EXPRESSION AND PARTIAL PURIFICATION OF THE VIRULENCE ANTIGENS OF YERSINIA PESTIS

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ABSTRACT

EXPRESSION AND PARTIAL PURIFICATION OF THE VIRULENCE ANTIGENS OF YERSINIA PESTIS

By

Robert John Zahorchak

Purification of the V and W antigens from cell extracts of Y. pestis was attempted. It was determined that more than 95% of the V antigen was associated with the cell. By employing conventional techniques of protein purification (ammonium sulfate fractionation, DEAE cellulose column chromatography, Sephadex gel filtration, calcium hydroxylapatite column chromatography, and preparative polyacrylamide gel electrophoresis), a preparation was obtained that contained two major proteins. One of these proteins was identified as the V antigen. W antigen was not purified from cell extracts by the use of similar methods due to the inability to recover the antigen after adsorption to calcium hydroxylapatite.

EXPRESSION AND PARTIAL PURIFICATION OF THE VIRULENCE ANTIGENS OF *YERSINIA PESTIS*

By

Robert John Zahorchak

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DEDICATION

To Kaye, the mountains, and the songs they brought.

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INTRODUCTION

Expression of virulence by Yersinia pestis, the causative agent of bubonic plague, is dependent upon the genetic potential to produce the virulence or V and W antigens described by Burrows and Bacon (8, 12). These antigens are selectively expressed in an environment that restricts the growth of virulent (Vwa^+) cells in vitro (21). Although the growth of Vwa^+ organisms that are cultured at 37 C in a defined medium is normal only if Ca^{2+} (replaceable by Sr^{2+} or Zn^{2+}) is supplied at a concentration of 2.5 mM, the expression of the virulence antigens is inhibited by the presence of this cation (6, 24, 32). Vwa^- mutants universally lack a nutritional requirement for Ca^{2+}. When shifted from permissive to restrictive conditions, Vwa^+ cells cease to divide and their synthesis of DNA is arrested after the current round of replication is completed (34, 41). The Vwa^+ determinant may promote resistance to phagocytosis by mouse neutrophiles and free macrophages; it may also enhance survival and multiplication of the bacteria during intracellular residence (8, 15, 29).

Lawton, Erdman, and Surgalla (33) have purified V and W from culture fluids (100 and 1000 fold, respectively). These authors reported that both antigens precipitated primarily between 1.3 and 2.2 M $(NH_4)_2SO_4$ (24 to 41% saturation at 4 C) and that V elutes from DEAE cellulose with 0.1 M NaCl and W elutes between 0.3 and 0.5 M NaCl. They also determined that V was a protein (90,000 MW) and W was a lipoprotein (145,000 MW). Although their preparations were not homogeneous, they were able to demonstrate that anti-V serum could passively protect mice against challenge with virulent organisms. Anti-W offered no protection.

Virtually nothing is known about the biochemical role that the virulence antigens play in restriction or virulence. The isolation and purification of V and W must precede an attempt to specifically identify their individual functions. Since the only assay system

that is presently available for the detection of the antigens is an immunological one, monospecific antiserum to both V and W would be invaluable to facilitate their purification. The studies presented in this thesis were initiated in order to prepare homologous antiserum to each antigen. Methods for the purification of V from cell extracts are described.

LITERATURE REVIEW

Yersinia pestis is a facultative intracellular parasite that is pathogenic for many mammalian species including man, in whom the disease is infamously known as the "black death" or bubonic plague (17). Although formerly classified as a species of Pasteurella, Y. pestis now shares a genus with Y. pseudotuberculosis and Y. enterocolitica due to the close relationship reported among these organisms with respect to phage susceptibility, DNA homology, and the common antigens (3). The members of the genus Yersinia appear to be closely related to the other enteric bacteria and have therefore been placed in the family Enterobacteriaceae (3).

Morphologically, the organism is a short blunt rod (0.5 to 0.8 X 1.5 to 2.0 um) (37). The cells are gram negative and are bipolarly stained when treated with Wayson's stain or other analine dyes (30). The bacteria can grow at temperatures from -2 to 45 C, but the optimal temperature for growth in vitro ranges from 27 to 28 C (38).

The organism is highly virulent. For example, an $L.D._{50}$ of less than ten is observed when mice or guinea pigs were challenged intraperitoneally with suspensions of wild-type Y. pestis (16). This high degree of virulence has been associated with the expression of the following determinants: 1) Pgm^+; loss of the ability to produce pigmented colonies on the hemin agar of Jackson and Burrows (27) or on the congo red agar of Surgalla and Beesley (39) increases the average lethal dose to greater than 10^7 for mice and greater than 10^8 for guinea pigs (16). 2) Fra^+; cells that are unable to produce the Fraction 1 or capsular antigen are decreased in virulence for guinea pigs ($L.D._{50}$ of 10^3 to 10^5) but are fully virulent for mice (16). 3) Pur^+; auxotrophy for purines resulting from a mutation eliminating guanosine monophosphate synthetase activity increased the average lethal dose to greater than 10^8 for both mice and guinea pigs, whereas lesions prior to the synthesis of inosine monophosphate lowered virulence only slightly ($L.D._{50}$ of approximately 10^2)

(2, 9). 4) Pst^+; non-pesticinogenic mutants are unable to produce coagulase and fibrinolysin (1). These mutants are decreased in virulence for both mice and guinea pigs if the animals are challenged with subcutaneous injections of Pgm^- cells (4, 7). 5) Vwa^+; the virulence antigens, V and W, are produced by virulent Y. pestis and Y. pseudotuberculosis (8, 11, 12). A considerable decrease in virulence is observed if this determinant is lost (16). These findings are summarized in Table 1.

Avirulence for mice due to lesions in pigmentation and purine independence was reversed by the injection of iron or purines, respectively, prior to or simultaneously with the challenge dose (9, 28). As already mentioned, Fra^- mutants retain their virulence for mice. Non-pesticinogenic Y. pestis also exhibited wild-type virulence if injected intravenously (4). However, avirulence due to the loss of the Vwa^+ determinant appears to be irreversible in the sense that virulence has not been phenotypically restored by the injection of any known nutritional supplement (3).

Burrows and Bacon (14, 15) first demonstrated a difference between virulent and avirulent cells of Y. pestis with respect to their ability to resist phagocytosis by polymorphonuclear leukocytes. Avirulent cells cultivated in vivo or in vitro at 37 C were shown to be much more susceptible to phagocytic injestion both in vivo and under defined conditions in vitro. Since phagocytosis-resistant cells became sensitive when homologous antiserum was added to the in vitro system, the investigators embarked on a search for an antigenic difference between sensitive and resistant isolates. Burrows (8) detected an antigen present in cultures of virulent Y. pestis that had been incubated at 37 C for three hours with aeration. This antigen, termed V_i, was not detectable in cultures of avirulent cells incubated at 37 C or in cultures of virulent cells incubated at 26 C. The antigen was redesignated V when further experimentation by Burrows and Bacon (12) revealed the presence of a second antigen, designated W, that was unique to cultures of virulent cells incubated at 37 C after an increase in incubation time to six hours. The short incubation time during which V and W were produced did not allow for the expression of a visible capsule, the presence of which rendered both Vwa^+ and Vwa^- cells highly resistant to phagocytosis (12).

Janssen et al. (29) later showed that the high resistance to phagocytosis by neutrophiles and free macrophages was indeed closely related to the Fra^+ determinant. Expression of V and W did render Fra^- cells somewhat more resistant to phagocytosis by mouse and guinea pig neutrophiles and free macrophages procured from mice. However, the susceptibility to phagocytosis by guinea pig free macrophages was not significantly different between $Fra^- Vwa^-$ and $Fra^- Vwa^+$ cells. These authors suggested that expression of the virulence antigens may be more important for the survival and multiplication of the bacteria during intracellular residence than for resistance to phagocytosis.

Fukui et al. (21) have shown that at the temperature at which V and W are expressed, aerated cultures of virulent cells became avirulent after serial transfers in a complex broth. The authors demonstrated that the lowered virulence was due to a shift in population resulting from selection for avirulent mutants. Studies on the growth of virulent and avirulent cells in a chemically defined medium (23) resulted in the description of a nutritional requirement for 2.5 mM Ca^{2+} (replaceable by Sr^{2+} or Zn^{2+}) by virulent Y. pestis grown at 37 C with aeration (24). Avirulent cells, however, showed no requirement for this cation under similar conditions.

The development of an agar medium consisting of blood agar base with added $MgCl_2$ and sodium oxalate (MgOx agar) enabled Higuchi and Smith (25) to assay for avirulent cells in virulent cultures. They determined that mutation from virulence to avirulence occurred at a spontaneous rate of 10^{-4}. This high spontaneous mutation rate plus the inhibition of growth of the virulent cells in a population cultured at 37 C in a medium deficient in Ca^{2+} accounted for the rapid attenuation of virulent cultures described by Fukui et al. (21). The mutation appears to be irreversible, although one occurrence of the reverse mutation has been reported (13). In this case, however, the investigators cautioned interpretation of the results due to the possibility of contamination by one of the many virulent strains of Y. pestis with which they were working at the time.

Due to the observations that avirulent cells lacked a nutritional requirement for Ca^{2+} and also failed to express the virulence antigens, there appeared to be a direct relationship between Ca^{2+}-

Table 1. The effect of the loss of various determinants on the virulence of Yersinia pestis.

