THE UNIVERSITY OF MANITOBA

IMMUNOCHEMICAL ANALYSIS OF SELECTED CYTOPLASMIC PROTEINS OF *NEISSERIA GONORRHOEAE*

BY

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ABSTRACT

The cytoplasm of clonal type 1 and type 4 Neisseria gonorrhoeae, obtained after ultrasonication of the bacteria and differential centrifugation, was subjected to analytical disc-electrophoresis and isoelectric focusing. Studied by both methods and by the intravenous toxicity test in 11-day old chicken embryos, the type 4 cytoplasm consisted of ten protein components whereas the type 1 cytoplasm contained eleven proteins of which one of them (β+t) was a unique toxic biopolymer. This toxic protein consisted of 16 amino acids, lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine, occurring in the molar ratios of 4 : 1 : 4 : 9 : 5 : 4 : 11 : 3 : 10 : 10 : 7 : 2 : 5 : 7 : 2 : 3.

After a further purification by the preparative polyacrylamide gel electrophoresis, a highly purified, non-toxic β(-t) protein was obtained. It exclusively consisted of a protein which amounted to 99.0% of its total dry weight. Only 7 amino acids were found to be present, lysine, aspartic acid, threonine, serine, glutamic acid, glycine, and alanine, combined at molar ratios of 1 : 1 : 1 : 3 : 2 : 7 : 1. This β(-t) biopolymer elicited a strong primary and secondary humoral as well as a cell-mediated response in the rabbit. Preliminary studies revealed that the immunity conferred by the detoxified β(-t) biopolymer was effective in the protection of rabbits against infection with Neisseria gonorrhoeae, as examined by injection of live gonococci into the anterior chamber.

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INTRODUCTION

The aim of this research project has been the elucidation of the biological role played by toxic and non-toxic immunogenic biopolymers present in the cytoplasm of Neisseria gonorrhoeae.

This study had been originated from the postulates, proposed in 1969 and published in 1974 (Kwapinski and Cheng, 1974), that (1) the immunogenicity of Neisseria gonorrhoeae depends upon a single immunochemical component present in the interior of the bacterium; (2) that the immunogenicity of this component is normally 'masked' by a co-existing, non-immunogenic, toxic component of the bacterial cell; and (3) that the removal of the latter constituent would free the gonococcal immunogen.

In order to test the above hypotheses, a step-wise approach had been adopted for this research. At the initial stage, an overall analysis of the cytoplasmic components (clonal types 1 and 4) of Neisseria gonorrhoeae had been carried out. This was followed by the separation and purification of immunochemical constituents and by the determination of their antigenic properties. Whenever applicable, the findings derived from the analyses above had been compared with the biologically active materials obtained by different methods and reported by other investigators. This information, gathered from the scientific literature, had been studied and critically evaluated before and during the period of the research. The literature review will be presented under the following headings: anatomical structure, chemical structure, antigenic structure, virulence, and host response.

LITERATURE REVIEW

ANATOMICAL STRUCTURE

The cell of Neisseria gonorrhoeae is composed of a wall, a cytoplasmic membrane with mesosomes of a simple form, and cytoplasm. The cell wall is a gently undulating, triple-layered component overlaying a very thin but dense and taut layer. This latter feature is missing in the large and lysing forms, in the L-forms, and in those gonococcal organisms altered by penicillin (Murray et. al., 1963). These findings have been confirmed by electron microscopy studies (Ovchinnikov et. al., 1968) with the additional demonstration of granules and a ribosomal apparatus within the cytoplasm, a nucleus with deoxyribonucleic acid threads, and mesosomes forming a continuation of the cytoplasmic membrane. Between the external wall and the cytoplasmic membrane there is a granular mass participating in cellular metabolism.

The cells of Neisseria gonorrhoeae divide by a combination of pinching and septation involving a simple type of membranous fold. Mesosomes are present in the cell periphery away from the plane of division. In spite of the separation of daughter cells by the membranous process, the completion of the cell wall often requires more time than membrane development. It is this delay in separation of the already divided cell which accounts for the high proportion of apparently dividing duplex forms seen in cultures of this organism (FitzJames, 1964).

Electron microscopic examination of negatively stained Neisseria gonorrhoeae also reveals that pili, with a diameter of 80-85 Å and a length of 0.5-4.0 μ, are present on the surfaces of types 1 and 2 virulent gonococci but not on avirulent types 3 and 4 (Swanson et. al., 1971; Jephcott et. al., 1971). Freeze-etch and freeze-fracture studies

have confirmed that the pili, of which there are about ten per cell and which may occur anywhere on the surface of the organism, maintain a close anatomical relationship with the cell wall (Swanson et. al., 1972).

Although it has been claimed that Neisseria gonorrhoeae is encapsulated (Israeli, 1921; Almaden, 1938; Bernstein, 1941), this remains controversial. Casper (1972) has argued that the capsule is a carbohydrate which disappears with in vitro cultivation. On the other hand Reyn (1949 c) has not been able to demonstrate capsular swelling, and electron microscopic studies have not shown a capsule (FitzJames, 1964; Ovchinnikov & Delektorskii, 1968). Perhaps the 'capsules' demonstrated by Casper and others are artifacts arising from the fixation or staining processes.

CHEMICAL STRUCTURE

The whole cell of Neisseria gonorrhoeae and its extracellular products have been analyzed chemically but less thoroughly than the endotoxins. Other components of the cell, including cytoplasm and pili, have only been studied with the availability of more sophisticated separation methods.

The Bacterial Cell

Microchemical analyses of six different strains of Neisseria gonorrhoeae grown in Douglas' broth (Stokinger et. al., 1944 a) reveal the dry mass of the gonococcus consists of an average of 60-65% nucleoprotein, 5-9% carbohydrate, and 10-14% lipid. Volatile and non-volatile matter constitutes an additional 13-18%. The protein portion consists of at least two major fractions: a relatively insoluble complex

containing approximately 25% bound lipid, and a minor, lipid-free, nucleoprotein which constitutes the major part of the gonococcal cell. The lipid recovered is highly complex and is separable into several crystalline substances which have been identified as lecithin, cephalin, and sphingomyelin.

By gas chromatography Moss et. al. (1970) and Yamakawa & Ueta (1964) have shown that the gonococcus contains the following major cellular fatty acids: lauric, myristic, palmitic, palmitoleic, hexadecenoic, octadecenoic, beta-hydroxylauric, and beta-hydroxymyristic. Glucose, galactose, and glyceromannoheptose are the major carbohydrates and alanine, methionine, glutamic acid, and phenylalanine occur as the major amino acids.

Neisseria gonorrhoeae elaborates several enzymes. A phosphatase is present in washed lyophilized gonococcal cells from broth cultures, but not in the supernatant (Leahy, Stokinger and Carpenter, 1940). A manometric oxygen uptake study of Neisseria gonorrhoeae (Tonhazy & Pelczar, 1953) in the presence of citric acid cycle compounds has demonstrated the presence of an oxaloacetic acid decarboxylase and a glutamic-aspartic transaminase, but not glutamic-alanine transaminase. The existence of a potent reduced diphosphopyridine nucleotide oxidase and a diphosphopyridine nucleotide-linked lactic dehydrogenase has been detected (Tauber & Russel, 1962) using the Thunberg technique with tetrazolium salt as the electron acceptor. This technique also has been useful in detecting the presence of a cyanide sensitive, aerobic, cysteine oxidase, and a cysteine desulfhydrase in the gonococcus. It has been shown that carbonic anhydrase is an enzyme produced by Neisseria gonorrhoeae (Veitch & Blankenship, 1963; Sanders & Maren, 1967) and that bicarbonate

is a growth requirement for Neisseria gonorrhoeae strains that contain carbonic anhydrase. Furthermore, the anti-bacterial activity of the sulfonamide carbonic anhydrase inhibitors results from their ability to inhibit bacterial carbonic anhydrase, reducing the rate of bicarbonate formation, and thus impeding the hydration of carbon dioxide.

Several investigators have reported on the isolation of different endotoxins from Neisseria gonorrhoeae when the cells are treated with various chemicals, including alkali, phenol-water, and aqueous ether. Extraction with alkali, followed by alcohol precipitation, yields a polysaccharide endotoxin containing 4.2% nitrogen (Miller & Boor, 1934). If cold acetone is incorporated with the alkali, a nucleoprotein endotoxin with a composition of 14.24% nitrogen and 1.08% phosphorus, is produced (Tauber & Garson, 1957). However, if the alkali treatment is followed by ion-exchange chromatography a pure acidic glycoprotein, known as the beta-antigen, is obtained (Apicella, 1973). This antigen has a $S_{20}W$ of 8.55 and contains four major amino acids (alanine, glutamic acid, glycine, and proline). Chemical analysis reveals its composition to consist of 11.2% nitrogen, 61.2-63.7% protein, 27.6-29.8% carbohydrate, and negligible amounts of phosphorus, pentose, methylpentose, KDO, and lipid. It has been suggested that the paucity of aromatic amino acids or the high carbohydrate content may be the cause of a negative color reaction with Folin-Ciocalteau reagent (up to 1 mg/ml antigen). The purified beta-antigen so obtained is sensitive to pronase treatment, whereas periodation and trypsin has little effect on the antigenic determinant. Furthermore, this antigen loses its ability to adsorb directly to erythrocytes as the crude antigen does, probably due to the loss of a portion of the molecule containing a lipid or carbohydrate

moiety; or to the loss of other charged groups. The location of this antigen has not been elucidated. However, it has been suggested that this is not likely a component of endotoxin due to the absence of 2-keto 3-deoxy sugar acids and heptoses.

Phenol-water extraction of Neisseria gonorrhoeae cells, on the other hand, yields a protein-free endotoxin with a nitrogen content of 8.5% consisting of polysaccharide (52%) and nucleic acid (53%) (Tauber & Garson, 1958). In contrast, Maeland and Kristofferson (1971) have isolated a lipopolysaccharide by the phenol-water method containing 35.6% lipid, 13.1% neutral sugars, 9% hexosamine, 4.4% nitrogen, 0.9% phosphorus, 8.1% 2-keto-3-deoxy-octulosonic acid (KDO), and 12.5% protein. This lipopolysaccharide can also be obtained by a hydrolytic extraction with phenol-water mixture, dialysis and differential centrifugation (Tauber & Garson, 1959). This fraction consists of 28.4% lipid, 13.8% hexosamine, 43% saccharide, 3.84% phosphorus, 3.3% nitrogen, and only 0.3% protein. The combination of phenol-water, dialysis and differential centrifugation appears to be superior to using phenol-water alone as less protein contamination is present in the latter procedure. It may be that dialysis removes the degraded protein product from the preparation. Further investigation has shown that the lipopolysaccharide endotoxin is a phosphoric acid ester, as demonstrated by its acidic property, high phosphorus content, and the release of phosphoric acid upon hydrolysis. Paper chromatographic analysis of this lipopolysaccharide endotoxin shows the presence of glutamic acid, glycine, alanine, ethanolamine, phosphoethanolamine, and trace amounts of α, ε-diaminopimelic acid as amino acids; D-glucosamine, glucose, and galactose occur as the only carbohydrate (Tauber & Russel, 1961).

In contrast to the production of a lipopolysaccharide endotoxin by extraction with phenol-water, aqueous ether-extracted endotoxin consists mainly of protein, and much less lipid and polysaccharide. Chemical analysis of this endotoxin reveals 82-88% protein, 1.4-2.1% neutral sugars, 0.7-1.1% hexosamine, 2.6-6.3% lipid, 12.6-14% nitrogen, and 0.3-0.4% phosphorus. This protein fraction contains all the commonly occurring amino acids, except cysteine, cystine, and diaminopimelic acid. Among the 16 amino acids detected, lysine, arginine, aspartic acid, glutamic acid, alanine, valine, and leucine appear to be the major components. Since diaminopimelic acid is absent, it would indicate that mucopeptide linkage is not present in the endotoxin preparation (Maeland, 1969 b).

The reason for the differences in chemical composition between the various endotoxin preparations is still largely unexplained. It may be that different chemicals attack different parts or layers of the outer cell structure of the gonococcus thus releasing endotoxins of different chemical structure. The chemical composition of the endotoxins extracted from Neisseria gonorrhoeae by the methods detailed above are summarized in Table I.

Cytoplasm

Fractionation of the gonococcal cells into cytoplasm and cell wall components by Ribi's pressure cell fractionator produces two endotoxins (Martin et. al., 1969), one derived from the cell wall, the other from the cytoplasm. The cytoplasmic endotoxin may be recovered as Fraction I by the Sephadex G-200 filtration method (Peacock & Schmale, 1969), and as the 0.4M fraction by the ion-exchange chromatography method (Schmale et. al.,

TABLE I

CHEMICAL COMPOSITION OF ORGANIC MATERIALS OF
NEISSERIA GONORRHOEAE ENDOTOXINS

Method of Extraction	Major Chemical Entity	Percentage of Organic Materials						Author
		Protein	Lipid	Carbohydrate	Nucleic Acid	N_2	P_2	
Phenol-water	Polysaccharide and nucleic acid	*	*	52	53	8.5	*	Tauber & Garson (1958)
Phenol-water	Lipopolysaccharide	12.5	35.6	22.1	*	4.4	0.9	Maeland & Kristofferson (1971)
Phenol-water, dialysis & differential centrifugation	Lipopolysaccharide	0.3	28.4	57	*	3.3	3.8	Tauber & Garson (1959)
Alkali + alcohol	Polysaccharide	*	*	*	*	4.2	*	Miller & Boor (1934)
Alkali + cold acetone	Nucleoprotein	*	*	*	*	14.2	1.08	Tauber & Garson (1957)
Alkali + ion-exchange chromatography	Glycoprotein	62.5	*	28.7	*	11.2	*	Apicella & Allen (1973)
Aqueous ether	Protein	85	4.5	2.7	*	13.4	0.35	Maeland (1969 b)

* Not reported

1969). It has been suggested that the cytoplasmic endotoxin has a different chemical composition from that in the cell wall as it is insoluble in the aqueous phase of the phenol extract although the cell wall endotoxin is soluble in this fraction; and that the aqueous phase of the phenol extract of the cell wall contains a lipopolysaccharide. However, no chemical analysis has been reported.

Pili

Purified pili have a molecular weight of 24,000 and a pI of 4.0. Sensitivity of pili to trypsin and inhibition of pilus formation by an inhibitor of protein synthesis prove their protein nature (Buchanan, 1972 and 1973).

Extracellular Materials

Gas chromatography of the fatty acids found in whole culture extracts of 3 strains of Neisseria gonorrhoeae shows that they are a homogeneous group. They can be distinguished from Neisseria meningitidis on the basis of production of acetic acid, failure to use propionic acid, and production of only a moderate amount of alpha-keto-isocaproic acid (Brooks et. al., 1971). This confirms Barron and Miller's (1932) and Barron's (1936) findings that Neisseria gonorrhoeae oxidizes pyruvate to acetate, and Tonhazy and Pelczar's (1953) finding that the gonococcus is unable to oxidize propionate.

The culture filtrate of Neisseria gonorrhoeae, purified by precipitation with ammonium sulphate, appears to contain a protein degradation product derived from the nucleoprotein of the cells. It consists of 15.7-16% total nitrogen, 0.70% amino nitrogen, 0.13% total phosphorus, 0.81% total sulphur, and approximately 2.8% carbohydrate

(Stokinger et. al., 1944 a). The virulent type 1 gonococcus grown on supplemented Frantz medium, extracted first with EDTA (ethylenediaminetetraacetic acid) and then with aqueous ether, yields a fraction which is devoid of endotoxic activity and produces 3-5 immunoprecipitation bands with the homologus antiserum (Foster et. al., 1973). Pronase and trypsin treatments eliminate 4 and 2 bands, respectively, whereas RNase and lipase have no apparent effect. The chemical composition of this fraction is 34.6% protein, 3.5% carbohydrate, 20% lipid, and 1-4% RNA. A similar preparation from type 4 Neisseria gonorrhoeae contains less protein and more carbohydrate (18.3%). Whether this difference in chemical composition is associated with the basic virulence characteristic of types 1 and 4 gonococci is still unknown.

ANTIGENIC STRUCTURE

Neisseria gonorrhoeae contains a number of antigens: some are located on the surface, some in the cytoplasm, while others are obtainable only through chemical extraction.

Surface Antigens

The first evidence of the existence of surface antigens is Chanarin's (1954) report that preparations obtained from freshly isolated strains of the gonococcus are adsorbed by red blood cells thus rendering them agglutinable by specific antibodies. This phenomenon as suggested by Chanarin, is due to a polysaccharide complex present on the surface of the smooth phase gonococcus which is lost when it undergoes the smooth-rough change.

Investigations, using a direct fluorescent staining and agglutination

test, have shown that these surface antigens prevent agglutination by acting as a protective cover for the underlying, thermostable, somatic antigens (Deacon et. al., 1961). These surface antigens appear to be species specific; possess characteristics similar to the Vi antigen of Salmonella typhi or the K antigens of the Escherichia group; develop fully only in freshly isolated cultures of infections exudates; are readily lost on subculture, and are destroyed by heating at 120 ^{o}C, but preserved by formalin treatment.

The presence of surface antigens has also been demonstrated by mixed agglutination, which detects only antigens on the cell surface and not those present inside the cell, and by hemagglutination tests (Maeland, 1967). However, the relationship of the surface K antigen to the erythrocyte-sensitizing antigen has not been investigated.

Investigation of the pili found on the surfaces of Kellogg's types 1 and 2 Neisseria gonorrhoeae (Buchanan et. al., 1972) has demonstrated that both types of pili are similar immunogenically and functionally. Purified pili and piliated type 1 gonococci also are found to stimulate the formation of antibodies in rabbits which: (1) precipitate purified pili in immunodiffusion, (2) react with piliated gonococci in the indirect fluorescent antibody technique, (3) inhibit the adherence of piliated gonococci to both erythrocytes and epithelial cells, (4) opsonize piliated gonococci. Furthermore, the purified pili can detect anti-pilus antibodies in convalescent human sera and in chimpanzees experimentally infected with gonococci.

Antigens Extracted with Chemicals

Tulloch (1922) first reported that strains of Neisseria gonorrhoeae

which are inagglutinable by an antiserum may still absorb agglutinins from that antiserum, and Nicolle (1919) that inagglutinable strains become agglutinable following acidulation and heating at 100 °C for 15 minutes. An antigen may be prepared by treating the gonococcus culture with alkali and precipitating the protein with acid. Unfortunately, the majority of gonococcal strains treated in this manner yield antigens which are either too highly anti-complementary and so produce false positive reactions, or may lack in breadth of antigenic valence and so give false negative results (Price, 1930, 1932, and 1933). Despite these findings, it has been postulated that Neisseria gonorrhoeae possesses both group- and type-specific antigens; and it has been suggested that the difficulties can be overcome by a lengthy and laborious procedure of selecting combinations of strains which have wide antigenic valence, but at the same time, are low in anti-complementary properties (Torrey, 1940).

With Chanarin's method (1954) of extraction by alkaline hydrolysis, two antigens have been demonstrated which appear to be type-specific. One of these is active in the gonococcal hemolysis test and behaves as a simple hapten, failing to react in the precipitin test and not fixing complement in the presence of an immune serum, but is able to adsorb onto red blood cells rendering them agglutinable by the specific antibodies. The second antigen appears to be primarily responsible for complement fixation.

Extraction of Neisseria gonorrhoeae with pyridine and subsequent ultrasonic treatment of the washed sediment produces a highly potent, genus specific, and stable antigen. This antigen is free of anti-complementary properties, possesses a broad antigenic valence, and has

a long range of working antigenic power (Labzoffsky & Kelen, 1961). The elimination of the lengthy and laborious procedure of strain selection makes it simpler and more practical than Torrey's modification of the Price method. It has been reported that Neisseria gonorrhoeae contains a variable combination of group-specific and type-specific antigens, present either in overt or, very frequently, masked form (Wilson, 1954); it would seem that pyridine treatment results in the unmasking of some of the latent antigenic factors and consequently in enhancing the potency and breadth of valence of the antigen.

A lipopolysaccharide antigen can be isolated from Neisseria gonorrhoeae by treatment with absolute alcohol, sodium acetate and acetic acid (Chacko & Nair, 1969), or with trichloroacetic acid (Boor & Miller, 1944). Both preparations appear to be non-specific, as is demonstrated by their cross-reactivity with the sera from patients suffering from meningococcal infection. However, hydrolysis of the trichloroacetic acid-extracted lipopolysaccharide removes the lipid component, revealing a carbohydrate fraction. This carbohydrate appears to be species-specific since it distinguishes the gonococcus from the meningococcus (Boor & Miller, 1944).

Extraction of freshly isolated strains of the gonococcus by repeated alcohol precipitation to remove water-insoluble protein yields two type-specific carbohydrates. These have been postulated to correlate with the two predominant types of Neisseria gonorrhoeae by a comparative agglutination test (Casper, 1937 a & b). However, other attempts to isolate these type-specific carbohydrates from Neisseria gonorrhoeae have failed (Miller & Boor, 1934; Stokinger & Carpenter, 1944 c).

These early, above-cited observations have provided certain

information on gonococcal antigens but Maeland's extensive studies (1966, 1967, 1969 a-d, and 1971) give more information about their distinctive characters. The treatment of Neisseria gonorrhoeae with aqueous ether, phenol-water, trichloroacetic acid, alkali, heat or by hemagglutination inhibition and absorption tests reveal two antigenic determinants. a and b (Maeland, 1968). Phenol-water endotoxin contains determinant a, while the other preparations contain both. Determinant a is polysaccharide in nature as shown by its resistance to digestion by proteolytic enzymes and its sensitivity to periodate oxidation. Determinant b appears to be a protein since it is resistant to the action of periodate and is destroyed by digestion with papain and pronase.

Experiments with aqueous ether preparations have provided evidence that both determinants a and b are constituents of the endotoxin complex, which is believed to be a lipopolysaccharide protein complex (Maeland, 1969 a). This complex contains a group-reactive, antigenic, determinant of protein nature, determinant b, and a serologically multi-specific, carbohydrate, component, determinant a, (Maeland, 1969 d). By means of an indirect hemagglutination technique, six factors have been demonstrated within determinant a. One of these factors appears to be common to all strains of Neisseria gonorrhoeae whereas the others occur in different combinations.

Further investigations have shown that galactose and alpha- and beta-lactose block the combination of three, a_2, a_5, and a_6, of the a-factors with their corresponding antibodies; thus it would appear that galactose is in the terminal position in the structures responsible for these particular factors. It is believed that the position of the linkage between glucose and galactose is of importance to the specificity since lactose

(alpha- and beta-galactosyl-1-4-glucose) inhibits whereas melibiose (alpha-galactoxyl-1-6-glucose) does not (Maeland, 1971 a & b). With respect to the a-determinant, the phenol-water preparation is serologically more active than the corresponding aqueous ether preparation, therefore it has been suggested that the endotoxin extracted by phenol-water is better suited for immunochemical analyses of the carbohydrate component of gonococcal endotoxin than the aqueous ether preparation.

