IMMUNOLOGICAL RESPONSES IN FERRETS INOCULATED WITH
INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS VACCINES

by

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DEDICATION

To my parents for their love and
encouragement, which is never ending.

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ABBREVIATIONS

ATCC	American Type Culture Collection
BUN	Blood Urea Nitrogen
BVDV	Bovine Virus Diarrhea Virus
$^{\circ}$C	Degrees Celsius
Ca	Calcium
CBC	Complete Blood Count
cc	Cubic Centimeter
Ci	Curie
Cl	Chloride
cm	Castrated Male
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DPM	Disintegrations per Minute
EDTA	Ethylene Diamine Tetraacetic Acid
F°	Fahrenheit
f	Female
FCS	Fetal Calf Serum
g	Gravity
H_2O	Water
Hb	Hemoglobin
HS	Horse Serum

^{3}H-TdR	Tritiated Thymidine
IBRV	Infectious Bovine Rhinotracheitis Virus
IM	Intramuscular
IN	Intranasal
K	Potassium
LA Strain	Los Angeles Strain
m	Male
MDBK	Madin Darby Bovine Kidney
MEM	Minimal Essential Media
mg	Milligram
ml	Milliliter
MLV	Modified Live Virus
mmole	Millimole
Na	Sodium
NaCl	Sodium Chloride
NADL	National Animal Disease Laboratories
ng	Nanogram
n	Sample Size
NS	Nonsignificant
P	Phosphorus
PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PFU	Plaque Forming Unit
PHA	Phytohemagglutinin
PSK	Penicillin-Streptomycin-Kanamycin
RNA	Ribonucleic Acid

RPMI	Roswell Park Memorial Institute
SDH	Sorbital Dehydrogenase
SGPT	Serum Glutamate Pyruvate Transaminase
SMA-12	Sequential Multiple Analysis
TCA	Trichloroacetic Acid
T.P.	Total Protein
uCi	Microcurie
ug	Microgram
UV	Ultraviolet
WBC	White Blood Cells

SECTION I

INTRODUCTION

INTRODUCTION

Infectious bovine rhinotracheitis (IBR), a herpes virus infection in cattle, is capable of producing a latent infection and a variety of clinical manifestations ranging from respiratory to reproductive syndromes (Kahrs, 1977; Owens, Chow and Mollelo, 1964; French, 1962; Abinati and Plumer, 1961; McKercher, et al., 1959; Madin, York and McKercher, 1956). Recrudescence or activation of latent IBRV infections can occur spontaneously during periods of stress when physiological hormone levels are altered or with the administration of corticosteroids (Davies and Carmichael, 1973). These characteristic syndromes in addition to the unbiquitous nature of IBRV in the United States and other parts of the world have attributed to an increasing concern to manage this disease. The detrimental effects of IBRV infections are manifested in cattle of all ages and cause economic losses in milk and meat production (Kahrs, 1977).

Presently, the prevention and control of IBRV is accomplished through proper animal management and most importantly through vaccination schemes. The availability of a variety of vaccines, such as modified live virus (MLV) or inactivated virus vaccines and the different routes of inoculation have left the practitioner and cattle owner with a seemingly difficult choice. Investigations evaluating these vaccines have elucidated some of the mechanisms by

which immunity to IBRV infections is achieved. It has been reported that the modified live IN vaccine provides protection for pregnant dams and elicits local antibody and interferon production (Todd, Volenec and Paton, 1972) but has no distinct advantage over ML-IM vaccines in humoral antibody production (McKercher and Crenshaw, 1971). Inactivated vaccines with and without adjuvant have been found to produce adequate levels of humoral antibody (Haralambiev, 1976; Karadjov, 1976; Juhasz, Palatka and Toth, 1973).

In influenza virus studies, the ferret, Mustela putorus furo, has been used to evaluate the efficacy of potential human vaccines, since ferrets closely simulate a similar course of disease observed in humans. These studies have provided much information on the local and humoral responses to various vaccines (Potter, et al., 1972a, 1972b). The National Animal Disease Center (1977) and Porter, Larsen and Cox, (1975) have studied the humoral response of IBRV infected ferrets, and have found that ferrets produce serum antibodies to IBRV.

The immune response is complex and it has been demonstrated that IBRV latency and spontaneous recrudescence occurred in the presence of humoral antibodies (Sheffy and Rodman, 1973). As a result, investigators are trying to determine the importance of the cell mediated immune responses to IBRV. The primary factors of the cellular immune system are the thymus (T) dependent lymphocytes. There are various techniques for measuring T cell stimulation or

blastogenesis to a mitogen or specific antigen. The method
reported by Sorenson, Anderson and Giese, (1969) measures
the incorporation of radioactive labelled DNA precursors as
an index of T-lymphocyte blastogenesis and is commonly used
as an indication of CMI responses.

