Development of a Vaccine for Bacterial Kidney Disease in Salmon

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by

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EXECUTIVE SUMMARY

Bacterial kidney disease (BKD) has been and remains a chronic contributory problem limiting the productivity of salmon in the Columbia River Basin. Control of this disease will not come easily, but it would lead to a tremendous increase in the health and numbers of our salmon populations. Vaccination of salmon to Renibacterium salmoninarum (KDB) is a potentially successful method of controlling this disease. To date, however, no successful vaccine has been developed for general use. A possible solution to this problem, and thus the goal of this research, is to isolate the antigenic components of KDB and enhance their ability to activate the host defenses. This will be accomplished by the chemical modification of these antigens with potent immunomodulatory substances. These modified antigens will then be tested for their effectiveness in inducing immunity to BKD and thereby preventing the disease.

The goal of the project's first year was primarily the isolation and characterization of the major antigens of KDB. A major antigen has been identified, which is found both in the bacterial cells and well as spent culture medium. Antisera from rabbits, mice and coho salmon all recognize this antigen as determined by immunodiffusion. Other major antigens generated from spent culture medium, and heat treated cells or sonicates of cells were also characterized,

Enzyme-linked immunosorbent assay (ELISA) systems were also developed for the detection of KDB soluble antigens and to the antibodies specific for the soluble antigens and whole KDB cells. These methods have been successfully used to screen for murine monoclonal antibodies to KDB

antigens.

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INTRODUCTION

Geographical Distribution

Bacterial kidney disease (BKD) is one of the most widespread diseases in the Columbia River Basin and occurs in many parts of the United States, Canada, Europe (Fryer and Sanders, 1981) and Japan (Kimura and Awakura, 1977). The first reported identification of what was most likely R. salmoninarum or kidney disease bacterium (KDB), was in 1930. Smith (1964) communicates that gram positive diplococci were isolated from Atlantic salmon (Salmo salar) found in Aberdeenshire in the River Spey in Scotland. The first recorded cases in the United States occurred in hatcheries in the state of Massachusetts as described by Belding and Merrill (1935). Not only is this disease a very grave problem for hatchery reared salmonids, but it has also been demonstrated to occur in wild populations (Evelyn, et al., 1973).

The Pathogen

The kidney disease bacterium (KDB) is described as a non-motile, non-spore forming diplobacillus. Original attempts to isolate the organism failed due to its rather fastidious requirements for growth in culture media, The first successful attempts at cultivation utilized media with such rich supplements as beef serum and fish extract (Earp et al., 1983). Later work of Ordal and Earp (1956) made use of cysteine blood agar, which contained 202 human blood as well as 0.1% cysteine-HC1. The most recent modifications obviates the need for blood

or serum, thus reducing the cost. This medium employs 0.1% L-cysteine-HCl in Mueller Hinton medium (Wolf and Dunbar, 1954).

Superficially the organisms, especially when seen within the tissues, resemble corynebacteria, thus they have been referred to as corynebacteria and the disease as corynebacterial kidney disease (Ordal and Earp, 1956; Hunn, 1964; Wedemeyer and Rose, 1973). Certain aspects of the pathology caused by this organism resemble not only corynebacterial disease, but to some degree diseases caused by mycobacteria and listeria. However, these rather circumstantial methods of characterization were not satisfactory for rigorous taxonomic classification. Fryer and Sanders (1980) explored this taxonomic problem on the molecular level, and determined that in regards to the guanosine/cytosine (GC) content, peptidoglycan and cell wall composition, these organisms were quite unique. Due to these singular molecular characteristics, these organisms have been placed in their own genus and species, Renibacterium salmoninarum. Isolates from various regions of the world also seem to share only a single serotype (Getchell, 1983).

Disease Pathology

The disease caused by KDB is considered to be a chronic systemic disease, with lesions occurring through much of the viscera and musculature in advanced cases. A common route of entry for the organism has not been demonstrated, and it seems plausible that infection may occur by various routes. Wood and Wallis (1955) have demonstrated that the ingestion of infected salmon flesh by chinook salmon leads to a lethal infection. Alternatively, eye trauma has also been suggested as a possible route of infection. Hendricks and

Leek (1975) found that chinook salmon possessing exophthalmia, demonstrated granulomatous lesions behind the affected eye which contained large numbers of leukocytes and KDB. Many of the fish which possessed this exophthalmia, had no other lesions internally. However, if all fish which exhibited this exophthalmia were held in aquaria, it was found that they all succumbed to BKD within two to three months. Upon necropsy, the animals were found to have disseminated BKD lesions. It was felt that the eye may serve a primary route of infection in hatchery-reared salmonids, due to the frequency of eye trauma when the animals are maintained in raceways. A similar, but perhaps a more common route of infection, may be through general abrasion of the body surface (Wolf and Dunbar, 1959).

Although there are many organs which become infiltrated with KDB, most investigators feel that the primary target is the kidney. The hematopoietic portion of the anterior kidney appears to be especially susceptible. It is a diagnostic feature of the disease to see white granulomatous areas of infection within the kidney. When examined microscopically these areas are seen to possess KD organisms. These foci of infection are not limited to the kidney, but they are found to appear also in the spleen and liver. As the disease progresses, the reproductive organs, musculature, and brain often become infected. Externally, besides the exophthalmia, pustules or blebs may be seen above the lateral fine and petechial hemorrhaging around the muscles of the peritoneum.

One of the most striking features of the internal pathology of BKD is the development of a white pseudomembrane composed of dead host tissue, bacteria, and leukocytes. This false membrane has been seen to cover the liver, reproductive organs, spleen, and

occasionally the swim biadder (Snieszko and Griffin, 1955). This phenomenon is not unlike the pseudomembrane produced in diptheria infections in man. The formation of this pseudomembrane, however, is quite temperature dependent. It is reported that it forms at temperatures below 8.3°C, whereas at higher temperatures only necrosis is found (Smith, 1964). Attempts to isolate an exotoxin as is expresssed by Corynebacteria diptheriae have not met with success.

On the cellular level, bacterial kidney disease appears to be more similar to listerial or mycobacterial infections. A common feature of these diseases are the granulomatous reactions that occur. Like listeria and mycobacteria, KDB organisms are phagocytized by macrophages, but are not always digested by the phagocytic cells (Young and Chapman, 1978). In fact, the KDB as well as listeria and mycobacteria have been observed to multiply within the macrophage itself. In the case of mycobacteria, the cellular arm of the immmune response is eventually activated to destroy the bacteria. The usual delay in this response, coupled with physiological mechanisms the pathogen uses to subvert the phagocytic response leads to a widespread infection. With regards to a specific antibody response, intracellular organisms such as KDB are thought to be relatively resistant, while within their host cell. In this situation, although the pathogen may be protected, the immune system of the host is still exposed to a continuous supply of antigen from the pathogens. As a result there is a continuous severe immune reaction which eventually destroys the surrounding host tissue in attempting to destroy the pathogen. This immune reaction leads to much necrosis and characteristically severe granulomatous lesions. Since KDB is harbored by macrophages of the fish and these severe granulomatous reactions occur in response to the antigen of the pathogen, it is not surprising that two of the organs that are most severely affected are the spleen and kidney. Both

of these organs are immune organs and contain a great number of macrophages.

Effect on the Kidney

It is felt that terminal cases of BKD may be fatal due to the destruction of the kidney which, in turn, may lead to an inability of the salmon to osmoregulate (Frantsi et al., 1975). This feature of BKD is extremely important in light of the evidence that salmon infected with BKD demonstrate marked increase in mortality when held in salt water as compared being held in fresh water (Banner et al., 1983). The possibility arises that even if fish seem relatively healthy or have recovered from BKD after antibiotic treatment, they may be at high risk once they enter the ocean, due to extensive kidney damage.

Kidney pathology, in salmon with BKD,looks quite similar to the pathology seen in glomerulonephritis in mammals. Glomerulonephritis could be mediated by either of two mechanisms: 1) immune complex formation beween KDB antigens and anti-KDB antibodies or, 2) by a reaction of anti-KDB with crossreactive kidney antigen on the basement membrane of the glomerulus. The precise mechanism by which this condition is elicited has yet to be discovered, however, due to the degree of bacterial infiltration that occurs throughout the body of the fish, it would seem most likely that an immune complex reaction could be occurring.

Tolerance

It seems obvious that in most cases of BKD the salmon are responding to the pathogen, but their response seems inappropriate and incapable of overcoming the pathogenic insult. Immunological tolerance may

play a role in BKD if it serves to delay or produce an inappropriate immune response.

If a foreign antigen is present within the body early in life, the animal may experience a state of immunological tolerance (Billingham et al., 1953). In this state the animal does not recognize that particular foreign antigen as being different from its own body and; therefore, it will not respond to it immunologically. This phenomenon may apply in some cases to BKD, It has been demonstrated by Evelyn et al. (1984) that eggs from females with BKD possess the pathogen within the yolk. This was demonstrated microscopically and by iodine treatment of the egg surface. The iodine treatment should have killed any pathogen on the surface of the egg or residing within the perivitelline space, but the organism persisted. If this evidence is correct, it would support the hypothesis of vertically transmitted of BKD and would indicate a route by which fry might experience conditions similar to that giving rise to neonatal tolerance in mammals.

Induction of Protective Immunity

The appearance of circulating antibody does not correlate with protection from the disease. Although this intracellualr location of the pathogen may seem inaccessible, it is not. Mycobacteria and listeria also reside within phagocytes and these pathogens can be controlled if the host is properly sensitized or immunized. Listeria, which possesses physical characteristics similar to those of KDB (Bullock et al., 1975), appears to be insensitive to specific antibody. Priming of the cell mediated immune response, however, results in the elimination of these pathogens. Induction of the cell mediated (T cell) response results in the activation of the phagocytic cell and digestion of the bacteria residing there (Mackaness, 1969).

It is possible to enhance this cell mediated immune rsponse, as well as the immune response in general though the use of adjuvants. Adjuvants utilizing bacteria such as mycobacteria or corynebacteria lead to an enhancement of the immune response to the admixed antigen. Such augmentation may aid in the control of BKD. It has been reported that intraperotineal injections of KDB emuldified in oil and mycobacteria (Freund's complete adjuvant) can lead to a reduction of BKD lesions and organisms (Paterson et al., 1981). Although such immunization procedures would be impractical for large scale vaccine programs, they do demonstrate that proper presentation of KDB antigen to the fish can lead to a protective state of immunity.

MATERIALS AND METHODS

Animals. Two-three year coho salmon (150-300 g) were kept in ambient (12°C) pathogen free well water, in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL). The fish were maintained on Oregon Moist Pellets.

Adult female New Zealand White (NZW) rabbits and BALB/C mice were maintained by the Laboratory Animal Resource Center at Oregon State University.

Bacterial strain. *R. salmoninarum* isolate Lea-l-74 (ATCC 33209), obtained from J. R. Rohovec, Oregon State University, Corvallis, Oregon, was used throughout the study.

Growth conditions. Bacteria were grown in either unfiltered Kidney Disease Medium-2 (KDM-2) or ultrafiltered KDM-2 (UF-KDM-2). Both media were modified by the elimination of the bovine serum supplementation that was originally specified by Evelyn (1977). The UF-KDM-2 was prepared by passage of KDM-2 through a PTGC-10,000 NMWL filter packet in a Minitan ultrafiltration apparatus (Millipore Corp., Bedford, MA). This filtration produced media free of molecules with molecular weights greater than 10,000. Cultures were incubated for one to three weeks in low form culture flasks with constant agitation. At the end of the incubation period, the bacterial cells were centrifuged at 6000 x g for 30 minutes (4°C) and the supernatant fluid was saved for soluble antigen extraction.

Soluble antigen extraction. Culture supernatants were filtered, as described above. The retentates, or high molecular weight fractions, were concentrated by 50% saturated ammonium sulfate (SAS) precipitation. After addition of the ammonium sulfate, the solutions were stirred for 3-4 hours at 4°C. The precipitate was removed by centrifugation at 6000 x g for 15 minutes (4°C) and suspended in 10-20 ml of 0.01 M phosphate buffered saline, pH 7.2 (PBS). The solution was reprecipitated twice and the resuspended precipitate dialysed extensively against PBS. The dialysed protein extract was assayed for protein by the method of Lowry et al. (1951).

Heat extraction. Heat extraction was performed as described previously by Kimura and Yoshimizu (1981). Briefly, bacterial cells, grown as described above, were washed 3x in PBS. After the final wash the cells were resuspended to a concentration of 10% (V/V). A flask of the suspension was then placed in a boiling water bath for 30 minutes. The cells were centrifuged at 5000 x g and the supernatant was filter sterilized (0.45 urn), and stored at 4°C until used.