The phenol-water extracted endotoxin does not appear to be homogeneous. Instead, two distinct antigenic populations are present, as has been shown by ion-exchange chromatography and immunodiffusion (Apicella, 1974). Finally, these antigens are immunogenic in rabbits; negatively charged; distributed in a diffuse band in pH 4-4.5 range by electrofocusing in acrylamide gels; carbohydrate in nature; free of protein and nucleic acids; and resistant to proteolytic enzymes but sensitive to periodation.

The properties of the various gonococcal antigens obtained through chemical extractions, and the different assay systems used, are summarized in Table II.

Cytoplasmic Antigens

It is now known that the cytoplasm of Neisseria gonorrhoeae is associated with antigenic reactivity as demonstrated by its reaction in complement fixation and gel-diffusion tests with sera from gonorrheal patients (Danielsson et. al., 1969). The cytoplasm does not possess cross-reactivity with anti-pneumococcal hyperimmune sera, but it does give a weak reaction with hyperimmune antisera to groups A and C meningococci. The purified β antigen, obtained by ion-exchange chromatography of alkaline-extracted gonococcal endotoxin, does not react

TABLE II

COMPARISON OF DIFFERENT GONOCOCCAL ANTIGENS
PREPARED BY CHEMICAL EXTRACTIONS

Method of Extraction	Properties of Antigen	Reaction Responsible	Author
Pyridine + Ultrasonication	Highly potent; Genus specific	Complement Fixation	Labzoffsky & Kelen (1961)
Absolute alcohol + sodium acetate + acetic acid	Useful in detecting asymptomatic female carriers; cross-reacts with meningococcus	Microprecipitation	Chacko & Nair (1969)
Repeated alcohol precipitation	Type-specific carbohydrate	Comparative agglutination	Casper (1937)
Trichloroacetic acid	Non-specific glucolipid	Precipitation	Boor & Miller (1944)
Trichloroacetic acid + hydrolysis	Species-specific carbohydrate	Precipitation	Boor & Miller (1944)
Phenol-water	Multi-specific carbohydrate (determinant a) with 6 (a) factors	Hemagglutination inhibition & absorption	Maeland (1968, 1969 a,d)
Phenol-water + ion-exchange chromatography	Subgroup-specific carbohydrate	Immunodiffusion	Apicella (1974)
Alkaline hydrolysis	Type-specific	Hemagglutination & complement fixation	Chanarin (1954)
Aqueous ether	Group-specific protein (determinant b)	Hemagglutination & inhibition & absorption	Maeland (1968, 1969 a,d)

with group-specific meningococcal hyperimmune sera but does react weakly with anti-pneumococcal types III, IV, and VIII sera (Apicella, 1973). The cytoplasm is also known to possess a number of antigens as illustrated by the several precipitation lines formed with anti-gonococcal serum in gel-diffusion (Danielsson, 1969) and counterimmunoelectrophoresis (Hoffman, 1974).

Some of the cytoplasmic antigens have been shown to elicit both humoral and cell-mediated responses and have been used in immunodiagnosis. Two antigens, 'A' and 'B', present in the cytoplasm, are responsible for the humoral response. The 'A' antigen is present in the supernatant of the cytoplasm, whereas the 'B' antigen is found in the sediment. The 'A' antigen may be partially separated from the other antigens by passing the supernatant of the cytoplasm through Sephadex G-200 (Danielsson et. al., 1969) and ion-exchange chromatography (Schmale et. al., 1969) where it locates in the first peak and the 0.4M peak, respectively. The 'A' antigen appears to be important in the complement fixation procedure when tested with human sera (Reising et. al., 1969), and is unrelated to the various cytoplasmic antigens employed by other investigators (Reising & Kellogg, 1965; Nair & Chacko, 1966).

The 'B' antigen, on the other hand, may be obtained from the sediment of 1.0M NaCl, 0.1M tris (hydroxymethyl) aminomethane, pH 8.0, buffer-treated cytoplasm (Lee & Schmale, 1970). In contrast to the 'A' antigen, the 'B' antigen appears to be important in a flocculation procedure.

Two antigens, 'a' and 'b', also have been described which apparently are associated with the humoral response of patients infected with Neisseria gonorrhoeae (Danielsson et. al., 1972). A fast-moving antigen

with a negative charge, designated antigen 'a' is responsible for the reaction in the immunoelectrophoresis test with patients' sera. Another antigen, designated antigen 'b', detects antibodies in the sera of patients with gonococcal septicemia. The relationship between these two antigens 'a' and 'b' and the 'A' and 'B' antigens described in the preceding paragraphs, has not been investigated.

Besides eliciting a humoral response, the cytoplasm has also the ability to produce a cellular response in patients with gonococcal infection, as demonstrated by lymphocyte transformation (Kraus et. al., 1970). The cytoplasmic antigens responsible for this phenomenon can be obtained by alkali extraction (Kearns et. al., 1973). They may also be separated by DE-52 ion-exchange chromatography (Esquenazi & Streitfeld, 1973). The bulk of the mitogenic activities is associated with eluates of 0.05 and 0.2M NaCl. However, whether or not these antigens are related to those responsible for humoral response, such as the 'A' antigen obtained from 0.4M NaCl by ion-exchange chromatography, is presently unknown.

Antigenic Groups and Types

Little progress has been made in the knowledge of the antigenic structure of the gonococcus. There is no unanimity of opinion as to whether antigenic variants exist within the group, or whether all strains are serologically identical.

The theory of the antigenic diversity of Neisseria gonorrhoeae was first advanced following studies of the agglutination and precipitation reactions in anti-gonococcal serum (Torrey, 1907) and later of the complement fixation test (Teaque & Torrey, 1907). The consensus

is that no distinction may be drawn between gonococcal strains by simple complement fixation or agglutination tests. Eight strains of Neisseria gonorrhoeae, obtained from patients, have only been separated into two different groups by means of the complement fixation test (Watabiki, 1910), whereas classification into adult and infant types has been reported by agglutination and complement fixation methods (Pearce, 1915). These two types are not well defined, as they only correspond to adult male urethritis and vulvovaginitis in small girls, respectively. Consequently it has been postulated that no distinct differences exist among the various strains of Neisseria gonorrhoeae, but only comparative differences; that the serological relationships within the group are complex; and that there exist all manner of intermediate forms and variations.

Further evidence to support this hypothesis has been: Torrey and Buckell's (1922) finding that no distinct serological types exist but only three ill-defined groups among 77 strains; Stokinger's et. al. (1944 c) observation that no distinct differences exist among 9 strains; and finally, Segawa's (1932) finding of no serologically distinct types by the agglutinin-absorption method among the 64 strains investigated by him.

However, other evidence suggests that the gonococci are a collection of organisms that fall into distinct, clear-cut, immunological types which have little relationship with each other. Even then, inconsistent findings as to the number of groups or types have been obtained by various researchers. Thus investigation of 27 strains of Neisseria gonorrhoeae by agglutination has revealed 4 serological subgroups, designated as A, B, C, and D (Jötten, 1920). Similar results

have been obtained by means of the complement fixation test (Thomsen & Vollmond, 1921; Uroma, 1943). Further evidence from agglutination and absorption studies have separated 85 strains of Neisseria gonorrhoeae into six very distinct serological types (Hermanies, 1921); 100 strains into five different groups (Tulloch, 1922); 200 strains into three distinct types (Vollmond, 1923); and finally, 60 strains into nine well-defined types (Reyn, 1949 c).

The investigations by Glynn and Ward (1970) of 60 strains of Neisseria gonorrhoeae showed that 4 main groups can be recognized by virture of their resistance to killing by human complement, together with either normal human or immune rabbit sera. Since bactericidal antibodies can be adsorbed from both normal and immune sera by autoclaved or trypsinized Neisseria gonorrhoeae, and by red cells coated with gonococcal lipopolysaccharide (LPS), it would appear that the antigens involved in the bactericidal reaction are LPS of several distinct specificities, and that surface blocking antigens may restrict access to the LPS in the intact organisms.

Colonial morphology has also been used as a criterion in the serological classification of gonococci (Atkin, 1925). Two morphologically different colony types have been observed when Neisseria gonorrhoeae is grown upon 'Trypagar' plates (alkaline pea-broth agar in a semi-solid state), which correspond to the two main serological groups. The Type I colony is large, irregularly spherical, flattened, transparent, and with papillae forming on its surface. The Type II colony is smaller, rounder, more convex, thicker, and without papillae. The colonies of each type are apparently different in chemical composition and possess serologically different soluble antigens, designated as antigens I and II, respectively.

Furthermore, strains isolated from cases of acute gonorrhea usually belong to serologic type I, whereas strains recovered from chronic cases of gonorrhea generally belong to type II. These two morphological types appear to be different from the four colonial types described by Kellogg et. al. (1963). Kellogg's type 1 and type 4 colonies fit into the morphological descriptions of Atkin's Type II and Type I cells, respectively. However, there is controversy as to the origin of these types. For example, Atkin's Type I cells arise mainly from acute cases of gonorrhea, whereas Kellogg's type 4 colonies are found mainly in old laboratory cultures. The four types described by Kellogg have been associated with virulence: types 1 and 2 being virulent and types 3 and 4 avirulent for human volunteers. The relationship between Atkin's two morphological types and virulence has not been investigated.

According to our present knowledge, Neisseria gonorrhoeae possesses a variable number of both group- and type-specific antigens. Some specific antigens are thermostable while some are thermolabile (Wilson, 1954; Reyn, 1949 a & c). By means of complement fixation and agglutination reactions, it has been shown that strains of Neisseria gonorrhoeae contain eight thermostable antigens, designated as A through G. Antigens A, B_1, B_2, and C behave as group antigens, whereas antigens D, E, F, and G appear to be type-specific. A strain may possess all four group-antigens and one of the type-specific antigens. It has been found also that besides these thermostable antigens, there is a thermostable species antigen common to all strains of Neisseria gonorrhoeae (Reyn, 1949 a). Both heat-labile and heat-stable antigenic factors take part in the fluorescent antibody staining and agar gel precipitation reactions, and there is a close relationship between different gonococcal strains.

Absorption experiments also indicate the occurrence of strain-specific antigenic factors (Danielsson, 1965 a). Results obtained by means of an indirect fluorescent antibody procedure (O'Reilly, Welch and Kellogg, 1973) have confirmed that Neisseria gonorrhoeae possesses thermostable species specific antigens similar to those previously described (Reyn, 1949 a; Wilson, 1954). The existence of strains of broadly representative antigenicity is also supported by previous findings that among the different serological groups and types classified, the majority of the strains tested usually fall into a single serological group (Hermanies, 1921; Tulloch, 1922; Uroma, 1943; Reyn, 1949 b; Wilson, 1954; Glynn & Ward, 1970).

As a whole, the information obtained from serological grouping and typing of Neisseria gonorrhoeae is confusing. The reason for the inconsistency of results may be the instability of the organisms and the use of rough strains. It is known that Neisseria gonorrhoeae, on first isolation from an acute case of gonorrhea, is 'smooth' and often inagglutinable; but when such a strain is subcultured, a reversible loss of various constituents of the organism can occur (Wilson, 1954). If the factor responsible for inagglutinability is lost and sufficient 'smooth' antigen remains, then the strain will change from the inagglutinable to the normal agglutinable state. It subsequently undergoes phase changes or becomes autoagglutinable (hyperagglutinable) only when a further loss of 'smooth' antigen occurs. Since the majority of previous workers have used strains which have been under cultivation for considerable periods, it seems probable that many of these strains are in the 'rough' phase, thus giving rise to the non-specific reactions and the inconsistent results.

The results obtained from the serological grouping and typing of Neisseria gonorrhoeae are summarized in Table III.

Immunodiagnosis

A good serological test is urgently needed for the diagnosis of gonorrhea, particularly in women in whom cultural methods are difficult. Such a test will also be important in epidemiological control of the disease where technical and psychological difficulties make routine cultural investigations impracticable.

Despite the increased attention to the study of the humoral immune response to gonococcal infection, there are problems. The complement fixation test was first used to detect gonococcal antibody in 1930 (Price, 1930), and has been used routinely since. However, after the introduction of the sulfonamide and penicillin treatments of gonorrhea, the use of this test decreased. Since the introduction of the complement fixation test several new serological tests for gonorrhea have been described, for example, immunofluorescence, hemagglutination, and precipitation, but none has come into routine use. Major difficulties with these tests include antigenic impurities with lack of both specificity and sensitivity, and test systems which are incapable of reproduceably detecting small differences in antibody levels between patients and controls. Following are a few examples which illustrate the different test systems and the complexity of gonococcal antigens employed for immunodiagnostic analyses during the past years.

When heated, whole organisms are used as antigen in a complement fixation test, positive results are obtained in 34% of females and 18% of males in uncomplicated gonorrhea, 41% of females in complicated cases,

TABLE III

SEROLOGICAL DIFFERENTIATION OF
NEISSERIA GONORRHOEAE

Number of strains tested	Method	Groups or types reported	Author
27	agglutination	4 subgroups	Jötten (1920)
100	agglutination and absorption	5 groups	Tulloch (1922)
85	agglutination and absorption	6 types	Hermanies (1921)
200	agglutination and absorption	3 types	Vollmond (1923)
60	cross-absorption	9 types	Reyn (1949)
26	complement fixation	4 groups	Thomsen & Vollmond (1921)
30	complement fixation	4 types	Uroma (1943)
60	resistance to bactericidal antibodies	4 groups	Glynn & Ward (1970)

and 2.5-6.5% of the control group (Ratnatunga, 1971). The same system when employed by other investigators detects seropositivity rates in 50% of females and 21% of males in uncomplicated gonorrhea, 65% in complicated cases, and 6% of controls (Magnusson & Kjellander, 1965); in 35-40% of females, 20-25% of males and 5% of the control group (Danielsson et. al., 1972); and in 31% of female patients with uncomplicated gonorrhea and 10% of normal controls (Rodas and Ronald, 1974). An antigen 'A', of unknown chemical nature, prepared by the fractionation of gonococcal cytoplasm on DEAE-Sephadex (Schmale et. al., 1969), has been also used in combination with the complement fixation test. This 'A' antigen reacts with 72% of sera from infected females, 20% from infected males, and with no normal sera; whereas the cytoplasm gives figures of 88%, 28%, and 4%, respectively (Reising et. al., 1969).

A micro-flocculation assay has been developed using cholesterol-lecithin particles sensitized with gonococcal cytoplasm. This test system, in two different studies, detects seropositivity rates in 50% of male and 79% of female patients with uncomplicated gonorrhea and in 6% of normal subjects (Reising, 1971); in 75% of female gonorrheal patients and in 11% of normal controls (Rodas and Ronald, 1974). However, when the cytoplasmic antigen 'B' is used as the test antigen, the same assay system reacts with 86% of female and 69% of male patients with gonorrhea and with 12% of normal controls (Lee & Schmale, 1970).

A protein antigen obtained by phenol-extraction, when used in a micro-precipitation test, reacts with the sera of 62% of either male or female gonorrheal patients and with no normal sera controls (Reising & Kellogg, 1965). If an ethanol extracted antigen is used, the sensitivity remains approximately the same (60%) but the specificity is decreased,

that is, there are 8% false positives (Chacko & Nair, 1969). By using a bentonite flocculation test, with bentonite particles sensitized with phenol-extracted and acetone-precipitated antigens, the sensitivity and specificity have been improved. It is found that 77% of male and 78% of female gonorrhea patients have circulating gonococcal antibodies, and only 4% false positives are recorded (Wallace et. al., 1970).

An indirect fluorescent antibody technique using formalin fixed Neisseria gonorrhoeae as antigen reacts with the sera of 80% of patients in the acute stages of gonococcal arthritis at a dilution of 1:100 (Hess et. al., 1965). Reactivity at this dilution is not present in normal sera, but is seen at lower dilutions. Using a similar technique, the presence of high titre antibody against formalin-fixed gonococci, in sera from patients with chronic or complicated gonococcal infections, has been confirmed (Cohen et. al., 1969). It has been suggested that reactivity in normal sera at low dilutions is due to the presence of natural antibody, directed primarily against a thermostable antigen on the gonococcal membrane. The indirect fluorescent antibody technique is also used to detect antibodies to Neisseria gonorrhoeae in sera from patients with uncomplicated gonorrhea. Instead of formalin-fixed gonococci, air-dried organisms are used as test antigens. This test system detects antibody to Neisseria gonorrhoeae in 79% of sera from culturally confirmed positive females and only in 4% of the sera from culturally negative females (Welch & O'Reilly, 1973). It has been suggested that formalin treatment may expose certain surface antigens that are normally veiled and against which natural and immune antibodies are reactive and that this results in the high seropositivity rates with normal controls observed by the former investigators. Further

studies have shown that the indirect fluorescent antibody technique also can be used to detect antibodies to Neisseria gonorrhoeae in urethral exudates (Kearns et. al., 1973 a). By this method the sera of 83% of males with uncomplicated gonococcal urethritis is found to be reactive.

By using the hemagglutination test with gonococcal lipopolysaccharide coated red blood cells as antigens, gonococcal antibodies are found to be significantly higher in gonorrheal patients than in normal subjects. This antigen detects antibody in the sera of 84% of female and 46% of male patients with uncomplicated gonorrhea and only gives 0-4% false positive reactions among controls (Ward & Glynn, 1972 a). When the passive hemagglutination technique is used, with Neisseria gonorrhoeae cytoplasm as the sensitizing material for tanned erythrocytes and Neisseria sicca cytoplasm as the absorbant, antibody can be detected in 77% of male and 88% of female patients with uncomplicated gonorrhea but also in 6% of controls (Logan et. al., 1970). It would appear that the cytoplasm of Neisseria sicca is unable to absorb out the cross-reactive antibody produced by the other saprophytic Neisseria.

The beta-antigen prepared by alkali extraction of gonococcal endotoxin, when used in a counterimmunoelectrophoresis system (Apicella, 1973), reacts with 69% of the sera from patients with uncomplicated gonorrhea to form precipitation lines which migrate towards the cathode. However, acute phase sera from disseminated gonococcal disease gives a precipitation line adjacent to the antibody well (Hoffman, 1974). It has been postulated that since immunoglobulin types show different electrophoretic mobilities, this system can be a sensitive method for the classification of gel precipitation lines to the beta antigen and other gonococcal antigens with anodal migration. However, this gel

diffusion system does not appear to be very sensitive as is shown by the mere 24% of positive reactions when the cytoplasm is used as the test antigen in the immunoelectrophoresis test (Danielsson, 1972).

In conclusion, an antigen-binding assay capable of precisely measuring antibodies to the gonococcal pili in human or rabbit serum has been developed (Buchanan et. al., 1973). Purified pili labelled with I^{125} are used in this assay. These are incubated with the test serum overnight and goat anti-human serum, or anti-rabbit immunoglobulin antiserum, is then added to precipitate the antibody. It has been found that the antibody titre against the purified pili is significantly higher in patients with gonococcal infection than in the normal control group. This assay system is very sensitive since it can detect as little as 100 pg of antibody. It has been suggested that this antigen-binding system to detect antibodies to gonococcal pili could be used to detect the large reservoir of asymptomatic female carriers (86%), thus reducing this source of infection.

As a whole, two major drawbacks are usually found in using the above-mentioned assay systems and test antigens. The first is the higher percentage reactivity of the sera in infected females as compared with infected males. The reason for this may be that males usually develop troublesome symptoms of the disease within a short time after infection and seek medical attention. Infected females, because of the relatively asymptomatic nature of their infection in most cases, may carry the organisms for prolonged periods and thus have a greater opportunity to develop detectable antibody levels. The second drawback is the false seropositivity rates detected in the normal control groups. This may be due to cross-reactions, for the test antigen, prepared

from Neisseria gonorrhoeae by various methods, is a mosaic of many antigens, some of which may be shared by other Neisseria or organisms of other genera, or both. Another possibility is that the antibodies detected in the normal control group are, in fact, due to previously treated infection or active infections which is undetected at the time the sera are drawn.

The results obtained in the immunodiagnosis of uncomplicated gonorrhea, using different test antigens and assay systems, are summarized in Table IV.

It is clear from Table IV that three combinations of test antigens and assay systems are best for the immunodiagnosis of both male and female patients in uncomplicated gonorrhea, namely: cytoplasm in the hemagglutination test (Logan, 1970); phenol- and acetone-precipitated antigens in a bentonite flocculation system (Wallace et. al., 1970); and cytoplasmic 'B' antigen in the micro-flocculation test (Lee & Schmale, 1970). However, each system has also demonstrated a certain percentage of false seropositivities among the normal control groups. Therefore, the use of a pure antigen may decrease the possibility of detecting cross-reacting antibody responses to organisms other than Neisseria gonorrhoeae, and is a prerequisite to measuring quantitatively the antibody response to a single antigen.

VIRULENCE OF NEISSERIA GONORRHOEAE

Intact Organism

The study of the nature of the virulence of Neisseria gonorrhoeae is hampered by the fact that its pathogenicity is peculiarly limited to man, and possibly, a few closely related primates. The first report

TABLE IV

SEROLOGICAL DIAGNOSIS FOR UNCOMPLICATED GONORRHEA

Method	Antigen Used	Percentage Positivity in Gonorrhea		Controls	Author
		Male	Female		
Complement Fixation	Heat killed whole cells	18	34	2.5-6.5	Ratnatunga (1971)
		20-25	35-40	5	Danielsson et. al. (1972)
		21	50	6	Magnusson & Kjellander (1965)
	Disrupted cells (mixture of cytoplasm and cell wall)		31	10	Rodas & Ronald (1974)
	Cytoplasm	28	88	4	Reising et. al. (1969)
	Cytoplasmic 'A' antigen	20	72	0	Reising et. al. (1969)
Micropre-cipitation	Protein from phenol extr-action	62*		0	Reising & Kellogg (1965)
	Antigen from ethanol ex-traction	60*		8	Chacko & Nair (1969)
Flocculation	Bentonite par-ticles sensi-tized with phenol and acetone pre-cipitated Ag	77	78	4	Wallace et. al. (1970)
	Cholesterol-lecithin par-ticles sensi-tized with cytoplasm	50	79	6	Reising (1971)
			75	11	Rodas & Ronald (1974)

(con't)

TABLE IV (con't)

Method	Antigen Used	Percentage Positivity in Gonorrhea		Controls	Author
		Male	Female		
	Cytoplasmic 'B' antigen	69	86	12	Lee & Schmale (1970)
Indirect immuno-fluores-cence	Air-dried whole cells	**	79	4	Welch & O'Reilly (1973)
		83	**	**	Kearns et. al. (1973 a)
			70	17	Rodas & Ronald (1974)
Hemagglu-tination	Lipopoly-saccharide	46	84	0-4	Ward & Glynn (1972 a)
	Antigen ex-tracted by alkali	23	49	2	Logan et. al. (1970)
	Cytoplasm	77	88	6	Logan et. al. (1970)
Immunoelec-trophoresis	Cytoplasm	24*		14	Danielsson et. al. (1972)
Counterimmu-noelectro-phoresis	β-antigen from alkali extracted endotoxin	69*		**	Apicella & Allen (1973)
Radioimmuno-assay	Pili	***		**	Buchanan et. al. (1973)

* Results obtained disregard sex

** Not reported

*** Detect 86% of asymptomatic female carriers

of a presumably successful experimental transmission of gonococcal infection to an animal other than man concerned the injection of an exudate from a human male with gonorrhea into the epididymis of a monkey, Lemur fulvus (Dodin, 1967). The subsequent urethral discharge obtained from the monkey contained polymorphonuclear leucocytes with intracellular diplococci; however, these organisms were not identified.

