Specificity of the serological response to rotavirus polypeptides by the avidin-biotin - Western blot procedure

by

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INTRODUCTION

The antigenic relationships between rotavirus strains have been investigated by a variety of serological tests. Recent studies have shown that the greatest discrimination between rotavirus strains can be obtained through the use of the hemagglutination-inhibition and serum neutralization tests. It has further been shown through the use of viral reassortants and monoclonal antibodies that the classification of rotavirus into serotype groups is dependent primarily on a glycoprotein located in the outer capsid shell. Employing these tests, four distinct serotypes of human rotavirus, at least two serotypes of bovine rotavirus, three distinct serotypes of avian rotavirus, and two distinct equine rotavirus serotypes have been established (Estes et al., 1983; Sabara et al., 1983). Despite the ability to serologically classify rotavirus isolates in vitro, recent studies by Woode et al. (1983) demonstrated a lack of cross-protection in gnotobiotic calves between two serologically related bovine rotavirus isolates, and one-way cross-protection between bovine rotavirus isolates of different serotypes.

In this study, investigations were conducted utilizing the Western blot technique, to immunologically probe the structural proteins of the bovine rotavirus isolates involved in the cross-protection experiments of Woode et al. (1983). Immunological probing of the structural proteins was accomplished using convalescent and hyperimmune antisera obtained from gnotobiotic calves, as well as hyperimmune guinea pig antisera produced against the relevant bovine rotavirus isolates. Visualization

of the antibody-antigen reactions was accomplished by the avidin-biotin system. The objective of this study was to determine if the interactions or lack of interactions of these sera with the structural proteins, and in particular with the major outer shell glycoprotein, correlates to a greater degree with the cross-protection results obtained *in vivo*, as compared with the serum neutralization test.

LITERATURE REVIEW

Rotavirus

Background

Rotavirus is an ubiquitous virus that has been isolated or serologically detected in humans, birds, and nearly all domestic and wild mammals from which it has been sought (Flewett and Woode, 1978; Estes et al., 1983; Holmes, 1983). The virus is one of the causative agents of acute diarrheal disease, which is typically manifested in infants or the young of animals. The disease is believed to be the major cause of infant mortality in many parts of the world, as well as being responsible for severe economic losses in livestock, particularly in calves and pigs, through death or retardation of weight gain in animals that recover (Editorial, 1975; Holmes 1983).

Historically rotaviruses have been referred to in the literature by several synonyms, due to the large number of animals from which they have been isolated, as well as the morphologic resemblance of the group to the reoviruses and orbiviruses. The earliest reported isolation of rotavirus was by Light and Hodes (1943, 1949) following several outbreaks of human gastroenteritis. Shortly thereafter, rotaviruses were isolated from several other animal species including the viruses responsible for epizootic diarrhea of infant mice (EDIM) (Cheever and Mueller, 1947; Kraft, 1957; Adams and Kraft, 1963), simian virus SA11 and the O (Offal) agent from Cercopithecus monkeys (Malherbe and Strickland-Cholmley, 1968;

Lecatsas, 1972) and Nebraska calf diarrhea virus (NCDV) from cattle (Mebus et al., 1969, 1971; Fernelius et al., 1972). Despite their resemblance to other members of the Reoviridae, rotaviruses were found to be significantly different both antigenically and in morphological detail. Consequently in 1978, the genus rotavirus was established in the family Reoviridae by the International Committee on The Nomenclature of Viruses (Matthews, 1979).

The name 'rotavirus,' derived from the Latin word rota, a wheel, was first suggested by Flewett et al. (1974), due to their wheel-like appearance when viewed by electron microscopy. The wheel-like appearance is due to the double shelled capsid, which is composed of an outer layer, an inner layer, and an icosahedral core. Double shelled particles measure 65-75 nm, while single shelled particles, which are seen in most negative stain preparations (Bridger and Woode, 1976) measure 55-65 nm (Woode et al., 1976; McNulty et al., 1979). Double-shelled particles represent the complete infectious virion (Bridger and Woode, 1976; Elias, 1977).

The general pattern of infection of rotavirus in animals has been reviewed by Holmes (1983) and Estes et al. (1983), and all exhibit similar pathological changes. Rotaviruses infect epithelial cells lining the villi of the small intestine. Infection causes vacuolation and shedding of the damaged cells into the small intestine, resulting in the release of large quantities of virus from the lysis of these cells, and shortening of the villi. Immature epithelial cells replacing those lost by shedding have reduced levels of lactase and other disaccharase

enzymes, and glucose-coupled sodium transport is impaired. Unabsorbed milk lactose and concurrent bacterial growth exerts an osmotic effect, and diarrhea results due to carbohydrate malabsoption. The disease is often self-limiting if diarrhea and dehydration are not so severe as to cause death, and villi return to normal within 3-4 weeks.

RNA

Rotaviruses have a segmented genome consisting of eleven segments of double-stranded RNA. These segments are grouped into four distinct classes based on contour-length measurements by electron microscopy, molecular weight, and electrophoretic patterns in polyacrylamide gel electrophoresis (PAGE) (Newman et al., 1975; Rodger et al., 1975; Kalica et al., 1976; Barnett et al., 1978). The RNA segments composing the four classes are characterized by four large segments (RNA segments 1-4, Class I), two medium sized segments (RNA segments 5 and 6, Class II), a closely running triplet (RNA segments 7-9, Class III), and two small segments (RNA segments 10 and 11, Class IV). This pattern is unique to rotaviruses, and is easily recognizable from those of reoviruses and orbiviruses (Schnagl and Holmes, 1976; Todd and McNulty, 1976). The molecular weight of bovine rotavirus RNA segments, as determined under nondenaturing conditions by comparison with reovirus RNA, have been reported to range from 0.2-2.2 x 10^6 (Newman et al., 1975; Rodger et al., 1975). A recent study however by Bernstein and Hruska (1981) comparing rotavirus RNA segments to ribosomal RNA under totally denaturing conditions, has reported molecular weights of 2.8-0.78 x 10^6 and

3.4-0.8 x 10^6 for the bovine and SAll rotaviruses, respectively. These values are considerably higher than those previously reported, due to the elimination of interference by secondary structure, which can alter electrophoretic migration and thus the values obtained for molecular weight.

Like other members of the Reoviridae, rotavirus particles contain an RNA-dependent RNA polymerase. Polymerase activity has been found to be optimal between 45 and 50 C at pH 8 in the presence of 8-10 mM magnesium ions (Cohen, 1977). Although polymerase activity is known to be associated with single-shelled virus particles, the proteins involved in this function are not known (Estes et al., 1983; Babiuk et al., 1985).

The electrophoretic patterns of rotavirus RNA have been recognized as a very useful method for distinguishing among different isolates. Segment pattern differences have facilitated the identification of viruses isolated from different species (Kalica et al., 1978; Rodger and Holmes, 1979), and single species, e.g., human (Kalica et al., 1978; Rodger and Holmes, 1979), and has helped in understanding the epidemiology of viral infections. Attempts however to correlate electropherotypes with serotypes has of yet not been thoroughly demonstrated. Studies by Kalica et al. (1981a) with human rotaviruses have shown that genomes with short electrophoretic patterns are characteristic of viruses belonging to subgroup 1, while viruses with long patterns correlate with subgroup 2. Beards (1982) however, has shown that viruses with different serotypes may have identical electropherotypes, while Gaul et al. (1982) have shown that serologically

related isolates may have diverse electropherotypes. It has further been demonstrated that inverting of RNA segments as determined by the gene-coding assignments of segments 10 and 11 between human rotaviruses, and RNA segments 7, 8, and 9 between bovine, simian, and human rotaviruses, can occur (Dyall-Smith and Holmes, 1981; McCrae and McCorquodale, 1982; Dyall-Smith et al., 1983; Mason et al., 1983; Dr. R. F. Ramig, Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas, personal communication).

Proteins

Numerous studies analyzing rotaviruses isolated from different animal species have resulted in conflicting data concerning the number of polypeptides contained in the rotavirus particle, as well as the molecular weights of the polypeptides observed. Although previous studies reported as many as 8-10 structural proteins (Matsuno and Mukoyama, 1979; McCrae and McCorquodale, 1982), it is currently believed that the true number may be closer to 5-6 (Newman et al., 1975; Esparza et al., 1983; Estes et al., 1983). Estes et al. (1983), attributed many of these discrepancies to the diversity of PAGE systems that have been used for analysis, the failure to use molecular weight standards, or the exposure of virus preparations to proteolytic enzymes before analysis, resulting in alterations of the polypeptide patterns. Many of the discrepancies however in the molecular weights reflect differences between rotavirus strains isolated from different animal species or the same species (Thouless, 1979; Espejo et al., 1980).

The inner capsid has generally been agreed to contain at least 1 minor and 2 major polypeptides with molecular weights in the area of 120, 94, and 42 kilodaltons (K) (Novo and Esparza, 1981; Estes et al., 1983; Holmes, 1983; Babiuk et al., 1985). The 94 and 42K polypeptides have been reported to comprise about 80% of the total virus protein (Obijeski et al., 1977), and 99% of the protein in single-shelled virus particles (Novo and Esparza, 1981). An additional 88K polypeptide has been described in studies with bovine rotavirus (Bican et al., 1982; Esparza et al., 1983; Babiuk et al., 1985). The outer capsid has been determined to contain a glycoprotein of molecular weight 34-42K, which is the most abundant protein in the outer shell. This protein is glycosylated with N-linked oligosaccharides of the high mannose type, and may occur in multiple forms within a single rotavirus isolate (Novo and Esparza, 1981; Estes et al., 1982). A second polypeptide of molecular weight 84 or 88K has been described in the outer capsid of both bovine and SA11 rotaviruses, respectively (Novo and Esparza, 1981; Estes et al., 1983). This protein is responsible for hemagglutination, and the increased infectivity of rotavirus particles following exposure to trypsin. Trypsin cleaves the 84 or 88K polypeptide into two polypeptides of molecular weights 60 and 28K.

The RNA coding assignments for the structural proteins have been extensively studied for the simian SA11 and bovine rotaviruses (Dyall-Smith and Holmes, 1981; McCrae and McCorquodale, 1982; Kantharidis et al., 1983; Esparza et al., 1983). As a result, the following correlation between the RNA segments and the polypeptides mentioned above has

evolved: 120K with RNA segment 1, 94K with RNA segments 2 and 3 (based on studies with SA11) or RNA segment 2 (based on studies with bovine), 88K (inner shell protein) with RNA segment 3 (based on bovine), 88 or 84K (outer shell protein) with RNA segment 4, 42K with RNA segment 6, and 34-42K with RNA segment 7, 8, or 9. The remaining RNA segments are believed to code for nonstructural proteins.

Antigenic determinants

Serological analysis of single and double-shelled rotavirus particles have revealed three types of antigens; common (group) antigens, subgroup (type) antigens, and serotype (neutralization) antigens. The common (group) antigen is shared by most rotaviruses isolated from various mammalian and bird species. The exception to the rule are the pararotaviruses isolated from chickens, pigs, and humans (McNulty et al., 1981; Bohl et al., 1982; Bridger et al., 1982). Although morphologically indistinguishable from the classic rotaviruses, the pararotaviruses lack the common antigen, and are characterized by differences in their RNA electrophoretic profiles. The common antigen is detectable by numerous serological tests including immunoelectron microscopy (IEM), complement fixation (CF), immunofluorescence (IF), enzyme-linked immunosorbent assays (ELISA), and immune adherence hemagglutination assay (IAHA) (Woode et al., 1976; Flewett and Woode, 1978; Estes et al., 1983; Holmes, 1983). Studies by IEM demonstrated that the common antigen is located on the inner capsid, as shown by the agglutination of only single-shelled virus particles with heterologous antisera (Woode et al., 1976; Bridger, 1978).

It has further been demonstrated through immunoprecipitation using monoclonal antibodies, that the 42K major inner-capsid polypeptide carries the common group determinant (Holmes, 1983; Greenberg et al., 1983).

The subgroup (type) antigen, which is detectable by ELISA or IAHA, discriminates between certain human and animal rotavirus strains. Subgroups 2 and 3 subdivide human strains, while subgroup 1 includes most animal rotaviruses and the human strain DS-1 (Kapikian et al., 1981; Zissis et al., 1981). Identification of the polypeptide that carries the subgroup antigenic determinant was revealed in a study by Kalica et al. (1981b). In this study, viral reassortants obtained following a mixed infection with a temperature sensitive bovine rotavirus mutant, and a noncultivatable or cultivatable human rotavirus, exhibited subgroup specificity that always correlated with genome segment 6. Previous studies correlating the 42K major inner capsid polypeptide to this RNA segment, identified the location of this antigen.

A greater degree of discrimination between rotaviruses can be accomplished by use of the neutralization or hemagglutination-inhibition tests, which detect the serotype antigens. These antigens were shown to be distinct from the subgroup and common antigens, by the finding that antiserum that was discriminatory by the neutralization test, was crossreactive by IF, CF, or IAHA (Woode et al., 1976; Thouless et al., 1977). Studies by IEM revealed that the neutralization specificity of antiserum was dependent on double-shelled particles (Bridger, 1978). Later studies by Kalica et al. (1981b) with reassortants correlated the

neutralization specificity of antiserum with RNA segment 9 in the Wa strain of human rotavirus and segment 8 or 9 in the United Kingdom strain of bovine rotavirus. As with the subgroup antigen, correlation of the 34-42K outer capsid glycoprotein with these RNA segments identified the antigen's location.

Although the common, subgroup, and serotype antigens have been correlated with polypeptides on the inner or outer shells, recent studies with monoclonal and monospecific antibodies have revealed that the antigenic determinant sites for these antigens may be more widely distributed. Recent experiments by Sabara et al. (1983) with monospecific antisera have shown that although the 34-42K glycoprotein is the major neutralizing antigen, low dilutions of antisera prepared specifically against the hemagglutinating protein in the outer shell and the 45K polypeptide of the inner shell (of bovine rotavirus) exhibit neutralizing abilities. The fact that antisera prepared against the 45K polypeptide of the inner shell reacts with double shelled virus particles, has led to speculation that this polypeptide may be partially exposed on the surface of the virus (Sabara et al., 1983). Holmes (1983) has further reported that monoclonal antibodies that neutralize SAll rotavirus and immunoprecipitate the 34-42K glycoprotein, also neutralizes other rotaviruses, indicating the presence of both shared and serotype specific antigenic determinants on the glycoprotein. He further reports the ability of neutralizing monoclonal antibodies to inhibit hemagglutination. It is apparent from these studies that the complete characterization of the antigenic makeup of rotavirus will await the

further developement of monoclonal and monospecific antibodies. Such characterization of rotaviruses will be necessary for the deployment of effective vaccines.