Determinant that is missing	LD_{50}[a] mice	guinea pig
Pgm	$>10^7$	$>10^8$
Fra	<10	10^3-10^6
Pur[b]	$\geq10^2$ ($>10^8$)	$\geq10^4$ ($>10^8$)
Pst[c]	$\sim10^5$	$\sim10^6$
Vwa	$>10^7$	$>10^8$
none	<10	<10

[a] Intraperitoneal challange.

[b] The values that are not in parentheses are the LD_{50} values observed with organisms that have biosynthetic blocks prior to inosine-5'-monophosphate. The values obtained when the challange organisms have no guanosine-5'-monophosphate synthase are enclosed within the parentheses.

[c] Intravenously injected Pst$^-$ Y. pestis exhibit wild-type virulence.

Table 2. The effect of the cultural conditions on the growth and virulence antigen expression of Vwa$^+$ and Vwa$^-$ Y. pestis.

Phenotype	Virulence	Cultural conditions					
		26 C		37 C + Ca^{2+}		37 C - Ca^{2+}	
		VW	growth	VW	growth	VW	growth
Vwa$^+$	+	-	+	-	+	+	-
Vwa$^-$	-	-	+	-	+	-	+

dependence, V and W production, and virulence (See Table 2). Supportive evidence for this hypothesis came when Fukui et al. (20) demonstrated that cells of Y. pestis grown at 37 C were phenotypically more virulent than those that were incubated at 5 C or 26 C. Furthermore, Lawton (32) and Brubaker and Surgalla (6) showed that the addition of Ca^{2+} to cultures of virulent Y. pestis growing at 37 C lowered the amounts of V and W produced.

The first evidence that Ca^{2+}-dependence may be a separate determinant of virulence was reported by Brubaker and Surgalla (5). By selecting for mutants in a virulent culture that were resistant to relatively high concentrations of streptomycin (5,000 units per ml), Ca^{2+}-independent mutants were isolated that retained the ability to express V and W. These mutants were shown to be avirulent, suggesting that Ca^{2+}-dependence is more closely associated with virulence than is the expression of V and W. On the other hand, growth at 37 C of one Ca^{2+}-independent Vwa^{+} isolate in a liquid medium devoid of free Ca^{2+} was intermediate between that of a Ca^{2+}-dependent Vwa^{+} cells and Ca^{2+}-independent Vwa^{-} cells. The authors later demonstrated that cells of the Ca^{2+}-independent Vwa^{+} phenotype expressed less V and W then did Ca^{2+}-dependent Vwa^{+} cells (6). The growth of Ca^{2+}-independent Vwa^{+} cells was similar to that of Ca^{2+}-independent Vwa^{-} cells if Ca^{2+} was supplied in the medium, indicating that the former phenotype was not completely devoid of a requirement for the cation. The presence of Ca^{2+} also inhibited the expression of V and W by the Ca^{2+}-independent cells as it does when it is present in cultures of wild-type cells.

When typical Vwa^{+} cells of Y. pestis were grown at 37 C in a modification of the medium of Higuchi, Kupferberg, and Smith (24) devoid of Ca^{2+}, the cells underwent a dramatic physiological change and entered a state called "restriction" (34). Restricted organisms failed to synthesize significant amounts of DNA when compared to growing cells (41). After Vwa^{+} cells were shifted from permissive to restrictive conditions, the cells underwent two doublings in mass during which time they divided once (34). Morphologically, restricted cells appeared to be elongated when observed microscopically (6) and lacked a dense nucleoid as elucidated by electron microscopy (22). As mentioned earlier, it is this re-

strictive environment that stimulates V and W production. However, if Ca^{2+} was included in the medium at a concentration of at least 2.5 mM, the cells divided normally and the virulence antigens were not expressed (6). Therefore, a direct relationship seemed to exist between cell division and the expression of the virulence antigens. This hypothesis was strengthened when Brubaker and Surgalla (6) showed a direct correlation between the stimulatory effect of various energy sources on growth under permissive conditions and on the expression of W under restrictive conditions. The more a substrate stimulated growth when Ca^{2+} was present, the more it stimulated W production in the absence of the cation.

In addition to the inhibitory effect of Ca^{2+} on the expression of V and W, Brubaker and Surgalla (6) observed a stimulatory effect of 20 mM Mg^{2+} on the production of these antigens. The W antigen could not be detected in cultures that did not contain this high concentration of Mg^{2+}. It is interesting to note that the concentrations of Ca^{2+} and Mg^{2+} that are necessary to promote the expression of the antigens are remarkably similar to those reported for mammalian intracellular fluids, whereas inhibitory concentrations of Ca^{2+} were present in the blood (31). These observations suggested that the response to Ca^{2+} and Mg^{2+} may be related to the ability of the bacteria to survive and multiply once it is phagocytized and that the V and W antigens may play a role in this stage of infection. However, the apparent paradox that cells cultured *in vitro* must be restricted in order for V and W to be fully expressed has not yet been explained. Perhaps the restricted state is an abberant manifestation of a physiological change which takes place *in vivo* that renders the bacterial cells resistant to host cell destructive mechanisms but still allows the bacteria to divide.

Attempts have been made to purify V and W in order to specify their biological functions. Lawton, Erdman and Surgalla (33) have purified V and W, 100 and 1,000 fold respectively, from the media supernatant in which Vwa^{+} *Y. pestis* were cultured. The chemical composition of each antigen has been determined. V is a protein of approximately 90,000 MW and W is a lipoprotein (38% lipid and 59% protein) of about 145,000 MW. These authors observed that anti-V but not anti-W protected mice against challenge with

MATERIALS AND METHODS

Bacteria. Strain M23, a Vwa^+ isolate of Y. pestis, and an isogenic Vwa^- mutant were employed throughout, unless stated otherwise. This isolate was chosen because it is Pgm^- and Fra^- and thus avirulent and unable to produce contaminating capsular antigen. Stock cultures were maintained at -20 C in a phosphate buffer-glycerol mixture as previously described (1), using a 0.033 M buffer at pH 7.0.

Buffers. In general the buffers employed in these studies were either 0.05 M tris(hydroxymethyl)aminomethane buffer (Tris) or phosphate buffered saline (PBS). The former was prepared by adding concentrated HCl to Trizma Base (reagent grade, Sigma Chemical Company, St. Louis, Mo.) until the pH was 7.85 at 4 C. PBS was prepared by adding one ml of a solution containing 12.36 g of anhydrous Na_2HPO_4 and 1.8 g of $NaH_2PO_4 \cdot H_2O$ per 100 ml of distilled water to 99 ml of 0.85% NaCl (final concentration of 6 mM PO_4 at pH 7.4).

Media. The oxalated N-Z amine medium used to cultivate the organisms for V and W production was prepared as follows. Stock solution A was prepared by bringing a 15% (wt/vol) solution of N-Z amine in distilled water to 0.02 M with solid sodium oxalate. The mixture was allowed to stand overnight at 4 C after which time the precipitate formed was removed by centrifugation at 14,000 X g for 20 min at 4 C in a refrigerated Sorvall centrifuge. The supernatant was carefully poured off and stored at 4 C with a small amount of chloroform added to prevent microbial growth during storage. The stock was routinely heated in a steamer and filtered through Whatmann No. 4 filter paper prior to use. Stock solution B consisted of 0.25 M K_2HPO_4, 0.1 M citric acid, 0.001 M $FeCl_2$, and 0.001 M $MnCl_2$ in aqueous solution. After addition of a few drops of chloroform, the solution was stored at room temperature. For each liter of medium, 200 ml of stock A were mixed with 100 ml of stock B plus 600 ml of distilled water. This solution was neutralized with NaOH and sterilized by autoclaving. When the medium had returned

to room temperature, 50 ml each of filtered sterilized 0.8 M potassium gluconate and separately autoclaved 0.8 M $MgCl_2$ were added aseptically to yield a final concentration of 0.04 M apiece.

Methods of bacterial cultivation. The starter cultures used to inoculate fermenters consisted of 200 ml of oxalated N-Z amine medium per 2 L flask into which was washed the growth from one slope of blood agar base previously incubated with the appropriate organism for 48 h at 26 C. The cultures were then cultivated overnight at 26 C during which time they were constantly shaken with the aid of a model R-25 NBS Gyrotory Shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) at a setting of 84 cycles per min, which promoted near optimal aeration. The resultant culture was aseptically poured into fermenters (prewarmed to 37 C) containing 3 L of the same oxalated N-Z amine medium until the cell concentration reached a desired optical density (read on a Beckman DU spectrophotometer at a wavelength of 620 nm). The concentration of cells obtained in this manner will be referred to as the inoculation density and is expressed in optical density units. The fermenters were then incubated at 37 C with agitation and aeration for 20 h. Sterile antifoam B emulsion (Dow Corning Corporarion, Midland, Michigan) was added as needed.

Preparation of the spent medium concentrates and crude cell extracts. The cells were harvested by centrifugation at 14,000 X g for 15 min at 4 C, washed in 0.033 M potassium phosphate buffer (pH 7.0), and resuspended in Tris buffer at a ratio of 0.5 ml of buffer per 1 g of wet packed cells. The cells were then fractured in a French pressure cell under a pressure of 7,000 psi and the soluble fraction was separated from particulate debris by centrifugation at 27,000 X g for 20 min at 4 C. The reclaimed supernatant was designated the crude cell extract. The pellet was resuspended in the original volume of buffer and the particulate material was once again pelleted by centrifugation. The supernatant from this step was designated the cellular debris wash.

