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Different Polypeptide Composition of Two Human Rotavirus Types

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Human rotaviruses, which are placed into two groups according to their ribonucleic acid patterns obtained by gel electrophoresis, were characterized both by polypeptide components from purified virions and by polypeptides translated from their denatured ribonucleic acids in rabbit reticulocyte lysates. Viruses assigned to different groups differed in the electrophoretic migration of the second largest of the polypeptides which compose the inner shell; polypeptides that had been synthetized in vitro from ribonucleic acid from each group showed this same difference, thus indicating that this is due to the genomic composition. This study suggests that there are differences in the third largest polypeptide of the inner shell and also in the three smaller polypeptides composing the outer shell. We also demonstrated that there are differences in genomic and polypeptide compositions between simian (SA11) and calf (Nebraska calf diarrhea virus) rotaviruses grown in tissue culture and human rotaviruses.

Rotaviruses, a major cause of gastroenteritis, consist of double-shelled virions approximately 75 nm in diameter (3), which possess double-stranded ribonucleic acid (RNA) genomes composed of 11 segments (21). The particles seem to be formed by eight or nine different polypeptides; five are in the inner shell and three or four are in the outer shell (22).

The existence of different types of rotaviruses causing acute gastroenteritis in humans has been shown by immunological and biochemical studies; two types have been distinguished by the enzyme-linked immunosorbent assay (23), complement fixation, and immunoelectromicroscopy techniques (33), whereas four have been distinguished by a neutralization test (9). Two types, designated 2s and 2l (7), have been found by analysis of the segmented double-stranded viral RNA by gel electrophoresis. Improvements in electrophoretic methods have permitted the observation of differences in migration in as many as seven segments between the two patterns and the distinction of small changes in migration of one or two segments within either of the two RNA patterns (8a).

By a similar technique, three distinct patterns differing in the electrophoretic migration of one, two, or three segments were distinguished among RNAs from eight human rotaviruses obtained during four successive epidemics (12). Since comparison of the RNA electrophoretic patterns from rotaviruses seems to be a useful approach for epidemiological studies (8, 12), the significance of the observed differences was explored by analysis of the viral proteins. The results reported in this paper show that rotaviruses with different RNA electrophoretic patterns differ in some of their structural polypeptides. These differences were observed in purified virions and in vitro transcripts of viral RNA.

Although human rotaviruses cannot be grown efficiently in cell culture, both Nebraska calf diarrhea virus (NCDV) and SA11, which are morphologically indistinguishable from human rotaviruses, can be grown in various cell lines (15, 17), are readily available, and are suitable standards for comparisons of different isolates of human rotaviruses. The differences that were found between these two rotaviruses, which were used as standards in this work, were useful in a comparative study of human and animal rotaviruses.

MATERIALS AND METHODS

Viruses. Patients were infants and children less than 3 years old who were admitted with acute gastroenteritis and diarrhea of 5 days duration or less to the Hospital de Pediatria, Instituto Mexicano del Seguro Social, during the period from October to December 1978. Stools (20 to 450 g) were obtained from each patient by continuous collection for 12 h, starting on the first day after admission, and the rotaviruses in each sample were purified separately.

Simian rotavirus SA11, obtained originally from H. H. Malherbe, University of Texas, and NCDV, obtained from R. S. Spendlove, Utah State University, were grown in MA 104 cells in petri dishes (125 cm^2)

as previously described (8), with the modification that for virus infection and propagation, minimal essential medium without serum but containing trypsin (1 μg/ml) was used. To prepare labeled virus for structure analysis, minimal essential medium was replaced 6 h postinfection with minimal essential medium containing L-methionine (0.3 mg/liter) and L-[^{35}S]methionine (0.86 mCi/liter).