It has been found that adrenal cortical hormones by
causing cytotoxic effects and altering the metabolism
and activity in the cellular components of the immune system,
suppress the CMI responses to either mitogen or specific
antigen. In vitro studies have illustrated the inhibition
of blastogenesis in lymphocyte cultures supplemented with
cortisol before cr simultaneously with the stimulus (Fauci
and Dale, 1974; Tormey, Fudenberg and Karmin, 1967; Stevens
and Dougherty, 1964; Nowell, 1961).

A better understanding of the complex mechanisms of the
immune response in an animal to an antigen and the suppression
observed in lymphocytes with cortisol, would provide insight
for establishing adequate resistance through vaccination
and controlling latency and the recrudescence of IBRV in-
fections. The objectives of this investigation are to
(1) study the hematological and serum profiles of ferrets,
(2) determine whether the ferret can be used as a suit-
able laboratory animal model for evaluating immunity to
IBRV infections by studying the humoral and cell mediated
immune responses of control and IBRV vaccine inoculated
ferrets and (3) study the in vitro effect of cortisol on
ferret lymphocytes in whole blood cultures.

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SECTION II

REVIEW OF LITERATURE

The Ferret as a Laboratory Animal.

The domestic ferret, _Mustela putorius furo_ L. in the United States includes the brownish-black and yellowish colored fitch ferret and the yellow-cream colored, ruby-eyed albino or English ferret (Willis and Barrow, 1971). As members of the weasel family, Mustelidae, these carnivores are small in size and are easily manageable in colonies for research purposes. Contrary to myths, the ferret has an agreeable disposition and can be handled without an animal restrainer and gloves, for procedures such as rectal temperatures, inoculations and weighings. The breeding, management, growth and development has been studied (Hammond and Chesterman, 1972; Willis and Barrow, 1971; Hammond, 1969; Pockson, 1956; Grinham, 1952; and Pyle, 1940), and the maintenance of laboratory colonies is conducive to ferrets.

Anesthesia for the Ferret.

Procedures causing prolonged discomfort and pain in ferrets require the use of anesthesia. In general, the mustelids are easily anesthetised with injectable agents and have similar responses as cats, except that adverse effects to morphine-like drugs are not encountered. Chemical restraint by alphoxolone-alphadalone, ketamine hydrochloride and fentanyl citrate-fluanisone have been found to provide safe and effective sedation (Green, 1978).

Increased doses of the above agents provide light anesthesia for surgical procedures. Fentanyl citrate and fluanisone supplemented with a halothane-oxygen mixture followed by nalorphine hydrobromide also produced satisfactory results (Harpur, 1978). Light ether induction provides short periods of immobilization and anesthesia, but should not be used for restraint purposes due to the difficulty in handling as a result of the animals sneezing and paddling reflexes (Pyle, 1940). Pentobarbitone sodium, although having a narrow safety margin given intraperitoneally (IP) for prolonged anesthesia or in conjunction with ether has been found to be satisfactory (Lumb, 1963). The use of chloroform is not recommended near the animal housing facilities, since low concentrations of vapor cause nursing ferrets to scatter the young about the cage (Hammond and Chesterman, 1971).

Blood Collection Procedures in Mustelids.

Small quantities of blood for studies such as packed cell volume and blood smears can be obtained by deeply pricking the skin at the tip of the ear (Pyle, 1940) or by overcutting the toenails (Bergman, Lodmell and Hadlow, 1972). Larger samples up to 12 ml can be drawn with relative ease by cardiac puncture when animals are anesthetised (Baker and Gorham, 1951). Safe multiple bleedings in minks Mustela vison, have been reported by Fetch and Wobeser, (1970) using a jugular venipuncture technique with a 20 or

22 guage, 1 inch needle. Bergman, _et al_. (1972) describes the surgical implantation of a polyethylene catheter into the jugular vein for studies which require numerous blood samplings over short time intervals. The coccygeal, saphenous and cephalic veins are small and not routinely used (Pyle, 1940).

Hematological and Serum Studies.

The majority of information on mustelid hematology and serology has been provided by studies on the mink. The blood parameters studied were packed cell volume (PCV), hemoglobin (Hb), total erythrocyte count (RBC), plasma and blood volume, total and differential white blood cell count (WBC), and mean corpuscular volume and density (Schalm, Jain and Carroll, 1975; Ringer, _et al_., 1974; Fletch and Karstad, 1972; Kennedy, 1935a, 1935b, 1935c). Various constituents in serum that have been studied in minks include total protein, albumin, globulin fractions, total lipids, B-lipoproteins, phospholipids, total, free and esterified cholesterols, glucose, calcium, nitrogen and carbon dioxide levels (Rotenberg and Jorgensen, 1971; Kubin and Mason, 1948). In the ferret, however, the information is limited to total RBC and WBC, and differential WBC counts (Hammond and Chesterman, 1972; Pyle, 1940; Fox, 1923). Preliminary blood chemistry studies on glucose, calcium and nonprotein nitrogen have been done by Pyle, (1940). Physiological parameters such as average normal rectal temperatures

(Woodnott, 1969), respiration rate ranges (Pyle, 1940), cardiac output (Kempf and Chang, 1949) and growth and development rates (Shump and Shump, 1978) have been studied.