An alternate method of heat extraction using an autoclave was also employed. Briefly, bacterial cells, grown as described above, were washed three times in phosphate buffered saline (PBS - see appendix). After a final wash, the cells were resuspended to a concentration of 10% (V/V). A flask of the suspension was then autoclaved for 30 minutes. The cells were then centrifuged at 5000 x g and the supernatant was filter sterilized (0.45 urn), and stored at 4°C.

Sonication. Sonicates of bacterial cells were prepared as described by Getchell (1983). Briefly, a suspension of 10% washed

cells in PBS were exposed to four bursts at 50 watts from a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Plainview,NY). Particulates were removed by centrifugation at 5000 x g and the supernate filter sterilized and stored at 4°C until used.

Antibody preparation. Female New Zealand white rabbits were injected subcutaneously between the scapulae (1.4 ml) and in the footpads (0.4 ml each) with a 1:l emulsion of immunogen and Freund's complete adjuvant (FCA). The rabbits werte rested 30 days then bled weekly. Seven weeks post-immunization the rabbits were boosted using the same protocol. The serum was aliquoted and stored at -70°C.

Immunogens used were formalin-killed *R. salmoninarum*, washed three times in PBS and resuspended to 1.0 OD_{520}; dialysed protein extract from UF-KDM-2 (1.5 mg/ml); and dialysed protein extract from KDM-2 (2.2 mg/ml).

Antisera to *R. salmoninarum* isolate K50 was kindly donated by J. R. Rohovec, O.S.U., Corvalli, Oregon.

Antisera from coho salmon were produced by monthly injections of 0.1 ml of a 1:l mixture of formalin-killed *R. salmoninarum* in FCA. Serum was aliquoted and stored at -70°C until used.

Gel filtration. Standard solutions of all antigen preparations were examined with respect to their molecular weight by the use of gel filtration. Briefly, each antigen preparation was eluted through a G-200 column (1.6 x 40 cm). Each sample was adjusted to a concentration of 2.2 mg in 500 ul. Elution was conducted using PBS as the elution buffer. The fractions were monitored for protein using the Lowry method. Molecular weight standards used for the calibration of the column consisted of human

IgG (150 kd), bovine serum albumin (68 kd), and cytochrome C (13 kd).

Immunodiffusion. Immunological methods employing immunodiffusion were performed as described by Getchell et al. (In press).

The Ouchterlony immunodiffusion test was performed on GelBond film (Marine Colloids, Inc., Rockland, ME) adsorbed to glass plates. Antigens (25 ul) and antisera (30 ul) were placed in the wells and allowed to react in a moist chamber for 48 hours (4°C). The dried gels were stained with 0.5% Comassie Brilliant Blue (w/v) and then photographed with background lighting using a macro lens and orange filter (Thirkill and Kenny, 1974).

Immunoelectrophoresis was performed on large glass plates (12 x 12 cm) covered with GelBond film. The supporting agarose matrix used in the following three methods was prepared as described by Thirkill and Kenny (1974). Antigens (5 ul) were placed in each 3 mm well and subjected to electrophoresis at 6 V/cm for 45 minutes. Antisera (200 ul) was then added to the 2 mm wide trough, and precipitation arcs were allowed to form in a moist chamber for 48 hours (4°C). Washing, staining, and photographing were performed as described previously.

Two-dimensional immunoelectrophoresis. The plates, gels, and buffer used for the two-dimensional immunoelectrophoresis were the same as those used in the immunoelectrophoresis experiments. The antigen (20 ul) was added to a 5 mm well cut out of the cathode corner of the gel plate. The first dimension electrophoresis was carried out at 7.5 V/cm for 1.5 hours. The upper portion of the gel (3/4) was then removed and replaced with 15 ml of a 5% antibody-agarose mixture, The second phase was run at 1.5 V/cm for 16 hours at right angles to

the electrophoretic direction of the first dimension. Washing, staining, and photographing were performed as described.

Isoelectric focusing. Isoelectric focusing was performed in a modified procedure of Jackson et al. (1980). Polyacrylamide gels (5%; 115 mm x 230 mm x 1 mm) were cast between glass plates and allowed to polymerize overnight. A pH gradient of 4-6.5 or 3-10 with a 5% carrier ampholyte (Pharmacia, Piscataway, NJ) concentration was used. The distance between the electrode wicks was 9.0 cm. The anolyte and catholyte were 0.04 M glutamic acid and 0.2 M (L)-histidine, respectively. Samples were applied near the cathode by the use of applicator masks (Pharmacia, Uppsala, Sweden). The running conditions were a constant power of 30 watts for 30 minutes. The gels were silver stained according to the method of Merrill (1981).

Growth study. *R. salmoninarum* was grown in nephalo flasks (Bellco, Vineland, NJ) containing either 100 ml of KDM-2 or UP-KDM-2 broth and incubated at 18°C on a shaker. Ten milliliters of this culture, in log phase, was used as a standard innoculum. The growth of the isolate was monitored daily by recording the absorbance at 520 nm.

Conjugation of horseradish peroxidase (HRPO) to antibody. Four mg of horseradish peroxidase (Sigma, St. Louis,MO) were dissolved in one ml of distilled water, To this solution, 200 ul of a 0.1 M solution of sodium meta-periodate (Sigma) in water was added and stirred at room temperature for 20 minutes, This mixture was then dialysed against 1 mM acetate buffer (pH 4.4) overnight at 4°C.

Simultaneously, a 50% saturated ammonium sulfate (SAS) globulin

precipitate of 1 ml rabbit antiserum was reconstituted in 1 ml of distilled water and dialysed overnight at 12°C in 0.01 M Na_2CO_3 pH 9.47. Twenty ul of this bicarbonate buffer was then added to the HRPO to raise it to a pH of 9.0-9.5. The rabbit globulins were then immediately added to the solution and stirred at room temperature for two hours. This conjugate was then dialysed against 0.01 M PBS with a dialysis memebrane having a pore size that included molecular weight species of greater than 50000. The conjugate was stored in the dark in 0.001% merthiolate.

ELISA for the detection of soluble antigens. A method for the detection of soluble KDB antigen was developed utilizing a capture antibody technique. Briefly, individual wells of Costar EIA l/2 well plate (Cambridge,MA) were coated with rabbit anti-soluble antigen (from either UF-KDM-2 or KDM-2. Antigen coating was accomplished by incubating the rabbit antibody diluted in carbonate buffer (0.05 M, pH 9.5) overnight at 4°C. The precise concentration of antibody varied depending upon the experimental protocol. Prior to use, the plates were washed three times in a Tris/Tween diluent buffer (see appendix), followed by three washes in Tris buffer. Solutions containing soluble antigens or unknowns were diluted in Tris/Tween the added to the wells of the plate in 100 ul aliquots and incubated for two hours at room temperature. Following this incubation, the wash sequence was repeated. At this point 100 ul of rabbit anti-soluble antigen-HRPO was added to each well and incubated for two hours at room temperature. This incubation was followed by another standard wash and the addition of 100 ul of the sustrate solution (see appendix). Elaboration of a colored product was read spectrophotometrically at 405 nM by a Biotek Automatic ELISA Reader (Burlington,VT). Absorbance readings were proportional to the amount of soluble antigen bound to the plate since

the conjugate was incubated in excess.

ELISA for antibody to KDB soluble antigen. This assay was basically prepared in a similar manner to the ELISA previously described. Briefly, 100 ul aliquots of soluble antigens were diluted in carbonate buffer and coated on the wells of an ELISA plate. After an overnight incubation at 4°C the plate was washed and a 100 ul aliquot of a test serum dilution or hybridoma supernatant was incubated in the wells for two hours at room temperature. Following this incubation the plates were washed, and 100 ul of the appropriate dilution of rabbit anti-mouse IgG-HRPO (Hyclone, Logan, Utah) was incubated on the plate for two hours at room temperature. After this incubation, the plates were washed and 100 ul of the substrate solution was added and read spectrophotometrically at 405 nM.

ELISA for antibody to KDB cellular antigens. This assay followed the identical procedures as described in the preceding section, except for the method of coating the antigen onto the plate. The antigen in this procedure is the whole bacterial cell and thus it does not readily adsorb to the ELISA plate. To facilitate binding of the whole KDB cell, the wells must first be coated with 100 ul of a 0.1 mg/ml solution of poly-L-lysine in water. The poly-L-lysine is allowed to adsorb for two hours at room temperature. Wells were then washed once in PBS and then incubated with dilutions of KDB in PBS. The cells were allowed to adsorb for one hour at room temperature. The wells were then washed twice in PBS. This wash was followed by an incubation of 100 ul of 0.25% glutaraldehyde for three minutes Following this incubation, the plates were washed three times

with PBS and the remaining reactive sites were blocked with the addition of a solution of 10 mg/ml bovine serum albumin (BSA) in 100 mM glycine. Plates were blocked overnight at room temperature before use.

Monoclonal antibody production. Adult female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were immunized with either 100 ug soluble antigen or 1 O.D. unit of formalin-treated KDB emulsified in Freund's complete adjuvant. Sera from immunized animals were tested at one month. Animals with positive titers, as determined by the previously described ELISA, were challenged intravenously (i.v.) with 10 ug of the same antigen in physiological saline. Animals with negative titers were boosted with the initial antigen preparation until a positive titer appeared.

Three days after i.v. challenge, single cell suspensions were prepared from the spleen and fused with a equivalent number of SP/2 myeloma cells by the aid of polyethylene glycol (Fisher, Fair Lawn, NJ). Hybridomas were selected by addition of hypoxanthine-thymidine -aminopterin (HAT) medium (Oi and Herzenberg, 1980). Productive fusions were ascertained by screening the hybridoma supernatants with the previously described ELISA. Cells from these fusions were cloned and stored in liquid nitrogen until used.

RESULTS AND DISCUSSION

Molecular weight and antigenic analysis of heat extracted antigens. Standard amounts of 2.2 mg heat extracted antigen were chromatographed on a G-200 column. Protein analysis of the fractions revealed similar protein profiles for boiled KDB (Fig. 1) and autoclaved KDB (Fig. 2), although the middle molecular weight protein peak was more pronounced than the two outer peaks from the autoclaved preparation. The left outer peak represents proteins found in the void volume (i.e. possessing molecular weights in excess of 600 Kd). The right outer peaks represent small peptide fragments of less than 10 Kd.

All fractions taken from the column were tested in a simple immunodiffusion for the presence of antigen. Precipitin lines (graphically represented in figures 3, 4, and 6) indicated the presence of the antigens. Each gel filtration figure also demonstrates the presence (+) or absence (-) of antigen in each fraction.

Examination of these diffusion patterns reveals another similarity between both forms of heat extraction. The antigenic activity seems to be in the same molecular weight range for both preparations (Figures 1 and 2). Both procedures yield antigens ranging from 37-150 kd with some antigenic activity occuring in the void volume.

