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Veterinary Science

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The Response of the Porcine Fetus Exposed to Porcine Enterovirus, Transmissible Gastroenteritis Virus or Parvovirus

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by


The Ohio State University

1973

Approved by

E. H. Bohl, Advisor
Department of Veterinary Preventive Medicine
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INTRODUCTION

A major problem confronting the swine industry is high mortality occurring during various stages of gestation and shortly after farrowing. Increasingly complaints are being reported regarding a high percentage of mummies, stillbirths, abortions, weak pigs at birth, breeding problems, and the "pseudo-pregnant" sow or pregnant sow syndrome. These problems are frequently reported by owners of newly established herds, or herds with a history of new additions. The problem is often associated with replacement gilts; however, older animals may be involved.

With emphasis on efficiency of production it becomes increasingly important to elucidate those factors that may influence reproductive performance. Changing management programs such as establishment of specific-pathogen-free breeding herds, development of artificial insemination procedures, and animal intensification with confinement rearing require additional insight into those factors that may affect reproductive performance.

Various viral agents have been suggested or proved to be involved in some of these reproductive problems. In
addition to the porcine enteroviruses, the viruses of Hog Cholera, Pseudorabies, Japanese encephalitis-B virus, Japanese hemagglutinating virus and parvovirus have been shown to cause fetal infection, resulting in stillbirths, malformation, embryonic death, or infertility (26,27,44,46, 52,67,68,131,132).

Too frequently, either because of lack of definitive diagnostic capabilities or because the more standard diagnostic procedures (e.g., Brucella and leptospira serologic tests) prove negative, certain viral agents are assumed to be involved with porcine reproductive problems when, in fact, this may not be true. Recent reports (46,47) have emphasized the porcine enteroviruses (SMEDI viruses) as being involved in intrauterine infections resulting in stillbirths, mummification, embryonic death and infertility. As a result of these reports and interest in the SMEDI viruses, diagnostic laboratories and clinicians tend to incriminate the enteroviruses in reproductive problems because of lack of evidence to substantiate other causes.

In a high percentage of cases the exact cause of fetal loss is not determined. In addition, the significance of porcine viruses as a cause of fetal infections or reproductive problems is not fully known. Too frequently evidence suggestive of a fetal viral infection is seen after
the fact, thus decreasing the chances of isolating viral agents from suspected cases.

This study was designed to help clarify the effect that certain porcine viruses (enteroviruses, transmissible gastroenteritis virus and parvovirus) have on the developing fetus with particular emphasis on the immune competence of the fetus. With knowledge of the immune capabilities of the fetus established, it is expected that retrospective studies regarding the role viral agents contribute to fetal infections could be established using the technique of "fetal serology".

Clarifying the role that certain viral agents might play with respect to reproductive performance and fetal losses of swine may lead to more extensive studies of other infectious or non-infectious factors involved in the problem of swine reproduction and abortion.
Infection of porcine fetuses with enteroviruses

Naturally occurring enterovirus infections.—Although several clinical features of porcine enteroviral infections, including polioencephalomyelitis (45,49,73,95,203), pneumonitis (13,124,169), myocarditis and pericarditis (105) have been recognized, one report (44) suggests that this class of viral agent may cross the placental barrier and infect the porcine fetus. The first association of porcine enteroviruses with reproductive failure in the United States was reported by Dunne et al. (44). They isolated an agent, cytopathic for porcine kidney cells, from a dead fetus obtained by hysterectomy at 75 days of gestation. The uterus also contained 7 live pigs, 1 other dead pig, and 3 mummies. Viruses were subsequently isolated from 4 separate herds where conditions of pigs characterized by stillbirth (S), mummification (M), embryonic death (ED), and infertility (I) were present. The virus isolates were therefore termed the "Smedi viruses". Virus characterization studies placed the "Smedi" isolates in the category of picorna viruses (enteroviruses) of swine.

Cartwright and Huck (26) in England isolated 15 enterovirus
viruses from material in herds affected with infertility, abortions, and stillbirths. Five of the isolates belonged to the V-13 group (Smedi-A), 3 to the F-34 group (Smedi-B), 2 to the Teschen disease virus group (Smedi-C) and 1 to the T80 group of enteroviruses. The most common isolate, however, was a small deoxyribonucleic acid (DNA) virus of the parvovirus group. Betts (14) reports that Steck and Addy of Switzerland isolated 30 viruses from 47 aborted fetuses originating in 28 litters from various parts of that country. The isolates belonged to 2 serological groups with some cross-reaction with Smedi-B or Smedi-C viruses.

Experimentally induced enterovirus infections.—In experimental studies 3 of 4 gilts inoculated with Smedi-A virus when 25 days pregnant subsequently had abnormal litters (14). Eleven mummified fetuses were delivered by 1 gilt, the second gilt had 4 live pigs, which died within 6 hours, and 5 mummified fetuses; while the third gilt delivered 1 pig with atresia ani, 1 mummified fetus and 6 normal piglets. Nine apparently normal piglets were delivered by the fourth gilt, although only 7 of these survived for as long as 5 days. Smedi-B virus also caused embryonic death and mummification when injected into pregnant gilts at the 25th day of pregnancy.

The comparative effects of Smedi viruses and Hog Cholera virus on porcine embryonic, fetal, and neonatal survival have been reported (17). Both viruses had an
effect upon the survival of the embryo (conception to 30
days), the fetus (from 30 days of gestation until birth)
and the neonatal pig (birth until 5 days after birth).
Embryonic infection was usually characterized by death and
absorption of the embryo with return to estrus. Fetal in-
fection resulted in death with mummification of one or more
fetuses and occasionally all fetuses in the uterus. In-
fection established in early gestation produced nondescript
effects until after birth and varied from a persistent
viremia (hog cholera infection) to an undefined lack of re-
sistance in the newborn (as in Smedi infection). Hog
Cholera vaccinal virus was more virulent than Smedi virus
and fetal infection could be established in the middle tri-
mester of pregnancy. In contrast, Smedi viruses were less
virulent and produced the most pathologic changes when the
dam was infected during the first 30 days of pregnancy.
Fetal deaths in immunized pregnant gilts exposed to the
viruses compared favorably with noninfected control animals.

Infected susceptible pregnant gilts with Smedi viruses
at 21 to 27 days gestation resulted in average litter size
of 6.7 pigs with 2.4 mummified fetuses and a 5-day post-
natal survival average of 2.9 pigs compared to 9.7, 0.31,
and 7.3 respectively for the controls (49). Infected sows
had 3.7 excess corpora lutea (CL) and the controls had 1.3
per litter. The embryonic loss associated with SMEDI virus
infection at 21 to 27 days gestation was calculated to be
2.4 per litter. No pathological effects were noted in the sows as a result of the infection. Virus isolation was difficult both in the field and experimental cases. Occasionally histopathological lesions were seen in the brains of neonatal pigs consisting of perivascular cuffing with round cells in the brain stem, and mild focal gliosis. Epizootiology as related to fetal infection.—Since Moscovici et al. (134), in 1956, reported isolating a cytopathic agent from the feces of a suckling pig with diarrhea, there have been several reports (11,12,70,111,126,127,141, 148,169,189,201) revealing the widespread distribution of enteroviruses in the porcine population based upon viral isolation from feces.

In addition to viral isolations, serologic surveys have revealed the worldwide distribution and high infection rate of enteroviruses in the porcine population. Significant antibody titers against enteroviruses were found by Wenner et al. (225) in 134 of 186 porcine serum samples. In this survey in Missouri, pigs from 38 of 41 farms gave positive reactions. In checking for a particular enterovirus, Webster in New Zealand (223) found 29 of 56 sera from an abattoir had titers in excess of 1:50. Studies reported from England have revealed the presence of serum-neutralizing (S-N) antibodies in high titer to several strains of porcine enteroviruses in serums of swine in England, United States, Africa, West Indies, Australia, and Sweden (25,77).
Another report revealed a high percentage of serums contained antibodies to 2 strains of porcine enteroviruses in several herds from California, Nebraska, and Hawaii (229). It is readily apparent, based on serological surveys and virus isolations, that various strains of enteroviruses are widely distributed throughout the porcine population.

**Sero**types of *porcine enteroviruses.*—Although earlier attempts at classification of porcine enteroviruses were based essentially on pathogenicity or biophysical properties, the tendency recently has been to follow current medical virology terminology and use the general term "porcine enteroviruses". The group is subdivided on the basis of antigenic differences and a letter or number is frequently used to distinguish the different serotype.

Bohl *et al.* (18) determined the presence of 5 antigenically different groups of viruses isolated from the feces of swine. These isolates have been designated ECPO (enteric cytopathic porcine orphan) -1, -2, -3, -4, -5. Subsequently, viral isolates designated as ECPO-6 and ECPO-7 were added to the list (181, 182, 183). At least 10 serologically distinguishable groups have been observed in Great Britain (5) and in Denmark (148). Eight groups have been reported from Sweden (171), 4 from Japan (128), and 16 types in Hungary (202). Dunne *et al.* (45) recorded 15 different serologic types in the United States. Petti of Germany (140, 142) determined 5 serologic groups among 52
porcine fecal viruses isolated.

Attempts have been made to compare and determine the antigenic relationships that exist among the porcine enteroviruses isolated in different laboratories or countries. Comparisons between strains have been made by Sibalin (170), Huck et al. (78), Szent-Ivanyi (202), Dunne et al. (45), Morimoto and Watanabe (129), Morimoto et al. (130) and Wang and Dunne (219).

Dunne et al. (45) determined the presence of 3 serologically distinguishable groups of viruses involved in the SMEDI syndrome of pigs. The Smedi viruses were not related serologically to any of the viruses reported in the United States or in Europe to be causes of reproductive failure or congenital infection (44). Subsequent serologic studies revealed the Smedi viruses to be serologically related to other porcine enteroviruses (219). Smedi-C virus was found to be related to the Teschen group of viruses, while Smedi-A virus was cross-neutralized by members of the V-13 group and Smedi-B by F-34 (26). Further serologic comparative tests revealed the Smedi viruses to be related to many porcine enteroviruses isolated in North America, Japan, and Europe (12,128). According to a report by Wang and Dunne (219) the ECPO-6 (122) virus used in this study belongs to the P-6 group of North American Porcine Picarnoviruses and is serologically related to Smedi-B (Pennsylvania) (44), F-34 (England) (26), PEI (Ontario, Canada) (104), and the
02b (Columbus, Ohio) (91) strains of enteroviruses. 

Occurrence of enterovirus infections with respect to age.--

Working with 1 strain of enteroviruses and 1 population of pigs, Beran et al. (11) concluded that the virus was widely distributed in pigs 5 to 10 weeks of age and less commonly in younger pigs. The suggestion was made that colostral antibodies prevented the infection during the first few weeks of life. Bohl et al. (18) reported isolating enteroviruses from a high percentage of normal or sick pigs most of which were made from pigs over 5 weeks of age. Wenner et al. (225) reported isolating enteroviruses from 4 of 13 pigs 5 to 6 weeks of age; 3 of 11 pigs with diarrhea and 1 of 2 apparently healthy pigs. In contrast, enteroviruses were not isolated from 28 pigs 1 to 2 weeks of age. Twelve of these were scouring and 16 were apparently healthy. 

Rasmussen (150) reported the results of experimental infections using a strain of enterovirus isolated from the intestinal tract of a 6-month-old pig which showed no signs of disease. 

Further studies indicated that at least 10 serologically different enterovirus types occurred quite frequently in Danish swine herds (149). The results indicated massive and extensive virus excretion in the feces from young pigs (2 months old) and a decrease of the excretion with age, confirming the findings of others (11). The fact that many older pigs excreted virus indicated the possibility of
virus transfer from generation to generation.

Derbyshire et al. (43) reported on virus isolation attempts from tissues of 24 healthy pigs representing 4 age groups from a closed conventional herd. An adenovirus was the only virus isolated from the tissues of 6 sows, 6 porkers, and 6 baby pigs. This was isolated from the ileum of 1 of the porkers. In contrast, each of 6 weanling pigs yielded "V-13 type" enteroviruses, 4 yielded "T-80 type" enteroviruses and 4 yielded adenoviruses. The above results on the infrequency with which viruses were isolated from the tissues of baby pigs, porkers, and adults as opposed to weanling pigs corresponds with the infrequent fecal excretion of viruses in these age groups as recorded in earlier studies (42). Izawa et al. (82,83) isolated 4 agents, 3 of which were from a group of 19 pigs, 30 to 40 days of age, from a herd repeatedly affected with enteritis. The fourth isolate was from 1 of 3 three-month-old apparently normal pigs purchased as kidney donors. The first 3 isolates from the younger pigs were serologically homogeneous but appeared to be distinct from the fourth isolate serologically, in plaque morphology and in heat stability.

Reports by Wenner et al. (225) and Szent-Ivanyi (201) provide insights into the pattern of enteroviral infections of pigs. In general, their results indicated that viruses could be frequently isolated from the feces of pigs near weaning time (about 8 weeks of age) but only occasionally from pigs under 3 weeks of age or from mature swine.
A recent report by Singh et al. (183) tends to confirm the results of the previous reports. In this study enteroviruses were first isolated from the feces between 34 to 64 days of age from each of 26 pigs representing 4 litters. Pigs from a given litter began shedding virus in their feces at about the same time, usually within 1 week, and the type of virus initially recovered was usually the same. During the observation period of 6 months subsequent waves of infection with different enteroviruses occurred. At least 6 antigenically different viruses were isolated from this herd over a 26-month period.

Singh et al. (183) concluded that the absence of detectable infection the first few weeks of life could probably be explained on the basis of protection provided by the presence of specific neutralizing antibodies (obtained from colostrum or milk) in the alimentary tract. Prenursing serum from a pig was negative for antibody against ECP0-5 virus but at 24 hours of age (post-nursing) the antibody titer was 1:256. The serum antibody titer of the pig gradually decreased to 1:16 at 119 days of age but increased to 1:256 on the 185th day. The second rise in antibody titer was presumably due to an active infection of the virus.

The onset of infection of pigs with enteroviruses appears to be much like the infection of mice with Theiler's virus and calves with enteroviruses. Olitsky (138) re-
ported that Theiler's virus was not detected in the feces of suckling mice up to 12 days of age but was present at the age of 20 days or later. Oliver et al. (30,31) were unable to isolate enteroviruses from calves less than 4 weeks of age although 11 of 12 calves were detected as viral shedders between 4 to 12 weeks of age, and again it was postulated that this delayed infection may have been due to neutralizing antibody in the ingested colostrum or milk.

In contrast to the naturally occurring situation, colostrum-deprived newborn pigs reared under pathogen-free conditions were readily infected with porcine enteroviruses (6,48). The results of these studies using ECP0-6 virus suggested an initial and rapid multiplication of the virus in the intestine and adjacent lymph nodes, followed by a viremia extending through the 5th day, localization in the CNS with the highest viral titer occurring at about the 6th day, and the presence of the virus only in the intestine after about the 11th day. Serum neutralizing antibodies were detected as early as the 6th day, at which time virus could no longer be detected in the blood.

Epidemiological studies regarding porcine fetal infections with enteroviruses have been reported by Dunne et al. (44,48). In an extensive study of herd cases evidence of infection with SMEDI viruses was first suggested by the presence of an abnormal number of stillborn pigs and mummi-
field fetuses, followed by breeding problems such as barren sows or repeat breeders. The condition appeared to recur only at periodic intervals, possibly following the introduction of new breeding stock or the introduction of new strains of virus. If repeat breeding was not a problem, sows that had farrowed abnormal litters subsequently conceived and delivered large normal litters without complications. On one farm 2 different viruses (Smedi-A and -B) were isolated from fetuses several months apart, suggesting possible alternate involvement with 2 or more SMEDI viruses. Dunne et al. (44) concluded the mode of transmission appeared to be either oral or respiratory. Observations on the random positions of dead fetuses in the uterus and the ages at which they died suggest that the virus in field cases must have entered the uterus through the blood stream. This theory is supported by experimental work using immune and susceptible pregnant animals for exposure studies (44,47).

Infection of porcine fetuses with parvovirus

Naturally occurring parvovirus infections.--Cartwright et al. (26) reported their results of virus isolation and serologic studies from herds with problems of infertility, abortions, and stillbirths in pigs. One-hundred-and-eleven viral isolations were made, 15 of which were classified as enteroviruses. The 96 remaining viral isolates were of the
same type, of which the prototype, FS 59e/63, was isolated from the colon of a stillborn pig. Studies on the physical and biological properties of the prototype virus suggested that it is a member of the picodna or parvovirus group (27). Tissues from which the virus was isolated included the colon, liver, lung, spleen, spinal cord, cerebellum, and kidney of stillborn or neonatal dead piglets. In common with Cartwright et al. (26, 27), others (87, 88) have reported isolating paroviruses from aborted, stillborn and normal pigs.

Serologic studies conducted on 430 sera from pigs in southeast England revealed 33% had positive titers to the FS 59e/63 virus (28). In herds experiencing infertility problems rising antibody titers to the virus were also demonstrated. The reported isolation of this virus from semen suggested the possibility that service by infected boars may contribute to the condition of "repeat breeders" (26). Mayr et al. (114) have reported on the morphological, physical and chemical characteristics of a virus isolated from uninoculated primary porcine kidney cell cultures derived from healthy 3-week-old pigs and proposed the virus as a member of the picodna virus group (Parvovirus).

Bachman (7) isolated a PPV agent from tissue cultures from the kidneys of 5 out of 40 conventionally reared 2- to 3-week-old piglets. Mengeling (116) in a recent report suggested the possibility of fetal infection with PPV based on serologic studies using prenursing serum. Morimoto,
from Japan, recently reported isolating parvovirus from brain tissue of stillborn pigs (132, 133).