Studies Using Ferrets.

An invaluable laboratory animal model, the ferret, has been used in a diversity of studies, including endocrinology (Wu and Chang, 1972), reproductive physiology (Chang, Casas and Hunt, 1971), pharmacology and toxicology (Steinberg, 1973) and microbiology (Rowlands, 1957). The ferret, because of its susceptibility to the canine distemper (Carpenter, 1976; Farrell, 1971) and human influenza viruses (Toms, et al., 1977, 1975; Small, 1976) have been used extensively for in vivo investigations and for the evaluation of vaccines for these viruses. In vitro cultures of respiratory and other ferret tissues have been developed (Kingsman, Toms and Smith, 1977a, 1977b; Toms, Rosztoczy and Smith, 1974; Gould et al., 1972) and are used for the localization of influenza viruses.

The Clinical and Immune Responses to Human Influenza Viruses in Ferrets.

The ferret is most frequently used in influenza virus studies, since the influenza virus, although highly transmissible to a variety of animal hosts, simulates a pathological response in ferrets most similar to that found in man. This is of particular interest for studying the pathogenesis and effects of human influenza virus vaccines.

Previous studies (Potter, Jennings and McLaren, 1973;

Potter, McLaren and Shore, 1973; Potter, et al., 1972a;
Potter, et al., 1972b) have compared the clinical (febrile)
and immunological responses of ferrets to live, attenuated
and inactivated influenza virus vaccines given either IN,
IM or IM with adjuvant. Comparisons between Freund's in-
complete adjuvant and adjuvant 65 were also made. The con-
centration of virus and the protein constituents collected
in the nasal washes were correlated with immunity to the
virus (Toms, et al., 1977; Potter, Shore, McLaren, 1972;
Shore, Potter and McLaren, 1972). Hemagglutinin inhibiting
(HI) and neutralizing antibodies detected in the nasal
washes and HI antibodies detected in the serum were titered
and found at high levels in live virus infected (IN) animals,
as compared to animals infected (IM) with attenuated or
inactivated influenza virus. Ferrets infected with live
influenza virus and challenged with the same virus were
found to be resistant to infection, as manifested by the
lack of a febrile response and the absence of an elevated
immune response. Live vaccines administered IN, produced
the most effective immunity, followed by attenuated virus
vaccines given intranasally. Inactivated virus administered
with adjuvant, IM, produced humoral immunity but to a lesser
degree. Inactivated virus given IM or IN with saline pro-
duced no immunity. Challenge with an influenza virus of a
different strain, elicited elevated antibody and febrile
responses in live virus infected (IN) ferrets, similar to
nonimmunized control animals (Potter, Jennings and McLaren,

1973; Potter, McLaren and Shore, 1973; Potter, et al., 1972a; Potter, et al., 1972b).

Isolation and Studies of IBR in Mustelidae.

Porter, Larsen and Cox (1975) reported the isolation of bovine herpesvirus I, commonly known as infectious bovine rhinotracheitis virus (IBRV) from minks and ferrets. An isolate of IBRV was obtained from a clinically normal, healthy ferret while a second was removed from the intestine of a mink displaying signs of clinical mink viral enteritis. Electron microscopic examinations of the cell cultures infected with these isolates showed viral particles with typical morphological characteristics similar to a herpes virus. Serum neutralization tests, using rabbit antisera prepared against the mink and ferret isolates prevented the formation of infectious foci. Indirect immunofluorescence tests using acetone-fixed ferret kidney cells, showed no reaction of isolates with antisera to a variety of herpes type viruses (human cytomegalovirus Ab 169, monkey cytomegalovirus GR 2757, varicellazoster virus, Epstein-Barr virus, equine herpes virus (type 1 and 2), feline herpes virus C27, herpes tamarinus virus, pseudorabies virus or Marek's disease virus). A weak cross-immunoflourescence reaction occurred between human herpes simplex type one serum and the mustelid isolates, but no cross-neutralization was observed. An extensive cross-immunofluorescence reaction occurred between human herpes simplex type one serum and the mustelid isolates, but no cross-neutralization was observed.

An extensive cross-immunofluorescence reaction was observed between the Cooper strain of IBR and the mink and ferret isolates, using antisera to the three viruses. Serum from the animals contained no detectable neutralizing antibodies, although one had weak reactions with IBRV by immunofluorescence. Porter, Larsen and Cox, (1975) also indicate that minks inoculated intraperitoneally (IP) with IBRV did not develop clinical signs of illness, but did produce antibodies three weeks post infection.

In more recent studies (NADC, 1977) young adult English ferrets were inoculated with a virulent field strain of IBRV, IN and IM in high and low doses. The ferrets were challenged six weeks post-inoculation with high doses of the virulent field strain and immunity was evaluated on the basis of antibody development and virus recovered from the nasal wash. It was found that only high doses of virulent field strains produced full immunity and protection against the challenge. Only varying degrees of low level protection were achieved for animals inoculated with low doses of the virus.