Another primate, the chimpanzee has also been used to demonstrate the virulence of Neisseria gonorrhoeae (Lucas et. al., 1971; Brown et. al., 1972). Results obtained from these experiments have shown that gonococcal urethritis can be transferred from man to the male chimpanzee by means of a gonococcal urethral exudate, and from male chimpanzee to female chimpanzee through natural transmission. In both cases, the presence of a purulent urethral exudate containing Gram-negative intracellular diplococci, and the recovery of Neisseria gonorrhoeae on bacteriological culture media, have been demonstrated. Despite these proofs that an animal model of gonococcal urethritis has been established, a less expensive and more readily obtainable animal model must still be found. Primate animals are beyond the reach of most research laboratories.

Other, smaller laboratory animals such as the mouse, guinea-pig, rabbit, and chicken embryo have been used to demonstrate the pathogenicity of Neisseria gonorrhoeae. When a suspension of gonococci and mucin is injected intraperitoneally into mice or guinea-pigs, the animals will die of toxemia in 1-3 days (Hill, 1944 b). Although the organisms may be recovered in culture media, there is no evidence of a true infection. The natural resistance of rats to a large number of human pathogens is known but not fully understood, but it has been suggested that this resistance is due to the activity of properdin (Pillemer, 1956). However,

attempts to lower both the cellular and humoral defences of the rats by feeding them with a special diet containing DL-ethionine have failed to reduce their natural resistance to infection with Neisseria gonorrhoeae (Flynn, 1972).

It appears that the infection of the chorio-allantoic membrane of the 10-day old chicken embryo reproduces all of the essential characteristics of acute urethritis in man (Morrow & Berry, 1938; Hill & Pitts, 1939; Bang, 1941). Following the initial multiplication and spread of the bacteria on the surface of the membrane, there follows a leucocytic exudate, desquamation of epithelium and later, phagocytosis (partial or complete). A thickening of squamous ectoderm and deep inflammation in the mesoderm, in the presence of few or no bacteria, are also observed. When the allantoic cavity is inoculated, the chicken embryo not only can maintain the virulence factor, but can also reconstitute virulence (Walsh et. al., 1963).

The chicken embryo can also be used to differentiate the virulence of the four types of Neisseria gonorrhoeae designated by Kellogg et. al. (1963). The association of these morphological types with virulence in human volunteers will be discussed later. When gonococci are placed on the intact chroioallantoic membrane of 10-day old chicken embryos (Buchanan & Gotschlich, 1973), the virulent types 1 and 2 organisms produce infection significantly more often (69%) than the avirulent type 3 and type 4 organisms (12%, $P>0.001$). Similar results have also been obtained by the intravenous inoculation of 11-day old chicken embryos (Bumgarner & Finkelstein, 1973). It appears that the differences in virulence are related to differences in the clearance of gonococci from the blood stream and subsequent multiplication of the virulent

colony types. Consequently it has been suggested that an animal model with the same correlation between colony types and infectivity observed in human volunteers is now established. Since the chicken embryo is without complement or antibodies until shortly after hatching (Beveridge & Burnet, 1946), it provides a model in which the direct effects of the gonococci and their interaction with phagocytic cells may be studied. It also provides a model to study the protective effects of passively-administered antibody in an environment where antibody-cell interactions, with or without complement, can take place.

There has been considerable success in infecting the anterior chamber of the rabbit's eye with Neisseria gonorrhoeae. When gonococci are introduced into the eye's anterior chamber the cornea will become diffusely turbid 24 hours later and accompanied by hypopyon (Maslovski, 1899). The gonococci later multiply and invade the intraocular tissues, particularly the lens and ciliary body, causing engorgement and edema. There is also an accumulation of polymorphonuclear cells overlying the iris of the anterior chamber (Miller et. al., 1945 a & b; Miller, 1948; Drell et. al., 1947).

Numerous studies of gonococcal virulence in man have been reported but most of the experiments have not been properly controlled. The experimental production of gonorrhea in man has only been successful occasionally when exudates from infected patients are transferred directly to human recipients (Hill, 1944 a; Mahoney, 1946). This lack of transmissability by patients' exudates or by in vitro isolates may be due to the use of avirulent strains as suggested by the study of Kellogg et. al. (1963; 1968) on human volunteers.

Despite the numerous investigations of the gonococcus, the nature

of its virulence still has not been fully elucidated. A surface component was first suggested as being responsible for the virulence of Neisseria gonorrhoeae (Spink et. al., 1937). Evidence obtained from indirect immunofluorescence (Deacon, 1959), resistance to bactericidal antibodies (Ward, 1970), and tissue culture experiments (Ward, 1972 b; Thongthai, 1973), have confirmed the existence of a surface virulence factor. This surface component appears to act as an anti-phagocytic protective cover enabling the gonococcus to resist killing by natural antibodies and complement. However, exposure of human volunteers to gonococci having large amounts of this surface component induced during in vitro cultivation, does not result in infection. Whether this surface component has any relationship to the pili present on the gonococcal surface still awaits further investigation.

Another aspect of the pathogenicity of Neisseria gonorrhoeae has been the association of virulence with genetically determined clonal types (Kellogg et. al., 1963; 1968). These specific, morphological, types are designated types 1 through 4 (Kellogg et. al., 1963; Jephcott et. al., 1971). Type 1 colonies are small (about 0.5 mm in diameter), convex, round, translucent, dark brown to black in color, slightly viscid, and with an entire edge. Type 2 colonies are similar to those of type 1, but slightly crenated, friable and with a sharper edge. Type 3 colonies differ from the previous two types in being low-convex, granular, light brown in color, larger (about 1 mm in diameter), and with a flat edge. Type 4 colonies are similar to type 3 except that they are colorless and amorphous. Type 1 is absent in laboratory strains, but a low percentage (0.01-0.1%) of type 2 is found in some laboratory strains; whereas old laboratory strains only consist of

types 3 and 4. In a study (Kellogg et. al., 1968) of intra-urethral inoculation of carefully selected isolated clonal types into human volunteers it has been determined that only gonococci from clonal types 1 and 2 cause gonorrhea. The standard inoculum of gonococci of types 3 and 4, on the other hand, failed to cause disease in volunteers. Other properties also vary among the different clonal types and are summarized in Table V.

Further evidence from electron microscopy has shown that pili are present on the surface of gonococci from type 1 and type 2 colonies, whereas those from types 3 and 4 colonies are devoid of pili (Swanson, 1971; 1972; Jephcott et. al., 1971). It has been suggested that the pili are responsible for the virulence of Neisseria gonorrhoeae, since they are involved in the resistance of the bacteria to phagocytosis by the host's leucocytes (Punsalang, 1973). Other research has shown that the attachment of Neisseria gonorrhoeae to amnion cells (Swanson, 1973) and to human epithelial cells (Thongthai & Sawyer, 1973) is facilitated if the gonococcus bears pili. However, some controversy has arisen over this hypothesis since pili are also present in several species of non-pathogenic Neisseria, such as Neisseria catarrhalis, Neisseria perflava, and Neisseria subflava (Wistreich & Baker, 1971).

Yet another aspect has been the association of the virulence of Neisseria gonorrhoeae with the toxins isolated from it. We shall discuss the gonococcal toxins under the general headings of endotoxin and exotoxin as is customary.

Endotoxins

Numerous reports exist concerning the isolation and extraction of

TABLE V

DIFFERENCES BETWEEN VIRULENT AND AVIRULENT TYPES OF NEISSERIA GONORRHOEAE

	Virulent Types (Types 1 and 2)	Avirulent Types (Types 3 and 4)	Author
Saline Autoagglutinability	+	-	Kellogg et. al. (1963)
Transient tenderness of inguinal lymph node	-	+	Kellogg et. al. (1968)
Presence of pili	+	-	Swanson (1971 & 1972) Jephcott et. al. (1971)
Infectivity on chorioallantoic membrane	High	Low	Buchanan & Gotschlich (1973)
and			
Infectivity by intravenous injection of chicken embryos	High	Low	Bumgarner & Finkelstein (1973)
Resistance to phagocytosis	+	-	Thongthai & Sawyer (1973) Punsalang & Sawyer (1973)

endotoxins with different chemical and biological characteristics from Neisseria gonorrhoeae. Most endotoxin preparations are found to be highly toxic for mice, but some investigators have reported that the endotoxin exhibits only low toxicity or is non-toxic for them. It appears that when an isolated endotoxin consists of either lipid or polysaccharide, this fraction generally possesses high toxicity for small laboratory animals. On the other hand, if an endotoxin is protein in nature, it is only weakly toxic.

There is now evidence which suggests that endotoxins obtained from Neisseria gonorrhoeae is immunogenic for rabbits and guinea-pigs. The only exception has been a polysaccharide fraction obtained from it by alkali extraction followed by alcohol precipitation (Miller & Boor, 1934). This polysaccharide is non-toxic, non-immunogenic, causes delayed type hypersensitivity in rabbits, and reacts strongly with anti-pneumococcal serum type III. In contrast, the beta-antigen, obtained by the extraction of gonococcal endotoxin with alkali, reacts only weakly (Hoffman, 1974). Other investigations with alkali extracts reveal endotoxins which consist mainly of protein (Boor & Miller, 1934; Tauber & Garson, 1957; Maeland, 1968; Apicella, 1973). This protein endotoxin is immunogenic and produces delayed type hypersensitivity in rabbits, but only exhibits low toxicity for mice. It appears that the low toxicity of alkali-extracted endotoxins is probably due to the detoxification of the endotoxin preparations by the alkali.

Either extraction by trichloroacetic acid (Boor & Miller, 1944) or extraction by phenol-water, followed by dialysis and differential centrifugation (Tauber & Garson, 1958) appears to be the most promising method to obtain a highly toxic endotoxin. Both methods yield a

lipopolysaccharide fraction which is highly toxic for mice and immunogenic for rabbits.

Whether the toxic moiety lies in the lipid or the polysaccharide molecule of the lipopolysaccharide complex is still largely unknown. It appears that the lipid fraction is mainly responsible for the toxicity, since alkali extraction followed by alcohol precipitation produces a non-toxic polysaccharide endotoxin (Miller & Boor, 1934). However, other investigators have suggested that the toxic action of the gonococcus is chiefly due to the nucleoprotein fraction (Boor & Miller, 1934; Tauber & Garson, 1957).

The epinephrine skin test also has been used as a parameter for demonstrating the in vivo biological activity of endotoxins (Maeland, 1968). Trichloroacetic acid, phenol-water, and aqueous ether extractions yield endotoxins which produce a positive epinephrine skin test, resulting in a hemorrhage and necrotic area around the local injection site. In contrast, alkali-extracted endotoxin is biologically inactive, as demonstrated by the negative results observed in this particular test.

An endotoxin of unknown chemical nature has also been reported to be formed by the autolysis of a gonococcus culture (Castellino, 1939; Öz, 1939). Although skin sensitivity to this endotoxin in patients with gonorrhea has been reported, it has not been confirmed by other investigators.

The gonococci have been separated into cell wall and cytoplasmic components by the Ribi pressure cell fractionator (Peacock & Schmale, 1969). The cytoplasm can be further fractionated into 4 fractions on Sephadex G-200 in a 1.0M NaCl, 0.1M TRIS HCl buffer at pH 8.0. Fraction I of the cytoplasm is found to be relatively toxic for 10 to 12-day old

chicken embryos injected intravenously. Whereas phenol extraction of Fraction I results in a non-toxic aqueous phase and an insoluble phenol phase, the aqueous phase of the phenol-extract of isolated cell walls contains the most toxic activity. This finding implies that two endotoxins are present in Neisseria gonorrhoeae, the first one is located in the cell wall, and the second one in the cytoplasm. It has also been postulated that the cytoplasmic endotoxin is a precursor of the cell wall endotoxin (Anacker et. al., 1966).

The biological activities of gonococcal endotoxins are summarized in Table VI.

Exotoxins

It is debatable whether Neisseria gonorrhoeae produces an exotoxin or not. Several investigators (DeChristmas, 1897; Herrold, 1927; Clark et. al., 1931) have reported the isolation of a toxic material from the broth in which gonococci have been grown. Several biological characteristics of this exotoxin have been demonstrated, for example: toxicity for guinea-pigs injected intracerebrally; in vivo and in vitro neutralization of the toxicity by serum from a rabbit injected with the exotoxin; and positive skin tests in patients with gonococcal infections. These findings have been interpreted as evidence that Neisseria gonorrhoeae elaborates an immunologically and biologically-active substance during growth in a broth medium. It was later suggested that this material might be a carbohydrate (Rossett, 1939) corresponding to the Type I substance of Neisseria gonorrhoeae (Casper, 1937 b), but no chemical proof has been given. However, a protein-like fraction isolated from broth cultures of Neisseria gonorrhoeae after removal of

TABLE VI

BIOLOGICAL ACTIVITIES OF ENDOTOXINS OF
NEISSERIA GONORRHOEAE

Chemical Entity	Method of Extraction	Biological Activities	Author
Polysaccharide & nucleic acid	Phenol-water	Toxicity for mice	Tauber & Garson (1958)
Lipopolysaccharide	Phenol-water	Positive epinephrine skin test	Maeland (1968)
Lipopolysaccharide phosphoric acid ester	Phenol-water, dialysis and differential centrifugation	Highly toxic for mice	Tauber & Garson (1959)
Nucleoprotein	Alkali	Delayed hypersensitivity and immunogenic in rabbits; toxicity for mice	Boor & Miller (1934)
*	Alkali	Negative epinephrine skin test; low toxicity for mice	Maeland (1968)
Polysaccharide	Alkali + alcohol	Delayed hypersensitivity in rabbits; non-toxic, non-immunogenic	Miller & Boor (1934)
Nucleoprotein	Alkali + cold acetone	Low toxicity for mice	Tauber & Garson (1957)
Glycoprotein	Alkali + ion-exchange chromatography	Immunogenic in rabbits	Apicella & Allen (1973) Hoffman (1974)

(con't)

TABLE VI (con't)

Chemical Entity	Method of Extraction	Biological Activities	Author
Glycolipid	Trichloro-acetic acid	Toxicity for mice; immunogenic in rabbits	Boor & Miller (1944)
*	Trichloro-acetic acid	Positive epineph-rine skin test	Maeland (1968)
*	Autolysis	Toxicity for guinea pigs; skin sensiti-vity for gonorrhea patients	Castellino (1939)
*	Autolysis	Immunogenic in guinea pigs and donkeys; skin sensitivity for gonorrhea patients	Oz (1939)
*	Ketene	Toxicity for mice	Boor & Miller (1939)
Protein	Aqueous ether	Positive epineph-rine skin test	Maeland (1968)
*	Ribi Cell Fraction-ator into cell wall and cyto-plasm	Toxicity for chicken embryos intrave-nously; immunogenic in rabbits	Peacock & Schmale (1969)

* Analysis not performed

the cells by centrifugation, is toxic for mice and rabbits and possesses antigenic activities (Stokinger et. al., 1944 b).

Other investigations on the culture filtrate produced by Neisseria gonorrhoeae have suggested that the exotoxin of this organism is derived solely from dead and disintegrated gonococci. This 'exotoxin' produces no immunity, but only causes anaphylactic shock in guinea-pigs (Torrey, 1908). Therefore, it would appear that the observations of DeChristmas and others suggest more likely an endotoxin produced by autolyzed cultures after prolonged incubation, rather than a true extracellular toxin.

THE QUESTION OF IMMUNITY IN GONORRHEA

Although gonorrhea is one of the most common of reported infections, the nature of the immune response to this disorder is poorly understood. Despite the demonstration of humoral and cellular antibody responses in persons infected with Neisseria gonorrhoeae, repeated infections can still occur. It has been postulated that this lack of protection against re-infection may be due to the short incubation period, three to five days, which does not allow the person infected sufficient time to establish an immune response to the gonococcal antigens. Perhaps the magnitude, rather than the mere presence of the immune response to gonococcal antigens, is the crucial factor in determining host resistance to this disease. Alternatively, it may be that men with repeated episodes of gonorrhea are a unique group from the population of all men exposed to gonorrhea, and are selected for repetitive disease because of an inherent inability to produce an antigonococcal response of suf-

ficient magnitude to obviate it. It is therefore possible that gonococcal infection occurs repeatedly because of a quantitative defect in the immune response.

It is customary to discuss the two main patterns of immune response as under the headings of humoral and cellular response.

Humoral Response

Antibodies of the IgG, IgM, and IgA classes present in the sera of both infected (with immune antibodies) and normal (with natural antibodies) persons were first demonstrated by an indirect fluorescent antibody technique to react with the heat-stable somatic antigens of Neisseria gonorrhoeae (Cohen, 1967). Immune IgG antibodies can be distinguished from natural IgG antibodies by the ability of the former to recognize heat-labile surface antigens and its greater resistance to heat. However, the distinctions between natural and immune IgM antibodies by these two criteria are less obvious. Immune IgA antibodies, on the other hand, react both with heat-labile and heat-stable antigens. In addition, their activity is increased by heating at 60 ^{o}C, and decreased at higher temperatures.

Further investigations have shown that a four-fold increase in IgG antibody activity against the heat-labile surface antigen occurs in nine out of ten human volunteers, whereas much less response is observed with IgM and IgA fractions. Heat-stable somatic antigens, on the other hand, stimulate predominantly IgA antibody (Cohen, Kellogg & Norins, 1969). Thus it appears that not all antigens of Neisseria gonorrhoeae stimulate similar responses in terms of the globulin classes of their respective antibody. In contrast to this has been the finding that

patients with gonorrhea have higher mean levels of IgG, IgM, and IgA antibodies than normal subjects (Scott & Rasbridge, 1972). Further studies by indirect immuno-fluorescence, using a conjugate mono-specific for IgA, have demonstrated the presence of IgA antibody in the urethral secretions of males with uncomplicated gonococcal urethritis and a higher percentage of positivity in repeated infections (Kearns et. al., 1973 a). That the major part of this local response is secretory IgA is later confirmed by the reduction in titer of this antibody by absorption with antibodies to secretory IgA. The data suggests that a secretory IgA system is operative in uncomplicated gonococcal urethritis.

It has been shown that two types of antibody appear in the sera of human volunteers, according to the data obtained from a vaccine trial using an autolyzed, unpurified preparation made from a virulent strain of Neisseria gonorrhoeae (F62) (Greenberg et. al., 1971). The first type is a precipitin antibody, whereas the second is an inhibitory antibody, as demonstrated by the bentonite floccultion test and a cell culture system, respectively. Although the precise immunoglobulin nature of these two types of antibody is still not known, it has been suggested that the precipitin antibody is an IgG, and the inhibitory antibody, IgM. It has also been postulated that the inhibitory antibody may develop after gonorrhea subsides and may account for the effective immunity that some people develop after one or more infections with gonorrhea.

Two ways have been suggested (Punsalang, 1973) by which an immune response against pili may help to protect man from gonorrhea. First, antibody directed against pili can inhibit attachment of bacteria to mucosal cells and hence reduce the likelihood of successful tissue invasion. Second, anti-pilus antibody of the appropriate immunoglobulin

class can opsonize gonococci on the mucosa or in tissue, thereby promoting their ingestion and destruction by phagocytes. It is further postulated that the reason why man does not acquire immunity to gonorrhea is probably due to the fact that the site of infection in nature is immunologically isolated; and that the virulent gonococcus possesses additional virulence determinants that require other host defence mechanisms for immunity.

Cellular Response

Perhaps the least known factor in gonococcal research is the host's cellular response to this organism. Whether or not cell-mediated hypersensitivity develops in the course of a naturally acquired gonorrheal infection is still largely unanswered. The pathological manifestations of abscess cavities and fibrosis, and the morbidity frequently attributed to gonorrhea, such as urethral strictures, chronic salpingitis and pelvic inflammatory disease, have suggested the possible existence of a cell-mediated response.

Although cell-mediated, delayed-type, skin hypersensitivity has long been known to occur in gonorrheal infection (Teaque & Torrey, 1907; Torrey, 1907), it is only through the recent development of a lymphocyte transformation technique that the existence of a cell-mediated immune response in this disease has been clearly demonstrated. Lymphocyte transformation induced by gonococcal cytoplasm occurs to a significantly greater extent in gonorrheal patients than in normal subjects, according to the observations of Kraus et. al. (1970). Further investigation has shown that besides the cytoplasm, an antigen extracted by alkali (Kearns et. al., 1973 b) and two fractions obtained by ion-exchange

chromatography (Esquenazi & Streitfeld, 1973) from Neisseria gonorrhoeae also cause lymphocyte transformation. Moreover, this response appears to subside within a period of 5 weeks after successful therapy and is only marginally greater for patients with a first infection, but is significantly greater for patients with multiple infections. The demonstration that the lymphocytes of some non-gonorrheal subjects also can be transformed by a variety of gonococcal antigens suggests that cross-reactive antigens with other Neisseria species are present. Despite the fact that no one antigen specific for the cell-mediated response in gonococcal infection has been identified, it is clear that there is a blastogenic response to gonococcal antigens in gonorrhea.

MATERIALS & METHODS

THE ORIGIN AND CULTIVATION OF NEISSERIA GONORRHOEAE

Two strains of Neisseria gonorrhoeae, designated as MB and ND, were freshly isolated from patients suffering from acute uncomplicated gonorrhea. Another strain F62 was supplied by D.S. Kellogg (CDC, Atlanta). The identity of the bacteria was established by Gram-staining, oxidase test, sugar fermentations and fluorescent antibody technique using a commercially prepared anti-gonococcal serum (Difco). The organisms were grown for 20 hours at 37 ^{o}C, under 5% CO_2, on GC agar base (Difco Laboratories, Michigan) supplemented with cocarboxylase, glutamine and dextrose (Lankford, 1950). Colonies of type 1 and type 4 were isolated and propagated for 60 passages or more as described by Kellogg et. al. (1963), and summarized in Table VII.

