Effects of Folic Acid or Zinc Malnutrition on Rotaviral Infection in a Murine Model

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EFFECTS OF FOLIC ACID OR ZINC MALNUTRITION ON
ROTA VIRAL INFECTION IN A MURINE MODEL

by

John Douglas Morrey

A dissertation submitted in partial fulfillment
of the requirements for the degree
of
DOCTOR OF PHILOSOPHY
in
Biology
(Virology)

Approved:

UTAH STATE UNIVERSITY
Logan, Utah
1984
ACKNOWLEDGMENTS

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Credit for my motivation, morale, and monetary support necessary for completion of my schooling and this project goes primarily to Lynne, my wife, and also to my parents, John and Barbara Morrey, who advocate the value of an education. Humble gratitude is also expressed to God, who supplied me with the faculties to pursue this endeavour.

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ABSTRACT

Effects of Folic Acid or Zinc Malnutrition on Rotaviral Infection in a Murine Model

by

John Douglas Morrey, Doctor of Philosophy
Utah State University, 1984

Major Professor: Dr. Gene W. Miller
Dissertation Advisor: Dr. Robert W. Sidwell
Department: Biology (Virology)

The influences of dietary deficiencies of folic acid or zinc on rotaviral disease in infant mice were studied. Preliminary studies indicated that bovine and simian rotaviruses, but not porcine rotavirus, caused diarrhea in infant mice. Bovine and porcine rotaviruses were not, however, sufficiently infectious to replace murine rotavirus in studies utilizing the murine model. It was also determined that murine rotavirus purified by a cesium chloride gradient was highly infectious and useful for subsequent studies on nutritional influences on rotaviral disease. In dietary studies, female Swiss Webster mice were fed diets containing deficient, moderately deficient, or adequate levels of folic acid or zinc. The quantity of specific nutrient added to the two diets were 0, 0.125, and 0.50 ug of folic acid per gram of diet, and 4,
12, and 60 ug of zinc per gram of diet. The infants from these mice were orally inoculated with approximately 100 ID₅₀ of purified murine rotavirus with sterile diluent as a control. Uninfected infants from dams fed the lowest folic acid diet gained weight at a significantly reduced rate compared with the two groups fed the higher dietary folic acid levels. Infants from the same low folic acid group had significantly less folate levels in their livers which indicated that a deficiency was achieved. A moderate enhancement of rotaviral disease was seen in these folic acid-deprived infants as determined by their decreased ability to gain weight, increased incidences of diarrhea, and an increased number which exhibited high rotaviral antigen titers in their intestines. Serum rotaviral antibody titers were below detectable levels in a significant number of animals fed the lowest folic acid diet.

Zinc deficiency in the infant mice was evidenced by significantly reduced thymus weight, inability to gain body weight, lower zinc concentration in whole infant mice, alopecia and presence of skin rashes.

The zinc deficiency produced in this study did not significantly affect weight gained by the infants, presence of diarrhea, titers of virus recovered from the intestine, rotaviral specific antibody in sera, rotaviral specific antibody in milk, ratio of intestine weight to whole mouse weight, and serum sodium levels. Since zinc deficiency did not alter the rotaviral disease, studies with athymic nude mice were done. After per os rotaviral exposure none of the athymic mice, while all of the mice with thymuses, had serum antibodies specific to rotavirus, demonstrating that elicitation of rotaviral specific antibodies was T-cell dependent.
INTRODUCTION

In this study the effects of malnutrition on rotaviral infection were determined, since there is a close association between diarrhea and malnutrition, in which malnourished children have a higher incidence of severe diarrhea resulting in increased mortality (World Health Organization 1978; Wray 1978). Considerable efforts have been made to study rotaviral disease, since the disease can be quite frequent and severe (Riepenoff-Talty et al. 1982). Noble et al. (1983) demonstrated in infant mice that general malnutrition, as established by nutrient dilution, or protein deficiency dramatically enhances the severity of the murine rotaviral disease. In this study was investigated the effects that folic acid or zinc deficient diets, which were given to dams throughout gestation and lactational periods, had on rotaviral disease induced in infants born to these dams.

Folic acid was chosen since it was one of the first limiting nutrients in human diets (Leevy et al. 1965) and deficiencies of this nutrient in mice have been shown to reduce the number of antibodies produced by the lymphocytes in the spleen and to severely impair hemagglutinin antibody in response to red blood cell challenge. In the studies discussed in this dissertation, folic acid deficiency in mice was found to have similar effects on rotavirus-induced disease.

Zinc deficiency was selected to study since this deficiency may have been more prevalent than previously thought (Hambidge et al. 1976)
and the cortex of the thymus from mice that consumed a zinc deficient diet was preferentially atrophied (Leucke et al. 1978; Fraker and Leucke 1977). Since the cortex contained immature thymocytes, lymphopoiesis was dramatically reduced. In consequence of these phenomena, reduced peripheral lymphocyte counts (Fraker and Leucke 1977), antibody levels (Beach et al. 1982), delayed hypersensitivity (Vadas et al. 1975), and T-cell blastogenesis (Zanzonica et al. 1981) have been observed in zinc deficient subjects. Due to these immune dysfunctions, the host immunity is depressed, subsequently rendering the host more susceptible to infection. Zinc deficiency profoundly raised the levels of blood Trypanosoma cruzi in mice (Fraker et al. 1982). Zinc deficiency also had similar effects with Francisella tularensis (Pekarek et al. 1977). It was found from the work of this dissertation, however, that zinc deficiency did not appreciably affect the rotaviral disease nor the immune response to that disease. It was realized, then, that zinc deficiency was not likely one of the specific nutritional factors that caused rotaviral disease to be more severe in the malnourished subject.

The mouse was used as a model for the study of human rotaviral disease, because mice had some obvious advantages for experimental studies when compared with other animals typically used for the study of rotaviral disease, e.g., calf, lamb, and piglet (Woode and Crouch 1978; Little and Shaddock 1982). The use of a mouse model also facilitated the measurement of various parameters which included the weight gain, presence of diarrhea, viral and antibody titers, ratio of large intestine weight to whole mouse weight, zinc and folic acid levels, and serum sodium levels. Serum sodium levels had not previously been used as a parameter to measure the status of the disease in the mouse model.
while some of the other parameters had been used only rarely (Noble et al. 1983, Smee et al. 1982, Schoub and Prozesky 1977, Little and Shaddock 1982). Another advantage of the mouse model utilized in the present study was the availability of athymic nude mice to study the influence that a lack of T-cell function had on rotaviral disease and elicitation of rotaviral-specific antibody. When all of these parameters were utilized, the mouse model became an effective tool for the study of rotaviral disease.

Preliminary work for the nutritional studies was also done, i.e., determination of the infectivity of bovine, simian, and porcine rotaviruses for infant mice, susceptibility of different aged mice to murine rotavirus, and animal end-point titration of cesium chloride-purified murine rotavirus.
Rotaviruses cause gastrointestinal disease in many animal species including man (Petrie et al. 1981; McNulty 1978). In the human host the rotavirus may affect all ages; however, the disease is most severe in children (Flewett et al. 1979; Vollet et al. 1979). The World Health Organization recently determined from numerous published studies that there were 744 to 1000 million episodes of diarrhea in which there were 4.6 million deaths in children under 5 years old in Asia (excluding China), Africa, and Latin America (Snyder anderson 1982). Other investigators have determined that up to 50% of hospitalized diarrhea is associated with rotavirus (World Health Organization 1978; Kapikian et al. 1975), demonstrating that rotaviral-associated disease is more frequent than previously thought. This high incidence correlates with a recent report of Riepenoff-Talty et al. (1982) that 73% of patients under 5 years old with virus-associated enteritis had rotavirus detected in the feces.

The economic losses due to rotavirus in the animal industry have been substantial: 3.2 to 9.7 percent of the losses caused by diarrheal disease agents in calves were due to rotavirus. This amounted to $3.1 to $8.7 million in the USA alone (House 1978).

It is known that there is a close association between diarrhea and malnutrition in which malnourished children have a higher incidence of severe diarrhea resulting in increased mortality (World Health Organization 1978; Wray 1978). Noble et al. (1983) demonstrated in
infant mice that general malnutrition, as established by nutrient
dilution or protein deficiency, enhances the severity of the murine
rotaviral disease. Conceptually, some specific nutrients may be
responsible for the adverse effect of malnutrition on rotaviral
diarrhea since it is known that deficiencies of certain specific
nutrients profoundly affect immunologic response (Gross and
Newberne 1980). It is realized that deficiencies of some specific
nutrients such as zinc and folid acid profoundly affect various
immunological responses (Gross and Newberne 1980).

Folic Acid Deficiency

If one is to understand the reasons for the effects of folic acid
deficiency on disease, the biochemical functions of folic acid must be
realized. Derivations of tetrahydrofolate are carriers for single carbon
units necessary for the synthesis of purines, thymine, glycine, and
choline (Metzler 1977). Since the products of synthesis are collectively
involved in nucleic acid, protein, lipid and hormone chemistry, it is
probable that the reduction of these functions in the event of folic
acid deficiency may adversely affect the ability of the host to ward
off infections.

The study of folic acid deficiency is clinically relevant since
the deficiency is common among the human population (Gross and Newberne
1980). Stores of folic acid are rapidly depleted during periods of
inadequate intake (Gross and Newberne 1980). Zemp et al. (1976) found
the approximate half-life for mouse liver and brain folate stores to be
1.7 and 5.9 weeks, respectively. The incidence of folic acid deficiency
in randomly selected hospitalized patients with hypovitaminosis in an urban US hospital was found to be 45% (Leevy et al. 1965). The deficiency may even be more prevalent in less developed parts of the world as evidenced by a recent study in the Zulu population of South Africa (Coleman et al. 1975). Of those surveyed, 44% of all pregnant women, 32% of nonpregnant women, and 19% of men were folate deficient. Many advocate that the prominent cause of folate acid deficiency is pregnancy because of fetus and placenta utilization (Chanarin et al. 1968; Lowenstein et al. 1966). Since rotaviral disease is primarily a disease of the young (McNulty 1978) and is, therefore, subject to immunological defenses of the mother through her breast milk and placenta, folate acid nutritional status may be of primary concern for the infant's immunological defense against rotaviral infection.

In the event of folate acid deficiency, humoral and cellular immune functions may be altered (Gross and Newberne 1980; Beisel 1982). In folate deficient rats the hemagglutinin antibody response to human erythrocytes (Ludovici and Axelrod 1951) and plaque forming units in the spleen against sheep red blood cells (Kumar and Axelrod 1978) are severely reduced from the normal values. Human studies (Gross et al. 1975) have shown that folate deficient subjects have a 50% and 67% reduction in delayed hypersensitivity and mitogenesis to phytohemagglutinin, respectively, when compared with controls. Patients with folate deficiency had reduced phagocytic and bactericidal activity due to neutrophilic dysfunction (Youinou et al. 1982). Because of these immunological impairments, infant guinea pigs fed folate deficient diet for two weeks had 89% mortality when inoculated with 10^9 Shigella flexneri, while the controls had no deaths (Nelson and Haltalin 1972).
Zinc Deficiency

Zinc deficiency is common in some human populations (Prasad et al. 1963; Hambidge et al. 1976). Even though zinc ions are usually at adequate levels in most foods, adsorption of zinc ions are inhibited through chelation by dietary phytates of high-fiber foods (Prasad et al. 1963; Gross and Newberne 1980). In fact, zinc deficiency is detected frequently in the Middle East, because the diet is high in grains, cereals and unleavened bread. It is interesting to note that the deficiencies are profound enough as to observe common clinical symptoms associated with this deficiency such as growth retardation, hypogonadism, rough skin, and anemia (Prasad et al. 1963). In more recent studies, 10 out of 132 children from middle to upper income households in the USA were found to have low zinc; 8 of the 10 were below 10 percentile of growth. Those individuals did respond to zinc supplementation (Hambidge et al. 1972), illustrating that this specific nutritional deficiency is worth studying.