Infectious Bovine Rhinotracheitis Virus.

Properties of the virion. The IBRV, classified in the herpes group, is a double stranded DNA virus that replicates in the nucleus. The IBR virion morphologically typifies the herpes virus family in having an icosahedral capsid with an envelope and is approximately 100-150 millimicrons in diameter (Bruner and Gillespie, 1973). Characteristic of the herpes viruses,

IBRV tends to remain cell associated after replication and contains both host and viral constituents in the envelope (Wagner, 1974).

Historical Appearance of Infectious Bovine Rhinotracheitis in Cattle.

Although known for many years in Europe as the cause of Blaschenausschlag, a mild genital tract infection in cattle, (Saxegaard, 1970; McKercher, 1963; Zwick and Gminder, 1913), IBR has been recognized as a clinical respiratory tract disease afflicting cattle in the U.S. only since 1950 (Miller, 1955; Schroeder and Mays, 1954). Isolation from nasal secretions of cattle and the subsequent culture in bovine embryonic kidney cells was first accomplished by Madin, York and McKercher, (1956). Later it was found that the causative agent of IBR was also implicated in infectious pustular vulvovaginitis (Kendrick, Gillespie and McEntee, 1958; McKercher, et al., 1959). Less than a decade after IBR was isolated, Armstrong, Pereira and Andrews, (1961) classified IBR into the herpes virus family. Subsequently, studies characterizing the morphology and biochemical features of the virus followed (Tousimis, et al., 1958; Hahnfeld and Hahnfeld, 1964; Jasty, et al., 1969). Although ubiquitous to most of the United States, (Kahrs, 1974), IBRV has also been reported in other areas of the world (St. George and Philpott, 1972). Reports indicate that IBR has been isolated from goats (Mohanty, et al., 1972), swine, (Nelson, Mare and Glock, 1972), water buffalo (St. George and Philpot, 1972) and mustelids (Porter,

Larsen and Cox, 1975).

Pathological Manifestations of Infectious Bovine Rhinotracheitis Virus.

The disease syndromes currently recognized as being caused by IBRV are rhinotracheitis, (Madin, York and McKercher, 1956), vaginitis, (Gillespie, et al., 1959; McKercher, et al., 1959), conjunctivitis, (Abinate and Plumer, 1961; McKercher, Wada and Straub, 1958), meningoencephalitis, (French, 1962), septicemia (Galloway, 1972), and abortion, (McKercher and Wada, 1964; Owens, Chow and Mollelo, 1964). Highly contagious, all forms of the disease are transmissible by contact (Bruner and Gillespie, 1973; Chow, Mollelo and Owen, 1964). The usual course of the disease extends from two to seven days (Runnels, Monlux and Monlux, 1967). Although most infections are rarely fatal for the adult bovine, the effects can be profoundly manifested by a decrease in feed efficiency, milk production and reproduction. Mortality can occur if the disease is complicated by secondary bacterial infections or viral infections (Kahrs, 1977).

Primarily a respiratory disease, IBRV commonly causes such clinical signs as pyrexia, anorexia, lethargy, dypsnea, and upper and lower respiratory tract congestion. Profuse ocular and nasal discharges, initially clear, often become mucopurulent (Kahrs, 1977). Necrotic lesions around the nostril and muzzle as well as in the pharynx, larynx, trachea and bronchi, (McKercher, et al., 1959) are found in severe cases.

Encephalomyelitis occasionally occurs in young cattle with chronic IBRV infections and results in incoordination instability, and occasional circling or licking at the hind quarters (Beck, 1975), followed by recumbency and death (McKercher, Bilbrack and Richards, 1970). Neonates infected in utero during late gestation or shortly after birth develop an acute systemic infection which is characterized by respiratory distress and necrotic lesions from the oral cavity down to and including the four stomach compartments. Peritonitis, causing diarrhea, may occur. In general, IBRV infections in newborn calves are fatal (Kahrs, 1977).

Infectious pustular vulvovaginitis (IPV), an IBRV infection of the vaginal and vulvar mucosa is characterized by pustule formation and a mucopurulent discharge. In mild cases, these conditions are not blatant (McKercher, 1963). This form of IBRV infection can be transmitted by natural breeding or other means involving direct contact (Kahrs and Smith, 1965). Careful diagnosis of IPV is warranted, since it is often confused with parturition or sadistic injuries, granular vaginitis and mycoplasma infections. Bulls that are bred to cows with IPV can develop severe balanoposthitis, which is characterized by lesions similar to those found in cows with IPV infections (Kendrick and McEntee, 1967).

Abortion caused by IBRV infections in pregnant and susceptible females occurs in approximately 25% of the cases under field conditions (Kahrs, 1977). The incidence increases in experimental situations when animals are given larger

doses of virus (Owen, Chow and Mollelo, 1964). Abortion
may occur as long as 100 days after IBRV is clinically
apparent in the herd (Kahrs, 1977).

<u>Histopathology of Infectious Bovine Rhinotracheitis Lesions</u>.