The medium from which the cells had been harvested was brought to 80% saturation with solid $(NH_4)_2SO_4$ and stirred at 4 C for 1 h. The precipitated material was retrieved by centrifugation at 14,000 X g for 20 min and dissolved in a minimal amount of Tris buffer.

This solution was then dialyzed against Tris buffer at 4 C and stored at -20 C. The dialyzed preparation was termed the spent medium concentrate.

Immunological methods. The antiserum used to detect the presence of the V and W antigens (anti-VW) was a gift from Dr. T. W. Burrows. Two dimensional agar gel immunodiffusion plates used in the qualitative assay system were prepared as described previously (33). After filling the wells with the appropriate antigen or antiserum, the plates were incubated at room temperature in a sealed plastic bag containing a wet paper towel to maintain a high humidity. Observations were made periodically with the final observations recorded after 24 h of incubation. Relative concentrations of antigen obtained during the course of purification were estimated by noting the position of the precipitation band. Since the antiserum concentration was constant, the closer the precipitation band was to the antiserum, the higher the antigen concentration was in the sample. This method of analyzing the plates aided in choosing fractions for further purification.

The semi-quantitative assay system employed in some of the experiments was performed on glass slides (1 by 3 in) overlayed with agar. The same agar medium was used as was for the plates except that merthiolate was omitted. To eliminate microbial growth, the slides were placed under an ultraviolet light for 10 min immediately after the agar had solidified and before filling the wells. The wells were punched with the aid of a Gelman immunodiffusion punch and the agar was removed with a Pasteur pipet attached to a vaccuum line. The well pattern was octagonal with the wells being 3 mm in diameter and 3 mm apart; the outer well were 6 mm from the center well. The slides were incubated for 24 h at room temperature in a plastic box with a small amount of water added to prevent dessication.

Antigen concentrations were determined as described by the method of Lawton, Erdman, and Surgalla (33) and consisted of placing serial two-fold dilutions of antigen in PBS in the outer wells. The center well was filled with anti-V serum. The reciprocal of the highest dilution showing a line of precipitation was designated the titer. Arbitrarily, a concentration of one unit per ml was

assigned to a sample that had a titer of one.

Column chromatography. Prior to any chromatographic step, the sample to be fractionated was dialyzed against the appropriate starting buffer. Samples were concentrated either by precipitation with 80% $(NH_4)_2SO_4$ and resuspension in a minimal amount of buffer or by ultrafiltration through a Diaflo UM 10 ultrafilter (Amicon Corp., Lexington, Mass.) under a pressure of 38 psi. Gradient elution of samples adsorbed to diethylaminoethyl (DEAE) cellulose (Whatman Ltd., Springfield Mill, Maidstone, Kent.) and hydroxylapatite (Bio-gel HTP, Bio-Rad Laboratories, Richmond, Ca.) was accomplished with the aid of an Autograd gradient maker (Technicon Instruments Corp., Chauncey, N.Y.). The method of using four of the cylinders to achieve a non-linear gradient has been described (26). Sephadex (Pharmacia Fine Chemicals, Piscastaway, N.J.) and DEAE cellulose fractionations were performed at 4 C. Hydroxylapatite column chromatography was performed at room temperature. The absorbancy at 280 nm of the eluant from each fractionation was routinely monitered with the aid of an ISCO Model UA-2 Ultraviolet Analyzer (Instrument Specialties Company, Lincoln, Nebraska).

Analytical Methods. Protein concentrations of samples were determined either by the method of Lowry et al. (35) using bovine serum albumin as the standard or by measuring the absorbancy at 280 and 260 nm and reading the protein concentration from a nomograph (40). Inorganic phosphate concentrations were determined by the method of Fiske and SubbaRow (19). NaCl concentrations were determined by measuring the conductivity of samples with a Wheatstone bridge (Model 31 Conductivity bridge, Yellow Springs Instrument Co., Yellow Springs, Ohio) and converting the value obtained to salt concentration with the aid of a standard curve. Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis (18).

Preparative disc gel electrophoresis. Preparative polyacrylamide disc gel electrophoresis was performed with the aid of a "Prep-Disc" electrophoresis apparatus, employing the PD-2/70 upper column (Canal Industrial Corp., Rockville, Md.). The separating system consisted of a 2.5% stacking gel (8 mm in length) and a 7% separating gel (97 mm in length). The gels were prepared as described

by Davis (18) with ammonium persulfate as the catalyst for polymerization of both gels. Tris-glycine buffer (the 10X stock consisted of 6 g tris(hydroymethyl)aminomethane and 28.8 g of glycine diluted to 1000 ml with distilled water) was used as both the top and bottom electrode buffer as well as the elution buffer. The current was maintained at 10 ma and 5 ml fractions were collected at a flow rate of one ml per min.

Chemicals. The sources of the chemicals used in these studies were: N,N'-methylenebisacrylamide and acrylamide, Eastman Kodak Company, Rochester, N.Y.; N,N,N',N',-tetramethylethylenediamine, Canal Industrial Corp., Rockville, Md.; potassium gluconate, Calbiochem, San Diego, Ca.; Ionagar No. 2, Colab Laboratories, Inc., Glenwood, Ill.; sodium oxalate, J. T. Baker Chemical Co., Phillipsburg, N.J.; N-Z amine type A, Sheffield Chemical, Norwich, N.Y.; Freund's compete adjuvant, Difco Laboratories, Detroit, Michigan.

RESULTS

Expression of the virulence antigens in oxalated N-Z amine. At least one antigen was produced by cells of Vwa^+ M23 during cultivation in the oxalated N-Z amine medium which was not detected in similar cultures of Vwa^- M23 (Fig. 1). After $(NH_4)_2SO_4$ fractionation, two unique antigens were resolved in the immunodiffusion system (Fig. 2). The material from the spent medium concentrate which precipitated between 20 and 50% saturated $(NH_4)_2SO_4$ (MC 20-50) and that from the cell extract which precipitated between 20 and 60% saturated $(NH_4)_2SO_4$ (CE 20-60) were used in subsequent fractionations.

Separation of the V and W antigens by DEAE cellulose column chromatography. Elution of MC 20-50 from DEAE cellulose with a linear gradient of NaCl in Tris buffer resulted in the profile shown in Figure 3a. The V antigen eluted at a NaCl concentration in the range of 0.1 to 0.15 M. W eluted in the range of 0.22 to 0.28 M NaCl. Chromatography of CE 20-60 resulted in similar separation of the antigens (Fig. 3b). It should be noted that the W antigen could be detected in fractions from DEAE cellulose column chromatography of CE 20-60 only if the anti-VW serum was diluted ten-fold. No difficulty was encountered in detecting W in the eluant of the MC 20-50 fractionation when undiluted anti-VW was employed. This finding suggests that the W antigen was present in much lower concentrations in the CE 20-60 fractionation even though almost four times as much protein was initially applied to the column.

Preparation of the crude V antigen. The assay system used in the previously described studies was required because of the limited quantity of anti-VW serum available. I thought it desirable, if not necessary, to prepare antiserum to each antigen individually in amounts large enough to be employed in a more quantitative assay system. I, therefore, proceeded to purify each antigen using the qualitative assay system so that antiserum to each antigen could be obtained.

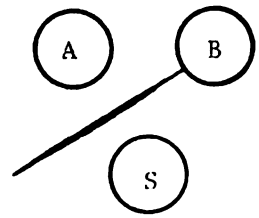


Fig. 1. Agar gel immunodiffusion analysis of the spent media concentrates obtained after the cultivation of (A) Vwa^{+} M23 or (B) Vwa^{-} M23 in oxalated N-Z amine. Well S contained anti-VW serum.

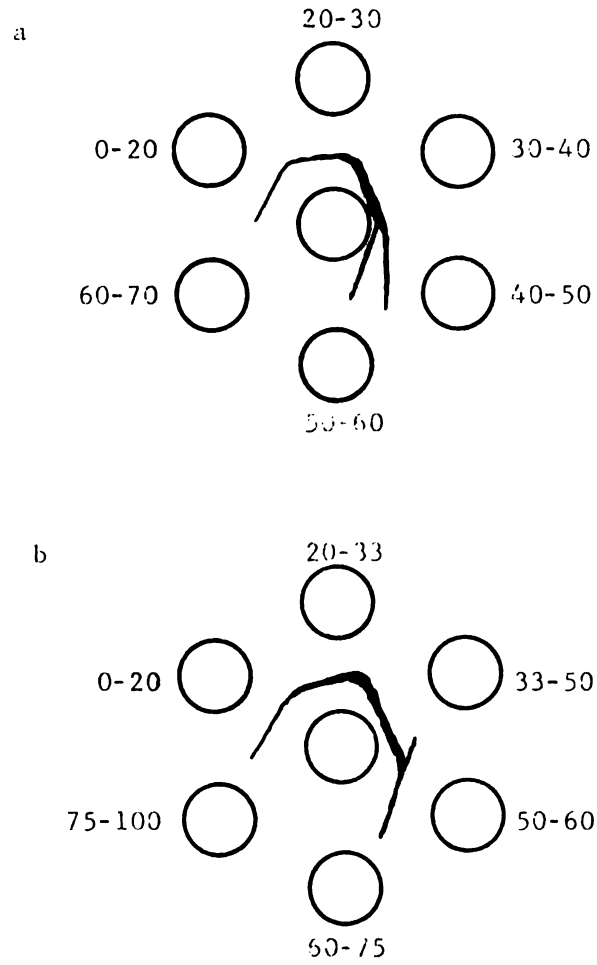
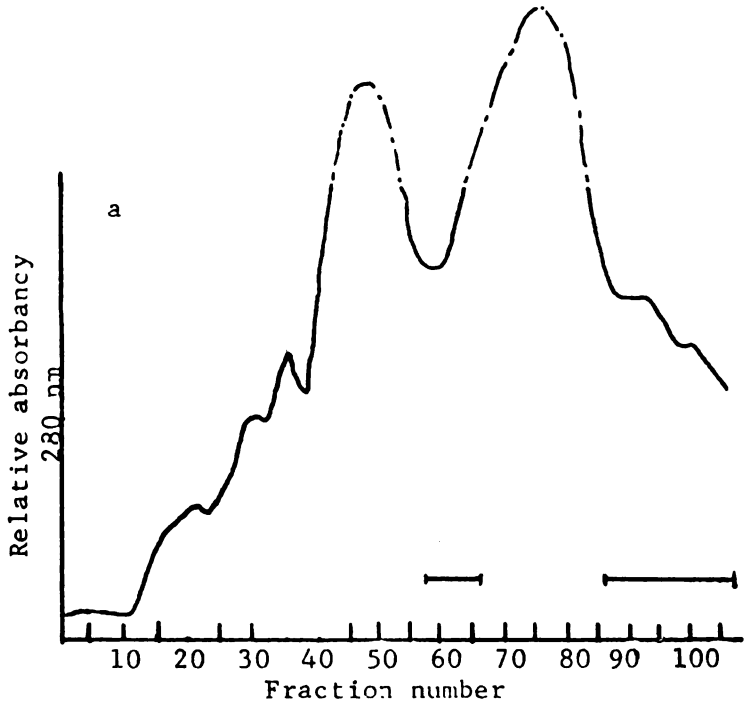