Dietary zinc deficiency in laboratory animals has a profound effect on the immune function, particularly on the thymus-derived lymphocytes (T-cells) (Gross and Newberne 1980). In zinc deficiency, the most atrophied organ in the mouse is the thymus (Leucke et al. 1978; Leucke and Fraker 1979), where the cortex is preferentially atrophied. Since the cortex contains immature thymocytes, lymphopoiesis is affected and T-cell function is diminished (Fraker et al. 1978). The thymus weight in I/J strain mice fed a zinc-deficient diet for 28 days was 14.4 g compared to 32.3 g (Fraker and Leucke, 1977). As would be predicted, then, antibody formation that requires helper T-cells is depressed in
the event of zinc deficiency (Fraker et al. 1978). Fraker et al. (1978) reported that zinc-deficient mice possessed 18% of the indirect Jerne plaques compared with the normal control mice. When these zinc-deficient mice were fed a zinc-adequate diet for only one week, the indirect Jerne plaque count raised to 68% of the normal values. Due to these immune dysfunctions, the host's immunity to infectious agents was depressed, subsequently rendering the host more susceptible to infection. Zinc deficiency profoundly raised the blood levels of *Trypanosoma cruzi* in mice (Fraker et al. 1982). Zinc deficiency also had similar effects with *Francisella tularensis* (Pekarek et al. 1977).

Zinc is an essential cofactor of more than 20 metalloenzymes which have key physiological functions (Karcioğlu and Sarper 1980). It is probable, then, that at least one of the major mechanisms for the effects of zinc deficiency on the immune response to disease process is the involvement of zinc as a cofactor. The attempt to determine these mechanisms is complex because of the role some of these enzymes play *in vivo*. The function of a number of the enzymes, however, allows for the formulation of some hypotheses. Carboxypeptidase A, a zinc-metalloenzyme, hydrolyzes aromatic or branched aliphatic amino acids from the C-terminus and is involved in protein processing (Karcioğlu and Sarper 1980). Alkaline phosphatases are also zinc metalloenzymes which enzymatically catalyze the hydrolysis of phosphate esters involved in lipid metabolism, and calcium and phosphate utilization (Karcioğlu and Sarper 1980). Zinc is necessary for growth in animals (Church and Pond 1982) and people (Hambidge et al. 1976). This phenomenon may be explained by the findings that a DNA-dependent
RNA polymerase (Scrutton et al. 1971), RNA-dependent DNA polymerase (Auld et al. 1974), and terminal deoxynucleotidyl transferase (Chang and Ballum 1970) are zinc dependent. The in vivo biochemistry of DNA and RNA, then, is dependent on zinc metalloenzymes. Since an immune response involves proteins, lipids and nucleic acids, it is conceivable that the mechanisms of zinc deficiency may be a reduced activity of these zinc metalloenzymes.

De Pasquale-Jardieu and Fraker (1980) demonstrated that zinc deficiency caused an in vivo elevation of corticosteroids, known to lyse T-cells, which may account for another mechanism of how zinc atrophies the thymus and reduces T-cell response. Their studies have provided data to support this hypothesis.

**Characteristics of the Rotaviral Disease**

In human rotaviral diarrhea, the stool is loose and yellow-green without the presence of blood (Shepherd et al. 1975). Leukocytes are not present in the feces as they are in some other diarrheal diseases. This suggests that there is not a cellular inflammatory response in rotaviral diarrhea (Ryder et al. 1976). Dehydration, electrolyte imbalance, raised temperature, abdominal cramps, and emesis also were often observed (Ryder et al. 1976; Middleton 1978). Symptoms usually last 5-8 days but occasionally up to 28 days (Flewett et al. 1975; Middleton 1978). Most of these signs are also observed in mice (Cheever 1956; Kraft 1958) which suggests that the mouse may provide a good model for the study of human rotaviral disease.
Characteristic histopathology has long been observed in mice (Adams and Kraft 1967) and more recently in man (Dolin 1979). Viral replication seems to be confined mainly to the epithelial cells (enterocytes) at the tips of the villi (Adams and Kraft 1967). Within these cells the virus is present in the dilated cisternae in vesicles of the endoplasmic reticulum. Vacuolization, evidence of cellular degeneration, is observed with a light microscope (Adams and Kraft 1967). As the infection proceeds, the epithelial cells at the tips of the villi are sluffed off and are subsequently replaced by more immature cells from the crypts of the villi that lack certain enzymes (Middleton 1978; Moon 1978; Mebus et al. 1971b). Unlike the cells normally at the tips of the villi, these immature cells do not have the ability to adsorb molecules from the lumen; as a result there is a net extracellular fluid-to-lumen flux of sodium ions, resulting in an electrolyte imbalance of not only sodium, but also potassium, chloride and carbonate ions. Experimental studies suggest that it is this disordered sodium transport system that causes the diarrhea. In serious cases, death can result from rotaviral disease and is likely due to electrolyte imbalance and dehydration leading to cardiac arrest (Middleton 1978; Moon 1978). For these reasons, this study has measured parameters that reflect electrolyte imbalance and dehydration to monitor the severity of the disease. Other investigators have not used electrolyte imbalance i.e., Na⁺ level, as an indication of the status of rotaviral disease using infant mice as a model. The most commonly used parameters have been rotavirus titer in intestine (Noble 1983), serum rotaviral antibody titer (Little and Shaddock 1982), presence of diarrhea (Little and Shaddock 1982), and histopathology (Little and
Shaddock 1982). Weight loss, due to dehydration, has been observed in diseased subjects (Middleton 1978) but has not been used to monitor the disease in mice except in one other study (Noble et al. 1983). Distension of the intestine from excess fluids in the lumen is also commonly observed in diarrheal diseases (Middleton 1978); this parameter was quantitatively measured in this study by ratio of large intestine to whole mouse weight as suggested by Dr. R. Orcutt (Personal communication, National Cancer Institute /Fort Detrick, Frederick, MD 21701).

**Characteristics of Rotavirus**

A distinction of the Reoviridae family, which includes rotavirus, is that the nucleic acid is segmented double-stranded RNA of which the rotaviruses possess 11 segments (Barnett et al. 1978). Considerable effort has been made to determine which specific segments code for the various structural and non-structural proteins of the virus (Estes et al. 1982; Kalica et al. 1981). These data have application in identifying different strains of the rotaviruses and also in the development of vaccines (Estes et al. 1982).

Morphologically the virus exists in various forms which occur during different stages of morphogenesis. It has been suggested (Petrie et al. 1981) that the virus enters by endocytosis and becomes sequestered in a lysosome. Uncoating then occurs in the lysosome producing a 50 nm subviral particle. Upon accumulation of viral proteins and RNA, a cytoplasmic viroplasm inclusion is formed. After assembly the virus leaves the viroplasm by budding through the endoplasmic
reticulum, thereby becoming enveloped. This envelope is later lost to produce a particle about 75 mm in diameter.

Viruses isolated in a cesium chloride density gradient are seen in two layers. The viruses in the less dense layer, designated as L-particles, are infectious, or at least potentially infectious, and possess two outer capsid layers (Clark et al. 1980). The infectivity of these L-particles can be enhanced by treatment with trypsin (Clark et al. 1979). Viruses of the denser layer, designated as D-particles, possess one capsid layer and are not infectious (Clark et al. 1980).

One of the difficulties of rotavirus research has been the inability to culture all rotaviruses in cell lines. Some progress was made when Mebus et al. (1971a) propagated bovine rotavirus in cell culture. Porcine and simian rotaviruses have also been propagated in cell culture (Theil et al. 1977; 1978). Recently, Sato et al. (1981) reported the cultivation of human rotaviruses in MA-104 cells. The murine rotavirus, however, has not been propagated in cell cultures and only with limited success in organ culture (Rubenstein et al. 1971).

Immunological Processes in the Intestine

Since rotavirus infects the intestinal mucosa, and since the effects of specific nutrients on the rotavirus infection were analyzed in this study, the immunological processes occurring in the gut will be discussed. Development of immunity to a specific antigen is initiated when the antigen is exposed to gut-associated tissue, particularly the Peyer's patches. Lymphocytes (T- or B-cells) are
sensitized to form lymphoblasts. These sensitized blast cells, then, migrate through the regional lymph nodes, to the thoracic duct, and into the peripheral blood. While circulating through the blood system they preferentially locate to either the mammary glands or the intestinal lamina propria. Upon transformation of the blast cell into an antibody-producing cell, specific antibodies are secreted into the milk and intestinal lumen, thereby providing protection to the adult animal and its infant (Ogra and Dayton 1979; Welliver and Ogra 1978; Doe 1979).

Sensitization to the specific antigen is best achieved by inoculation of the antigen at the site of infection (Welliver and Ogra 1978; Ogra and Dayton 1979), e.g., the intestine in the case of rotavirus. This concept can best be illustrated by the historical event of the development of the polio vaccine. It was observed that parenteral inoculation of susceptible individuals with inactivated polio virus did not prevent subsequent infections even though the serum had specific antibody. The only effective way to induce protection to this intestinal virus was to inoculate the subject in the intestine through oral administration (Ogra and Karzon 1969). These observations led to the concept that antibody formation in the gut is an independent process from the antibody formation in other parts of the body (Ogra and Dayton 1979).

In selecting assays as tools to study the immune response to a gastrointestinal infectious agent such as rotavirus, it would be advantageous to measure the specific immune response in the gut. Lymphocytes producing rotaviral specific antibodies have been detected (Immunofluorescent anti-rotaviral antibody producing cell test, APC Test)
in lymph nodes of rabbits (Vonderfecht and Osburn 1982a) and in the intestinal lamina propria of calves infected with rotavirus (Vonderfecht and Osburn 1982b). This assay has been used to study the local and systemic humoral immune response to rotavirus in neonatal calves (Vonderfecht and Osburn 1982b) and could conceivably be used in nutritional studies of the nature presented in this dissertation.

Even though the formation of the antibodies in the gut may be independent, leakage of intestinal antibodies into sera or from sera into the intestinal lumen can occur to a limited degree (Snodgrass and Wells 1976). The suggestion that vaccine inoculation through the intestine is the only way to offer effective protection to the infant has been challenged. An alternate method was illustrated when ewes were stimulated to have high antibody titers in the colostrum by inoculating the ewes with Freund's incomplete adjuvant and rotavirus (Snodgrass and Wells 1978). It is emphasized that serum antibodies alone will not offer effective protection (Kapikian et al. 1975). It is known that 80% of children possess rotaviral antibodies by the time they are three years old (Kapikian et al. 1975), but the presence of this pre-existent serum antibody does not correlate with resistance to infection in children or adults (Snodgrass and Wells 1978). These concepts could also apply to rotaviral infections in the intestine (Snodgrass and Wells 1978; Acres and Babiuk 1978). Adult animals are immunologically sensitized through the gut to rotavirus and consequently pass antibodies through the colostrum and milk to the infants. The infants then possess passive intestinal antibodies to protect against rotaviral infections (Snodgrass and Wells 1978). Evidence (Snodgrass and Wells 1978; Snodgrass and Wells 1976) has accumulated to support the idea that infants receiving
Antibody-containing colostrum are protected from rotaviral infections more than infants receiving no antibody-containing colostrum.

The classes of antibodies present in the intestine are different from those found in the sera. The ratio of IgA:IgG:IgM producing cells in the gut of man was reported to be 20:3:1 (Doe 1972). The predominant immunoglobulin in the milk of humans and other animals is also IgA. The question of how secretory IgA (slgA) protects the host against invading organisms is an intriguing one. Complement components probably do not play the major role they render in the serum immunity, because they are in very low concentrations in intestinal secretions (Watson and McMurray 1979). Some investigators suggest that slgA binds to the virus prohibiting the invader from attaching to the mucosal surface. The antigen-antibody complexes are consequently washed away (McMurray et al. 1977; Tomasi 1972). Ogra and Dayton (1979) advocate that slgA selectively binds to the intestinal epithelial cells, thus acting as an immunologic barrier to the virus.

Colostrum or milk might also provide many other antimicrobial factors other than antibodies (Otnaess and Orstavik 1980). Certain populations of maternal T-cells, including helper and suppressor T-cells, B-cells and macrophages programmed to mediate mucosal immunity are present in the milk, and some are able to enter the infant's circulation (Ogra and Dayton 1979). Non-specific viral inhibitors may be present in the milk, e.g. low pH, bile acids, and glycocalyx. Glycoprotein fraction of bovine milk has been shown specifically to have antirotaviral effects (Snodgrass and Wells 1978).
Detection of Rotavirus and Rotavirus-specific Antibodies

Because of the difficulty of culturing rotaviruses, early investigators used methods for detection of the virus not requiring in vitro cultivation, such as electron microscopy (Adams and Kraft 1967). Techniques utilizing immunological identification include immune electron microscopy (McNulty 1978), complement fixation (Kapikian et al. 1975), enzyme-linked immunosorbent assay (ELISA) (Yolken et al. 1977), radioimmunoassay (McNulty 1978), hemagglutination (Bishai et al. 1978), and immunofluorescent antibody techniques (Wilsnack et al. 1969). Methods for measuring the infective virus are widely utilized but are restricted to those rotaviruses capable of being cultured in cells. These methods include fluorescent antibody technique (Barnett et al. 1975), plaque assay (Matsuno et al. 1977), immune adherence hemagglutination test (Gary et al. 1982), and an enzyme labelling test (McNulty et al. 1979) in which infected cells are labelled with peroxidase. Since murine rotavirus has not yet been cultivated in cell lines, methods not requiring in vitro cultivation can only be utilized.