Cross-protection

Cross-protection studies performed in this laboratory have previously been reported (Gaul et al., 1982; Woode et al., 1983). Initial studies by Gaul et al. (1982) investigated the ability of rotaviruses of various serotypes and animal origins to protect a given animal against infection with another rotavirus, regardless of the viruses' serotype. Investigations were conducted with rotaviruses isolated from canine, simian, porcine, human, and bovine species, which were classified into serotype groups on the basis of neutralization titer differences of 20-fold or greater. Three of the isolates were classified into separate serotype groups, while a fourth serotype consisted of the simian and canine rotaviruses. Vaccination of gnotobiotic piglets with the rotavirus isolates and subsequent challenge with virulent porcine rotavirus, resulted in a lack of cross-protection, with the exception of those animals previously inoculated with the porcine isolate. Animals vaccinated however with canine rotavirus, were found to be protected against challenge with simian rotavirus, indicating that cross-protection may be most likely conferred only between viruses of the same serotype group.

Cross-protection studies were further investigated by Woode et al. (1983) between bovine rotavirus isolates of the serotype I and II groups.

In this study, the serological relationships of bovine rotavirus isolates B641 and B223 to NCDV (bovine serotype I group) were established by comparison of homologous and heterologous neutralization titers, using hyperimmune guinea pig antisera. Bovine isolate B641 was shown to be neutralized to the same titer as NCDV with NCDV antisera, but showed an approximately 10 fold higher homologous neutralization titer with B641 antisera. Bovine isolate B223 differed from B641 and NCDV by at least 48 fold with their respective antisera, and 32- and 60-fold from B641 and NCDV respectively, with B223 antiserum. Based on the premise that a difference of 20-fold or greater in neutralization titers is necessary to differentiate viruses by serotype, it was concluded that NCDV and B641 were serologically related, despite the broader neutralizing activities of the NCDV antisera, while rotavirus isolate B223 was serologically distinct, and representative of a second bovine serotype group.

Cross-protection experiments performed in gnotobiotic calves with the above isolates did not correlate with the in vitro serum neutralization results. Although vaccination of gnotobiotic calves with bovine isolates B641 and NCDV failed to protect animals against challenge with B223 as expected, a lack of cross-protection was observed between the vaccine virus NCDV and the challenge virus B641. It was further observed that one-way protection occurred between the vaccine virus B223 and the challenge virus B641. Cross-protection experiments performed in this study have been repeated in additional gnotobiotic animals, confirming the results obtained in this study (Dr. Gerald N. Woode, Department of Veterinary Microbiology and Preventive Medicine, Iowa State

University, Ames, Iowa, unpublished data). Thus, cross-protection has proven to be a more sensitive method for distinguishing antigenic differences between rotaviruses than the in vitro serum neutralization test.

Western Blot

Rotavirus has been characterized serologically to a limited extent by immunoelectrophoresis (Middleton et al., 1976; Tufvesson and Johnsson, 1976; Grauballe et al., 1977). Although antigenic differences were observed, the range of separation by the system was limited, due to the pore size of the gels and the diffusion efficiency of antibody. With the advent of SDS-PAGE electrophoresis and the discontinuous buffer system, many of the problems in protein separation were overcome. Progressive improvements in this technique through the use of one or two-dimensional gel systems, has allowed the efficient resolution of hundreds or even thousands of polypeptides found in protein sample mixtures. Although proteins have been characterized immunologically directly in PAGE gels (Adair et al., 1978; Gershoni and Palade, 1983), the procedure generally requires long incubation periods, large amounts of reagents, and multiple manipulations of the gels, often leading to accidental breakage.

In 1975, Southern introduced a technique for transferring DNA fragments from agarose slab gels to nitrocellulose sheets. This technique, named Southern blot, involved the elution of DNA from gels by the mass flow of solvent, by placing nitrocellulose filters and dry

filter paper consecutively on top of the gels, resulting in the movement of buffer from a reservoir through the gels toward the nitrocellulose filters. This technique was quickly adapted to RNA (Northern blot) and proteins (Western blot) by Alwine et al. (1977) and Renart et al. (1979), respectively. In 1979, Towbin and coworkers devised a method for electrophoretically transferring proteins from polyacrylamide gels to nitrocellulose filters (Towbin et al., 1979). The electrophoretic blotting procedure produced a faithful replica of the separated proteins on the filters, which had the advantages of being easy to handle, readily accessible to probing by small quantities of various ligands, quickly processed, and storable for several months prior to use. In addition, multiple replicas of electrophoretically resolved proteins could be made using a single polyacrylamide gel, and protein transfers could be reused for probing with different ligands (Renart et al., 1979; Legocki and Verma, 1981; McLellan and Ramshaw, 1981; Erickson et al., 1982).

A number of filter matrices have been used for blotting, including diazobenzyloxymethyl (DBM) modified cellulose paper, diazophenylthioether (DPT) paper, nitrocellulose, and nylon-based membranes, e.g., Zetabind (AMF/CUNO, Meriden, CT). Of all the matrices, nitrocellulose has been the most widely used. The interaction of proteins with nitrocellulose is not clearly understood (Wallis et al., 1979; Gershoni and Palade, 1983). Nitrocellulose filters were originally designed for microfiltration, in which particles such as bacteria were removed by sieving and retained near the surface of the filter. However, in their current use with blotting, macromolecules are adsorbed throughout the filter matrix and

are predominantly retained by chemical interactions. At pH 8, the pH at which electrophoretic transfer is usually performed, nitrocellulose and the proteins being electroeluted are both negatively charged. Hydrophobic interactions are believed to play a role in protein retention at high pH, due to the ability of nonionic detergents and chaotropic salts, which weaken hydrophobic interactions, to elute proteins absorbed to nitrocellulose filters (Farrah et al., 1981).

Proteins have been blotted to filter matrices from a variety of gels, including urea gels, SDS gels, lithium dodecyl sulfate-containing gels, nondenaturing gels, two-dimensional gels, and agarose gels (Renart et al., 1979; Towbin et al., 1979; Sabara et al., 1983; Gershoni and Palade, 1983). As a result a variety of transfer buffers of low ionic strength have been devised, including 25 mM phosphate buffer, pH 6.5 (Bittner et al., 1980), 7.5 mM Tris-1.2 mM borate, pH 8.9 (McLellan and Ramshaw, 1981), and 25 mM Tris, 192 mM glycine, 20% V/V methanol, pH 8.3 (Towbin et al., 1979). The original transfer buffer developed by Towbin et al. (1979) for SDS-gels, containing methanol, has been widely used, and has several advantages and disadvantages. Methanol tends to increase the capacity of nitrocellulose for binding protein, as well as preventing swelling of the gels during transfer (Burnette, 1981; Nielsen et al., 1982). The addition of methanol however prevents efficient elution of proteins from SDS-PAGE gels, resulting in longer transfer times in order to elute high molecular weight proteins (Nielsen et al., 1982). The dependency of elution on the molecular weight of the polypeptides being transferred is a common problem in the Western blot procedure (Burnette,

1981; Howe and Hershey, 1981). Several methods that have been used for reducing this phenomenon include the use of reversible gel crosslinkers coupled with gel depolymerization prior to transfer, limited protease digestion of high molecular weight proteins, and the addition of 0.1% SDS to Towbin's transfer buffer (Renart et al., 1979; Gibson, 1981; Erickson et al., 1982).

Although the Western blot procedure has primarily been utilized for the immunological or immunochemical detection of antibody reactions with proteins, several new applications, as reviewed by Gershoni and Palade (1983), have been reported. These include the analysis of protein-nucleic acid and protein-cell interactions, identification of enzyme subunits, and affinity purification of monospecific antibodies. With further developement and refinement, other usage will most certainly be shown.

Avidin-Biotin

The affinity of avidin for biotin was discovered during investigations by Boas (1927), in which rats fed a limited diet consisting of egg white as the primary source of protein, developed a striking dermatitis leading to alopecia, nervous disorders, and death. Subsequent studies determined that this effect was due to the presence of a glycoprotein of molecular weight 68,000 present in egg white, with a high affinity (dissociation constant: 10^{-15} M) for biotin (vitamin H) (Green, 1963; Green, 1975; Elo and Korpela, 1984). Avidin is a basic tetrameric glycoprotein with a carbohydrate content of about 10%, and an

isoelectric point at pH 10. It has been isolated from the oviducts and egg white/jelly of many species of birds, reptiles, and amphibians, as well as many other tissues of oviparous animals following acute inflammation due to mechanical and thermal injury, retrovirus-induced cell transformation, and septic bacterial infection and toxic drugs (Elo, 1980; Elo and Korpela, 1984). An avidin analog, streptavidin, has also been isolated from the culture filtrates of many Streptomyces species. Streptavidin has been shown to have antimicrobial activities against gram-negative bacteria. Avidin is currently believed to play an immunological role in vivo, due to the above observations, and the demonstration of avidin secretion by yolk sac macrophages as well as macrophages and fibroblasts in vitro (Green, 1975; Elo and Korpela, 1984). Avidin has not been detected in mammals (Green, 1975; Elo, 1980; Elo and Korpela, 1984).

The ability of avidin to firmly bind one biotin molecule to each of the four identical subunits of the protein has also played a role in immunology in vitro. This is due to the capability of covalently coupling avidin and biotin to a variety of biologically active or detectable molecules, resulting in conjugates that retain their original activity. The avidin-biotin complex has been used for a variety of applications, including the delivery of drugs via tumor-specific antibodies, immunoassays, detection of proteins or nucleic acids on nitrocellulose blots, and immunohistochemical localization of various surface constituents (Bayer et al., 1979; Kendall et al., 1983; Leary et al., 1983; Wilchek and Bayer, 1984).

The application of the avidin-biotin complex toward immunological detection in vitro was first reported by Bayer et al. (1976), in which biotin labeled antibody in conjunction with ferritin-avidin conjugates were utilized for the localization of erythrocyte surface antigens. Following this study, three basic techniques using the avidin-biotin system evolved. Two of the techniques, developed by Guesdon et al. (1979), are the labeled avidin-biotin (LAB) technique, and the bridged avidin-biotin (BRAB) technique. Both techniques may be performed using biotin-labeled primary antibody, or indirectly with the use of biotin-labeled secondary antibody. With LAB, biotin-labeled antibody and enzyme-labeled-avidin are used sequentially. In the BRAB technique, unlabeled avidin acts a bridge between biotin-labeled antibody and biotin-labeled enzyme. A third technique, developed by Hsu et al. (1981), is the avidin-biotin-peroxidase complex (ABC). This technique involves the formation of a complex, in which avidin serves as a bridge between several biotin-labeled peroxidase molecules, which is reacted with biotin-labeled primary or secondary antibodies. The availability of biotin-binding sites in the complex is insured by the addition of a slight excess of avidin when reacted with biotin-labeled peroxidase.

The avidin-biotin technique, due to its sensitivity, has provided a useful alternative to many procedures formerly requiring the use of radiolabeled compounds. An ever increasing variety of modifications have been developed, including further amplification by anti-biotin or anti-avidin antibodies (Wilchek and Bayer, 1984). The avidin-biotin system however is not ubiquitously suitable for all applications, due to the

fact that avidin is a basic glycoprotein which is recognized by various lectins, and is subject to electrostatic interaction with acidic macromolecules leading to nonspecific reactions (Morris and Saelinger, 1984; Wilchek and Bayer, 1984). Furthermore, the use of the avidin-biotin system in histochemical applications may be limited due to endogenous avidin and biotin. Applications of the avidin-biotin system must be used with caution.

MATERIALS AND METHODS

Rotavirus Isolates

Rotavirus isolates used for this study were supplied by G. N. Woode. Nebraska calf diarrhea virus (NCDV), a rotavirus of the bovine serotype I group, was originally isolated by Mebus et al. (1969) and adapted to continuous cell culture by Fernelius et al. (1972). It was further subcultured and cloned by R. Wyatt, and following submittal to this laboratory, was obtained for use in this study following 5 passages in Ma-104 cells. The origin and serologic characterization of rotavirus isolate's B641 and B223 were previously reported by Woode et al. (1983). Briefly, rotavirus isolate B641, also of the bovine serotype I group, was isolated from a diarrheic calf in Florida. It was adapted to cell culture in this laboratory and cloned by diluting to extinction. Rotavirus isolate B641 was used in this study following 14 passages in African green monkey kidney cells (BSC-1 cells). Rotavirus isolate B223, a rotavirus of the bovine serotype II group, was isolated by this laboratory during an outbreak of diarrhea in beef calves in Iowa. It was adapted to cell culture in Ma-104 cells in the presence of hyperimmune guinea pig antisera to NCDV and B641. Virus utilized in this study was cloned twice via plaque selection.

Rotavirus isolate B14 was previously described by Gaul et al. (1982). Briefly, rotavirus isolate B14 was obtained from a diarrheic calf in Iowa and adapted to cell culture in this laboratory. This

isolate, as with rotavirus isolate B641, was antigenically related but serologically distinguishable from NCDV. Following adaptation to cell culture, rotavirus isolate B14 was cloned three times by diluting to extinction. Once cloned, rotavirus isolate B14 was used for the production of hyperimmune bovine antiserum.

Water

Water used for tissue culture media and all solutions and buffers, unless otherwise stated, was of reagent grade 1 quality. Water purification was achieved with a Culligan Reagent I Water System TD83-Type I (Culligan, Owatonna, MN). Purification was achieved by passing tap water through a carbon filter and reverse osmosis membranes, to remove organic contaminants, and mixed bed ion-exchange resins, for removal of inorganic ions. Purified water had a minimum resistivity of 10 megohm/cm.

Tissue Culture

Fetal rhesus monkey kidney (Ma-104) cells were utilized for propagation of rotavirus due to the ability of this cell line to support the replication of rotaviruses isolated from many animal species. Ma-104 cells were originally developed by Microbiological Associates Bioproducts, Walkersville, MD.