Experimentally induced parvovirus infections.—In one study 5 of 6 pregnant gilts infected with parvovirus by the intravenous route 21 to 28 days after service farrowed normally; the sixth proved barren (26). The virus was isolated from tissues of stillborn piglets and from live piglets killed before nursing. In another study susceptible pregnant gilts infected orally or intravenously during the last half of pregnancy farrowed without any difficulty (88). There were only 3 stillbirths recorded in 52 piglets born and these occurred in a litter of 16. The virus was regularly isolated from kidneys, liver, and testicles of representative members of each litter sacrificed between 1 and 7 days of age, and irregularly between 7 and 9 days of age. This virus isolation was possible despite the presence of antibody in the serum. An interesting result of this study was the finding of very high levels of H-I and SN antibody to the parvovirus in 2 or 3 stillborn, and 24 of 28 newborn piglets bled before nursing. The antibody levels remained high in the piglets, independent of whether they received colostrum, for up to 4 months of age. Cartwright et al. (28) and Johnson (88, 89) have reported results of experimentally infecting pregnant swine with parvovirus and concluded that the virus can infect the fetus by the transplacental route in susceptible animals. A common
feature of both studies was absence of disease in the infected sows, normal farrowings, little or no effect on the piglets, and detectable antibody in some piglets at birth.

Cartwright et al. (28) infected 2 serologically negative gilts at the time of service by mixing parvovirus with the semen. One gilt farrowed 3 normal piglets on the 115th day. However, the ovaries of the gilt contained 16 CL and a possible reabsorption site was detected in the right uterine horn. The second animal was examined at term. Each uterine horn contained 1 large fully formed fetus and 1 mummified fetus. Seventeen CL were present on the ovaries. In contrast, Johnson (89) was unable to affect litter size or fetal development by instilling the virus into the uterus of serologically positive gilts coincident with service. The serologic status of the gilts in these two studies may have accounted for the differing results.

Serotypes of porcine parvovirus.--Although there are several reports (7,26,27,87,114,116,133) in the literature on the isolation and characterization of a parvovirus of pigs, extensive investigation into the antigenic comparisons were not noted. Cartwright et al. (27) did demonstrate cross-inhibition between their 59e/63 virus and antisera to PRP virus (38) "Wavre" virus (81) and PPV virus strain G10/1 (114). All 7 viral isolates reported by Johnson et al. (88) from normal, aborted and stillborn piglets were antigenically similar and classified as parvoviruses. In
recent reports from the United States (116) and Japan (132,133) of isolating PPV from swine no mention is made of the antigenic relationship with the parvoviruses from Europe.

**Occurrence of parvovirus infections with respect to age.**

Based upon serologic studies there would appear to be a definite age incidence of infection due to parvovirus. One study reported a sharp decline in antibody titers of pigs around 4 months of age, followed by a sharp rise in titers in stock of breeding age (27). The first response was presumably due to declining passive immunity of maternal origin and the second response was probably caused by active infection. Although parvoviruses are apparently widespread (26), Johnson *et al.* (88) concluded from their field and experimental studies that the virus is probably of little significance unless infection occurs in serologically negative gilts or sows at the time of service or during the early part of pregnancy. A recent study in the United States reported that 40.7% of 423 porcine serum samples originating from several states contained hemagglutinating inhibiting antibodies for porcine parvovirus (116). No mention was made on the age distribution of the animals tested. The serum of newborn pigs in 2 of 20 litters of pigs obtained by hysterectomy and deprived of colostrum contained H-I antibody in titers of >160 for parvovirus. In a sero-epizootiological survey Morimoto
(132) demonstrated that 10 gilts had no antibodies against PPV before breeding, but detected antibodies at the time of farrowing. The results suggested that all the gilts were infected with PPV during the period of pregnancy.

**Infection of porcine fetuses with other viruses**

The viruses of hog cholera, pseudorabies, Japanese encephalitis, Japanese hemagglutinating virus, are known to be associated with transplacental infection of the porcine fetus. Circumstantial evidence has been used to incriminate the viruses of TGE and swine influenza as possibly being involved in fetal losses of swine.

**Hog Cholera.**—The hog cholera virus has been strongly implicated in transplacental infection resulting in fetal death, malformation and stillbirth (24,52,53,166,188,234, 235). Young et al. (235) were successful in initiating embryonic infection with attenuated hog cholera virus during the first third of the gestation period. Additional evidence has shown that the hog cholera virus could invade the uterus of nonimmune sows at practically any stage of pregnancy. During the first 30 days of gestation injected virus caused embryonic death with absorption and return to estrus or small litters. The greatest numbers of mummified fetuses resulted from exposure to the virus at 60 days of gestation, and exposure at 90 days caused the greatest number of stillborn. Within 1 week of term, in-
fection did not affect viability of the newborn pig, but the virus was isolated from them (33,46). Immune sows were not affected by viral injection and normal litters resulted (46,199). Carbrey et al. (23) suggest the possibility that immunologic tolerance may be involved in the condition when the hog cholera virus infects the fetus and persists. One of the viral strains used by Carbrey et al. (23) produced a syndrome resembling immunologic tolerance similar to the state observed in mice infected with lymphocytic chorio-meningitis (LCM) virus (75,137).

Sautter et al. (163) reported that edema, ascites, mottling of the liver, asymmetry of the head, and lengthening and twisting of the snout were the most common abnormalities observed as a result of exposing the sow at the 70th day of gestation. Several workers (52,71,79) have observed congenital tremors, cerebellar hypoplasia, and hypomyelogenesis as a result of prenatal infection with hog cholera virus. Other lesions in neonatal pigs as a result of in utero infection included hemorrhages of the skin, focal necrosis of the liver, and widening of the epiphyseal line at the costochondral junction. Degenerative changes were seen in the placenta in the areas where dead fetuses were found.

Under experimental conditions the hog cholera virus could be isolated from the spleens of malformed fetuses, but not from the spleens of their dams (235). Specific
areas of damage or mechanisms were not determined. Stair et al. (190) reported the value of the fluorescent antibody test for studying embryonic infection with hog cholera virus. Others have reported the use of the fluorescent antibody tissue culture technique for detecting hog cholera virus both from stillborn pigs and from pigs born alive but living for only a short time (24, 33, 46).

Pseudorabies.—Pseudorabies was reported from Ireland as the cause of many weak pigs at birth which died soon after birth, and the delivery of stillborn and mummified fetuses (65). There have been similar reports associating pseudorabies virus with abortion in swine from the Netherlands (3, 205). A similar condition associated with pseudorabies has been described in the United States (67, 161).

Infection of swine is often inapparent (51, 110). Experimentally the natural route of infection has been shown to be by way of the susceptible cells in the nasopharynx, laryngopharynx, and oropharynx (35, 108, 112, 162). Venereal transmission of pseudorabies infection to either sex during copulation has been reported (4).

Experimental exposure of a susceptible sow 10 weeks pregnant resulted in 5 days of illness with recovery; however, six weeks later at the end of gestation, 11 mummified fetuses were delivered (65). In another study susceptible pregnant sows were injected with pseudorabies virus 3 to 5 weeks before farrowing which resulted in the intrauterine
death of the fetal pigs (235).

Gustafson et al. reported that abortions were common following infection of pregnant swine during the first month of pregnancy. However, infections later in gestation resulted in fetal death and mummification (67,68). Newborn pigs from infected sows often died within 3 days (67, 76). Gustafson and Mitchell (66) did not find virus-neutralizing antibodies present during the first week of life of colostrum-deprived piglets born alive from infected sows. Stair (191) reported results similar to others (36,65,68) in which pseudorabies virus was isolated from fetal tissue. Using a conjugated serum and immunofluorescent procedures (190) Stair (191) reported the greatest concentration of pseudorabies antigen to be present in the chorionic epithelium but it was also found in fetal brain, lymph nodes, and spleen. The pseudorabies antigen was not detected in tissues from viable fetuses.

**Japanese encephalitis virus.**—Subsequent to a report by Matumoto et al. (113) a number of strains of Japanese encephalitis virus have been isolated from apparently congenitally infected stillborn fetuses (99,167,204,210). Experimental work by Shimizu et al. (168) confirmed Japanese encephalitis virus as the causative agent of swine stillbirth observed in Japan during the summer months.

Morimoto (131) reviewed the literature regarding the
epizootiological and etiological aspects of swine still-birth associated with Japanese encephalitis virus.

As a result of experimental vaccination trials (92) a research committee was established on the prevention of stillbirth in sows due to Japanese encephalitis virus (152). Kawakubo et al. (93) reported encouraging results with their experimental vaccination trials. The average number of normal young delivered was larger by 2.3 to 3.7 per litter in the vaccinated than in the control sows. Of the 2 strains studied, there was no appreciable difference either in the prevention effect on stillbirth or in the development of antibodies.

Japanese hemagglutinating virus.--Another example of virus-induced fetal death is Japanese hemagglutinating virus isolated from stillborn pigs. The virus is classified as a myxovirus parainfluenza I and has been isolated from humans (228), swine (159,222) and mice (60). Sasahara et al. (160) experimentally produced 3 cases of stillbirth by intranasal instillation of the virus in pregnant sows. Of 6 inoculated animals, 1 sow delivered 7 mummified fetuses following exposure to the virus 39 days after breeding. Another sow exposed 43 days after breeding, delivered 5 mummies and 4 stillborn fetuses. The 4 remaining sows were infected with the virus at later stages of gestation and delivered normal living pigs, except for 1 sow that produced 1 stillborn fetus. Hydrocephalus or cerebromalacia,
sometimes seen in experimental cases of stillbirth with Japanese encephalitis virus (168), was not observed with Japanese hemagglutinating virus.

Although it is clear that the hemagglutinating virus of Japan has the capability of producing stillborn in pregnant swine, the true significance of the virus in field outbreaks of stillbirth among swine remains unknown. 

Influenza.--Ray (151) incriminated swine influenza virus as the cause of fetal death in swine based upon clinical evidence. Experimental infection of pregnant sows resulted in frequent occurrence of dead and/or mummified fetuses and dead piglets soon after birth (117,118). In these studies influenza virus was isolated from the organs of stillborn piglets and from the placenta of the sow. Other workers have considered the effect of swine influenza virus on the developing fetus and the potential influence the influenza virus contributes to suckling pig mortality (230,231,232, 233). They concluded that the most pronounced effect of the virus on the fetus occurred when the dams were inoculated during the first month of pregnancy. Woods (227) mentions that intravenous exposure of pregnant gilts to influenza virus resulted in farrowing of weak pigs, higher mortality than in controls during the first 3 days after farrowing, and stillbirths and mummified fetuses.

Transmissible gastroenteritis (TGE).--Regarding TGE virus, Dunne (49) suggests that there is strong support of a
clinical nature to incriminate the virus as being capable of disturbing embryonic growth if sows are infected during the first month of pregnancy. Additional research is necessary to substantiate the claim.

**Immunologic response of the porcine fetus**

In recent years it has been shown that the fetuses of several mammalian species are able to produce an immune response if stimulated at the proper age with adequate amounts of certain antigens (125,198). Comparatively little is known about the capacity of the porcine fetus to produce antibodies. The fetal pig should be an excellent model for studying fetal antibody production. The epitheliochorial placenta of the pig does not allow passage of immunoglobulin molecules from the dam (97,144,197). Sterzl et al. (194) could not find any trace of antibodies being transferred from either passively or actively immunized sows.

The observation of Myers and Segre (135) that antibody activity could be found in concentrated sera from newborn pigs was not confirmed by Sterzl et al. (197) using the same antigens and concentration procedures. Kim et al. (96) using very sensitive antibody-detecting techniques also concluded that transplacental transfer of immunoglobulin does not occur in the normal sow, but it can occur when placental tissues are damaged by *Escherichia coli* endotoxin. Traces of small molecular weight gamma-globulins have,
however, been detected by Sterzl et al. (193) in newborn piglet sera but they did not possess any demonstrable antibody activity. Kim et al. (97) concluded that the de novo synthesis of antibody in utero does not occur and, therefore, the colostrum-deprived germfree pig appears to be "immunologically virgin".

If immunoglobulins are found in fetal or newborn pig serum, it would appear that they must be of fetal origin following antigenic stimulation or be passively transferred from the dam across a damaged placenta. Sterzl et al. (195) detected antibody formation in newborn piglets after immunizing the fetus in utero one month before term with sheep erythrocytes, but not in newborn piglets similarly stimulated with Salmonella paratyphoe B and Brucella suis. By using various attenuated Leptospira cultures intra-amniotically on the 60th day of pregnancy, Fennestad et al. (56) concluded that the porcine fetus in utero is able to produce gamma-globulin and leptospira agglutinin at least 2 weeks before term. Hajek et al. (69) reported on the antibody-forming capacity of fetal pigs following injection of ØX174 bacteriophage via the umbilical vein. Four days later $^{131}$I-labelled bacteriophage exhibited immune-type elimination from the blood and neutralizing antibody was detected in the serum. Antibody production appeared to be directly proportional to antigen dose and the antibody was susceptible to 2-mercaptoethanol (2-ME) treatment.
Mensik (119) detected antibodies against *Myxovirus influenzae suis* by the hemagglutination-inhibition (H-I) test in precolostral serum of all 10 (sow II) piglets from the second litters of 2 sows. Sow I was inoculated with the myxovirus sub-cutaneously once during the fourth month of gestation and sow II on 4 occasions at 7-day intervals during the first month of pregnancy. Antibodies were not demonstrable in pre- or post-colostral serum of piglets from 2 serologically negative sows. The above work confirmed the observations of Young et al. (231) regarding the presence of specific antibodies to viral antigens (*Influenza suis*) in the precolostral serum of piglets immediately after birth. Further work by Mensik and Franz (120) revealed that H-I antibodies insensitive to 2-ME were present in a tenfold concentrated pooled pre-colostral serum sample of piglets from a persistently serologic positive sow which had been inoculated with live myxovirus when 14 days old. The presence of gamma globulin was confirmed by immunoelectrophoresis. Antibodies sensitive to 2-ME were found in the precolostral serum of 4 of 11 piglets from a sow similarly inoculated when 4 months old and again 18 days before farrowing. On gel filtration of a pooled precolostral serum sample with Sephadex G-200, antibodies detected in fractions of the second peak were also sensitive to 2-ME. The possibility of active fetal antibody production or transplacental transference was discussed.
Recent work has shown that in 1 gilt inoculated with PPV 78 days after service, 9 of 11 unsuckled piglets had H-I titers ranging from 20 to 1280 (28). The titer rose after suckling to the level of the colostral titer but failed to follow the usual pattern of declining maternal antibody, since titers of 5120 were recorded at 4 months. One surviving negative piglet had no detectable antibody at 95 days. This suggests that the initial titer was due to an active intrafetal stimulus rather than a passively acquired maternal antibody.

Binns (17) reported on the immune response of pig fetuses injected with allogenic white cell suspensions at 60, 80, or 104 days of gestation. Definite differences were found between homograft rejection patterns in untreated piglets and in piglets exposed in utero or at birth to the transplantation antigens of an unrelated or distantly related donor. Immunelectrophoretic analysis of presuckled serum showed the presence of a clear immunoglobulin line in all piglets injected with cells at 60 or 80 days gestation. Control uninjected newborn litter mates rarely showed more than a trace of immunoglobulin. A clear immunoglobulin line was not found in the piglets injected with cells 8 days before birth. With the antigen used, 8 days was presumably too short a period for the development of sufficient serum immunoglobulin.

Using a flagellar antigen of *Salmonella oranienberg* in
55- or 60-day fetuses, Binns (16) failed to prime the fetuses for a challenge injection of the antigen after birth. In contrast, injection of the antigen at 80 to 104 days of gestation resulted in detectable antibody when the serum was tested at birth. These piglets were also primed and exhibited a typical secondary response when challenged with the antigen at 10 days of age. A recent report by Schultz et al. (165) indicates that the pig fetus is immunologically competent, as determined by the Jerne hemolytic plaque technique, to produce antibodies to sheep red blood cells (SRBC) by the 74th day of gestation. In this study the serum hemagglutinins of fetuses and newborn pigs were tentatively classified as belonging to the immunoglobulin M class. In contrast, pig fetuses inoculated at the 58th and 65th days with SRBC did not have plaque forming cells (PFC) in their spleens 6 days later. Age equivalence data of Solomon (187) suggests that the antibody-forming ability in fetal pigs should appear at about 58 days of gestation.

There are a number of reviews in the literature concerning the serum proteins or classes of immunoglobulins present in fetuses and newborn pigs (21,145,187). Opinion on the presence of $\gamma$ globulin in fetal or newborn pigs varies considerably from author to author. Brummerstedt-Hansen (21) presents an excellent review of the literature concerning the controversy.
Franck and co-workers (57,58) found that the molecular weight of newborn pig immunoglobulin was lower than that of adults and further studies revealed single polypeptide chains which corresponded in chromatographic and electrophoretic behavior to heavy and light chains of normal IgG. This finding was not confirmed by Kim et al. (97,98). A recent report by Prokesova et al. (146) determined the presence of an immunoglobulin with antigenic specificity corresponding to IgG in the serum of precolostral germfree piglets. This immunoglobulin had a sedimentation constant of 4S and carried antigenic determinants of both heavy and light chains. Antibody activity has not been demonstrated to the small amount of IgG present in newborn piglet serum. Further work by Prokesova et al. (147) confirmed previous results that newborn piglet serum does not contain any demonstrable IgM and that the first immunoglobulin detected in newborn colostrum-deprived piglets is of the IgG antigenic type.