The whitish necrotic lesions and pustules found in
severe IBRV infections consist mainly of leucocytes, fibrin
and necrotic epithelial cells (Kahrs, 1977). Inclusion
bodies within cell nuclei have been reported by Crandell,
Cheatham and Maurer, (1959).

<u>Diagnosis and Confirmation of Infectious Bovine Rhino-
tracheitis</u>.

Serological tests, detecting the presence of IBRV anti-
bodies, virus isolation or fluorescent antibody tests,
coupled with a description of the appropriate clinical signs
and history are commonly employed in the laboratory diagnosis
of IBRV infections.

<u>Types of Vaccines Developed Against Infectious Bovine
Rhinotracheitis</u>.

a) Modified live IBRV vaccines

Modified live IBRV for IM administration was the first
IBRV vaccine developed (Swartz, <u>et al</u>., 1957). The vaccine
is widely used but is restricted from pregnant dams as the
virus can induce abortion (Kelling, <u>et al</u>., 1973). Bartha,
(1973) reports on the production and use of heat attenuated
IBRV vaccine for IM use, which does not cause abortion in
pregnant cows and can be inoculated in calves. His study

indicate that repeated inoculations of this vaccine result in increased serum neutralizing antibody levels. In general, vaccinated calves, less than six weeks old, should be re-vaccinated, since colostrally acquired maternal antibodies may interfere with the immune response against the initial inoculation of the antigen. Vaccination of calves suckling pregnant cows is contraindicated, since virus may be shed and cause the dam to abort (Kelling, et al., 1973).

A single successful immunization usually results in the development of humoral antibodies lasting three to six years, (Chow, 1972; Rosner, 1968), and provides partial protection against challenge with virulent virus and abortion, (Kahrs, 1974). Annual vaccinations may be needed to insure cell mediated immune defenses (Studer, 1973).

There are relatively minimal undesirable effects resulting from MLV given IM. Aside from causing abortion in pregnant dams (Kelling, et al., 1973), Frank, Marshall and Smith, (1973) report the occurrence of fever and leukopenia without clinical signs or virus shedding. Straub, (1972) found that heifers vaccinated IM, shed virus when challenged with virulent IBRV.

Field trials by Schell, et al., (1971) have demonstrated the high level of antigenicity in multivalent vaccines for IM use (IBR/bovine virus diarrhea (BVD)/para influenza-3 (PI-3) with Pasteurella and IBR-PI-3 with Pasteurella) and the production of antibodies to all components.

Todd, Volenec and Paton, (1971) developed the first

MLV vaccine against IBR for IN administration. The relative safety of this vaccine for inoculation into all cattle, including pregnant dams has attributed to its widespread acceptance. Utilizing a natural route of infection, IN vaccines provide rapid protection (within 40-72 hours post vaccination) against virulent IBRV challenge (Todd, 1974). Resistance was attributed to the production of interferon and secretory antibodies at the mucosal surfaces (Todd, Volenec and Paton, 1972; Todd, Volenec and Paton, 1971). McKercher and Crenshaw, (1971); however, demonstrated the production of serum neutralizing antibodies in cattle vaccinated with IN vaccine comparable to levels produced in animals inoculated with IM vaccine and report no distinct advantage of IN vaccine over the vaccine produced for IM use. Although not completely evaluated, annual inoculations of IN vaccines are recommended for restimulating humoral and cellular immune responses (Kahrs, 1977). Adverse side effects which are rare and often mild, include fever, nasal discharge, virus shedding (Hyland, Easterday and Pawlisch, 1975) and lesions in the nasal mucosa (Kahrs, Hillman and Todd, 1973). Laborious restraint of the bovine head while inoculating may be an undesirable task (Kahrs, 1977).

Multivalent vaccines for IN use containing IBRV/PI-3/ bovine adenovirus-3 and IBRV/PI-3 have been found to induce antibody production in calves and adults, to all components of the vaccine (Zygraich, Vascoboinic and Huygelen, 1976; Todd, 1976).

b) Inactivated IBRV vaccines

The efficacy of inactivated IBR vaccines remains contro-
versial and animals usually require annual vaccination to
achieve adequate levels of antibodies. It has been inter-
mittently available as a product containing two Pasteurella
spp and the inactivated PI-3 virus (Kahrs, 1977). Schipper
and Kelling, (1974) tested a commercially available inacti-
vated vaccine for subcutaneous (SC) use and found no detect-
able serum neutralizing antibodies. Kendrick and Osburn,
(1973) found commercially inactivated aluminum hydroxide
adjuvated IBR vaccine produced levels of serum antibody in
bovine fetuses comparable to the levels in similarly vaccin-
ated adults. Judhasz, Palatka and Toth, (1972) report
formalin inactivated IBRV adjuvated with aluminum hydroxide
gel absorbent administered SC, resulted in high titers of
serum neutralizing antibodies. Only mild symptoms were ob-
served when animals were simultaneously challenged IM and
intratracheally or IN and by scarification into the vaginal
mucosa with large quantities of virulent virus. Karadjov,
(1976) describes the high level of immunogenicity elicited
by an inactivated vaccine administered with an oil adjuvant,
as determined by the resistance to challenge with virulent
IBRV. Haralambiev, (1976) reports of the production of virus
neutralizing factors and subsequent resistance to challenge
with virulent virus in calves vaccinated with an ethanol and
saponin inactivated IBRV given in the nasal submucosa. The
efficacy of the IN route was found superior compared to the

SC inoculation of the vaccine.