Fig. 2. Agar gel immunodiffusion analysis of $(NH_4)_2SO_4$ fractions of (a) the spent medium concentrate and (b) the cell free extract of Vwa^+ M23. The center wells contained anti-VW serum. The numbers assigned to each of the outer wells indicate the upper and lower $(NH_4)_2SO_4$ concentrations, in percent saturated $(NH_4)_2SO_4$, between which the material precipitated.

Fig. 3. DEAE cellulose column chromatography of the V and W antigens. In (a) 564 mg of protein of MC 20-50 were adsorbed to the resin and in (b) 2,200 mg of CE 20-60 were adsorbed. The sample was eluted with a linear gradient from 0.0 to 0.5 M NaCl in 0.5 M Tris buffer, pH 7.85, at a flow rate of 2 ml per min. The gradient was applied as soon as the sample was adsorbed to the column. The packed resin dimensions were 2.5 by 40 cm. In (a) the relative absorbancy at 280 nm was continuously monitered. In (b) the absorbancy of each fraction was measured at 280 nm with the aid of a Beckman DU Spectrophotometer. The horizontal bars indicate the fractions (5 ml) which exhibited antigenic activity when tested against anti-VW serum.


a
Relative absorbancy
280 nm
10 20 30 40 50 60 70 80 90 100
Fraction number

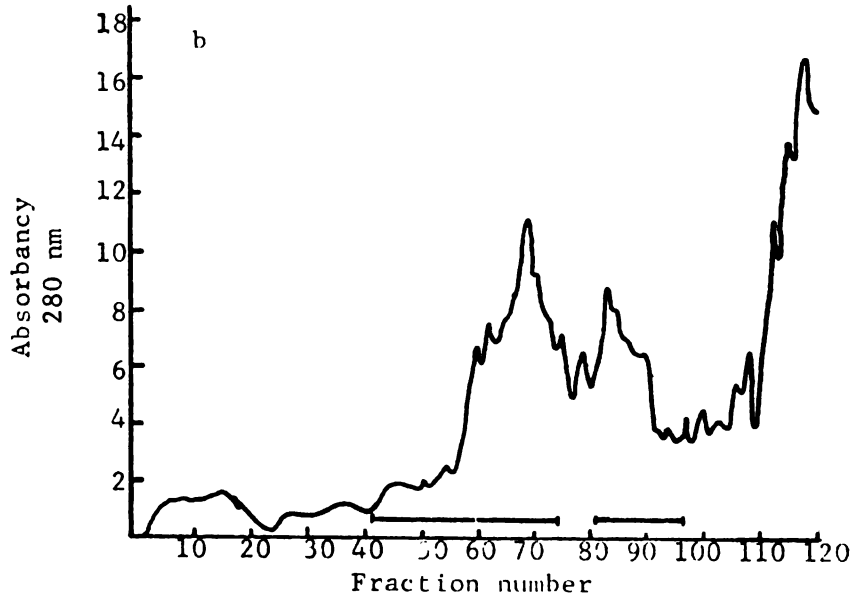

b
Absorbancy
280 nm
18
16
14
12
10
8
6
4
2
10 20 30 40 50 60 70 80 90 100 110 120
Fraction number

Fig. 3.

be obtained.

The V preparation obtained from DEAE cellulose chromatography was first filtered through Sephadex G-200. Antigenic activity eluted in a broad peak, indicating that optimal separation was not achieved by this technique (Fig. 4). However, this step did provide a means to eliminate contaminating molecules of both very high and low molecular weights. The V positive fractions were pooled and concentrated. This concentrate was then applied to an hydroxylapatite column and eluted with a linear sodium phosphate buffer gradient (0.001 to 0.5 M, pH 6.8). V was detected in fractions containing between 0.075 and 0.15 M phosphate (Fig. 5a). When a more shallow non-linear gradient was used to elute the sample, the antigen was detected in fractions that reflected eluting protein (Fig. 5b). These fractions were pooled, concentrated, and dialyzed against PBS. The resultant preparation, containing 2.5 mg of protein per ml, was analyzed by polyacrylamide gel electrophoresis. Stained gels revealed the presence of one very prominant band and at least 13 minor bands of protein (Fig. 6). This preparation was termed the crude V antigen.

Similar manipulations of the W antigen did not yield positive results. After Sephadex G-200 fractionation, the antigen was adsorbed to hydroxylapatite. No W activity was detected in the eluant when a linear sodium phosphate gradient was applied (0.001 to 0.5 M, pH 6.8). Various other methods of elution, including 2.0 M sodium phosphate buffer and NaCl solutions at concentrations up to 3.0 M, failed to yield detectable W activity, although these processes did release some UV absorbing material.

Production of anti-V serum. Anti-V serum was obtained by inducing antibody formation to the crude V antigen in a New Zealand white rabbit. Prior to immunization, the preparation was mixed with an equal volume of rabbit anti-Vwa^- M23 cell extract and incubated at 37 C for 1 h. The immunoprecipitate formed was pelleted by ultracentrifugation at 149,000 X g for 30 min at 4 C. An emulsion of the supernatant and Freund's complete adjuvant (mixed in equal proportions) was injected according to the schedule presented in Table 3. The rabbit was bled through the marginal ear vein on the sixth, eighth, tenth, and twelfth days after the final injection. Anti-V activity was discerned after absorbing out contaminating antibodies with 100 mg of lypholized Vwa^- cell extract per 1.0

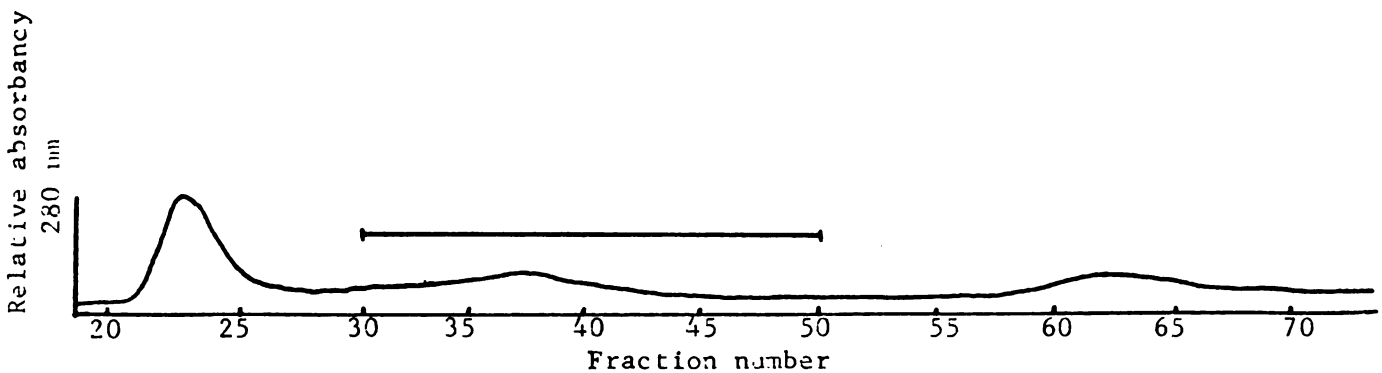


Fig. 4. Sephadex G-200 gel filtration of V antigen. A sample of 3 ml containing 60 mg of protein in 0.05 M Tris buffer (pH 7.85) was applied to the column (bed dimensions of 2.5 by 60 cm) and eluted under a hydrostatic pressure of 10 cm. Fractions of 5 ml were collected and the absorbancy at 280 nm was continuously monitered. A tracing of the elution profile is presented. The fractions containing antigenic activity are indicated by the horizontal bar.