Confluent monolayers of Ma-104 cells grown in 150 cm^2 flasks

(Corning Glass Works, Corning, NY) were removed with trypsin-ethylenediaminetetraacetic acid (EDTA) solution and resuspended in growth medium at a ratio of 2 flasks of cells per 100 ml of medium. Trypsin-EDTA solution used for removing cells consisted of 2 g trypsin, 8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, and 1 g EDTA per liter of water. Ethylenediaminetetraacetic acid was included in the trypsin solution because of its role in chelating divalent ions that interfere with the enzymatic actions of trypsin. To facilitate the production of large quantities of virus, 100 ml of cell suspension was transferred to each of 850 cm^2 roller bottles (Corning Glass Works) and incubated at 37 C. The growth medium consisted of Eagle Minimum Essential Medium (Modified) (MEM) (Flow Laboratories, Inc., McLean, VA) supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY), 100 IU/ml penicillin, 100 ug/ml streptomycin, and 3 ug/ml amphotericin B [Fungizone (E. R. Squibb & Sons, Inc., Princeton, NJ)]. After incubation for 72 hours, monolayers were washed twice with 50 ml of serum-free (SF) MEM supplemented with 0.25% lactalbumin hydrolysate (LAH) (DIFCO Laboratories, Detroit, MI) in preparation for inoculation with virus.

Virus Culture and Purification

Monolayers were infected with 0.3-0.5 ml of tissue culture virus (virus titers were in the range of 10^7 to 10^8) suspended in SF MEM supplemented with LAH, antibiotics, and 0.05% pancreatin [4X N.F. 2.5% (10X) (GIBCO Laboratories)]. Virus-infected cells were incubated 24 to

72 hours at 37 C until a cytopathic effect (CPE) involving > 90% of the cell monolayer was observed. Culture fluid was harvested and clarified by centrifugation for 20 minutes at 1500 RPM (IEC PR-6000 Centrifuge, 253 rotor, Damon/IEC Division, Needham Heights, MA). The virus in the resulting supernatant was purified by high speed centrifugation at 122,000 x g (L5-65 Ultracentrifuge, SW27 rotor, Beckman Instruments, Inc., Palo Alto, CA) for 3 hours at 15 C through a 40% W/V sucrose cushion containing 10 mM $CaCl_2$. The virus pellets obtained were overlayed with 1 ml of 0.1 M tris buffer, pH 7.2 (0.1 M Tris, 10 mM $CaCl_2$, 0.01% sodium ethylmercurithiosalicylate [Thimerosol]) and held overnight at 4 C. The virus was resuspended by sonication for 30 seconds at a power setting of 1 (Sonifier Cell Disrupter, Model 350, Branson Sonic Power Co, Danbury, CT) and then pooled. Concentrated virus was centrifuged again at 122,000 x g as above, resuspended in 0.5 ml of tris buffer by sonication, and stored at -70 C until used.

Serum

Convalescent antisera to bovine isolates NCDV, B641, and B223 were prepared by the methodology previously described by Woode et al. (1983). Briefly, convalescent antisera were obtained from gnotobiotic calves which had been inoculated by the oral and/or nasal route with 5 ml of tissue culture-grown or fecal derived rotavirus preparations. Calves were vaccinated at one day of age, following which observations were made for signs of anorexia, diarrhea, and color changes in the

feces. Three weeks post inoculation the animals were bled, and the sera collected were aliquoted and stored at -70 C until used.

Hyperimmune bovine and guinea pig antisera to rotavirus isolates B14 and NCDV, B641, and B223, respectively, were prepared by the methodology previously described by Gaul et al. (1982). Briefly, hyperimmune bovine antiserum was prepared by the inoculation of gnotobiotic calf #5 (GC5) at one day of age by the oral and/or nasal route with 5 ml of a tissue culture-grown preparation of rotavirus isolate B14. Three weeks post inoculation, GC5 was immunized intramuscularly with a mixture of B14 tissue culture-grown virus and Freund's incomplete adjuvant. The animal was bled out three weeks after the second immunization, and the serum collected was aliquoted and stored at -70 C until used. Hyperimmune guinea pig antisera were obtained from guinea pigs which had been inoculated twice in the footpad at 3-week intervals with 0.1 ml of rotavirus inoculum. Inoculum was prepared by emulsifying equal volumes of purified cell-culture rotavirus and Freund's incomplete adjuvant. The animals were bled three weeks after the last injection and the sera collected were stored as above.

The convalescent and hyperimmune antisera used in this study are presented in Table 1.

Table 1. Antisera used for probing the structural proteins of the bovine rotavirus isolates

Animal No.	Animal	Antisera To:	(Antisera) Convalescent or Hyperimmune
GC[a]47	Calf	B223	Convalescent
GC36	Calf	B223	Convalescent
GC30	Calf	B223	Convalescent
GC55	Calf	B223	Convalescent
GC54	Calf	NCDV	Convalescent
GC25	Calf	NCDV	Convalescent
GC5	Calf	NCDV	Hyperimmune
GP[b]70	Guinea Pig	B223	Hyperimmune
GP55	Guinea Pig	NCDV	Hyperimmune
GP47	Guinea Pig	B641	Hyperimmune

[a]GC signifies gnotobiotic calf.

[b]GP signifies guinea pig (conventionally raised).

Rotavirus RNA

Gel electrophoresis of rotavirus RNA is recognized as a very useful method for both the rapid detection of the presence of rotavirus in fecal specimens, as well as for distinguishing different rotavirus strains for epidemiological purposes (Rodger and Holmes, 1979; Croxson and Bellamy, 1981; Herring et al., 1982; Estes et al., 1984). Its use in epidemiology is due to the ability of RNA electrophoretic patterns, or fingerprints, to discriminate between rotavirus isolates from different species or a single species. Due to the great stability of rotavirus in the extracellular phase, cross-contamination in the laboratory between strains is well known. In order to constantly monitor the purity of each rotavirus used in the study, the virus was extracted from the feces of each vaccinated calf and from each passage in roller bottle tissue culture, the RNA extracted, and the electropherotype pattern determined. This was compared by coelectrophoresis with the laboratory standard RNA extracted for the particular rotavirus strain.

Extraction

Extraction of RNA from fecal excreted virus was performed by the procedure of Herring et al. (1982), with the following modifications for tissue culture virus. Aliquots of 4-5 ml of culture fluid from roller bottles used for bulk production of rotavirus were processed as previously mentioned to obtain pelleted virus. Virus pellets were

resuspended in 1 ml of 0.1 M sodium acetate buffer (pH 5) containing 1% sodium dodecyl sulfate. Virus suspensions were then transferred to 15 ml Corex tubes coated with silicone (Sigmacote, Sigma Chemical Co., St. Louis, MO). To each suspension was added 1 ml of a 3:2 (vol/vol) phenol solution-chloroform mixture. The phenol solution consisted of 26.56 M phenol, 3.23 M m-cresol, and 0.017 M 8 hydroxyquinoline. The virus suspension was vortexed for 1 minute and then centrifuged at 1200 x g for 10 min. (IEC Model B-20 Centrifuge, Type A-147 fixed angle rotor, International Equipment Co., Needham Heights, MA). The upper clear aqueous layer containing rotavirus RNA was removed and stored in 1.5 ml microcentrifuge tubes at -70 C until used.

Electrophoresis

Electrophoresis of RNA was performed in general by the methods of Laemmli (1970) but as modified by the methods of Sabara et al. (1982). Sodium dodecyl sulphate (SDS) was omitted from the polyacrylamide gel, running buffer, and sample buffer. Electrophoresis was performed in a 7.5% polyacrylamide resolving slab gel with no stacking gel. Acrylamide used for preparing resolving gels was obtained from United States Biochemical Corp., Cleveland, OH, and was of electrophoresis grade. Ammonium persulfate, N,N'-Methylene-bis-acrylamide (bis-acrylamide), and N,N,N',N'-Tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories, Richmond, CA. The solutions used for RNA electrophoresis are shown below:

Running Buffer, pH 8.3:

tris base	24.24 g
glycine	115.36 g
thimerosol	40 mg
water	4000 ml

Resolving Gel Buffer, pH 8.8:

tris base	15.39 g
tris-HCl	3.69 g
water to	100 ml

Acrylamide Solution, 30%:

acrylamide	29 g
bis-acrylamide	1 g
water to	100 ml

7.5% Resolving Gel:

acrylamide solution	7.6 ml
resolving gel buffer	7.6 ml
water	14.6 ml
TEMED	30 ul
ammonium persulfate (10%)	150 ul

Sample Buffer, 10X, pH 6.8:

tris base	7.56 g
water to	100 ml

Tracking Dye:

sample buffer, 10X, pH 6.8	1 ml
water	1.4 ml
glycerol	7.5 ml
bromphenol blue	10 mg

The 7.5% polyacrylamide resolving slab gels were prepared by mixing acrylamide solution, resolving gel buffer, and water, degassing the mixture with agitation for 5 minutes, and then adding TEMED and freshly prepared 10% ammonium persulfate. The gels cast were 1.5 mm thick, 14 cm wide, and 12.5 cm long. Once cast, the gels were held at room

temperature for 2 hours before electrophoresis to allow complete polymerization of the acrylamide.

RNA samples to be run were diluted 1:1:1 with 10X sample buffer and tracking dye. The diluted samples were incubated for 2 minutes at 55 C before loading onto the gel to break up any aggregates of RNA. Samples were loaded onto the gel using a 50 ul Hamilton syringe. Although samples were not assayed for RNA concentration, trial-and-error showed an RNA sample size of 15-20 ul to be sufficient for visualizing all RNA segments.

Electrophoresis of RNA samples was performed using a Bio-Rad Model 220 Dual Vertical Slab Gel Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA). Cooling during the run was provided by the running buffer and by filling the center of the cell with cold tap water. Electrophoresis was carried out at a constant current of 10 mA per gel for 16 hours, with a typical run characterized by an increase in voltage from 26 V to 76 V.

Staining

Following electrophoresis, gels were stained by the silver stain methodology of Sammons et al. (1981) with modifications by Herring et al. (1982). Pyrex trays 28 x 14 x 4 cm (Corning Glass Works, Corning, NY) were used for containing the gels in the various developement solutions. All steps were performed at room temperature and lighting, and gels were agitated in the solutions by placement of the trays on a rocking platform

(Bellco Glass, Inc., Vineland, NJ). Manipulation of the gels among trays was performed using a nylon spatula and while wearing plastic gloves.

Fixation of the gel immediately following electrophoresis was accomplished in 400 ml of a 10% ethanol-0.5% acetic acid solution for 30 minutes. The gel was then transferred to 300 ml of 0.011 M silver nitrate solution and allowed to soak 2 hours. Preceding transfer of the gel into reducing solution, the gel was rinsed briefly in distilled water. The gel was then reduced in 400 ml of solution containing 0.75 M sodium hydroxide, 0.1 M formaldehyde (USP 37%), and 0.0023 M sodium borohydride. During the reduction step, the RNA segments became visible in the gel. Reduction was allowed to continue until RNA segments were clearly visible with a maximum developement time of 10 minutes. To stop reduction of the gel, the gel was transferred into 400 ml of a 5% acetic acid solution and allowed to soak for a minimum of 1 hour before photographing, or overnight.

Gels were photographed by placing them on a pre-wetted clear Plexiglas board suspended above a fluorescent light box. Photographs were taken using a Polaroid MP-4 Land Camera and Polaroid Type 665 Positive/Negative film (Polaroid Corp., Cambridge, MA).

Rotavirus Proteins

Protein assay

In order to quantitate the amount of viral protein obtained from different passes of virus, virus suspensions were tested by Read and Northcote's (1981) modification of Bradford's (1976) protein assay procedure. This procedure is based on the color change associated with the binding of Coomassie blue dye with protein. Although determination of protein concentration among different virus suspensions did not accurately correlate with antigen concentration, it did provide a rough basis for comparison, and provided a numerical value for determining volumes of virus suspension for use during electrophoresis. Viral suspensions were utilized at 100 ug of protein per lane in the SDS-polyacrylamide gels.

Standard dye-reagent solution was prepared by dissolving 50 mg of Coomassie brilliant blue G-250 (Eastman Kokak Co., Rochester, NY) into 50 ml of 16 M phosphoric acid and 46.7 ml absolute ethanol. The dye mixture was then diluted to 1 L with water and filtered through Whatman No. 1 filter paper. Following filtration, the standard dye reagent solution was stored in the dark in a brown glass bottle at room temperature until used.

Bovine serum albumin (BSA) was used as a protein standard, and dissolved in the same buffer used for resuspending purified virus pellets. Aliquots of virus suspensions and BSA standard were mixed with

standard dye reagent solution at a ratio of 50 ul of virus suspension/BSA standard per 950 ul of standard dye reagent solution. Assays were performed in 3.5 ml polystyrene cuvettes (Bio- Rad Laboratories, Richmond, CA), and absorbance was read at a wavelength of 595 nm on a Beckman Model DB-G spectrophotometer (Beckman Instruments, Inc, Fullerton, CA).

Electrophoresis

Virus proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli (1970). Electrophoresis of viral proteins was performed for both the estimation of molecular weight, and as a preliminary step preceding the electrophoretic transfer of protein from PAGE to nitrocellulose (Western blot). SDS used in SDS-PAGE was of electrophoresis grade, and was obtained from United States Biochemical Corp., Cleveland, OH. The solutions used for protein electrophoresis are shown below:

Running Buffer, pH 8.3:

tris base	24.24 g
glycine	115.36 g
SDS	4 g
thimerosol	40 mg
water	4000 ml

Acrylamide Solution, 30%:

acrylamide	29 g
bis-acrylamide	1 g
water to	100 ml

Resolving Gel Buffer, pH 8.8:

tris base	15.39 g
tris-HCl	3.69 g
water to	100 ml

7.5% Resolving Gel:

acrylamide solution	6.3 ml
resolving gel buffer	6.3 ml
water	12 ml
SDS (10%)	250 ul
TEMED	12.5 ul
ammonium persulfate (10%)	125 ul

Stacking Gel Buffer, pH 6.8:

tris base	6.057 g
water	50 ml
adjust pH to 6.8	
water to	100 ml

3% Stacking Gel:

acrylamide solution	2 ml
stacking gel buffer	3.8 ml
water	9 ml
SDS (10%)	150 ul
TEMED	15 ul
ammonium persulfate (10%)	75 ul

Sample buffers used with protein samples in this study, with the exception of Bromphenol blue dye concentration, were the same as Laemmli's (1970) original formulation. In some cases, water was substituted for 2-mercaptoethanol in order to obtain "nonreducing" sample buffers, although SDS was still included in these formulations. Three times concentrated (3X) sample buffer formulations, with or without 2-mercaptoethanol, were used for mixing with protein samples at a ratio of 1:2, respectively. Sample buffers used in this study are shown below:

3X Sample Buffer (Nonreducing):

1 M tris, pH 6.8	1.875 ml
SDS (20%)	3 ml
water	1.5 ml
glycerol	3 ml
bromphenol blue (0.5%)	625 ul

3X Sample Buffer (Reducing):

1 M tris, pH 6.8	1.875 ml
SDS (20%)	3 ml
2-mercaptoethanol	1.5 ml
glycerol	3 ml
bromphenol blue (0.5%)	625 ul

1X Sample Buffer:

0.5 M tris, pH 6.8	1.25 ml
SDS (10%)	2 ml
glycerol (80%)	1.25 ml
water	5 ml
bromphenol blue (0.5%)	500 ul

The methodology used for electrophoresis of proteins was similar to RNA electrophoresis previously discussed, particularly concerning preparation of gels and electrophoresis apparatus used. Differences, however, between the two procedures included the addition of SDS in running buffer, sample buffer, and PAGE gel formulations, the use of a stacking gel, and changes in electrophoretic running conditions.