PREPARATION OF CYTOPLASM

The bacteria from type 1 and type 4 colonies were harvested from the enriched GC Medium with 0.85%, pH 7.1 phosphate-buffered saline. Whole cells, obtained as sediment after the centrifugation of the pure cultures obtained, were washed repeatedly with the above-mentioned buffer, followed by centrifugation until no trace of amino acids and carbohydrate were revealed in the washings, as determined by Ninhydrin (Schiffman, 1958) and Molisch (Dische, 1955) reactions, respectively. To the final washing, 5 times volume of the same buffer was added, and the suspension was sonicated by a Biosonik BP-III Ultrasonicator (Bronwill Scientific, Rochester, N.Y.) for 15 minutes at 80% maximum output (2.2 Amp.). The supernatant fluid was then processed according to Table VIII.

TABLE VII

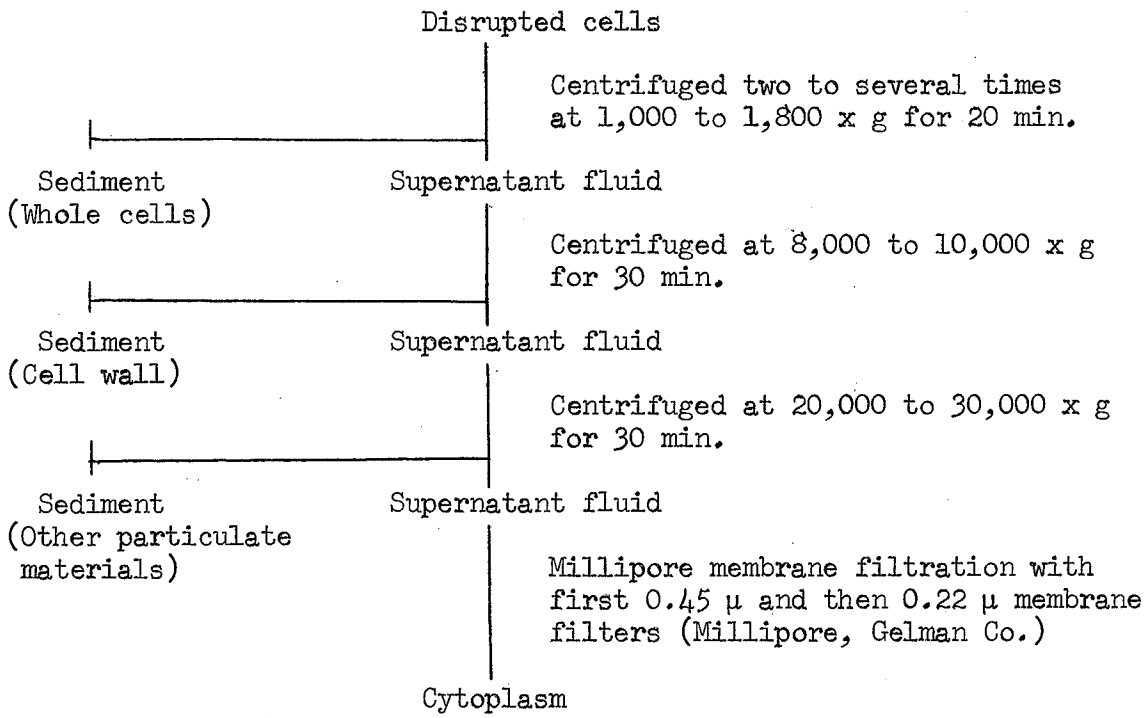
MORPHOLOGICAL CHARACTERISTICS OF
TYPE 1 AND TYPE 4 COLONIES OF NEISSERIA GONORRHOEAE

	Type 1	Type 4
Colonial Morphology		
Size	Small (0.5 mm)	Large (1.0 mm)
Color*(single light source, with diffused, angled, transmitted light through the medium from below the plate)	Dark gold to black	Colorless
Convexity*(combined light source, with diffused, angled light transmitted from below, and edge lighting 15° above plate)	Convex (glistening)	Less convex (dull)
Number of selective transfer required to obtain almost pure cultures (99%) of types	9	69

* Observed under a dissecting microscope at 10 x magnification

TABLE VIII

PROCEDURE FOR THE PREPARATION OF CYTOPLASM



The whole preparation was done at 4 °C using an International Refrigerated Centrifuge Model B-20 (International Equipment Co., Mass., U.S.A.). The homogeneity of the cytoplasm was then checked for by electron microscopy at 25,000 x magnification to reveal any contamination with particulate material.

CHEMICAL EXAMINATION

Qualitative Determination of Polymer-Categories by Disc-Electrophoresis

Different fractions of the cytoplasm of Neisseria gonorrhoeae were identified preliminary using the comprehensive disc-electrophoresis technique (Kwapinski, 1972) using the basic acrylamide-gel electrophoresis of Ornstein (1964) 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 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 cytoplasm which could have migrated faster than the tracking dye. Different components of the cytoplasm were then identified by staining the gel slabs 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:

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

The proteinaceous fractions were revealed by scanning in the Chromoscan MK-II (Allied Scientific Co. Ltd., Canada).

Preparative Separation and Purification of the Different Polymer-Categories

The following physical procedures were employed at 4 °C to separate and isolate the biologically-active components of the cytoplasm.

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 0.01M phosphate-buffered saline, pH 7.1 added to the swollen gel. The dilute slurry of the Sephadex gel in phosphate 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 Ltdl) 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 cytoplasm were applied to the column and 10 ml

fractions were collected, using the Buchler Fractomat fraction collector with built-in refrigeration system (Buchler Instruments Inc., Fort Lee, New Jersey). The effluents collected were scanned at 280 nm using the ISCO Dual Beam Ultraviolet Analyzer Model UA-2 with a built-in recorder (Instrumentation Specialties Co., Lincoln, Nebraska).

The Isoelectric Focusing Technique

The procedure of Vesterberg et. al. (1967) was followed. The experiments were performed on LKB 8101 (110 ml) and 8102 (440 ml) columns (LKB—Produkter AB, Stockholm) utilizing different ampholytes with different pH ranges (pH ranges of 3-10 and 3-6). The power supply was adjusted to 500 V at constant voltage with anode at top and increased to 700 V after 24 hours of the experiment. The eluate was collected in 3 ml fractions. The pH value of each fraction was measured at 4 ^{o}C immediately after the elution of the column, and each fraction was dialyzed exhaustively for 48 hours (5 changes of 10 litres of the buffer) against 0.01M PBS, pH 7.1 at 4 ^{o}C to remove sucrose and ampholyte. The absorbance was subsequently measured at 280 nm in a Unicam SP1800 spectrophotometer (Pye Unicam Ltd., Cambridge, England).

Each fraction was then concentrated by lyophilization in a Virtis Lyophilyzer (Virtis Co., Gardiner, N.Y.).

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 unique biologically-active cytoplasmic fraction obtained at pI 3.2 from five isoelectric focusing runs (pH range 3-6) were pooled. After dialysis overnight against 0.01M, pH 7.1 phosphate-buffered saline and concentration by lyophilization,

the sample was 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 initially to 30 mA at constant current with anode at bottom and increased to 80 mA after 1 hour of the electrophoresis.

The eluate was collected at 5 ml portions in the Fractomat fraction collector. The absorbance of the materials thus passing from the column was continuously monitored at 280 nm wavelength and recorded automatically on an ISCO absorbance recorder. All the fractions were also measured for the presence of saccharides using Dubois' et. al. (1956) and Fairbairn's (1953) method. The quantity of protein was measured by Lowry's et. al. (1951) method and Weichselbaum's biuret (1946) method modified by Dittebrandt (1948).

The Chemical Analysis

The chemical composition of the purified unique biologically-active cytoplasmic fraction was determined quantitatively using the following methods:

Dry Weight Determination - A 5 ml solution or suspension of the individual 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 ^{o}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.

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 minimum 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 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 ^{o}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.

Amino Acid Analysis - The amino acid content of the purified antigens was determined by using the Beckman Automatic-Analyzer Model 120C,

using the method of Dus et. al. (1966). Before the assay, the antigens (approximately 1 mg protein) were hydrolyzed in 5 ml of 5N HCl for 18 hours in a sealed vial at 110 °C in an oven. The hydrolyzates were decolorized with charcoal, centrifuged and evaporated in vacuo in the presence of P_2O_5. The times for elution of the various amino acids from the samples were then compared directly against the amino acid standards.

Determination of Physicochemical Characteristics

The heat sensitivity of the whole cytoplasm and of the biologically-active biopolymers was examined by heating the materials in a water bath at 37 °C for 24 hours, 56 °C for 30 minutes and 100 °C for 5 minutes, and repeating the toxicity testing.

The enzyme sensitivity was investigated by exposing aliquots of the cytoplasm or the toxic fractions containing 100 LD_{50}, to DNase 1 (Bovine Pancreas Sigma Chemical Co.), RNase A (Worthington Biochemical Corp.), and alpha chymotrypsin (Worthington), in the following conditions:

1) nucleases and the substrate (1:10) in 0.1M, pH 5.0 acetate buffer, incubated at 37 °C for 60 minutes.

2) trypsin or alpha chymotrypsin and substrate (1:10) in 0.01M, pH 7.1 phosphate-buffered saline, incubated at 37 °C for 10 hours. After the treatment with the proteolytic enzymes, their activities were abolished by incubating the resulting mixture with an equal amount of soybean trypsin inhibitor (Sigma) for 2 hours at 37 °C.

The sensitivity to periodate was examined using equal volumes of the cytoplasm (or of the isolated biopolymers) and 0.02M periodic acid in 0.01M, pH 7.1 phosphate-buffered saline, and incubating the mixture in the dark at 25 °C for 5 hours. The reaction was then stopped by adding 0.2 ml of 10% dextrose. The toxicity assay was then repeated.

SEROLOGICAL EXAMINATION

Sources of Hyperimmune Rabbit Sera and Human Sera

Albino rabbits weighing from 5 to 6 pounds were injected with the filtered type 1 and type 4 cytoplasms according to the schedule as follows (Kwapinski, 1971):

Subcutaneously	0.3 ml
Intramuscularly	0.4 ml
Foot pad	0.4 ml
Another foot pad	0.4 ml
I.V.	0.4 ml
Subcutaneously	0.5 ml
Intramuscularly	0.5 ml
I.V.	0.5 ml

The cytoplasmic 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 at -20 °C.

Human sera, as followings, were obtained from Winnipeg General Hospital: three 'normal' sera from people without a previous history of gonorrhea, and ten sera from patients with untreated, acute gonorrhea. The sera were maintained at -20 °C.

Immunodiffusion

The agarose micro-immunodiffusion technique of Wadsworth (1957) was employed for antigenic analysis of the cytoplasm. A 1.0% melted agarose (Mann Laboratories) was prepared by dissolving in 0.002M TRIS

buffer of pH 7.2, by autoclaving at 121 °C for 30 minutes. One ml of an 0.1M sodium azide was added to 100 ml of the molten agarose. Microscope slides (2.5 x 7.5 cm) were placed on a level surface and 3.0 ml of the molten agarose preparation was pipetted onto each slide. A cover was placed over the slides to allow the agarose to solidify at 23 °C without drying out in the process. The agarose slides were stored in a moist chamber at 4 °C for at least 5 hours before the wells were cut. The wells were cut in the agarose slide using a gel-cutter (Shandon). By using suction, the agarose from the wells only was removed. The diameter of the wells was 3.0 mm. The distance between the center and the outside wells was that of 4.0 mm apart; whereas the distance between individual outer well was 5.0 mm. For this assay, each fraction obtained by the above preparative methods and lyophilized, was diluted in 0.01M phosphate-buffered saline to contain 1 mg/ml solids. Each well received 15 µl of the antigen or the antiserum. The plates were incubated in a moist chamber at 23 °C for a period of 3 days. Precipitate bands were recorded as lines of identity, non-identity, and partial identity of the different antigens with the antiserum.

For preservation, the slides were washed at 4 °C with 1 litre of phosphate-buffered saline (0.01M, pH 7.2) for 48 hours with two changes of the buffer. After the washing, filter paper strips of the same size as the slides were slightly pressed on top and left for a minimum of 8 hours in a 37 °C incubator. After the agarose had been dried to a thin film, the slides were then stained for 30 minutes in a 0.1% Amido-black solution in 7% glacial acetic acid. The residual dye was removed by destaining in 7% acetic acid.

Agarose -gel Immunoelectrophoresis

The immunoelectrophoresis was essentially carried out according to Scheidegger's (1955) micro-method, except that the current of 5 mA per slide was applied for 3 hours.

In this test, the agarose slides were prepared as before. The electrophoresis apparatus (Colab, Canada) was filled with 0.1M Veronal buffer, at pH 8.6. The wells were filled with 15 µl of the appropriate antigens. Whatman 3M filter paper wicks were used. After the electrophoresis, a trough (2 x 55 mm) was made in the middle of the slide with a distance of 3 mm from the antigen wells. The trough was then filled with an antiserum, and the slide was placed in a level humid chamber for 3 days, at 23 ^{o}C.

Absorption

The absorption of hyperimmune rabbit sera with the cytoplasm was carried out as described by Kwapinski's et. al. (1971) method.

This was carried out by adding 2 parts of the crude cytoplasm, adjusted to the O.D. of 1.5 at 280 nm with 0.01M PBS, pH 7.1, to 3 parts of the antiserum. The mixture was incubated for 3 hours at 37 ^{o}C with gentle shaking on a mechanical shaker. The absorbed serum was then centrifuged at 10,000 x g for 20 minutes. The resulting clear supernatant was subjected to a second absorption and centrifugation under the same conditions. Finally, it was concentrated to half the original volume by lyophilization.

Radial-Immunodiffusion

The procedure modified from Pereira's et. al. (1972) method was employed. In this method, the preparation of agarose was the same as

before except a 1.2% concentration was used. The agarose, glasswares and the test antigens were kept in a 40 °C water bath. A 0.1 ml solution of the antigen (containing 50 μg protein) was added to 3 ml agarose, followed by thorough mixing. The mixture was then pipetted onto a pre-warmed microscope slide. Fifteen microlitres of the antiserum in serial dilution was then applied to individual well (3 mm diameter). Precipitate zones were measured perpendicularly in 2 directions after an incubation period of 3 days at 23 °C, in a moist chamber.

The Homogeneity Determination

The β(-t) fraction (obtained on the preparative polyacrylamide electrophoresis), as well as the initial β(+t) fraction (obtained on the isoelectric focusing), were examined against the antisera produced in rabbits by injections of: (i) the original type 1 cytoplasm, (ii) the β(+t) fraction, and (iii) the β(-t) fraction recovered from the major peak area on the polyacrylamide electrophoresis.

Whereas the anti-cytoplasm antisera were obtained by immunizations of rabbits according to the previously described Kwapinski's et. al. (1971) method, the remaining antisera were produced by intradermal injections of a total of 0.2-0.6 ml (containing 20-60 μg protein) of the β(+t) or β(-t) materials, divided in small volumes and injected into two skin areas.

The Immunogenicity Assays

The immunogenicity of β(+t) and β(-t) fractions was investigated in the following manner: Albino male rabbits, obtained from a single litter and weighing between 3.5 and 3.6 lbs, were intradermally injected with a total of 0.2, 0.4, or 0.6 ml of the antigens (containing 100

μg protein/ml PBS), divided into two equal portions and applied into two different areas on the closely shaved back. Three rabbits were used for each material.

Blood samples (3-5 ml) were taken from the peripheral ear vein before the injections and at 3-day intervals thereafter.

The content of antibodies in the rabbit sera was determined by means of a radial-immunodiffusion procedure as described earlier, on incubation at 23 °C for 3 days.

The cell-mediated response was examined on the 21st day after intradermal injections of 0.1 ml of the corresponding antigens, diluted 1:2, 1:4, and 1:8. The skin reactions were observed and measured in two perpendicular directions after 3, 6, 12, 24, 48, and 72 hours.

After the intradermal challenge, further samples of blood were taken at 7-day intervals, for a total period of 6 weeks, and the sera were examined as above.

BIOLOGICAL EXAMINATION

The Toxicity Assay

The biological effect of the cytoplasms and of the isolated biopolymers was examined by an intravenous toxicity test performed in 11-day old chicken embryos according to a modified Finkelstein's (1964) method.

Embryonated eggs were obtained from a single flock of Leghorn hens (Strain Shaver 288). They were kept in a humidified incubator at 37 °C until inoculated, and were candled daily for 3 days after inoculation although specific deaths usually occurred within 24 hours. Rectangular

area of the egg shell (2 x 7 mm) was removed over a prominant, preferably straight allantoic vein (while candling) using a hand drill (Moto-Tool Model, Dremel Mfg. Co., Wisconsin, U.S.A.). Inoculations were performed while candling using a 1 ml tuberculin syringe fitted with a 27-gauge disposable needle. Eggs which were observed to bleed internally (under the membrane or into the allantoic cavity) after inoculation were discarded.

Prior to the injections, the concentration of each material was measured in terms of protein contents by Lowry's et. al. (1955) method. The solutions were then filtered through an 0.45 μ Millipore membrane. In order to determine the LD_{50} dosage, each material found to be toxic on the preliminary test was made into serial two-fold dilutions, and injected in an 0.1 ml volume to 20 chicken embryos. The LD_{50} dosage was computed by Probit Analysis (Finney, 1971) using the University of Manitoba Health Science Centre Computer Program ST36.

Neutralization Assay

The neutralization test was performed by adding equal volumes of the toxic biopolymer (containing 100 LD_{50}) to different dilutions of either the hyperimmune rabbit sera or the human sera. The mixture was incubated for 2 hours at 37 ^{o}C with gentle shaking on a mechanical shaker. Any precipitate was removed by centrifugation at 10,000 x g for 15 minutes. The clear supernatant was then filtered through a 0.45 μ Millipore membrane prior to the toxicity assay. The untreated toxic biopolymers or PBS alone were used as controls.

The Assay of the Immuno-Protective Power

The immuno-protective power of the β(-t) and β(+t) biopolymers

was determined by a slightly modified Sery and Nagy's (1971) technique. In this assay, the Albino rabbits were twice injected intradermally with the pure β(-t) or β(+t) fraction, as described before. After a period of time which varied from 6-12 weeks (because of practical considerations), 0.1 ml of a suspension of live type 1 Neisseria gonorrhoeae (containing about 5×10^7 organisms) was injected into the anterior eye chamber of the rabbits above and of rabbits which had not been pre-injected. Another group of rabbits also received the same dose of live gonococci which had been pre-incubated (37 °C for 5 minutes) with either the anti-β(-t) antiserum or normal rabbit serum. Control rabbits received 0.1 ml PBS.

The eyes were then examined at 24-hour intervals for a week. The aqueous humour was inoculated onto the enriched GC culture medium, and the corneas were examined histologically.

Prior to the injection, the rabbits were anaesthetized by ether. It was also found helpful to apply local anaesthesia to the eye by 1 drop of 0.5% Ophthaine solution.

RESULTS

I PHYSICOCHEMICAL PROPERTIES

Qualitative and Quantitative Separation of Different Polymer-Categories

Comprehensive Disc-Electrophoresis Pattern

By means of the analytical disc-electrophoresis, ten protein components were separated from the type 4 cytoplasm, whereas the type 1 cytoplasm of Neisseria gonorrhoeae yielded eleven bands. Ten of these bands were identical with those present in the cytoplasm of type 4, whereas an additional constituent possessing an R_s of 0.71 was only found in type 1 cytoplasm (Figures 1 and 2).

The Sephadex G-200 Gel Filtration Pattern

On the gel filtration, each cytoplasm was resolved into three major areas of polymer concentration (Figure 3). The fastest peak was found to come out almost immediately after the void volume (V_o) and was collected between the 120 and 180 ml volume of the eluate. The two other concentration zones were collected between the 350-420 ml and 460-510 ml volume of the eluate, respectively.

Purification of the Unique Cytoplasmic Biopolymer

Isoelectric Focusing

The leading, fast-moving fraction of type 4 cytoplasm, eluted from the gel filtration column, and then concentrated and electrofocused at pH 3-10 range, yielded a single major peak at pI 4.4 (herein designated α) (Figure 4); whereas two major zones of the polymer concentration, at pIs 3.2 and 4.4 (herein designated $\beta(+t)$ and α, respectively), were found on the isoelectric focusing of type 1 cytoplasm (Figure 5). The $\beta(+t)$ fraction, obtained from the pI 3.2 area by isoelectric focusing at 3-10 range was further purified by

Figure 1

Proteinaceous materials separated by the disc-electrophoresis from type 1 cytoplasm (tubes 2, 4, 6, and 7) and type 4 cytoplasm (tubes 1, 3, and 5).

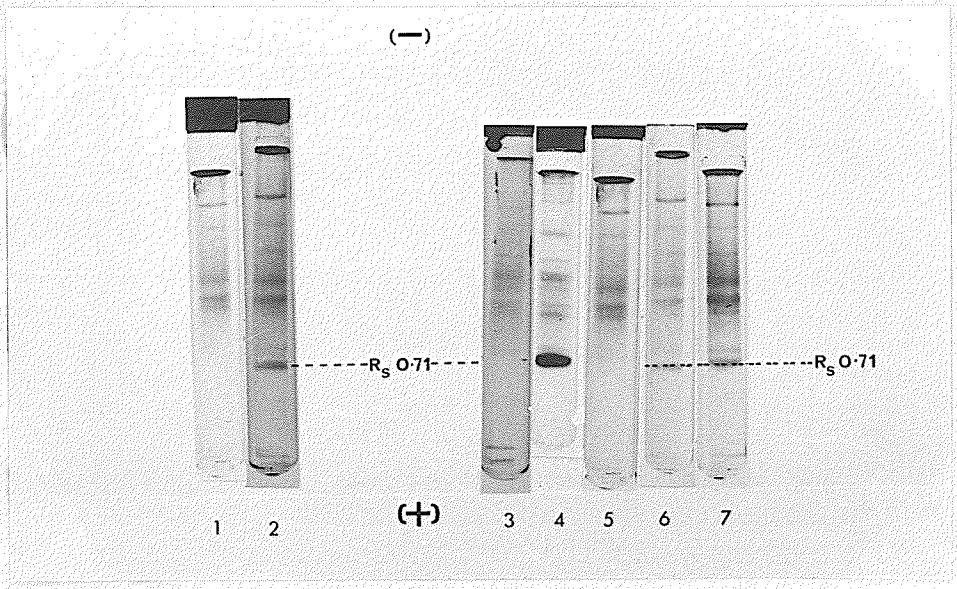

(—)
R_S 0·71
R_S 0·71
1
2
(+)
3
4
5
6
7

Figure 2

Contours of the proteinaceous fractions separated by the disc-electrophoresis from type 1- (top curve) and type 4 cytoplasm (bottom curve), revealed by scanning in the Chromoscan.