Fig. 5. Calcium hydroxylapatite column chromatography of the V antigen. After $(NH_4)_2SO_4$ fractionation, DEAE cellulose column chromatography, and Sephadex gel filtration, the V antigen preparation was adsorbed to a column containing packed calcium hydroxylapatite (column dimensions of 1.5 by 26 cm). The sample was then eluted with either (a) a linear gradient (0.001 to 0.5 M sodium phosphate buffer at pH 6.8) or (b) a non-linear gradient formed by using four cylinders of the gradient maker (cylinders one through four contained 0.001, 0.1, 0.1, and 0.25 M sodium phosphate buffer at pH 6.8, respectively). In (a) 55.5 mg of protein were adsorbed to the resin and in (b) 15 mg of protein were adsorbed. The flow rate was maintained at 1 ml per min, 5 ml fractions were collected, and the absorbancy at 280 nm was continuously monitered. Tracings of the elution profiles are presented. Fractions containing V activity are indicated by the horizontal bars.

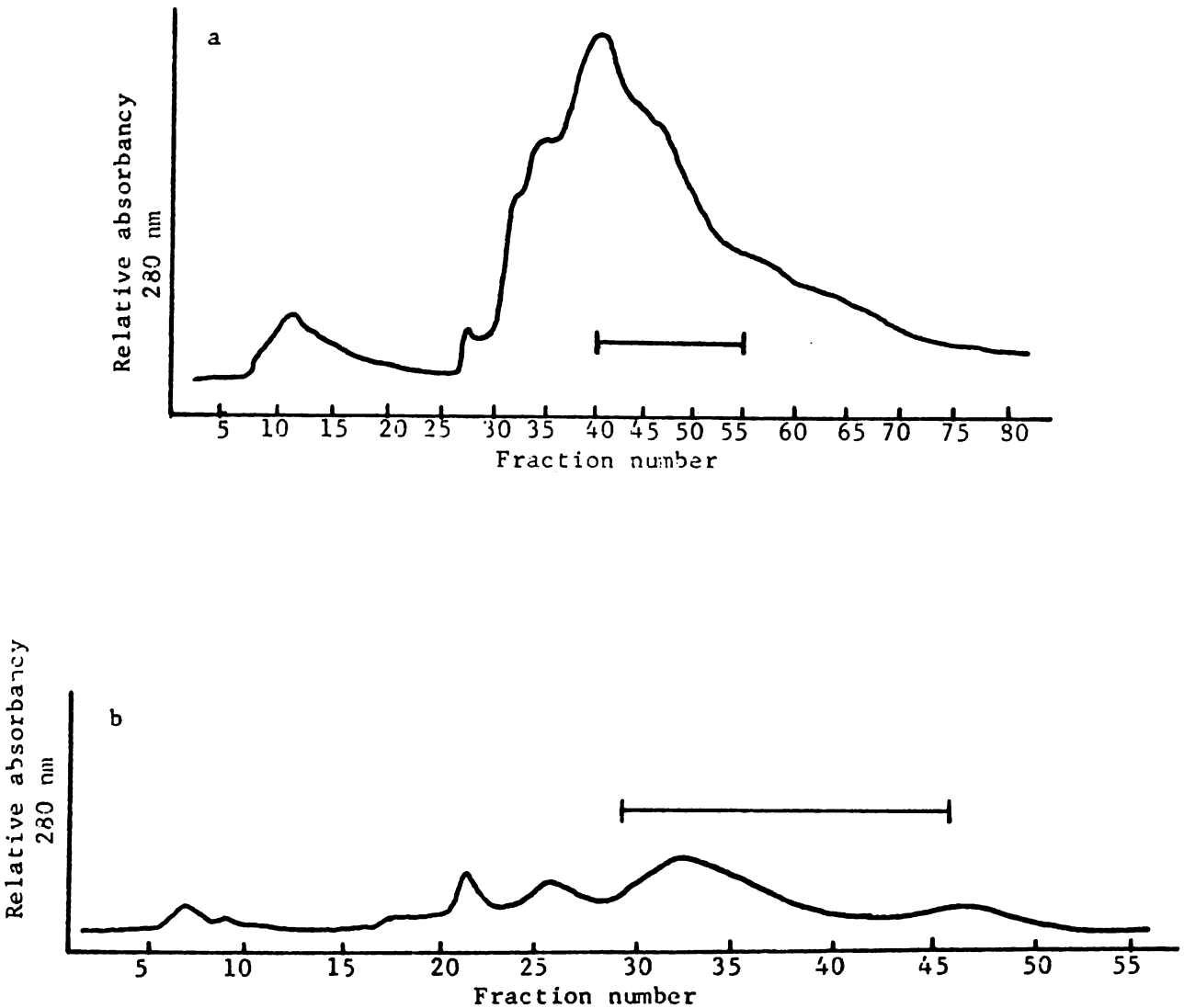

a
Relative absorbancy
280 nm
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Fraction number
b
Relative absorbancy
280 nm
5 10 15 20 25 30 35 40 45 50 55
Fraction number

Fig. 5.

Fig. 6. Analytical polyacrylamide gel electrophoresis of the crude V antigen. The sample analyzed contained 496 µg of protein. The positive electrode was at the bottom. The gel was stained with 1% Amido black in 7% acetic acid after electrophoresis was completed, as indicated by the fact that the tracking dye had migrated to the bottom of the gel.

Table 3. Inoculation schedule for the immunization against the crude V preparation.

Day	Volume	Route[a]	Location
1	0.1 ml	S.Q.	each foot pad[b]
8	0.1 ml	S.Q.	each foot pad
15	0.3 ml	I.M.	each thigh[c]

[a] S.Q., subcutaneously; I.M., intramuscularly.

[b] total of 0.4 ml injected.

[c] preparation not mixed with adjuvant.

ml of serum and incubating the mixture at 37 C for 1 h. The absorbed antiserum showed a line of identity with anti-VW serum in the Ouchterlony test (Fig. 7). A single arc of precipitation was observed when absorbed anti-V was diffused against a polyacrylamide gel into which was electrophoresed the crude V antigen (Fig. 8). It was not possible to identify which band of protein contained the antigenic activity, although the major protein was ruled out as the source of activity.

To show that the antigen which was being detected was not unique to strain M23, cell extracts of various strains of Y. pestis and Y. pseudotuberculosis were prepared by the standard proceedure described in Materials and Methods and tested against the anti-V serum. The results of those tests are tabulated in Table 4. Only the extracts of Vwa^+ cells showed a reaction with the absorbed anti-V. This antiserum made it possible to employ a semi-quantative assay system in the following studies.

Determination of the relative amounts of V present extracellularly and associated with the cell. I performed one experiment to determine the relative amounts of V that were present either extracellularly or associated with the cell. Fermenters containing 3 L of oxalated N-Z amine were inoculated at an optical density of 0.35 and incubated at 37 C for 20 h. After harvesting the cells, the medium supernatant volume was measured. A 100 ml aliquot of the supernatant was filtered through a Nalgene filter unit (pore diameter of 0.20 um) to remove any residual cells. A crude medium concentrate was prepared from the filtrate. The cells were ruptured in a French pressure cell in order to obtain the extract as described in Materials and Methods. The concentration of V antigen in both preparations was determined and the total units per culture was calculated. A total of 98 units were present extracellularly, whereas 1700 units were detected in the cell extract.

To eliminate the possibility that these results were due to adsorbtion of V to the filter, a reconstruction experiment was performed. After diluting 1.0 ml of a protein preparation containing V to 100 ml with distilled water, the solution was filtered in the same manner as described above. The filtrate was concentrated by adding $(NH_4)_2SO_4$ to 80% saturation. The precipitate was pelleted

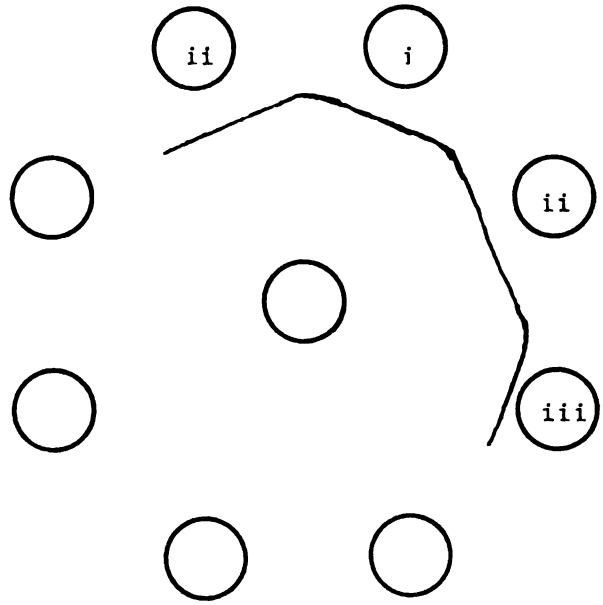


Fig. 7. Agar gel two dimensional immunodiffusion of antiserum prepared against the crude V preparation. A volume of 1 ml of antiserum was absorbed with 100 mg of lyophilized Vwa$^-$ M23 whole cell extract as described in Materials and Methods. Symbols: i, absorbed anti-VW; ii, absorbed anti-V; iii, absorbed anti-V diluted 1:2 in PBS. The center well contained crude V antigen.