The detection of the rotaviral antigen can be reduced or blocked upon addition of rotavirus-specific antibody. It is this concept that is widely used for the detection of rotavirus-specific antibodies. Some of the tests that utilize this concept are blocking ELISA (Yolken et al. 1978), plaque reduction assay (Matsuno et al. 1977), neutralization of immunofluorescence (Thouless et al. 1977), reverse passive hemagglutination inhibition (Sanekata et al. 1982), and hemagglutination inhibition (Spence et al. 1976). RIA and ELISA antibody tests have also
been used to measure the class of antibody (Yolken et al. 1978, McLean and Holmes 1980). Rotaviral specific antibody-producing lymphocytes in the gut can also be measured (Vonderfecht and Osburn 1982a).

The detection of coproantibodies has been difficult because of the interfering nature of the samples. Successful methods have been described (Watanabe and Holmes 1977; Watanabe et al. 1978; Riepenhoff-Talty et al. 1981; Corthier and Vannier 1983) in which the basic concept is to concentrate the specific antibody, often by centrifugation, and then detect the antibody serologically with antibodies labelled with radioisotopes or fluorescent molecules.
MATERIALS AND METHODS

**Mice**

Specific pathogen-free Swiss Webster mice used in all of the experiments, except when otherwise specified, were designated Crl: CFW (SW) BR by the supplier, Charles River Laboratories, (Wilmington MA). These mice were previously found to be susceptible to murine rotavirus infection (Noble et al. 1983). Immediately upon arrival of the adult mice, precautions were taken to not inadvertently expose the mice to rotavirus by placing them in a limited-access disinfected room. All materials used in the room were sterile. The ventilation of the room was independent from the ventilation of the rest of the building, thereby reducing the possibility of extraneous disease agents from entering the room. The mice were housed in disposable cages having filter tops (Lab Products, Inc., Federalsburg, MD), with bedding changed frequently to minimize coprophagy. The animal care personnel wore disinfected clothing when caring for the animals. Mice infected with rotavirus were kept in a building separate from the housing of the uninfected mice, and personnel working with these infected animals were denied access to rotavirus-free areas. The lighting was automatically maintained at a 12 hour cycle. The temperature was maintained at 72°F to 75°F.

Athymic nude male mice and heterozygous female mice, with a CD-1 background, were obtained from Charles River (Wilmington, MA). The same
precautions were taken as described above to reduce extraneous infections. The temperature was maintained at 74°F to 78°F.

**Diets**

The amounts of folic acid in three different diets were determined using data of the National Academy of Sciences (National Academy of Sciences 1972). Folate in concentrations of 0, 0.125 and 0.50 ug per gram of basal diet was added to make respectively, a deficient, marginal, and normal folic acid diet as recommended for normal growth. The basal diet contained (g/kg diet): casein 150, corn oil 60, mineral mix (Appendix) 11.6, vitamin mix (Appendix) prepared without folate 20, CaCO₃ 16.75, NaHPO₄·H₂O 29.8, cellulose 50, corn syrup 50 and dextrose to make 1 kg. Diets were pressed into pellets approximately 3/4 inch diameter.

The amount of zinc, in the form of zinc sulfate, for three different diets was determined by personal communication with Dr. P. J. Fraker (Department of Nutrition, Michigan State University, East Lansing, MI). Elemental zinc, 4 ug, 12 ug, and 60 ug per gram of basal diet, was added to make respectively, a deficient, marginal and normal amount of zinc diet as recommended for normal physiological function. Zinc-free diet was purchased from United States Biochemical Corp. (Diet # 23566, Cleveland, OH). The protein source was egg albumin since it contained a minimal amount of zinc. Composition of the basal diet by percentage was celufil 3.0, corn oil 10.0, dextrose 68.3, egg albumin (spray dried) 15.0, and zinc free salt mix 3.7. Biotin was added at 2 mg/kg of diet. The zinc-basal diet and administration of
the diets to the animals were the same as used in folic acid diet. The mice were given pelleted diets and distilled water ad libitum.

**Rotavirus**

Murine rotavirus (MRV) was obtained from Dr. M. Collins of Microbiological Associates, Inc. (Bethesda, MD). RNA band patterns of the virus were similar to patterns of bovine and human rotaviruses (Smee et al. 1982), demonstrating that it was a rotavirus. Stock virus was prepared by inoculating mice per os (p.o.) with MRV, then harvesting the intestines 4 days later. These were homogenized to a 10% w/v suspension of 0.01 M phosphate buffered saline (PBS), pH 7.2, which was then freon-extracted and purified further by centrifugation in a CsCl-density gradient. The virus was collected at a density of 1.3 to 1.4 g/ml and dialyzed against 0.01 M PBS, pH 7.2. The virus stock was titered in infant mice to determine the 50% infectious dose (ID\textsubscript{50}), and the inoculum was prepared by diluting the stock virus in PBS to achieve an approximate 100 ID\textsubscript{50} dose.

The cells used to propagate Lincoln strain bovine rotavirus (BRV), SA-11 strain simian rotavirus (SRV), and porcine rotavirus (PRV) were Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection, Rockville, MD) and embryonic rhesus monkey kidney (MA-104) cells (M. Estes, Baylor College of Medicine, Houston, TX, originally obtained from Microbiological Associates, Bethesda, MD). Cells were grown in Eagles Minimum Essential Medium (MEM) (Gibco, MD) supplemented with 0.19% NaHCO\textsubscript{3}, 50 units/ml penicillin-G (Sigma), 100 ug/ml streptomycin sulfate (Sigma) and 10% heat-inactivated fetal bovine
serum. Bovine rotavirus, PRV, and SRV were obtained from Norden Laboratories (Lincoln, NB), from a porcine isolate from Dr. Edward Bohl (Ohio Agricultural Research and Development Center, Wooster, OH) and from Dr. Mary Estes (affiliation above), respectively. These rotaviruses were propagated by allowing virus to adsorb for a period of one hour, then washing the cells twice with MEM without serum. Extensive cytopathic effects were observed at least by 24 hours, then the cells were frozen and thawed 3 times, removed from the petri plates, and freon extracted. The viruses were then stored at -80°C. These stocks of bovine, porcine, and simian rotaviruses were used to study the comparative infectivity in infant mice. Bovine rotavirus was also used as a standard for ELISA rotaviral antigen test. The viral antigen used in the immunofluorescent anti-rotaviral antibody producing cell (APC) test (Vonderfecht and Osburn 1982a) was the BRV stock, concentrated by reverse dialysis using polyethylene glycol 8000 (Sigma). The stock was assayed using the immunofluorescent cell count test (Barnett et al. 1975) and was determined to be 3.1x10^8 fluorescing units/ml.

Animal Titration of Murine Rotavirus

The main purpose of titration of MRV was to determine if the newly prepared MRV stock (see procedure above) produced typical clinical signs of rotaviral infection in mice, and to also obtain an ID_{50}. The virus stock was diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in PBS. Uninoculated controls, sham-inoculated with PBS, were present in each litter to determine that the litter was not inadvertently exposed to extraneous virus present in the room. Swiss Webster mice were used at 2
days old and orally inoculated with a virus suspension. So that the susceptibility of the infant mice to the various dilutions of the virus would not be biased as to which litter was used, ten litters were randomly assigned to 3 treatment blocks. These treatment blocks were litters infected with $10^{-1}$ and $10^{-2}$ of the virus and PBS control, $10^{-3}$ and $10^{-4}$ virus dilution and PBS control, and $10^{-5}$ and $10^{-6}$ virus dilution and PBS control. The respective virus dilution were marked on each infant mouse with a permanent ink marker. The presence of diarrhea was noted at days 1, 2, 3, and 4 after virus infection. The results were then tabulated and the Reed-Muench Method (Reed and Muench 1938) was used to calculate the ID$_{50}$ on day 3 post inoculation (p.i.).

**Susceptibility of Infant Mice to Bovine, Porcine, and Simian Rotaviruses**

Infant mice were orally infected with cell culture-propagated (described above) BRV, PRV, and SRV. Two litters were infected with each virus. Pooled intestinal homogenates from each virus group were then used to orally infect a second group of mice. This procedure was repeated again to obtain 3 passages of each virus. The presence of diarrhea on day 2 post inoculation (p.i.), and the intestinal rotaviral titer on day 3 p.i. were determined for the mice in each viral passage. Mice were housed in filter top cages as previously described. Care was taken to not cross-infect one viral group to another viral group by changing gloves between the handling of groups and separating each group to different parts of the room.
Numerous attempts were made to adapt the APC test to detect rotaviral specific antibody producing cells in the intestine of the mouse. The basic principle of the test (Vonderfecht and Osburn 1982a; Pierce and Gowan 1975) is that anti-rotaviral antibody producing cells in the intestine, or any other tissue containing immunological cells, are detected by making a section of the tissue, adding rotaviral antigen to the histological preparation, washing the slide, and then adding a fluorescein-conjugated rotaviral-specific antibody on the sections. The antibody producing cells are then detected with an epiluminescent fluorescent microscope by observing fluorescing cells.

The following controls and variations of the test were done:
-- PBS was added, instead of rotaviral antigen, as a negative control.
-- Fluorescein-conjugated reoviral specific antibody, instead of the antirotavirus-conjugate, was added as a negative control.
-- Intestines from mice not previously exposed to rotavirus or possessing any specific antibody were used as a negative control.
-- Unconjugated antimouse immunoglobulin was added previous to the addition of the antirotavirus-conjugate in an attempt to block the fluorescence.
-- Unconjugated rabbit antirotavirus antibody was added previous to the addition of the antirotavirus-conjugate in an attempt to block the fluorescence.
-- 20% fetal bovine sera, 2% mouse sera in PBS was used as a diluent for the antirotavirus-conjugate in an attempt to block the non-specific fluorescence.

-- Paraffin-embedded tissue (Vonderfecht and Osburn 1982a) at a thickness of 4 um, as well as cryostat-prepared tissue (Pierce and Gowan 1975) at a thickness of 7 um was used.

-- BRV was added to the antirotavirus-conjugate in an attempt to block non-specific fluorescence.

**Enzyme Linked Immunosorbent Assay**

Viral antigen in homogenized intestine or cell culture fluid was assayed by ELISA. The test was performed by first coating 96-well microtiter plates (Polyvinyl V-well, Dynatech, Alexandria, VA) with guinea pig anti-SRV antibody, previously purified by ammonium sulfate precipitation (Herbert and Pittman 1965), and then incubating for 18-24 h at 4°C. The wells were then washed 9 times for 30 seconds each with distilled water. All subsequent washings were the same. Serial dilutions of the samples in brain heart infusion broth (BHI, BBL Microbiology Systems, Cockeysville, MD) were added to the wells and incubated 18-24 h at 4°C. After washing the wells again, rabbit anti-human rotavirus antibody conjugated to peroxidase (DAKO, Accurate Chemicals, Westbury, NY) diluted 1/2000 in PBS containing 20% fetal bovine serum and 2% normal guinea pig serum was added to the wells and incubated 3 hours at 37°C. After washing, substrate (o-phenylenediamine * 2 HCl, Rotazyme, Abbott Laboratories, North Chicago, IL) was added and incubated 30 minutes at room temperature. After stopping the
reaction with 1 N HCl, the absorbance at 490 nm was read on a Microelisa Reader MR590 (Dynatech Laboraties, Inc., Alexandria, VA). Titers were expressed as the highest dilution giving a P/N > 2.1, where P is the absorbance of test sample and N is the average of 6 wells containing rotavirus negative intestinal homogenate.