Purification of viruses. The initial steps of purification were as previously described (3). Briefly, either a 20% (wt/vol) suspension in TSM buffer [0.01 M tris(hydroxymethyl)aminomethane, 0.15 M NaCl, 0.001 M $MgCl_2$] of the stools from a single patient or a culture of infected cells showing 80 to 100% cytopathic effects was extracted with trifluorotrichloroethane, and the virus in the aqueous phase was subsequently precipitated with 8% (wt/vol) polyethylene glycol 6000 (Union Carbide). The precipitate was resuspended in TSM buffer (1/100 the volume of the original sample) and then sedimented in a discontinuous gradient containing 0.5 ml of a CsCl solution having a density of 1.4 g/cm^3, 0.5 ml of a CsCl solution having a density of 1.3 g/cm^3, and 1 ml of a 45% (wt/vol) sucrose solution in polyallomer tubes which fit the SW50.1 rotor of a Beckman ultracentrifuge. After centrifugation at 40,000 rpm for 1 h, the opalescent band of virus that formed immediately above the CsCl solution having a density of 1.4 g/cm^3 was collected and mixed with 2 volumes of TSM buffer. The virus was then once more sedimented in a discontinuous gradient containing 0.5 and 1.0 ml of CsCl solutions having densities of 1.4 and 1.3 g/cm^3, respectively. After collection of the opalescent band, the virus was subsequently centrifuged to equilibrium at 40,000 rpm for 17 h in a CsCl solution with an initial density of 1.36 to 1.37 g/cm^3 in a type 50.1 fixed-angle rotor or an SW50.1 rotor and a Beckman ultracentrifuge. After centrifugation, the tubes were punctured, fractions were collected, and the refractive index and absorbance at 260 nm of each fraction were measured. Those fractions containing either light or heavy virus were pooled separately and dialyzed against TSM buffer. Further purification of [^{35}S]methionine-labeled virus was achieved by centrifugation of the virus in a 15 to 45% sucrose gradient at 19,000 rpm for 145 min in an SW40 rotor and a Beckman ultracentrifuge. The yields of human rotavirus varied from 3 to 23 μg of virus per g of stools. Yields of calf and simian rotaviruses varied between 10 and 30 μg of virus per culture plate. To estimate the amount of rotavirus, it was assumed that the specific absorbance of rotavirus was equal to that of reovirus (5.1 absorbance units at 260 nm per mg of virus) (27).

RNA and protein extraction. The purified virus was disrupted with sodium dodecyl sulfate and 2-mercaptoethanol as described previously (8). The disrupted virus was then treated twice with both 1 volume of phenol saturated with TE buffer [0.1 M tris(hydroxymethyl)aminomethane, 0.001 M ethylenediaminetetraacetate] and 1 volume of chloroform (11). To precipitate the RNA in the separated aqueous layer, 0.04 volume of 5 M NaCl and 2 volumes of ethanol were added, and the solution was kept at −20°C overnight. The RNA which precipitated was centrifuged at 10,000 rpm for 30 min, and the pellet was then suspended in TE buffer. The protein remaining on top of the phenol-chloroform layer was precipitated with ethanol (24), and the pellet obtained after centrifugation was dissolved in TE buffer containing 0.1% sodium dodecyl sulfate.

Gel electrophoresis. For electrophoresis of RNA, 5% (wt/vol) polyacrylamide–0.125% (wt/vol) bisacrylamide slab gels were prepared as described previously (8). Samples containing approximately 0.5 μg of viral RNA were used, and electrophoresis was carried out at 20 mA/slab gel (35 V). Since the pH of the buffer in the reservoirs began to change after 10 to 12 h of electrophoresis at this voltage, the buffer was changed at appropriate times. Electrophoresis of the viral polypeptides was performed in 11% (wt/vol) polyacrylamide–0.3% (wt/vol) bisacrylamide slab gels by the method of Laemmli (14). Protein was dissociated by treatment with Laemmli sample buffer (14) in a boiling water bath for 2 min. Polypeptides were stacked at 10 mA/gel, and then electrophoresis was continued at 20 mA/gel. Gels were subsequently fixed and stained with 0.06% (wt/vol) Coomassie brilliant blue in 30% methanol–10% acetic acid. After destaining in 10% acetic acid, gels were photographed and, when appropriate, processed for fluorography by the method of Bonner and Laskey (4).