Possible adverse effects include hypersensitivity reactions such as anaphylaxis from adjuvated vaccines and other less severe, non-fatal allergic syndromes (Kahrs, 1977).

Cell Mediated Immunity and Blastogenesis.

The host response to a viral infection involves both humoral and CMI responses. In the past the main focus of viral immunity studies, centered around the humoral component of the immune system. It is becoming increasingly apparent that resistance to and recovery from certain viral infections involves the CMI responses (W.H.O., 1973). There is mounting evidence that infections caused by herpes and parainfluenza viruses are more severe in individuals with deficiency syndromes of the CMI responses but essentially normal in those incapable of producing humoral antibody (Allison, 1972; Merigan and Stevens, 1971).

Until recently, the lack of suitable quantitative in vitro assays has hampered investigations in CMI. One method for testing CMI to study the blastogenic transformation of lymphocytes by the mitogen, phytohemagglutinin (PHA), a glycoprotein extract from the red kidney bean, was first reported by Nowell, (1960). The stimulation of lymphocytes, resulting in their transformation or blastogenesis (Stites, 1976), is characterized by the synthesis of deoxyribonucleic acid in thymus-dependent (T dependent) lymphocytes (Alhaji, Muscoplat and Johnson, 1974; Oates, et al., 1972; Piquet and Vasalli, 1972; Blomgren and Svedmyr, 1971; Janossy and

Greaves, 1971).

In earlier studies, the lymphocyte response to PHA was evaluated microscopically on the basis of cellular morphology (Pospisil et al., 1975) and by autoradiographic techniques. Sorenson, et al., (1971) developed a method for measuring the incorporation of ^{3}H-thymidine into PHA-stimulated cells. Since then, there have been a number of methods for quantitative assessment of the lymphocyte response to PHA (Lazary, et al., 1974; Faquet, 1974; Fernald and Metzgar, 1971; Phillips and Zweinmann, 1970).

It has been found that responsiveness of lymphocytes to PHA is diminished by in vivo or in vitro infection with viruses such as Newcastle disease virus (Montgomery, et al., 1967), herpes virus (Willems, et al., 1969), bovine viral diarrhea virus (Johnson and Muscoplat, 1973) and rubella virus (McMorrow, et al., 1973).

Infectious Bovine Rhinotracheitis Virus and Blastogenesis.

Rouse and Babiuk, (1974), demonstrated the blastogenic effect of specific antigen (UV irradiated IBRV) on the peripheral blood lymphocytes from cattle immunized or infected with IBRV by quantitating the cellular uptake of ^{3}H-thymidine (^{3}H-TdR). The failure of lymphocytes from these animals to incorporate ^{3}H-TdR when cultured with two other herpes virus antigens, herpes simplex and equine rhinopneumonitis, demonstrates the specificity of lymphocyte transformation. Blastogenesis was prevented by reacting the IBRV antigen with IBRV specific antibody before addition to the cultures.

An assay (Rouse and Babiuk, 1975) utilizing peripheral blood lymphocytes from IBRV immunized cattle, confirms the cell-mediated specificity of these lymphocytes to inhibit cyto-pathic effects by IBRV in cell cultures.

Suppression of Cell Mediated Immunity and Adrenal Corticoids.

Herpes viruses can be present as latent infections without clinical manifestations (Sprinkle, Vettri and Wade, 1976) and result in spontaneous recrudescence despite the presence of neutralizing antibodies (Snowden, 1965). The recrudescence of IBRV infections in cattle has been experimentally demonstrated by the administration of synthetic corticoids, as long as four years after the initial infection (Sheffy and Davies, 1972).

Corticosteroids have been found to suppress CMI responses to bacterial antigens (North, 1972); however, suppression of CMI as measured by lymphocyte transformation was not detected when cattle infected with IBRV were treated three months post infection with synthetic corticosteroid to induce recrudescence (Davies and Carmichael, 1973).

The adverse effects of certain adrenal cortical hormones on lymphocytes has been attributed to the actual cytotoxic activity of the hormones (Claman, 1972). It has been demonstrated that glucocortocoids cause destruction of small and medium-sized lymphocytes and the depression of mitosis in lymphatic tissues (Stevens, Colossides and Dougherty, 1966; Moore, 1966; Bellamy, Hanssens and Leonard, 1966). Claman,

(1972) further reported that cortisol produced an inhibitory effect on cellular metabolism by altering glucose uptake and nucleic acid synthesis. Studies measuring the incorporation of radioactive labelled precursors of the DNA molecule have demonstrated the inhibitory effect of cortisol on DNA synthesis in rat lymphatic tissues (Hofert and White, 1968a, 1968b) and the depletion of nucleic acid in mouse lymphatic tissues (Brinch-Johnsen and Dougherty, 1959). Stevens and Dougherty, (1964) reported the decrease of nucleic acid when measured by the incorporation of thymidine-2-14C into lymphatic tissue nucleic acid after cortisol addition.

