulaceum*) and *Dermatophilus*. It is reasonable to assume that *Mycococcus* might have evolved as a progenitor for these four branches of the *Actinomycetales*.

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