Immunologic response of fetuses of other species

As early as 1904 evidence began to accumulate indicating that the fetus of certain species was able to respond to antigenic stimulus in utero (103). It has generally been thought, however, that the mammalian fetus does not engage in the active production of gammaglobulin in utero (20,50). The early notion that the mammalian fetus was
immunologically incompetent was supported by evidence on the inability of neonates to form antibodies, and on their susceptibility to the induction of immunologic tolerance (72,186) and to the inhibition of immunogenesis as a result of thymectomy (64). During the last few years the evidence has been accumulating quite rapidly that the mammalian fetus from a variety of species is able to respond to an antigenic stimulus (63,177,198). These studies indicate that developing animals, still immature from other points of view, are able to elicit immunological reactivity. It has been shown that the fetal lamb and fetal Rhesus monkey are able to form antibodies during the first half of gestation (174,178) while the very immature oppossum in the pouch forms antibody as early as the fifth day (157), at a time when its lymphoid tissues are still relatively immature. The human premature newborn may be stimulated to form antibody (213) and to develop delayed hypersensitivity (212), while the human fetus responds to congenital infection with plasma cell formation (173). The calf in utero (54,55) and the immature oppossum in the pouch (90) are able to produce antibody, the fetal guinea pig develops delayed hypersensitivity (211), and the fetal lamb is able to reject homografts specifically (176).

Various methods or systems both in vivo and in vitro have been employed in studying the immune response of fetal
animals. Determining the kinetics of antigen elimination and early appearance of antibody (179, 180, 214, 215), the rejection of orthotopic skin homografts (41, 115, 176, 180) and the cellular kinetics of the primary antibody (85, 180) response have been used to test the concept of immunologic immaturity of the developing mammalian fetus. By studying the incorporation of $^{14}$C labelled amino acid into immunoglobulins by tissues in vitro it has been concluded that various mammalian species synthesize immunoglobulins during the gestation period (74, 207, 226).

Following the conclusion that fetal animals may respond to antigenic stimuli, it became necessary to study the variations of the response, as a function of gestational age of the fetus, the nature of the antigen used, and the duration of the stimulus. Silverstein et al. (174) reported on the nature of the antibody response in fetal lambs as a function of gestational age, duration of stimulus, and type of antigen. Using an antigen mixture containing viable Bacillus Calmette-Guerin (BCG), heat-killed Salmonella typhosa, ovalbumin, and ferritin, all emulsified in Freund's adjuvant, fetal lambs at about 70 days were injected intramuscularly. Fetuses were obtained at intervals of 10 to 60 days after injection and the serum examined for antibody response. Antibodies against the Salmonella or BCG components of the mixture could not be detected in any of the fetal serum. Antiovalbumin was not found in any of the
fetuses within the first 40 days following injection, and only 60 days after injection did a fetus show evidence of small amounts of antibody to the antigen. Ferritin appeared to be a better stimulus since all fetal lambs responded with anti-ferritin production as early as 10 days after the injection of the antigen. This same procedure was repeated in 89- to 101-day fetal lambs with essentially the same results.

It is apparent from the previous tests that the nature of the antigen may influence the fetal antibody response. Using bacteriophage ØX174 and diphtheria toxoid, Silverstein et al. (174) studied the response of stimulated fetal lambs ranging from 60 to 118 days gestational age. Circulating diphtheria antitoxin was not found in any of the immunized lambs regardless of the duration of the stimulus. All fetal lambs responded with circulating antibody against the bacteriophage. Even the 60-day-old fetus produced small amounts of antibody to the phage within 6 days. It is apparent that the fetal lamb does not become competent to all antigens at the same time. Competence with respect to S. typhosa and BCG is known to develop as the animals grow older (10). Even among those antigens to which the fetal lamb is capable of responding by the production of antibody in utero, there appears to be a hierarchal rank to the response. The earliest response is found against bacteriophage at a given time in gestation. With longer dura-
tion of stimulus antiferritin appears, and finally in the case of the 90-day fetus, antiovalbumin some 3 weeks later. In general, the amounts of antibody produced against the three antigens followed the same order: anti OX > antiferritin > antiovalbumin (174). The development of immunologic competence against different antigens at different stages of gestation appears also to be true for the fetal Rhesus monkey (180).

In an experiment using pregnant sheep exposed to bovine virus diarrhea-mucosal disease virus Ward (221) found fetal death, mummification, central nervous system lesions, and specific bovine virus diarrhea-mucosal disease (BVD-MD) antibodies present in the offspring. It has also been shown that calves born to cows experimentally exposed to BVD-MD may have typical lesions as well as serum antibody titers (220). The BVD-MD antibody titers in these calves did not decline with time suggesting active antibody production instead of passive immunity (220). Classick et al. (29) confirmed the ability of the bovine fetus to become infected with BVD virus and to produce neutralizing antibodies.

McKercher et al. (109) provided evidence, based on fetal serology, of an etiologic role for Bluetongue (BT) virus in hydroencephaly of calves. The information was disclosed through the circumstances that 2 of 3 hydroencephalic calves failed to nurse. The 1 calf that did
nurse possessed the same complement of serum antibody (BT, BVD, and IBR) as did its dam. The 2 calves that did not nurse had antibody to BT virus only. On this basis they concluded that the 2 calves had been exposed to Blue-tongue virus in utero.

Biberstein et al. (15) in an experiment using ram epididymitis organism (Brucella ovis) concluded that fetal lambs can respond immunologically to this naturally occurring infectious disease. In order to study the non-reactivity of the newborn lamb to Brucella antigens Richardson et al. (154) immunized fetal lambs with Brucella antigens. They concluded that the ovine fetus can synthesize high levels of anti-Brucella agglutinins when immunized late in gestation in contrast to the low levels attained when the response was initiated in newborn lambs. Further work indicated that the fetal lamb could be stimulated by the oral route with the Brucella antigen and that a secondary response comparable to that of adult sheep could be elicited in fetal and newborn lambs by killed Brucella abortus (155, 156).

Thorbecke et al. (207) and Van Furth et al. (217) reviewed the data on the initiation of immunoglobulin synthesis in various mammalian species. By using an in vitro cell culture system of studying the incorporation of C¹⁴ labelled amino acids into immunoglobulins one can detect the synthesis of small amounts of these proteins. In the
human fetus, IgG and IgM production is regularly found in the spleen after the 20th week of gestation (217). The possibility that antigenic stimulus may have occurred in these prematurely delivered fetuses was discussed. At about the 90th day of gestation the spleen of normal sheep fetus starts to produce IgM (175). Induction of other immunoglobulins is usually not observed unless intrauterine immunization has occurred. By the 58th day of gestation the guinea pig spleen is producing IgM and this remains the only immunoglobulin produced until 1 to 2 weeks after birth (206). Spleen cultures from rabbits never showed synthesis of IgG at birth but some IgM production was detected (192). Gamma M production could not be detected from the spleens of newborn mice or rats (192). The responsiveness to certain antigens has been established in some species. In bovine embryos (54,55) the earliest responsiveness is found at approximately 80 days before birth, and in pigs (196) at approximately 30 days before birth. The opossum with a gestation period of 12.5 days can be induced to produce antibodies 8 days after birth (157).

Kelly (94) demonstrated morphologic evidence of prenatal development of lymphoid tissue during the final third of gestation in the fetal dog suggesting that dogs should be capable of responding to antigenic stimuli at least several weeks prior to birth. Jacoby et al. (84) and Dennis et al. (40,41) studied the ontogeny of the humoral response,
cell-mediated responses and the role of the thymus to test the possibility that fetal dogs could be stimulated. Their results indicated that in dogs as in other species (32, 174, 198) immune competence to different antigens develops sequentially rather than simultaneously. The fetal dog responded to stimulation with bacteriophage ØX174 as early as the 40th day of gestation, to ovine erythrocytes at about the 48th day, but failed to react against bovine serum albumin (BSA) even in postnatal life unless it was combined with an adjuvant (84).

Since it has been concluded that the immature animal in utero is capable of being stimulated by a variety of antigens the next step was to study the globulin response and the nature of the antibody.

By studying the kinetics of the primary antibody response in fetal lambs to bacteriophage (179), it has been shown that the antigen disappeared within 59 hours after immunization. As early as 54 hours after immunization IgM antibody was evident, increasing with a doubling time of about 9.5 hours. Gamma G antibody was first detected during the fourth day after immunization, and the titer slowly increased during the next 11 days. The kinetics of the primary antibody response of fetal lambs appear to be qualitatively similar to those reported for premature human newborn and older children (185, 213), for newborn and adult guinea pigs (214, 215) and for rabbits (1). The features of
the primary immune response include the immune elimination of antigen during the second day after immunization, the formation of 19S IgM antibodies with a rapid initial doubling time with no appreciable IgG antibody formation if small doses of antigen are used. Larger doses of antigen elicit the formation of IgG antibody.

It has been postulated that the time of immunologic responsiveness in the human fetus occurs about the 20th week of gestation (172). This dating is based largely on the morphologic observation that despite the presence of *Treponema pallidum* within fetal organs, morphologic inflammatory lesions associated with plasmacytoid or plasma cells do not appear until the 20th week of gestation in congenital syphilis (172). The combination of the presence of plasmacytoid cells and delayed tissue responsiveness has been interpreted as indicating immunological competence on the part of the human fetus (172).

The human IgM immunoglobulins of maternal origin are excluded from the fetal circulation; however, IgG immunoglobulins can pass through to the fetus (62). By using $^{131}$I labelled gamma globulin it has been shown that labelled gamma globulin is transferred from the maternal to fetal circulation at about 3 months gestation (37). The IgG immunoglobulin levels are low through the fourth month of gestation, thereafter the values increase, equaling the maternal values at around the ninth month, and depending
upon the mode of delivery exceed them slightly at term
(39,102,216).

Specific IgM immunoglobulins are readily demonstrable
when sought for in fetal, cord, and neonatal serum from
cases of congenital rubella (2,22,224). Since IgM immuno-
obulins of maternal origin are excluded from the human
fetal circulation, the finding constitutes presumptive
evidence that the human fetus responds in utero to the
antigenic determinants of rubella virus. McCracken et al.
(107) have shown that 25 percent of infants with congenital
rubella syndrome have appreciable elevations of their IgM
immunoglobulins. In general, they found a positive correla-
tion between these elevations and the severity of the cli-
nical involvement.

In a review regarding Herpesvirus hominis infections of
newborns, it has been stated that IgM Herpes antibody can
be produced by the human fetus and total serum IgM levels
may increase (136). Since IgM and its antibody components
do not cross the placenta under normal circumstances, de-
tection of specific IgM herpes antibody in the umbilical
cord or in serum obtained shortly after birth is indicative
of fetal Herpetic infection. It was further suggested that
increased levels of total IgM in neonatal serum offer pre-
sumptive evidence of infection in the infant and indicate
the need for further diagnostic tests.

It was suggested in a report by Gardiner (61) that the
appearance of immunoglobulins (7S IgG) in a significant proportion of pre-colostral sera, from lambs with experimental or natural Border disease (8,80), was indicative of fetal immune response to the yet uncharacterized infective agent. This observation promised the possibility of a means to identify infected lambs in any Border disease experiment. Work by Patterson and Sweasey (139) confirmed the presence of IgG in the sera from lambs out of ewes previously injected with experimental material. However, they concluded that without the support of specific serology, the mere presence of IgG in pre-colostral lamb sera can be regarded only as a general confirmation of a fetal immune response. Additional work by Gardiner's group (9) found the 7S gamma globulin evident in a greater proportion of experimental than control animals. However, they agreed with Patterson and Sweasey (139) that the relationship of this globulin to Border disease remains questionable.

Reports have repeatedly indicated that serum of the newborn calf before nursing does not contain immunoglobulin (59,86,153,184,200). However, there are a few reports mentioning the detection of traces of immunoglobulins in pre-suckling sera of calves (101,106,143). Klaus et al. (100) have identified the presence of low levels of both IgM and IgG in prenursing sera of calves. Merriman (121) demonstrated the presence of IgM, IgG1 and IgG2 but not IgA in
in the serum of newborn calves before the ingestion of colostrum. In contrast, all 4 classes of immunoglobulins were demonstrated in the serum of the calves after the ingestion of colostrum and in much higher levels. Schultz et al. (164) have also reported the identification of serum immunoglobulin in a few bovine fetuses.
MATERIALS AND METHODS

Animals

The pregnant swine used in this study were obtained from the Department of Animal Science of the Ohio Agricultural Research and Development Center (OARDC), Wooster, Ohio or occasionally from Frankenstein Farms, West Salem, Ohio. Known breeding dates were available for all animals used in this study. The animals were delivered to the isolation facilities of the Department of Veterinary Science prior to experimental exposure of the fetuses.

Surgical procedure

Food and water were withheld for 12 hours prior to the scheduled surgery. Premedication consisted of atropine (1 mg /100# body weight) given intramuscularly approximately 20 minutes prior to giving the anesthetic agent. Twenty-five ml of a 4% solution of sodium thiampylal (Surital, Parke-Davis, Detroit, Mich.) was then injected fairly rapidly into the anterior vena cava. Prior to injecting the anesthetic agent a blood sample was collected to be used as a reference specimen for various serologic procedures. The animal was placed in lateral recumbency and an inflatable cuffed endotracheal tube was instilled using a mouth
speculum and a large laryngoscope (North American Drager, Telfor, Pa.). Occasionally it was necessary to inject additional sodium thiamylal into the marginal ear vein in order to obtain adequate anesthesia for passing the endotracheal tube. Anesthesia was maintained using a closed system with a mixture of nitrous oxide, oxygen and halothane (Fluothane, Ayerst Lab. Inc., New York, N.Y.).

After induction the animal was placed in a surgical cradle and elevated to operating height. Following a surgical preparation and application of sterile towels and drape, a 20 cm long mid-ventral incision was made continuing from the umbilicus caudally. The pregnant uterus was readily exposed and individual fetuses were then injected with the experimental material. The uterus was then returned to the abdominal cavity and the incision closed in an accepted manner.

The sow was allowed to recover from the anesthesia, and was returned to the isolation pen where she remained until the termination of pregnancy, either by natural farrowing or by hysterectomy.

Viral infection of the fetus

A porcine enterovirus (ECPO-6), a porcine coronavirus (transmissible gastroenteritis (TGE)), or a porcine parvovirus was used for intrafetal infection. The ECPO-6 virus used in this study was the same as that reported previously
and was originally isolated from the feces of apparently
healthy pigs (6,122,182). This virus is reported to be
serologically related to Smedi-B virus (219). The virus
was propagated on monolayer cultures of porcine kidney (PK)
cells and represented 16 passages including 2 plaque iso-
lations. Fetuses to be infected were injected with 0.1 ml of
culture fluid containing 20X10^5 plaque-forming units (PFU)
of virus.

The TGE virus was the Purdue strain of the 111th cell
culture passage. The inoculum contained 2X10^5 PFU in 0.1 ml
of culture fluid. Characteristics of this virus have been
reported and because of its high passage in tissue culture
systems it is considered an attenuated virus (19).

The porcine parvovirus was obtained from Dr. Ben
Sheffy⁠¹ and was referred to as PPV 12 PK Cornell. Serial
10-fold dilutions of the viral preparation were inoculated
onto bottles of PK cells for the purposes of purifying the
virus by the terminal dilution technique. Viral propaga-
tion was determined by testing the fluid in the inoculated
PK cells after 9 days for hemagglutinating activity (HA).
The viral preparation which was propagated at the terminal
dilution was used for inoculating several bottles of PK
cells. These later PK cells were derived from germfree

⁠¹PPV virus kindly furnished by Dr. Ben Sheffy, Cornell
Univ., Ithaca, N.Y. under federal permit.
pigs so as to minimize the introduction of extraneous viruses through the cell culture system. After an incubation of 8 days the cell culture fluid and cells were harvested, pooled, centrifuged and dispensed in 2 ml or 5 ml amounts and stored at -20 C. This virus pool was designated PPV(14PK-11/71) and had a HA titer of 1:320.

Prior to use all material (virus, saline, and media) used for injecting into fetuses was filtered through a 0.45 μm membrane (Millipore Corp., Bedford, Mass.) and fetuses were injected directly into the biceps femoris muscle using a tuberculin syringe with a 23 ga 1-inch needle. Usually 2 fetuses were injected with 0.1 ml of sterile media or saline, and 2 with 0.1 ml of the virus material.

**Identification of injected fetuses**

Trypan blue (2.5% suspension) that had been autoclaved and filtered 0.45 μm membrane or a nonabsorbable suture material (Vetafil, Bengen and Co., West Germany) were used to identify injected fetuses. The dye (0.1 ml) was injected subcutaneously in various locations of the fetus, such as legs, base of tail and ears for reference points.

**Collection and handling of pigs**

Pregnancy was terminated either by natural farrowing at term or by a hysterectomy at a predetermined stage of gestation. Blood samples for serum were obtained from the
sows and newborn pigs. Blood was not collected from piglets if there was any doubt that the pig might have nursed. Selected piglets from a litter were euthanitized and various tissues were aseptically collected and frozen for further processing for virus isolation procedures. The remainder of the pigs were either returned to the sow or were reared in cages for further studies. In a few cases piglets were derived by a hysterectomy technique as previously reported and reared in sterile isolators (123).

Cell cultures

PK cell cultures were grown in 4-ounce prescription bottles. The procedures were similar to those described by Hancock et al. (70). The growth medium consisted of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (HBSS), 10% inactivated bovine serum, and antibiotics (100 units penicillin, 100 ug of streptomycin, 25 units mycostatin/ml). Confluent cell monolayers were usually obtained after 7 to 9 days of incubation and were then used for neutralization studies. Younger cell cultures were used for virus isolation attempts and for detection of cytopathic effect (CPE). The CP agents isolated in cell cultures from experimental animals were not identified but the CPE was typical of that seen with ECPO-6 or TGE and it was assumed that the isolated CP agent was the one that had been injected.
Serologic tests

Enterovirus and TGE.--The serum neutralization test was used to determine the serum antibody titer for ECP0-6 and TGE viruses. The test involved the neutralization of a determined amount of ECP0-6 or TGE virus by varying dilutions of heat inactivated serum, or serum fractions. The amount of unneutralized virus was detected by the plaque reduction method using PK cell-culture monolayers. Neutralizing antibody titers are reported as the reciprocal of the serum dilution which resulted in a plaque reduction of 80%. Graphic intrapolation was used to reach end point values of titers in different samples. Titers of less than 1 are reported as negative in the results. The technique of conducting the plaque-reduction test for determining neutralizing antibody has recently been reported (19).