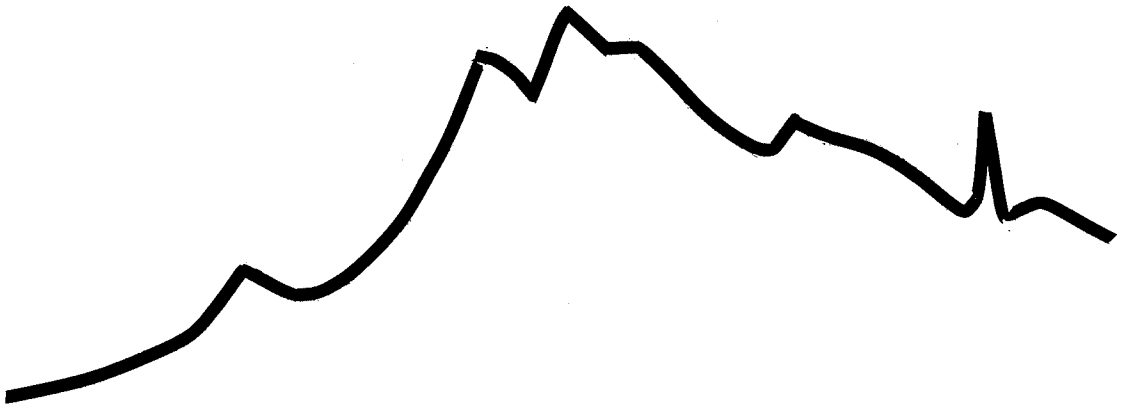
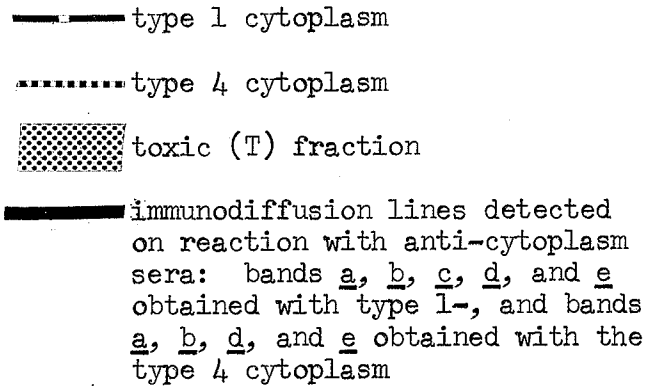


Figure 3

Proteinaceous fractions separated by Sephadex G-200 gel filtration.



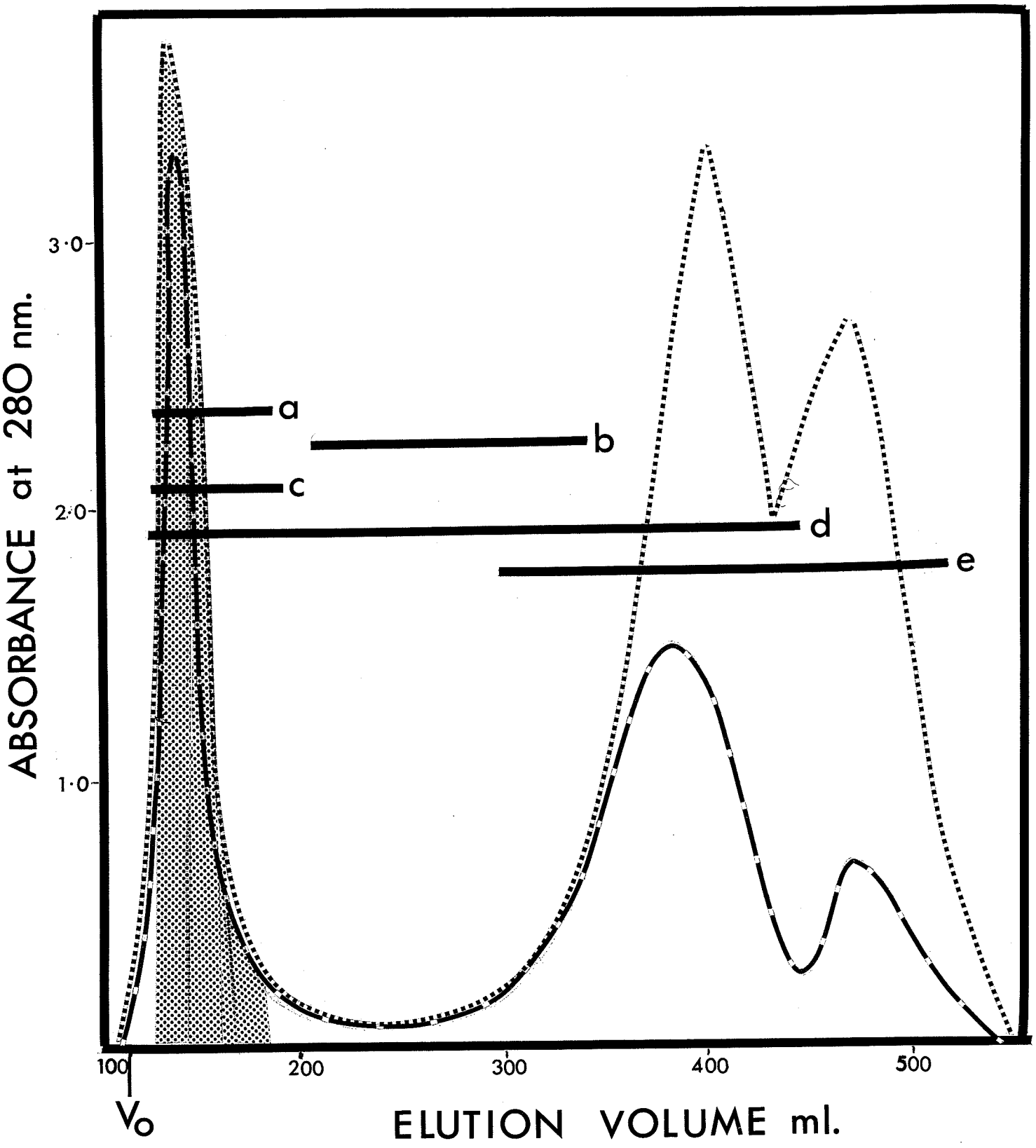

ABSORBANCE at 280 nm.
3·0
2·0
1·0
a
b
c
d
e
100
200
300
400
500
Vo
ELUTION VOLUME ml.

Figure 4

Distribution of proteinaceous fractions separated by the isoelectric focusing (pH 3-10 range with anode at top) of the leading fraction of type 4 cytoplasm, eluted from Sephadex G-200 column.

▒▒▒ toxic α biopolymer

• • • • pH curve

▬▬ immunodiffusion lines detected on reaction with antisera against types 1 and 4 cytoplasm

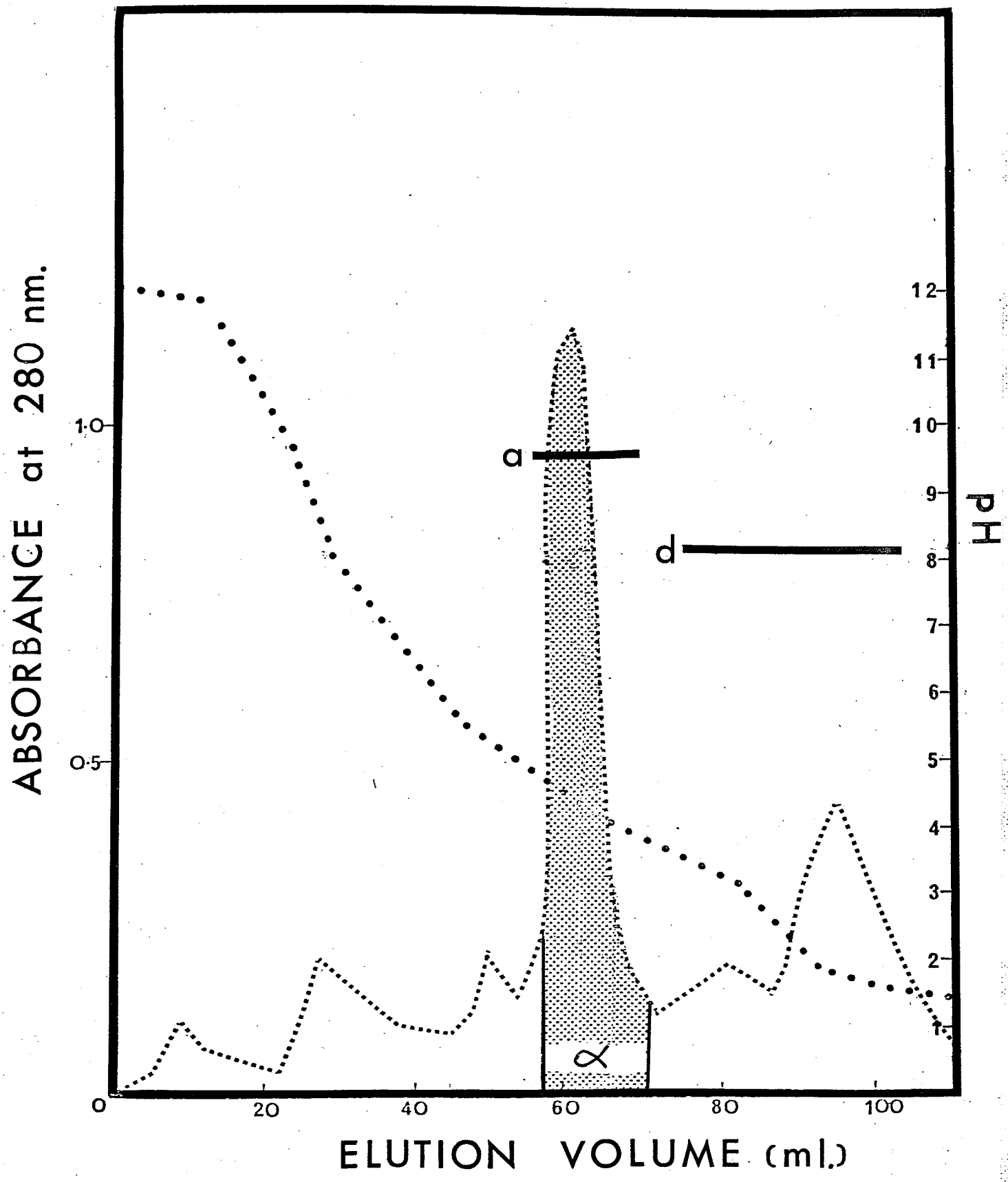

ABSORBANCE at 280 nm.
1·0
0·5
0
20
40
60
80
100
ELUTION VOLUME (ml.)
pH
12
11
10
9
8
7
6
5
4
3
2
1
a
d
α

Figure 5

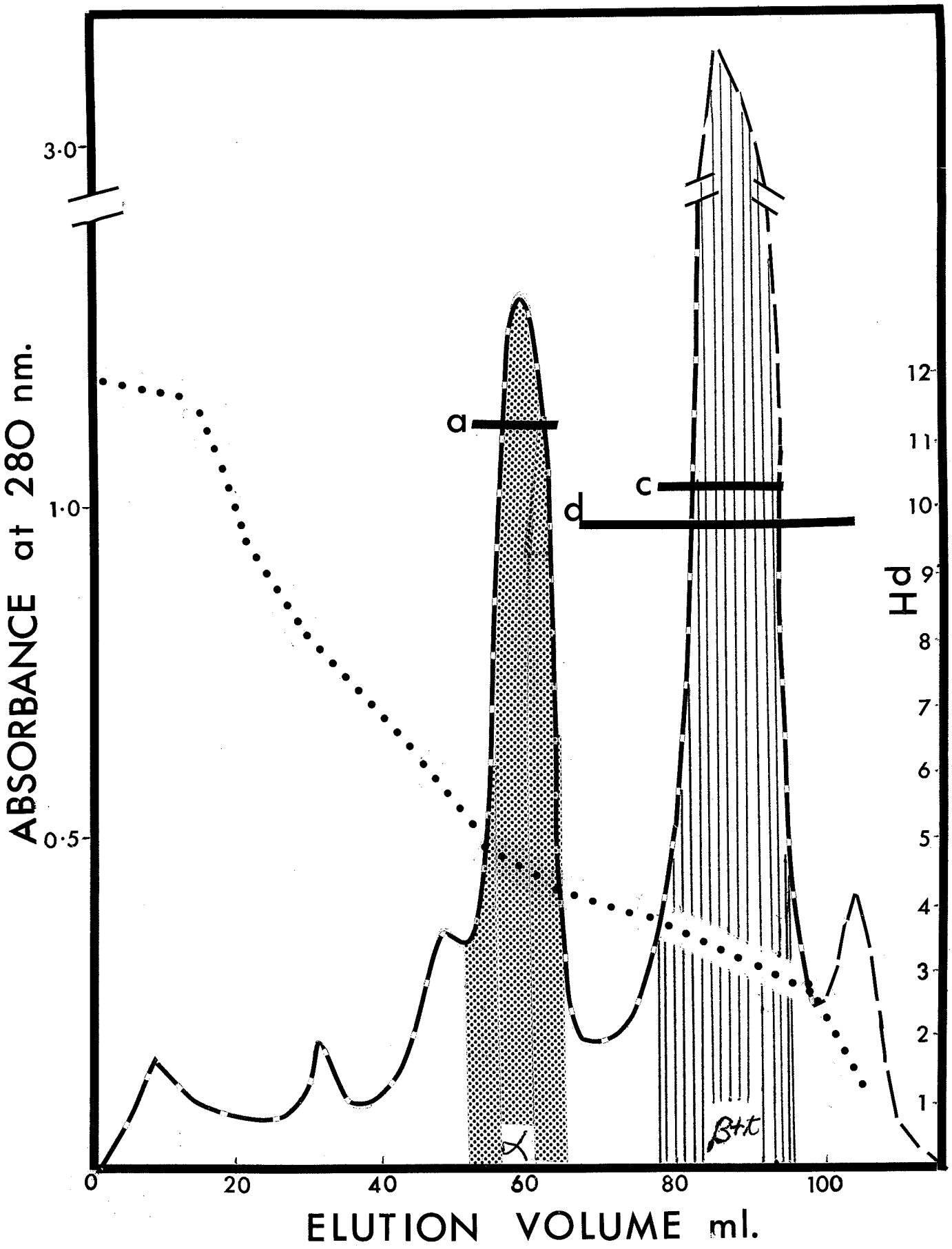
Distribution of proteinaceous fractions separated by the isoelectric focusing (pH 3-10 range with anode at top) of the leading fraction of type 1 cytoplasm, eluted from Sephadex G-200 column.

toxic α biopolymer

toxic β(+t) biopolymer

pH curve

immunodiffusion lines detected on reaction with antiserum against type 1 cytoplasm


3·0
1·0
0·5
ABSORBANCE at 280 nm.
a
c
d
12
11
10
9
8
7
6
5
4
3
2
1
pH
0
20
40
60
80
100
ELUTION VOLUME ml.
γ
β+t

electrofocusing at a pH range of 3-6. A single major peak with a pI of 3.2 was again obtained (Figure 6).

Preparative Polyacrylamide Gel Electrophoresis

The biopolymer β(+t), collected from pI 3.2 region of the isoelectric focusing (pH range 3-6) and passed through a preparative polyacrylamide gel column, yielded a single major peak collected between the 120 and 160 ml volume of the eluate (herein designated as β(-t)), and a very small peak at 310-330 ml volume of the eluate (Figure 7).

Chemical Analyses of the β(+t) and β(-t) Biopolymers

The chemical analyses of the β(-t) fraction revealed that it exclusively consisted of a protein which amounted to 99.0% of its total dry weight, as compared with 97.8% protein in the β(+t) fraction. The β(-t) biopolymer consisted of 7 different amino acids, lysine, aspartic acid, threonine, serine, glutamic acid, glycine, and alanine occurring in the molar ratios of 1 : 1 : 1 : 3 : 2 : 7 : 1. The β(+t) fraction was composed of 16 different amino acids, lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine, combined at molar ratios of 4 : 1 : 4 : 9 : 5 : 4 : 11 : 3 : 10 : 10 : 7 : 2 : 5 : 7 : 2 : 3 (Table IX).

Figure 6

Distribution of proteinaceous fractions separated by the isoelectric focusing (pH 3-6 range with anode at top) of the β(+t) biopolymer.


toxic β(+t) biopolymer


pH curve


immunodiffusion line detected on reaction with antiserum against type 1 cytoplasm

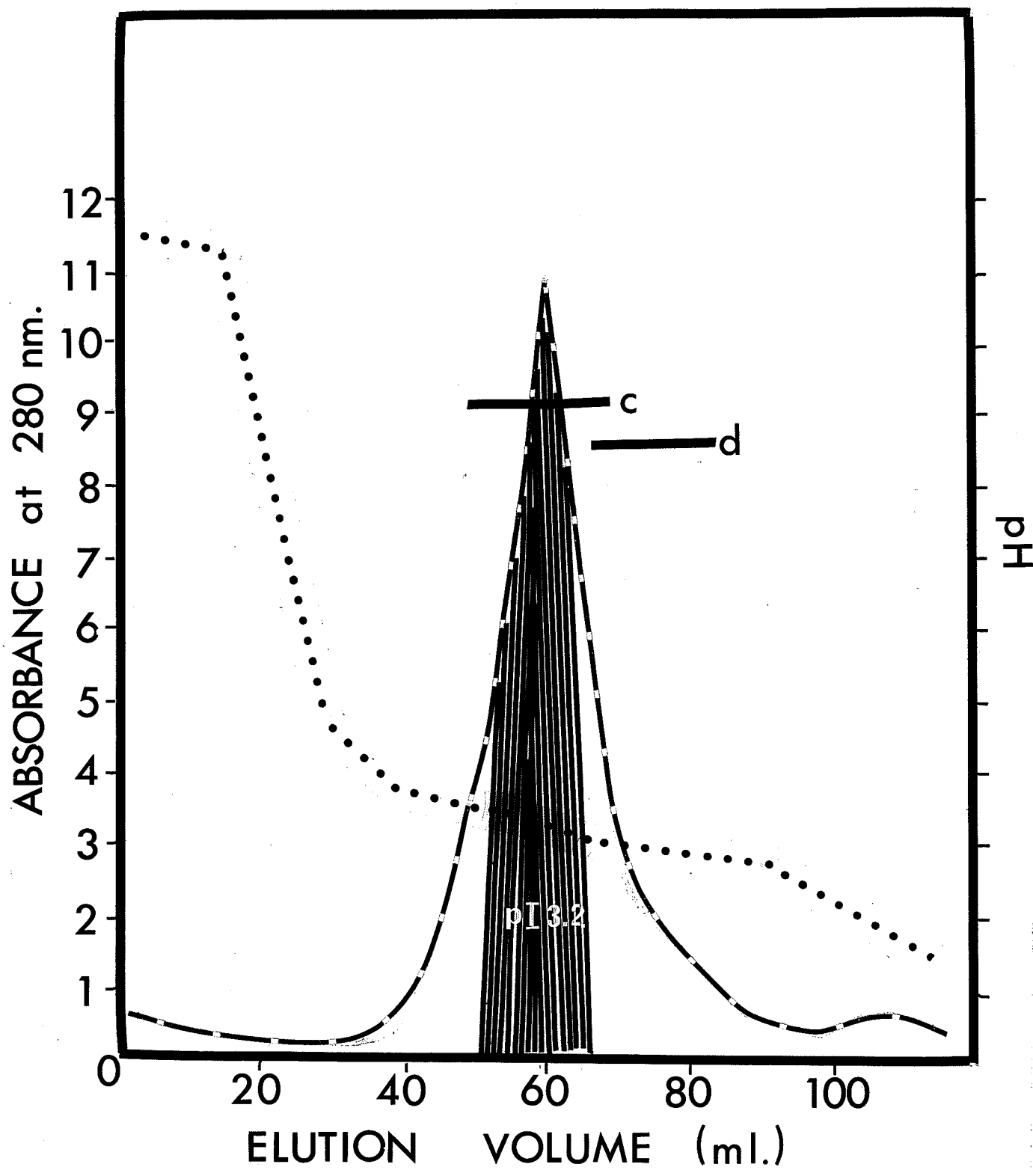

ABSORBANCE at 280 nm.
12
11
10
9
8
7
6
5
4
3
2
1
0
c
d
pI 3.2
pH
20
40
60
80
100
ELUTION VOLUME (ml.)