The 7.5% polyacrylamide resolving slab gels were prepared by mixing acrylamide solution, resolving gel buffer, water, and SDS, degassing the mixture with agitation for 5 minutes, and then adding TEMED and freshly prepared 10% ammonium persulfate. Resolving gels were cast to a height of 8.8 cm and overlayed with water-saturated isobutanol. After polymerizing for 1 hour at room temperature, resolving gels were washed with water, dried, and overlayed with stacking gels prepared in a manner analogous to the resolving gels. The stacking gels measured 0.7 cm in height, and both stacking and resolving gels cast were 1.5 mm thick and 14 cm wide. Once cast, stacking gels were held at room temperature for 2 hours before electrophoresis to allow complete polymerization of the acrylamide.

Viral protein to be used for electrophoretic transfer to nitrocellulose was prepared for electrophoresis by diluting 100 ug of virus, in 0.1 M tris buffer, 2:1 with 3X sample buffer (nonreducing), and boiling the sample for 2 minutes to degrade the virus and solubilize the proteins. For molecular weight estimations, viral proteins were completely denatured by diluting 100 ug of virus, in 0.1 M tris buffer, 2:1 with 3X sample buffer (reducing), and boiling for 2 minutes.

Molecular weight protein standards were run in all gels with viral proteins. High and low molecular weight protein standard mixtures were purchased from Bio-Rad Laboratories, and collectively contained myosin (MW 200,000), β-galactosidase (MW 116,250), phosphorylase B (MW 92,500), bovine serum albumin (MW 66,200), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), soybean trypsin inhibitor (MW 21,500), and lysozyme (MW 14,400). Protein standards were prepared for electrophoresis by mixing 2 ul each of high and low molecular weight standards, containing 2-mercaptoethanol, with appropriate amounts of 1X sample buffer, and boiling for 2 minutes. All protein samples were loaded onto gels using a 50 ul Hamilton syringe fitted with Intramedic polyethylene tubing [I.D. 0.58 mm, O.D. 0.0965 mm (Clay Adams, Division of Becton Dickinson and Company, Parsippany, NJ)].

Electrophoresis of viral protein was accomplished using a Bio-Rad Model 220 Dual Vertical Slab Gel Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA). Cooling during the run was provided by the running buffer and by circulating cold tap water through the center well. Electrophoresis was performed at a constant power setting of 1.5 W until the bromphenol blue tracking dye was within 0.5 cm of the bottom of the gel.

Molecular weight estimations

In order to visualize proteins for calculating molecular weight, SDS-PAGE gels were stained immediately following electrophoresis.

Staining solution consisted of 300 ml of 0.1% Coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid. Gels were soaked in staining solution for 1 hour to overnight, and then destained in a solution of 5% methanol-10% acetic acid until background color was reduced.

Gels were photographed by placing them on a pre-wetted clear Plexiglas board suspended above a fluorescent light box. Photographs were taken using a Polaroid MP-4 Land Camera (Polaroid Corp., Cambridge, MA) in conjunction with a Tiffen #58 green filter (Tiffen, Hauppauge, NY) and Polaroid Type 655 Positive/Negative film (Polaroid Corp.).

Migration distance of molecular weight standards, viral proteins, and bromphenol blue dye, measured from the top of the separation gel, were recorded and used for calculating molecular weights.

Western Blot

Transfer of rotavirus proteins and molecular weight standards from SDS-PAGE gels to nitrocellulose was accomplished electrophoretically using the procedures of Towbin et al. (1979). This procedure has been termed Western blot in order to conform to terminology used for similar transfers of macromolecules, e.g., Southern blot for DNA and Northern blot for RNA. Theadvantage of this procedure is the accessibility of immobilized proteins on the surface of a solid phase filter for probing with various ligands. The Western blot procedure was used in this study for probing rotavirus proteins with specific convalescent bovine and hyperimmune bovine and guinea pig antisera.

"Electroblotting sandwich"

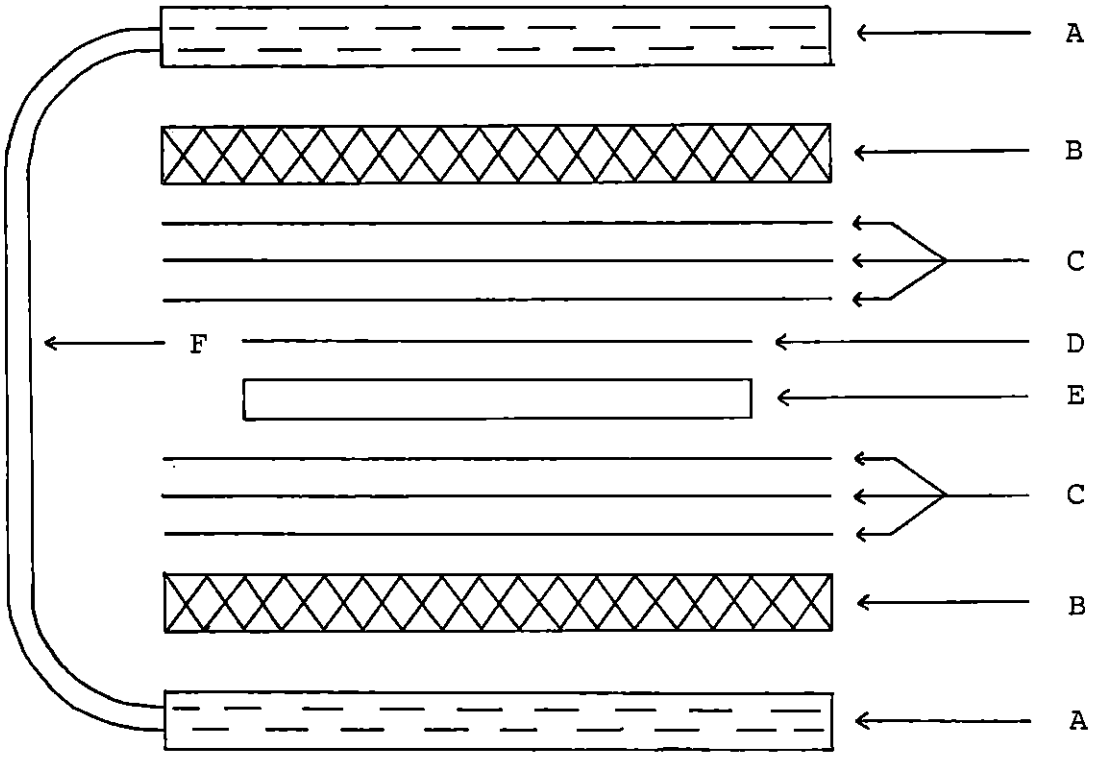
In order to avoid excessive diffusion of protein bands in SDS-PAGE gels, proteins were blotted immediately following electrophoresis. The dimensions of SDS-PAGE gels containing lanes to be transferred were measured, and nitrocellulose sheets (0.45 um, Sartorius Filters Inc., Hayward, CA) of equal size were cut and soaked in electroblotting buffer for 20 minutes prior to use. Electroblotting buffer, pH 8.3, consisted of 20 mM tris, 190 mM glycine, and 20% methanol. An "electroblotting sandwich," presented in Figure 1, was assembled by layering on top of a plastic grid, which was attached by a hinge to another plastic grid to form a "cassette," the following: 1 Scotch-Brite pad (Bio-Rad Laboratories, Richmond, CA), 3 sheets of Whatman #1 filter paper (W & R Balston LTD., England) cut to the same size as the Scotch-Brite pad, the SDS-PAGE gel, the presoaked nitrocellulose sheet, 3 more sheets of Whatman #1 filter paper of the same dimensions as the Scotch-Brite pad, and a second Scotch-Brite pad. The plastic grid which made up the other half of the cassette was layed flush against the second Scotch-Brite pad and completed the assembly. Scotch-Brite pads and Whatman #1 filter papers were soaked briefly in electroblotting buffer immediately before assemblage of the "sandwich." Care was taken during construction of the "sandwich" to remove air bubbles between the filter paper, the gel and the filter paper, and particularly between the nitrocellulose and the gel. Plastic gloves were worn at all times when handling nitrocellulose to prevent contamination with extraneous protein.

Figure 1. Diagrammatic representation of the "electroblotting sandwich"

(A) plastic grid
(B) Scotch-Brite pad
(C) Whatman #1 filter paper
(D) nitrocellulose
(E) SDS-polyacrylamide gel
(F) "cassette" hinge

The distance between the components of the "electroblotting sandwich" have been exaggerated for visualization purposes only.

(+)


A
B
C
F
D
E
C
B
A

(—)

Transfer

Electrophoretic blotting of the proteins was performed in a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA). The "electro-blotting sandwich" containing the gel to be blotted was placed in the Trans-Blot Cell immediately following assembly. The "cassette" was oriented so that the nitrocellulose sheet was closest to the anode. Electrophoretic blotting was carried out at a constant current of 200 mA for 5 hours 45 minutes. Blotting time was chosen so that sufficient transfer of all viral proteins and molecular weights had occurred.

Several steps were taken to prevent overheating and thus denaturation of the proteins during the transfer. The Trans-Blot Cell was placed in a styrofoam box packed with ice, and ice was constantly repacked around the Cell throughout the run. The styrofoam box was situated above a magnetic stirrer, which was used to drive a small magnet placed inside the Trans-Blot Cell to circulate electroblotting buffer. Electroblotting buffer used for the transfer was chilled to 4 C before the run. Finally, a coiled plastic tube through which cold tap water was passed was placed in the Trans-Blot Cell alongside the "cassette."

Following blotting, the "cassette" was opened to expose the nitrocellulose sheet and the gel. The nitrocellulose sheet was cut into strips corresponding to lanes of electrophoretically separated viral protein by using the wells in the stacking gel as a guide. Nitrocellulose strips were placed on Whatman #1 filter paper to dry, and

then numbered in the lower left and upper right corners using an extra soft #1 lead pencil. Dried nitrocellulose strips were placed in whirl-bags and stored at room temperature in the dark until used.

Confirmation of transfer and staining of molecular weights

In order to confirm transfer of proteins to nitrocellulose, selected lanes of SDS-PAGE gel from each run were stained using the Coomassie brilliant blue staining procedure previously described for estimating molecular weights. In addition, molecular weight standards transferred to nitrocellulose were also stained for confirmation of transfer of protein, as well as for use in comparison with immunologically reacted nitrocellulose strips. Molecular weight standards were stained using the India ink staining procedures of Hancock and Tsang (1983). In this procedure, nitrocellulose strips, ca. 9.5 x 3 cm, were washed 4 times for 10 minutes each in 50 ml of 0.01 M phosphate buffered saline (PBS) containing 0.05% polyoxy-ethylene sorbitan monolaurate [Tween 20 (Sigma Chemical Co., St. Louis, MO)] (PBS-Tween 20). Phosphate buffered saline (0.01 M), pH 7.2, was prepared by adding 1.1350 g Na_2HPO_4, 0.2715 g KH_2PO_4, and 8.5 g NaCl per liter of water. Washes in PBS-Tween 20, and staining, were performed at room temperature with agitation on a rocking platform (Bellco Glass, Inc., Vineland, NJ). Between washes, nitrocellulose strips were rinsed briefly in distilled water. Staining was performed overnight in 50 ml of PBS-Tween 20 containing 50 ul of Pelikan fount India drawing ink for fountain pens (Pelikan AG, D-3000

Hannover 1, Germany). Following staining, nitrocellulose strips were washed for 5 minutes in distilled water, and then air-dried.

Dot Immunobinding

Reagents used for immunological probing of electrophoretically separated and blotted rotavirus proteins were first utilized on a dot immunobinding system. Dot immunobinding involves the application of small quantities of antigen directly to a solid paper support as a spot, followed by immunological detection. The spot application of antigen specifically to nitrocellulose paper was first described by both Herbrink et al. (1982) and Hawkes et al. (1982). The advantages of this procedure include the ability to use minute quantities of antigen for antibody detection, reduced exposure of proteins to harsh chemicals for fixation which may alter antigenic sites, and the ability to screen many antigen and antibody samples at one time. Dot immunobinding was used in this study to determine: 1. workable dilutions of serum, secondary antibody, and peroxidase related reagents, 2. satisfactory buffers and quenching solutions, and 3. the specificity of the reagents used in the test. The ability of the dot immunobinding system to utilize small quantities of antigen for immunological detection was of particular interest for this study, due to the cost and time involved in *in vitro* production of virus, as well as the large amount of viral protein necessary for detection of all viral proteins of interest by the Western blot procedure.

Application of antigen

Nitrocellulose (0.45 um, Sartorius Filters Inc., Hayward, CA) used for dot immunobinding was cut into approximately 1.5 x 1.5 cm squares. Each square was numbered in the lower left and upper right corners using an extra soft #1 lead pencil in order to provide a method for identification during the test. Plastic gloves were worn at all times when handling the nitrocellulose to avoid contamination with extraneous proteins, and forceps were used for most manipulations of the squares. Antigen was applied to the nitrocellulose using a 50 ul Hamilton syringe fitted with 1-2 inches of Intramedic polyethylene tubing [I.D. 0.58 mm, O.D. 0.965 mm (Clay Adams, Division of Becton Dickinson and Company, Parsippany, NJ)]. The antigen applied was in the form of whole intact virus particles suspended in 0.1 M tris buffer (0.1 M Tris, 10 mM $CaCL_2$, 0.01% Thimerosol), and the amount of protein used ranged from 3.2 to 4.5 ug. The volume of rotavirus antigen applied at one time to the nitrocellulose squares was limited to 5 ul. This is due to the fact that the visibility of the immunoreacted antigen dot depends on the contrast of the color generated against the background, and thus is dependent on the density of antigen in the dot rather than the amount of antigen applied to the nitrocellulose. Antigen preparations used in this study which were not as concentrated as the terms dictated above, were utilized by applying successive volumes of 5 ul or less to the same spot on the nitrocellulose squares, allowing sufficient time for the preceding drop to dry. All antigen dots applied to nitrocellulose were allowed to dry

at least 30 minutes before use in immunoreactions.