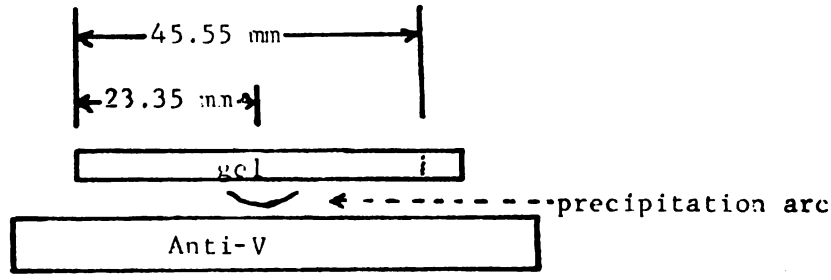


Fig. 8. Immunodiffusion of an analytical polyacrylamide gel of the crude V antigen against anti-V. A sample of the crude V antigen was electrophoresed into a polyacrylamide gel until the tracking dye migrated to the bottom of the gel (dotted line at the right of the gel). The gel was then placed in a glass petri dish and molten agar was poured to cover the bottom of the dish. After the agar had solidified, a rectangular well was cut out of the agar parallel to the gel. The well was filled with anti-V diluted 1:2 in molten agar. The plate was then incubated for 24 h at room temperature. The calculated mobility of the antigen antigenic activity was approximately 0.5. This mobility is not the same as that observed for the major protein shown in Fig. 6 (mobility = 0.25).

Table 4. Demonstration that absorbed anti-V reacts with only Vwa$^+$ strains of Y. pestis and Y. pseudotuberculosis.

Species[a]	Strain	Virulence Determinant[b]				Reaction[c]
		Vwa	Pgm	Pst	Fra	
p	M23	+	-	+	-	+
p	D1	+	-	+	+	+
p	G32	+	-	-	+	+
ps	MD31	+	0	0	0	+
p	A12	-	-	-	+	-
p	A4	-	-	-	+	-
p	KIM	+	-	+	+	+
p	EV76	+	-	+	+	+
p	KIM	-	-	+	+	-
p	KUMA	-	+	+	+	-
p	Salazar	-	+	+	+	-
p	M23	-	+	+	-	-
p	A1122	-	-	+	+	-
p	JAVA	-	-	-	-	-
p	G35	-	-	-	-	-
ps	PB1/0	-	0	0	0	-
p	KUMA	-	-	+	+	-
p	G32	-	+	+	+	-
p	TS	-	+	+	+	-
p	Salazar	-	+	+	+	-
p	Yokohoma	-	+	+	+	-
p	Kimberly	-	+	+		-
p	G32	-	-	-	+	-
p	M23	-	-	+	-	-
p	Dodson	-	+	-	+	-
p	A12	-	+	-	+	-
ps	PB1/+	+	0	0	0	+

[a] p, Y. pestis; ps, Y. pseudotuberculosis.

[b] +, expressed; -, not expressed; 0, not expressed by the species.

[c] +, cell extract shows a line of precipitation in the Ouchterlony test when diffused against anti-V; -, no reaction observed under identical conditions.

by centrifugation, redissolved, and dialyzed against PBS. The volume of the dialysate was measured and the concentration of V was determined. After diluting 1.0 ml of the original protein preparation to a volume equal to that of the filtered sample, the V concentration was determined. There was no decrease in the antigen concentration due to filtration and subsequent $(NH_4)_2SO_4$ precipitation as indicated by the fact that the V titer in the two preparations was identical.

Determination of the optimum inoculation density for batch culture production of the V antigen. I thought it desireable to inoculate the cultures at as high a concentration of cells as possible in order to obtain the maximal amount of V per batch of cells cultivated. Therefore, fermenters were inoculated at different densities and then incubated at 37 C for 20 h to see if the inoculation density had an effect on the expression of the V antigen. Determination of the concentration and specific activity of the antigen revealed that this parameter did affect the relative amounts of V produced in the culture. Although the amount of V produced per culture appeared to be proportional to the inoculation density except at the highest density, the specific activity of the antigen was higher at an inoculation density of 1.4 than at the other densities tested (Table 5).

Quantitative studies of the purification of the V antigen. The purification scheme that resulted in the least contaminated preparation of V is outlined in Fig. 9. With the use of the semi-quantitative assay system it was observed that approximately 90% of the antigen precipitated between 30 and 50% saturated $(NH_4)_2SO_4$ (Table 6). Therefore, a 30 to 50% saturated $(NH_4)_2SO_4$ fraction of Vwa^+ M23 cell extract (3.7 g of protein) was applied to a DEAE cellulose column (2.5 by 43 cm). The column was then washed with Tris buffer at a flow rate of 2 ml per min until no protein was detected in the eluant. At this time, a linear gradient was applied (0.0 to 0.3 M NaCl in Tris buffer) maintaining the flow rate. Fractions of 10 ml were collected and analyzed for V (Fig. 10a). V antigen eluted along with the first protein peak. Although the elution profile from DEAE suggested that this technique was desireable to include in the purification of the antigen, a more stringent analysis revealed that no net purification of V was acheived and more

Table 5. The effect of inoculation density on the production of V antigen by M23 in oxalated N-Z amine.

Inoculation density[a]	Total V[b]	Specific activity[c]		
		CE	CD	CE+CD
0.385	1,695	1.8	1.9	ND
0.389	3,008	1.8	1.8	ND
0.743	ND	ND	ND	3.1
1.4	ND	ND	ND	4.5
1.4	12,096	3.3	7.1	ND
2.65	9,660	2.0	2.5	ND

[a]Optical density at 620 nm.

[b]Total units of V calculated for both CE and CD.

[c]Units of V per ml / mg of protein per ml; CE, crude cell extract; CD, cellular debris wash (see Materials and Methods).

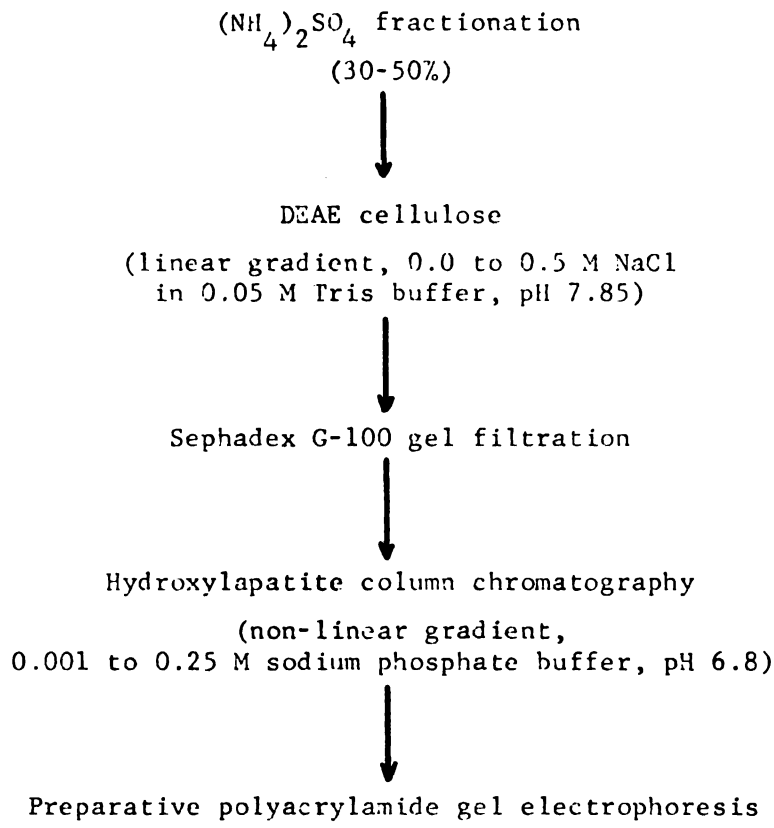


Fig. 9. Outline of the methods used to purify the V antigen.

Table 6. Ammonium sulfate fractionation of M23 cell extract.

Fraction[a]	Resuspended volume (ml)	V titer (units/ ml)	Total units of V
20-25	5.7	16	91.2
25-30	5.6	32	179.2
30-35	8.0	128	1,024.0
35-40	8.1	128	1,126.4
40-45	6.6	128	844.8
45-50	5.3	64	339.2
50-55	5.1	16	81.6
55-60	5.2	2	10.4

[a]A 20-60% $(NH_4)_2SO_4$ fractionation of M23 cell extract was brought to 25% saturation with solid $(NH_4)_2SO_4$ and the solution was stirred on ice for 1 h. The precipitate was pelleted by centrifugation at 39,000 X g for 15 min and resuspended in 5 ml of 0.05 M Tris buffer at pH 7.85. This was designated the 20-25 fraction. The supernatant was then brought to 30% $(NH_4)_2SO_4$ saturation and treated similarly (fraction 25-30). The other fractions were prepared and designated in a similar fashion. All fractions were dialyzed against 0.05 M Tris buffer at pH 7.85 before being analyzed for V antigen.

Fig. 10. Elution profiles of the V antigen from (a) DEAE cellulose, (b) Sephadex G-100, and (c) hydroxylapatite. The methods used in these fractionations are detailed in the Results section of this thesis. The relative absorbancy at 280 nm was continuously monitered in (a) and (b). The protein concentration in each fraction from hydroxylapatite (c) was determined by measuring the absorbancy at 280 and 260 nm and reading the protein concentration from a nomograph. Tracings of the elution profiles are presented. The vertical bars indicate the titer of V in each fraction.