The molecular weights of SA11 polypeptides were determined by comparing their electrophoretic mobilities in the same slab gel with the following markers: β-galactosidase (grade IV), bovine serum albumin (fraction V), ovalbumin (grade V), pepsin (from hog stomach mucosa), and lysozyme (grade I, from egg white) (all obtained from Sigma Chemical Co.); these markers had molecular weights of 130,000, 67,000, 42,000, 34,000, and 14,300, respectively. The results obtained with lysozyme were not considered in the calculations because under the conditions used, this compound migrated close to bromophenol blue and deviated significantly from the linear relationship obtained with the other standard polypeptides, when log of molecular weight was plotted versus electrophoretic migration. Once the molecular weights of SA11 polypeptides were determined, these were used as references for the molecular weight calculations of the polypeptides which were either isolated from other rotaviruses or synthesized in vitro. The coefficient r for the linear regression of the standard polypeptides was 0.995 and that obtained in different electrophoresis for SA11 polypeptides was between 0.99 and 1.000.

The molecular weights of the different RNA segments were determined in a manner analogous to that described above for polypeptides by using the values obtained by Rodger and Holmes (personal communication) for SA11 RNA segments 1, 2, 5, and 6; these values are 2.04×10^6, 1.62×10^6, 0.96×10^6, and 0.79×10^6, respectively. These segments were chosen because they are very well resolved by electrophoresis and because their molecular weights are within the range of the molecular weights of the RNA segments of the Abney strain of reovirus type 3, which was used as a reference by Rodger and Holmes.

Preparation of rabbit reticulocyte lysate and

conditions for protein synthesis. Cells were obtained from the blood of New Zealand white rabbits which had been made anemic by subcutaneous injection of 2.5% (wt/vol) neutralized phenylhydrazine hydrochloride in water; 10 mg of phenylhydrazine per kg of body weight was injected for 5 successive days, and the rabbits were bled on either day 6 or day 7. Cells were washed and lysed by the procedure of Villa-Komaroff et al. (30) and were stored in small samples at −70°C. The lysate was made messenger dependent by treatment with micrococcal nuclease, and protein synthesis was assayed by the method of Pelham and Jackson (19), with the following modifications: 100 mM KCl, 19 unlabeled amino acids (each at 0.25 mM), micrococcal nuclease (30 μg/ml), and ethylene glycol-bis(β-aminoethylether)-*N,N*-tetraacetic acid (final concentration, 8 mM) were used. Ethanol-precipitated viral RNA was dissolved in 90% dimethyl sulfoxide and denatured at 50°C for 15 min (16); this solution was then added to the lysates in an amount sufficient to obtain a concentration of 20 to 50 μg of RNA per ml but not to exceed 1.5% dimethyl sulfoxide. [^{35}S]methionine was used at a final concentration of 0.07 mCi/ml of incubation mixture. The incubation was carried out for 90 min at 30°C, and protein synthesis was assayed as described previously (30). Polypeptide samples were analyzed on slab gels, as described above.

Buffers and reagents. TS buffer contained 0.1 M tris (hydroxymethyl)aminomethane and 0.15 M NaCl. TS, TSM, and TE buffers were adjusted to pH 8.2 with HCl. Minimal essential medium with Earle salts (autoclavable) was obtained from GIBCO Laboratories, Grand Island, N.Y., micrococcal nuclease was from P. L. Biochemicals, Inc. Milwaukee, Wis., creatine kinase was from Miles Research Products, Westhaven, Conn., ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid and creatine phosphate were from Sigma Chemical Co., St. Louis, Mo., and L-[^{35}S]methionine (>500 Ci/mmol) was from New England Nuclear Corp., Boston, Mass.