Reaction with antibody and substrate

The following buffers and solutions were utilized for reaction of antigen spotted nitrocellulose squares with antibody and substrate:

Quenching Solution:

ovalbumin	5 g
thimerosol	5 mg
Tween 20	0.5 ml
water	500 ml

Primary Antibody Buffer, pH 7.5:

$NaH_2PO_4 \cdot H_2O$	0.0483 g
Na_2HPO_4	0.6601 g
NaCl	29.22 g
Tween 20	1 ml
water	1000 ml

Wash Buffer, pH 7.5:

$NaH_2PO_4 \cdot H_2O$	0.0897 g
Na_2HPO_4	0.6175 g
NaCl	9 g
Tween 20	1 ml
water	1000 ml

Substrate Buffer, pH 7.5:

tris base	0.236 g
tris-HCL	1.27 g
NaCl	14.61 g
water	500 ml

Nitrocellulose squares on which rotavirus antigen was spotted, was quenched before reaction with antiserum in order to saturate all remaining protein binding sites. Quenching was accomplished by prewetting the nitrocellulose squares first in water, followed by incubation with agitation in quenching solution for a minimum of 1 hour at 37 C. Quenching solution was used at approximately 5 ml for every nitrocellulose square. Following quenching, nitrocellulose squares were washed briefly in primary antibody buffer, and incubated with bovine convalescent, hyperimmune guinea pig, or hyperimmune bovine antisera diluted in the same buffer. One milliliter of diluted primary antibody

was used per square. Incubation with antiserum, as well as secondary antibody and avidin-biotin complex (ABC) (Vector Laboratories, Burlingame, CA), was performed in Seal-A-Meal bags (Dazey Corporation, Industrial Airport, KS) in a 37 C water bath for a minimum of 1 hour. The bags were gently squeezed during the incubation periods to allow mixing, and new Seal-A-Meal bags were used for each incubation step.

Following incubation in antiserum, nitrocellulose squares were washed for 30 minutes with three changes of wash buffer. They were then incubated with biotinylated secondary antibody specific for host IgG (H+L) of the animal from which primary antiserum was obtained. Two biotinylated secondary antibody preparations were utilized in this study; biotinylated goat anti-guinea pig (Vector Laboratories, Burlingame, CA) and biotinylated rabbit anti-bovine (Zymed Laboratories, Inc., South San Francisco, CA). Both secondary antibody preparations were diluted in wash buffer before incubation with nitrocellulose squares.

Following incubation with secondary antibody, nitrocellulose squares were again washed as described above. They were then incubated with ABC, which was prepared from a Standard Vectastain ABC Kit (Vector Laboratories). Preparation of ABC involved the mixing of equal volumes of unlabeled avidin and biotinylated horseradish peroxidase into 10 ml of wash buffer, and allowing the complex to form for 30 minutes prior to use. Following incubation with ABC, nitrocellulose squares were again washed as described above, with the exception of an additional 10 minute wash in substrate buffer to remove Tween 20. Substrate used for reaction with nitrocellulose squares was then prepared by dissolving 30 mg of

4-chloro-1-napthol (Sigma Chemical Co., St. Louis MO) in 10 ml of ice cold methanol. This solution in turn was mixed with 50 ml of substrate buffer at room temperature, to which 30 ul of ice cold 30% hydrogen peroxide had been added. Nitrocellulose squares were added immediately, and incubated at room temperature with agitation for 30 minutes. Color developement was stopped by washing the nitrocellulose squares for 10 minutes in distilled water. The nitrocellulose squares were then air-dried on paper towelling, and sealed in plastic and stored in the dark for future reference. Selected photographs were taken using a Polaroid MP-4 Land Camera (Polaroid Corp., Cambridge, MA) and a Tiffen #58 green filter (Tiffen, Hauppauge, NY) with Polaroid Type 655 Positive/Negative film (Polaroid Corp.).

Immunodetection of Rotavirus Proteins On Nitrocellulose Strips

Nitrocellulose strips obtained by the Western blot procedure were reacted with antibody and substrate in a manner analogous to the dot immunobinding method. Briefly, nitrocellulose strips representing lanes of electrophoretically separated rotavirus proteins were cut in half lengthwise before use. Cut nitrocellulose strips were then quenched in quenching solution with 10 ml per strip for a minimum of 1 hour at 37 C. Following a brief wash in primary antibody buffer, nitrocellulose strips were incubated in appropriate antiserum diluted in the same buffer overnight at 37 C. One milliliter of diluted antiserum was used per strip. The following morning, nitrocellulose strips were incubated

consecutively in appropriate biotinylated secondary antibody and ABC. Both incubations were performed using 2 ml of reagent per strip for a minimum of 2 hours at 37 C. Incubations in antiserum, biotinylated secondary antibody, and ABC were followed by 30 minute washes with 3 changes of wash buffer. An additional 10 minute wash in substrate buffer followed incubation in ABC. Nitrocellulose strips were then reacted with substrate at room temperature for a maximum of 1.5 hours. When viral protein bands became visible, nitrocellulose strips were immediately removed from the substrate and photographed as previously described. In those reactions in which heterologous antisera failed to recognize viral proteins, the nitrocellulose strips were re-reacted with homologous antiserum in order to confirm the presence of the protein band in question.

RESULTS

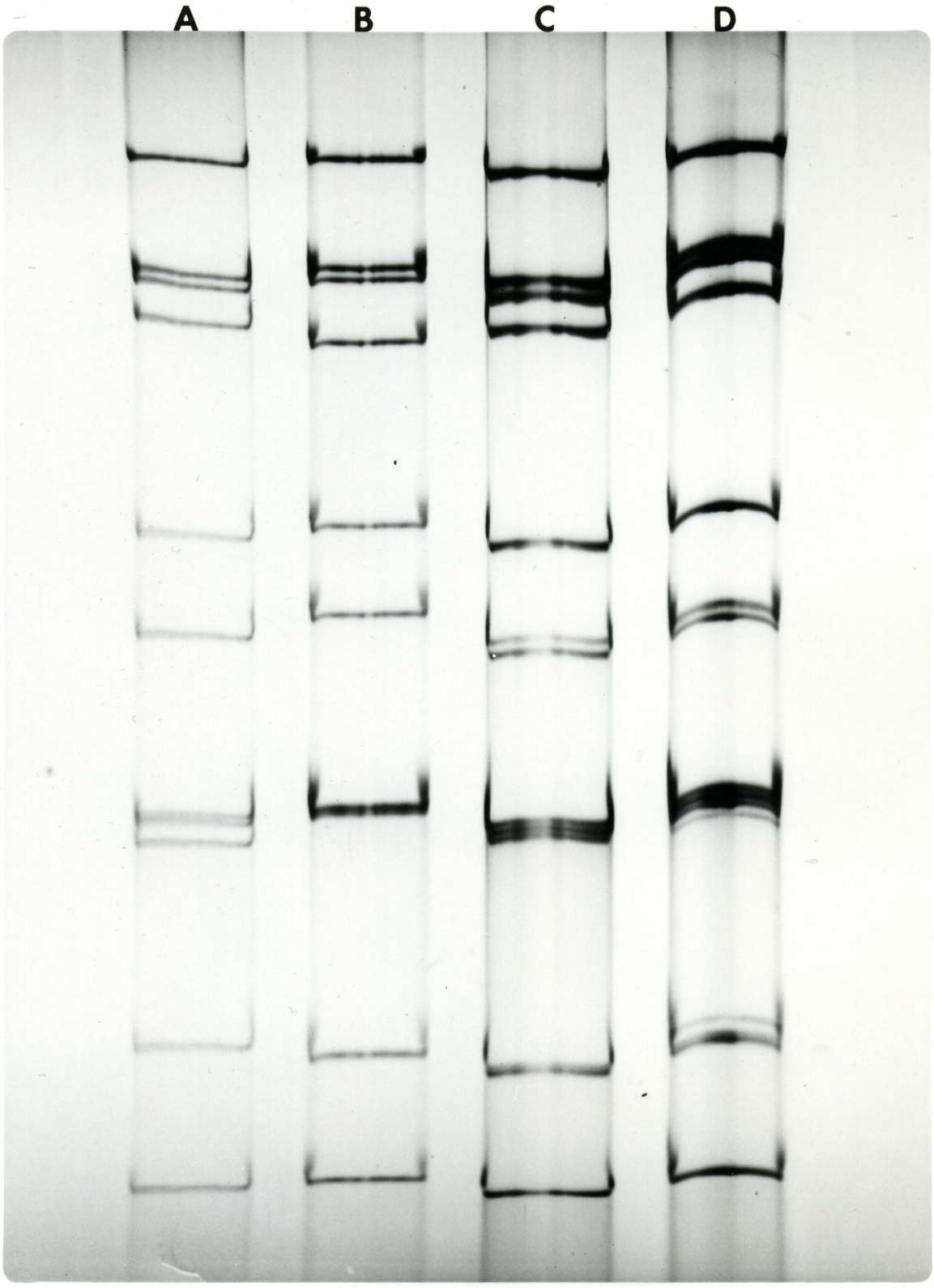
RNA Electrophoresis

The RNA electrophoretic patterns of all virus isolates harvested, following passage in cell culture or excretion in the feces of gnotobiotic calves, were identical to the RNA electropherotype of the inoculum viruses. The electrophoretic patterns of the three bovine rotavirus isolates used in this study are shown in Lanes A, B, and C in Figure 2. Bovine isolate B641, which was cloned by diluting to extinction, was found to consist of two subpopulations of rotavirus as evidenced by the double band at RNA segment 6. Bovine isolates NCDV and B223 consisted of one virus population. Although rotavirus isolates NCDV and B641 were serologically related and classified in the bovine serotype I group, differences were observed in the electrophoretic migration of RNA segments 2, possibly 3, 4, 6, possibly 7, 9, and 11 [based on results obtained in Figure 2 and the coelectrophoresis of RNA from both isolates (data not shown)]. In contrast, the electrophoretic migration of B641 RNA segments showed greater similarities with the RNA segment migration of isolate B223, a member of the bovine serotype II group. Coelectrophoresis of RNA from isolates B641 and B223, in Lane D, revealed differences between the 789 RNA segment complex, and RNA segment 10. The RNA segment migration differences observed between B641 and B223 agreed with previous observations noted by Woode et al. (1983). The inability of the RNA electrophoretic patterns to predict antigenic relatedness has

Figure 2. RNA electrophoretic patterns of the bovine rotavirus isolates as visualized by silver stain

Lane A. Bovine rotavirus isolate B223
Lane B Bovine rotavirus isolate NCDV
Lane C Bovine rotavirus isolate B641
Lane D Coelectrophoresis of bovine rotavirus isolates B223 and B641

Bovine rotavirus isolate B641 contained a double band at RNA segment 6 indicating two subpopulations of rotavirus. Coelectrophoresis of bovine rotavirus isolates B223 and B641 showed differences in the relative migration mobilities of RNA segments 789, and 10.


A
B
C
D

previously been reported (Rodger and Holmes, 1981; Gaul et al., 1982; Sabara et al., 1982; Schroeder et al., 1982; Estes et al., 1983).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Proteins

Bovine rotavirus isolates NCDV, B223, and B641 were analyzed by SDS-PAGE to determine and characterize the electrophoretic profile of the structural proteins of each isolate. Analysis of the structural proteins was performed under both reducing and nonreducing conditions, in order to facilitate the estimation of the molecular weight of the structural proteins observed, and for use as a visual reference for comparing with immunologically reacted proteins transferred to nitrocellulose. The electrophoretic profile of the three isolates following electrophoresis and staining with Coomassie brilliant blue is presented in Figure 3. Lanes A, B, and C represent the electrophoretic profile of the three isolates under nonreducing conditions, while lanes F, G, and H represent the same isolates under reducing conditions. Low and high molecular weight protein standard mixtures were electrophoresed in lanes D and E, respectively.

To estimate molecular weight, a calibration curve was constructed by plotting the relative mobility of the protein standards against the $\log_{10}$ of their known molecular weights. This is presented in Figure 4. Relative mobility of the protein standards was determined by dividing the distance of migration of the proteins, by the distance of migration of the bromphenol blue dye. All of the molecular weight protein standards

Figure 3. SDS-PAGE electrophoresis of the structural proteins of bovine rotavirus isolates B223, B641, and NCDV under reducing and nonreducing conditions. Protein bands were visualized by staining with Coomassie brilliant blue

Nonreducing Conditions

Lane A Bovine rotavirus isolate B223
Lane B Bovine rotavirus isolate B641
Lane C Bovine rotavirus isolate NCDV

Molecular Weight Standards

Lane D High molecular weight markers: (Top to bottom) phosphorylase B 92,500, bovine serum albumin 66,200, ovalbumin 45,000, and carbonic anhydrase 31,000

Lane E Low molecular weight markers: (Top to bottom) myosin 200,000, β-galactosidase 116,250, phosphorylase B 92,500, bovine serum albumin 66,200, and ovalbumin 45,000

Reducing Conditions

Lane F Bovine rotavirus isolate B223
Lane G Bovine rotavirus isolate B641
Lane H Bovine rotavirus isolate NCDV