Fauci and Dale, (1974) and Webel, et al., (1974) observed that corticosteroid administration significantly altered the absolute number of circulatory lymphocytes, monocytes and eosinophils, while increasing the number of neutrophils. The absolute numbers of T lymphocytes were proportionately more affected than the B lymphocytes. Craddock, et al., (1967) showed that cortisone treatment impaired leukocyte or macrophage migration in certain CMI responses. This was supported by the studies of Weston, et al., (1973) demonstrating the inhibitory effect of cortisol on the ability of macrophages to respond to the sensitized small (T) lymphocyte in cellular immunity.

Corticosteroids have been shown to inhibit T cell responses to mitogens and specific antigens when added to lymphocyte cultures (Heilman and Leicher, 1972). In other studies (McIntyre, et al., 1969; Stefani and Oester, 1967;

Tormey, et al., 1967; Nowell, 1961), found that glucocorti-
coids added to lymphocyte cultures inhibited PHA induced
transformation. The most effective inhibition occurred
when corticosteroids were added before or simultaneously with
a specific antigen or mitogen whereas no inhibition was
observed when steroids were added after the lymphocytes had
been stimulated (Bach, 1975). There is evidence that some
cell populations or animal species as well as functions of
T cells may be more resistant than others to corticosteroids
and may require higher doses for inhibition to occur (Fauci,
1975; Heilman, 1972; Esteban, 1968).

SECTION III

HEMATOLOGICAL AND SERUM PROFILES OF FERRETS

Abstract

Normal hematological and serum profiles were determined in ferrets (<u>Mustela</u> <u>putorius</u> <u>furo</u>). The arithmetic mean, standard deviation, 90% confidence limits, 90% tolerance limits and simple linear correlation coefficients with respect to age or body weight were calculated on these variables.

Introduction. The recent development of technical and auto-
mated laboratory analysis of hematological and serum chemistry
profiles has been invaluable in many animal investigations.
Comparisons of these parameters between normal and disease
suspected cases may provide insight or rapid confirmation
of the clinical diagnosis. Some hematological and serum pro-
files (Rotenberg and Jorgensen, 1971) have been established
for the mink (Mustela vison); however values for ferret para-
meters are few (Pyle, 1940). The main objective of this
investigation was to determine hematological and serum
chemistry profiles in clinically normal ferrets (Mustela
putorius furo) and to determine the relationship of these
parameters with respect to age or body weight.

Materials and Methods.

Animals. Thirteen fitch ferrets, Mustela putorius furo
(Marshall Research Animals, North Rose, NY), four to eight
months of age were used in these studies. All ferrets were
vaccinated against canine distemper prior to shipment from
Marshall Research Laboratories. The ferrets were housed in
individual stainless steel cages (67 x 61 x 67 cm, Harford
Metal Products, Aberdeen, MD) under artificial lighting
(12 hours photoperiod), at 25° C, and provided with feed
(Purina Main Meal Cat Chow, Checkerboard Square, MO) and
water ad libitum. All animals were acclimatized for at least
four weeks prior to initiation of the experiment. Ketamine

hydrochloride, (Bristol Laboratories, Syracuse, NY) admini-
stered intramuscularly (IM) at a dose of 12-15 mg/lb of body
weight was used to induce anesthesia. Blood was drawn by
cardiac or jugular vein puncture, using a 22 guage, one
inch needle. A maximum of eight ml of blood was drawn from
the animals at approximately weekly intervals. (This corres-
ponds to 20% of the total blood volume as reported by Hahn
and Wester, 1969).

Serum and hematological profiles. Serum profiles were
determined in the Sequential Multiple Analyzer (SMA-12,
Technicon Instruments Corporation, Tarrytown, NY) using one
ml serum samples. Complete blood counts (CBC's) were deter-
mined using one ml blood samples collected in one mg of
K_2EDTA. Both analyses were performed at the Clinical Patho-
logy Laboratory, Department of Infectious Diseases, Kansas
State University, Manhattan, KS.

Hemolysis test. Serum containing varying degrees of hemolysis
(0-4+ and 10+) was analyzed for the serum chemistry profiles.
Degrees of hemolysis in serum were simulated with serum pre-
pared from frozen blood (hemolysis +10) and serum containing
slight hemolysis (1+) as follows: hemolysis designated 2+,
contained one part 10+ hemolyzed serum to 99 parts 1+ hemolyzed
serum and hemolysis designated 4+, contained one part 10+
hemolyzed serum to 19 parts 1+ hemolyzed serum.