RESULTS

Polypeptide components of different human rotavirus particles. Rotaviruses purified from the stools of individual patients and differing in their segmented double-stranded RNAs were further characterized by their polypeptide compositions. Figure 1A shows the patterns obtained by gel electrophoresis of RNAs extracted from rotaviruses assigned to the two different groups (2s and 2l) previously described (7, 8). Relatively large differences in mobility were observed between the two groups in segments 2, 5, 10, and 11. Differences in migration in segments 7 and 9 were not constant; the mobilities of these segments may differ among isolates from either group (Espejo, unpublished data). Figure 1B shows the polypeptides obtained from the same preparations of purified rotavirus particles employed in Fig. 1A; the two human rotaviruses can be distinguished by the electrophoretic mobility of polypeptide 2, a structural component of the inner capsid (22). No differences in the

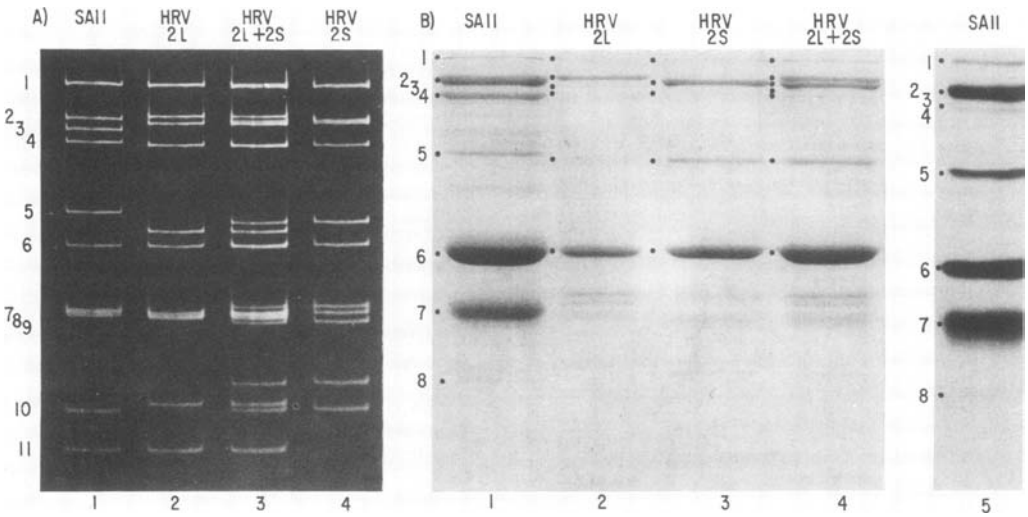

FIG. 1. *Electrophoretic patterns of RNAs (A) and proteins (B) of different human rotaviruses (HRV) and of simian (SA11) rotavirus. (A) Track 1, SA11 RNA; track 2, RNA from human rotavirus of type 2l (HRV 3222); track 3, RNA from human rotavirus of type 2l plus RNA from human rotavirus of type 2s; track 4, RNA from human rotavirus of type 2s (HRV 5859). (B) Track 1, Polypeptides from partially purified double-shelled SA11; track 2, polypeptides from HRV 3222; track 3, polypeptides from HRV 5859; track 4, polypeptides from HRV 3222 and HRV 5859; track 5, polypeptides from purified [^{35}S]methionine-labeled double-shelled SA11 (fluorography). Numbers at the left of each group of tracks are the numbers assigned to RNA segments or structural polypeptides; dots at the left of each track indicate corresponding bands. The number given to each human rotavirus was that assigned at the hospital to the patient excreting that virus.*

migrations of polypeptides 1 and 6 were observed, even when smaller amounts of samples were used to resolve polypeptide 6 as a thin band. Comparison of polypeptides 3 and 4 was difficult because only polypeptide 3 or polypeptide 4 was observed with the preparation of group 2s. The differences in the bands which migrated ahead of band 6 might reflect differences in the compositions of the outer layer; however, it is not certain that these bands are due to viral proteins. Further purification of the virus to clarify this point was hindered since the majority of stool samples extracted by us contained very few double-shelled particles when examined by electron microscopy and had a single viral band at a density of 1.38 to 1.39 g/cm^3 in a CsCl gradient when subjected to the purification procedure. To further explore the differences observed in the polypeptides of the inner capsid, we did an analysis of purified viruses which had little or no outer layer polypeptides and which were obtained from different patients with gastroenteritis. Figure 2 shows the RNA (Fig. 2A) and polypeptide (Fig. 2B) elec-