Molecular weight and antigenic analysis of sonicated antigens. A 2.2 mg protein amount of sonicated KDB revealed no large mid molecular range of protein as did the heated preparations (Figure 5). Most of the proteins were present as large molecular weight material or small peptides. This is not unusual in that sonication would cause solubilization of large molecular weight complexes, that may not be

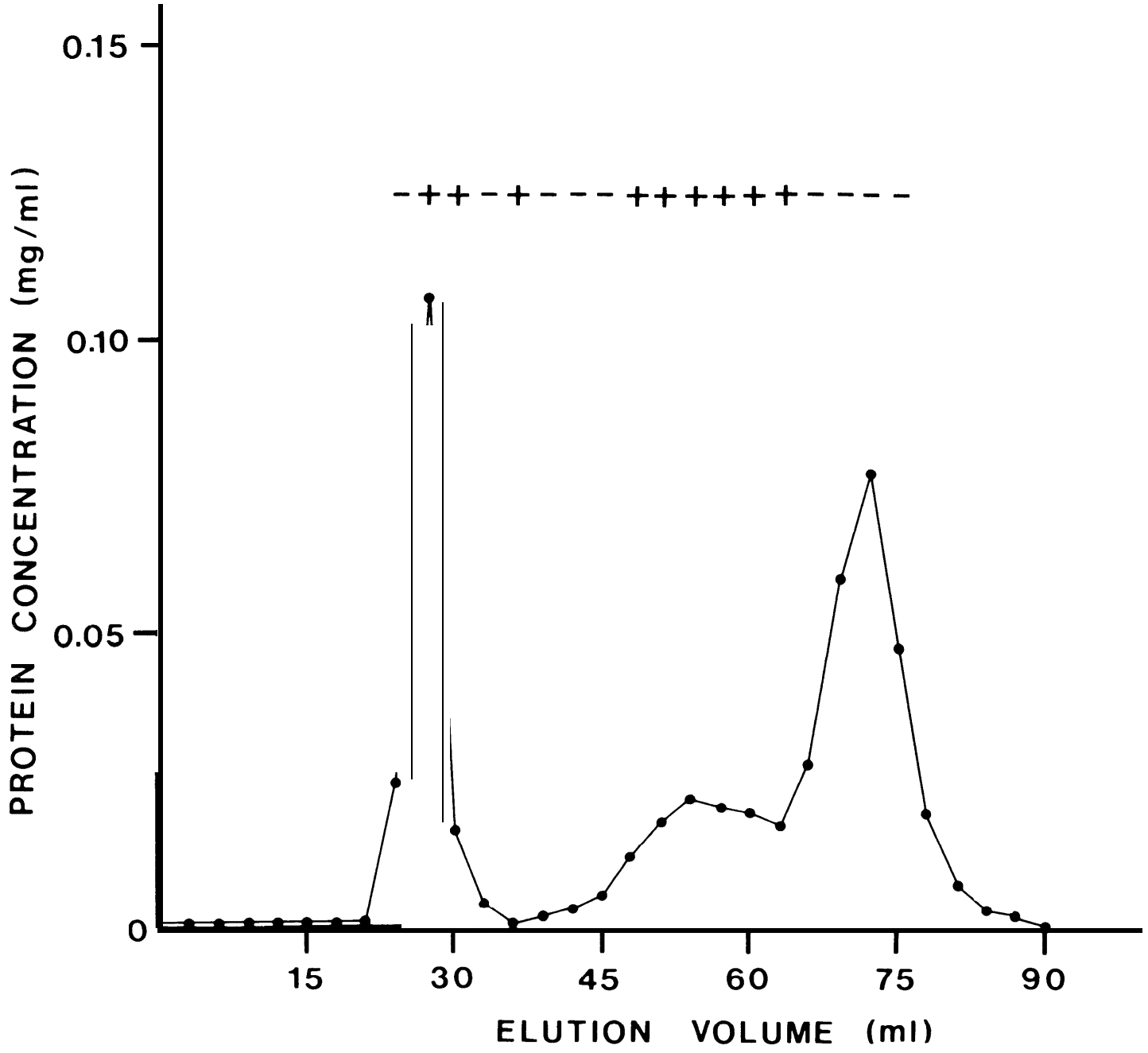

Figure 1. Molecular weight fractionation of KDB heat extract antigens using Sephadex G-200 column chromatography. A 2.2 mg aliquot of an extract obtained from a 10% suspension of KDB in PBS placed for 30 minutes in a boiling water bath was applied to a G-200 column (1.6 x 40 cm). The flow rate was 10 ml/hour and the elution buffer was PBS. The protein concentration was determined by the Lowry method. The presence (+) or absence (-) of antigenic activity are given.

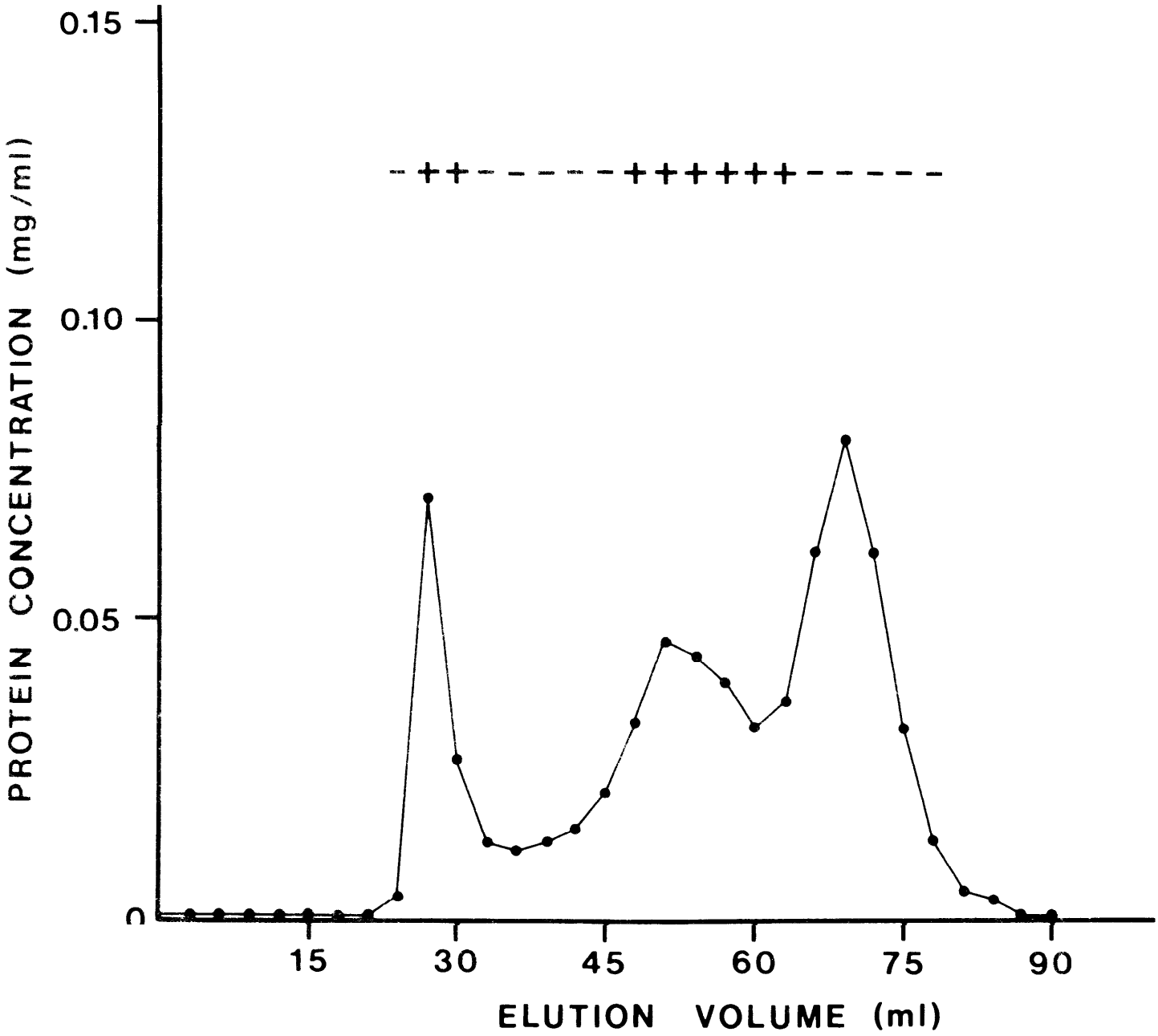

Figure 2. Molecular weight fractionation of KDB autoclaved extract antigens using Sephadex G-200 column chromatography.

A 2.2 mg aliquot of an extract obtained From autoclaving a 10% suspension of KDR in PBS for minutes was applied to a G-200 column (1.6 x 40 cm), The flow rate was 10 ml/hour and the elution buffer was PBS. The protein concentration was determined by, the Lowry method. The presence (+) or absence (--) of antigenic activity are given,

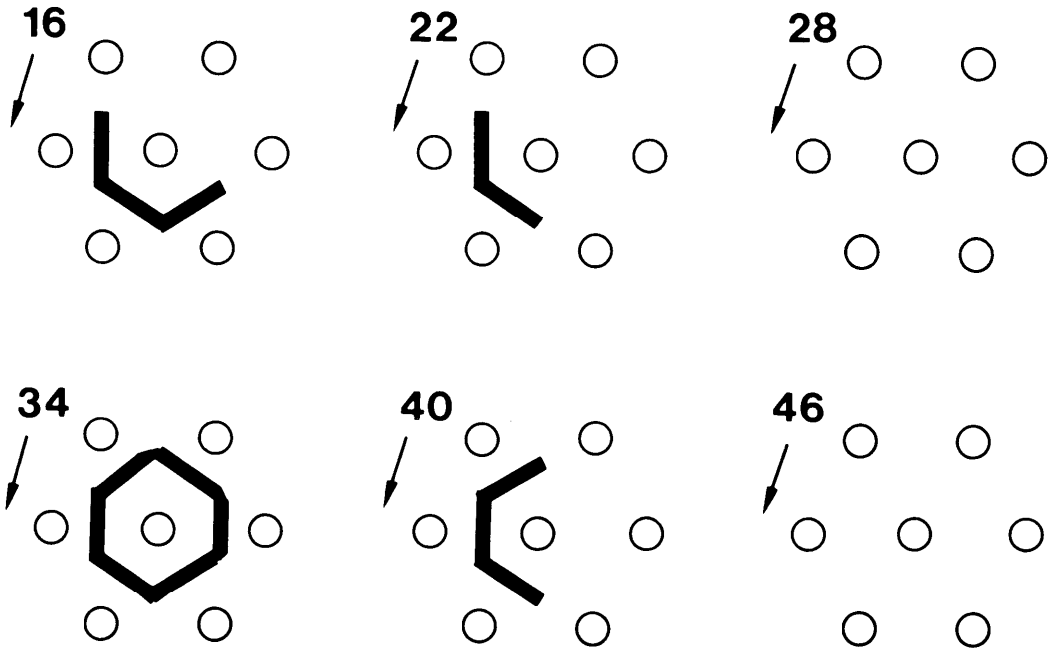

Figure 3. Identification of antigens in the G-200 fractions of the heat extract as assessed by immunodiffusion. Ouchterlony immunodiffusion analysis of KDB heat extract fractions (Fig.1) from the G-200 column. Well numbers correspond to their respective fraction numbers. The center wells contain rabbit anti-sonicate serum. The lines represent the precipitates produced upon the reaction of the antisera with a fraction containing KDB antigen.

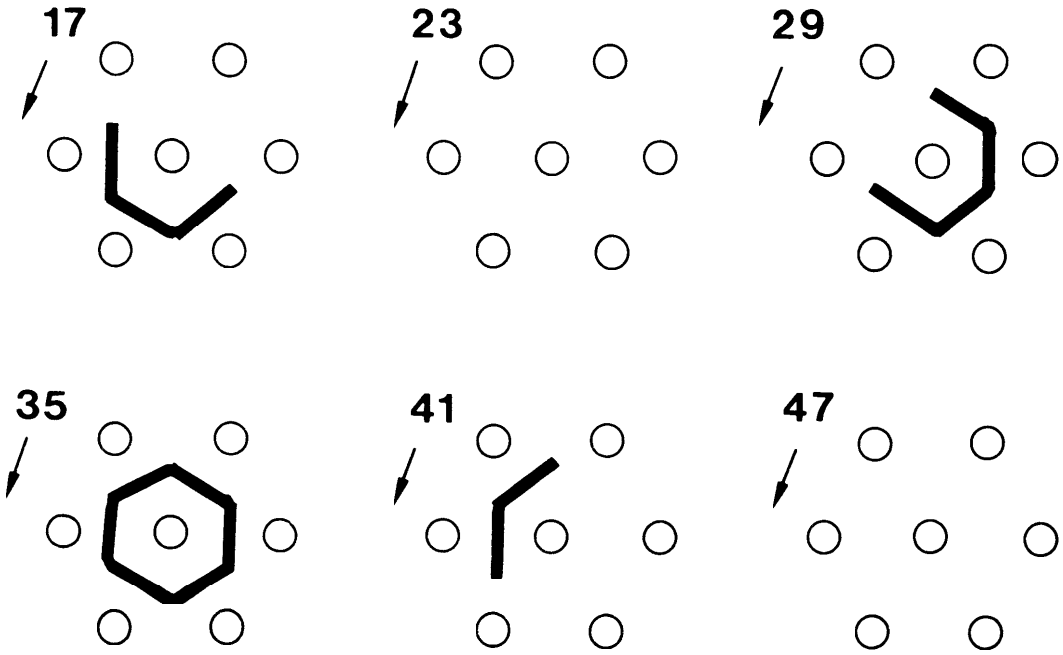

Figure 4. The identification of antigens in the G-200 fractions of the autoclaved extract as assessed by immunodiffusion. Ouchterlony immunodiffusion analysis of KDB autoclaved extract fractions (Fig.2) from the G-200 column. Well numbers correspond to their respective fraction numbers. The center wells contain rabbit anti-sonicate serum. The lines represent the precipitates produced upon the reaction of the antisera with a fraction containing KDB antigen(s).