Hemagglutination inhibition test for porcine parvovirus antibodies

Various serum samples were tested for the presence of antibodies against a porcine parvovirus. The parvovirus antigen used in the test was designated PPV(PK14-11/71).

All serum samples were heat inactivated at 56°C for 30 minutes, then treated with kaolin and guinea pig red blood cells (rbc) to remove non-specific inhibitors.

A. Treatment with kaolin to remove non-specific inhibitors.
1. 0.3 ml of serum was added to 1.2 ml of saline in a small tube, resulting in a 1:5 dilution of serum.

2. 1.5 ml of a 25% suspension of kaolin was then added to the same tube.

3. Contents were mixed and allowed to incubate at room temperature for 20 minutes.

4. The tube was then centrifuged at approximately 1000 rpm for 10 minutes.

5. 2 ml of the supernatant were aspirated and transferred to another tube.

B. Treatment of serum with guinea pig rbc to remove agglutinins.

6. 0.2 ml of a 50% suspension of washed guinea pig rbc was added to the aspirated fluid from above.

7. The mixture was incubated for 18 hours (overnight) at 4°C.

8. The tube was then centrifuged.

9. The supernatant was harvested and used for the H-I test. The material was designated as a 1:10 dilution of the original serum sample.

**Technique for conducting the H-I test**

Doubling dilutions of the treated serum were made ranging from 1:10 to 1:5120 in 0.2 ml volumes. To each tube
was added 0.2 ml of the parvovirus antigen (containing 4 HA units). After incubating for 30-60 minutes at room temperature, 0.4 ml of a 0.5% suspension of guinea pig rbc was added. Appropriate serum, saline, and virus controls were also run with each test. The material was mixed, incubated at room temperature for 2 to 3 hours and read for hemagglutinating activity.

The reciprocal of the highest dilution eliciting a complete H-I response was recorded as the end point. Titers were not determined below 10 or above 5120 dilutions. Serums that were negative in 1:10 dilution are reported as negative in the results.

**Virus isolation**

For virus isolation attempts various tissues were aseptically collected from necropsied pigs. The samples were placed into sterile bottles and stored at -20°C until tested for virus content. The usual tissues collected from the ECP0-6-infected pigs were the colon, brain, and lumbar cord. The intestinal contents were collected from the TGE-infected pigs and the kidneys or intestinal contents from the parvovirus-infected pigs.

Tissues were transferred aseptically into a sterile mortar and ground using a pestle and a small amount of sterile sand. A 10% suspension of the ground tissue or intestinal contents was made using Hanks' lactalbumin
hydrolysate (HLH) medium, containing 0.044% sodium bicarbonate plus antibiotics (500 units penicillin, 500 ug streptomycin and 125 units mycostatin/ml). The fluid suspension was poured into a sterile centrifuge tube and centrifuged at 2000 rpm in a refrigerated centrifuge for 20 minutes.

Two-tenths ml of the supernatant fluid was inoculated onto each of two bottles containing a confluent monolayer of cells. The nutrient medium had previously been removed, prior to inoculation. After an adsorption period of 0.5 hr at 37°C, 15 to 18 ml of maintenance medium (HLH) were added. The bottles were incubated at 37°C and examined periodically for CPE or for the presence of HA against guinea pig red blood cells. Negative bottles were subjected to freezing and thawing and passed at least one time before being declared negative.

**Gel filtration**

Two or 3 ml of selected fetal or newborn pig serum samples were passed through a system of 2 columns in a series. The columns (2.5X45.0 cm) were packed with Sephadex G-200 (Pharmacia, Upsala, Sweden). Flow through the first column was in a downward direction and through the second column in an upward direction. A flow rate of 16 ml/hour was maintained by a peristaltic pump (Buchler Instruments, Fort Lee, N.J.) hydrostatic pressure. The
buffer system used was 0.1 M tris (hydroxymethyl) amino methane (Fisher Scientific Co., Fairlawn, N.J.) in 0.2 M NaCl, adjusted to pH 8 with HCl.

The eluate was collected in fractions of 3 ml in each tube and the optical density (OD) at 280 nm was determined in a Gilford Model 240 spectrophotometer. The OD values were plotted against tube numbers. The G-200 chromatograms illustrated in this dissertation do not include fractions preceding the void volume.

Contents of individual tubes or pools of several tubes from selected areas of the chromatograms were selected, heat inactivated (56°C for 30 min), .45 µm membrane filtered and stored at -20°C. Portions of the eluate from these tubes were examined by immunodiffusion techniques for presence of immunoglobulins and were tested for the presence of neutralizing or H-I antibodies.

**Immunodiffusion**

Serum samples and Sephadex G-200 fractions of serum were tested for the presence of IgM, IgA and IgG by the double micro-immunodiffusion technique. This procedure is a slight modification of Ouchterlony's method described by Wadsworth (218). The technique utilized 1% Noble agar (Difco), 1% NaCl, and 1:10,000 Merthiolate. Monospecific rabbit antisera against porcine IgM, IgG, or IgA were utilized for immunoglobulin determinations. The procedures for preparing the monospecific antisera have been reported (158).
PROCEDURES AND RESULTS

Fetal Exposure with ECPO-6 Virus

Fetal pigs or porcine embryos ranging from 21 to 106 days of gestation were injected by the transuterine route with ECPO-6 virus. Inoculated fetuses were identified with trypan blue or a vetafil suture. A listing of the experimental animals and the number of days from infection to farrowing or hysterectomy are presented in Table 1. Table 2 lists the farrowing data and condition of the pigs at the time of delivery. The dose of virus and number of fetuses injected varied and is noted under each animal.

Exposure of 105-and 106-day fetal pigs

Twenty $10^6$ PFU of ECPO-6 virus in 1 ml of fluid were injected into each of three 106-day fetal pigs of sow 51-7. Seven live and 2 stillborn pigs were delivered prematurely 5 days post-infection. The 2 stillborn pigs were identified as having been virus-injected. The third virus-injected pig (1) was very weak at birth and was laid on by the sow. The remaining pigs appeared normal except for pig 6 which was much smaller than the rest. Pigs 7, 8 and 9 had nursed; therefore, were allowed to remain with the sow, and blood was not collected. Pigs 4, 5 and 6 were
<table>
<thead>
<tr>
<th>Sow no.</th>
<th>Days pregnant at exposure</th>
<th>No. fetuses(^a) injected</th>
<th>Days pregnant at delivery(^b)</th>
<th>Days from injection to delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>51-7</td>
<td>106</td>
<td>3</td>
<td>F111</td>
<td>5</td>
</tr>
<tr>
<td>20-9</td>
<td>105</td>
<td>4</td>
<td>F109</td>
<td>4</td>
</tr>
<tr>
<td>38-9</td>
<td>102</td>
<td>3</td>
<td>F110</td>
<td>8</td>
</tr>
<tr>
<td>7-3</td>
<td>91</td>
<td>2-2</td>
<td>F111</td>
<td>20</td>
</tr>
<tr>
<td>13-5</td>
<td>89</td>
<td>4</td>
<td>F111</td>
<td>22</td>
</tr>
<tr>
<td>5-9</td>
<td>84</td>
<td>2-2</td>
<td>F113</td>
<td>29</td>
</tr>
<tr>
<td>140</td>
<td>84</td>
<td>2-2</td>
<td>H112</td>
<td>28</td>
</tr>
<tr>
<td>80</td>
<td>76</td>
<td>3</td>
<td>F111</td>
<td>35</td>
</tr>
<tr>
<td>20-2</td>
<td>64</td>
<td>2-2</td>
<td>H115</td>
<td>51</td>
</tr>
<tr>
<td>19-6</td>
<td>64</td>
<td>4</td>
<td>H112</td>
<td>48</td>
</tr>
<tr>
<td>12-1</td>
<td>63</td>
<td>2-2</td>
<td>H79</td>
<td>16</td>
</tr>
<tr>
<td>19-3</td>
<td>49</td>
<td>3</td>
<td>H120</td>
<td>71</td>
</tr>
<tr>
<td>54-7</td>
<td>45</td>
<td>2-2</td>
<td>H115</td>
<td>70</td>
</tr>
<tr>
<td>7-2</td>
<td>21</td>
<td>3</td>
<td>H108</td>
<td>87</td>
</tr>
</tbody>
</table>

\(^a\) Numbers indicate 2 fetuses virus-injected and 2 injected with saline or sterile media

\(^b\) F = natural farrowing; H = hysterectomy
Table 2. Farrowing data after intrafetal ECP0-6 viral infection.

<table>
<thead>
<tr>
<th>Sow #</th>
<th>Number of pigs</th>
<th>Condition of pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live</td>
<td>Stillborn</td>
</tr>
<tr>
<td>51-7</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>20-9</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>38-9</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>7-3</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>13-5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5-9</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>140</td>
<td>6</td>
<td>...</td>
</tr>
<tr>
<td>80</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>20-2</td>
<td>7</td>
<td>...</td>
</tr>
<tr>
<td>19-6</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>12-1c</td>
<td>10</td>
<td>...</td>
</tr>
<tr>
<td>19-3</td>
<td>6</td>
<td>...</td>
</tr>
<tr>
<td>54-7</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>7-2</td>
<td>8</td>
<td>...</td>
</tr>
</tbody>
</table>

<sup>a</sup>C-H = cerebellar hypoplasia  
<sup>b</sup>Arthrogryposis  
<sup>c</sup>Fetuses 79 days of age at time of hysterectomy
artificially reared. Pig 6 was found dead 3 days after delivery; pig 4 was euthanized 5 days after delivery and pig 5 was used for other purposes.

Sow 51-7 had an S-N titer of 400 against ECPO-6 virus on the 106th day of gestation and the titer was 600 seven days post-infection. The S-N titer of pigs 1 and 2 (virus-injected) were 52 and 60 five days post-infection. The S-N titer of pig 4 (non-injected) was 100 at 5 days of age. IgM and IgG were present in the sera of pigs 1 and 4 as detected by immunodiffusion. The sera were not checked for IgA.

Virus was isolated from various tissues, including the small and large intestines, brain, spleen, liver, adrenal, kidneys and thoracic fluid of pigs 1, 2, 5 and 6. Virus was isolated from intestinal contents but not from brain tissue of pig 4 when necropsied at 5 days of age.

Four 105-day fetal pigs of sow 20-9 were each injected with 10 X 10^6 PFU of ECPO-6 virus in 0.5 ml of fluid, one-half the dose used in the previous experiment. The sow farrowed 12 live pigs on the 109th day or 4 days post-infection. The pigs appeared to be slightly premature and weak; otherwise, they were all bright, alert and active. Serum samples were obtained from 3 of the 4 virus-injected pigs (2, 3, and 4) and all 3 were serologically negative at the 1:1 dilution for ECPO-6 virus. Likewise, sera from 2 non-injected pigs were serologically negative at the 1:1
dilution. Based upon immunodiffusion, IgG was detected in all 5 samples; however, they were negative for IgM and IgA.

Virus was isolated from various tissues, including the heart, axillary lymph node, kidney, lumbar spinal cord, brain; large intestine and spleen of all 4 virus-injected pigs as well as 1 non-injected fetus.

Exposure of 102-day fetal pigs

Three fetuses of sow 38-9 were injected on the 102nd day of gestation with $4 \times 10^5$ PFU of ECPO-6 virus in 0.2 ml of fluid. The sow farrowed 5 live pigs on the 110th day of gestation. Pigs 4 and 5 (non-injected) appeared normal and were allowed to nurse and remain with the sow. In contrast, signs of varying degrees of incoordination and ataxia were evident in the 3 virus-injected pigs (1, 2 and 3). Pig 1 was unable to stand or to walk at birth. Pig 2 was more active than pig 1; however, evidence of partial paralysis was present in the rear legs. The pig was reared for 3 days at which time it was found near death and was euthanized. Internal hydrocephalus was present and a large amount of fluid was released upon excising the brain. Pig 3 was very active; however, ataxia, incoordination and hyperexcitability were evident. The pig always went to the right and fell over after attempting to stand. On the 4th day the pig was paralyzed in the rear legs and was euthanitized.

The results of immunologic and virologic studies of this
litter are presented in Table 3. All 3 virus-injected pigs had a S-N titer to the ECPO-6 virus ranging from 115 to 410 at birth. The S-N titer of pig 2 was 1100 within 3 days and the titer of pig 3 was 1600 within 4 days after birth. Although the sow had an H-I titer of 1280 against PPV at the time of farrowing, the sera of the 3 virus-injected pigs were negative for H-I antibodies against PPV.

IgM and IgG were present in the sera of pigs 1 and 2, but not IgA. Results of Sephadex G-200 separation and immunodiffusion studies on the sera of both pigs are presented in Figs. 1 and 2. The serum profiles are very similar except for the middle peak shown in Fig. 2. The serum represented in Fig. 2 was obtained 3 days after birth.

Virus was isolated from the brain and colon of pig 1 and the brain and lumbar cord of pigs 2 and 3. Histopathologic studies revealed glial foci, hyperemia and petechia of the medulla, and gliosis around the 4th ventricle of pig 1; gliosis around the 4th ventricle of pig 2; gliosis around the 4th ventricle and focal gliosis in the gray matter of the lumbar cord of pig 3.

**Exposure of 89- and 91-day fetal pigs**

Two fetuses of sow 7-3 were injected with saline and 2 were injected with 20 X 10^5 PFU of the ECPO-6 virus on the 91st day of gestation. The sow farrowed on the 111th day or 20 days post-infection. Eight live pigs and 4 dead pigs (no mummies) were delivered. Results of this pregnancy are
Table 3. Immunologic and virologic results of injecting three 102-day fetuses with ECPO-6 virus.  

<table>
<thead>
<tr>
<th>Animal</th>
<th>Antibody titers</th>
<th>Ig class</th>
<th>Virus isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>38-9(^b)</td>
<td>70  1280</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>38-9(^c)</td>
<td>300  1280</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1(^d)</td>
<td>410</td>
<td>-</td>
<td>++ -</td>
</tr>
<tr>
<td>2</td>
<td>115</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>340</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5) non-injected, nursed, left with sow</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2(^e)</td>
<td>1100</td>
<td>-</td>
<td>++ -</td>
</tr>
<tr>
<td>3(^e)</td>
<td>1600</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Sow farrowed 8 days after fetal injection. 
\(^b\) Sow at the time of fetal injection. 
\(^c\) Sow at the time of farrowing. 
\(^d\) Pigs 1, 2, 3 virus injected. Samples collected at the time of farrowing. 
\(^e\) Samples collected 3 and 4 days after farrowing. 
\(^f\) - for porcine parvovirus (PPV) indicates no antibody detected at 1:10 dilution.
Fig. 1. Gel filtration on Sephadex G-200 of serum from newborn pig (110th day) injected 8 days previously with ECPD-6 virus. S-N titer of 410 for ECPD-6 virus. Indicated are ECPD-6 antibody titers, represented by vertical bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgA was not detected in the serum.
Fig. 2. Gel filtration on Sephadex G-200 of serum from 3-day-old pig. Pig was ECPO-6 injected on 102nd day of gestation and born 8 days later. S-N titer for ECPO-6 at time of birth was 115 and 3 days later 1100. Indicated are ECPO-6 antibody titers, represented by vertical bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgA was not detected in the serum.
presented in Table 4. One of the dead pigs (2) had been injected with saline and one (4) with virus. All remaining pigs appeared normal except virus-injected pig 3. This pig was very wobbly and ataxic at birth, kept rolling over to the right and was unable to stand.

An S-N titer of 1400 was present in the serum of pig 1 at birth against ECP0-6 virus. Pig 3 had a titer of 1350 and was negative for H-I antibodies against PPV. Pig 9, a non-injected control, was serologically negative for ECP0-6 virus. Virus was isolated from colon contents of all 4 injected pigs (1, 2, 3, 4) as well as pig 11. Virus was not isolated from tissues of pig 9 which was serologically negative for ECP0-6 virus.

Sephadex G-200 chromatogram for pig 1 is presented in Fig 3. IgM, IgG and IgA were detected in the serum. Neutralizing antibodies were present in various fractions tested. The titer of the fractions ranged from 9 to 150 with the highest titer in the first peak of the chromatogram. The fraction with the highest titer also contained IgM and IgG based on immunodiffusion. Correlation of the presence of immunoglobulins in the fractions with the level of neutralizing antibodies present is shown in Fig. 3. Although IgG was present in fraction 28, this fraction was not tested for neutralizing antibodies. The Sephadex G-200 chromatogram profile on the serum of pig 9 (serologically negative) was essentially the same as that shown in Fig. 3.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Cerebellar hypoplasia</th>
<th>Virus isolated</th>
<th>Antibody titers</th>
<th>Ig class</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-3b</td>
<td>-</td>
<td>colon</td>
<td>1400</td>
<td>++</td>
</tr>
<tr>
<td>1d</td>
<td>-</td>
<td>colon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-dead</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>col on</td>
<td>1350</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-dead</td>
<td>col on</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>-</td>
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<td>7</td>
<td>-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>-dead</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sow farrowed 20 days after fetal injection.*

*Sow at the time of fetal injection.*

*Sow at the time of farrowing. Serum not collected.*

*Pigs 1 and 2 saline injected; 3 and 4 virus injected.*

*e - for PPV indicates no antibody detected at 1:10 dilution; for ECPO-6 indicates no antibody detected at 1:1 dilution.*
Fig. 3. Gel filtration on Sephadex G-200 of serum from newborn pig (111th day) injected 20 days previously with sterile saline. Two litter mates were injected with ECPO-6 virus. S-N titer of 1400 for ECPO-6 virus. Indicated are ECPO-6 antibody titers, represented by vertical bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA present in the serum; IgA was not detected in the fractions.
However, IgM and IgA were not present but IgG was detected in the serum of pig 9. Likewise, all fractions tested were negative for neutralizing antibodies and for IgM and IgG.