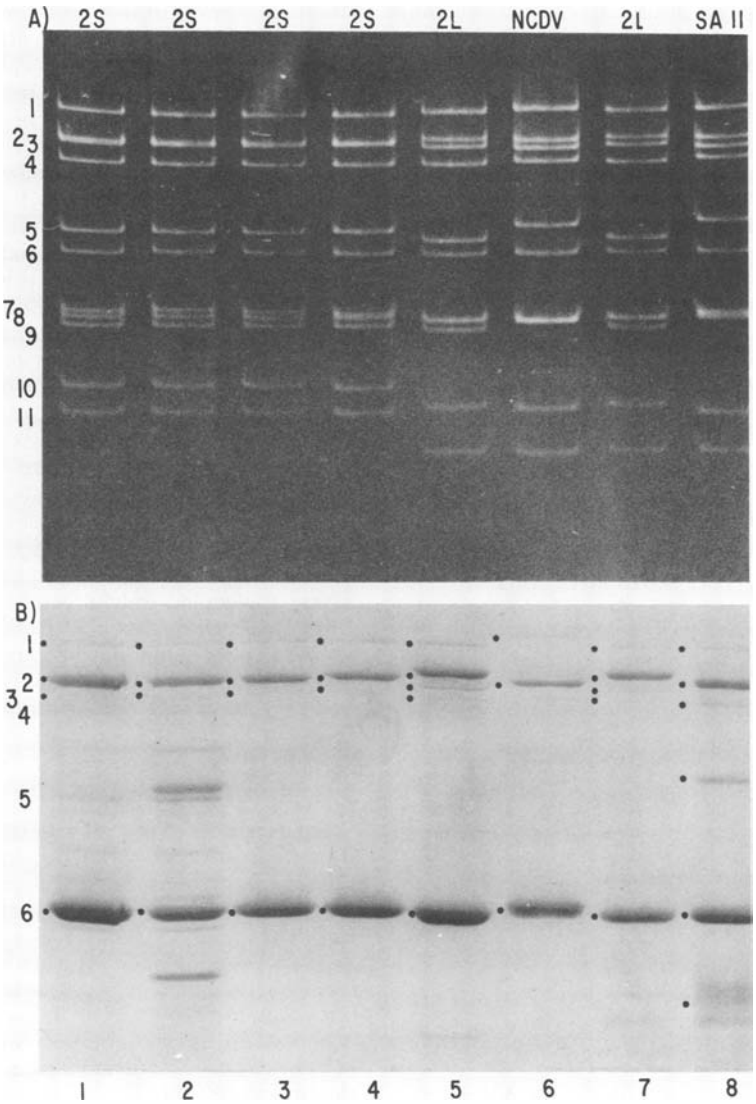

FIG. 2. *Electrophoretic patterns of RNAs (A) and proteins (B) extracted from simian, calf, and different human rotaviruses. Tracks 1 to 4 show the patterns obtained with human rotaviruses of type 2s; tracks 5 and 7 show the patterns obtained with human rotaviruses of type 2l; and tracks 6 and 8 show the patterns obtained with single-shelled NCDV (density, 1.39 g/cm^3) and double-shelled SA11 rotavirus, respectively. Track 1, HRV 0454; track 2, HRV 2017; track 3, HRV 9923; track 4, HRV 1383; track 5, HRV 1601; track 7, HRV 5148.*