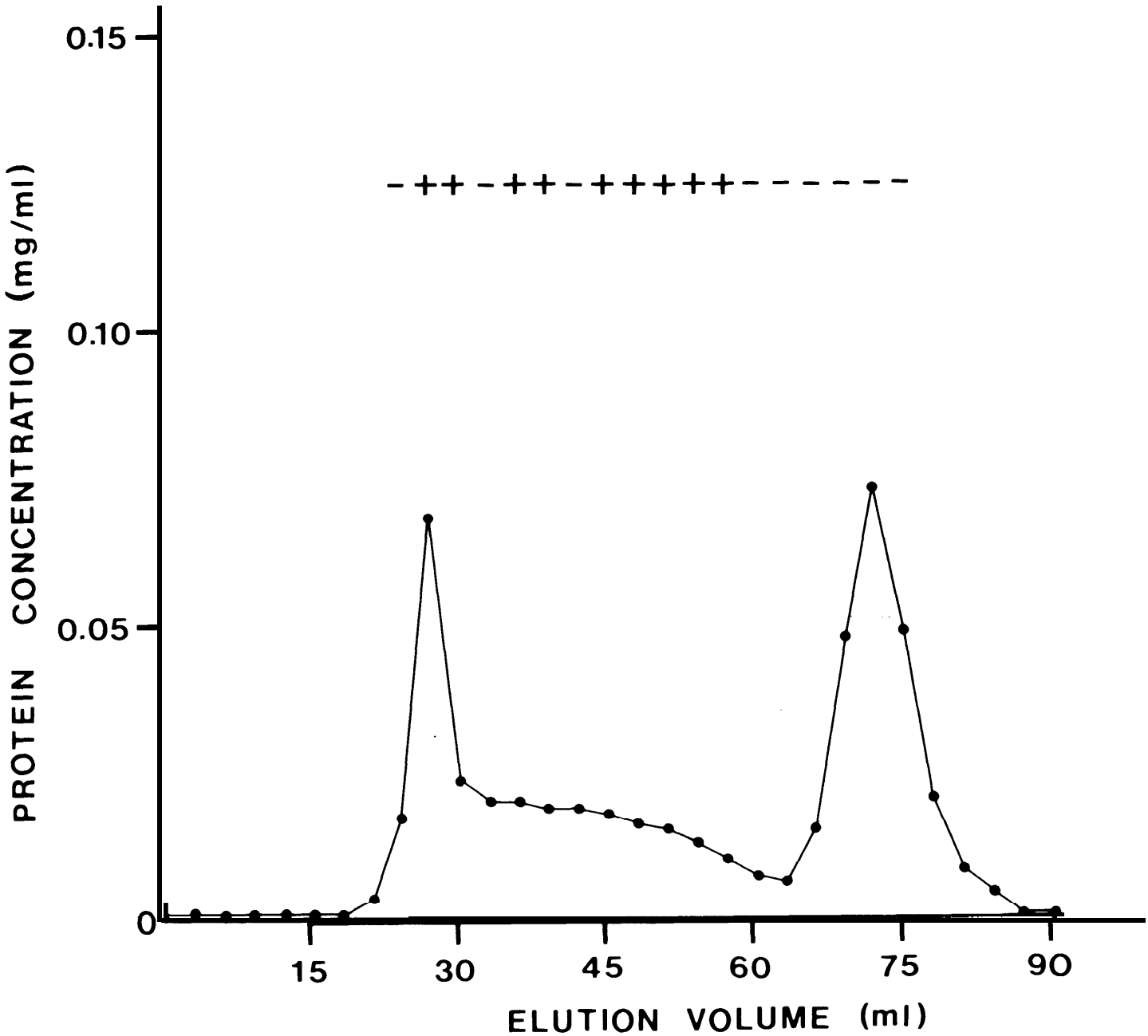

Figure 5. Molecular weight fractionation of sonicated KDB antigens using Sephadex G-ZOO column chromatography. A 2.2 mg aliquot of a sonicated preparation of KDB in PBS was applied to a C-200 column (1.6 x 40 cm). The flow rate was 10 ml/hour and the elution buffer was PBS. The protein concentration was determined by the Lowry method. The presence (+) or absence (-) of antigenic activity are given.

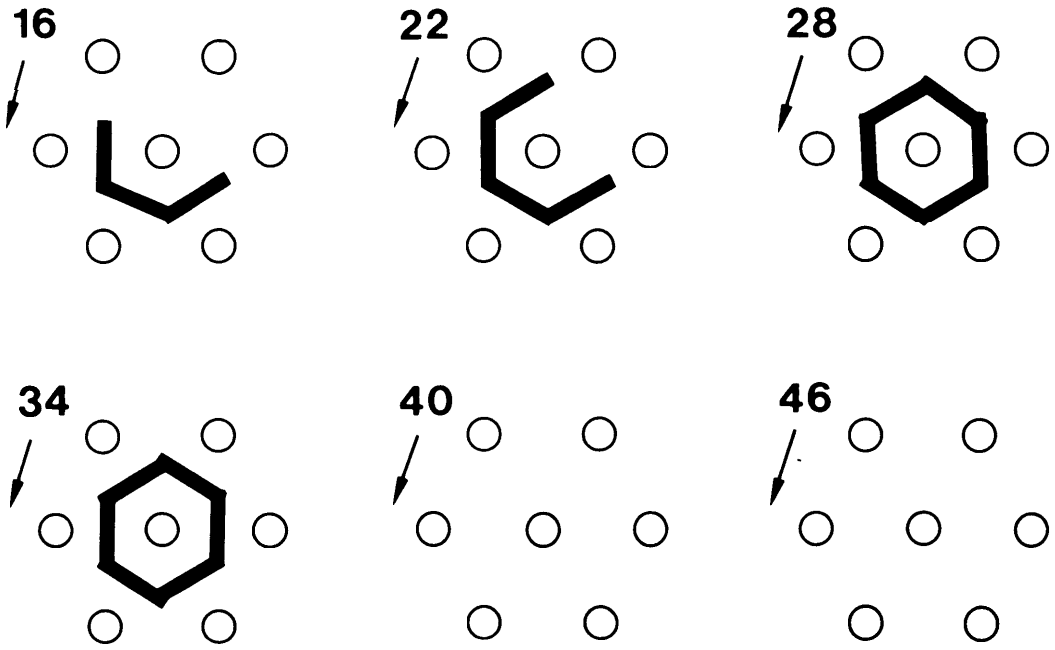

Figure 6. The identification of antigens in the G-200 fractions of the sonicated preparation as assessed by immunodiffusion. Ouchterlony immunodiffusion analysis of KDB sonicate fractions (Fig. 5) from a G-200 column. Well numbers correspond to their respective fraction numbers. The center wells contain rabbit anti-sonicate serum. The lines represent the precipitates produced upon the reaction of the antisera with a fraction containing KDB antigen(s).

liberated by simple heating. The immunodiffusion profile (Figures 5 and 6) reveal a relatively scattered range of molecular weights for these antigens. Sonication yields 69-200 kd, 350-450 kd, and greater than 600kd ranges for the antigens.

Characterization of soluble antigen. The following section examines thoroughly, by various methods,the soluble antigens, both produced in UF-KDM-2 and KDM-2. These antigens, produced in culture media, represent strongly immunogenic antigens as recognized by the rabbit and coho salmon (*Oncorhynchus kisutch*, data not shown). Most of the following studies make comparisons between the soluble antigens harvested from ultrafiltered KDM-2 (UF-KDM-2) and unfiltered KDM-2. This procedure of ultrafiltration was used in order to prevent contamination of the antigen preparations by any high molecular weight components from the medium.

Molecular weight and antigenic analysis. As can be seen from figure 7, a considerable amount of a SAS (50%) precipitable material (■) can be derived from KDM-2 alone. Addition of 50% SAS to UF-KDM-2 causes no precipitation, therefore, ultrafiltration appears to eliminate SAS precipitable material that could be derived from unfiltered KDM-2. Growth of KDB on UF-KDM-2 produced a narrower range of protein molecular weights (A) than did growth of KDB on KDM-2. As the figure also reveals, antigenic activity is also found in a much narrower range of molecular weights (51-350 kd vs >69-600 kd, for KDM-2). It is apparent from this figure that ultrafiltration produces a much purer preparation of antigen.

SDS-PAGE anlysis of soluble antigens. Both forms of soluble antigen were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The running of this material revealed two important facts (Figure 8); 1) both preparations seem to reveal three similar bands of 60 kd, 34 kd, and 26 kd and 2) no higher molecular weight

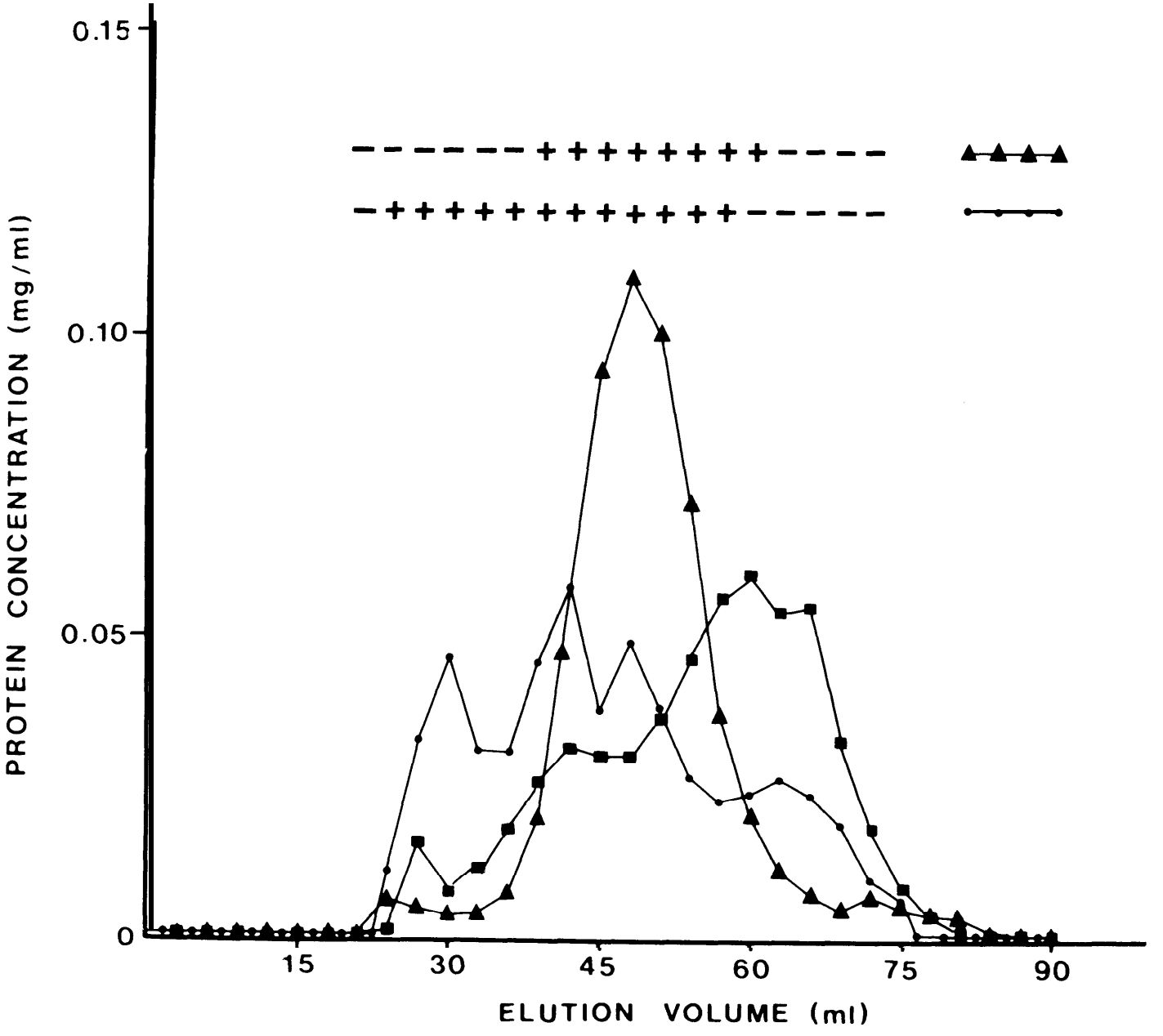

Figure 7. Molecular weight fractionation of KDB soluble antigens using Sephadex G-200 column chromatography. A 2.2 mg aliquot of the soluble antigens from KDM-2 (●) or UF-KDM-2 (A) were applied to the G-200 column and eluted at a flow rate of 10 ml/hour in PBS. The protein concentration was determined by the Lowry method. The medium control (■) represents the precipitated components of an uninoculated sample of KDM-2. The presence (+) or absence (-) of antigenic activity in each fraction are given.