Four fetal pigs, 89 days old, of sow 13-5 were injected with $20 \times 10^6$ PFU of ECP0-6 virus in 1 ml. Seven pigs were delivered naturally on the 111th day, or 22 days after exposure. Of the 7 pigs, 4 were in various stages of decomposition and 3 were fully developed but 1 was stillborn, 1 died shortly after delivery and 1 was alive but weak. The dye markings were not recognizable; however, it was assumed that the 4 decomposed pigs were the virus-injected fetuses. Virus was isolated from the colon contents of pig 2 (virus-injected) and from pig 5, a live, non-injected pig. An S-N titer of 1150 was detected in pig 5 at term. The S-N titer of the sow was 400 at the time of surgery and 27 days later was 10,000. Results of the histopathologic examination of the brains of pigs 5 and 6 were normal.

**Exposure of 76- and 84-day fetal pigs**

Sow 5-9 was subjected to surgery on the 84th day of gestation and 2 fetuses were injected with saline and 2 pigs with $20 \times 10^5$ PFU of the ECP0-6 virus in 0.1 ml. The sow farrowed 8 pigs on the 113th day of gestation or 29 days post-infection. One pig was weak and near death and 3 were fairly normal in appearance. Both saline-injected pigs (1 and 2) were alive. However, pig 2 could not stand, showed hyperextension of the rear legs and appeared near
death. The 2 virus-injected pigs (3 and 4) plus pigs 5 and 6 were in varying stages of autolysis. Pigs 7 and 8 were alive, normal and were observed nursing; therefore, no tissues were collected. Pig 1, saline-injected, was serologically negative for ECPO-6 virus at the 1:1 dilution, in contrast with pig 2, also a saline-injected pig, which had a titer of 1024. In addition, both pigs were serologically positive for PPV with titers in excess of 1024. IgM, IgG and IgA were present in the serum of both pigs. Virus was isolated from the colon of pig 1; colon, brain and lumbar cord of pig 2 and the colon of pigs 5 and 6.

A similar experimental procedure was conducted on sow 140 on the 84th day, as in the previous animal. Two pigs were injected with saline and 2 pigs with the same dose (20 x 10^5 PFU) of ECPO-6 virus. Pregnancy was terminated by a hysterectomy on the 112th day or 28 days post-exposure. Six live pigs were obtained. Pigs 5 and 6, non-injected control pigs, appeared normal and tissues were not collected. Results of immunologic and virologic studies on the remaining pigs are presented in Table 5. The S-N titer for ECPO-6 virus of the sow was 480 at the time of intrafetal exposure, and 110 at the time of hysterectomy. The 2 saline-injected pigs (1 and 2) were serologically negative for ECPO-6 virus at the 1:1 dilution and virus was not isolated from the lumbar cord, brain, or colon of these 2 pigs. Pigs 3 and 4 (virus-injected) had S-N titers of 420 and 500, respectively,
Table 5. Immunologic and virologic results of injecting two 84-day fetuses with ECPO-6 virus.a

<table>
<thead>
<tr>
<th>Animal</th>
<th>Antibody titers</th>
<th>Ig class</th>
<th>Virus isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECP0-6</td>
<td>PPV</td>
<td></td>
</tr>
<tr>
<td>140b</td>
<td>480</td>
<td>2048</td>
<td></td>
</tr>
<tr>
<td>140c</td>
<td>110</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>e</td>
<td>2048</td>
<td>++ +</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>2560</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>420</td>
<td>1280</td>
<td>++ +</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>2560</td>
<td>colon</td>
</tr>
</tbody>
</table>

5) returned to sow, no samples collected

6) - antibody titer at 1:1 dilution.

aPigs hysterectomy derived 28 days post-injection.
bSow at the time of fetal injection.
cSow at the time of hysterectomy.
dPigs 1 and 2 saline injected; 3 and 4 virus injected.
e - for ECP0-6 indicates no antibody detected at 1:1 dilution.
for ECPO-6 virus and virus was isolated from the colon, but not the brain or lumbar cord of either pig. The sow was serologically positive for PPV with a titer of 1024 at the time of hysterectomy. All 4 injected pigs had titers for PPV ranging from 1280 to 2048. IgM, IgG and IgA were detected in the sera of pigs 1 and 3. The serum chromatogram for pig 3 is presented in Fig. 4. Immunoglobulins were not detected in the first fraction collected; however, IgM and IgG were present in the fraction representing the first peak. A neutralizing titer of 25 was present in this fraction. Of the other 3 fractions saved all were negative for neutralizing antibody at the 1:1 dilution for ECPO-6. However, IgG was detected in the fraction representing the second peak. The serum profile for pig 1 was essentially the same as that shown for pig 3 in Fig. 4. Immunoglobulins IgM and IgG were located in the same fractions as for pig 3. Although both pigs were serologically positive for PPV the fractions were not tested for antibodies against this virus.

Three fetuses of sow 80 were injected with 20 x 10^5 PFU of ECPO-6 virus on the 76th day of gestation. The sow farrowed 3 mummies, 1 stillborn and 4 live pigs on the 111th day, 35 days post-injection. The 3 mummies were the virus-injected pigs. Pigs 4 and 5 appeared normal at birth, pig 6 had been dead for 1 to 2 days, pig 7 was weak and unable to stand, and pig 8 was very wobbly and spastic in the rear legs. The S-N titers for ECPO-6 virus were 1900, 540 and
Fig. 4. Gel filtration on Sephadex G-200 of serum from newborn hysterectomy-derived pig (112th day) injected 28 days previously with ECPO-6 virus. S-N titer of 420 for ECPO-6 virus. Indicated are ECPO-6 antibody titers, represented by vertical bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA present in the serum; IgA not detected in the fractions. Antibody titer of 1280 for PPV; however, the fractions were not tested for PPV antibodies.
270 for pigs 4, 5 and 8 respectively. IgM and IgG were present in the serum of all 3 pigs, but not IgA. These 3 pigs had a titer against PPV of 160, >320 and >320 respectively. Although a serum sample was not obtained from the sow at the time of farrowing, she was serologically negative for PPV at the time of fetal exposure. Virus was isolated from the colon contents of pigs 4, 6, 7 and 8 as well as the lumbar cord and brain of pig 8.

**Exposure of 63- and 64-day fetal pigs**

On the 64th day of gestation 2 fetuses of sow 20-2 were injected with saline and 2 with $20 \times 10^5$ PFU of ECPO-6 virus. A hysterectomy was performed at 115 days or 51 days following viral exposure. Two mummies and 7 live pigs were obtained. The 2 mummies (pigs 3 and 4) were the virus-injected pigs and based on crown-rump length were judged to be 100 days and 75 days of age when death occurred. Pig 2, saline-injected, and pig 5, non-injected control, were reared in a sterile isolator for 20 days and then euthanized. Pigs 8 and 9 were likewise reared in isolators for 12 days and then euthanized. Pig 1 (saline-injected) was weak in the pasterns, ataxic, shaky, and was therefore euthanized at the time of delivery. Pig 7 died within 5 minutes of delivery. A blood sample was collected from pig 6 which died soon thereafter.

A summary of the clinical observations, immunologic and virologic studies of the pigs from this sow are presented
in Table 6. Varying degrees of cerebellar hypoplasia were observed in 4 of the piglets examined. Fig. 5 is a photograph of the longitudinal section of the head of pig 5, showing cerebellar hypoplasia. This was a non-injected piglet which was reared in a sterile isolator and was euthanized 20 days following hysterectomy. The S-N titer of this animal for ECPO-6 was 1800 at the time of necropsy and virus was isolated from colon contents at that time. Fig. 6 is a photograph of the heads of pigs 8 and 9 taken 12 days after birth; pig 8 showing cerebellar hypoplasia and pig 9 with a normal appearing cerebellum. At 12 days of age the S-N titters of the pigs were 7200 for pig 8 and 2030 for pig 9. Virus was not recovered from the colon contents of either pig. Pig 1 had an S-N titer for ECPO-6 of 1550 at term, was ataxic, and revealed cerebellar hypoplasia at necropsy.

Virus was not isolated from the various tissues cultured. In contrast, pig 6 did not have an S-N titer to the virus at the 1:1 dilution and virus was recovered from the colon, brain, and lung tissues. The pig did not show signs of ataxia and the cerebellum appeared normal. H-I antibodies to PPV were not detected in any of the 6 serum samples available from the above litter at the 1:10 dilution. IgM, IgG and IgA were present in the serum of pig 1 and only IgG was detected in the serum of pig 6.

Fetuses of sow 19-66 were likewise subjected to the virus
Table 6. Clinical, immunologic and virologic results of injecting two 64-day fetuses with ECPO-6 virus.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Animal</th>
<th>Arthro-</th>
<th>Cerebellar</th>
<th>Virus isolated</th>
<th>Antibody titers</th>
<th>Ig class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gryposis</td>
<td>hypoplasia</td>
<td></td>
<td>ECP0-6</td>
<td>PPV</td>
</tr>
<tr>
<td>20-2\textsuperscript{b}</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>530</td>
<td>-</td>
</tr>
<tr>
<td>1\textsuperscript{c}</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>1550 \textsuperscript{f}</td>
<td>+ + +</td>
</tr>
<tr>
<td>2\textsuperscript{d}</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1500</td>
<td>-</td>
</tr>
<tr>
<td>3 - mummy, died approximately 100th day</td>
<td>-</td>
<td>-</td>
<td>colon</td>
<td>1800</td>
<td>-</td>
</tr>
<tr>
<td>4 - mummy, died approximately 75th day</td>
<td>-</td>
<td>-</td>
<td>lung, colon</td>
<td>-</td>
<td>- + +</td>
</tr>
<tr>
<td>5\textsuperscript{d}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>lung, colon</td>
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</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>brain</td>
<td>-</td>
<td>- + +</td>
</tr>
<tr>
<td>8\textsuperscript{e}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7200</td>
<td>-</td>
</tr>
<tr>
<td>9\textsuperscript{e}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2030</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Pigs hysterectomy derived 51 days post-injection
\textsuperscript{b} Sow at the time of hysterectomy
\textsuperscript{c} Pigs 1 and 2 saline injected, 3 and 4 virus injected
\textsuperscript{d} Pigs reared in sterile isolators and samples collected at 20 days of age
\textsuperscript{e} Pigs reared in sterile isolators and samples collected at 12 days of age
\textsuperscript{f} For PPV indicates no antibody detected at 1:10 dilution; for ECPO-6 indicates no antibody detected at 1:1 dilution
Fig. 5. Longitudinal section of the head of pig 5 from sow 20-2 showing cerebellar hypoplasia at 20 days of age. Litter mates had been injected with ECPO-6 virus on the 64th day of gestation.
Fig. 6. Longitudinal section of the heads of pigs 8 (right) and 9 (left) from sow 20-2. Pigs were necropsied 12 days after birth. A normal cerebellum is evident in pig 9 in contrast to pig 8 showing cerebellar hypoplasia. Litter mates had been injected with ECP0-6 virus on the 64th day of gestation.
on the 64th day of gestation. In this experiment 4 fetuses were each injected with $20 \times 10^5$ PFU of the ECPO-6 virus in 0.1 ml amounts. Instead of identifying with the trypan blue, a knot of vetafil suture was incorporated in the myometrium over each injected fetus. The duration of pregnancy was uneventful and was terminated by hysterectomy at 112 days or 48 days post-infection.

Eight live pigs, 4 small mummies (75-130 mm) and 1 dead fetus near term were obtained. The 4 mummies were the virus-injected pigs as identified by the sutures. Signs of CNS disturbances were noted in a number of the live pigs. Pigs were observed with inability to right themselves, hyperextension of the legs, and arthrogryposis. Upon necropsy cerebellar hypoplasia was noted in 5 of the 8 live pigs. Fig. 7 shows the degree of cerebellar hypoplasia seen in pig 2.

Table 7 presents a summary of the clinical observations, gross examination of the brains, virologic and immunologic studies from the pigs of this litter. Arthrogryposis was observed in the left forelegs of pigs 5 and 7. Fig. 8 shows the degree of arthrogryposis present in pig 5. Virus was isolated from the colon of pigs 6 and 8. All living pigs had an S-N titer to the ECPO-6 virus ranging from 600 to 2800. The S-N titer of the sow was 320 at the time of exposing the fetuses and 1300 at the time of hysterectomy 48 days later. All pigs and the sow were serologically
Fig. 7. Longitudinal section of the head of pig 2 from sow 19-6 showing cerebellar hypoplasia. Four litter mates had been injected with ECPO-6 virus on the 64th day of gestation.
Table 7. Results of injecting four 64-day fetuses with ECPO-6 virus.$^a$

<table>
<thead>
<tr>
<th>Animal</th>
<th>Arthro-</th>
<th>Cerebellar</th>
<th>Virus isolated</th>
<th>Antibody titers</th>
<th>Ig class</th>
</tr>
</thead>
<tbody>
<tr>
<td>gryposis</td>
<td>Ataxia</td>
<td>hypoplasia</td>
<td></td>
<td>ECP0-6</td>
<td>PPV</td>
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<tr>
<td>19-6$^b$</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>320</td>
</tr>
<tr>
<td>19-6$^c$</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1300</td>
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</tr>
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<td>6</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>colon</td>
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</tr>
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<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>2800</td>
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<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>colon</td>
<td>2400</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>dead full term</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,11,12,13$^d$</td>
<td>-</td>
<td>mummies (75mm - 130mm)</td>
<td></td>
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</tr>
</tbody>
</table>

$^a$Pigs hysterectomy derived 48 days post-injection
$^b$Sow at the time of fetal injection
$^c$Sow at the time of hysterectomy
$^d$The 4 mummies were the virus-injected pigs
$^e$- for PPV indicates no antibody detected at 1:10 dilution
$^f$NT = not tested
Fig. 8. Arthrogryposis of left foreleg of pig 5 from sow 19-6. Four litter mates had been injected with ECPO-6 virus on the 64th day of gestation. Cerebellar hypoplasia was evident at necropsy.
negative for PPV. The sera from 3 pigs (4, 5 and 6) were examined for the presence of immunoglobulins. IgM and IgG were present in the sera of these pigs. The serum of pig 6, being the only one tested, was positive for IgA.

A chromatogram for pig 6 is shown in Fig. 9. An S-N titer of 730 against ECPO-6 virus was present in the serum of this pig. Neutralizing titers, ranging from 1.5 to 33 were present in several of the fractions beginning at the first peak and continuing through to the start of the last peak. IgM and IgG were detected in fractions representing the decline of the first peak and IgG only was detected in the slightly elevated region between the two peaks. Although IgA was present in the serum of pig 6, it was not evident in any of the fractions tested.

Two fetuses of sow 12-1 were injected with $20 \times 10^5$ PFU of the ECPO-6 virus and 2 with 0.1 ml saline on the 63rd day of gestation. Ten live and 3 mummified fetuses were obtained by hysterectomy 16 days post-exposure. Pigs 1 (saline-injected) and 4 (virus-injected) were readily identified by the dye markings. Pigs 2 and 3 (one saline-injected and one virus-injected) were apparently mummified and it was difficult to distinguish if dye was present; however, it was thought to be. The dye markings were not seen in any of the other live pigs. The pigs were small and it was difficult to collect blood. Blood samples were obtained from pigs 1 and 4 and the remaining pigs...
Fig. 9. Gel filtration on Sephadex G-200 of serum from hysterectomy-derived pig (112th day). Four litter mates had been injected with ECPO-6 virus 48 days previously. S-N titer of 730 for ECPO-6 virus. Indicated are ECPO-6 antibody titers, represented by vertical bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA present in the serum. IgA was not detected in the fractions.
were sampled and the sera pooled.

An S-N titer to ECPO-6 virus was not detected in the serum of pig 1 at the 1:1 dilution. Virus was isolated from the colon of this pig. Precipitin lines for IgM, IgG and IgA were not present in the immunodiffusion studies. Virus-injected pig 4 had an S-N titer of 70 to the enterovirus and virus was isolated from the colon and brain tissue. IgM and IgG but not IgA were detected. The pooled serum sample of the remaining 8 live pigs had an S-N titer of 280 against the virus. As in pig 4 the pooled serum contained IgM and IgG but not IgA.

**Exposure of 45-and 49-day fetal pigs**

Three fetuses of sow 19-3 were each injected with 20 X 10^5 PFU of ECPO-6 virus on the 49th day of gestation. A knot of vetafil suture in the myometrium was used to identify the injected fetuses. A hysterectomy was done on the sow at the 120th day of gestation, 7 days overdue. Nine mummified fetuses were found ranging in crown-rump length from 50 mm to 150 mm. Two of the 3 suture knots were readily located. The smallest mummified fetus found was one that had been injected with the virus. Tissues were not collected for virus isolation purposes.

The next sow (54-7) was 45 days pregnant at the time of injecting the fetuses. As in previous experiments 2 were injected with saline and 2 with 20 X 10^5 PFU of the enterovirus. Because of fetal size both the saline and virus
were injected intra-amnionically. Trypan blue was injected sub-serosally in the uterine wall over each injected fetus. Pregnancy was terminated by hysterectomy on the 115th day of gestation. Seven live pigs and 3 mummies were obtained. At the time of the hysterectomy the dye injections in the uterus were not apparent; therefore, the injected piglets could not be identified.