**Rotaviral Antibody Blocking of Enzyme Linked Immunosorbent Assay**

Anti-rotaviral antibodies in serum and milk were assayed using blocking of bovine rotavirus detection by ELISA (Yolken et al. 1978). The virus used in the test was extracted from the aqueous phase of an homogenized solution of 1 part of Freon 113 to 4 parts of cell culture-propagated bovine rotavirus. Equal volumes of freon-extracted cell culture-propagated BRV and varying 2-fold dilutions of the mouse serum or the aqueous fraction of milk to be tested for anti-rotavirus antibody were incubated together at 37°C. The stomach containing milk was prepared by homogenizing the stomach in a 1/10 dilution of PBS and centrifuging the solution to recover the aqueous phase. After 1 hour incubation, titration of bovine rotavirus in these mixtures was carried out as described above for titration of viral antigen. A 50% reduction in absorbance at 490 nm of the mean of 3 positive controls containing no sera compared to the mean of 3 negative controls (BHI) was considered to be positive for rotaviral-specific antibody. The end point of the sample being tested was the highest titration that was positive for antibody.
Folic Acid Assay

The liver folic acid levels were assayed (Bennett et al. 1964) by placing a portion of homogenized liver in media without folic acid. Commercially prepared Folic Acid Casei Medium was used (Difco Laboratories, Detroit, MI). *Lactobacillus casei* ATCC 7469, which depends on extraneous folic acid for growth, was seeded to the media. The degree of growth, as measured by absorbance at 670 nm, was proportionate to the amount of folic acid in the liver. A standard curve was calculated to quantitate the folic acid in the liver.

Electron Microscopy

Cell culture-propagated BRV was allowed to adsorb for 15 minutes onto formvar-coated copper grids. After removal of the excess liquid and drying of the grids, the samples were stained with 2% phosphotungstic acid and viewed with a Zeiss EM-9A electron microscope.

Zinc Assay

Zinc content of whole infant mice was assayed by atomic absorption spectrophotometry using an Atomic Absorption/Atomic Emission Spectrophotometer 457, (Instrument Laboratories, Andover, MA). The assay, set at a wave length of 213.9 nm, used an oxidizing, lean flame and air-acetylene gas mixture. The values of the samples were calculated from a set of zinc standards. Whole infant mice were prepared by weighing each individual mouse, placing it in a crucible, and burning
the specimen on a hot plate until no more smoke was evolving from the sample; the samples were then placed in a furnace overnight at 450°F. After cooling, 6N HCl was added to each crucible and heated slightly to dissolve the contents. Contents were filtered in volumetric flasks, and the flask was filled with deionized water to contain 200 ml. The solution was then assayed for zinc and the values calculated from the standard curve.

**Serum Sodium Assay**

The same instrument described above for the zinc assay was used for the sodium assay; flame emission was used instead of atomic absorption. The same fuel mixture and lean flame were used as above and the wavelength setting was 589 nm. The serum samples of the infant mice were diluted into deionized water. The solution could then be assayed for sodium content and the absolute concentration of serum sodium determined.

**Influence of Age on Susceptibility of Mice to Murine Rotavirus**

A study was done to determine the susceptibility of different aged mice to rotaviral infection by determining the incidence of diarrhea. Infant mice at the age of <1 day, 2, 5, 8 and 11 days were orally inoculated with the 100 ID$_{50}$ of MRV. There were 5 litters per age group in the study. The proportion of mice with diarrhea was recorded 1, 2, 3, 4, 5, and 10 days post inoculation (p.i.) for the
different aged mice.

Experimental Design for Folic Acid and Zinc Dietary Studies

Soon after arrival of the mice, one or two sexually mature females were bred with one male. The mice were randomized to the diets. At least 25 female mice were fed each diet group. Fifty percent of litters were infected p.o. with $100 \text{ID}_{50}$ of MRV stock at 2 days of age for mice from the folic acid dietary experiment, and 5 days of age for mice from the zinc dietary experiment. Controls were sham-infected with PBS pH 7.2, the buffer for the stock virus. The sham-infected control mice were kept separate from the infected mice to maintain an uninfected status in the control animals. The schedule for parameters obtained for the folic acid and zinc dietary studies, and the approximate number of samples in each dietary group are summarized in Table 1. The presence of diarrhea in the infant mice was measured on specified days (Table 1) p.i. by slightly pressing the abdomen of the infant 3 times and observing for the presence or absence of yellow diarrhea. Each litter was also weighed on specified days p.i. (Table 1). Mice were sacrificed equally from each litter at designated times to obtain specimens throughout the course of the experiments. The number of mice, then, in each litter at any time during the experiment was approximately equal. Intestines from infants were excised from the pyloric valve to the anus, diluted 1/10 in PBS pH 7.2, homogenized, and assayed for rotavirus by ELISA on the days listed in Table 1. Portions of the duodenum, ileum, and jejunum from three animals in each dietary group at 20 days p.i.
Table 1. "Schedule for Obtained Parameters" for Zinc and Folic Acid Dietary Studies

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Zinc dietary study</th>
<th>Folic acid dietary study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1-5, 6, 10, 20</td>
<td>1-5, 10, 20</td>
</tr>
<tr>
<td>(12 litters)</td>
<td>(10 litters)</td>
<td></td>
</tr>
<tr>
<td>Histological sections of intestines</td>
<td>20 (3 mice)</td>
<td>20 (3 mice)</td>
</tr>
<tr>
<td>Incidence of diarrhea</td>
<td>2-5, 7, 10</td>
<td>2-5, 10</td>
</tr>
<tr>
<td>(12 litters)</td>
<td>(10 litters)</td>
<td></td>
</tr>
<tr>
<td>Intestinal rotavirus titer</td>
<td>1, 6 (30 mice)</td>
<td>4 (15 mice)</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer in infants</td>
<td>6, 20 (25 mice)</td>
<td>20 (16 mice)</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer in dams</td>
<td>30 (12 mice)</td>
<td>20 (7 mice)</td>
</tr>
<tr>
<td>Rotaviral antibody titer in infants' stomachs</td>
<td>6 (20 mice)</td>
<td>4 (15 mice)</td>
</tr>
<tr>
<td>Intestine weight/whole mouse weight</td>
<td>6 (30 mice)</td>
<td>--</td>
</tr>
<tr>
<td>Zinc level in whole infant mice</td>
<td>6 (20 mice)</td>
<td>--</td>
</tr>
<tr>
<td>Thymus weight in infant mice</td>
<td>20 (25 mice)</td>
<td>--</td>
</tr>
<tr>
<td>Serum sodium level in infant mice</td>
<td>6 (30 mice)</td>
<td>--</td>
</tr>
<tr>
<td>Folic acid levels in infant mice</td>
<td>--</td>
<td>1, 4 (5 mice)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average number of measurements per dietary group. Numbers between groups are uneven and may vary slightly.

<sup>b</sup>Percentage of weight gained from day of infection.
were also collected, placed in formalin, imbedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological examination.

In the zinc dietary study the whole infant mouse at 6 days p.i. was weighed, and the large intestine was excised from the anus to the cecum. The ratio of weight of large intestine to weight of whole mouse was then calculated and used as a quantitative evaluation of the amount of diarrheal fluids in the intestines.

Sera from infants were collected 20 days p.i. from the folic acid and zinc dietary experiments for the assay of rotaviral-specific antibody. Stomachs from infants were collected 4 days and 6 days p.i. for the folic acid and zinc dietary studies, respectively. At least 20 stomachs from each diet group were processed for antibody assays by homogenizing the curdled milk contents in a 1/5 dilution of PBS. To determine that antibodies can be detected if contents were prepared in this way, a female mouse was inoculated p.o. with MRV stock about 5 days after gestation and rotaviral specific antibodies were detected in the milk from the stomachs of her offspring at endpoint titers ranging from 1/16 to 1/64.

In the folic acid dietary experiment, livers were excised from uninfected infant mice at 1 and 4 days p.i., homogenized in a tissue grinder, and assayed for folic acid.

In the zinc dietary study, thymus weight, whole body zinc level (carcass of the mouse without intestines and stomachs), and serum sodium level in 25, 12, and 12 day-old mice, respectively, were assayed. Also in the zinc dietary study each individual mouse, intestine and serum was
numbered so that a correlation of zinc content of the animal could be made to the ratio of large intestine to mouse weight, rotavirus titer in intestine, and serum sodium levels. Upon collection of the tissues, all specimens were stored at -20°C. The assays were performed soon after the collection of the sample.

**Experimental Design of**

**Athymic Nude Mouse Study**

Two experiments were done with athymic nude mice. In experiment #1, homozygous (nu/nu) nude mice were crossed with heterozygous (nu+/+) female mice to obtain both homozygous and heterozygous offspring in the same litter. Since the control heterozygous mice were in the same litter as the test animals, the results were not biased as to which litters were studied. At 5 days after gestation the female mice were challenged p.o. with 100 ID$_{50}$ of purified murine rotavirus so that at time of birth the infants would be passively exposed to the virus, and the dams would have elicited an antibody response to the virus by the time the infants were born. When the infant mice were 3, 5, and 7 days-old, they were examined for the presence of diarrhea. At eight days of age, 1/2 of the homozygous and 1/2 of the heterozygous mice were sacrificed to determine the ratio of the large intestine weight to whole mouse weight and to measure rotavirus in the intestine. The serum from each infant was also obtained to measure rotaviral specific antibodies. The remainder of the infant mice was sacrificed to obtain sera for measuring rotavirus-specific antibodies.

In experiment #2, 4 adult (approximately 25 g) athymic nude mice...
and 4 adult thymic mice were challenged 2 times, 17 days apart, p.o. with the MRV stock. Twelve days after the challenge, the sera from these mice were assayed for rotavirus-specific antibody using the blocking assay previously described.

**Statistical Analysis**

The percent of the weight gained from day of virus challenge was analyzed by expressing the experimental design in the form of a model, \( y = I_i + D_j + C(ij)k + T_l + ID_{ij} + IT_{il} + DT_{lj} + IDT_{ijl} + E \), where \( I \) was infected or sham-infected mice designated as 1 or 2, \( D \) was diet designated as 1, 2, or 3, \( C \) was the cage number and \( T \) was days after inoculation. The analysis of variance was computed by rummage program (Bryce, G.R. Data analysis in rummage--A User's Guide. Brigham Young University, Provo, Utah).

Means for antigen and antibody titers were calculated by obtaining the \( \log_{10} \) of the inverse of each titer, averaging those values, obtaining the antilog of that mean, and obtaining the inverse to arrive at the mean for the antigen or antibody titers. The data for serum antibodies, intestinal antigen, and presence of diarrhea were analyzed by chi square analysis (Christensen 1977) with Yates' correction (Croxton 1953). Chi square analysis for antibody data was performed by obtaining the mean of the antibody end point titers from mice in all three dietary groups and using the mean value as a division line; those values above the line were connoted (+) and those below were
connoted (-). The numbers of end point titers that were (+) and (-) were then used to calculate chi square analysis.

The Student's t-Test (Christensen 1977) was also used to analyze the ratio of large intestine weight to mouse weight in the athymic nude mouse experiment and to analyze the data for thymus weight of uninfected animals.
RESULTS

Animal Titration of Murine Rotavirus

Infant mice were infected per os (p.o.) with varying dilutions of stock MRV. The cesium chloride-purified MRV produced the signs of a typical rotaviral infection, i.e., presence of yellow diarrhea in 2 days and a reduced ability to gain weight. Table 2 lists the proportion of mice with diarrhea that were infected with serial dilutions of stock MRV on days 2, 3, and 4. Sham-infected controls (PBS-inoculated), present in each litter, showed no presence of diarrhea in 2 days while the litter mates infected with the virus had diarrhea. Utilizing the Reed and Muench Method (Reed and Muench 1938) and the percentage of mice with diarrhea 3 days, a 50% infectious dose ($10^{5.3}$) was calculated to be $10^{-5.3}$.

Susceptibility of Infant Mice to Bovine, Porcine, and Simian Rotaviruses

Infant mice were inoculated with cell culture-propagated BRV, SRV, or PRV to observe the ability of the viruses to produce clinical signs in infant mice. The cell culture-propagated BRV can be viewed from the electron micrograph in Figure 1. Upon careful inspection, one will notice the spoke-like appearance of the virus, confirming that the virus used in the ELISA blocking antibody test is indeed a rotavirus.
Table 2. Titration of Cesium Chloride-purified Murine Rotavirus Stock Using Infant Mouse Susceptibility\textsuperscript{a}

<table>
<thead>
<tr>
<th>Days post-virus infection</th>
<th>% Diarrheic Mice</th>
<th>PBS (uninfected control)</th>
<th>MRV Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10\textsuperscript{-1}</td>
<td>10\textsuperscript{-2}</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>3\textsuperscript{c}</td>
<td></td>
<td>18</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>18</td>
<td>50</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Assayed by percent of diarrheic positive mice upon oral inoculation with CsCl-purified MRV stock at 2 days old.