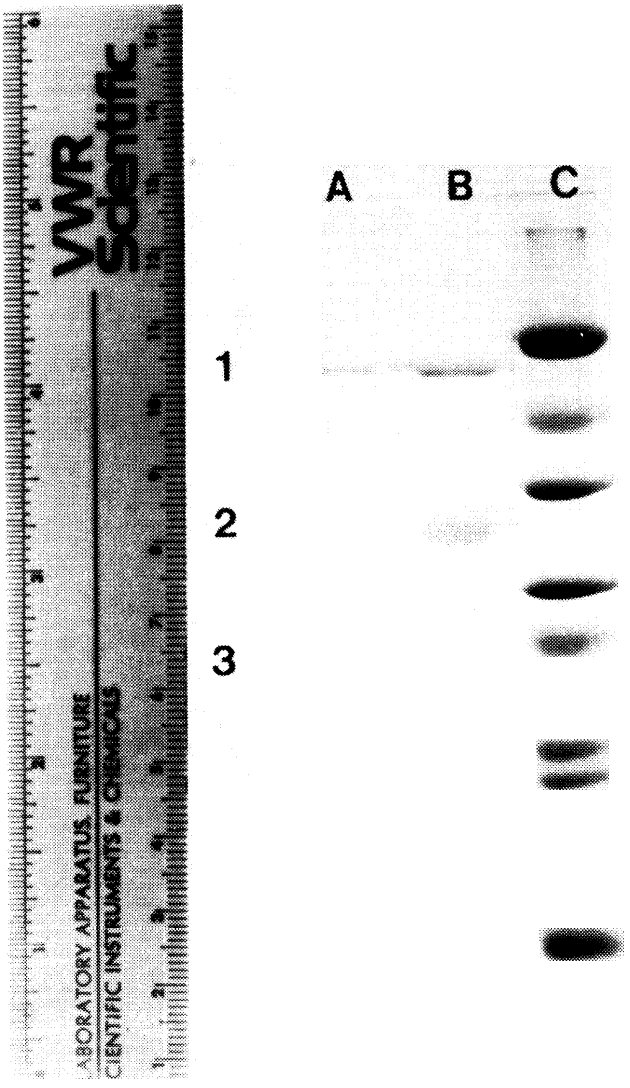

Figure 8. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of BKD soluble antigens. Soluble antigens of KDB were extracted from KDM-2 or UF-KDM-2 spent culture media. Lanes: A) UF-KDM-2 antigens; B) KDM-2 antigens; C) molecular weight standards -66 kd (10.5 cm), 45 kd (9.6), 36 kd (8.7), 30 kd (7.2), 24 kd (6.69, 20 kd (5.1), and l4kd (2.4). The three BKD soluble proteins are identified as 1 (60 kd), 2 (34 kd) and 3 (26 kd). Protein 3 (26 kd) from the UF-KDM-2 was very faint, but easily visualized by direct examination of the gel.

protein bands were revealed. This could indicate that either all the higher molecular weight species may be simply aggregates of the 60 kd or smaller molecules. Alternatively, the large molecular weight molecules may have been too large to enter the gel. This last hypothesis seems untenable because a portion of the stacking gel above the UF-KDM-2 and KDM-2 channels revealed no protein. It, therefore, seems most plausible that the proteins in the soluble antigen preparations consist of only the three distinct molecular weight species described above. It is also of interest to note that together the two lower molecular weight species would equal the molecular weight of the 60 kd molecule, suggesting the possibility that the two may be break down products or subunits of the larger molecule.

Immunological comparison of soluble antigens. Simple double diffusion reveals that there appears to be three distinct bands which are shared by UF-KDM-2 and KDM-2 preparations (Figure 9). The fact that there are three precipitin bands and three molecular weight species argues very strongly, yet circumstantially, for each of the protein bands seen on SDS-PAGE being a separate antigen.

Immunoelectrophoresis. Immunoelectrophoresis of both soluble preparations, again reveals no distinctly different immunoprecipitate bands between the two preparations (Figure 10).

Tandem two-dimensional immunoelectrophoresis. In an attempt to space the antigens differentially, and thereby increase the sensitivity in the detection of antigenic differences, tandem two-dimensional immunoelectrophoresis was performed on the preparations (Figure 11). Regardless of whether the wells were spaced closely (Figure 11) or distant (Figure 12), the precipitin bands always appeared totally identical.

Isoelectric focusing. The most exacting method used to

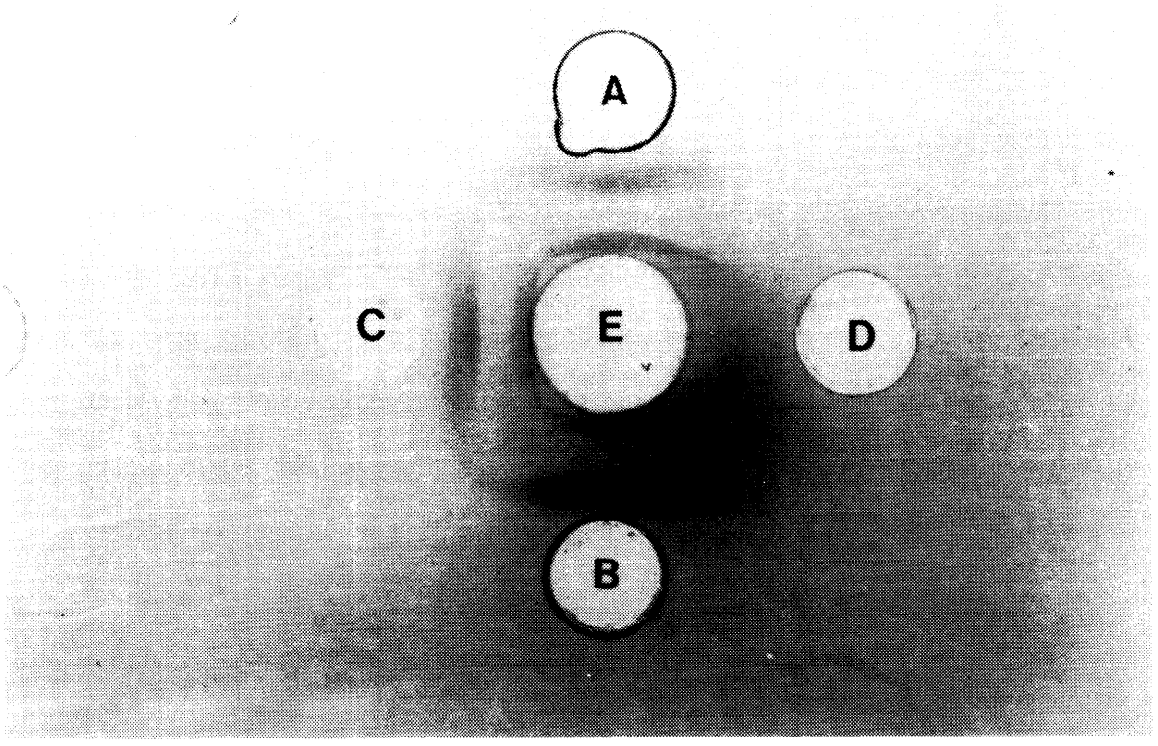

Figure 9. Ouchterlony immunodiffusion analysis of KDB soluble antigens. Wells were cut in an agarose gel and filled with UF-KDM-2 antigens (A and B), KDM-2 andtigens (C), or KDM-2 medium control (D). The center well (E) was filled with rabbit anti-sonicate serum. The filled gel was then allowed to incubate for two days at 4°C. Precipitin lines demonstrate the three crossreactive antigens.

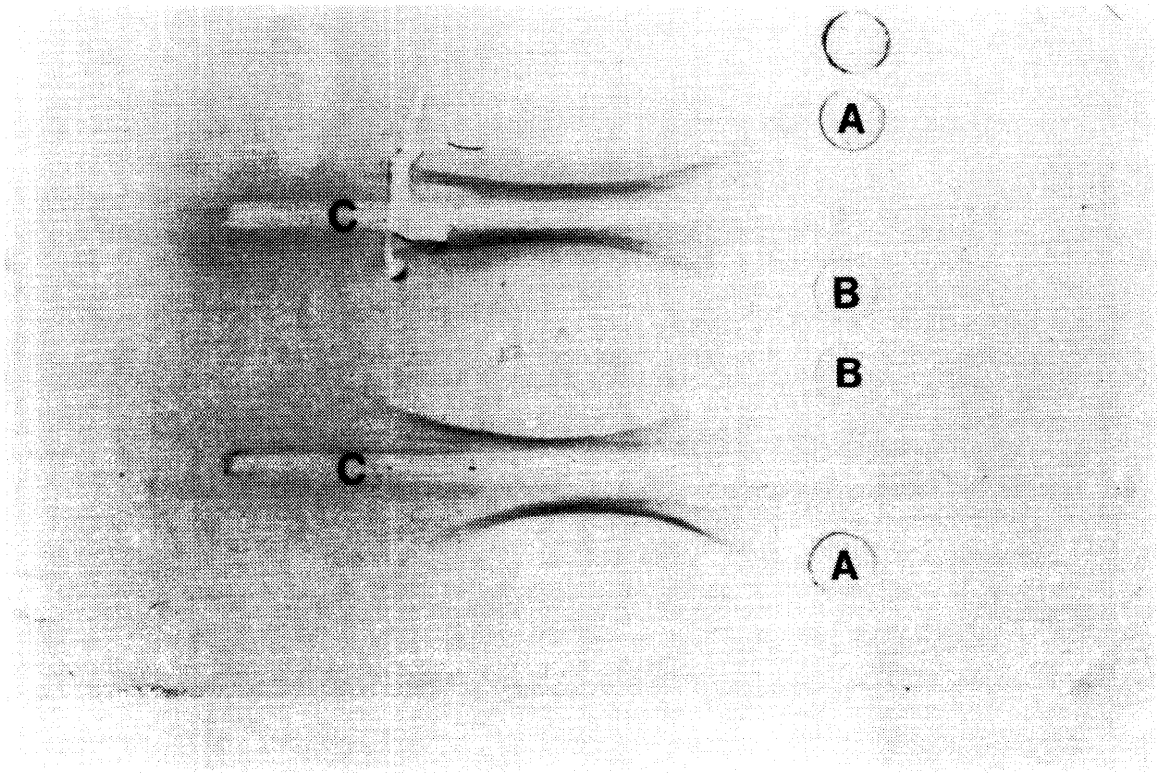

Figure 10. Immunoelectrophoresis of KDB soluble antigens. Wells were cut in an agarose gel and filled with KDM-2 antigens (A) or UF-KDM-2 (B), The antigens were then electrophoresed. Following electrophoresis, rabbit anti-KDM-2 antiserum was placed in the troughs (C). Immunodiffusion was allowed to occur for two days at 4°C. Precipitin lines indicate the multiple antigens recognized by the antiserum.

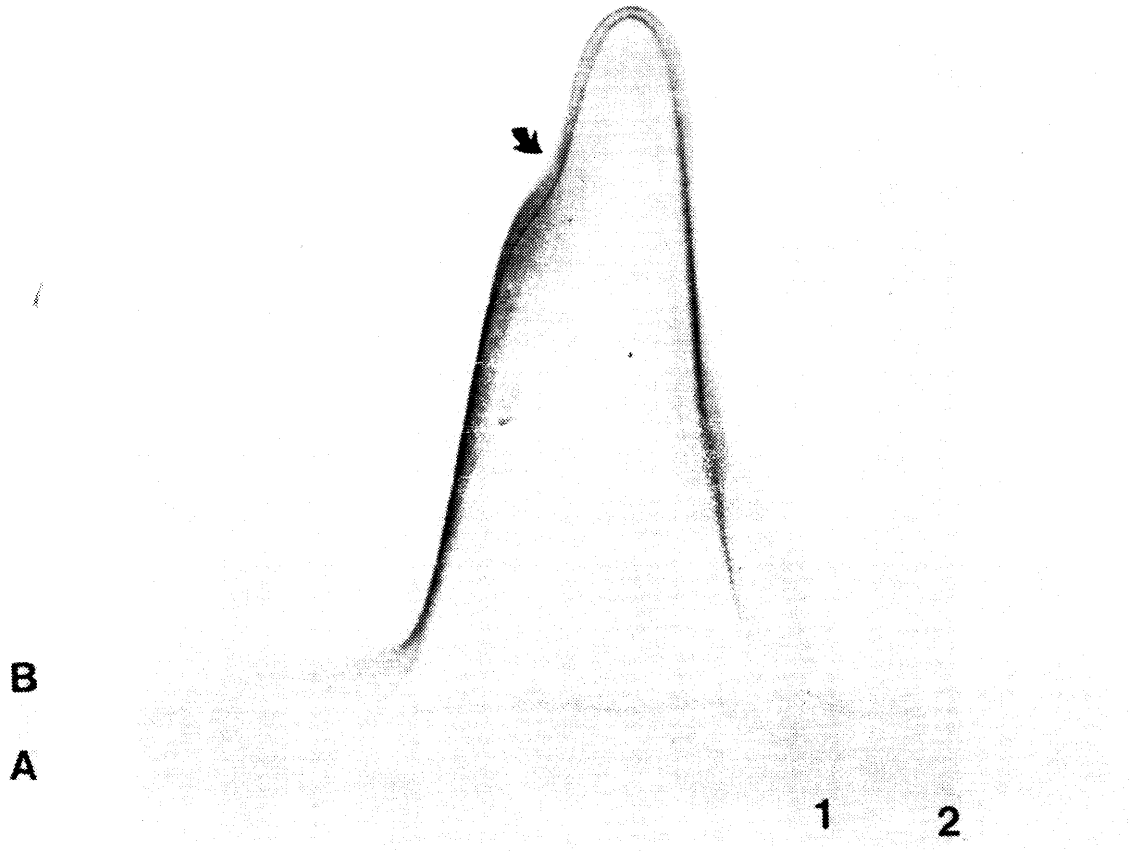

Figure 11. Tandem two dimensional immunoelectrophoresis. Wells were cut in an agarose gel and filled with KDM-2 antigens (1) or UF-KDM-2 antigens (2). The antigens were then electrophoresed. Following this initial elctrophoresis, the antigen containing gel (A) was set next to another gel slab containing 5% anti-sonicate serum (B). The antigens were then electrophoresed into the antibody-containing gel slab. The reaction of immunological identity between the two antigen preparations is noted by the arrow.