Figure 7

The profile of β(-t) protein on the preparative polyacrylamide gel electrophoresis.

 non-toxic β(-t) biopolymer

 immunodiffusion line detected on reaction with antiserum against type 1 cytoplasm

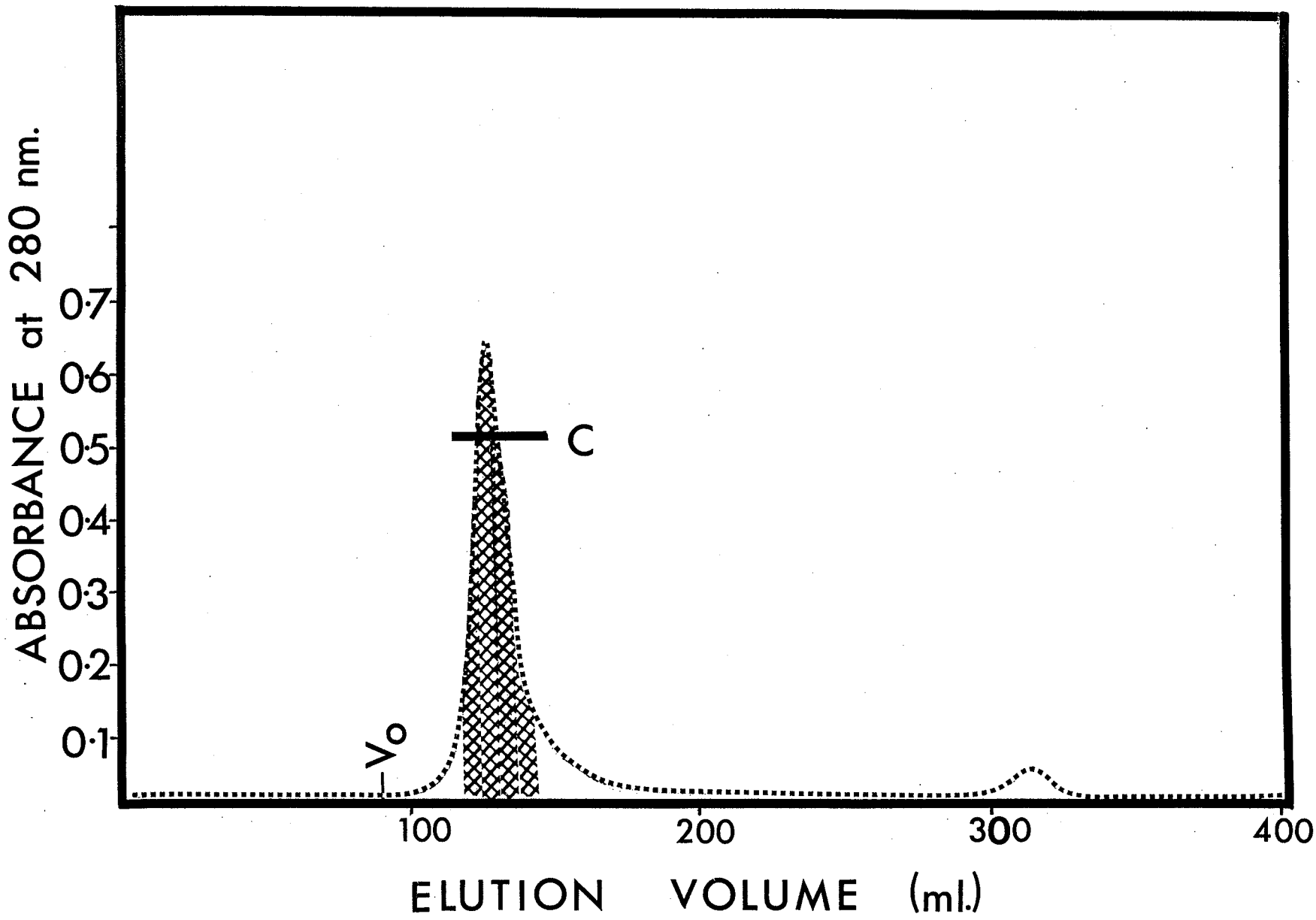

ABSORBANCE at 280 nm.
0·7
0·6
0·5
0·4
0·3
0·2
0·1
C
Vo
100
200
300
400
ELUTION VOLUME (ml.)

TABLE IX

AMINO ACIDS DETECTED IN THE CYTOPLASMIC PROTEINS OF NEISSERIA GONORRHOEAE

Types of Amino Acids	Percentage Molar Composition of: β(+t) protein	β(-t) protein
Lysine	5.03	7.45
Aspartic Acid	10.1	7.56
Threonine	5.63	7.17
Serine	5.03	17.55
Glutamic Acid	11.87	13.00
Glycine	11.24	41.35
Alanine	11.52	5.94
Histidine	1.12	0
Arginine	4.87	0
Proline	3.60	0
Valine	8.03	0
Methionine	2.31	0
Isoleucine	5.33	0
Leucine	8.19	0
Tyrosine	2.53	0
Phenylalanine	3.63	0

II IMMUNOLOGICAL PROPERTIES

Immunodiffusion

On the immunodiffusion, type 1 cytoplasm produced at least 5 precipitin lines (a, b, c, d, and e, starting at the antigen well) when examined with its homologous antiserum (Figure 8), and only 4 lines (a, b, d, and e) when tested with the anti-type 4 cytoplasm serum (Figure 9). The cytoplasm of type 4 also produced 4 lines analogous to a, b, d, and e, as shown by the fusion of the corresponding precipitin lines (Figure 9).

After absorption of the anti-type 1 antiserum with type 4 cytoplasm, the absorbed antiserum only reacted with the type 1 cytoplasm and the β(+t) and β(-t) biopolymers (Figure 10).

On the gel filtration, the toxic biopolymers of type 1 cytoplasm produced 3 precipitin bands (a, c, and d) when examined against the homologous antiserum, and 2 bands (a and d) with the anti-type 4 cytoplasm serum (Figure 11).

The toxic cytoplasmic biopolymers of type 4 separated by gel filtration alone, produced 2 bands (a and d) on the examination with both types of anti-cytoplasm sera. The non-toxic fractions of both types of cytoplasm formed precipitin bands b, d, or e (Refer to Figure 3).

The toxic α biopolymers of type 1 and type 4 cytoplasm, purified by the electrofocusing, yielded a single precipitin band a with both the homologous and heterologous antisera. The β(+t) biopolymer (unique of type 1 cytoplasm), on the other hand, formed two bands, c and d, with the homologous antiserum (Figure 12).

The β(-t) biopolymer purified by preparative polyacrylamide electrophoresis behaved as a homogeneous material. This pure fraction

Figure 8

Precipitin bands formed on reaction between the rabbit antiserum produced against type 1 cytoplasm and the cytoplasms of Neisseria gonorrhoeae.

A = antiserum against type 1 cytoplasm
B, C, and F = type 1 cytoplasms
D and E = type 4 cytoplasms

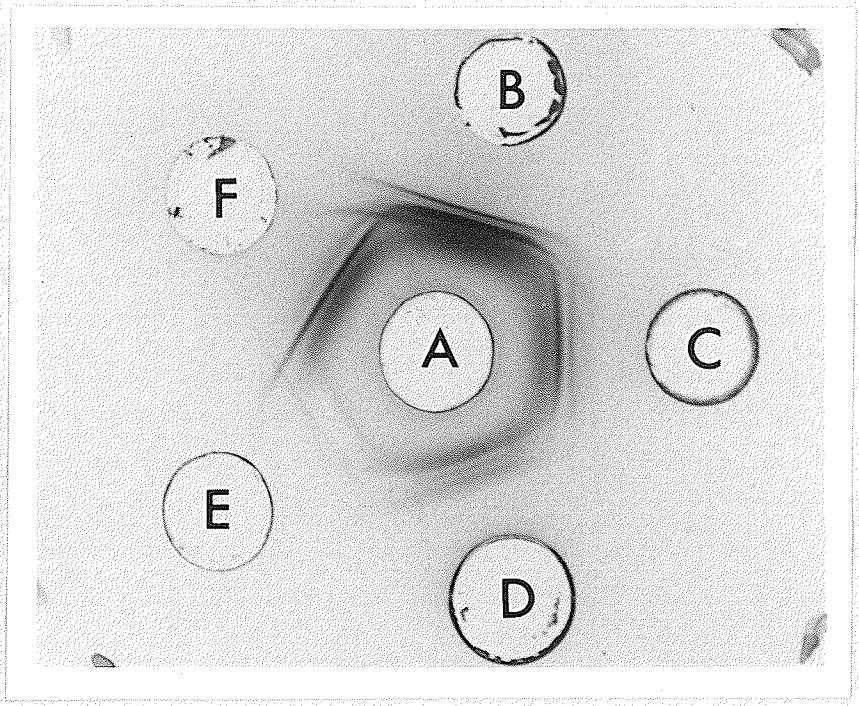

B
F
A
C
E
D

Figure 9

Precipitin bands formed on reaction between the rabbit antiserum produced against type 4 cytoplasm and the cytoplasms of Neisseria gonorrhoeae.

C = antiserum against type 4 cytoplasm

A = type 1 cytoplasm

B = type 4 cytoplasm

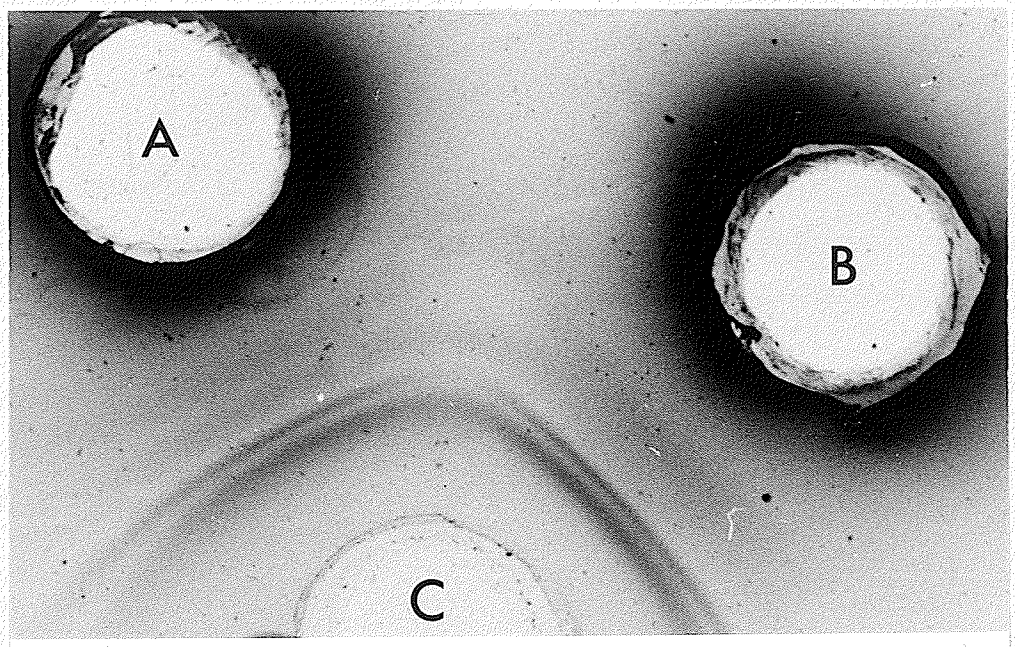

A
B
C

Figure 10

Precipitin bands formed on reaction between the absorbed anti-type 1 cytoplasm serum and the cytoplasms of *Neisseria gonorrhoeae*.

A = type 1 cytoplasm
B = anti-type 1 cytoplasm serum absorbed with type 1 cytoplasm
C = anti-type 1 cytoplasm serum
D = anti-type 1 cytoplasm serum
E = anti-type 1 cytoplasm serum absorbed with type 4 cytoplasm
F = anti-type 4 cytoplasm serum

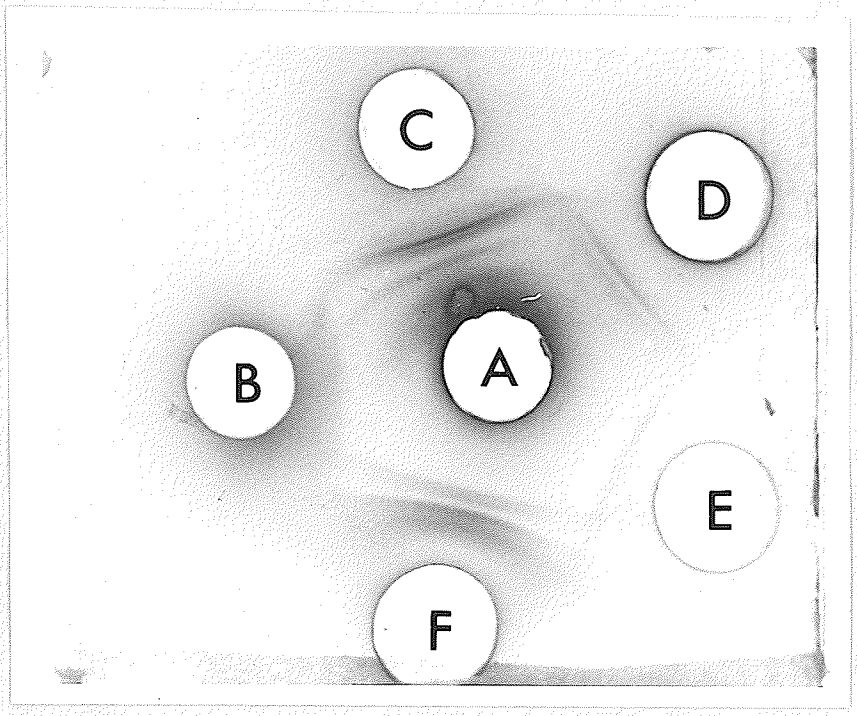

C
D
B
A
E
F

Figure 11

Precipitin bands formed on reaction between the rabbit antiserum produced against type 1 cytoplasm and the toxic biopolymers of Neisseria gonorrhoeae cytoplasms.

A = toxic biopolymer of type 4 cytoplasm separated by gel filtration

B = toxic biopolymer of type 1 cytoplasm separated by gel filtration

C = antiserum against type 1 cytoplasm

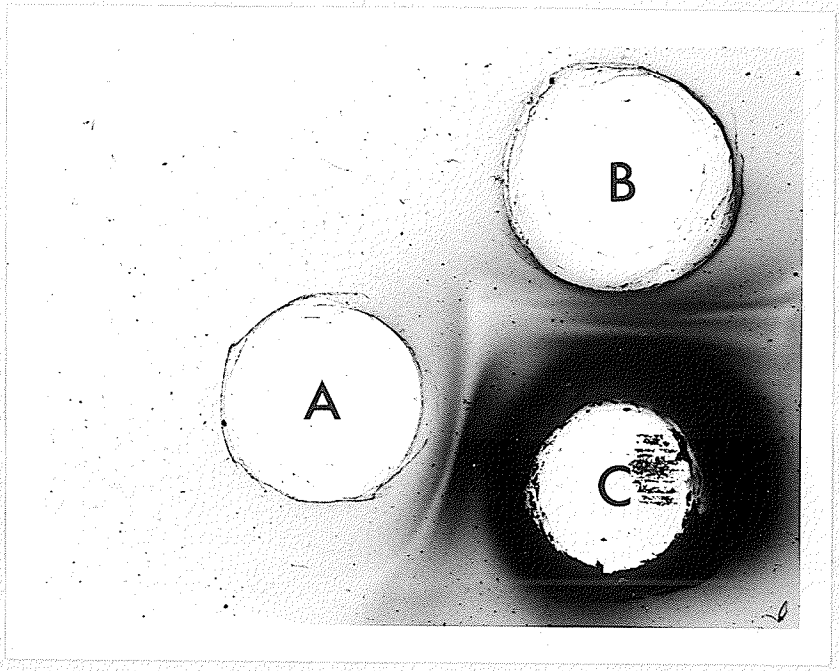
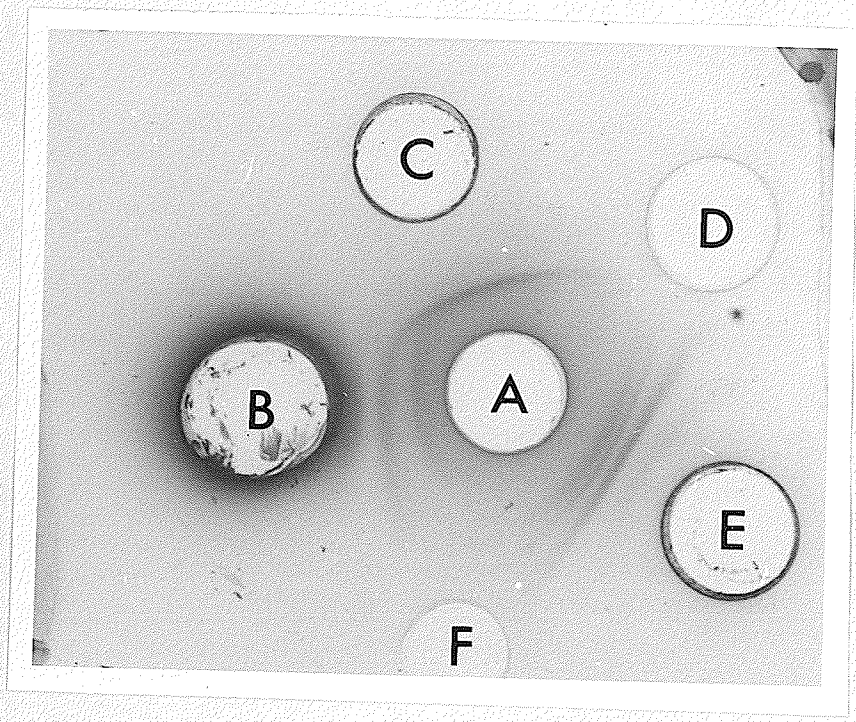

B
A
C

Figure 12

Precipitin bands formed on reaction between the rabbit antiserum produced against type 1 cytoplasm and the antigens prepared from Neisseria gonorrhoeae cytoplasms.

A = antiserum against type 1 cytoplasm
B = β(+t) biopolymer
C = β(-t) biopolymer
D = phosphate-buffered saline
E = type 1 cytoplasm
F = pI 3.2 area obtained from the type 4 cytoplasm by isoelectric focusing


C
D
B
A
E
F

yielded a single precipitin line with the anti-type 1 cytoplasm serum but not with the antiserum against type 4 cytoplasm (Figure 12). The homogeneity of the β(-t) biopolymer was also demonstrated by its production of a monospecific antiserum. This anti-β(-t) antiserum reacted with different preparations of type 1 gonococcal cytoplasm to form a single precipitin line but not with the type 4 cytoplasm (Figure 13).

Immunodiffusion reactions between Neisseria gonorrhoeae cytoplasms and human sera revealed that 6 of the 10 sera obtained from gonorrhea patients reacted with the type 1 cytoplasm to form a single precipitin line. This immunoprecipitin line corresponded to line c as shown by the fusion when the same cytoplasm reacted with the homologous hyperimmune rabbit antiserum (Figure 14). In contrast, the type 4 cytoplasm showed no reaction with the patients' sera tested. Both type 1 and type 4 cytoplasm did not react with sera obtained from people without a previous history of gonorrhea.

Immunoelectrophoresis

The cytoplasm of type 1 Neisseria gonorrhoeae, upon micro-immunoelectrophoresis, revealed 4 antigens (corresponding to lines a, c, d, and e in immunodiffusion) which migrated towards the anode. One of the antigens (corresponding to line b in immunodiffusion) was shown to be positively charged, as demonstrated by its migration towards the cathode in an electrical field.

The toxic α biopolymers of type 1 and type 4 cytoplasm, purified by the electrofocusing, yielded a single precipitin line which migrated towards the anode upon reaction with both the homologous and heterologous antisera (Figure 15).

Figure 13

Precipitin bands formed on reaction between the rabbit antiserum produced against the β(-t) biopolymer and the cytoplasms of Neisseria gonorrhoeae.

A = antiserum against β(-t) biopolymer
B = type 1 cytoplasm
C = type 1 cytoplasm
D = type 1 cytoplasm diluted 1:10
E = type 4 cytoplasm
F = type 1 cytoplasm diluted 1:10

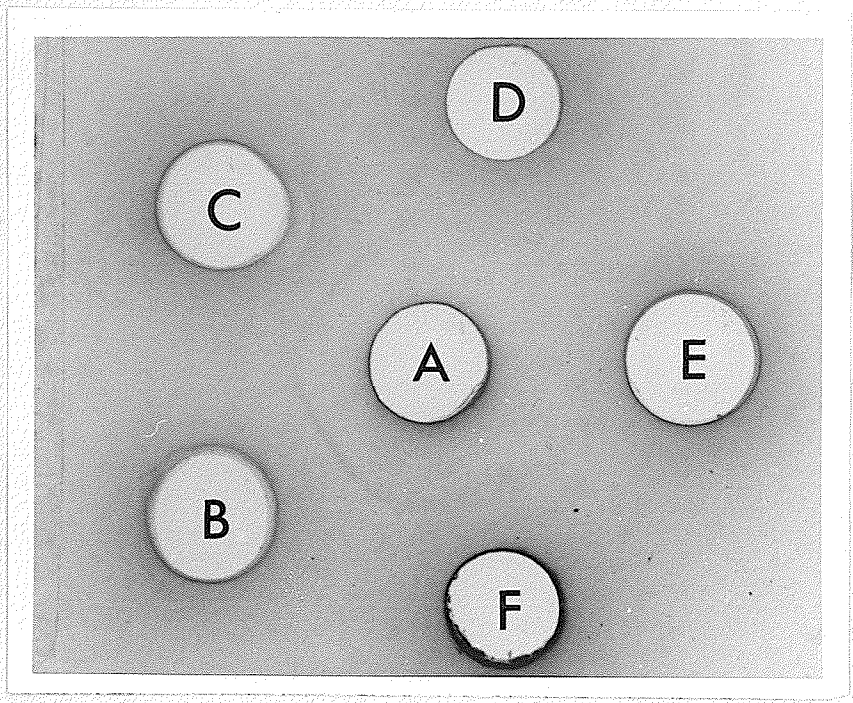

D
C
A
E
B
F

Figure 14

Precipitin bands formed on reaction between the hyperimmune rabbit antiserum and human sera and the cytoplasms of Neisseria gonorrhoeae.

A = type 1 cytoplasm
B = type 4 cytoplasm
C = antiserum against type 1 cytoplasm
D = serum obtained from patient with acute uncomplicated gonococcal infection
E = antiserum against type 1 cytoplasm
F = serum obtained from normal human

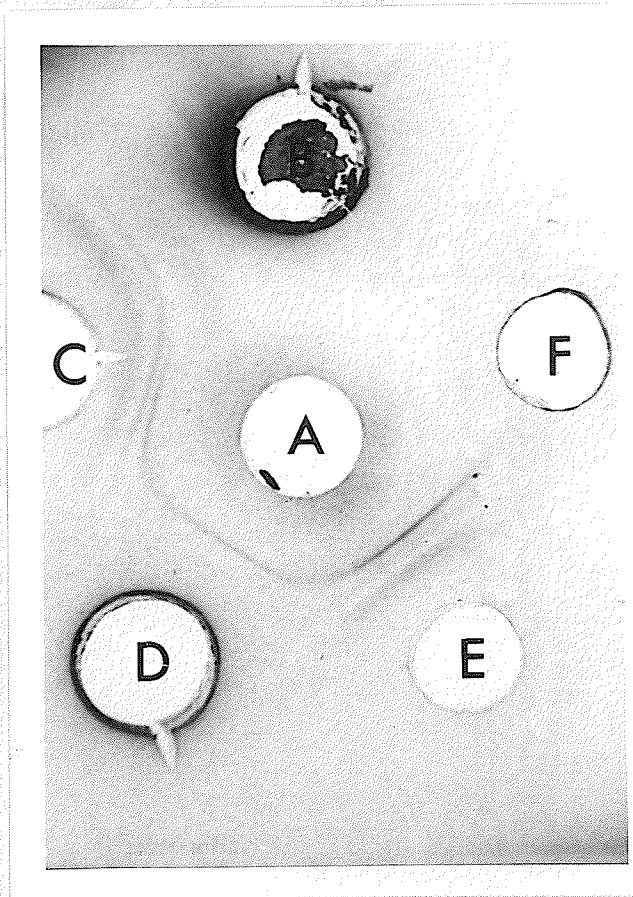
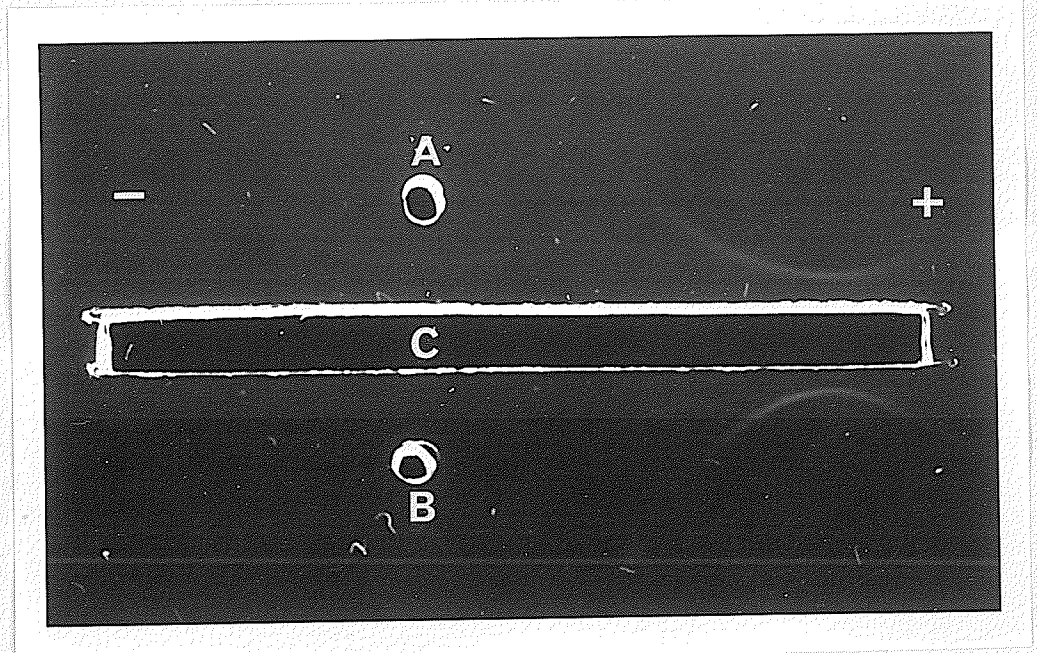

C
F
A
D
E

Figure 15

Immunoelectrophoresis of the toxic α biopolymers of Neisseria gonorrhoeae cytoplasms.