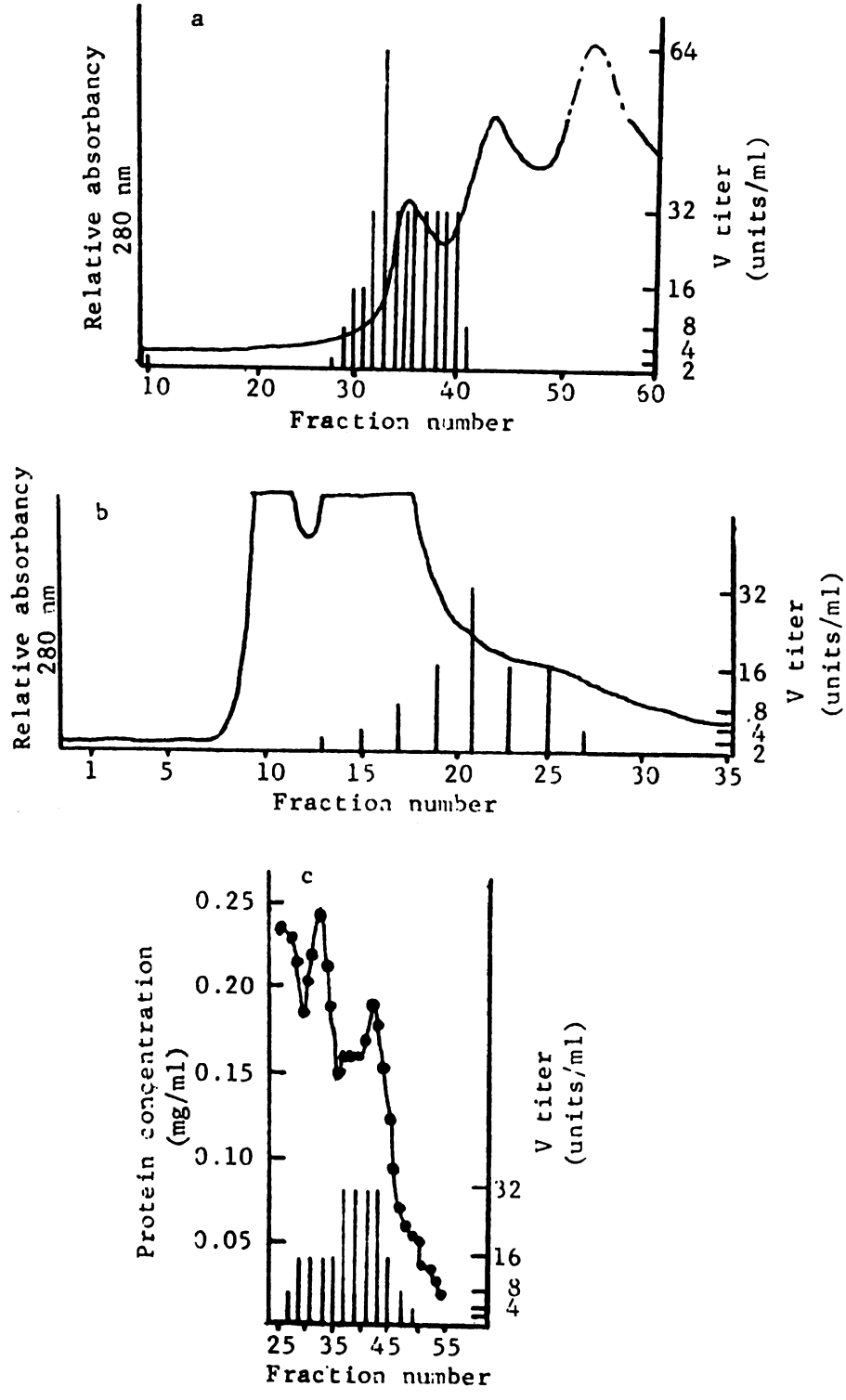

a
Relative absorbancy
280 nm
64
32
16
8
4
2
V titer
(units/ml)
10
20
30
40
50
60
Fraction number
b
Relative absorbancy
280 nm
32
16
8
4
2
V titer
(units/ml)
1
5
10
15
20
25
30
35
Fraction number
c
Protein concentration
(mg/ml)
0.25
0.20
0.15
0.10
0.05
32
16
8
4
V titer
(units/ml)
25
35
45
55
Fraction number

Fig. 10.

than 70% of the antigen adsorbed to the column was not recovered (Table 6).

The fractions from DEAE that contained the highest concentrations of antigen were pooled, concentrated and dialyzed. This concentrate was filtered through Sephadex G-100 (2.5 by 90 cm) in two separate batches (2.0 ml each, containing 145 mg of protein) under an operating pressure of 30 cm. The fractions containing the bulk of the antigen were pooled and, after concentrating the solution, the preparation (69 mg of protein) was adsorbed to an hydroxylapatite column (1.5 by 28 cm). A gradient (non-linear with cylinders one through four containing 0.001, 0.1, 0.1 and 0.25 M sodium phosphate buffer at a pH of 6.8, respectively) was applied at a flow rate of one ml per min. Each cylinder contained 100 ml of buffer resulting in a total gradient volume of 400 ml. The 5 ml fractions from this separation that contained V were concentrated and dialyzed against PBS. The elution profiles from Sephadex G-100 and hydroxylapatite are presented in Fig. 10b and 10c. The antigen eluted as a single symmetrical peak from each of the columns. Both techniques resulted in a net purification of V (Table 7). Preparative disc gel electrophoresis of the V antigen obtained by the methods described above resulted in an alteration of the relative amounts of the proteins in the preparation (Fig. 11).

In order to obtain a better separation of the two major proteins and identify the band associated with V activity, 80 ug of the purified preparation were electrophoresed into a 10 cm polyacrylamide gel (10% acrylamide) for 5.5 h. The gel was scanned at 280 nm and then sliced into 1 mm slices. Each slice was placed in the cold overnight in a test tube containing 0.1 ml PBS to allow the protein to diffuse out of the gel. The concentration of V in each tube was determined. A peak of antigenic activity was detected in the region of the gel that contained the major protein when the slices were matched up with the scan (Fig. 12). Although the evidence is not conclusive, it appears that the major protein in the preparation exhibits V activity (see Fig. 11 and 12).

Table 7. Purification data for the V antigen.

Step		V (units/ml)	Total units	Protein (mg/ml)	Total protein (mg)	Specific activity	Purification (fold)	Percent recovery
cell extract	280	64	17,920	38	10,640	1.7	1	100
$(NH_4)_2SO_4$	54	256	13,824	68	3,672	3.8	2.2	77
DEAE cellulose	4.2	256	1,075	69	290	3.7	2.2	6
Sephadex G-100	6.3	128	806	11	59	11.6	6.8	4.5
calcium hydroxyl-apatite	2.4	64	154	4	10	16.0	9.5	1

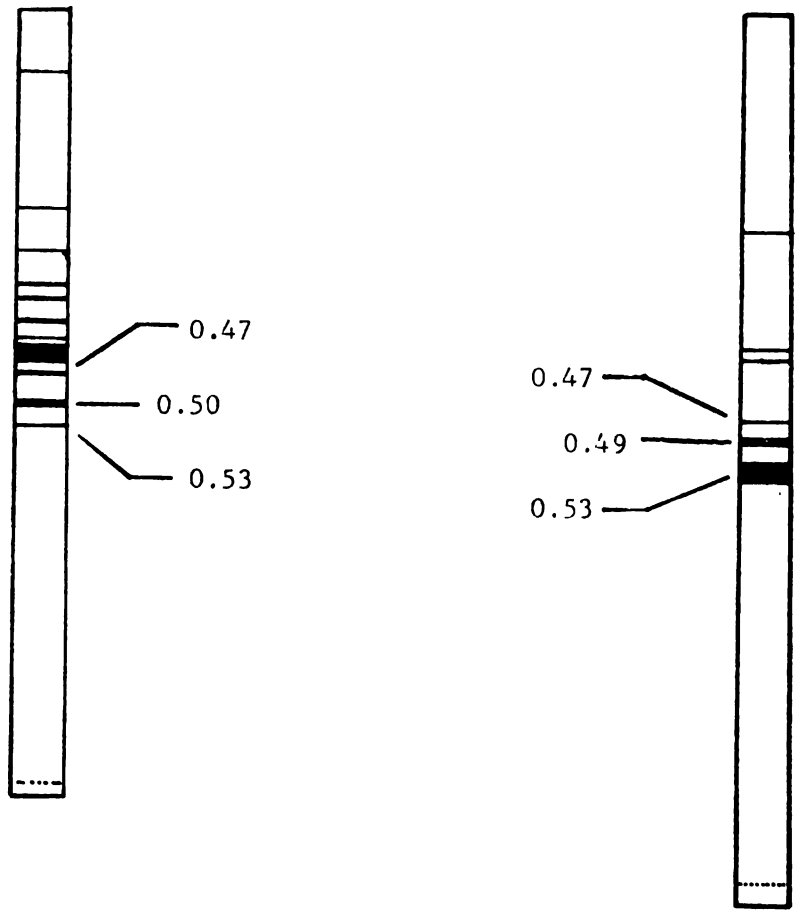


Fig. 11. Analytical polyacrylamide gel electrophoresis of the V antigen. A sample containing 160 ug of protein obtained either before (left gel) or after (right gel) preparative polyacrylamide gel electrophoresis was electrophoresed into the analytical gel until the tracking dye reached the bottom of the gel. After electrophoresis, the gels were stained with 1% Amido black in 7% acetic acid. Presented above are full scale drawings of the stained gels. The mobilities of the proteins that migrate similarly to the V antigen (see Fig. 8) are indicated.

Fig. 12. Identification of a band of protein containing V activity by polyacrylamide gel electrophoresis. The figure is a tracing of a gel scan made with the aid of a Beckman DU Spectrophotometer with an attached linear transport. The inlay indicates the titer of the slices that contained V activity (vertical bars). The diagram on the top depicts a stained gel of the same preparation that was electrophoresed for a shorter period of time (ca. 2 h). The arrows indicate the staining band that correlates with the 280 nm absorbing band. For a more complete description of the methods see the text.