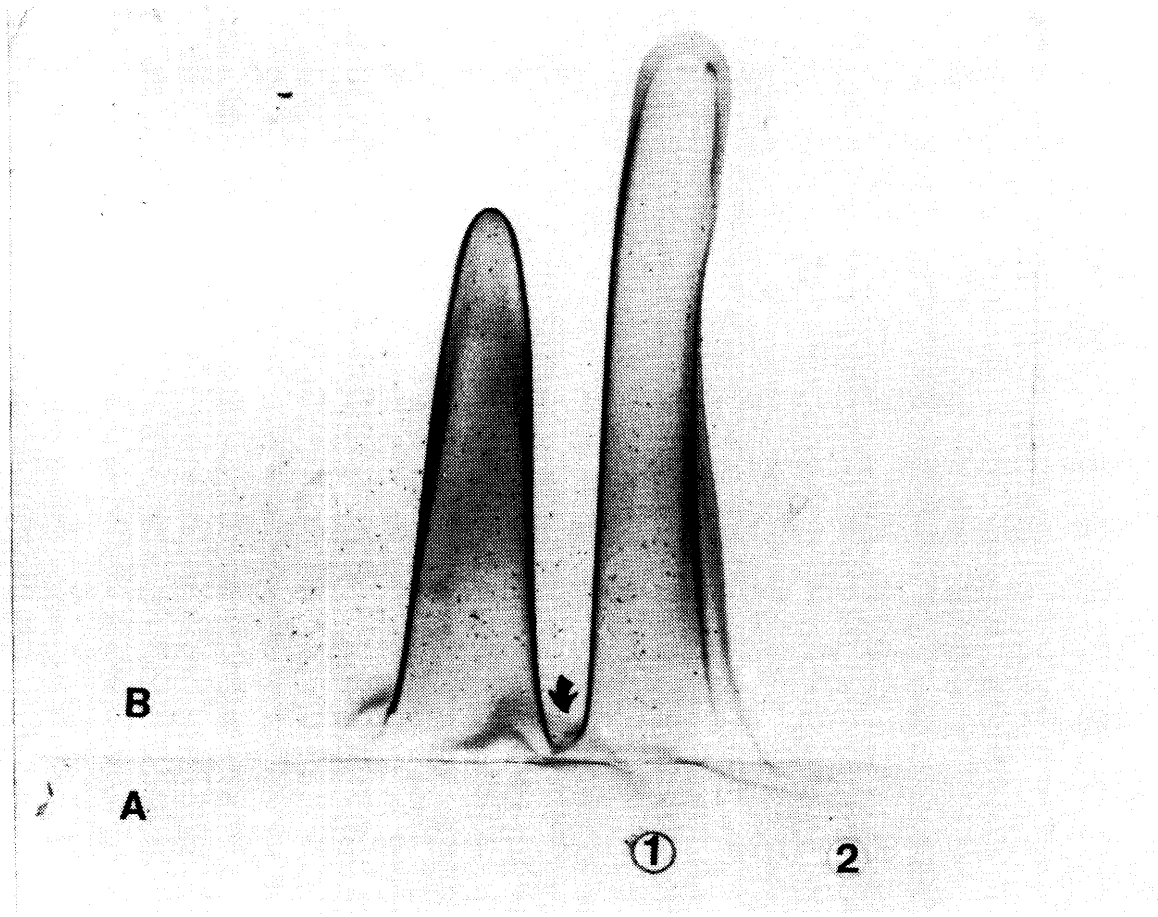

Figure 12. Tandem two dimensional immunoelectrophoresis (alternate). Gels were prepared and run as described in figure 11 , with the exception that the wells were spaced at a distance of 17 mm rather than 5 mm. Reaction of identity between the major antigens is noted by the arrow.

differentiate proteins is isoelectric focusing (IEF). The power of this technique is that it separates proteins on the basis of the proteins' isoelectric points (pIs). The gel shown in figure 13 has a pH range of 3-10. The standards have a 5-10.5 pI range, the top most being beta-lactoglobulin (pI 5.2). Both the UF-KDM-2 and KDM-2 generated antigens have pIs equivalent to or less than this marker.

Isoelectric focusing was also conducted with ampholytes producing a more restricted pH range of 4-6.5 (Figure 14). Again, it can be seen that the p1 of the soluble antigens from either preparation fall very close to that of the beta-lactoglobulin marker. The most striking feature of these IEFs are the increased number of bands seen with KDM-2 generated soluble antigen. This does not represent a concentration difference in the amount of protein applied to the gel, for both lanes received 10 ug of the corresponding antigen. In light of the SDS-PAGE and immunodiffusion studies, it becomes difficult to explain the presence of more than three protein bands in the KDM-2 IEF gels. A possible explanation may be that the multiple bands may represent different glycosylated versions of the same protein antigens seen with the UF-KDM-2 preparation.

Growth rates. Growth comparisons between the two media demonstrated that R. salmoninarum took 2-5 days longer to reach log phase when grown in UF-KDM-2 as opposed to KDM-2 (Figure 15). Pier et al. (1983), reported reduced growth of Pseudomonas aeruginosa when using UF-TSB (ultrafiltered typticase soy broth), This extended lag phase, when culturing R. salmoninarum in UF-KDM-2, may be due to the loss of a nutrient or growth factor during ultrafiltration. Once R. salmoninarum began to multi ply, however , it. rel eased the same antigcnic products and attained the same optical density as when cultured in KDM-2.

Comparison of antigens by SDS-PAGE. Figure 16 shows the protein

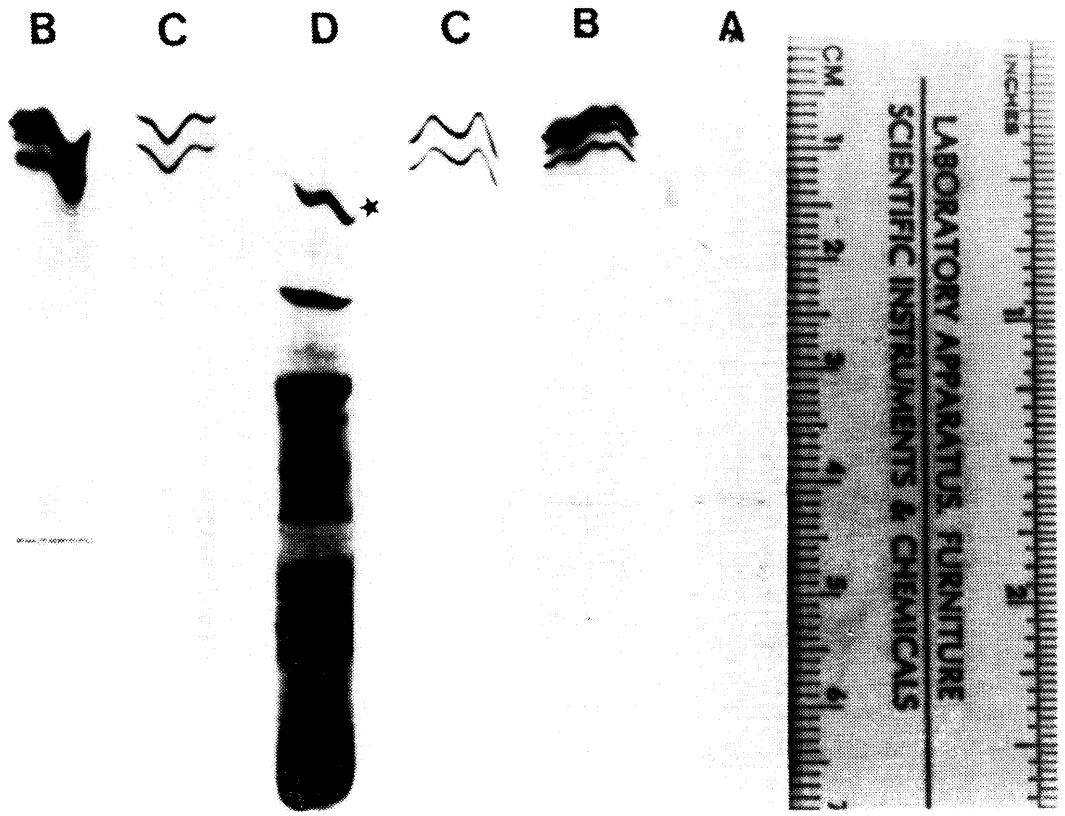

Figure 13. Isoelectric focusing of soluble antigens, Samples of KDM-2 medium control (A),, KDM-2 antigens (B), UF-KDM-2 antigens (C), and isoelectric point (PI) marker proteins (D) were electrophoresed into a gel containing 5% ampholytes which generated a pH range of 3.0 (top) to 10.0 (bottom), The pI of the top most marker (beta-lactoglobulin) was 5.2(✶).

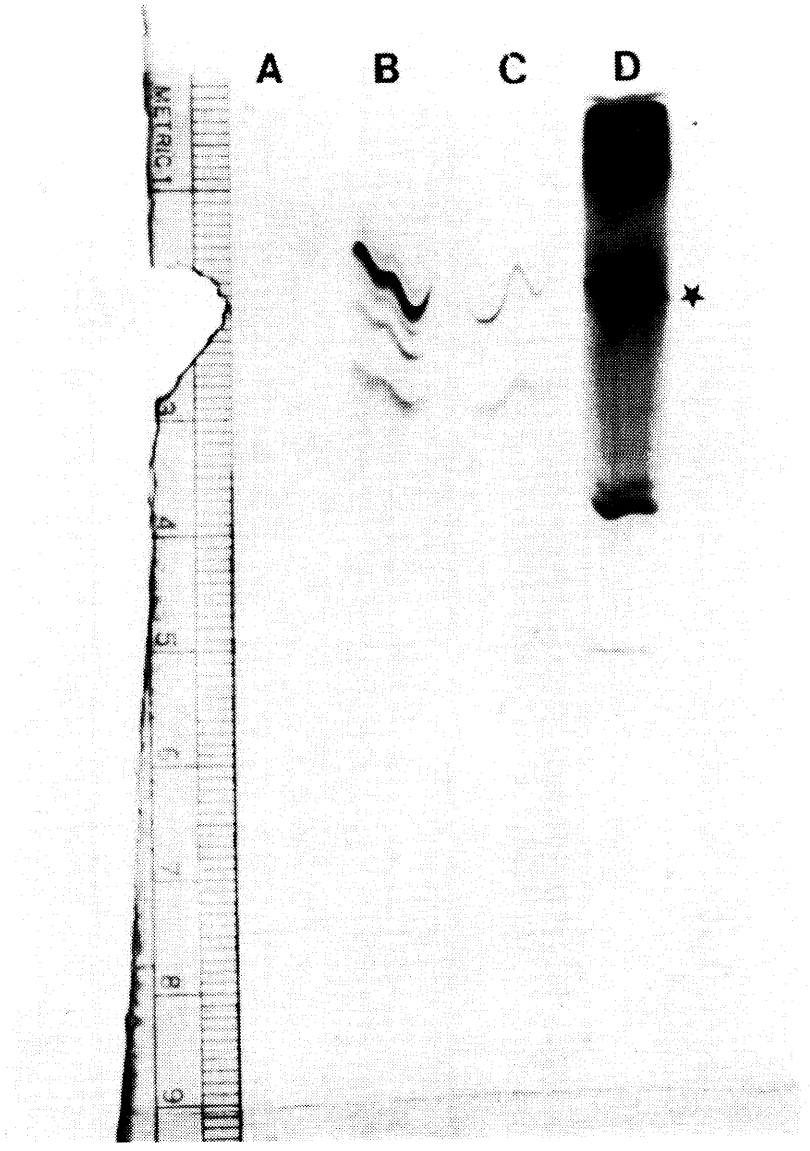

Figure 14. Isoelectric focusing of soluble antigen (narrow pH range). The gel presented contained 5% ampholytes with a pH range of 4.0-6.5. Lanes: (left to right), A) KDM-2 medium control (no bands detected); B) KDM-2 antigens; C) UF-KDM-2 antigens; D) pI markers. The center pI marker band (✶) represents a pI of 5.2.