Of the 7 live pigs, 3 were observed with gross deformities of arthrogryposis, 2 with marked signs of CNS disturbance, such as hyperextension of the legs, shaking of the head, and wobbly, ataxic gait, and 1 pig with mild locomotor disturbance. One pig (3) appeared normal in all respects and was exsanguinated immediately after delivery with blood and tissues being collected. Figs. 10 and 11 are photographs of part of the litter showing the varying degrees of developmental defects.

The most severe signs of CNS disturbance was seen in pig 5. This animal was reared artificially for 50 days. Its ataxic condition never improved and, early, the pig appeared blind. This animal was euthanitized and severe cerebellar hypoplasia was observed. Fig. 12 is a photograph of a longitudinal section of the head of pig 5 showing the cerebellar hypoplasia. Normal histology was seen in sections of the spinal cord, eye and optic nerve.

Pig 7 had malformed segmented kidneys resembling bovine kidneys. Histologic examination of the kidney revealed long renal papillae with uneven distribution of the medullary rays. The brain appeared normal on histological
Fig. 10. Arthrogryposis of 3 live pigs from sow 54-7. Two fetuses of this litter had been injected with ECPO-6 virus on the 45th day of gestation.
Fig. 11. Three mummified fetuses and 4 live, deformed, ataxic pigs from sow 54-7. Two fetuses of this litter had been injected with ECP0-6 virus on the 45th day of gestation.
Fig. 12. Longitudinal section of the head of pig 5 from sow 54-7 showing cerebellar hypoplasia at 50 days of age. Two fetuses of this litter had been injected with ECPO-6 virus on the 45th day of gestation.
examination with edema present around the ventral horn neurons of the lumbar cord.

A summary of the clinical observations, immunologic and virologic studies of this litter is presented in Table 8. The sow had an S-N titer of 140 at the time of fetal exposure and 2600 seventy days later. Of all the pig sera tested only 1 (pig 3) was serologically negative for ECPO-6. However, virus was isolated from the colon of this pig. Pig 6, which was not tested serologically, revealed the presence of virus in colon, brain, and lung tissue. Virus was also isolated from the colon of pigs 1, 4 and 7.

Except for pigs 3 and 4 which had S-N titers of <1 and 8 respectively, the remaining pigs had high S-N titers, ranging from 1250 to 2800. The S-N titer of pig 5 was 10,000 when 11 days old and 3020 when euthanitized at 50 days of age. All pigs were serologically negative for PPV.

Immunodiffusion tests were conducted on the sera of pigs 2, 3, 5 and 7. Pig 3 serum, negative for ECPO-6 antibodies, produced a strong precipitin line with IgG antiserum but not with IgM or IgA antiserum. The other 3 serum samples which were positive for ECPO-6 antibodies elicited precipitin lines with all 3 classes of anti-immunoglobulins. The serum chromatogram for pig 7 is presented in Fig. 13. The profile for pig 3 was similar to that shown in Fig. 13. Immunoglobulins were not detected in any of the fractions of pig 3 and the fractions were negative for neutralizing
<table>
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<th>Antibody titers</th>
<th>Ig class</th>
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</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>colon</td>
<td>1250</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>colon</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<td>colon, brain</td>
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<td>10</td>
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<td>+</td>
<td>-</td>
<td>lung</td>
<td>3020</td>
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</tr>
<tr>
<td>7</td>
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<td>+</td>
<td></td>
<td>colon</td>
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<td>-</td>
</tr>
</tbody>
</table>

8,9,10 - mummies

<sup>a</sup>Pigs hysterectomy derived 70 days post-injection. The injected pigs could not be identified at term.

<sup>b</sup>Sow at the time of fetal injection.

<sup>c</sup>Sow at the time of hysterectomy.

<sup>d</sup>Sample collected at 11 days of age.

<sup>e</sup>Sample collected at 50 days of age.

<sup>f</sup>- for PPV indicates no antibody detected at 1:10 dilution; for ECP0-6 indicates no antibody detected at 1:1 dilution.
Fig. 13. Gel filtration on Sephadex G-200 of serum from newborn, hysterectomy-derived pig (115th day). Two fetuses of this litter had been injected with ECPO-6 virus on the 45th day of gestation. S-N titer of 1750 for ECPO-6 virus. Indicated are ECPO-6 antibody titers represented by vertical bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG, IgA present in the serum; IgA was not detected in the fractions.
antibodies. A few fractions of pig 7, which had an S-N titer of 1750, also contained neutralizing antibodies ranging in titer from 13 to 110. IgM and IgG were detected in a few of the fractions as shown in Fig. 13. IgA was not detected in any of the fractions of pig 7.

**Exposure of 21-day fetal pigs**

The last animal exposed to ECPO-6 virus was only 21 days pregnant at the time of surgery. Because the embryos could not be readily identified through the uterine wall the virus was injected directly into 3 sacculations. The dose was $20 \times 10^5$ PFU in 0.1 ml of fluid. A hysterectomy was performed on the 108th day of gestation. Eight live pigs were obtained and there was no evidence of mummies. However, seven corpus luteums were counted on each ovary. Virus was not isolated from any tissues of these 8 pigs. Although the sow had a titer of 1500 against ECPO-6 virus and 2560 against PPV at the time of hysterectomy, all 7 pig sera checked were serologically negative for both viruses. Sera from 2 of the pigs were checked for immunoglobulins by immunodiffusion and they were positive for IgG but negative for IgM and IgA.

**Fetal Exposure with TGE Virus**

Two fetuses from each of 3 sows were injected with $2 \times 10^5$ PFU of a modified live TGE virus. An additional 2 fetuses of each sow were injected with saline or sterile...
medium. All material was filtered (0.45 μm membrane) prior to injecting the fetuses. All injected fetuses were identified with trypan blue. The sows (numbers 189, 260 and 267) were 95, 77, and 74 days pregnant, respectively, at the time of fetal injections. Hysterectomies were performed on the sows at 110, 91, and 111 days of gestation or 15, 14, and 37 days after injecting the TGE virus. All pigs were alive at delivery with the virus- and diluent-injected piglets readily identified.

All the pigs from sows 189 and 260 were euthanized at the time of hysterectomy and various tissues were collected. The piglets of sow 267 were derived by germfree techniques (123) through a sterile surgical isolator and transfer unit. Six of these piglets were transferred to sterile rearing isolators for subsequent challenge with TGE virus, 2 were reared in clean cages and 3 were euthanized at the time of hysterectomy for collection of specimens.

Exposure of 95-day fetal pigs

The immunologic results of infecting two 95-day fetuses of sow 189 with TGE virus are presented in Table 9.

Seven live pigs were obtained by hysterectomy from sow 189 at the 110th day of gestation. S-N titers (70 and 310) against TGE virus were detected in the virus-injected fetuses but not in the 2 saline-injected fetuses, nor a non-injected control fetus. The S-N titer of the sow was
Table 9. Immunologic results of injecting two 95-day fetuses with TGE virus.a

<table>
<thead>
<tr>
<th>Animal</th>
<th>Antibody titer</th>
<th>Ig class</th>
</tr>
</thead>
<tbody>
<tr>
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<td>TGE&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>28</td>
</tr>
<tr>
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</tr>
<tr>
<td>2</td>
<td>1280</td>
<td>310</td>
</tr>
<tr>
<td>3</td>
<td>2560</td>
<td>-&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>2560</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>640</td>
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</tr>
<tr>
<td>6</td>
<td>NT&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

aPigs hysterectomy derived 15 days post-injection.
bSow at the time of fetal injection.
cSow at the time of hysterectomy.
dPigs 1 and 2 virus injected, 3 and 4 saline injected.
eIntestinal villous atrophy present.
fNormal intestinal villi.
gNT = not tested.
h - for ECP0-6 and TGE indicates no antibody detected at 1:1 dilution.
28 at the time of fetal exposure and 90 fifteen days later. Neutralizing antibodies against ECP0-6 virus were not detected in any of the fetal pig serum samples. However, a titer of 256 against ECP0-6 virus was present in the serum of the sow at the time of hysterectomy. All 5 fetal serum samples tested had H-I antibodies against PPV with titers ranging from 640 to 2560. This response against PPV was present in serum from TGE virus-injected, saline-injected and a non-injected control pig. IgM, IgG and IgA were present in all 5 pig sera.

Examination of the intestines revealed a thin-walled, fluid-filled lumen in the TGE-infected pigs in contrast to a thicker wall and thicker intestinal contents in the non-injected pigs. Sub-gross examination of the intestinal villi of pigs 1 and 2 (virus-injected) revealed villous atrophy, as is typical for TGE infection (34,208), in contrast to the normal appearing villi in pigs 4 and 6 (non-infected).

Sephadex G-200 chromatograms of TGE virus-injected pig 1 and non-injected pig 5 of sow 189 are illustrated in Figs. 14 and 15. Although pig 1 was serologically positive for TGE with a titer of 70, both pigs were serologically positive with titers of 640 for PPV. All 3 classes of immunoglobulins were detected in the serum of both pigs. IgM and IgG were present in a few of the serum fractions of both pigs while IgA was not present. Results of testing various
Fig. 14. Gel filtration on Sephadex G-200 of serum from newborn, hysterectomy-derived pig (110th day). Pig had been injected 15 days previously with TGE virus. Antibody titers of 70 for TGE and 640 for PPV were present in the serum. Indicated are TGE antibody titers, represented by vertical bars, PPV antibody titers represented by dotted bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA present in the serum; IgA was not detected in the fractions.
Fig. 15. Gel filtration on Sephadex G-200 of serum from newborn, hysterectomy-derived pig (110th day). Two litter mates had been infected 15 days previously with TGE virus. Antibody titers were negative for TGE and 640 for PPV. Indicated are PPV antibody titers represented by dotted bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA present in the serum; IgA was not detected in the fractions.
fractions for the presence of antibodies against TGE and PPV are also shown in Figs. 14 and 15.

**Exposure of 77-day fetal pigs**

Two 77-day fetuses of sow 260 were injected with an aliquot of the same TGE virus as used in sow 189. The same dose of virus was injected. Twelve live pigs were obtained by hysterectomy 14 days post-exposure at 91 days of fetal age. The 4 injected pigs (2 with virus and 2 with diluent) were readily identified. A summary of the immunologic results are presented in Table 10. Of the serum samples tested only the sera of the 2 virus-injected pigs contained S-N antibodies against TGE. The titer was 54 in 1 pig and 140 in the other. All fetal sera examined were negative for ECP0-6 and PPV antibodies even though the dam was serologically positive for both of these agents. Virus was isolated from the intestinal contents of pig 4, the only animal where virus isolation was attempted.

Examination of the intestines of the 2 virus-injected pigs revealed a distended, thin-walled, fluid-filled gut in contrast to the normal appearing intestines of all other pigs. Subgross examination of the intestines of the 2 virus-injected pigs revealed a marked shortening and broadening of the villi. Villous atrophy was not observed in any of the other pigs. Fig. 16 shows the villous atrophy present in the mid-section of the small intestine of a virus-injected pig. This is in contrast to the normal appearing
Table 10. Immunologic results of injecting two 77-day fetuses with TGE virus.\textsuperscript{a}

<table>
<thead>
<tr>
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</thead>
<tbody>
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<td>256</td>
</tr>
<tr>
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<td>-</td>
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<td>2</td>
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</tr>
<tr>
<td>3\textsuperscript{e}</td>
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<td>-</td>
</tr>
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<td>4\textsuperscript{e}</td>
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</tr>
<tr>
<td>12</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Pigs hysterectomy derived 14 days post-injection.
\textsuperscript{b}Sow at the time of fetal injection.
\textsuperscript{c}Sow at the time of hysterectomy.
\textsuperscript{d}Pigs 1 and 2 media injected, 3 and 4 virus injected.
\textsuperscript{e}Villous atrophy present only in the two virus injected fetuses. TGE virus isolated from intestinal contents of pig 4.
\textsuperscript{f} - for PPV indicates no antibody detected at 1:10 dilution; for ECP0-6 or TGE indicates no antibody detected at 1:1 dilution.

\textit{NT} = not tested.
Fig. 16. Villous atrophy in section of mid-small intestine from fetal pig (pig 3, sow 260) injected with TGE virus on the 77th day of gestation. Hysterectomy-derived 14 days later. AGE (Azure and Eosin) stain; X120.
villi of a non-injected litter mate shown in Fig. 17.

The Sephadex G-200 chromatograms of pig 3 (virus-injected) of sow 260 is presented in Fig. 18. Based on immunodiffusion, IgM and IgG were detected in the sera of both virus-injected pigs. IgG and IgM were not detected in the serum of the 6 litter mates of the above dam. IgA was not detected in any of the 8 pig sera examined. IgM and IgG were present in the fraction representing the first peak of the chromatogram for pig 3 (Fig. 18). This fraction also had a titer of 20 for TGE. All other fractions tested had titers of less than 1; however, IgG was evident in one other fraction. The chromatogram for the virus-injected pig 4 was essentially the same as shown in Fig. 18, except that the titer of the first peak was only 1.5 and immunoglobulins were not detected in any of the fractions.

**Exposure of 74-day fetal pigs**

Two 74-day fetuses of sow 267 were injected with the same TGE virus preparation used above. Eleven live pigs were delivered into a sterile surgical isolator on the 111th day of gestation, 37 days post-injection. The pigs were small and slightly premature. The 2 virus- and 2 diluent-injected pigs were readily identified. One virus-injected pig (3), one diluent-injected pig (1), and a non-injected pig (5) were euthanized immediately after the hysterectomy and tissues were collected. Four non-injected pigs (6, 7, 8 and 9) and the other virus (4) and diluent (2)
Fig. 17. Normal villi in section of mid-small intestine from non-injected litter mate of pig represented in Fig. 16. A&E (Azure and Eosin) stain; X120.
Fig. 18. Gel filtration on Sephadex G-200 of serum from newborn hysterectomy-derived pig (91st day) injected 14 days previously with TGE virus. Antibody titer of 140 for TGE virus. Indicated are TGE antibody titers represented by vertical bars and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM and IgG were present in the serum; IgA was not detected.
injected pigs were passed into 3 sterile rearing isolators. The pigs were paired in the following manner—2 and 6, 4 and 7, 8 and 9—to each of the 3 isolators. Two pigs (10 and 11) were artificially reared in 2 open top cages. All pigs were slow to start eating. Pigs 10 and 11 were found dead on the second day and were not examined.

Two days after birth virus-injected pig 4 was in poor condition and, therefore, euthanized. This pig was very weak from the start and never ate well. Pig 2, media-injected, exhibited diarrhea on the 4th day, was very dehydrated and weak by the 7th day and was therefore euthanized. Rectal swabs from all pigs in the isolators taken on the 7th and 32nd day were bacteriologically negative after culturing on blood agar, eosin methylene blue, and thioglycollate media.

Blood samples were collected from the remaining 4 pigs in the isolators on the 15th and 32nd day of life. A summary of the results of exposing these pigs intrafetally to TGE virus is presented in Table 11.

Sephadex G-200 chromatograms of serum samples from pigs 1 and 3, of sow 267, are presented in Figs. 19 and 20. Antibodies to TGE were not detected in the serum of pig 1 on the day of hysterectomy. Although IgM and IgG were evident in the serum of pig 1, the chromatographic fractions were not examined for immunoglobulins or neutralizing antibodies. The chromatogram profiles for the 2 virus-injected
Table 11. Immunologic results of injecting two 74-day fetuses with TGE virus.a

<table>
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<tr>
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</tr>
<tr>
<td>11</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

aPiglets hysterectomy derived 37 days post-injection.
bSow at the time of fetal injection.
cSow at the time of hysterectomy.
dPigs 1 and 2 media injected, 3 and 4 virus injected, normal intestinal villi in pigs 1 thru 5.
eSample collected at 7 days of age.
fSample collected at 2 days of age.
gRaised in sterile isolators and sample collected at 15 days of age.
h - for PPV indicates no antibody detected at 1:10 dilution; for TGE or ECPO-6 indicates no antibody detected at 1:1 dilution.
NT = not tested.
Fig. 19. Gel filtration on Sephadex G-200 of serum from newborn, hysterectomy-derived pig (111th day). Pig 1 was injected with medium and 2 litter mates were injected with TGE virus 37 days previously. Pig 1 was serologically negative for TGE, ECPO-6, and PPV. IgM and IgG were present in the serum; IgA was not detected. All fractions were negative for immunoglobulins.
Fig. 20. Gel filtration on Sephadex G-200 of serum from newborn, hysterectomy-derived pig (111th day) injected 37 days previously with TGE virus. Indicated are TGE antibody titers represented by vertical bars and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA were present in the serum; IgA was not detected in any fractions.
pigs were similar.

Pig 3 had an S-N titer of 330 for TGE. The greatest titer for TGE in the fractions was located in the slight elevation between the two peaks as shown in Fig. 20. IgG was the only immunoglobulin evident in any of the fractions and that was located in the eluate with the highest titer for TGE. Immunoglobulins were not identified in the intestinal contents of pig 3; however, a faint reaction for IgG was detected in the stomach contents. All three classes of immunoglobulins were noted in the serum of pig 4, a virus-injected litter mate, 2 days after delivery. Only IgG was noted in the serum of pig 5, a non-injected control litter mate euthanized at birth. No attempt was made to isolate viruses from tissues of any of the pigs from sow 267. The intestinal villi of pigs 1, 2, 3, 4 and 5 appeared normal upon subgross examination.