A = toxic α biopolymer of type 1 cytoplasm
B = toxic α biopolymer of type 4 cytoplasm
C = antiserum against type 1 cytoplasm


A
−
+
C
B

After preparative polyacrylamide electrophoresis, the pure β(-t) biopolymer produced a single precipitin line located close to the antigen well (Figure 16). The β(-t) fraction also migrated towards the anode, but with a slower migration speed than the α biopolymer.

Immunogenicity of the β(+t) and β(-t) Biopolymers

Results obtained by radial-immunodiffusion showed that rabbits responded to the β(-t) biopolymer much more rapidly than to β(+t) fraction (Figure 17). A considerable increase of precipitating antibodies to the β(-t) biopolymer was produced within 10-12 days whereas the primary response to the β(+t) fraction was negligible. The secondary humoral response to both biopolymers was noted to occur almost immediately and reached the peak within 15-20 days after the booster injections.

The dermal hypersensitivity to the biopolymer β(+t) occurred within 3-4 hours reaching its highest intensity in 12-16 hours and declining rapidly in 24-48 hours. In contrast, the dermal hypersensitivity to the β(-t) biopolymer was only noted after 36-48 hours; it increased slowly and persisted for a period of 48-72 hours thereafter (Figure 18).

Figure 16

Immunoelectrophoresis of the non-toxic β(-t) biopolymer of type 1 cytoplasm of *Neisseria gonorrhoeae*.

A = β(-t) biopolymer

B = type 1 cytoplasm

C = antiserum against type 1 cytoplasm

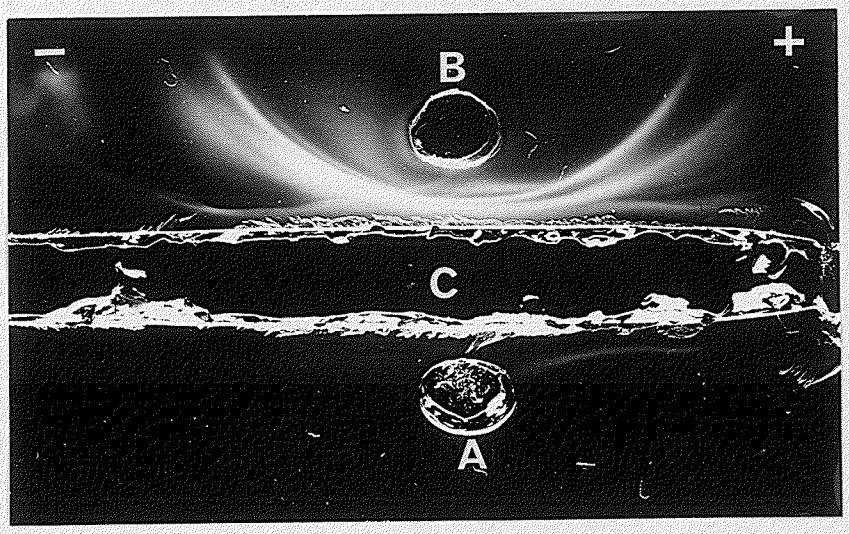

−
+
B
C
A

Figure 17

Profile of antibody response of the β(+t) and β(-t) proteins, as shown by radial-immunodiffusion.

——— β(-t) biopolymer

..... β(+t) biopolymer

↑ booster injection

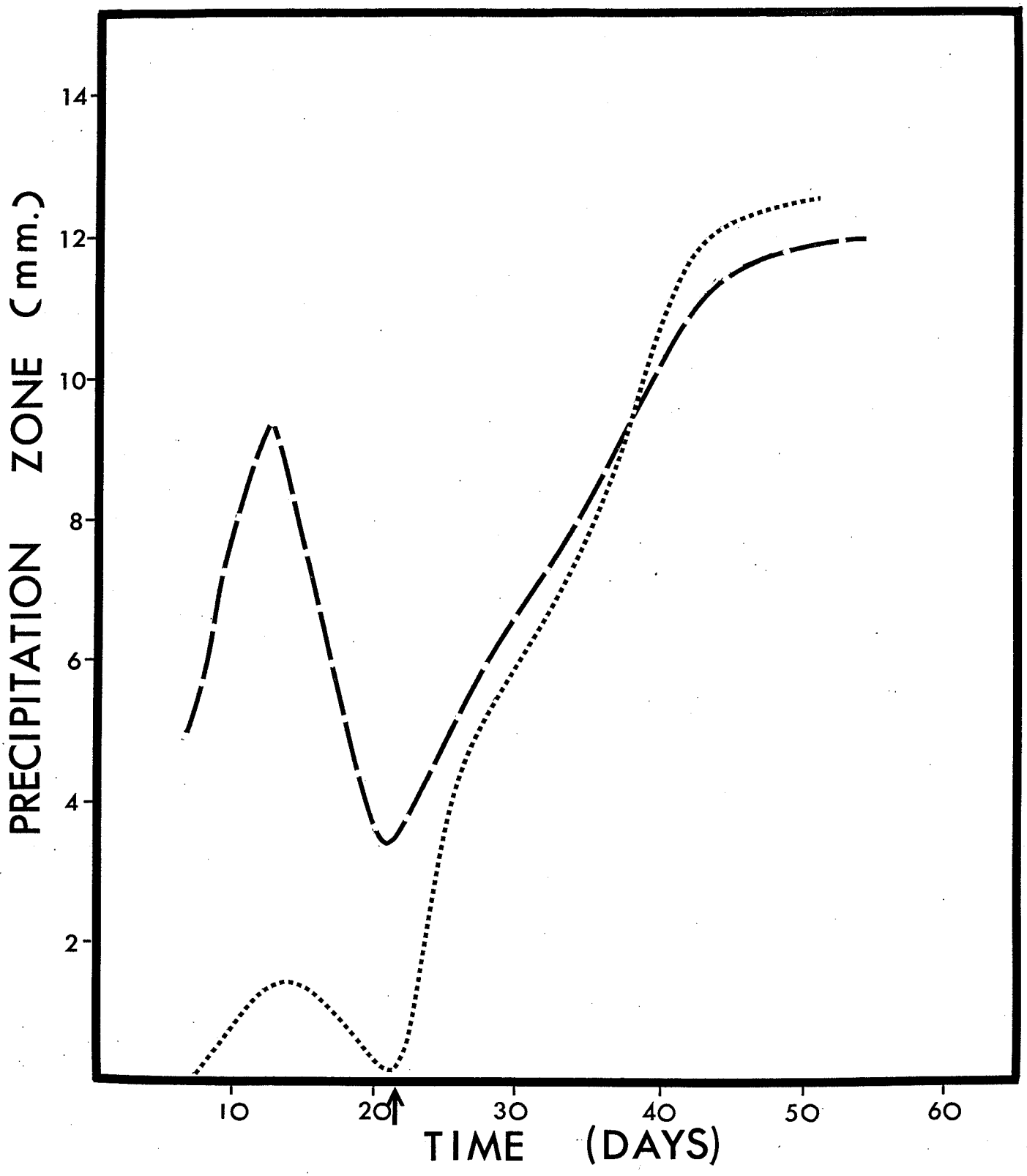

PRECIPITATION ZONE (mm.)
14
12
10
8
6
4
2
10
20
30
40
50
60
TIME (DAYS)

Figure 18

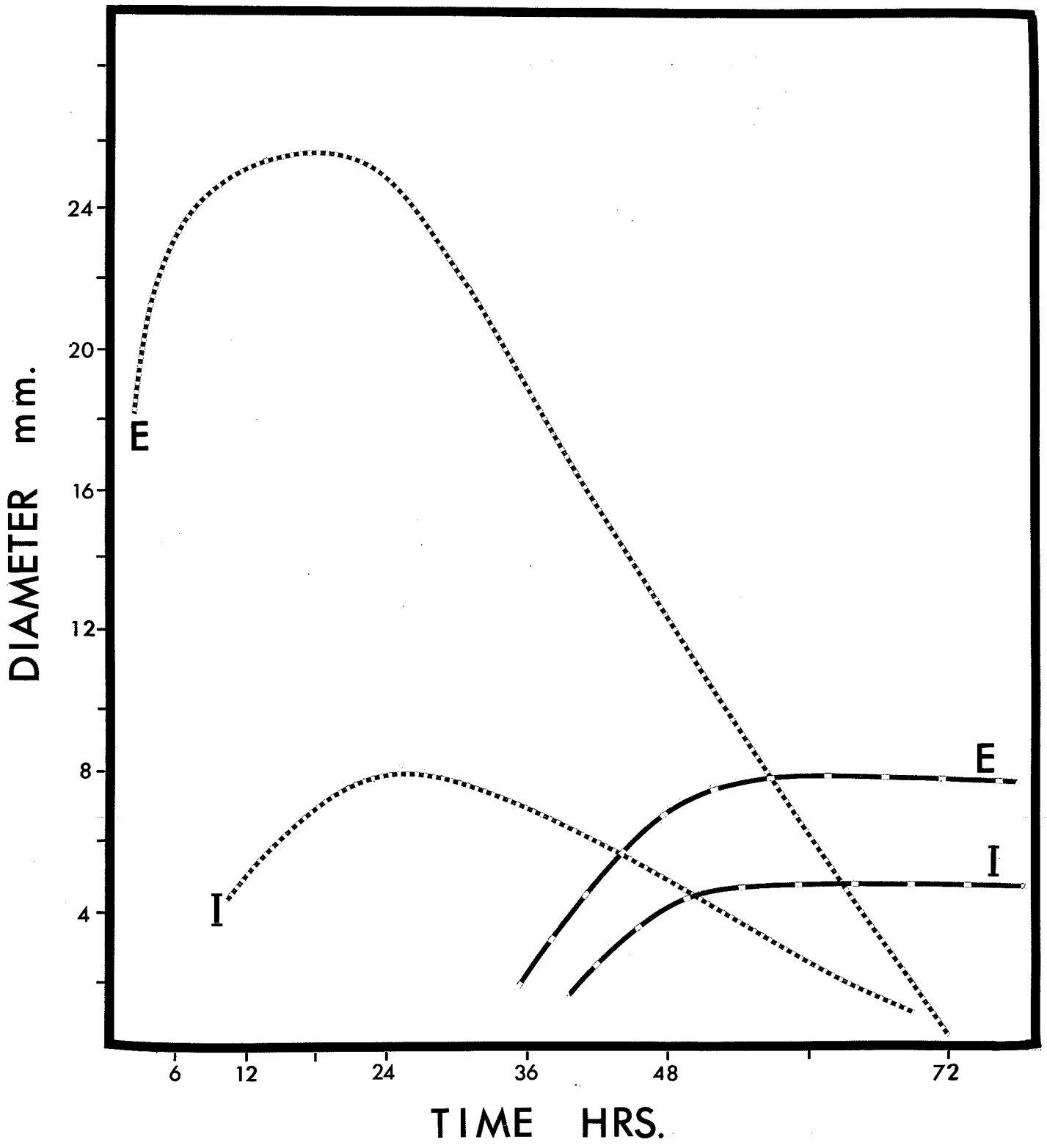
Patterns of dermal hypersensitivity response to the β(-t) and β(+t) biopolymers.

——— β(-t) biopolymer

..... β(+t) biopolymer

E = erythema

I = induration


DIAMETER mm.
24
20
16
12
8
4
E
I
E
I
6
12
24
36
48
72
TIME HRS.

III BIOLOGICAL PROPERTIES

Toxicity Assay

By the intravenous toxicity test performed on 11-day old chicken embryos, the cytoplasms of Neisseria gonorrhoeae was found to be lethal. However, type 1 cytoplasm was 4 times more toxic than that of the type 4 cytoplasm, as shown by the LD_{50} values of 1.667 and 6.695 per μg protein, respectively.

On the gel filtration of type 1 and type 4 cytoplasm, the fastest peak proved to contain a material toxic for chicken embryos; whereas the 2 other concentration zones contained non-toxic materials (Refer to Figure 3).

The α fractions of type 1 and type 4 cytoplasm, obtained after electrofocusing at pH 3-10 range (Figures 4 & 5), proved to be toxic to chicken embryos at the LD_{50} values of 3.0 and 2.1 per μg protein, respectively. The toxicity of the β(+t) biopolymer, expressed in terms of LD_{50} value per μg protein, was about 4 fold that of the α biopolymer obtained from the same cytoplasm and about 3 fold greater than that of the α fraction from type 4 cytoplasm (Table X).

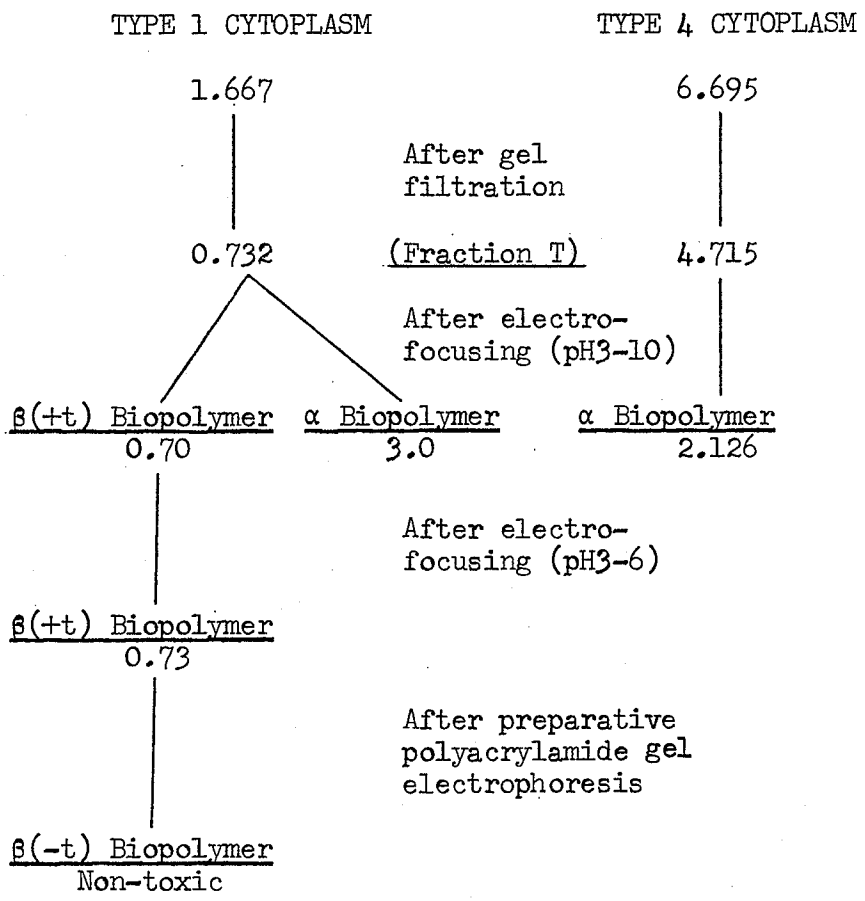
The toxicity of the β(+t) fraction obtained from electrofocusing at pH range of 3-6 was no greater than that from electrofocusing at pH 3-10 range, as shown by the LD_{50} values of 0.73 and 0.70 per μg protein, respectively.

The major peak material β(-t), obtained after further purification of the β(+t) biopolymer by preparative polyacrylamide electrophoresis, proved to be non-toxic.

TABLE X

THE TOXICITY OF α AND β(+t) FRACTIONS FOR CHICKEN EMBRYOS

LD_{50} (μg protein) of:



Physicochemical Properties of the Toxic Biopolymers

The toxic α fractions isolated from both types of Neisseria gonorrhoeae proved to be sensitive to periodate oxidation but not to the proteolytic enzymes or to the temperature of 56 °C. In contrast, the toxic β(+t) biopolymer was heatlabile and sensitive to proteolytic enzymes, but it was resistant to periodate oxidation. Such differences were not noted when crude cytoplasms or the materials separated by gel filtration were examined (Table XI). None of the materials was destroyed by nucleases.

Neutralization with Hyperimmune Rabbit Sera

Results obtained from neutralization test revealed that the hyperimmune rabbit anti-cytoplasm sera possessed neutralizing activity. Anti-type 1 cytoplasm serum, even diluted 10 times, neutralized completely the toxicity of both type 1 and type 4 cytoplasm. In contrast, antiserum prepared against type 4 cytoplasm only neutralized the toxicity of type 4 cytoplasm but not that of type 1 (Table XII). Normal rabbit serum had no neutralizing activity.

Neutralization with Human Sera

The sera from patients with gonococcal infections either abolished or reduced the toxicity of the cytoplasms and their toxic biopolymers α and β(+t). The neutralizing power of these patient's sera was 75-100%, as calculated from the survival rates of the chicken embryos. The serum obtained from a patient (MB) who also was the source of one of the gonococcal strains, was found to neutralize completely the toxicity.

TABLE XI

PHYSICOCHEMICAL PROPERTIES OF NEISSERIA GONORRHOEAE CYTOPLASM AND ITS BIOPOLYMERS

Sensitivity to	Death Rate on Challenge of Chicken Embryos with				
	Crude Cytoplasm		Gel-filtration	Electrofocusing	
	Type 1	Type 4	Fraction T	α	β(+t)
56 °C, 30 min.	18*/20**	17/20	18/20	20/20	1/20
Trypsin	14/20	15/20	15/20	10/20	1/20
Periodate	18/20	9/20	17/20	0/20	18/20
DNase	20/20	20/20	20/20	20/20	20/20
RNase	20/20	20/20	20/20	20/20	20/20

* The numerator represents number of chicken embryos died

** The denominator represents number of chicken embryos injected

TABLE XII

NEUTRALIZATION WITH HYPERIMMUNE RABBIT SERA

Rabbit Antiserum against	Anti-serum Dilution	Death Rate on Challenge of Chicken Embryos with Type 1 cytoplasm	Type 4 cytoplasm
Type 1 cytoplasm	1:10	0*/40**	0/20
	1:50	6/40	2/20
	1:100	20/20	12/20
Type 4 cytoplasm	1:1	20/20	0/20
Normal Rabbit Serum	1:1	20/20	20/20

* The numerator represents the number of chicken embryos died

** The denominator represents the number of chicken embryos injected

The normal sera also demonstrated slight neutralizing activity. In contrast, this activity was significantly lower (15-25%) than that of the patients' sera (Table XIII).

Immunoprotection Assay

In the group of rabbits pre-injected with the β(-t) biopolymer, the corneas became diffusely cloudy during the initial 36-48 hours after the injection of live gonococci into the anterior eye chamber. These symptoms were accompanied by a profuse discharge of a serous fluid from the conjunctivas which lasted for about 72 hours. At approximately 48 hours, the cloudiness began to subside and the corneas were entirely clear within 4-5 days after the challenge with live gonococci (Figure 19). A slower and partly incomplete disappearance of corneal lesions was observed in normal rabbits that received an injection of 0.1 ml of filtered anti-β(-t) antiserum pre-incubated at 37 °C for 5 minutes with the same dose of live gonococci as used for other rabbits.

In contrast, the corneas of rabbits pre-injected with the biopolymer β(+t), and then challenged with live gonococci, as well as normal rabbits that received an anterior chamber injection of live gonococci pre-incubated with a normal rabbit serum, and normal rabbits injected with live gonococci alone, showed a rapidly progressing cloudiness of the entire cornea with the pus accumulated in the centre. These symptoms were aggravated during the 7-10 day observation period and would persist and cause blindness (Figure 20).

In the group of rabbits injected with PBS, the corneas showed no signs of infection.

Samples of aqueous fluids aspirated from the anterior eye chambers

TABLE XIII

NEUTRALIZATION WITH HUMAN SERA

	Death Rate on Challenge of Chicken Embryos with				
	Crude Cytoplasm		Gel-filtration	Electrofocusing	
	Type 1	Type 4	Fraction T	α	β(+t)
Normal Sera:					
AC	32*/40**	18/20	17/20	15/20	16/20
AR	15/20	17/20	14/20	16/20	13/20
Patient's Sera:					
MB	0/20	0/20	0/20	0/20	0/20
MK	1/20	1/19	2/35	0/20	0/20
VV	3/20	5/20	5/20	3/20	4/19
RR	6/20	4/20	6/20	4/20	1/20
FB	4/20	5/20	2/18	1/20	1/20

* The numerator represents the number of chicken embryos died

** The denominator represents the number of chicken embryos injected

Figure 19

The cornea of a rabbit immunized with the β(-t) biopolymer and challenged with live type 1 Neisseria gonorrhoeae.