trophoretic patterns of six rotaviruses purified from the stools of six different patients and those of calf rotavirus (NCDV) without outer layer and double-shelled simian rotavirus (SA11). The differences in the RNA electrophoretic patterns of human, calf, and simian rotaviruses will be reported and discussed elsewhere. Although the presence of nonviral proteins is noticeable in some samples, especially those in Fig. 2B, tracks 1 and 2, viral polypeptides 1, 2, and 6 may be easily identified. The slower migration of polypeptide 2 from virus of group 2l was also observed with this preparation; no difference was observed for polypeptide 1. Even though a minor difference in polypeptide 6 was apparent, no difference was observed when less sample was used to resolve this polypeptide as a thin band (data not shown). As in Fig. 1, four large polypeptides were observed with rotaviruses of group 2l, whereas only three were observed with group 2s rotaviruses. Polypeptides 1, 2, and 6 of the inner capsid of SA11 appear very similar to the corresponding polypeptides of human rotavirus of group 2s, whereas polypeptides 1 and 6 of NCDV are larger than the corresponding polypeptides of human or SA11 rotaviruses. By using less sample for better resolution of band 6, the patterns shown in Fig. 3 were obtained by coelectrophoresis of NCDV and human rotavirus type 2l (HRV 2l); this confirmed the above observations.

Polypeptides translated from denatured RNA of different rotavirus strains. Denatured reovirus RNA may be translated in an in vitro system (16). In an attempt to compare outer layer and nonstructural viral proteins, the polypeptides synthesized in rabbit reticulocyte lysates upon addition of denatured viral RNA were examined. After denaturation in dimethyl sulfoxide, 2 to 5 μg of rotavirus RNA which had been obtained from different stool samples was added to reticulocyte lysates; these lysates had been made messenger RNA dependent by incubation with micrococcal nuclease and were supplemented with [^{35}S]methionine. Figure 4 shows that the difference observed for polypeptide 2 obtained from purified human rotavirus is also seen with the polypeptides synthesized in vitro from these viral RNA types. The fidelity of the in vitro translation may also be observed when comparing polypeptides 1, 2, and 6 synthesized from NCDV and from human rotavirus 2l (Fig. 4B); just as with the polypeptides obtained from purified viruses, polypeptides 1 and 6 of NCDV are larger than the corresponding polypeptides of human rotavirus. Since partial degradation of the particles and their polypeptides might have taken place in the stool samples, comparison of the human rotavirus proteins synthesized in vitro was considered important to show that the differences observed between types 2l and 2s are not artifacts.

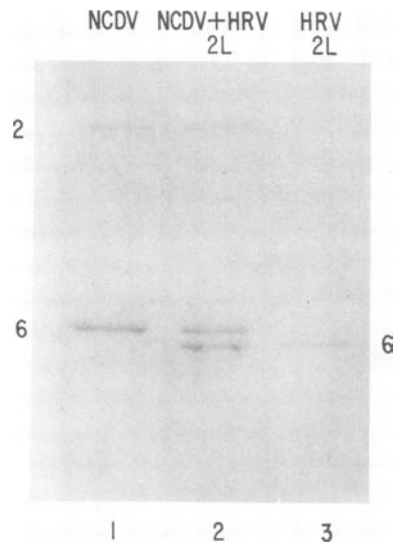

FIG. 3. *Protein electrophoretic patterns of NCDV and human rotavirus (HRV) of type 2l. Track 1, NCDV; track 2, NCDV plus HRV 1601; track 3, HRV 1601. Both rotaviruses were devoid of the outer layer.*

RNA and polypeptide patterns of different rotavirus strains. Figure 5 shows the assigned molecular weights for all RNA segments, isolated structural polypeptides, and polypeptides synthesized in vitro which were observed with the different rotavirus strains examined. These molecular weight estimates, calculated from Fig. 1 through 4 and from other electrophoretic runs (data not shown), are intended only to illustrate the differences among the RNA and polypeptide species of the different rotaviruses.