\textsuperscript{b} Number of mice.

\textsuperscript{c} \textit{ID}_{50} was calculated to be 10\textsuperscript{-5.3} using the data from day 3 after infection.
Figure 1. Negative stained phosphotungstic acid electron micrograph of cell culture-propagated bovine rotavirus.
Homogenized intestines from those mice were used to inoculate a second group of mice. The process was repeated for a third passage. Note in Table 3 that for the first passage of BRV and SRV, the viruses caused diarrhea in about 30% of the mice while those mice infected with PRV had no signs of infection. There were no further signs of the disease in subsequent passages with the cell culture-propagated viruses tested. Homogenized intestines from mice infected with either BRV, PRV, or SRV for the first, second, and third passages of the virus were assayed for rotavirus by ELISA. No viral antigens were detected for any of the samples, not even from mice that showed clinical signs.

Influence of Age on Susceptibility of Mice to Murine Rotavirus

The susceptibility of different aged mice to MRV (Figure 2) was measured by presence of diarrhea. All ages of mice through age 11 days were susceptible to MRV infection; however, the days and frequency in which the animals developed diarrhea were different for the mice of various ages. Notice that the mice infected at birth (age <1) developed diarrhea 3 days post inoculation (p.i.) which was later than the mice of other ages. These mice, <1 day-old, exhibited diarrhea up to 10 days p.i., longer than any of the older mice. Conversely, the older mice got diarrhea sooner, but cleared the disease in a shorter period of time. The 11 day-old mice exhibited diarrhea for only 3 days while the <1 day, and 2 day-old mice had diarrhea for at least 8 and 9 days,
Table 3. Susceptibility of Infant Mice to Infection with Cell Culture-propagated Bovine, Porcine or Simian Rotaviruses

<table>
<thead>
<tr>
<th>Passage</th>
<th>PRV</th>
<th>SRV</th>
<th>BRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>0 (17)(^c)</td>
<td>31 (13)</td>
<td>33 (13)</td>
</tr>
<tr>
<td>2nd</td>
<td>0 (14)</td>
<td>0 (15)</td>
<td>0 (16)</td>
</tr>
<tr>
<td>3rd</td>
<td>0 (19)</td>
<td>0 (18)</td>
<td>0 (17)</td>
</tr>
</tbody>
</table>

\(^a\)As determined 4 days post-virus exposure in 2 day old mice.

\(^b\)The infant mice of the first passage were infected with cell culture-propagated PRV, SRV or BRV. The infant mice of subsequent passages received an oral inoculum of homogenized intestine from mice of the previous passage.

\(^c\)Number of mice per group.
Figure 2. Susceptibility of <1 day, 2 day, 5 day, 8 day, and 11 day-old infant mice to 100 IU of murine rotavirus stock at various days after inoculation.
respectively. The 5 day-old mice had the highest frequency of diarrhea which occurred at days 3, 4, and 5 p.i.

**Immunofluorescent Anti-rotaviral Antibody Producing Cell Test**

The immunofluorescent anti-rotaviral antibody producing cell (APC) test for detecting rotaviral specific antibody-producing cells did not work because of the inability to eliminate non-specific fluorescing particles. Variations of the APC test and controls, as described in Materials and Methods, did not eliminate non-specific fluorescing particles. Non-specific fluorescing particles were observed in the lamina propria, and to a lesser extent, scattered randomly throughout the tissue section. Initially it was suspected that these particles were antibody-producing cells. Upon closer inspection, using a variety of controls, it was concluded that the particles were fluorescing non-specifically. A number of attempts were made to eliminate this non-specific fluorescence, but none were successful (see Materials and Methods).

**Effect of Dietary Folic Acid on Murine Rotavirus Infection**

Female mice were fed diets that contained low levels of folic acid to induce a deficiency in their offspring. The infant mice were orally inoculated with MRV stock; then the disease and immunological status of
infants were monitored. The data measuring the effects of alterations in dietary folic acid deficiency on rotaviral infection are summarized in Table 4. Infants from mice receiving 0 ug folic acid/ g diet had reduced quantities of folate in the livers. Mice that received the higher dietary levels of folic acid exhibited no apparent decrease of folate in their livers. It is shown by these data that the 0 ug folic acid/ g diet given to the dams was sufficient to bring about a reduced folic acid concentration in the livers of the infants born to those mice.

Weight gain data of infected and sham-infected infant mice from each dietary group are summarized in Figure 3. The sham-infected mice from dams receiving the lowest folic acid diet gained less weight (p<0.05) than the sham-infected mice from the dams fed the two diets having higher levels of folic acid. This decreased weight gain was especially apparent on day 2 p.i., but was still seen by day 20. The mice whose dams were fed the marginal folic acid diet appeared to gain less weight than the mice whose dams received the highest folic acid diet on every day except day 4 p.i. The difference between weight gain of the mice from the marginal and adequate diet groups, however, was not statistically significant. More obvious in Figure 3 is the substantially reduced weight gain of the rotaviral infected infants as compared with the uninfected infants (p<0.001). Although infected infants from mice fed the 0 ug folic acid/ g diet had the overall lowest weight gains, this difference as compared with the infected animals in the higher folic acid diet groups was not statistically significant.

The percentage of mice exhibiting diarrhea on days 2-5 and 10 p.i. is seen in Figure 4. Moderate increases in incidence of diarrhea were seen particularly on day 2 in the infants from the lowest folic acid
Table 4. Effects of Alterations in Dietary Folic Acid on Rotaviral Infection in Infant Mice.a

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observation Timeb</th>
<th>Concentration of Folic Acid Added to Dietsc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid content in livers of uninfected infant mice (µg/g)d</td>
<td>3 d old 3.8* ± 1.5e</td>
<td>0 µg/g 5.1 ± 1.5 5.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>6 d old 3.7* ± 0.9 4.5 ± 1.0</td>
<td>0.125 µg/g 6.2 ± 1.2</td>
</tr>
<tr>
<td>Incidence of diarrhea</td>
<td>2 d pi 73%*</td>
<td>0.5 µg/g 64% 58%</td>
</tr>
<tr>
<td></td>
<td>3 d pi 74%*</td>
<td>62% 63%</td>
</tr>
<tr>
<td>Weight gainf</td>
<td>10 d pi 198%</td>
<td>219% 232%</td>
</tr>
<tr>
<td>Intestinal rotavirus titerg</td>
<td>4 d pi 544*</td>
<td>740* 386</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer in infantsg</td>
<td>20 d pi 2.2*</td>
<td>3.7 3.2</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer in damsg</td>
<td>20 d pi 10.4</td>
<td>9.3 12.7</td>
</tr>
</tbody>
</table>

Mice infected p.o. at 2 days old.

Indicated as either age of mouse (old) or post-inoculation (pi) in days (d).

Diets of dams from which infants were taken.

By microbiological assays.

Mean ± SE.

Percentage of weight gained from the weight on the day of inoculation.

Reciprocal of the mean.

* P <0.05 compared to 0.5 µg/g diet group.
Figure 3. Percentage of weight gained from day of inoculation to 1, 2, 3, 4, 5, 10 and 20 post inoculation in infant mice whose dams received varying levels of dietary folic acid during pregnancy and lactation. Two day-old infants were infected with 100 ID$_{50}$ of murine rotavirus.
Figure 4. Percentage of rotaviral infected infant mice with diarrhea on days 2, 3, 4, 5, and 10 post inoculation whose dams received varying levels of dietary folic acid during pregnancy and lactation. Two day-old infants were infected with 100 IU of murine rotavirus. The (*) connotes p<0.05 compared to the 0.500 ug/g diet group.
dietary group (76% compared to 60% in the mice from the highest folic acid diet group). These differences were still seen on day 3 and were found to be significant at p<0.05. The incidences of diarrhea between any group on days 4, 5 or 10 p.i. were not found to be statistically significant.

The individual rotaviral antigen titers in intestines from infected infants killed on day 4 p.i. are presented in Figure 5. Reciprocal mean antigen titers were, respectively, 544 and 740 in the infants from mice fed the lowest and marginal folic acid deficient diets as compared with 386 for the animals receiving the highest folate diet (Table 4). By utilizing chi square analysis where those reciprocal titer values greater than or equal to 1280 were connoted (+) and those below 1280 were connoted (-), the reciprocal titers of the groups who received the lowest and marginal folic acid diets were considered to be significantly higher (p<0.05) as compared with the highest folic acid diet group, indicating the folate deficient diets may have enhanced the MRV infection in a significant number of the infants.

No serum rotaviral antibody was found in uninfected dams and infants.

A small degree of cytoplasmic vacuolization in the epithelial cells at the tip of the villi was seen in infected mouse intestines from all three dietary groups. There was no difference in the severity of this degeneration between any of the three dietary groups. In Figure 6 are two photographs illustrating the small degree of cytoplasmic vacuolization present in the tip of the villi from the ilium of a diseased animal and of normal villi of the uninfected control animal.
Figure 5. Reciprocal rotavirus titer in intestines obtained 4 days post inoculation from infants whose dams received varying levels of dietary folic acid. Two day-old infants were infected with 100 $10^6$ of murine rotavirus.
Figure 6. Photograph of: (a) cytoplasmic vacuolization present in the tips of villi from a rotaviral infected animal, and (b) the tips of villi from the ilium of an uninfected control animal.
Milk in the stomachs of infected infants at 4 days p.i. was assayed for rotaviral specific antibody, but no antibodies were detected at the lowest dilution (1/5) tested. Serum rotaviral antibody titers of infected dams and infants from each dietary group killed on day 22 p.i. are shown in Figures 7 and 8, respectively. Sera from infants in the lowest folic acid dietary group had overall lower reciprocal titers (mean=2.2) than did those from either marginal or normal folic acid groups (mean=3.7 and 3.2 respectively, Table 4). The increased number having reciprocal titers of <4 was statistically significant (p<0.05). The rotaviral reciprocal antibody titer of the dams' sera was not appreciably altered in any group. These animals were not directly infected with rotavirus but presumably exposed to the virus by being in close proximity to their experimentally infected infants.

Effect of Dietary Zinc on Murine Rotavirus Infection

Female mice were fed diets containing various levels of zinc to induce a zinc deficiency in their offspring. The infant mice were inoculated p.o. with MRV stock; then the disease and immunological status of the infants were monitored. The data measuring the effects of alterations in dietary zinc deficiency on rotaviral infection are summarized in Table 5. The infant mice from dams receiving the deficient zinc diet (4ug zinc/g) were deficient in the mineral as evidenced by reduced zinc levels in uninfected whole infant mice (p<0.01). The mice from the deficient dietary zinc group had a mean of 15.2 + 0.3 (+ SE) ug zinc per g of diet compared with 20.9 + 0.4
Figure 7. Reciprocal rotaviral antibody titer in sera obtained 20 days post inoculation from dams who received varying levels of dietary folic acid during pregnancy and lactation.
Figure 8. Reciprocal rotaviral antibody titer in sera obtained 20 days post inoculation from infants whose dams received varying levels of dietary folic acid during pregnancy and lactation. Two day-old infants were infected with 100 ID\textsubscript{50} of murine rotavirus.
Table 5. Effects of Alterations in Dietary Zinc on Rotaviral Infection in Infant Mice.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observation Time\textsuperscript{b}</th>
<th>Concentration of Zinc in Diets\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 μg/g</td>
</tr>
<tr>
<td>Zinc level in uninfected whole infant mice (μg/g)</td>
<td>12 d old</td>
<td>15.2 ± 0.3\textsuperscript{d}</td>
</tr>
<tr>
<td>Thymus weight in uninfected infant mice (mg)</td>
<td>25 d old</td>
<td>80.1 ± 2.9</td>
</tr>
<tr>
<td>Serum sodium (meq/l)\textsuperscript{f}</td>
<td>12 d old</td>
<td>142 ± 1.5</td>
</tr>
<tr>
<td>Incidence of diarrhea</td>
<td>4 d pi</td>
<td>71%</td>
</tr>
<tr>
<td>Intestine wt/whole mouse wt\textsuperscript{g}</td>
<td>7 d pi</td>
<td>96.9 ± 2.6</td>
</tr>
<tr>
<td>Incidental rotavirus titer\textsuperscript{i}</td>
<td>1 d pi</td>
<td>5.1</td>
</tr>
<tr>
<td>Weight gain\textsuperscript{h}</td>
<td>4 d pi</td>
<td>34%</td>
</tr>
<tr>
<td>Intestinal rotavirus titer\textsuperscript{j}</td>
<td>7 d pi</td>
<td>3596</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer in infants\textsuperscript{l}</td>
<td>20 d pi</td>
<td>8</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer in dams\textsuperscript{l}</td>
<td>30 d pi</td>
<td>135</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mice infected p.o. at 5 days old.
\textsuperscript{b} Indicated as either age of mouse (old) or post-virus inoculation (pi) in days (d).
\textsuperscript{c} Diets of dams from which infants were taken.
\textsuperscript{d} By atomic adsorption spectrophotometry.
\textsuperscript{e} Mean ± SE.
\textsuperscript{f} By atomic emission spectrophotometry.
\textsuperscript{g} Quantitative measurement of diarrhea.
\textsuperscript{h} Percentage of weight gained from day of infection to 4 days pi.
\textsuperscript{i} Reciprocal of the mean.