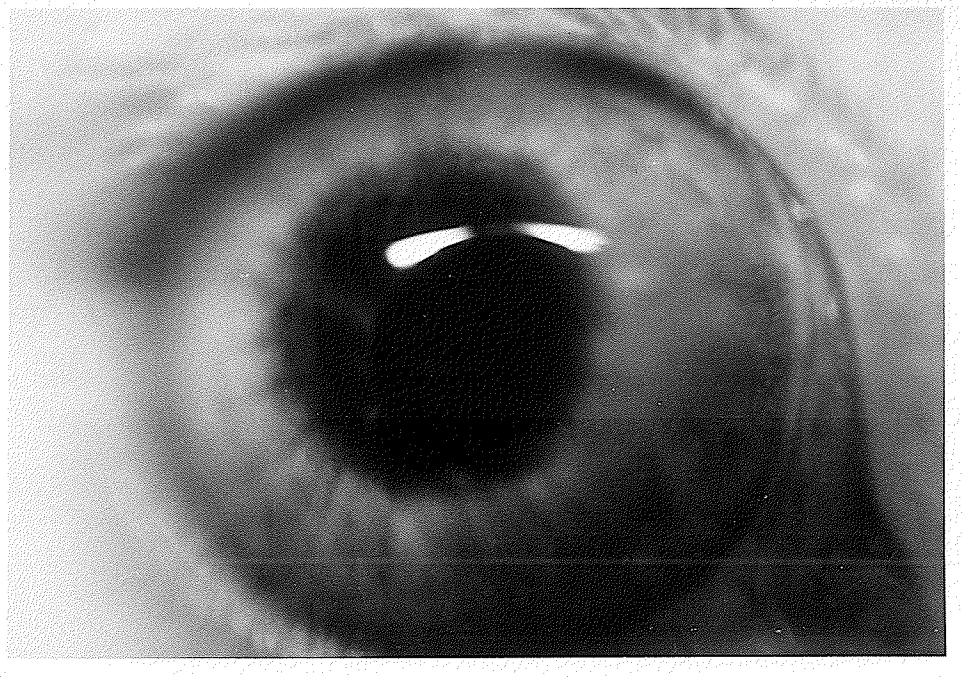
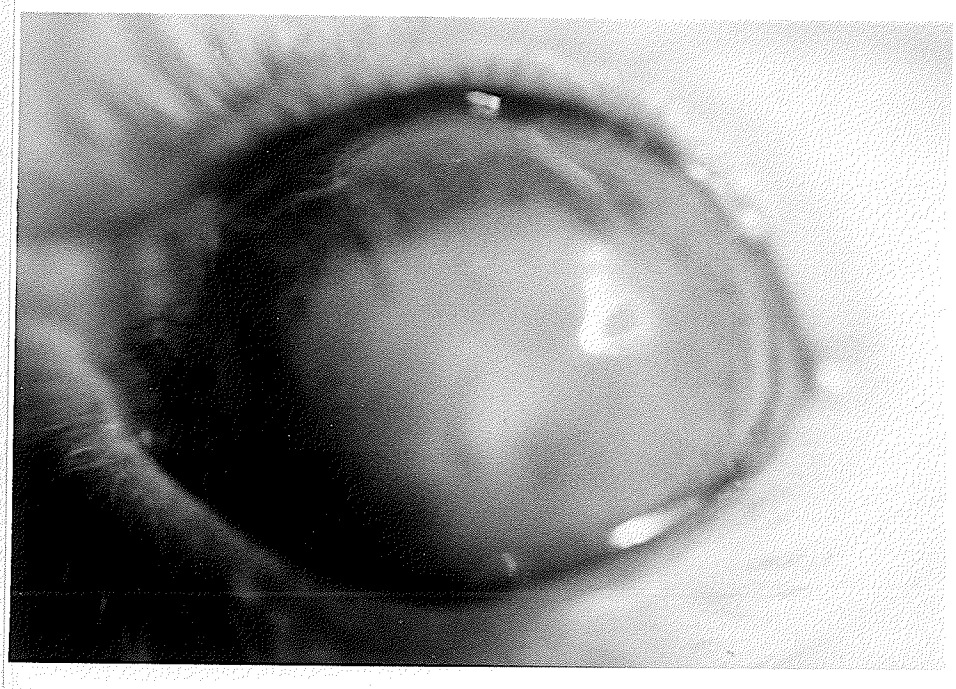


Figure 20

The cornea of a rabbit pre-injected with the β(+t) biopolymer and challenged with live type 1 Neisseria gonorrhoeae.



of rabbits immunized with the β(-t) biopolymer, inoculated on the enriched GC medium, yielded no growth of gonococci. In contrast, the samples obtained from the anterior chambers of the others, including those animals that received live gonococci pre-incubated with anti-β(-t) antiserum, produced cultures of type 1 Neisseria gonorrhoeae (Table XIV).

TABLE XIV

PROTECTION OF RABBITS INJECTED WITH β(-t) AND β(+t) BIOPOLYMERS AGAINST ANTERIOR CHAMBER INFECTION WITH NEISSERIA GONORRHOEAE

	No. of Eyes Injected	No. of Eyes Infected	+ve Culture from Aqueous Humour
Rabbits pre-injected with the β(-t) biopolymer and challenged with type 1 gonococci	4	0	0
Rabbits pre-injected with the β(+t) biopolymer and challenged with type 1 gonococci	4	4	2
Normal rabbits challenged with type 1 gonococci pre-incubated with anti-β(-t) antiserum	2	2	2
Normal rabbits challenged with type 1 gonococci pre-incubated with normal rabbit serum	2	2	2
Normal rabbits challenged with type 1 gonococci	4	4	4
Normal rabbits challenged with PBS	4	0	-

DISCUSSION

Although the virulence of Neisseria gonorrhoeae was found to be associated with the clonal types (Kellogg et. al., 1963, 1968; Buchanan and Gotschlich, 1973; Bumgarner and Finklestein, 1973), the chemical basis of the biological difference has not been elucidated. Our investigations have revealed electrophoretic difference between clonal type 1 and type 4 cytoplasm of Neisseria gonorrhoeae. It has been found by isoelectric focusing and disc-electrophoresis that the clonal type 1 alone contained a biopolymer which was lethal for chicken embryos and that it accounted for the increased toxicity of type 1 crude cytoplasm compared with that of type 4.

Two toxic moieties a heat-stable, periodate sensitive α fraction and a heat-labile periodate resistant $\beta(+t)$ factor were isolated in our studies from clonal type 1 cytoplasm. These two biopolymers may be similar to those previously reported by Maeland (1967, 1968, 1969, and 1971) who detected two immunochemical components, one carbohydrate and one protein, in an aqueous ether extract of gonococci. The α biopolymer isolated from both types of cytoplasm in our investigation was also similar to the subgroup antigens reported by Apicella and Allen (1973 and 1974). The similarities were based on the findings that these subgroup antigens were polysaccharide in nature and were located at pI 4-4.5 region in electrofocusing. Although Maeland considered his antigens to be derived from endotoxin, and the subgroup antigens were still of unknown origin, these preparations may have contained cytoplasm.

Our investigations revealed that the cytoplasm and the toxic $\beta(+t)$ biopolymer preparations did not contain endotoxin contaminations, as shown by the evidences that:

1) the toxicity of the cytoplasms was neutralized by the homologous

antisera,

2) the toxicity of the β(+t) biopolymer was neutralized only by the anti-type 1 cytoplasm serum, and to a significant extent by the patients' sera, and

3) the β(+t) biopolymer was a heat-labile protein.

A correlation between the presence of pili and the virulence has been shown by Punsalang and Sawyer (1973), and Swanson et. al. (1973). It was noted that the clonal type 4 Neisseria gonorrhoeae is not piliated and that pili enable the gonococci to attach to epithelial cell surfaces and confer resistance to phagocytosis. In our investigation, pili appeared to be sheared from the organism early in the prepartion of cytoplasm, and none was seen on electron microscopy in a crude cytoplasm preparation. Furthermore, high titre antiserum against the purified β(-t) antigen failed to bind purified 2868 pili in an antigen binding assay (Buchanan, T.M., Personal Communication, 1974).

Further differences between virulent and avirulent types of Neisseria gonorrhoeae were demonstrated immunologically in our studies. With the aid of absorption, it was revealed that the β(+t) component of type 1 accounted for this difference. Danielsson et. al. (1969) and Peacock and Schmale (1971) identified protoplasmic constituents from clonal type 1 and type 4 with Sephadex G-200 gel filtration and reported major areas of polymer concentrations similar to that shown in Figure 3. Although these investigators did not observe antigenic differences between the clonal types, they did find a high molecular weight fraction present in the first peak which was toxic for chicken embryos and reacted with sera from patients infected with gonococci. This heat-labile constituent was designated antigen A and may be identical with the β(+t) fraction.

Antigen A was also readily isolated from soluble gonococcal protoplasm with ion-exchange chromatography.

Purification by preparative polyacrylamide gel electrophoresis and the evidence obtained from chemical analysis revealed the presence of two distinct types of proteins in the β(+t) fraction, one of which was non-toxic. The non-toxic β(-t) biopolymer consisted of only 7 different types of amino acids and produced a single immuno-precipitation line with only the anti-type 1 cytoplasm serum. The exact nature of the other component has not yet been elucidated, but possibly it should account for the following 9 amino acids missing from the β(-t) protein, namely: histidine, arginine, proline, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine.

Theoretically, the loss of toxicity that occurred after subjecting the β(+t) biopolymer to preparative polyacrylamide gel electrophoresis may depend on one or more of the following phenomena: 1) depolymerization of the macromolecule, 2) the loss of certain toxic amino acids during the purification procedure, or 3) the loss of a chemical linkage or a change of spatial arrangements of certain amino acids responsible for a toxic effect.

It must be emphasized that the toxicity elicited by the cytoplasm and the β(+t) biopolymer had only been studied in chicken embryos. Other systems such as tissue culture, mice, and guinea pigs have not been tested due to the limited amount of material and time.

The purified β(-t) biopolymer behaved as a single and homogeneous entity was provided by the following experimental observations:
1) according to charge and molecular size criteria, the β(-t) biopolymer migrated as a single band in both preparative polyacrylamide and analytical

disc-gel electrophoresis,

2) according to charge and pH criteria, the β(-t) biopolymer appeared as a single peak at a pI of 3.4 in isoelectric focusing performed at different pH ranges,

3) according to immunological criterion, the β(-t) biopolymer reacted with the anti-cytoplasm serum to form one precipitation band. The antiserum against β(-t) biopolymer also formed a single precipitation band with either the cytoplasm and the β(-t) protein,

4) finally, after the treatment with sodium dodecyl sulphate (SDS), the β(-t) biopolymer still migrated as a single component in polyacrylamide gel electrophoresis. This clearly indicates that the β(-t) protein occurs as a single entity and is not composed of subunits of polypeptide aggregates.

The finding that the β(-t) biopolymer is present only in type 1 but not in type 4 gonococcal cytoplasm may have several implications:

1) it may help to elucidate the association of colonial morphologies with various types of Neisseria gonorrhoeae,

2) it may serve to explain the linkage between the differences in clonal types and virulence of the organism, and

3) it may also be useful for a more extensive understanding of the nature of the immune response of the host in gonorrhea, the pathogenic mechanisms of the infecting agent, and the procedures in the development of an effective prophylactic immunogen.

Since only three strains of Neisseria gonorrhoeae (MB, BY, and F62) had been tested so far, the distinction of the β(-t) biopolymer present in type 1 Neisseria gonorrhoeae cannot be concluded as yet at the present time. Three possibilities may exist as pertaining to the presence of this non-toxic β(-t) protein:

1) the β(-t) biopolymer really exists as a type-specific antigen, thus, it occurs exclusively in the virulent types of Neisseria gonorrhoeae,

2) the β(-t) fraction only occurs as a strain-specific antigen and may be absent from other strains, and

3) the β(-t) protein may also be present in type 4 cytoplasm in very minute quantity.

However, within the limits of the detection methods employed in this project, the presence of a unique β(-t) biopolymer was found to be the rule.

It has been shown that during the course of an uncomplicated gonococcal infection, the gonococci induce in the infected patient an antibody response of circulating immunoglobulin G, to a lesser extent immunoglobulin M, and also of local secretory immunoglobulin A (Cohen, Kellogg, and Norins, 1969). These antibodies appear with infection and disappear when infection subsides. The immunoglobulin G has been suggested to be a precipitin antibody and is more indicative of active disease, whereas the immunoglobulin M is an inhibitory antibody which is more reflective of the ability to resist gonorrhea and may confer some measure of long-term protection (Greenberg, Diena, Kenny, and Znamirowski, 1971). It is still unknown whether these antibodies influence gonococcal-host cell interaction in any way. However, these humoral antibodies do not seem to play an important part in gonococcal immunity, although volunteers with circulating antibodies are more resistant to experimental infection than those without it. This is shown by the fact that patients with gonorrhea who demonstrate a significant level of humoral antibodies may still be re-infected. The acute disease has also been produced experimentally in human volunteers suffering from chronic gonorrhea, despite the presence of anti-gonococcal antibodies in their sera.

Cell-mediated response, on the other hand, has been shown to occur during the second week of infection and increase in strength of reaction with the duration of infection or the presence of clinical complications (Kraus, Perkins, and Geller, 1970; Grimble and McIllmurray, 1973). Although this cell-mediated response, as suggested by the above authors, is rather insignificant and does not seem to protect patients against further infection with gonorrhea, it may have played an important role in limiting the attack period when specific therapy is unavailable. Furthermore, it has to be emphasized that the antigens used for the delayed-type skin hypersensitivity and lymphocyte transformation experiments were crude, non-purified materials.

The use of crude materials in the form of killed whole-cell vaccine may also explain the only partial success of different vaccine trials in human volunteers (Tulloch, 1929; Greenberg et. al., 1971) and in chimpanzees (Arko, Kraus, Brown, Buchanan, and Kuhn, 1974). Systemic immunization with this crude vaccine preparation only partially protects the experimental subjects from getting the infection. This protective immune response appears to be related to the challenge inoculum dosage and strain-specific. The number of such immunogens present in the virulent types of gonococcus is still unknown. It is possible that the β(-t) biopolymer obtained from the three strains of virulent Neisseria gonorrhoeae is an antigen of this type.

The immunity conferred by the β(-t) factor against rabbit anterior chamber gonococcal infection may arise from an action of immunocompetent cells rather than from humoral antibodies. This is supported by the evidence that no gonococci could be subcultured after 7 days from the anterior chamber of rabbits immunized with the β(-t) biopolymer; whereas

gonococci could be recovered from the aqueous humour of rabbits that received live gonococci pre-incubated with anti-β(-t) antiserum. Other *in vitro* tests (macrophage migration inhibition and lymphocyte transformation) and *in vivo* test (corneal hypersensitivity) to show that the β(-t) biopolymer indeed produces a cell-mediated immune response are being carried out currently in another project.

CONCLUSION

The cytoplasms of type 1 and type 4 Neisseria gonorrhoeae, passed through 0.2 μ membrane filters, were resolved on Sephadex G-200 columns, followed by repeated isoelectric focusing of peak-zone materials. By both isoelectric focusing and disc-electrophoresis, the type 1 cytoplasm contained one extra component (β+t). The partly purified, unique biopolymer of type 1 cytoplasm proved to be three to four times more toxic for chicken embryos than was another toxic material (α) found in both cytoplasms. The toxicity of the β(+t) biopolymer was neutralized only by the anti-type 1 but not anti-type 4 cytoplasm serum. Furthermore, the toxicity of the β(+t) fraction was neutralized to a significant extent by the sera of patients with uncomplicated gonorrhea, but not by normal human sera. The β(+t) biopolymer was identified as a protein that had no immunological counterpart among the remaining cytoplasm components.

Further purification of the toxic β(+t) fraction by preparative polyacrylamide electrophoresis produced a pure non-toxic β(-t) protein. Chemical evidence showed that the β(-t) biopolymer consisted of 7 amino acids (lysine, serine, aspartic acid, threonine, glutamic acid, glycine, and alanine); whereas the β(+t) biopolymer contained, in addition, nine more amino acids (histidine, arginine, proline, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine).

The immune response in rabbits elicited by the β(+t) and β(-t) biopolymers revealed the following differences:

1) the primary humoral response to β(-t) was much greater than that to β(+t) biopolymer which caused negligible primary response,

2) the secondary humoral response to both biopolymers was very strong but the level of antibodies elicited by β(+t) was slightly higher than

that caused by β(-t) biopolymer,

3) the β(+t) biopolymer evoked immediate dermal hypersensitivity whereas the β(-t) fraction only elicited delayed (cell-mediated) hypersensitivity.

Finally, results obtained from the immuno-protection assay showed that only the detoxified β(-t) biopolymer conferred a complete immunity against anterior chamber gonococcal infection in rabbits.

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APPENDIX

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS, Matheson, Coleman, and Bell) was carried out to determine the homogeneity and the molecular weight of the β (-t) protein.

MATERIALS AND METHODS

The standard proteins with known molecular weights used were obtained from Mann: myoglobin (horse heart, M.W. 17,200), lysozyme (egg white, M.W. 14,300), ribonuclease (bovine pancreas, M.W. 13,700), serum albumin (bovine, M.W. 68,000), and pepsin (hog stomach, M.W. 35,000); from Worthington: papain (papaya latex, M.W. 23,000), and ovalbumin (egg white, M.W. 43,000).

The experiment was carried out following the procedure of Weber, Pringle, and Osborn (1972). The standard proteins and the β(-t) biopolymer were prepared as follows: one ml of the protein solutions containing 1 mg was added to 9 ml of sample buffer consisting of 0.01 M sodium phosphate, pH 7.0, with 1% SDS and 1% 2-mercaptoethanol. The tube was capped and the mixture was incubated in a boiling water bath for 2 minutes. The sample was then cooled to room temperature and was dialyzed against the sample buffer overnight at room temperature. Other conditions including preparation of the gels, staining and destaining techniques were identical to those described in the previous analytical disc-gel electrophoresis experiment. The electrophoretic mobilities (Rs) of different standard proteins were plotted against the known molecular weights expressed on a logarithmic scale.

RESULTS

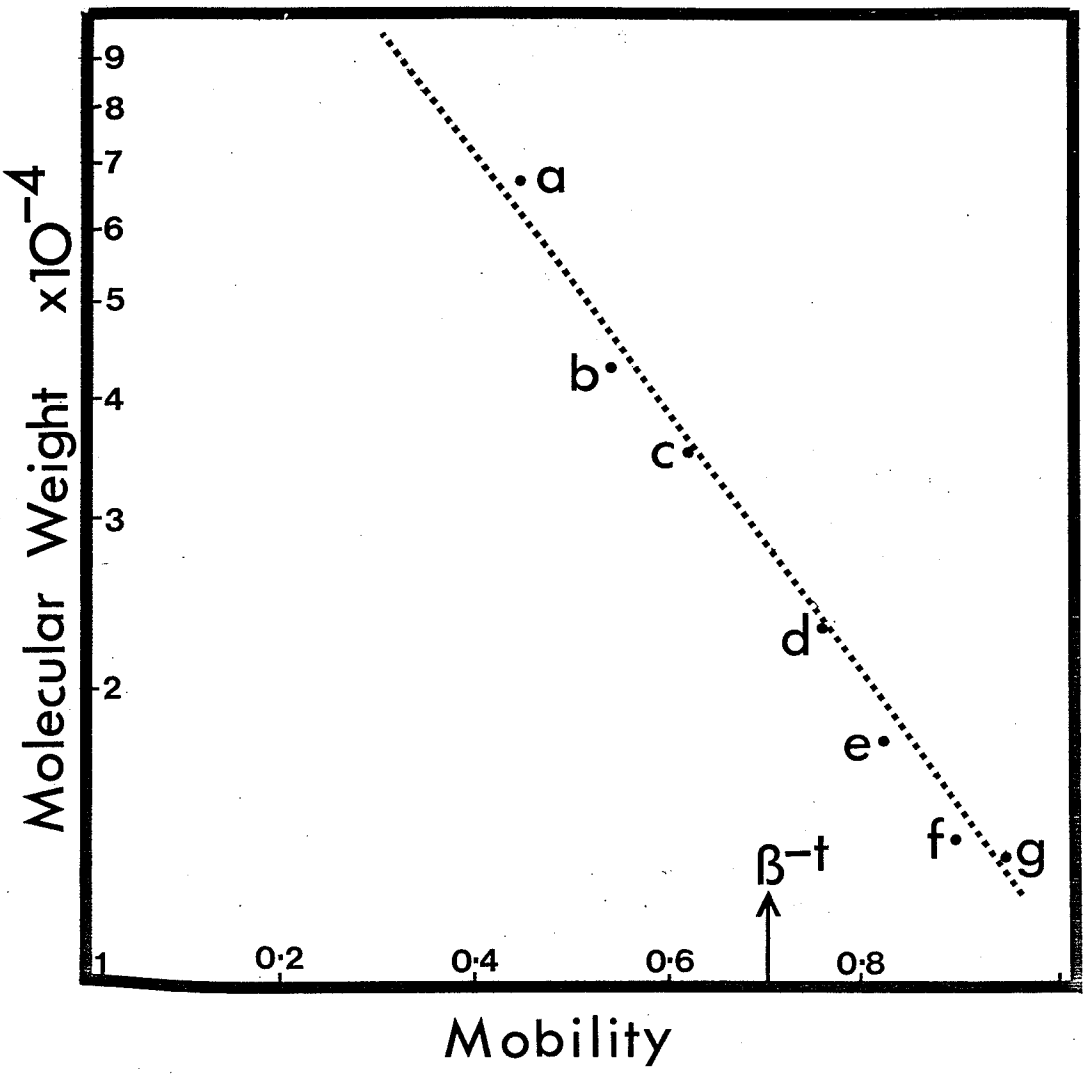
The β (-t) protein migrated down the gel columns after polyacrylamide gel electrophoresis at an Rs of 0.71 before and after sodium dodecyl sulfate treatment. This finding further confirmed that the β (-t) biopolymer indeed was homogeneous and was not composed of aggregates of different subunits.

The plot of the Rs of different standard proteins against their respective molecular weights produced a smooth linear line (Figure 21). From the electrophoretic mobility of the β (-t) protein, a molecular weight of 28,000 was found.

Figure 21

Molecular weight determination of the β(-t) biopolymer by SDS-polyacrylamide gel electrophoresis from a set of 7 standard proteins.

A = serum albumin (M.W. 68,000)
B = ovalbumin (M.W. 43,000)
C = pepsin (M.W. 35,000)
D = papsin (M.W. 23,000)
E = myoglobin (M.W. 17,200)
F = lysozyme (M.W. 14,300)
G = ribonuclease (M.W. 13,700)


Molecular Weight x10⁻⁴
9
8
7
6
5
4
3
2
a
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e
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g
β-t
1
0·2
0·4
0·6
0·8
Mobility