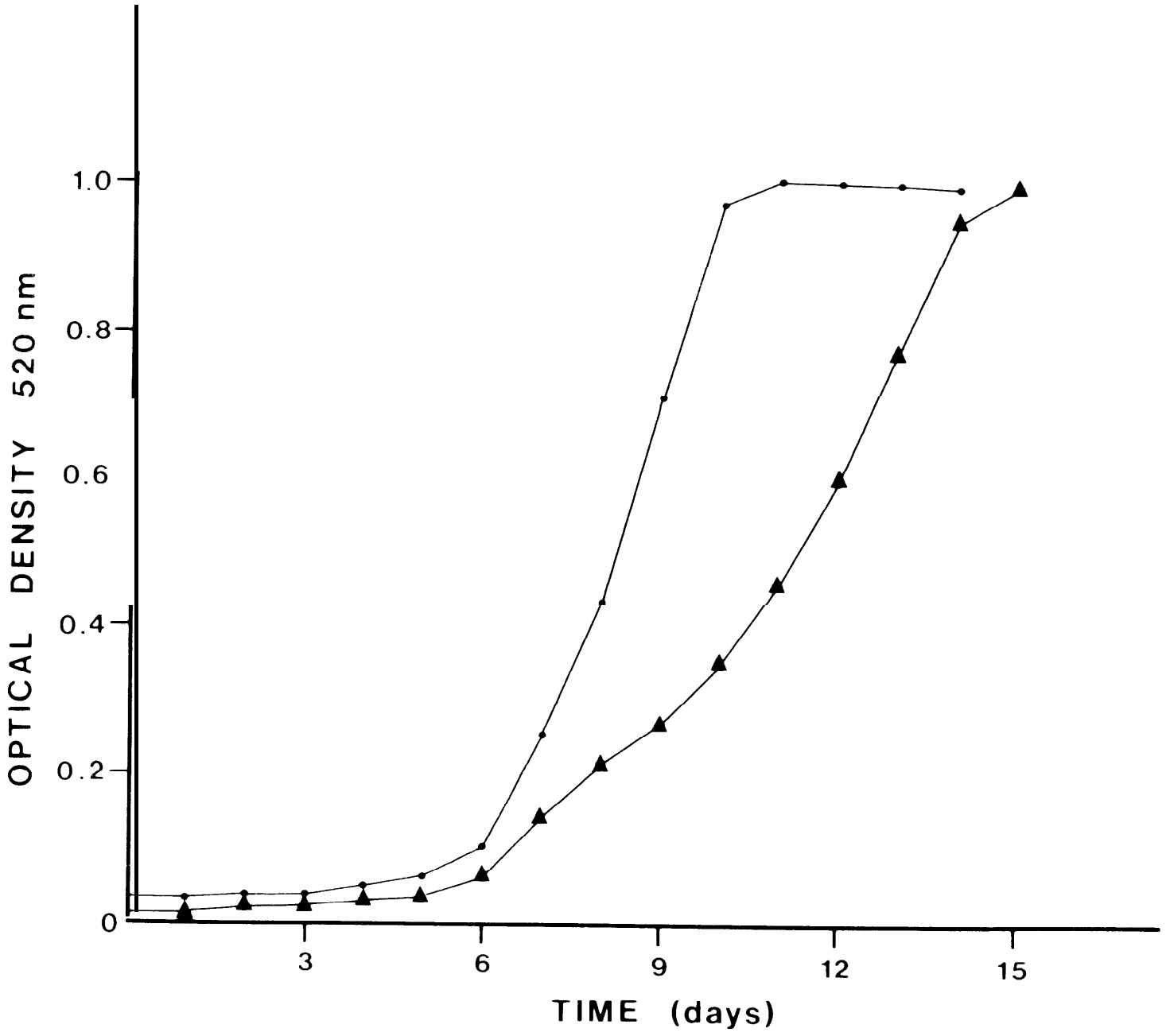

Figure 15. Growth of KDB on KDM-2 and UF-KDM-2. The growth of KDB on either KDM-2 broth (ı) or UF-KDM-2 (A) was measured daily by absorbance at 520 nm. Test organisms were grown to log phase in UF-KDM-2 broth. Ten ml were then inoculated into 100 ml of either broth. Cultures were grown at 18°C.

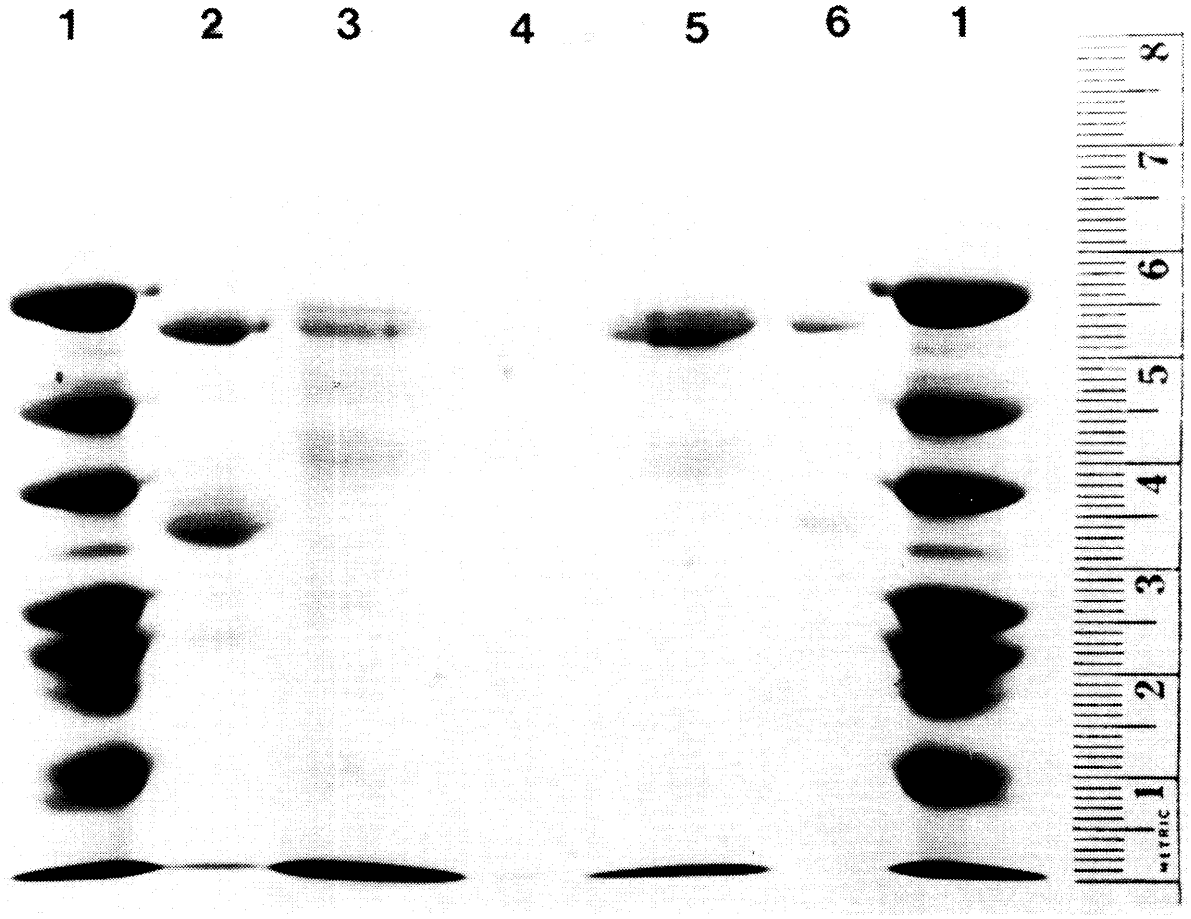

Figure 16. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis comparison of the antigen BKD antigen extracts. Lanes: (left to right), 1) molecular weight standards; 2) UF-KDM-2 antigens; 3) boiled antigens; 4) sonicate antigens; 5) autoclaved antigens; 6) KDM-2 soluble antigens; molecular weight standards - 66 kd (5.6 cm), 45 kd (4.5) 36 kd (3.8) 30 kd (2.6) 24 kd (2.1), 20 kd (1.7) and 14 kd (1.0).

bands for UF-KDM-2 soluble antigen, boiled extract, sonicate, autoclaved extract and KDM-2 antigen. The most consistently observed antigen appears to be the 60 kd antigen, which can be found in all preparations. UF-KDM-2 and KDM-2 antigens demonstrate the same two 34 kd and 26 kd proteins. The boiled whole cells preparation reveals the greatest heterogeneity of molecular weights, with a second major band appearing at 40 kd, but other bands appearing at 14, 20, 24, 32, and 46 kd. The sonicated antigen preparation showed very little antigen, other than the 60 kd species. Autoclaved whole cells produced a major band at 40 kd, as did the boiled preparation. Thus, it would seem likely that boiling and autoclaving would cause the release of similar antigens, however, the boiling may be slightly more harsh and thus release a variety of other proteins or protein fragments of various molecular weights.

Comparison of antigens by immunodiffusion. Figure 17 represents an immunodiffusion reaction between an anti-sonicate antiserum and autoclaved, boiled, sonicated and soluble antigens. This diffusion demonstrates that boiled and soluble antigens show total identity with the sonicated preparation, the autoclaved preparation appears to have no homology with any of the boiled antigens. The boiled preparation reveals at least four distinct antigens, two of which are homologous with the sonicate antigens. The immunodiffusion gel utilizing the rabbit anti-soluble antigen (Fig. 18) reveals that a common major antigen can be found in boiled, autoclaved, sonicated and soluble antigen preparations. Preliminary evidence also demonstrates that coho anti-KDB serum produces a precipitin band when reacted with UF-KDM-2 antigens.

ELISA for soluble antigen. Figure 19 demonstrates that

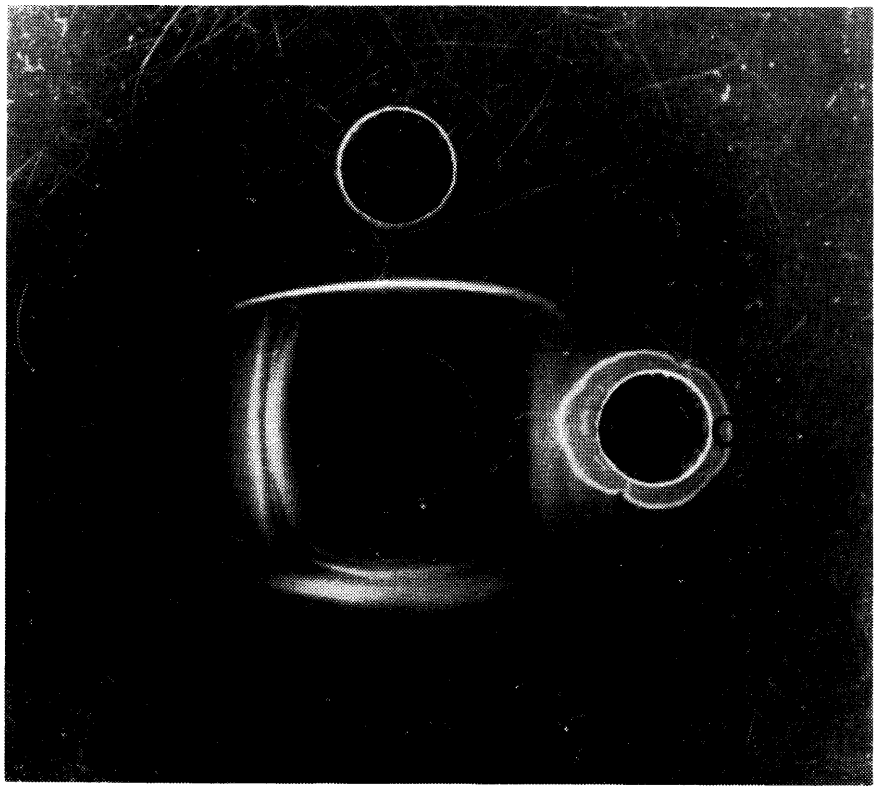

Figure 17. Ouchterlony immunodiffusion analysis of KDB antigen extracts using anti-sonicate serum. Wells were cut in an agarose gel and filled with autoclaved extract (A - top), heat extract (B - left), UF-KDM-2 antigens (C -right), or sonicate (D - bottom). The center well (E) was filled with rabbet anti-sonicate serum. The gel was then allowed to incubate for two days at 4°C.