Pigs 6, 7, 8 and 9 were reared in sterile isolators up to 46 days after delivery. Serum samples from all pigs except pig 7 were negative for TGE neutralizing antibodies on days 15 and 32 post-delivery. Serum from pig 7 had a TGE antibody titer of 550 fifteen days post-hysterectomy and 580 on the 32nd day post-delivery. All 4 pigs were infected with virulent (Miller #3) TGE virus on the 32nd day. Typical symptoms of TGE were evident in pigs 6, 8 and 9 beginning as early as 24 hours after viral exposure and lasting 6 to 8 days. All 3 pigs had severe diarrhea.
vomiting, dehydration and weight loss. Pig 7, in contrast, which had a TGE antibody titer of 580 at the time of challenge did not exhibit any signs of clinical TGE. Pigs 6 and 7 were euthanitized 7 days following exposure to TGE virus and pigs 8 and 9 fourteen days after challenge. An anamnestic response was not detected in the serum of pig 7 within 7 days after challenge. Pigs 6, 8 and 9 did respond serologically as evidenced by the S-N titers of 45, 150 and 180 respectively. Serologic response of these 4 pigs as a result of exposure with TGE virus at 32 days of age is presented in Table 12.

**Fetal Exposure with PPV**

Two sows were subjected to a surgical laparotomy and 2 fetuses of each sow were injected by the transuterine route with porcine parvovirus. One tenth ml of fluid from a virus pool with an HA titer of 320 was used. Sterile medium (0.1 ml) was used to inject 2 litter mates. The injected fetuses were identified with trypan blue. The sows were put in an isolation room for the duration of the pregnancy which was uneventful.

**Exposure of 101-day fetal pigs**

Two fetuses of sow 22-10 were injected with the virus on the 101st day of gestation and 2 litter mates were injected with sterile medium. The sow farrowed 10 live, normal pigs on the 112th day, 11 days post-injection.
Table 12. Immunologic results of pigs 6 thru 9 from Sow 267 after challenge with TGE virus at 32 days of age.

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<thead>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>9&lt;sup&gt;b)&lt;/sup&gt; 14 days post-TGE</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reared in sterile isolators and sample collected at 32 days of age just prior to challenge with TGE virus. Two litter mates were injected with TGE virus in utero on the 75th day of gestation.

<sup>b</sup>Pigs 6,8, and 9 develop typical clinical signs of TGE 24 to 48 hours post-challenge.

<sup>c</sup>- for TGE and ECPD-6 indicates no antibody detected at 1:1 dilution; for PPV indicates no antibody detected at 1:10 dilution.
Pigs 1 and 2 (media-injected) and pigs 3 and 4 (virus-injected) were readily identified by the blue dye. None of the pigs had nursed at the time of collection of specimens. The sow's H-I titer for PPV at the time of exposure was 1280 and at the time of farrowing it was 2560.

Pigs 1 through 5 were sacrificed immediately and tissues aseptically collected. The kidneys from pigs 1, 2 and 5 were pooled, ground in sand, and a 10% suspension made in tissue culture media. The kidneys from pigs 3 and 4 were pooled and treated in a similar manner. The fluids from the kidney tissues were inoculated onto young porcine kidney-cell sheets in an attempt to re-isolate the parvovirus. After several attempts and passages no HA activity or evidence of cytopathology were observed. Blood was also collected from these 5 pigs. The remaining 5 pigs (6 through 10) were returned to the sow.

Table 13 presents the immunologic results at birth from the sow and the 5 pigs examined. H-I antibodies were not detected in the serum of the 2 medium-injected pigs (1 and 2); in contrast, H-I titers of 1280 and 320 were present in the sera of the virus-injected pigs (3 and 4). Pigs 2 and 3 were adjacent in the uterus and, based on H-I antibody response, the virus apparently did not spread from pig 3 to pig 2, pig 1 or pig 5.

Although the dam had an S-N titer of 18 against ECP0-6 virus, antibodies against this virus were not detected in
Table 13. Immunologic results after injecting two 101-day fetuses with parvovirus.

<table>
<thead>
<tr>
<th>Animal</th>
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<th>TGE</th>
<th>ECP0-6</th>
<th>Ig class</th>
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<td>- + -</td>
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<tr>
<td>3</td>
<td>1280</td>
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<td>+ + -</td>
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<td>4</td>
<td>320</td>
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\textsuperscript{a}Sow farrowed 11 days after fetal injection.

\textsuperscript{b}Sow at the time of fetal injection.

\textsuperscript{c}Sow at the time of hysterectomy.

\textsuperscript{d}Pigs 1 and 2 diluent injected, 3 and 4 virus injected.

\textsuperscript{e} - for TGE and ECP0-6 indicates no antibody detected at 1:1 dilution; for PPV indicates no antibody detected at 1:10 dilution.
the sera of pigs 2, 3 and 4. Sera of pigs 1, 3, 4 and 5 were tested for antibodies against TGE and all were negative. However, the sow had a titer of 64 against TGE virus.

Sephadex G-200 chromatogram of the serum of pig 3 is presented in Fig. 21. The H-I titers of the fractions ranged from 2 to 256. IgG and IgM were detected in the serum of this animal. The first 3 fractions contained IgM and the next 3, IgG (Fig. 21). The fractions with IgM present were also the ones with the highest antibody titers for PPV. IgA was not present in the serum or any of the fractions of pig 3.

The chromatogram profile for pig 1 (non-injected and serologically negative) was essentially the same as that for pig 3. All the fractions tested were negative for immunoglobulins. The sera of all 5 pigs tested contained IgG but not IgA. IgM was found only in the serum of the 2 virus-injected pigs.

**Exposure of 62-day fetal pigs**

A second sow (0-0) was subjected to surgery on the 62nd day of gestation and 2 fetuses were injected with 0.1 ml of PPV and 1 fetus with 0.1 ml of sterile media. An H-I titer of 2560 was present in the serum of the sow at the time of surgery. A hysterectomy was performed at the 113th day of gestation, or 51 days post-infection. At this time the serologic titer of the sow for PPV was 1280.
Fig. 21. Gel filtration on Sephadex G-200 of serum from newborn pig (112th day) injected 11 days previously with PPV. Antibody titer of 1280 for PPV. Indicated are PPV antibody titers represented by dotted bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM and IgG were present in the serum. IgA was not present in the serum or fractions.
Nine live pigs and 2 mummies were obtained. One mummy was intact and the second one was disintegrating. Based upon crown-rump length it was assumed the intact mummy died around the 70th to 75th day of gestation.

The single diluent-injected pig was readily identified based upon dye markings. Since evidence of trypan blue was not seen in the other living pigs, it was assumed the 2 mummies were the virus-injected fetuses.

Immunologic results on the serum samples examined are presented in Table 14. Of the 9 live pigs present, 4 were serologically negative for PPV antibodies and 5 were positive. The titers in the positive specimens ranged from 640 to greater than 5120.

Although the dam was serologically positive with a titer of 70 against ECP0-6 virus, the sera of 8 pigs were tested and all were negative for ECP0-6 antibodies. Both the sow and 5 of the pigs' sera tested negative for antibodies against TGE virus.

The serum chromatogram for pig 1 is presented in Fig. 22. IgM, IgG, and IgA were present in the serum. None of the fractions revealed IgA. IgG and IgM were present in a few of the fractions as shown in Fig. 22. Treatment of the fractions with kaolin and guinea pig red blood cells eliminated all H-I activity for PPV in the various fractions. All 3 classes of immunoglobulins were detected in the sera of the 5 serologically positive pigs. In contrast, the 4
Table 14. Immunologic results of injecting two 62-day porcine fetuses with parvovirus.a

<table>
<thead>
<tr>
<th>Animal</th>
<th>Antibody titer</th>
<th>Ig class</th>
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<tr>
<td></td>
<td>PPV</td>
<td>TGE</td>
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<tr>
<td>0-0b</td>
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<td>0-0c</td>
<td>1280</td>
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<td>1d</td>
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<tr>
<td>11</td>
<td>5120</td>
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aPigs hysterectomy derived 51 days post-injection.
bSow at the time of fetal injection.
cSow at the time of hysterectomy.
dPig 1 diluent injected, 2 and 3 assumed to be the virus injected pigs.
e - for TGE and ECPO-6 indicates no antibody detected at 1:1 dilution; for PPV indicates no antibody detected at 1:10 dilution.
Fig. 22. Gel filtration on Sephadex G-200 of serum from newborn hysterectomy-derived pig (113th day). Pig 1 was injected with medium and 2 litter mates were injected with PPV 51 days previously. Pig 1 had an antibody titer of >5120 for PPV. Indicated are PPV antibody titers represented by dotted bars, and classes of immunoglobulins in selected unconcentrated eluate fractions. IgM, IgG and IgA were present in the serum; IgA was not detected in any fractions.
serologically negative pigs did not have IgA and 1 of the 4 did not have IgM.

SeroLogic Survey for PPV Antibodies in Pigs

Of 116 pre-nursing serum samples from newborn piglets representing 52 litters, PPV antibodies were present in 17 pigs from 6 different litters. Fifteen of these 17 pigs were positive for IgM and IgG by immunodiffusion while 12 were positive for IgA. All 116 pre-nursing samples were negative for ECP0-6 antibodies and all those tested were negative for TGE. The dams of all 52 litters were serologically positive for ECP0-6 virus. Two of the 6 litters were from a herd that sporadically experiences reproductive problems, including mummies, weak pigs, and breeding difficulties. The remaining 4 litters were from a herd in which reproductive problems have not been a complaint; however, occasional mummies were observed.

Serum samples were also tested from 23 swine farms throughout Ohio and from 5 packing plants. Of 129 samples from Ohio farms 77% were positive for PPV antibodies. Twenty-one or 91% of these 23 farms had animals in which part or all of the samples were positive for PPV antibodies. Titers were as high as 16,000. Eighty-two percent of the 96 samples from 5 Ohio packing plants were positive for PPV.
Dye Control

Injection of trypan blue into fetal pigs

Selected fetuses of 2 sows (51-4 and 7-3) were each injected with 0.1 ml of .45 μm membrane filtered trypan blue. This procedure was done to determine the effect of the dye on the fetus or the outcome of the pregnancy.

Four fetuses of sow 51-4 were injected with the dye subcutaneously on the 101st day of gestation. The duration of pregnancy was uneventful and the sow farrowed 11 days later. Ten live, fully developed normal appearing pigs and 1 dead pig were delivered. The 4 dye-injected pigs were readily identified. Evidence of arthrogryposis or ataxia was not noticed in any of the pigs. The pigs were allowed to remain with the sow and tissues were not collected.

Three fetuses of the second sow (7-3) were injected with the same amount and preparation of trypan blue on the 80th day of gestation. The duration of pregnancy was uneventful and the pigs were hysterectomy-derived on the 113th day or 33 days following injection of the dye. Four live, normal, good-sized pigs were delivered and the 3 dye-injected pigs were readily identified. Evidence of ataxia or arthrogryposis was not seen in any of the pigs and the brains of pigs 1 and 3 appeared normal. Pigs 2 (dye-injected) and 4 (non-injected) were reared in sterile
isolators and used for other experimental purposes.

At the time of hysterectomy the serum titer of the dam to ECP0-6 and PPV was 520 and 2560, respectively. Both pigs 1 and 3 were serologically negative for the 2 viral agents.

The Sephadex G-200 chromatogram for pig 1 of sow 7-3 is presented in Fig. 23. Immunodiffusion studies revealed IgG but not IgM or IgA in the serum of pigs 1 and 3. The fractions of pig 1 were negative for the 3 classes of immunoglobulins (Fig. 23).
Fig. 23. Gel filtration on Sephadex G-200 of serum from newborn, hysterectomy-derived pig (113th day) injected 33 days previously with trypan blue. Pig was serologically negative for PPV and ECPO-6. IgG was the only immunoglobulin present in the serum with all fractions negative for immunoglobulins.
This study was conducted to help clarify the effect that certain porcine viruses (enteroviruses, TGE, PPV) have on the developing fetus, with particular emphasis on the immune competence of the fetus. Fetal pigs at varying stages of development were injected by the transuterine route with one of the three viruses. Litter mates were injected with either sterile medium or saline. In the course of this study it was determined that fetal pigs were immunologically competent to the three viruses used and that the technique of "fetal serology" could be a useful tool for retrospective studies regarding the role viral agents contribute to fetal infections.

Fetal exposure with ECPO-6 virus

Early in the course of this investigation, abortions frequently resulted 3 to 10 days following the transuterine injection of fetal pigs with ECPO-6 virus. Subsequently, the dose of virus injected into each fetus was reduced and only 2 fetuses of each litter were injected with the virus. This proved beneficial since full term pregnancies resulted. Apparently the dose of the virus injected and the number of
fetuses infected influenced the outcome of the pregnancy. Although there are several reports incriminating porcine enteroviruses as a cause of stillbirths, mummification, embryonic death and infertility (44,46,47) this writer knows of no reports regarding developmental defects in swine being caused by the enteroviruses. There are, however, reports of other viral agents including hog cholera virus, causing cerebellar hypoplasia, hypomyelogenesis and congenital tremor in newborn pigs (52,53). In the course of the present investigation such abnormalities as ataxia, arthrogryposis and cerebellar hypoplasia were observed following exposure of fetal pigs with ECP0-6 virus (Figs. 5,6,7,8,11). The above mentioned abnormalities were not observed in any pigs infected after the 102nd day of gestation. Likewise these abnormalities were not observed following fetal exposure with TGE or PPV virus, although only a limited number of animals was used for the latter studies. Ataxic pigs were observed in litters that had been infected from the 45th to the 102nd day of gestation. Cerebellar hypoplasia was noted only within those litters in which exposure with ECP0-6 occurred on the 45th and the 64th day of gestation.

Although not conclusively proven, the evidence supports the view that the trypan blue used to identify injected fetuses was not involved in causing the defects observed.
Examination of Table 2 reveals that cerebellar hypoplasia and ataxia were evident within the 2 litters infected on the 64th day (sows 20-2 and 19-6). However, the dye was not used to identify the injected fetuses of sow 19-6 yet the defects were observed. This suggests that trypan blue was not involved in producing the developmental defects. Secondly, trypan blue by itself did not produce evident abnormalities in fetal pigs injected on the 80th or 101st day of gestation.

Although extensive histopathologic studies were not conducted in the present investigation, it is of interest to note the lesions observed in virus-injected pigs from sow 38-9. These pigs were exposed on the 102nd day of gestation with ECP0-6 virus and the sow farrowed 8 days later. Glial foci, hyperemia and petechia of the medulla, gliosis around the 4th ventricle and focal gliosis in the gray matter of the lumbar cord were noted in the 3 virus-injected pigs of this litter. This observation is similar to that reported after infecting newborn germfree colostrum-deprived pigs with ECP0-6 virus (6,182).

Also of interest are the observations made on the litter infected on the 21st day of gestation (sow 7-2, Tables 1 and 2). Eighty-seven days later 8 live normal appearing pigs were obtained by hysterectomy. Evidence of mummification or other abnormalities were not seen. Likewise, there was
no evidence of viral infection in these 8 pigs based on virus isolation procedures, absence of antibody or classes of immunoglobulins. One can only speculate what might have occurred within this litter. Since 14 corpus lutea were present and only 8 pigs delivered, embryonic death with reabsorption and lack of spread of the virus could have occurred. It is also possible that actual infection of the embryo did not occur since embryo size at the time of injection precluded precise identification of the embryo. Therefore the virus could have been placed directly into a sacculatum in the area of the embryo.

Although the duration of antigenic stimulus before antibody can be detected in fetal pigs has not been extensively investigated, it appears from the present studies that a minimum of 5 days is necessary with ECPO-6 virus before there is any detectable antibody produced. ECPO-6 antibody was present in 111-day fetal pigs that had been injected 5 days previously. Likewise, IgM was evident in the serum, also suggestive of antibody production. In contrast, antibody or IgM was not present in 109-day fetal pigs which had been injected with virus 4 days previously. With a minimum of 5 days after virus injection to a maximum of 70 days, antibody was detected in a part of all litters of pigs that had been exposed with the virus. In a few cases antibody was present only in the virus-injected pigs. In other cases, particularly with longer exposure
periods, antibodies were also detected in non-injected litter mates, apparently due to inter-fetal transmission of virus. Antibody was not detected in any of 7 litter mates in which 3 embryos were virus injected 87 days previously. The possibility exists that immunologic tolerance could have developed. If this is the case one might expect to isolate the virus from fetal tissues; however, virus was not isolated from any tissues of the 8 pigs delivered.

Based on the detection of fetal ECPO-6 antibody, and also virus isolation, the virus apparently spreads from one fetus to another. It was not determined in the present studies if this was a direct spread to adjacent fetuses or by some other route.

PPV antibodies were present in pre-nursing sera of the litters of sows 5-9, 140 and 80 used in the ECPO-6 studies. Although ECPO-6 antibody was not detected in one of the 2 saline-injected pigs of sow 5-9, virus was isolated from the colon. Isolating virus but not detecting antibody suggests that the pig had been recently infected. The interesting observation was the detection of IgM, IgG and IgA in the serum of this pig even though it was negative for ECPO-6 antibodies. Subsequent studies revealed that both saline-injected fetuses were serologically positive for PPV. Likewise, both virus-injected and saline-injected pigs of sow 140 were positive for PPV antibodies and all 3 classes of immunoglobulins were present in pigs 1 and 3,
a saline-injected and a virus-injected pig respectively (Table 5). In the case of sow 80, 3 pigs were serologically positive for PPV; however, only IgM and IgG, but not IgA, were detected on immunodiffusion. The fact that IgA was not detected in the latter case suggests that the fetus had been recently infected with PPV. Studies discussed elsewhere revealed that IgA was not evident in fetal pigs 11 days after exposure with PPV but was detected in term pigs exposed 51 days previously with PPV. Although a serum sample was not available from the sow at the time of farrowing, she was serologically negative for PPV 35 days previously. Since IgA was not detected in the serologically positive fetuses and the sow was negative for PPV antibodies 35 days prior to farrowing, this would suggest a recent infection of the fetus.