DISCUSSION

Examination of the polypeptides synthesized by rotaviruses with different genomic compositions revealed substantial heterogenity of the polypeptides of the inner layer. Human rotavirus, SA11, and NCDV cross-react to a high degree in complement fixation (13, 29) and in the enzyme-linked immunosorbent assay (32), but do not cross-react or do so to a very low extent when specific antigens are measured, as in neutralization or in inhibition of hemmaglutination (5, 28, 29).

Since these specific antigens are thought to reside in the outer layer, heterogeneity for the polypeptides comprising the outer layer was expected. Even though the inner layer polypep-

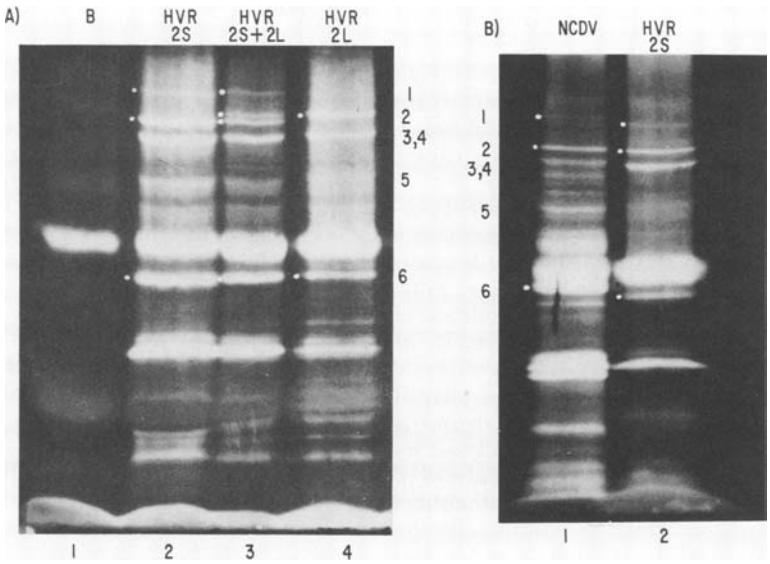

FIG. 4. *Polypeptides synthesized in vitro from denatured RNA isolated from different human rotaviruses. Slab gels were fluorographed after electrophoresis of [^{35}S]methionine-labeled polypeptides synthesized in reticulocyte lysates which were supplemented with the following RNAs. (A) Track 1, None; track 2, HRV (type 2s); track 3, mixture of lysates shown in tracks 2 and 4; track 4, HRV 1601 (type 2l). (B) Track 1, Lysate supplemented with NCDV; track 2, lysate supplemented with HRV 0464 (type 2s).*

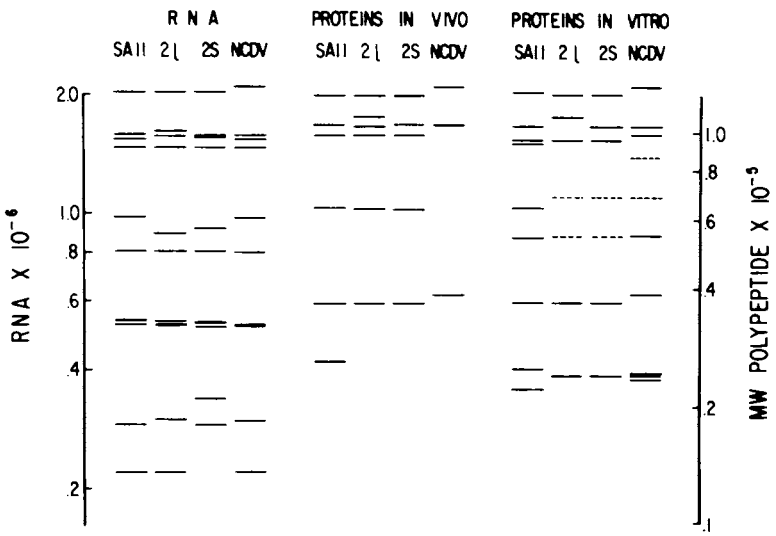

FIG. 5. *Diagramatic presentation of the assigned molecular weight (MW) for each RNA segment, isolated structural polypeptide, and polypeptide synthesized in vitro observed with NCDV, SA11, and human rotaviruses of types 2l and 2s.*