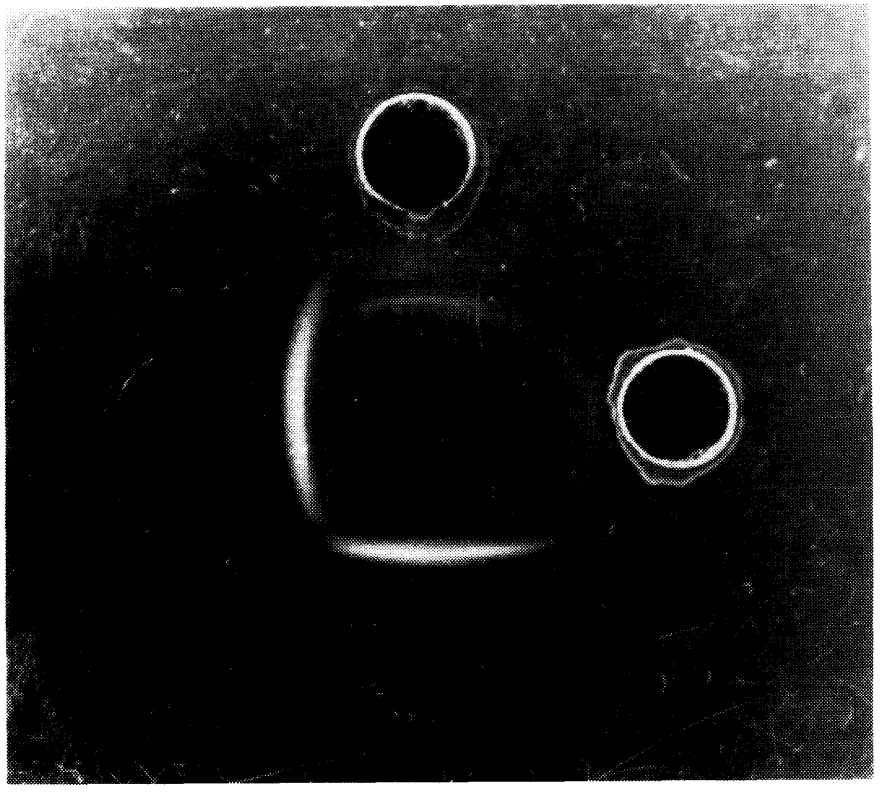

Figure 18. Quchterlony immunodiffusion analysis of KDB antigen extracts using anti-UF-KDM-2 serum. Wells were cut in an agarose gel and filled with autoclaved extract (A - top), heat extract (B - left), UF-KDM-2 antigens (C - right), or sonicate (D - bottom), The center well (E) was filled with rabbit anti-UF-KDM-2 serum. The gel was then allowed to incubate for two days at $4^{o}C$.

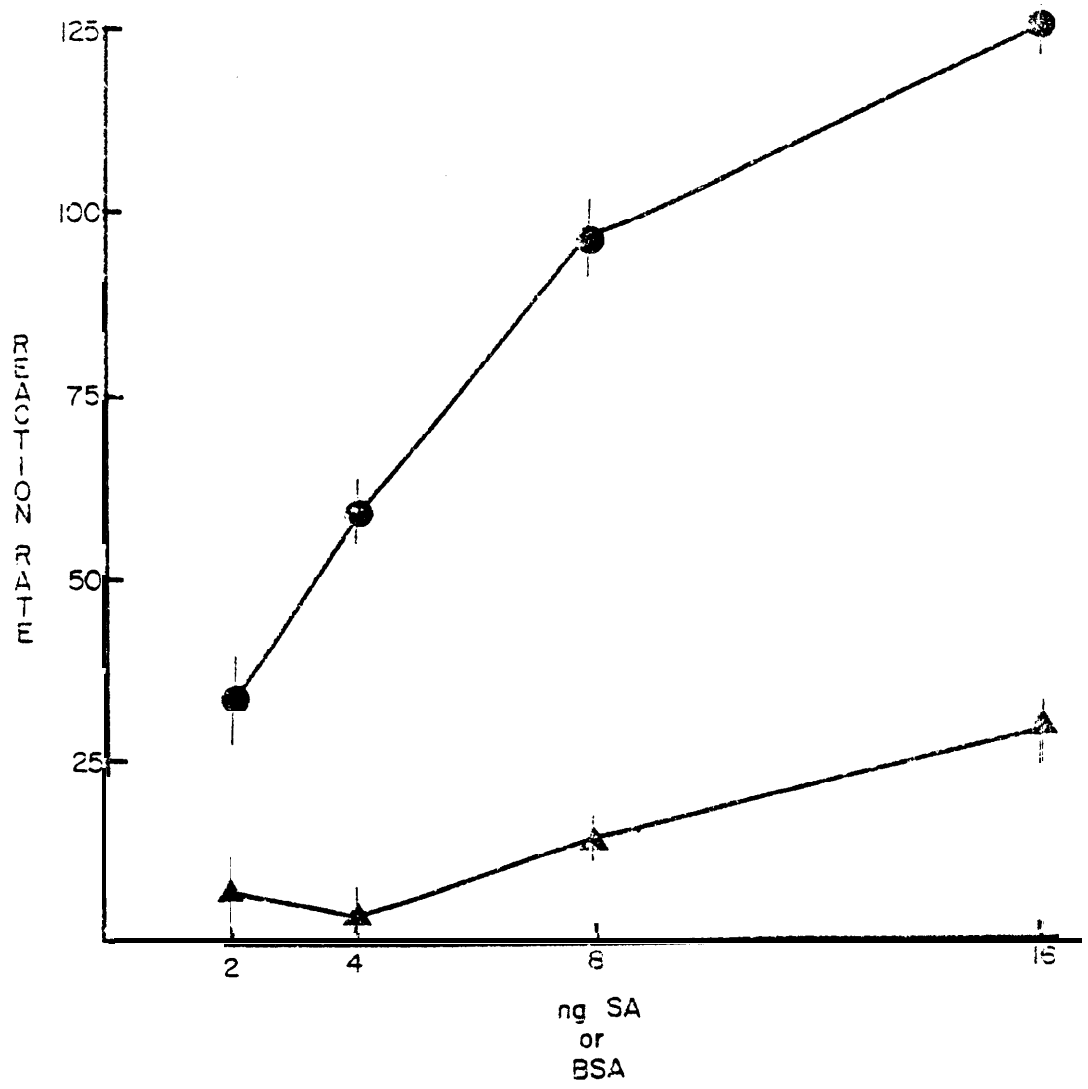

Figure 19. Detection of soluble antigen by the ELISA. The capture antibody technique was utilized, where 10 ug/ml of anti-soluble antigen was adsorbed to the wells of an ELISA plate. Various concentrations of soluble antigen (●) or bovine serum control (A) were then incubated in the wells in the second phase. Rabbit anti-soluble antigen-horse radish peroxidase (HRPO) was added in the third phase of the reaction. The reaction rate, upon addition of the substrate, is proportional to the amount of soluble antigen bound. The reaction rate is equal to A_{405} / time(min) x 1000. Error bars represent one standard error.

levels of2.0 ng/100 ul UF-KDM-2antigens can be detected by the capture antibody technique. This assay can now be used as a measure of kidney disease form the analysis of serum or other body fluids and tissues of infected fish. Future vaccine trials in this study will utilize this technique to monitor the progress of the KD infection without the necessity of terminating the experimental animal.

ELISA for antibody to soluble and cellular antigens. This system has been used to screen hybridoma supernatants for the presence of mouse anti-soluble antigen antibodies. Supernatants were screened for the generation of O.D. reading above that found with control SP/2 supernatants. Cells elaborating this activity have cloned and preserved for future analyses.

This ELISA system has also been used to screen for monoclonal antibodies directed toward whole KDB cells. Together both of the ELISA systems have enabled our laboratory to produce two monclonal antibodies to the soluble antigen and one to a cellular antigen.

SUMMARY AND CONCLUSIONS

The data presented here demonstrate that there is some variability to the antigenic structure of KDB. Although gel filtration of all antigenic preparations revealed a wide range of sizes for antigens, resolution on a denaturing gel revealed relatively few protein bands and immunological assays revealed the same (3) low number of antigens. It is of particular interest that there seems to be a protein of 60 kd in all preparations, but that there are not larger individual molecular species. This, in turn indicates that the larger molecular weight species detected in gel filtration are most likely aggregates or membrane fragments composed of a lower molecular weight subunit.

Use of ultrafiltration of KDM-2 medium appears to be successful in eliminating contamination of high molecular weight material found in KDM-2. There appears to be no alteration in the number of soluble antigens produced by growth in either medium, nor in the number of proteins, as detected by SDS-PAGE. However, soluble antigens isolated from UF-KDM-2 does appear to have greater heterogeneity in their isoelectric focusing (IEF) patterns than those from UF-KDM-2. Also, although there does appear to be an extended lag period in KDB growth on UF-KDM-2, there is no alteration in final O.D. or wet weight of cells. Thus, it appears that UF-KDM-2 may be an alternate medium for those wishing to isolate purified bacterial proteins or antigens.

ELISA assays have been developed for the detection of soluble KDB antigens. This system is currently being developed as a sensitive measure of the presence of soluble antigen in serum and tissues of fish. Such a sensitive assay may also allow for the detection of KD+ spawners by the testing of ovarian fluid or serum.

ELISA assays have also been developed to detect antibodies to soluble and cellular antigens of KDB. These systems have been proven successful in the detection of rabbit and murine monoclonal antibodies against KDB antigens. Future work will develop the use of anti-fish immunoglobulin (Ig) reagents to detect the presence of fish antibodies to KDB. This would be an extremely useful tool to be used in monitoring the immune response of salmon to the various test vaccines.

The various antigens characterized in this study, along with whole KDB cells are currently being conjugated to various immunopotentiating agents. Testing of these prototype vaccines is currently under study.

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APPENDIX A - REAGENTS

1. Tris buffer (diluent buffer)

   6.07 g Trizma, 0.37 g EDTA, and 8.7 g NaCl brought to one liter in distilled water and a final pH of 8.0.

2. Tris/Tween

   Tris buffer supplemented with 0.1% polyethylene sorbitan monolaurate (Tween 20)

3. Citric acid buffer

   0.2 g of citric acid in 100 ml distilled water and brought to a final pH of 4.0.

4. Substrate solution

   75 ul of a lmg/ml solution of 2,2'-azino-bis(3-ethylbenz-thiazolenesulfonic acid (ABTS) in water, 5 ul of 30% H_2O_2, in 10 ml of citric acid buffer.

5. Phosphate buffered saline

   8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4.12H_2O$, 0.2 g KC1 in 1 liter of distilled water, adjusted to a pH of 7.4

SUMMARY OF EXPENDITURES

1.	Salaries (including personnel and benefits)	$ 30,260.93[a]
2.	Travel and transportation (including per diem)	846.21
3.	Nonexpendable equipment and material (greater than $1000 per item)	13,333.41
4.	Expendable equipment and material (sensitive in nature)	12,617.12
5.	Operations and maintenance (including computer services and publications)	2,195.49
6.	Overhead	13,675.23
7,	The currently approved budget	113,043.00
8.	Current budget period	7/1/84-6/31/85
9.	Cumulative expenses to date	72,923.39

a. through 4/30/85

LIST OF MAJOR PROPERTY PURCHASED

1. Analytical balance - Satorius
3. Refrigerated centrifuge - IEC
3. UV spectrophotometer - Bausch and Lomb
4. Fraction collector - Eldex
5. Ultrafiltration system - Millipore
6. Ultralow freezer - ASP
7. Cell Harvester - Skatron
8. Room rennovation - physical plant