Fetal exposure with TGE virus

Fetal pigs from 3 different sows were infected with TGE virus on the 95th, 77th, and 74th day of gestation. The pigs were obtained by hysterectomy 15, 14, and 37 days, respectively, after injecting the TGE virus. In the first two cases, with infection 14 to 15 days previously, typical villous atrophy, as reported for TGE (34, 208), was detected only in the virus-injected pigs (Figs. 16 and 17). In contrast, villous atrophy was not observed in either virus-injected or non-injected pigs that had been infected 37 days previous. In comparing the results from these 3 sows it
may be concluded that in the fetal pigs infected 37 days previously, villous atrophy had probably occurred and subsequently repaired. As long as the pig in utero is receiving nourishment by the transplacental route, TGE virus might not be lethal. If this is the case this antigen might prove effective in defining a model system for studying the development of immune competency by the fetal pig.

Indirect evidence would suggest that TGE virus did not spread to non-injected litter mates from those pigs exposed 14 to 15 days prior to hysterectomy. The TGE antibody was detected only in the virus-injected fetuses of these 2 litters as shown in Tables 9 and 10. If the virus did infect the non-injected litter mates, there was not adequate time to stimulate detectable levels of antibody. This assumption may or may not be valid in the case of pigs exposed with TGE virus on the 74th day followed by hysterectomy 37 days later. It will be noted from Table 11, that of the samples tested at birth only the virus-injected pigs were positive for TGE antibodies; however pig 7, reared in a sterile isolator, was serologically positive for TGE at 15 days of age. It is not known if this pig became infected in utero or after birth. Since this pig did not develop clinical signs of TGE infection it might be assumed that infection occurred in utero, suggesting that the virus had spread within the uterus. It must be remembered, however, that the TGE virus used for fetal
injection was attenuated and usually does not make newborn pigs very sick.

Table 12 presents the data regarding challenge studies with TGE virus on the 32nd day of age. Of the 4 pigs used, 3 were serologically negative for TGE on the 32nd day of life but pig 7 had a titer of 580. All 3 serologically negative pigs developed typical signs of TGE 24 to 48 hours following viral exposure. However, pig 7 remained clinically normal.

If the assumption is true that pig 7 had been infected in utero and since it was shown to be resistant to challenge with the virus, this procedure of fetal immunization might prove beneficial in controlling baby pig losses from TGE. However, the feasibility of such a procedure remains in question. Many problems and the technique of trans-uterine immunization on a practical scale would have to be developed.

Although both sows, 260 and 267, were serologically positive for ECPO-6 and PPV, antibodies against these 2 viruses were not detected in any of their pigs at the time of terminating the pregnancies (Tables 10 and 11). In contrast, antibodies against PPV but not ECPO-6 were present in all of the newborn pigs examined from sow 189 (Table 9). This was a surprising finding and would make one suspicious that the TGE virus used to inoculate the fetuses 15 days previously may have been contaminated with
PPV. This possibility was ruled out since the same virus pool had been used to infect the other 2 litters and they did not have detectable PPV antibody present. Additional work using germfree animals also confirmed that the viruses used in these studies were not cross contaminated. The possibility exists that the PPV antibodies detected in the litter of sow 189 were of maternal origin transplacentally acquired or were fetal in origin. The latter assumption would appear the most valid. This conclusion was made because the serum profile of the dam was not compatible with that of her pigs as would be the case if the fetal antibodies were of maternal origin. Examination of Table 9 reveals the sow was serologically positive for ECPO-6, TGE and PPV. However, all the pigs were negative for ECPO-6 antibodies, only the 2 TGE-infected pigs had TGE antibodies, yet PPV antibodies were detected in all of the pigs. The most likely explanation for this observation is that sow 189 was in a state of PPV viremia sometime during pregnancy which resulted in fetal infection and fetal antibody against PPV. Sow 189 originated from a herd in which newborn pre-nursing sera of other litters have been positive for PPV antibodies.

The detection of PPV antibodies in the pigs of sow 189 was somewhat retrospective in nature. It will be noted from Table 9 that all 3 classes of immunoglobulins (IgM, IgG and IgA) were detected in all pigs irrespective of
being virus-injected, medium-injected, or non-injected. However, TGE antibodies were present only in the virus-injected pigs. As will be discussed later, IgM and IgA are usually not present in newborn pre-nursing pig sera unless an antigenic stimulus has occurred. Since IgM and IgA were evident in pigs serologically negative for TGE it was decided to test for PPV antibodies.

Fetal exposure with parvovirus

Mummies or other abnormalities were not detected in term pigs of sow 22-10 injected with PPV on the 101st day of gestation. In contrast, 2 mummified fetuses were found at term from the litter of sow 0-0 injected with the virus on the 62nd day of gestation. Based upon the crown-rump length of the 2 mummies, it was ascertained they died sometime after fetal exposure. Since evidence of the trypan blue was not seen in the other living pigs it was assumed the 2 mummies were the virus-injected fetuses.

PPV antibody was detected only in the 2 virus-injected fetuses of sow 22-10 and not from the medium-injected or non-injected litter mates. Indirect evidence would suggest that the virus had not spread to other pigs at least with sufficient time to stimulate any detectable antibody. The second case, however, in which the pigs had been injected for a much longer period of time the virus apparently spread to litter mates as 5 of the 9 live pigs born were
serologically positive for PPV and these 5 pigs had not been injected with the virus.

Sows 22-10 and 0-0 were serologically positive for ECPO-6 virus and sow 22-10 was positive for TGE virus. However, none of the pigs from these 2 sows had detectable antibodies against these 2 viruses. This information would suggest that transplacentally acquired maternal antibody was not present in the fetuses, either as a result of the surgery or the infection, and that the PPV antibody detected was probably of fetal origin.

The data on the class of immunoglobulins detected in these 2 litters (Tables 13 and 14) would suggest that infection for 11 days was insufficient time to stimulate the IgA system in the fetus, as IgA was not detected in the 2 virus-injected pigs of sow 22-10. In contrast, IgA was present in all pigs serologically positive for PPV of sow 0-0, in which fetal exposure occurred 51 days previously.

Another interesting observation is that 3 pigs (4, 7 and 10) of sow 0-0 (Table 14) were serologically negative for PPV; however, IgM was detected in their sera on immunodiffusion. This discrepancy of finding IgM but not PPV antibody may represent a very recent infection and the immunodiffusion procedures may be more sensitive than H-I techniques. It is also possible the animal may have been
exposed with an antigen not tested for.

Although not much is known regarding the importance of PPV in fetal infections of swine, the results of the serologic survey on newborn pre-nursing sera are in agreement with others (28,89,116,132), suggesting that this virus may transplacentally infect the fetus resulting in fetal loss or reproductive problems. In the present study 17 of 116 pre-nursing sera were positive for PPV antibodies, yet all 116 were serologically negative for ECPO-6. This observation would suggest that PPV could be a major contributing factor in fetal loss or breeding problems of swine in the United States. The data suggest that PPV may be more important in naturally occurring fetal infections than the enteroviruses, at least the ECPO-6 virus used in this study. Most of the reports regarding this virus have been from other countries (7,27,132) and only recently has there been a report of isolating PPV from swine in the United States (116).

Seventy-seven percent of 129 serum samples from Ohio farms and 82% of 96 samples from slaughter plants in Ohio were serologically positive for PPV. This information indicates that PPV infection may be fairly common in the swine population. The serologic results reported in this study compare favorably with those recently reported by Mengeling (116) who also suggested the widespread distri-
bution of serologic positive animals in the swine population in the U.S.A.

Additional information is needed regarding the epidemiology and pathogenicity of PPV so its significance to porcine fetal losses or reproductive problems in the United States might be ascertained.

Fetal exposure with trypan blue

Early in the course of this study it was deemed advisable to determine if trypan blue had any effect on the developing fetus either in respect to developmental abnormalities or influence on the presence of immunoglobulins. Although only 2 animals were used, one in which fetuses were dye-injected on the 101st day of gestation and the other on the 80th day of gestation, evidence of mummies, stillbirths, ataxia or arthrogryposis was not seen in any of their term pigs. Cerebellar hypoplasia was not detected in those brains that were examined.

Serologic studies were not conducted on newborn pigs that had been dye-injected on the 101st day of gestation. In the case of the pigs that were dye-injected on the 80th day of gestation, only 4 pigs were obtained by hysterectomy 33 days later. However, no evidence of mummification was detected and all injected fetuses were identified by the dye marking. Although the sow was positive for ECPO-6 and PPV antibodies, the 2 dye-injected pigs that were checked
were serologically negative for these 2 viruses. Likewise only IgG, and not IgM or IgA, was detected in the sera of these 2 pigs.

Information from this study would suggest that trypan blue did not contribute to any detectable developmental defects when injected into 80- or 101-day fetal pigs. Likewise, the technique of injecting the dye transuterinely apparently did not affect passage of maternal antibodies across the placenta as antibodies present in the dam were not detected in the fetuses. Trypan blue injected subcutaneously in various locations proved to be an effective means of fetal identification.

**Immune response of the fetus to injected antigens**

IgG was present in all newborn full term pigs used in this study independent of their history of antigenic stimulation. However, IgG was not present in the sera of pigs obtained on the 79th (sow 12-1) and 91st (sow 260, Table 10) day of gestation, unless antigenic stimulation had occurred. This observation is compatible with other reports in that IgG is not detected in fetal pigs until the 102nd day of gestation (209). The finding of IgG in full term pre-nursing sera is in agreement with others, who have studied the active synthesis of immunoglobulins in the fetal pig (57, 58, 146, 147). With the exception of 1 case (sow 0-0, Table 14) discussed previously, IgA or IgM was not present in pre-nursing sera unless there was a history of antigenic
stimulation or antibody was detected. Travnicek et al. (209) could find no trace of IgM or IgA in the sera of fetuses of different ages. The detection of IgM after antigenic stimulation is in agreement with Prokesova et al. (146, 147) who reported finding IgM in newborn piglets only if intrauterine immunization of the fetuses or intrauterine infection had occurred.

Gel filtration (Sephadex G-200) studies were conducted on newborn or hysterectomy-derived pigs which had been intrafetally infected and were serologically positive (Figs. 1, 2, 4, 9, 13, 14, 15, 18, 20, 21, 22). The purpose was to determine if antibody activity was associated with any particular class of immunoglobulins. The results indicated that antibody detected within 8 to 28 days post-exposure was associated with high molecular weight Ig, either IgM or the 19S IgG class. The technique used did not provide a differentiation between the 2 classes of Ig. There is evidence, as indicated by Figs. 1, 2, 3, 4 and 18, that early antibody activity was not associated with low molecular weight 7S Ig. Although IgA was occasionally detected in the sera of fetal pigs following antigenic stimulation, at no time was IgA detected in any of the Sephadex G-200 fractions of these sera. The reason for this is not fully known, however it is speculated the IgA was in low concentration in the serum and the dilution factor after separation in the columns did not permit the detection of IgA by
The results of this study would suggest that simple immunodiffusion techniques using monospecific antisera against porcine IgM or IgA could be a means of screening pre-nursing piglet sera for evidence of fetal infection. If either one of these two classes of immunoglobulins were detected one could use additional immunologic techniques in an attempt to specifically identify the infecting agent.

Except in 4 cases, the antibody response of the injected fetuses was apparently specific for the antigen injected. In the case of sows 5-9, 140 (Table 5), 80 (Table 1) and 189 (Table 9) antibodies against PPV were detected in their offspring at birth. Fetuses of sows 140, 80, and 5-9 had been injected with ECPO-6 virus and fetuses of sow 189 with TGE virus.

The possibility that the virus inoculum had been contaminated with PPV was ruled out based upon three facts. The same pool of each virus was used throughout these studies and the four mentioned cases are the only litters where PPV antibodies were detected in newborn pig sera. Secondly, the other 2 viruses used in these studies have been injected into germfree pigs without eliciting a PPV antibody response. In addition, PPV antibodies have been detected in newborn pigs from sows of the same herds from which the experimental animals originated, indicating that infection with PPV was endemic in these herds. These
observations suggest that the litters of sows 140, 80, 5-9 and 189 were naturally infected intrafetally with PPV.

The possibility of maternal antibody being present in the transuterine injected pigs was ruled out by comparing the serum profile of the dam with that of the offspring. By using the three antigens (ECPO-6, PPV, TGE) it was demonstrated that the antibody detected in the fetuses was not of maternal origin; and secondly, the technique of transuterine inoculation following surgery apparently did not result in any leakage of maternal antibody across to the fetus.

It would appear that the technique of "fetal serology" using pre-nursing sera may have potential value in determining if an intrauterine antigenic stimulus has occurred. There may, however, be certain limitations to this technique. When the placenta is damaged, it is possible that maternal antibodies may appear in the fetus. Such damage can be induced by hog cholera virus (234). Mechanical and functional defects may occur which would also permit the transfer of macroglobulins. Data by Kim et al. (97) suggested that damage to the placental barrier occurred when pregnant sows were given multiple injections of antigens containing endotoxin. The possibility of maternal antibodies being present in the newborn pig, either by way of a damaged placenta or absorbed from maternal fluid during birth, needs to be known and accounted for before a
definite diagnosis is made using the technique of "fetal serology".
SUMMARY

Fetal pigs at varying stages of development were exposed by the transuterine route with one of three porcine viruses: ECPO-6 which is an enterovirus, transmissible gastroenteritis (TGE), or a parvovirus (PPV). Litter mates were injected with either sterile medium or saline and injected fetuses were identified with trypan blue.

The immunologic response was determined by employing serum-neutralization or hemagglutination-inhibition tests against the antigen used as well as determining the class of immunoglobulins present by the agar gel immunodiffusion technique using monospecific antisera. The immunologic responses of the virus-injected, medium-injected, and non-injected pigs were compared. Comparisons were also made between the antibodies present in the fetus and those present in the dam.

Abnormalities such as ataxia, arthrogryposis, and cerebellar hypoplasia were observed following exposure of fetal pigs to ECPO-6 virus. Ataxic pigs were present within litters exposed between the 45th and the 102nd day of gestation. Cerebellar hypoplasia and arthrogryposis were detected within those litters exposed on the 64th and the
45th day of gestation. These abnormalities were not observed in any pigs exposed with the virus before the 45th or after the 102nd day of gestation. Likewise, they were not seen following fetal injection with TGE, PPV or trypan blue. It appeared that trypan blue was not acting as a teratogenic agent in these studies.

ECP0-6 antibody and IgM were present in 111-day fetal pigs that had been injected 5 days previously with ECP0-6 virus. In contrast, antibody or IgM was not present in 109-day fetal pigs which had been injected with virus 4 days previously. From this data it was concluded that a minimum of 5 days after exposure with ECP0-6 virus is necessary before there is any detectable antibody. In a few cases antibody was detected only in the virus-injected pigs. When ECP0-6 antibody was detected in non-injected litter mates, it was usually associated with longer exposure periods and was apparently due to inter-fetal transmission of the virus.

Villous atrophy of the intestinal mucosa was observed in 110- and 91-day fetal pigs which were injected 14 days previously with TGE virus. Villous atrophy was not seen in medium-injected or non-injected litter mates. The TGE antibody response was limited to the virus-injected pigs. Intestinal villous atrophy was not present in term pigs, positive for TGE antibodies, exposed 37 days previously. It was concluded that in the fetal pigs injected 37 days
previously villous atrophy had probably occurred with subsequent repair of the intestine.

Exposing 101-day fetuses with PPV resulted in detecting PPV antibody 11 days later only in the virus-injected pigs. In contrast, PPV antibody was detected in 5 non-injected term pigs in which 2 litter mates were injected with PPV on the 62nd day of gestation. The classes of immunoglobulins detected in the 2 litters exposed with PPV were presented and discussed. It was concluded that infection for 11 days was an insufficient length of time to stimulate the IgA system with PPV, as IgA was not detected in the two serologically positive virus-injected pigs. In contrast IgA was present in all pigs serologically positive for PPV in which fetal exposure of 2 litter mates occurred 51 days previously.

IgG was present in the pre-nursing sera of all full-term pigs irrespective of their history of antigenic stimulation. In contrast, IgG was not present in the sera of 91- or 79-day fetal pigs unless they were antigenically stimulated and detectable antibody was evident. The detection of IgM or IgA in newborn pre-nursing sera was highly correlated with a history of antigenic stimulation or the detection of specific antibodies. In 4 experimental litters, IgM or IgA was detected in serologically negative pigs in which litter mates were exposed with ECP0-6 or TGE virus. Subsequent studies revealed these pigs to be sero-
logically positive for PPV. It was concluded from this indirect evidence that 4 of the litters used in these studies were naturally infected intrauterinely with PPV.

Gel filtration studies in an attempt to determine the class of immunoglobulins to which the fetal antibody activity belongs did not lead to any conclusive results. Antibody was present only in the first peak in those pigs exposed for 8 to 28 days with viral antigen suggesting a high molecular weight Ig, either IgM or the 19S IgG class. The early antibody activity was not associated with low molecular weight 7S IgG. In contrast, exposure for 37 days or longer to the viruses resulted in antibody only in the peak representing low molecular weight 7S IgG.

In a serologic survey 17 of 116 pre-nursing pig sera were positive for PPV antibodies. All 116 samples were negative for ECP0-6 antibodies and all those tested for TGE were negative. Data is presented and discussed suggesting that PPV infection is widespread in the swine population. The importance of this observation in relation to fetal infections and reproductive problems is also discussed.

By comparing the antibody profile of the dam to that of her offspring it was concluded that the antibodies detected in the pre-nursing sera were of fetal origin and were not transplacentally acquired. It was also determined that the technique of transuterinely injecting fetuses did not result in placental damage with subsequent passage of maternal
antibodies across the placenta.

The detection of IgM or IgA in pre-nursing sera by simple immunodiffusion technique proved a useful way of screening for evidence of antigenic stimulation in fetal pigs. Subsequent determination of specific antibodies could prove a useful tool in attempting to identify agents responsible for fetal infections.