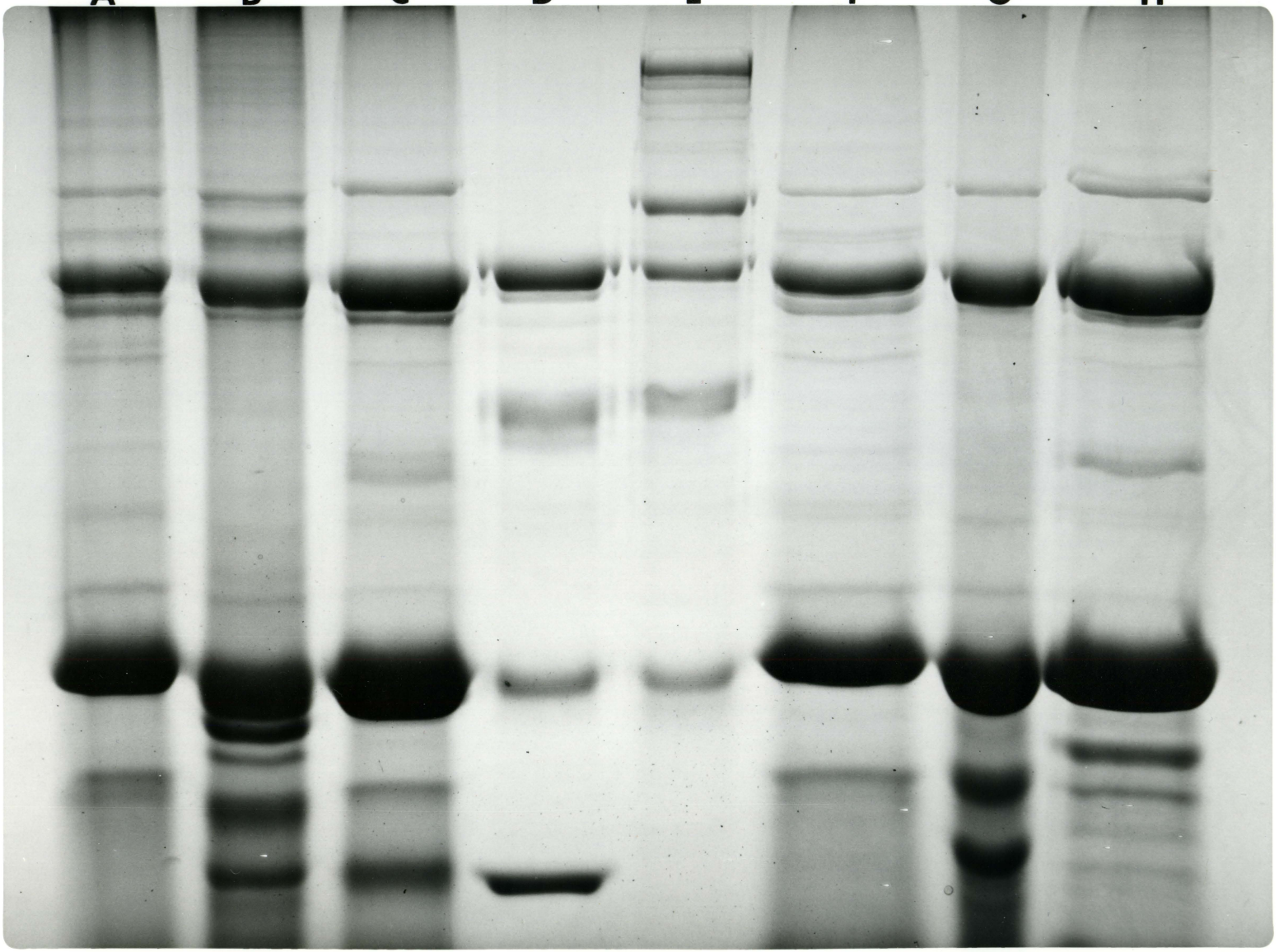
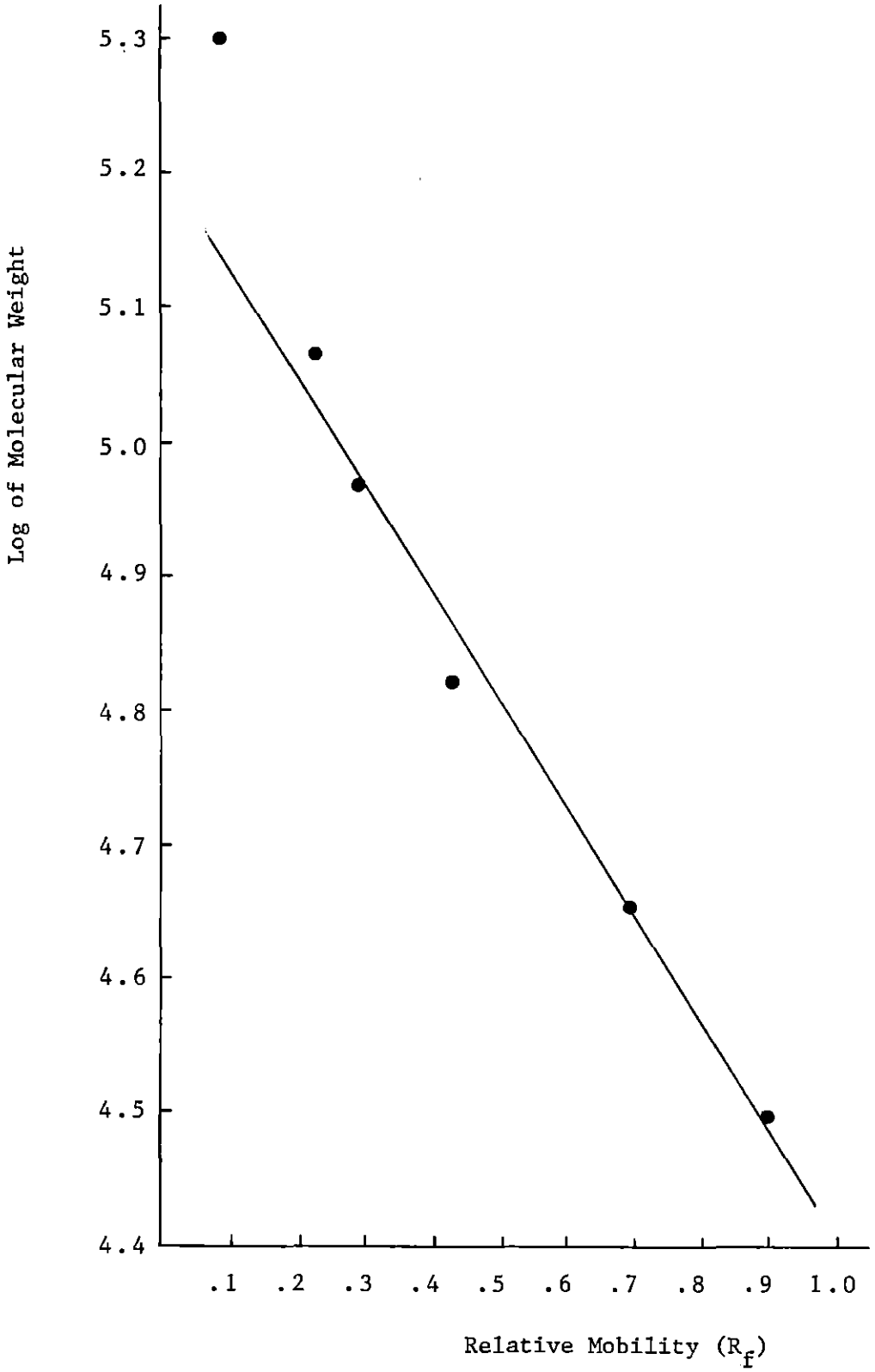

A
B
C
D
E
F
G
H

Figure 4. Calibration curve used for calculating the molecular weight estimates of the bovine rotavirus structural proteins

The molecular weight protein standard Myosin (MW 200,000) was excluded when constructing the curve due to the fact that the relative mobility of a 200,000 molecular weight protein in a 7.5% gel lies outside of the limited range in which mobility is linearly related to molecular weight.


Log of Molecular Weight
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Relative Mobility (R_f)

visualized in lanes D and E of Figure 3 were used for construction of the calibration curve with the exception of myosin. Myosin was excluded due to the fact that the relative mobility of a protein of MW 200,000 in a 7.5% separation gel lies outside of the limited range in which mobility is linearly related to molecular weight.

Analysis of the electrophoretic profile of the three bovine rotavirus isolates revealed 6 to 7 structural proteins. These included the two major and one minor inner capsid polypeptides of approximate molecular weights 43-45K, 90-94K, and 110-111K, and the two outer capsid polypeptides 31-38K (the glycoprotein), and 87-89K. The third outer capsid polypeptide of approximate molecular weight 80K (the trypsin cleavable hemagglutinating protein) was observed in the reduced and nonreduced protein profiles of isolates B223 and NCDV, but was absent in the protein profile of isolate B641. This protein was presumed to have been exposed to trypsin and to have undergone proteolytic cleavage during purification of the viruses, as evidenced by the presence of the known proteolytic breakdown product, a polpeptide of 60-66K, detected in the reduced protein profiles of all three isolates. The structural proteins detected, along with the values obtained for the molecular weights of the reduced proteins, agreed with the findings previously reported by other investigators (Estes et al., 1983; Holmes, 1983; Novo and Esparza, 1981; Esparza et al., 1983; Babiuk et al., 1985; Bican et al., 1982).

The discrepancy in the number of structural proteins detected, as mentioned above, occurred between rotavirus isolate B223 and isolates NCDV and B641, in which 6 and 7 structural proteins were observed,

respectively. This discrepancy pertained to the major outer capsid glycoprotein, which was present in the capsids of NCDV and B641 in two forms. The occurrence of 2 or more glycoproteins of differing molecular weights within the outer capsid of a single rotavirus isolate has previously been reported in both bovine and simian rotaviruses (Estes et al., 1982; Novo and Esparza, 1981). The number and molecular weights of the polypeptides of all three isolates above 40 K were essentially the same.

Developement and Optimization of the Methodology for the Immunodetection of Rotavirus Proteins on Nitrocellulose Strips

Several quenching agents were compared for use in this study. Comparisons were made using the dot immunobinding assay. The quenching agents investigated were 3% bovine serum albumin fraction V (GIBCO Laboratories, Grand Island, NY), bovine lacto transfer technique optimizer (Blotto) (Johnson et al. 1984), 1% and 3% ovalbumin grade V (Sigma Chemical Co., St. Louis, MO), 1% and 3% ovalbumin hydrolysate (Sigma), and 1% ovalbumin hydrolysate with 0.1% Tween 20. The latter quenching agent proved to be the most economical and efficient in preventing background staining, and was used for this study with the addition of 0.001% Thimerosol to control bacterial growth.

Preliminary selection of the primary antibody dilutions for use in reactions with nitrocellulose strips, were performed using the dot immunobinding assay. The final selection of the optimal dilutions were

determined following reaction of the antisera with homologous viral proteins transferred to nitrocellulose strips. Initial reactions of convalescent bovine antisera to NCDV with blotted proteins proved difficult due to low antibody titers. Since data from serum neutralization tests performed in this laboratory indicated equivalent or lower antibody titers with B641, blot experiments were confined to comparisons between B223 (four convalescent animals) and NCDV (one hyperimmunized and two convalescent animals). In contrast, the relatively strong reactions of the guinea pig hyperimmune antisera to the blotted proteins enabled comparisons to be made between the bovine isolates B223, NCDV, and B641 (one hyperimmunized animal for each isolate). Optimal dilutions of convalescent bovine antisera were 1:50 and 1:100 for all NCDV and B223 sera, respectively. Hyperimmune bovine antiserum (GC5) was found optimal at a dilution of 1:60, and guinea pig hyperimmune antisera was optimal at 1:600 and 1:200 for B223, and NCDV and B641, respectively.

Several conjugated secondary antibody preparations were utilized for reaction with primary bovine antisera. These included peroxidase labeled goat anti-bovine IgG (H+L) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), biotinylated rabbit anti-goat IgG (H+L) (Vector Laboratories, Inc., Burlingame, CA), and biotinylated rabbit anti-bovine IgG (H+L) (Zymed Laboratories Inc., S. San Francisco, CA). Peroxidase labeled goat anti-bovine IgG provided insufficient amplification for the immunological detection of bovine antisera reactions with antigen, therefore, biotinylated secondary antibody conjugates were examined. The

unavailability of biotinylated anti-bovine conjugates at the beginning of this study, prompted the utilization of biotinylated rabbit anti-goat, due to reported cross-reactivity of this conjugate with bovine immunoglobulins (Vector Laboratories Inc., Burlinggame, CA, personal communication). Although cross-reactivity with primary bovine antisera was observed, significant nonspecific interactions with control sera prompted the use of biotinylated anti-bovine conjugate when it became commercially available. Biotinylated rabbit anti-bovine IgG was utilized at a dilution of 1:167.

A biotinylated secondary antibody conjugate was also utilized to detect the interaction of hyperimmune guinea pig antisera with rotavirus antigens. Biotinylated goat anti-guinea pig IgG (H+L) (Vector Laboratories, Inc.) was utilized at a dilution of 1:250.

Two avidin-peroxidase conjugates were compared for reaction with biotinylated secondary antibodies. These included avidin-horseradish peroxidase (avidin-HRP) and avidin-biotinylated horseradish peroxidase complex (ABC) (Vector Laboratories, Inc.). Avidin-HRP posed many problems due to nonspecific binding to proteins spotted or electrophoretically transferred to nitrocellulose. This is due to the net positive charge of avidin at normal physiological pH, which results in electrostatic binding to negatively charged proteins (Morris and Saelinger, 1984). Although the nonspecific binding can be limited by careful selection of buffers, Figure 5, the need for further amplification for visualization of the bovine and guinea pig antisera interactions prompted the use of ABC. Avidin-biotinylated horseradish

Figure 5. Nonspecific reactions encountered during the developement of the avidin-biotin immunoassay system, as demonstrated by dot-immunobinding

1. Effect of salt concentration on the nonspecific binding activities of the conjugate avidin-horseradish peroxidase (avidin-HRP) with rotavirus proteins and nitrocellulose. Diluent used for the avidin conjugate consisted of a 10mM phosphate buffer, pH 7.5, 0.05% Tween 20, and NaCl.

   NC[1] square 23: specific reaction [1° Ab, biotinylated 2° Ab, avidin-HRP, and 4-chloro-1-napthol (0.154 M NaCl c.a. 0.9% NaCl)].
   NC square 24: reaction with avidin-HRP and 4-chloro-1-napthol only. (0.154 M NaCl).

   NC square 21: specific reaction, same as above (0.5 M NaCl).
   NC square 22: reaction with avidin-HRP and 4-chloro-1-napthol only. (0.5 M NaCl).

   NC square 7: specific reaction, same as above (0.7 M NaCl).
   NC square 8: reaction with avidin-HRP and 4-chloro-1-napthol only. (0.7 M NaCl).

2. Nonspecific absorption of the substrate 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) by rota-virus spotted on nitrocellulose. Diluent used for the avidin conjugate consisted of 10 mM phosphate buffer, pH 7.5, 0.05% Tween 20, and 0.5 M NaCl.

   NC square 5: specific reaction (1 Ab, biotinylated 2 Ab, avidin-HRP, and ABTS).
   NC square 2: reaction with ABTS only.