\* p < 0.05 compared to 60 μg/g control group.
ug zinc per g of diet in the control animals (Table 5). Notice also from Figure 9 that the zinc levels from infected mice in all three dietary groups were higher than the values of the uninfected animals. The differences were statistically different (p<0.05) for the infected compared to the uninfected animals in the 4 and 12 ug zinc/g diet groups, but not the 60 ug zinc/g diet group. The thymus weight, reflective of the thymus hypoplasia due to zinc deficiency, was significantly lower in the uninfected infant mice whose dams were fed zinc deficient diet compared with those of the control group (p<0.01) (Figure 10). Clinical signs of zinc deficiency were also apparent in the zinc deficient diet animals, i.e., scaly skin, reduced ability to gain weight, and alopecia (loss of hair). Alopecia has been easily observed in swine with zinc deficiency (Church and Pond 1982). Also, mice in the 4ug zinc/g diet group of either the uninfected or infected mice, gained significantly less weight over a 20 day period than the mice from the 12 and 60 ug zinc/g diet groups at a p<0.05 (Figure 10). Even though the animals from the 12 ug zinc/g diet group on the average gained more weight than the 60 ug zinc/g diet group, there was no significant difference between these groups in either the infected or uninfected animals. It has been shown by these data that the mice in the 4 ug zinc/g diet group were indeed zinc deficient.

It was most apparent from weight gain data on days 3 and 4 p.i. that infected mice gained less weight than the uninfected mice (Figure 11). This type of parameter was effective in the mouse model as a measure of the disease.

The infected mice of the three dietary groups had an elevated mean
Figure 9. Zinc level in rotaviral infected (6 days post inoculation) and sham-inoculated whole infant mice whose dams received varying levels of dietary zinc during pregnancy and lactation. Five day-old infants were infected with 100 ID$_{50}$ of murine rotavirus. Bars are shown with ± SE.
Figure 10. Weight of thymuses from uninfected 25 day-old infant mice whose dams received varying levels of dietary zinc during pregnancy and lactation. Bars are shown with \(_{\pm} SE\).
Figure 11. Percentage of weight gained from day of inoculation to 1, 2, 3, 4, 5, 6, 10, and 20 post inoculation in infants whose dams received varying levels of dietary zinc during pregnancy and lactation. Five day-old infants were infected with 100 IU of murine rotavirus.
serum sodium level of 148 ± 1.5 meq/l while the mean serum sodium level in the infected mice of the three groups was 141 ± 1.3 meq/l. (Table 5). There was no difference, however, in the serum sodium level of the 4 ug zinc/g diet group compared with the 12 or 60 ug zinc/g diet group in either the infected or uninfected mice (Figure 12).

The same pattern (Figure 13) also was seen with the ratio of large intestine weight to whole mouse weight. The ratios of the uninfected mice were nearly twice as low as the ratios of the infected mice. The difference was highly significant (p<0.001), thus indicating this parameter was indeed a measure of degree of diarrhea. There was no difference, however, when comparing the ratios between any of the three dietary groups. Figure 14 is a photograph of a distended large intestine from an 11 day-old mouse infected 6 days p.i. with murine rotavirus and the smaller intestine from a control mouse of the same age. When measuring the incidence of diarrhea on days 2-5, 7 and 10 p.i. as a monitor of the disease (Figure 15), we did not observe any statistically significant difference between the infected zinc deficient dietary groups and the infected control dietary group.

There were also no statistically significant differences between the dietary groups in the viral titers in the infant intestines, day 1 or day 8 p.i. (Figure 16), or in the antibody titers in the sera from infants 20 days, or dams 30 days p.i. (Figure 17). The virus titer, serum sodium concentration, and ratio of intestine weight to mouse weight were plotted for each mouse whose body zinc content was known so that a correlation of zinc content to various experimental parameters could be plotted (Figure 18a, b, and c). No correlation appeared to exist between zinc content of the mouse and the various parameters.
Figure 12. Serum sodium level in rotaviral infected (6 days post inoculation) and sham-inoculated infant mice whose dams received varying levels of dietary zinc during pregnancy and lactation. Five day-old infants were infected with 100 ID$_{50}$ of murine rotavirus. Bars are shown with ± SE.
Figure 13. Ratio of large intestine weight to whole mouse weight in rotaviral infected (6 days post inoculation) and sham-inoculated infant mice whose dams received varying levels of dietary zinc during pregnancy and lactation. Five day-old infants were infected with 100 \(10^{5}\) of murine rotavirus. Bars are shown with ± SE.
Figure 14. Photograph of a distended large intestine from an 11 day-old mouse infected 5 days of age, and the smaller intestine from a sham-infected control mouse of the same age.
Figure 15. Percent of rotaviral infected infant mice with diarrhea on days 2, 3, 4, 5, 7, and 10 post inoculation whose dams received varying levels of dietary zinc during pregnancy and lactation. Five day-old infants were infected with 100 IU 50 of murine rotavirus.
Figure 16. Reciprocal rotavirus titers in intestines from infants 1 day and 8 days post inoculation whose dams received varying levels of dietary zinc during pregnancy and lactation. Five day-old infants were infected with 100 ID_{50} of murine rotavirus.
Figure 17. Reciprocal rotaviral antibody titers in sera of infants 20 days post inoculation whose dams received varying levels of dietary zinc during pregnancy and lactation, and also reciprocal antibody titers in the sera of those dams 30 days post inoculation. Five day-old infants were infected with 100 ID$_{50}$ of murine rotavirus.
Figure 18a. Plot of whole body zinc content versus ratio of large intestine to whole mouse weight in rotaviral infected infant mice (6 days post inoculation) whose dams received varying levels of dietary zinc during pregnancy and lactation.
Figure 18b. Plot of whole body zinc content versus rotavirus titer in intestine in rotaviral infected infant mice (6 days post inoculation) whose dams received varying levels of dietary zinc during pregnancy and lactation.
Figure 18c. Plot of whole body zinc content versus serum sodium level in rotaviral infected infant mice (6 day post inoculation) whose dams received varying levels of dietary zinc during pregnancy and lactation.
mentioned above.

A small degree of cytoplasmic vacuolization in the epithelial cells at the tip of the villi was seen in infected mouse intestines from all three dietary groups. There was no difference, however, in the severity of this degeneration between any of the three dietary groups.

**Athymic Mouse Experiments**

Athymic and thymic mice (Experiment #1) were challenged 2 times, 17 days apart p.o. with 100 ID$_{50}$ of cesium chloride-purified MRV to determine if elicitation of rotaviral specific antibodies was dependent on T-cell function. Twelve days after the second challenge, the sera were assayed for rotavirus-specific antibody. No specific antibody was detected in the four athymic mice while specific antibody was detected in all four of the thymic mice (Table 6).

In athymic nude mouse experiment #2, one litter that contained both 2 day-old athymic and thymic mice was sacrificed. Rotaviral specific antibody at a reciprocal titer of >128 was found in the sera of the thymic dam which had been infected p.o. with MRV stock about 5 days after gestation and in the milk from infants' stomachs at a mean reciprocal titer of 32. It is assumed that the infants were exposed to residual virus left by inoculation of the dams. These high serum and milk antibody titers demonstrated that dams inoculated p.o. by rotavirus were likely to have specific antibodies when the litters were born. Rotavirus-specific antibody was also detected in the sera of other athymic and thymic infant mice whose thymic dams were infected p.o. with
Table 6. Comparison of Rotaviral Disease Parameters In Athymic and Thymic Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observation time</th>
<th>Athymic mice (controls)</th>
<th>Thymus-containing mice (controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum rotaviral antibody titer</td>
<td>12</td>
<td>&lt;4, &lt;4, &lt;4, &lt;4</td>
<td>8, 8, 16, 16</td>
</tr>
<tr>
<td>Infant mice</td>
<td>Experiment #1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence of diarrhea</td>
<td>3</td>
<td>4/35 (11%)</td>
<td>7/42 (17%)</td>
</tr>
<tr>
<td>Incidence of diarrhea</td>
<td>5</td>
<td>15/35 (43%)</td>
<td>21/42 (50%)</td>
</tr>
<tr>
<td>Incidence of diarrhea</td>
<td>7</td>
<td>19/35 (54%)</td>
<td>22/38 (58%)</td>
</tr>
<tr>
<td>Intestine wt/whole mouse wt</td>
<td>6</td>
<td>0.015±0.003 (12)</td>
<td>0.014±0.003 (13)</td>
</tr>
<tr>
<td>Intestinal rotavirus titer</td>
<td>8</td>
<td>356 (12)</td>
<td>297 (15)</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer</td>
<td>8</td>
<td>13 (10)</td>
<td>13 (8)</td>
</tr>
<tr>
<td>Serum rotaviral antibody titer</td>
<td>20</td>
<td>20 (16)</td>
<td>59 (12)</td>
</tr>
</tbody>
</table>

aDesignated CD-1, either athymic nude homozygous (nu/nu) or thymic heterozygous (nu+/+) mice.
bDays post-exposure.
cAdult mice were challenged 2 times, 17 days apart p.o. with MRV stock. Twelve days post inoculation, the sera of these mice were assayed for rotavirus-specific antibody.
dReciprocal antibody titer for each individual sample.
eDams of these infant mice were infected p.o. with MRV stock. The infants presumably acquired the disease from the dams, probably via the residual virus in the cage.
fPositive/total (percent positive).
gMeasurement of diarrhea, mean ± S.E. (number of mice/group).
hReciprocal of mean (number of mice/group).
MRV stock about 5 days after gestation. The average sera antibody titers were the same in both the 8-day old athymic and thymic mice, whereas on day 10 the average titers were less than one-half the average titers of the thymic mice (Figure 20). The incidence of diarrhea, ratio of intestine weight to mouse weight and intestinal rotavirus titers were the same in athymic and thymic infant mice (Table 6).
Figure 19. Athymic Nude Mouse Experiment #2. Reciprocal rotaviral serum antibody end point titers in athymic nude mice and thymic heterozygous control mice 8 and 20 days post inoculation.
DISCUSSION

The primary purpose of this study was to determine if dietary folic acid or zinc deficiencies would affect the disease status of rotaviral infection in infant mice. This study was prompted since both of these nutritional factors have previously been shown to alter the immune responses to certain antigens (Beisel 1982; Gross and Newberne 1980) and also affect the status of some specific infections (Nelson and Haltalin 1972; Fraker et al. 1982). An enhancement of rotaviral disease was seen in folic acid deprived infants as determined by decreased ability to gain weight, increased incidence of diarrhea, and high rotaviral antigen titers in their intestines. Serum antibody titers were also below detectable levels in a significant number of mice fed the lowest folic acid diet. Zinc deficiency in the infant mice, however, had no apparent effect on any of the parameters measuring immunological response or status of the rotaviral infection.

Folic acid deficiency was observed in the infant mice born to dams fed the most deficient folic acid diet, as evidenced by the reduced concentrations of folic acid in the infant's livers. Also, the uninfected animals fed these diets had progeny that gained less weight than the mice in the normal folic acid diet group. It is noteworthy to compare the values of the folic acid concentrations in the livers with the studies of Klipstein and Lipton (1970). They observed the normal range of folic acid in mouse livers to be 6.0 to 13.4 ug/g (ug folic acid/g wet liver) and the range for deficient animals to be below 5.3
We also observed similar values of 5.3 to 6.2 ug/g and 3.7 to 5.1 ug/g for the respective normal and deficient folic acid liver concentrations. The observation in the Klipstein and Lipton (1970) studies, that weight retardation does occur in infant mice from dams fed a folic acid deficient diet was also observed in our study.