tides are thought to contain the common antigens (31, 32), differences were found for the three inner layer polypeptides which were clearly distinguished in this study; polypeptides 1 and 6 of NCDV are larger than those of either SA11 or human rotavirus, and polypeptide 2 is distinct from the two types of human rotaviruses.

Differences in electrophoretic mobilities of polypeptides, however, do not necessarily reflect differences in immunogenicity or antigenicity, and polypeptides shown to be different in rotaviruses of distinct origin may still represent common antigens. In fact, our attempts to distinguish serologically particles from the different rotaviruses employed in this study when devoid

of their outer layer have been unsuccessful (8a). A similar observation has been reported previously for the three reovirus types. Although these viruses share common antigens which are thought to reside in the core, substantial heterogeneity was observed among the inner capsid polypeptides of the different types (20).

Although the degree of cross-reactivity of proteins is related to the degree of resemblance between their amino acid sequences, it is possible that some changes may have no measurable effect, whereas a change in only one amino acid can have a drastic effect on the antigenicity of the whole protein (6).

According to our results, the heterogeneity of the inner capsid polypeptides of rotaviruses is not reflected in antigenicity when analyzed by the currently available techniques. Analyses of RNA and protein compositions of rotaviruses provide information which is unattainable by standard immunological screening of viruses. The differences described may become useful markers to study the evolution and epidemiology of rotaviruses; for example, the most likely source of a rotavirus might be indicated better by its analysis of RNA composition than by immunological techniques, as shown with the "swine" influenza virus isolated at Fort Dix, N. J., in 1976 (18). Since efficient gene reassortment in mixed infections has been described in reovirus (25) and orbivirus (10), screening of these markers may be valuable in exploring possible gene reassortments of genome segments between different rotaviruses.

The synthesis of rotavirus polypeptides in rabbit reticulocytes is a promising system to correlate genome segments with their products by using individual segments isolated by gel electrophoresis as an exogenous RNA; in fact, the products of 10 of the 11 genome segments of NCDV have been identified (C. Arias, S. López, and R. Espejo, submitted for publication). Immunoprecipitation of the polypeptides synthesized in this system also is a promising technique for the identification of the immunologically related polypeptides present in different rotavirus serotypes or in rotaviruses isolated from different species.

The assignment of molecular weights to RNA segments was performed by using the molecular weights reported by Rodger and Holmes for those SA11 RNA segments migrating close to the RNA segments of reovirus type 3 (Abney strain) (I. H. Holmes, personal communication), which were used as standards. Since the assignment of molecular weights for the reovirus type 3 RNA segments involved several assumptions (2, 26), the estimates presented here are intended to illustrate relative differences and not absolute values. The estimates obtained by this method for NDCV RNA segments are in close agreement with those obtained by Barnett et al., who employed reovirus type 1 (Lang strain) as a standard (1). Our estimates for the molecular weights of the polypeptides of SA11 agree well with those reported by Rodger et al. (22), with the exception that protein 5 gave a consistently larger value under the conditions which we employed.

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ADDENDUM

After this manuscript was submitted for publication, we learned of the following three recent reports on rotavirus polypeptides: M. E. Thouless, J. Gen. Virol. **44**:187-197, 1979; S. Matsuno and A. Mukoyama, J. Gen. Virol. **43**:309-316, 1979; and A. R. Kalica and T. S. Theodore, J. Gen. Virol. **43**: 463-466, 1979. There is a disagreement as to the exact number of rotavirus structural polypeptides, their molecular weights, and their location in the virion; however, these reports do not change the conclusions reached in this paper.

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