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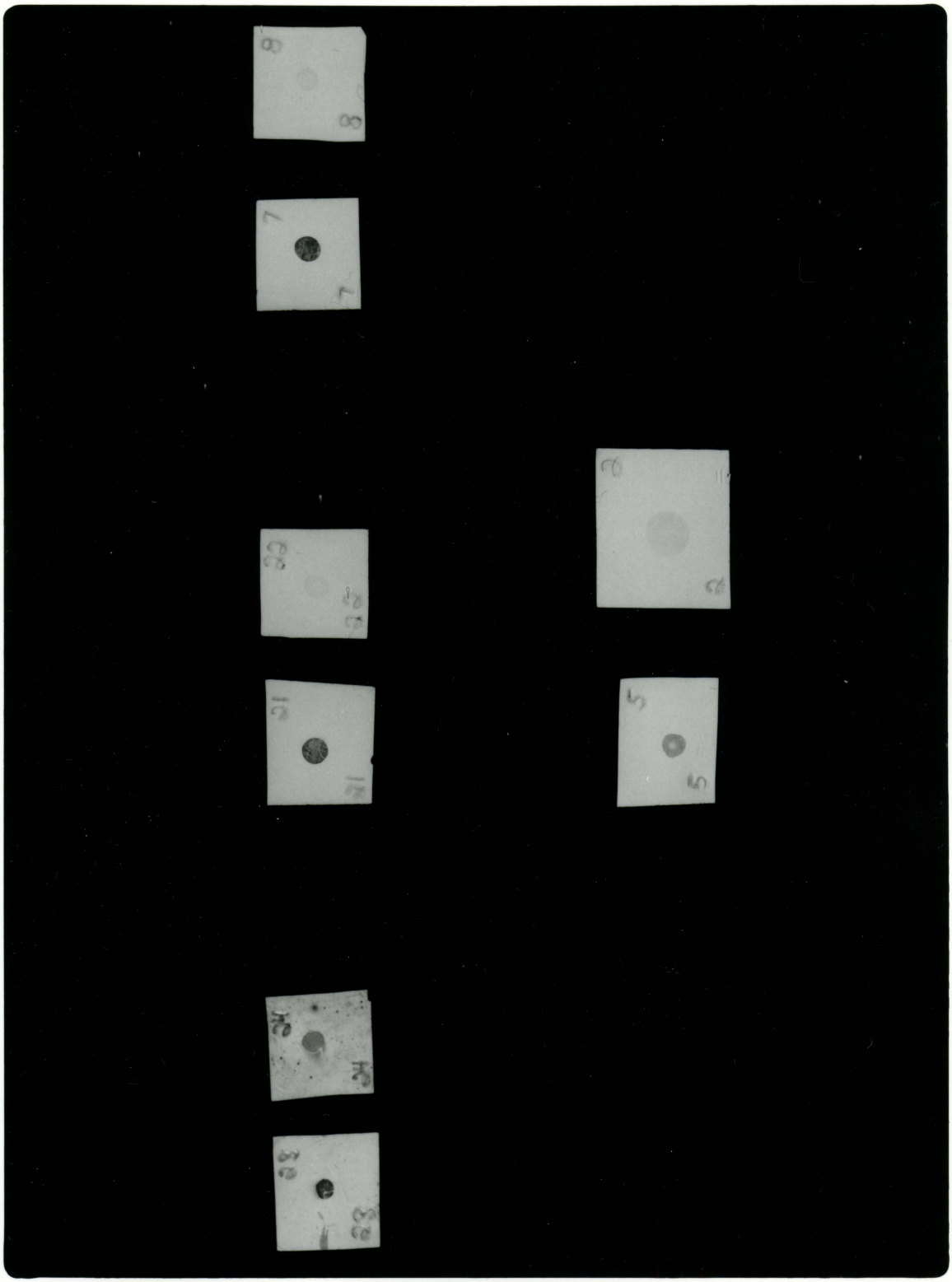
[1] NC - nitrocellulose.



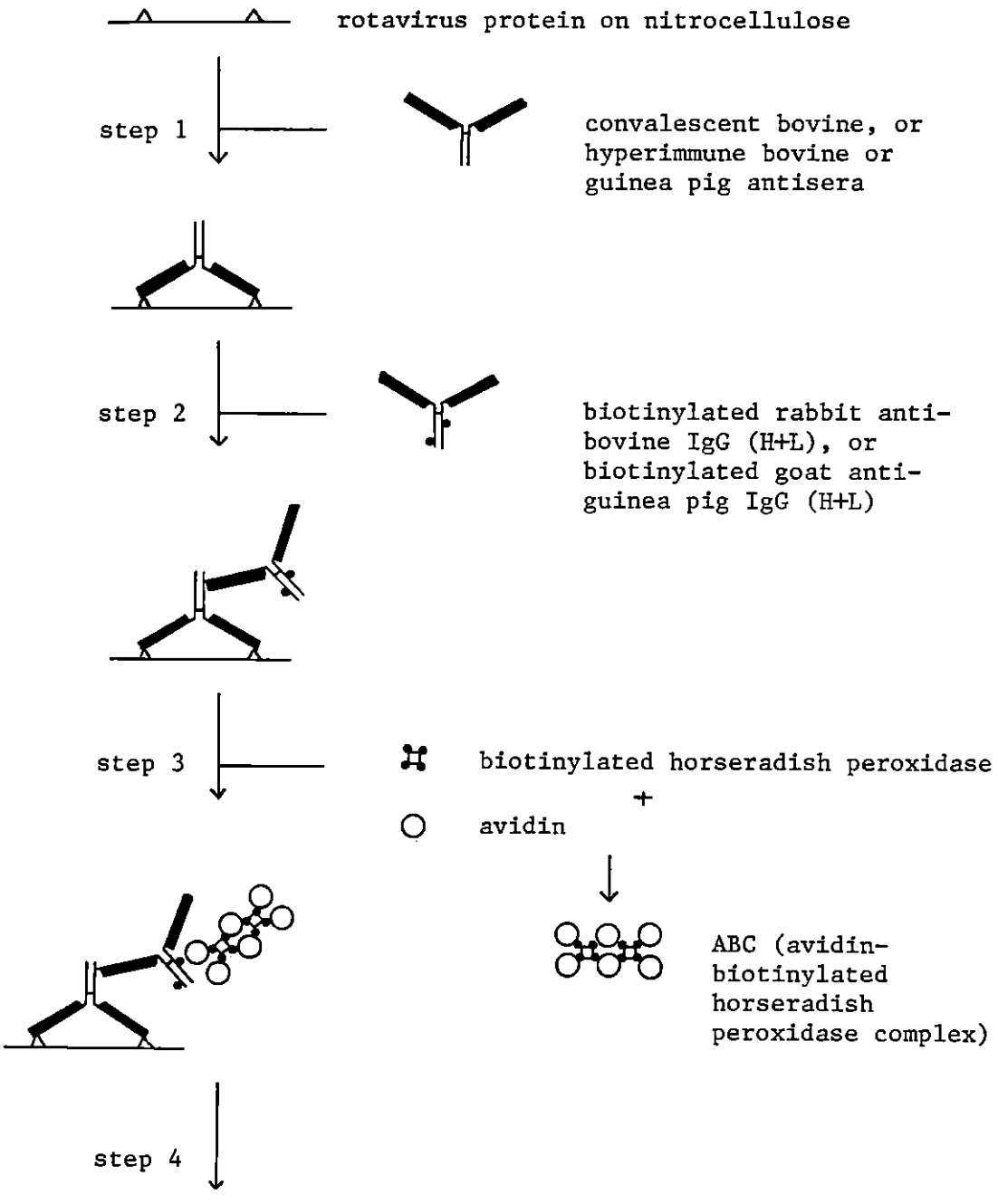
peroxidase complex was prepared according to the manufacturer's instructions, and utilized at a dilution of 1:5. Contrary to avidin-HRP, ABC did not exhibit significant nonspecific binding to proteins.

Although a wide variety of peroxidase substrates are available, three substrates were selected for comparison in this study. These included 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), 3,3'-dimethoxybenzidine [dihydrochloride (O-dianisidine)], and 4-chloro-1-napthol (Sigma Chemical Co.). The substrate ABTS proved to be ineffective for immunological detection of antibody-antigen reactions on nitrocellulose. This was due to the fact that the soluble blue-green color produced by ABTS was absorbed nonspecifically by proteins on nitrocellulose, whether or not they had been previously probed by reagents labeled with horseradish peroxidase. This is shown in Figure 5. Although the substrate color produced by O-dianisidine was not absorbed nonspecifically by protein, it was absorbed to a greater extent by nitrocellulose as compared with 4-chloro-1-napthol, producing a higher background color. As a result, 4-chloro-1-napthol was found to be the optimal substrate for use in the detection of immunological reactions on nitrocellulose, and is summarized in Figure 6 along with the optimal avidin-biotin reagents.

Careful selection of buffers overcame many of the problems with nonspecific staining encountered in the developement of this assay. As noted above, problems were encountered with the use of the reagent avidin-HRP. Two approaches were used to control this problem; elevated pH and increased concentration of NaCl. Successful utilization of

Figure 6. Summary of the optimal avidin-biotin reagents and substrate used in this study

Avidin-Biotin



reaction with substrate - 4-chloro-1-napthol

buffers with high pH (pH 9.6) was reported by Bussolati and Gugliotta (1983) to overcome nonspecific staining of mast cells in immunohistochemical studies with the avidin reagent ABC. The strategy for using buffers with elevated pH is due to the net positive charge of avidin below pH 10.5. As the pH is raised toward the isoelectric point, the net charge of avidin approaches neutrality. Ten millimole phosphate buffers of pH 7.5, 9.5. 10.0, and 10.5 were tested with rotavirus antigens on the dot immunobinding system. Although specific reactions were observed with buffers having a maximum pH of 10.0, test squares exposed to avidin-HRP and substrate alone produced peculiar reactions in which the rotavirus antigens appeared as white dots against a dark purple background. Greater success in reducing nonspecific reactions was achieved with the use of buffers containing high concentrations of NaCl [0.5 to 0.7 M as opposed to 0.145 M (approximately 0.85%, normal saline)]. The ability of NaCl to disrupt electrostatic interactions has previously been reported (Shields and Farrah, 1983), and its application to the problems of nonspecific binding by avidin has been described (Fritz et al., 1984; Vector Laboratories Inc., Burlingame, CA, personal communication). Although high NaCl concentrations were found to be necessary for controlling the reagent avidin-HRP, phosphate buffers containing 0.154 M NaCl were found to be sufficient for use with ABC.

The ability of buffers containing high concentrations of NaCl to prevent nonspecific reactions was also applied to similar problems encountered with the use of convalescent or hyperimmune antisera as the source of primary antibodies. As with avidin, buffers containing 0.5 M

NaCl were effective in reducing nonspecific reactions, while having little or no effect on specific reactions.

The specificity of all reagents used in the assays were tested by dot immunobinding, and are exemplified in Figure 7 with the findings for guinea pig 55 hyperimmune antiserum. In addition to the controls compared on the dot immunobinding assay system, all reactions of test sera with the nitrocellulose strips were performed simultaneously with a serum control. Control sera for the bovine convalescent and hyperimmune antisera consisted of anti-breda virus or anti-Cryptosporidium sera prepared in gnotobiotic calves in this laboratory. Convalescent antiserum from a three week old nonvaccinated gnotobiotic calf was also used as a control, and was subsequently shown to be nonreactive against rotavirus proteins. Control sera for the guinea pig hyperimmune antisera consisted of the prebleeds of the hyperimmunized animals. The prebleed antisera were found to contain low levels of antibody against rotavirus.

Reactions of Bovine and Guinea Pig Antisera with Electrophoretically Blotted Proteins

The reactions of the four bovine convalescent antisera to B223 with homologous and heterologous virus proteins, B223 and NCDV, respectively, is presented in Figure 8. The antisera reacted with the structural proteins of both isolates, and in particular with the major neutralizing glycoproteins, and thus were found not to be discriminatory. A similar cross-reactivity was observed with bovine convalescent and hyperimmune

Figure 7. Application of the dot immunobinding system for monitoring the specificity of reagents used for immunological detection

Nitrocellulose squares (NC)	Antigen	Primary Antibody	Secondary Antibody	ABC	Substrate
NC 1,2	X (R)	X (R)	X (B)	X	X
NC 3,4	X (R)		X (B)	X	X
NC 5,6	X (R)	X (R)		X	X
NC 7,8	X (R)	X (R)	X (B)		X
NC 9,10	X (R)			X	X
NC 11,12	X (R)	X (R)			X
NC 13,14	X (R)				X
NC 15,16		X (R)	X (B)	X	X
NC 17,18	X (M)	X (R)	X (B)	X	X
NC 19,20	X (M)	stained: India ink			
NC 21,22	X (C)	X (R)	X (B)	X	X
NC 23,24	X (C)	X (C)	X (G)	X	X

Antigen: (R) = B223 rotavirus (M) = Ma-104 cells (C) = Corona virus

Primary antibody: (R) = GP55 (NCDV) hyperimmune guinea pig antiserum
(C) = GC78 (Coronavirus) hyperimmune bovine antiserum

Secondary antibody: (B) = biotinylated rabbit anti-bovine
(G) = biotinylated goat anti-guinea pig