Numerous investigators (Beisel 1982; Gross and Newberne 1980; Kumar and Axelrod 1978; William et al. 1975) have reported that reduced levels of folic acid affects host immunity which might consequently influence the status of the disease. These observed effects are likely due to the role derivatives of tetrahydrofolate play in protein, lipid, nucleic acid, and hormone chemistry (Metzler 1977). The reported normal liver folic acid values in this study agreed with the values of other studies. Bennett et al. (1964) reported normal rat liver folic acid levels to be 5.0 to 9.0 ug folate/g diet. Our mean values for mice 3 days old and 6 days old were 5.3 and 6.2 ug folate/g diet, respectively.

Most investigators (Kumar and Axelrod, 1978; William et al. 1975) have studied the effects of folic acid deficiency on immune response to mitogens or sheep red blood cells to derive conclusions about effect of the deficiency on disease. Few, (Nelson and Haltalin 1972) however, have applied their research to study the effect of folic acid deficiency on specific diseases; this study was designed to do that.

To correlate the results obtained from such a study, one needs to experimentally induce signs of folic acid deficiencies that represent the signs of deficiencies seen in human populations. The folic acid deficiency observed in the infants of the deficient and marginal folic acid diet groups was not believed to be a severe deficiency.
weight retardation was the only clinical manifestation of the deficiency which probably simulates more realistically a diet-acquired deficiency in man since most folic acid deficiencies are predominantly mild or subclinical rather than severe (Herbert 1968). From the results of this study one may infer that dietary folic acid deficiency may be a factor in influencing the severity of rotavirus-induced diarrheal disease in humans.

It appears that weight gain of the infant mice is a good parameter to measure the presence and intensity of the rotaviral disease. The reduced folic acid diet seemed to retard the weight gained by the infant mice because the percent of weight gained from day 1 p.i. to day 4 p.i. was about 100% for the uninfected animals and about 60% for the infected animals. The infected animals gained less weight than the uninfected animals possibly because of dehydration due to the diarrhea (Middleton 1978).

It was concluded that the controls were uninfected since none of the animals sampled had rotavirus in the intestine, rotaviral antibodies in the sera, or presence of diarrhea. Therefore, our methods of handling the mice were adequate to exclude MRV from the control mice. Uninfected controls were important to include in the experiment since they were used to delineate which observed effects were as a result of a folic acid deficient diet or the rotaviral infection.

It is recognized that immunity to rotavirus can be conferred to the infant by the mother through the milk (Snodgrass and Wells 1976). She develops an immunological response upon gastrointestinal exposure to the virus (Snodgrass and Wells 1976). Sensitized lymphocytes may then migrate to other portions of the body i.e., spleen, lamina propria of
the intestinal villi, lymph nodes and mammary glands (Ogra and Dayton 1979). In this study rotaviral antibodies were assayed in the sera of the dams and in the milk of the stomachs of the infants in an attempt to monitor the immunological status, which in turn, would reflect the degree of immunity that might be transferred to the infants. There was no difference, however, in the maternal serum antibody titers between the deficient diet groups and control diet group. This may have been because the adult female mice may not have had a large enough folic acid deficiency to affect the rotaviral-specific antibody titer of the serum as it did in the infants. The serum rotaviral-specific antibody titers of the folic acid deficient infant mice were lower than the titers of the control mice. This effect was likely due to the reduced folic acid levels since others (Kumar and Axelrod 1978) have observed similar humoral responses in folic acid deficient hosts. There was no rotavirus-specific antibodies detected in the milk. It is possible that the animals were not sensitized long enough to have an antibody titer large enough to be detected at day 4 p.i.

The rotaviral disease was enhanced in infants from the deficient and marginal folic acid diet groups compared with the adequate diet group. This was apparent by the increased intestinal rotavirus titers of the infant mice in the deficient and marginal folic acid diet groups. Conceptually, the increased virus may result in increased diarrhea in the animals. There was indeed an increased proportion of mice with diarrhea in the deficient folic acid diet group compared to the normal folic acid diet group. It should be noted that the effect appeared to be most pronounced early in the infection—days 2 and 3. Nelson and Haltalin
(1972) observed that folic acid deficiency also affects disease status. Infant guinea pigs fed folate deficient diet for 2 weeks showed 89% mortality when inoculated with $10^9$ *Shigella flexneri* while those mice fed normal diet had no mortality.

The zinc deficient diet as administered in this study appeared to be effective in causing a deficiency in the infant mice since a zinc deficiency was seen in the infant mice by assaying the whole body zinc content. The values obtained from the 4 ug zinc/g diet group were significantly lower than those for the 12 and 60 ug zinc/g diet groups.

It is important to realize that the animals did have signs of zinc deficiency during the course of the rotaviral infection i.e., the zinc deficiency occurred not only at periods before or after, but also during the time the animal had the disease. Before, and through 15 days p.i., the animals had alopecia (Church and Pond 1982) and by day 2 p.i. growth retardation was quite evident. At day 6 p.i., near the peak of the disease, the animals were shown to have hypoplasia of the thymus and depleted body zinc levels.

It has long been known clinically that sodium is one of the serum ions involved in electrolyte imbalance (Tietz 1976). In the present study, the infant mice infected with rotavirus had a definite electrolyte imbalance, possibly due to dehydration (Middleton 1978) as represented by the elevated serum sodium levels in the infant mice.

It was felt that one of the best means of measuring the illness of the host would not necessarily be ability to gain weight or presence of diarrhea but a parameter that would indicate if the disease was severe enough as to cause death. It has been observed that human patients near death had serum sodium levels as high as 190 mEq/l (Middleton 1978),
whereas normal values range from 135 to 148 mEq/l. It is believed that rotavirus causes death on a basis of electrolyte imbalance leading to cardiac arrest (Middleton 1978). Note, however, that potassium ion imbalance is the cause of cardiac arrest (Tietz 1976). An abnormal sodium level indirectly suggests an imbalance in other electrolytes, including potassium, which may subsequently lead to cardiac arrest. Serum sodium level was an effective means for measuring the status of the disease in a mouse model since they also had elevated serum sodium levels, even as high as 178 mEq/l.

Hypernatremia (high sodium level) is much more rare than hyponatremia (Tietz 1976). To understand a possible reason for the hypernatremia observed in this dissertation, an understanding is needed of the diarrheal mechanism and physiological alterations that occur during severe dehydration due to rotaviral disease. Rotavirus denudes the tips of villi of columnar epithelial cells which possess digestive enzymes. The main purpose of these cells is to digest and adsorb nutrients and electrolytes (Moon 1978). The cells at the crypts of the villi are more immature and do not demonstrate digestive or adsorptive properties; in fact, these younger cells perform secretory functions. The result then, in rotaviral diarrhea, is a net flux of electrolytes and fluids into the lumen of the gut. Theoretically, the diseased host should have hyponatremia. Some of the diseased mice in our study did have hyponatremia; however, on the average the diseased mice had hypernatremia. Human subjects that had severe rotaviral disease and died from it, exhibited marked dehydration and hypernatremia (Middleton 1978). It is likely, then, that in severely diseased subjects
hypernatremia results from severe dehydration even though they had episodes of diarrhea causing an intralumen flux of sodium into the intestine. Perhaps hypernatremia, instead of hyponatremia, is a sign of a more progressed, severe, disease. Further studies may reveal this parameter to be of more predictable value of the status of the disease than presently realized.

Other parameters to illustrate that the mouse model was effective were that mice with zinc deficiency displayed clinical signs such as scaly skin, reduced weight gain, reduced thymus weight, and alopecia (loss of hair). Similar clinical signs and weight gain have also been observed in human subjects suffering from zinc deficiencies (Hambidge et al. 1976; Oleske et al. 1979).

Investigators of a previous study (Noble et al. 1983) were able to qualitatively score the degree of diarrheal fluids by visually scoring the intestines. In this study we have found that the ratio of large intestine weight to whole mouse weight was effective for quantitatively measuring the degree of diarrheal fluids. The ratios for the infected animals were twice that of the uninfected animals, suggesting that this was an effective parameter for measuring the status of the disease. It had a definite advantage in being less subjective than visual observation of the intestine.

The infants from the dams fed the deficient zinc diet (4ug zinc/g diet group), compared to the control mice, were zinc deficient as evidenced by depressed whole body zinc content, reduced thymus weight, and reduced ability to gain body weight. Even though zinc deficiency has previously been shown (Gross and Newberne 1980) to dramatically affect the laboratory animal's immunological response and subsequently alter
the ability of the host to eliminate the antigen, the dietary zinc deficiency obtained in this study apparently did not alter the status of rotaviral disease in the infant mouse model. This was seen because the proportion of mice with diarrhea, serum sodium level, ratio of large intestine weight to whole mouse weight, intestinal virus titer, or intestinal histopathology were not significantly different between the animals of the three dietary groups. The zinc deficiency also did not appear to alter the immunological response of the mouse to the rotaviral antigen since the rotavirus-specific antibodies in the sera from the dams of infants were not noticeably affected.

Zinc is important in the tissue healing process (Porries et al. 1974); therefore, it is possible that this phenomenon could result in prolonged rotaviral disease in zinc deficient individuals. This prolonged disease was not observed since intestinal histopathological signs obtained 20 days p.i. were not more apparent in the zinc deficient mice compared to the control mice.

Considering previous studies by other investigators that zinc deficiency depresses the immune responses to certain antigens other than rotaviral antigens, the apparent question to ask is why did not the zinc deficiency in the infant mice affect the rotaviral disease in these mice. A recent study by Beach et al. (1982) used the same diet formulation which contained approximately the same concentration of zinc and the same species of infant mice used in this study to measure the antibody response to sheep red blood cells (SRBC). The infant mice from their study also had similar clinical manifestations as the mice in this study. The infants were smaller and the zinc levels
were less in the deficient infant mice. In addition, they observed a higher mortality, something that we did not observe. This indicated that the mice in the Beach et al. (1982) study had at least a slightly higher zinc deficiency compared with those in this study. Thus, differences in the experimental results between the two studies may possibly be explained by the slight difference in the degree of zinc deficiency in the mice.

It may be difficult to find two independent studies that were exactly the same, but an empirical comparison between the study of Beach et al. (1982) and studies presented here can be made. In their study, there was a dramatic reduction in the SRBC-specific antibody level in the zinc deficient animals compared to the controls. The results were explained by the fact that it was known that the specific antibody formation to SRBC was highly dependent on T-helper cells (Barrett 1978). Since zinc deficiency atrophied the thymus, it was presumed that T-helper cell activity was depressed (Fernandes et al. 1979); subsequently, the ability of the animal to elicit a response to SRBC antigens was likewise depressed. The zinc deficient infant mice in this study, however, did not have a lower rotaviral specific antibody end point titer, or a more severe disease than the control mice.

Studies were done with athymic mice and thymic control mice to investigate why zinc deficiency did not affect the rotaviral infection. This was accomplished by determining how dependent the rotavirus-specific antibody formation may be on T-cell function. Such a study would aid in elucidating how zinc deficiency may affect the ability of the host to respond to rotavirus infection.

If rotaviral antibody response were thymus independent, decreased
thymic function, due to zinc deficiency (Fraker et al. 1978), may not have an effect on rotaviral infection. This reasoning could then be used to explain the experimental results that zinc deficiency had no observable effect on rotaviral infection. Adult athymic mice and thymic mice were challenged p.o. with MRV, and the sera were assayed for rotaviral specific antibodies (Athymic nude mouse experiment #1). It was determined that rotaviral specific antibodies were thymus dependent since the athymic mice did not have specific antibodies while the mice that possessed a thymus did (Table 6). Hence, the lack of effect that zinc deficiency had on rotaviral infection may not be explained by the fact that humoral immune response was T-cell independent.

In athymic nude mouse experiment #1, the result that infant athymic nude mice responded the same as control mice to rotaviral challenge was similar to a study by Letvin et al. (1981). They observed that athymic nude mice, challenged per os or intraperitoneally with reovirus, eliminated the virus as effectively as the thymic control mice. They demonstrated that \textit{in vivo} reovirus clearance did not depend on T-cell function even though such cells were stimulated \textit{in vitro} by reovirus. Since rotaviruses and reoviruses are similar morphologically and belong to the Reoviridae family (McNulty 1978), both may be acted upon by the host immune system in a similar fashion. Like \textit{in vivo} reovirus clearance from mice, T-cell function may not be paramount to the elimination of an \textit{in vivo} challenge of rotavirus. Other factors may be more important in elimination of the disease.