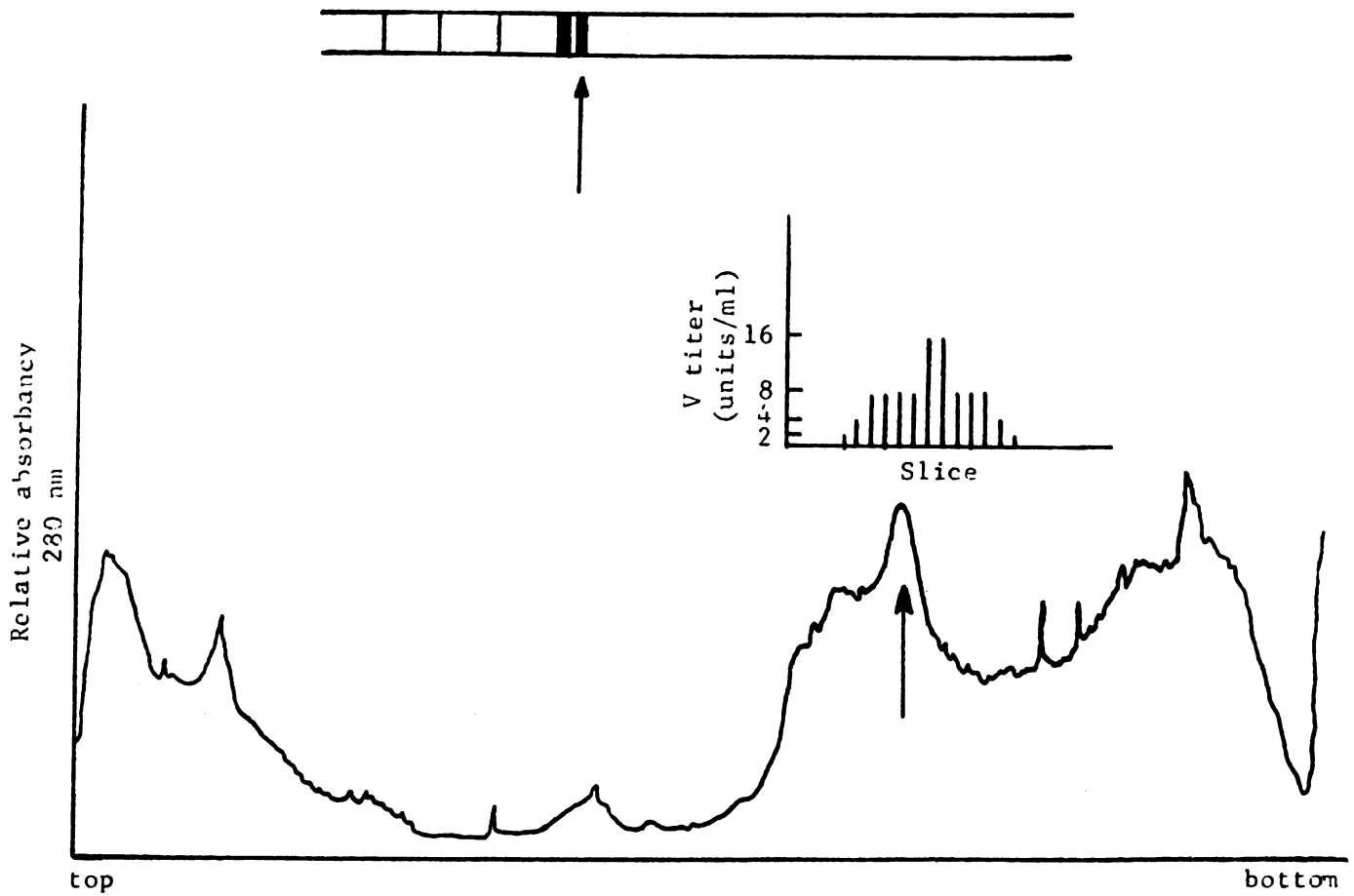

Relative absorbancy
280 nm
V titer
(units/ml)
16
8
2
Slice
top
bottom

Fig. 12.

DISCUSSION

In my first attempts to purify the virulence antigens, I cultivated the bacterial cells in the medium of Lawton, Erdman, and Surgalla (33) and used the spent medium concentrate as the source of the antigens. However, many medium constituents precipitated upon $(NH_4)_2SO_4$ fractionation. This resulted in difficulties in resuspending the precipitate in a workable volume and in subsequent separation of the antigens from the medium constituents. I, therefore, employed the oxalated N-Z amine described in the Material and Methods section of this thesis. The medium proved to be desirable in that; (i) the virulence antigens are produced in the medium, (ii) it contains few constituents that precipitate upon $(NH_4)_2SO_4$ fractionation, (iii) any Ca^{2+}, which would inhibit the expression of V and W, was removed by the addition of sodium oxalate, and (iv) the N-Z amine is less expensive than the Bacto-casitone previously employed for such studies. Although no studies were done comparing the efficiency at which V and W were produced in oxalated N-Z amine verses their production in Bacto-casitone, it is obvious that the former promotes the expression of sufficient V and W to warrent its use for purposes of antigen production and purification.

The observation that the V antigen is cell associated to some extent has been reported by other investigators. Burrows (10) states that 50% of the antigen was deposited with the cells upon centrifugation. Pirt, Thackeray, and Harris-Smith (36) were unable to detect V extracellularly in studies on antigen production in continuous culture. They observed that the antigen was associated with the cell. On the other hand, Brubaker and Surgalla (6) were unable to detect V and W in lysates of virulent cells. These authors were able to demonstrate the presence of both antigens in whole cultures.

The discrepancies in the results reported may be due to differences in the methods used to prepare the culture fluids.

The presence of 0.2 mM Ca^{2+} in the medium of Pirt, Thackeray, and Harris-Smith (36) should not allow significant growth of the Vwa^{+} cells (24). However, this concentration of Ca^{2+} may inhibit V antigen expression to some extent. It is also possible that these authors were unable to detect extracellular V due to the continuous dilution of the culture. The expected transition of the cell population to the Vwa^{-} phenotype may also have adversly affected their results.

Under the conditions specified in this thesis, more than 95% of the V antigen was associated with the cells. The lysates of all the Vwa^{+} strains tested in this work exhibited V activity. The antigen, therefore, is probably associated with the cell.

The assay system used in this research is useful in that it is reliable,, specific, semi-quantitative, and economical. The employment of this assay system allowed various methods of protein separation to be evaluated as to their applicability to the purification of V. It was observed that the DEAE cellulose step was responsible for the loss of a large amount of the antigen. In addition to the poor recovery of V, the method resulted in no net purification. Lawton, Erdman, and Surgalla (33) experienced similar difficulties in their attempts to purify V by batch elution of culture fluids from DEAE cellulose. They report acheiving an average of a 3.4 fold purification with recoveries ranging from 5 to 63%. The recovery of the antigen in their studies appeared to be a function of the amount of protein initially adsorbed to the resin. More recent experiments done in this laboratory have indicated that the phenomenon is also true for the recovery of V from cell extracts; increasing the amount of protein adsorbed to DEAE cellulose decreased the amount of antigen recovered.

I also observed that a large amount of the antigen is lost upon hydroxylapatite column chromatography. Two common characteristics of DEAE and hydroxylapatite column chromatography are that (i) localized high concentrations of protein occur during adsorbtion and (ii) a certain amount of physical stress probably occurs during elution. Possibly, antigenic activity is lost due to one or both of these phenomena. The former may promote aggregation of the antigen whereas the latter may result in the removal of subunits or in the

alteration of the conformation of V. In any case, these methods should not be used in the purification of the antigen unless the conditions are altered in such a way that recoveries and purifications are optimized.

It has been demonstrated that antisera prepared against a partially purified preparation of V passively protects mice against virulent Y. pestis (33). It is not likely that anti-V protects against plague in the manner that antibody to somatic antigens protects against infection by many gram negative organisms. In these cases the reaction between antibody and antigen promotes subsequent phagocytosis or even direct killing of the invading organism via the action of the complement system (17). In that the V antigen is a cytoplasmic component, it is difficult to envision these types of mechanisms occuring with the V-anti-V system. Also, anti-V probably does not act by neutralyzing an exotoxic activity of V because Vwa^+ organisms that lack one of the other virulence determinants do not appear to be toxic to the host (except when injected in very high concentrations where the toxicity is attributed to endotoxin or murein toxin) (3). Toxicity studies with purified V should reveal the relationship of this antigen to other bacterial toxins.

Although the precise role that V plays in the pathogenesis of plague remains obscure, the protective capacity of anti-V strongly suggests that its function it necessary to the disease process. It will now be possible to use the purified preparation of V that was obtained to stimulate the production of monospecific anti-V in rabbits. This antiserum will be useful in studying the pretective role of anti-V as well as in studying the role of V in the pathogenesis of plague. The development of a quantitative assay system, such as quantitative immunoelectrophoresis, would make studies on the expression of the antigen possible. Such an assay system requires monospecific antiserum. It would also be possible to employ this assay system which, along with optimized or deleted DEAE and hydroxylapatite steps and the other techniques described herein, should result in the purification of enough homogeneous V to extensively characterize the antigen both physically and chemically. The information obtained from these proposed investigations should aid not only

in the understanding of the relationship of the V antigen to virulence but also in the understanding of the role of host defense mechanisms, such as phagocytosis, in the prevention of disease.

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LIST OF REFERENCES

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