ABC: avidin-biotin complex

Substrate: 4-chloro-1-napthol

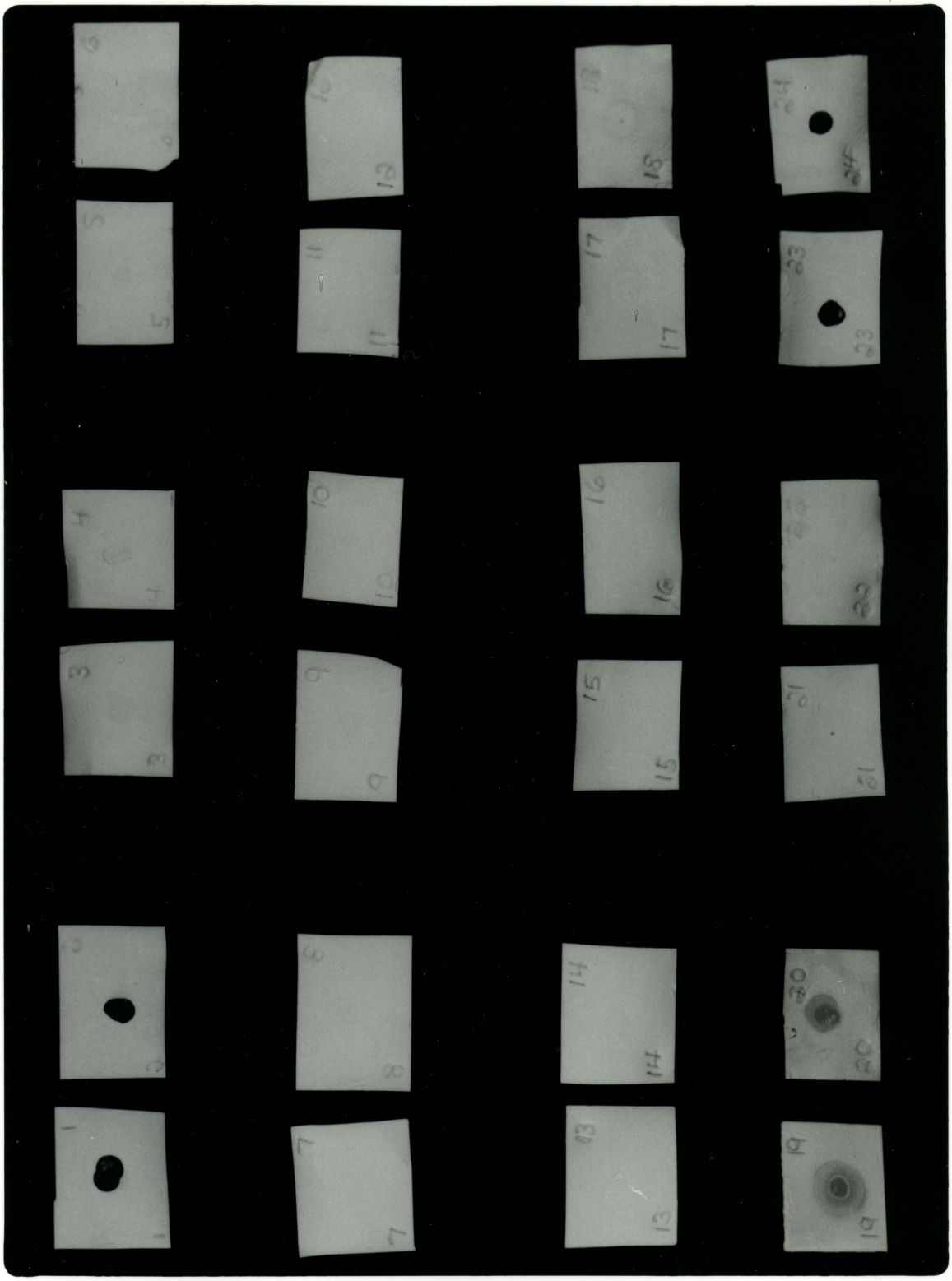


Figure 8. Composite of optimal reactions of bovine convalescent antisera to B223 against homologous and heterologous (NCDV) rotavirus structural proteins

Lane A High molecular weight markers
Lane B Low molecular weight markers

Lane C Control: Crytosporidium antiserum
Lane D Control: Convalescent antiserum from a 3 week old nonvaccinated gnotobiotic calf
Lane E Control: Breda virus antiserum

Lane F GC47 antiserum against B223
Lane G GC47 antiserum against NCDV

Lane H GC36 antiserum against B223
Lane I GC36 antiserum against NCDV

Lane J GC30 antiserum against B223
Lane K GC30 antiserum against NCDV

Lane L GC55 antiserum against B223
Lane M GC55 antiserum against NCDV


A
B
C
D
E
F
G
H
I
J
K
L
M
200,000
116,250
92,500
66,200
45,000
31,000

antisera to NCDV, when reacted with the structural proteins of NCDV and B223. This is presented in Figure 9. Although bovine convalescent and hyperimmune antisera failed to discriminate between virus strains, strain specificity was observed using hyperimmune guinea pig antisera. This is presented in Figure 10. The specificity observed concerned the failure of antisera to NCDV and B641 to react with the major neutralizing glycoprotein of bovine rotavirus isolate B223. This specificity was noted on two separate reactions of NCDV antisera from guinea pig 55 with B223 viral proteins. In contrast, antisera to bovine rotavirus isolate B223 was reactive with both homologous glycoprotein as well as the glycoproteins of bovine rotavirus isolates NCDV and B641. Antisera to NCDV and B641 were reactive against their respective homologous and heterologous glycoproteins.

In order to confirm the presence of the B223 glycoprotein on the nitrocellulose strips which failed to react with NCDV and B641 antisera, the nitrocellulose strips were re-reacted with homologous B223 antiserum. Due to excessive background staining, the protein bands present on the nitrocellulose strips previously reacted with B641 antiserum and one of the NCDV antisera were obscured, and no observations could be made. The major neutralizing glycoprotein of B223 however was visualized on the second nitrocellulose strip that had previously been reacted with NCDV, and is presented in Figure 11.

Figure 9. Composite of optimal reactions of bovine convalescent and hyperimmune antisera to NCDV against homologous and heterologous (B223) rotavirus structural proteins

Lane A High molecular weight markers (as in Figure 8)
Lane B Low molecular weight markers (as in Figure 8)

Lane C Control: Cryptosporidium antiserum
Lane D Control: Convalescent antiserum from a 3 week old nonvaccinated gnotobiotic calf
Lane E Control: Breda virus antiserum

Lane F GC54 (Convalescent) antiserum against NCDV
Lane G GC54 antiserum against B223

Lane H GC25 (Convalescent) antiserum against NCDV
Lane I GC25 antiserum against B223

Lane J GC5 (Hyperimmune) antiserum against NCDV
Lane K GC5 antiserum against B223


A
B
C
D
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F
G
H
I
J
K

Figure 10. Composite of optimal reactions of guinea pig hyperimmune antisera against rotavirus structural proteins

Lane A High molecular weight markers (as in Figure 8)
Lane B Low molecular weight markers (as in Figure 8)

Lane C GP71 Prebleed against B223
Lane D GP55 Prebleed against NCDV
Lane E GP47 Prebleed against B641

Lane F GP71 (B223 antiserum) against B223
Lane G GP71 against NCDV
Lane H GP71 against B641

Lane I GP55 (NCDV antiserum) against NCDV
Lane J GP55 against B223
Lane K GP55 against B641

Lane L GP47 (B641 antiserum) against B641
Lane M GP47 against B223
Lane N GP47 against NCDV


A
B
C
D
E
F
G
H
I
J
K
L
M
N

Figure 11. Re-run of B223 viral proteins against GP71 (B223 antiserum) following reaction with GP55 (NCDV antiserum)

Lane A High molecular weight markers (as in Figure 8)
Lane B Low molecular weight markers (as in Figure 8)

Lane C Rerun B223 viral proteins


A
B
C

DISCUSSION

The Western blot procedure, in conjuction with the efficient resolving power of sodium dodecyl sulfate polyacrylamide gel electrophoresis, has proven to be a powerful tool for detecting the interaction of antiserum with specific structural polypeptides of many organisms (Erickson et al., 1982; Nielsen et al., 1982; Hedstrom et al., 1984). Continuous improvements in the technique, including the ability to partially renature proteins following blotting onto filter matrices (Mandrell and Zollinger, 1984), indicate that the Western blot methodology may be quite useful in further antigenic characterization of rotavirus particles. Indeed, recent studies utilizing the Western blot procedure for studying the interaction of monoclonal antibodies with the antigenic determinants of rotavirus particles have recently been published (Coulson et al., 1985; Gunn et al., 1985), and further studies utilizing this approach may be expected in the future.

The application of the avidin-biotin system in this study toward the detection of antibody-antigen reactions with Western blot has proven to be a satisfactory and effective alternative to radiolabeled reagents. Although difficulties were encountered with the detection of convalescent antisera prepared against NCDV, the problems incurred were related more to the antibody titers of the antisera, than with the detection abilities of the avidin-biotin system. The ABC complex was shown in this study to be a far more sensitive reagent for detecting antibody-antigen reactions, and was less troublesome than avidin-enzyme conjugates concerning

nonspecific reactions. Through the use of high concentrations of NaCl (0.5–0.7 M) in the buffers employed for diluting reagents, many of the nonspecific reactions of avidin-peroxidase conjugates, as well as convalescent and hyperimmune antisera when utilized as the source of primary antibody, can be overcome.

Although a variety of serological tests have been used to differentiate and classify rotavirus strains, the serum neutralization test has established itself as the best indicator of antigenic relatedness. Despite these findings, recent investigations by Woode et al. (1983) concerning cross-protection in gnotobiotic calves demonstrated a lack of cross-protection between two bovine rotavirus isolates of the same serotype, and one-way cross-protection between serologically unrelated bovine rotavirus isolates. In order to investigate these findings, the recently developed technique of Western blot was utilized to immunologically probe the structural proteins of the bovine isolates involved, using convalescent and hyperimmune bovine antisera obtained from the gnotobiotic calves. It was hoped that the interactions or lack of interactions of these sera with the structural proteins, and in particular with the major outer shell glycoprotein, might provide an explanation for the cross-protection results observed, and thus provide a more accurate in vitro test for predicting cross-protection as compared with serum neutralization.

The broad cross-reactivity of both convalescent and hyperimmune antisera to rotavirus has previously been described by other investigators, when utilized in ELISA and immunofluorescence tests

(Estes et al., 1983; Holmes, 1983). This is due to the presence of common antigenic determinant sites located on the 42K inner capsid polypeptide. Sometime after this study began, several papers were published indicating that additional common antigenic determinant sites had been located by monospecific and monoclonal antibodies on the structural proteins of different rotavirus strains, including the outer shell glycoprotein (Estes et al., 1983; Holmes, 1983). Despite these findings, the present study was continued on the rationale that high dilutions of antisera might discriminate between the virus structural proteins. This was based on data from in vivo cross-protection studies which indicated convalescent animals could indeed discriminate between various rotavirus isolates, and the fact that low dilutions (1:100) of hyperimmune antisera were cross-reactive in serum neutralization tests in vitro, while high dilutions were discriminatory.

The results obtained in this study demonstrated cross-reactivity of both hyperimmune and convalescent bovine antisera (B223 and NCDV) with the homologous and heterologous structural proteins of the two isolates, including the outer shell glycoproteins. The failure to differentiate between the structural proteins of the two isolates was believed to be due to the low dilutions of antisera needed to visualize antibody-antigen reactions, thus precluding any discrimination as a result of the interactions with both specific and common antigenic determinant sites.

The inability of bovine convalescent and hyperimmune antisera to detect differences by Western blot led to investigations utilizing guinea pig hyperimmune antisera, which had previously been successful in

distinguishing between the isolates in serum neutralization tests. Using these antisera, discrimination was observed by the failure of antisera to NCDV and B641 to react with the outer shell glycoprotein of rotavirus isolate B223. The discrimination observed was believed to be due to the utilization of the two antisera at high enough dilutions (1:200), at which interactions with common antigens no longer occurred. Support for this conclusion also comes from cross-protection results *in vivo*, in which animals vaccinated with NCDV and B641 were not protected against challenge with B223. In contrast, antiserum to B223 was cross-reactive with both homologous and heterologous glycoproteins of rotavirus isolates B641 and NCDV. The cross-reactivity observed may be due to the presence of two neutralization epitopes (one major antigenic epitope and another less important epitope) on the glycoprotein, only one of which may be shared (Sabara et al., 1983; Sonza et al., 1984; Lazdins et al., 1985).

Hyperimmune guinea pig antiserum to NCDV was cross-reactive in this study with the glycoprotein of rotavirus isolate B641, and the reverse was also true. This result did not correlate with cross-protection observed *in vivo*, since vaccination of calves with B641 did not protect the calves against challenge with NCDV. It did, however, correlate with serum neutralization results *in vitro*, in which the two viruses were found to be serologically related utilizing both NCDV and B641 antisera.

In conclusion, it was found that these initial studies involving the reaction of guinea pig hyperimmune antisera with the structural proteins of the rotavirus isolates by Western blot, did not correlate with the *in vitro* serum neutralization test. Although further repetitions with other

guinea pig antisera should be performed, efforts to develope this method to improve its reliability may require the utilization of monoclonal antibodies, or cross-absorption of polyclonal antiserum to remove reactivity with common antigenic determinant sites.

SUMMARY

An immunoblotting procedure was developed using the avidin-biotin system for detecting the reactions of bovine convalescent, and hyperimmune bovine and guinea pig antisera, against the structural proteins of bovine rotavirus isolates transferred to nitrocellulose by the Western blot procedure.

The RNA electrophoretic profiles of the three bovine rotavirus isolates NCDV, B641, and B223 were monitored throughout the study to assure that cross-contamination of the virus isolates passaged in cell culture or used for inoculation of gnotobiotic calves did not occur. Although NCDV and B641 were shown to be antigenically related by the serum neutralization test, comparison of their RNA electrophoretic profiles revealed differences in the migration of RNA segments 2, possibly 3, 4, 6, possibly 7, 9, and 11. In contrast, B641, which was shown to be serologically distinct from B223, revealed differences between the 789 RNA segment complex, and RNA segment 10. The differences observed confirms the findings of other researchers who have shown that RNA segment migration may not correlate with serotype.

Observations concerning the structural proteins of the bovine isolates revealed two glycoproteins in the capsids of B641 and NCDV. The occurrence of two or more glycoproteins in the rotavirus outer shell has previously been reported for other bovine and simian rotaviruses.

Nonspecificity problems incurred through the use of avidin-peroxidase conjugates, as well as convalescent and hyperimmune antisera

as the source of primary antibody, were found to be controllable by increasing the concentration of NaCl in buffer solutions to 0.5-0.7 M. Increased sensitivity as well as reduced nonspecific reactivity was achieved through the use of the avidin-biotin reagent ABC (containing horseradish peroxidase) as opposed to avidin-horseradish peroxidase conjugate.

The homologous and heterologous reactions of the bovine convalescent and hyperimmune antisera with the structural proteins of B223 and NCDV by Western blot, were unable to differentiate between the individual proteins (particularly the glycoprotein) of each isolate. In contrast, hyperimmune guinea pig antisera to NCDV and B641 were discriminatory, as observed by the failure of these antisera to react with the glycoprotein of rotavirus isolate B223. Differences in the differentiating capabilities of the antisera were believed to be dependent on the finding by chance of dilutions of the antisera at which antibody capable of reacting with common epitopes was no longer found, but yet serotype specific antibody-antigen reactions could still be detected.

LITERATURE CITED

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