The other potential modes of defense of the animal i.e., natural killer cells (Letvin et al. 1981), epithelial integrity, acid
secretions, bile salts, enzyme secretions, or antiviral components in
the maternal milk, may be the first lines of defense to eliminate the
rotavirus. It was assumed that the dams in the dietary studies may not
have had previous exposure to the virus before they were exposed through
their artificially inoculated infants; therefore, they elicited a primary
immunological response, i.e., an immune response after the first exposure
to the virus. As a result their infants would not have received any
immediate passive maternal immunity. The infant's antibody response
probably would not occur until after the disease manifested itself. The
reason why the rotaviral infection did not seem to be altered by
nutritional zinc deficiency may have been that any effect zinc had on
the late arrival of the humoral immune response would be superseded by
the primary lines of defense; if zinc deficiency did not affect these
lines of defense, the deficient animal would recover from the disease as
effectively as the control animals.

The investigators of the reovirus study cited above (Letvin et al.
1981) support this hypothesis by suggesting that natural killer cell
activity (Herberman et al. 1975) may have been responsible for
elimination of the virus in the athymic or thymic mice since reduced
T-cell activity of the athymic mice did not alter the ability of the
mice to eliminate the reovirus.

The use of athymic mice also allowed the study of the role of
maternal immunity in the response of infants to rotaviral disease. In
the athymic nude mouse experiment #2, female adult mice were infected
p.o. with MRV about 5 days after gestation which allowed for an
antibody response before infants were born. At birth, the infants
probably contracted the disease from residual virus in the cages. Since
it was determined from adult athymic mice that rotaviral antibody response was thymus-dependent, and since any mouse not possessing a thymus would not produce specific antibody, one might presume that sera from athymic infant mice also would not contain any specific antibody. The infant athymic mice did, however, possess rotaviral specific antibody in the serum at comparable levels to thymic control infant mice. These antibodies, it was presumed, must be maternal-derived. It was shown that the dams did possess the rotaviral-specific antibody which consequently might be passed to the infants through the milk (Snodgrass and Wells 1978) or the yolk sac (Porter) while the infant was in utero. The milk not only contains antibodies (Ogra and Dayton 1979), but also lymphocytes and macrophages have been shown to traverse the intestine into the circulatory system (Otnaess and Orstavik 1980).

In athymic nude mouse experiment #2, the infant mice still developed diarrhea when challenged by the rotavirus. Other studies (Snodgrass and Wells 1978) have shown that specific antibody-containing colostrum protects the neonate from rotaviral disease. Possible explanations as to why the mice in this study still exhibited the disease, even after they received the antibody-containing milk, may be that they only received a slight manifestation of the disease compared to that which they would have received if they had not been protected through the milk at all. Unfortunately, the disease parameters from these mice and data from other mice described in this dissertation who received milk that contained no specific antibodies to rotavirus cannot be properly compared. The main reason for the inability to compare the data was that in the athymic mouse experiment, the mice were passively infected at birth,
whereas in the dietary study the mice were orally inoculated with the rotavirus at least several days after birth. Since the age at which the mice were inoculated would make a difference in the intensities of the disease, a well controlled experiment would need to be conducted to determine if specific antibodies from the dam's milk could offer protection to the murine infant from the rotaviral disease.

The main purpose of this research was to delineate which nutritional factors may be responsible for the increased severity of rotaviral disease in malnourished subjects. Future approaches to this problem would be to survey the nutritional deficiencies of populations of people with severe rotaviral disease, thereby being able to identify a correlation between specific nutritional factors and the disease. Studies should also be done to determine if the elicitation of rotaviral antibody in the gut occurs by the same process as immune responses to other, more thoroughly studied, gastrointestinal infectious agents such as polio (Ogra and Dayton 1979). It would be important to know if cellular components such as natural killer cells are involved in the elimination of the virus. Much work (Gross and Newberne 1980; Beisel 1982) has already been done to determine which immunologic or non-immunologic host defenses are affected by specific nutritional factors, therefore, with the information that would be acquired from the above proposed areas of research, one ought to be able to more accurately predict which nutrients are likely to effect rotaviral infection in the host.

One of the advantages with the use of mice as an experimental model is that relatively large numbers of mice can be utilized when compared to other animals typically used to study rotaviral disease e.g., calf,
lamb, and piglet. The statistical advantages are profound when one considers that approximately 2500 mice were used in this study. It is also obvious, for logistical reasons, that the mouse model is better for the measurement of weight of the animal and weight of large intestine. Other researchers have also used the mouse as a model of rotaviral disease (Little and Shaddock 1982; Dolin 1979; La Bonnardiere and de Vaureix 1979; Noble et al. 1983).

Since MRV cannot be effectively propagated in cell lines (Theil et al. 1978), and since the use of cell culture-propagated virus would provide a pure viral inoculum for animals and more methods for viral assay, the infectivity of cell culture-propagated SRV, BRV and PRV were evaluated in infant mice. BRV and SRV, but not PRV, induced diarrhea in 30% of the animals. There were no further signs of the disease, however, in 2 subsequent passages of the viruses. Also, no viral antigens were detected for any of the samples. It was then determined that none of these cell culture-propagated viruses could replace MRV in the nutritional experiments.

The questions arise as to why BRV and SRV induced diarrhea in the first passage, but upon subsequent passages no diarrhea was detected; and why were no viral antigens detected in animals presenting diarrhea. There is a possibility that the BRV and SRV do infect the cells of the intestine and enough damage may be obtained to induce diarrhea in the animals; however, the infection might be incomplete, not allowing the virus to propagate. Upon subsequent passages no virus would be available to infect the intestinal cells. The explanation for the second question might be that since viral replication might be incomplete, resulting in
no virus propagation, the antigen might be present below detectable levels.

Since a purer cell culture-propagated rotavirus was not available to infect infant mice, MRV propagated in vivo in mouse intestines was purified by freon extraction and cesium chloride density centrifugation. A titration of this purified virus was done in infant mice to obtain a 50% infectious dose and to confirm that the purification process did not inactivate the virus. The animals did contract diarrhea demonstrating that the virus was not inactivated, and a reasonably high \( 10^{50} \) was determined. For the animal titration of MRV, it was important that uninfected controls were included in each cage of mice since it has been previously observed by me that unexposed mice can be placed in the room used to infect animals and they will eventually get diarrhea. It was important to realize that if mice contracted the disease before the uninfected controls, they obtained the virus from the inoculum and not extraneous virus from the room.

Different aged mice were infected with purified MRV to determine what age of mouse was most susceptible, i.e., which age had the greatest proportion of mice with diarrhea. It was this age of mouse that was infected for the zinc dietary study. Chronologically, the folic acid dietary study had already been done; as a result, the mice in that study were inoculated at 2 days old. A greater proportion of 5-day-old mice had diarrhea; therefore, this age mouse was used to infect mice for the zinc dietary study. Other observations of interest were made. The youngest mice, <1 day-old, got diarrhea later after infection compared to older mice; however, they had diarrhea longer than animals of the
older age groups. This has also been observed in other studies (Little and Snaddock 1982; Noble 1983). Succinctly, the younger the mouse, the later it got the disease and the longer the disease was sustained. Conversely, the older mice developed diarrhea sooner but had the disease for a shorter period of time.

Attempts to adapt the APC test for the detection of rotaviral specific antibody-producing cells were unsuccessful since fluorescing particles, that might be construed as cells associated with antibody, were also seen in negative controls. The fluorescence could not be blocked by various methods designed to eliminate specific fluorescence of cells associated with antibody. This information is not necessarily significant, except that other investigators (Personal Communication, S. L. Vonderfecht, Dept. of Pathology, Johns Hopkins School of Medicine, Baltimore, MD) have had difficulty in adapting the APC test specifically to murine tissue. It may be possible that some factor, unique to the murine species, prevents the development of the APC test.

It was not anticipated that these attempts to develop the APC test in the murine model would be futile since valuable data about the gut immune response to rotaviral exposure has been obtained in the lymph nodes of rabbits (Vonderfecht and Osburne 1982a) and in the intestinal lamina propria of calves (Vonderfecht and Osburne 1982b).

The dietary studies were designed to be amenable to statistical analysis. This was important because, traditionally, experiments using animals have high variability which lessens the accuracy of data interpretation. As a result, the variability needed to be kept at a minimum. Among the precautions taken were to use the same inbred mice
throughout all of the studies. Another source of variability is that litters having more infants compared to litters having less infants will gain weight differently, therefore, when litters were born, the numbers of mice were equalized in the litters. For example, if one litter were born with 10 infants and one litter was born the same day with 8, they were equalized so that the litters would both contain 9 infants. Notice that the use of multiple litters was used as repetitions of the experimental units, which also reduced variability.

Considering the observations that an animals' physiology may be different at one part of the day when compared to another, an effort was made to collect the specimens at the same time each day. In this way the values obtained for those parameters would not be biased as to what time of the day the samples were collected.

Throughout the course of all experiments, the animals were randomly assigned to the various groups. In this way experimental results were not biased as to which mice were first selected to be assigned to a certain group. The other precaution taken to reduce experimental variability was the controlling of the environmental conditions existing in the animal containment rooms, such as lighting and temperature.

For future studies, it would be of particular interest to investigate not only the effects of nutritional factors on a primary rotaviral exposure but also on a secondary rotaviral exposure while monitoring non-immunologic responses and rotaviral antibody in the gastrointestinal tract of the infant and milk of the dams.
CONCLUSION

1. Folic acid malnutrition increased the severity of rotaviral disease and decreased the humoral immune response. This disease enhancement was evidenced by decreased ability to gain weight, increased incidence of diarrhea, increased number of mice exhibiting high rotaviral antigen titers and decreased serum rotaviral antibody titers.

2. Zinc malnutrition did not alter the severity of the rotaviral disease or humoral immune response.

3. The lack of effect that dietary zinc had on rotaviral disease is not due to a T-cell independence of rotaviral antibody formation, since it was observed, using athymic mice, that the antibody response is T-cell dependent.

4. A cesium chloride-purification of murine rotavirus can be used to infect infant mice since the virus is not inactivated by the purification process. Such a purified virus was found to be highly infectious, with a dilution of over $10^{-6}$ still inducing recognizable disease.

5. Cell-cultured strains of bovine, simian, but not porcine rotavirus will induce diarrhea in a small proportion of infant mice; however, the viruses were not demonstratively propagated in the mouse intestines. Therefore, the viruses could not be used to replace murine rotavirus in these dietary studies.

6. Younger mice, <1 and 2 days-old, infected with murine rotavirus, contracted the diarrhea later but sustained the disease for a longer period of time than older mice. A greater percentage of
5 day-old mice got diarrhea than 0, 2, 8, and 11 day-old mice.

7. Serum sodium levels, ratio of large intestine weight to whole mouse weight, ability to gain weight, presence of diarrhea, virus titer of the intestine, and rotaviral specific antibodies in a mouse model are effective for evaluation of rotaviral disease.


Church, D. C.; Pond, W. G. Basic animal nutrition and feeding. 2nd ed. New York: John Wiley and Sons; 1982: 159-163.


APPENDIX
Table 7. Mineral and Vitamin Mixes Used in the Formulations of Diets

<table>
<thead>
<tr>
<th>Item</th>
<th>g/kg diet</th>
<th>Vitamin mix</th>
<th>g/kg diet</th>
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</thead>
<tbody>
<tr>
<td>KCl</td>
<td>296.9</td>
<td>Vitamin A (200,000 IU/g)</td>
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<tr>
<td>MgCO₃</td>
<td>121.0</td>
<td>Vitamin D (400,000 IU/g)</td>
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<td>MnSO₄ · H₂O</td>
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<td>CoCl₂</td>
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<td>ascorbic acid</td>
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<td>KI</td>
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<td>myo-inositol</td>
<td>5.0</td>
</tr>
<tr>
<td>NaMoO</td>
<td>0.1</td>
<td>chlorine · HCl</td>
<td>75.0</td>
</tr>
<tr>
<td>FeSO₄ · 7H₂O</td>
<td>15.02</td>
<td>menadion (Vitamin K)</td>
<td>2.25</td>
</tr>
<tr>
<td>Dextrose</td>
<td>550.98</td>
<td>p-aminobenzoic acid</td>
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</tr>
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<td>CuSO₄ · 5H₂O</td>
<td>1.8</td>
<td>niacin</td>
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<td></td>
<td></td>
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<td>pyridoxine · HCl</td>
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<td></td>
<td></td>
<td>thiamin · HCl</td>
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<td></td>
<td>Vitamin B₁₂ · HCl</td>
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<td></td>
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</tr>
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VITA

John D. Morrey

Candidate for the Degree of

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