Statistical analysis. The arithmetic means, standard

deviations, 90% confidence limits, limits on 90% of the individual measurements in the designated population stated with 90% confidence, and simple linear correlation coefficients with respect to age or body weight were determined by standard statistical techniques.

Results. In samples with varying degrees of hemolysis, appreciable differences were observed only in total protein, albumin, sorbital dehydrogenase (SDH) and phosphorus (Table 1). Serum from frozen blood demonstrated increased levels of hemoglobin (Hb), potassium (K), serum glutamate pyruvate transaminase (SGPT), total protein and albumin; however chloride (Cl), sodium, (Na), carbon dioxide (CO_2), calcium (Ca), phosphorus (P) and glucose levels decreased.

Tables 2 and 3 present the arithmetic mean, standard deviation and 90% confidence intervals on the population mean of the hematological and serum chemistry profiles for female, male and castrated males. The mean value for white blood cells (WBC's) in females was observed to be less as compared to the males and castrated males; however, the mean values determined for the other parameters in the three groups of animals did not differ appreciably.

Table 4 lists the 90% tolerance limits on 90% of the population of the various hematological and serum parameters for the same groups of animals, assuming an essentially normal distribution of values.

There was a significant age related correlation with

neutrophils (+0.409, p = 0.07) and lymphocytes (-0.448, p = 0.04). Other hematological variables did not change significantly with respect to age or body weight (Table 5).

The serum enzymes, alkaline phosphatase, SGPT and SDH were significantly decreased, while glucose, blood urea nitrogen (BUN) and albumin were significantly elevated with age.

Sodium and chloride were positively correlated with body weight; however, phosphorus, CO_2 and glucose were negatively correlated. Significant correlations were not found between body weight and other serum parameters that were measured.

Discussion. The degree of hemolysis in the blood was directly proportional to the amount of hemoglobin present in the serum sample (Table 1). The hemolysis associated changes in the serum chemistry was a direct result of the damage to blood cell constituents, especially the erythrocytes. These results can be useful for interpreting serum profiles, since the degree of hemolysis and differences in the profiles may depend on the sample collection rather than on abnormal condition in the animal.

Pyle (1940) reported a neutrophil:lymphocyte ratio of 65:33 as compared to a ratio of 34:54 observed in our studies. In studies of minks a 38:53 ratio was reported by Rotenberg and Jorgensen (1971). Higher glucose and lower calcium levels were also demonstrated as compared to the mean values observed in the present study; however, there

were no appreciable differences in the other blood cell con-
stituents. There appeared to be distinct similarities in the
hematological and serum profile values of mink reported by
Kennedy (1935a), Kubin and Mason (1948) and Rotenberg and
Jorgensen (1971) and the ferrets in this study.

This data provides information on the normal hematologi-
cal and serum profiles of ferrets which have not been studied
in detail previously. This may be extremely useful with
regard to the undertaking of scientific investigations in
these species.

TABLE 1

EFFECT OF HEMOLYSIS ON SERUM PROFILES

	0	0	1+	2+	4+	10+ (Frozen)
Sorbital Dehydrogenase (SDH) I.U.		1.2	2.0	5.2	16.1	
Chloride (Cl) meq/L	119		108	120	108	108
Hemoglobin (Hb) gm%			0.2GM%	0.4GM%	0.7GM%	13.2GM%
Sodium (Na) meq/L	153	154	156	154	153	133
Potassium (K) meq/L	5.1	5.3	5.1	5.1	5.1	>8
Carbon Dioxide (CO_2) meq/L	21	22	26.5	25.5	24	5
Blood Urea Nitrogen (BUN) mg%	19	20	19	18.5	18	16
Alkaline Phosphatase mu/ml	45	45	45	45	45	43
Serum Glutamate Pyruvate Transaminase (SGPT) mu/ml	111	114	112	117	140	>500
Total Protein (T.P.) gm%	5.5	5.5	6.0	6.2	6.6	>12.0
Albumin gm%	3.2	3.4	3.6	3.6	4.0	>8.0
Calcium (Ca) mg%	8.6	8.6	8.8	8.9	8.5	5.7
Phosphorus (P) mg%	5.1	5.9	7.3	7.4	2.4	5.7
Glucose mg%	100	104	110	110	110	85
Creatinine mg%	0.4	0.4	0.4	0.4	0.5	0.5

TABLE 2

HEMATOLOGICAL AND SERUM PROFILES IN FERRETS

Variable	Arithmetic Mean			Estimated Standard Deviation		
	$f(n_i)$	$m(n_i)$	$cm(n_i)$	f	m	cm
PCV	43.38 (8)	43.40 (5)	49.12 (8)	1.77	6.47	3.48
Hb	15.88 (8)	14.34 (5)	16.06 (8)	0.650	2.15	0.848
WBC	5.88 (8)	11.32 (5)	7.29 (8)	1.78	2.93	1.95
Bands	1.88 (8)	1.00 (5)	2.88 (8)	1.55	1.00	3.09
Neutrophils	31.25 (8)	39.20 (5)	33.38 (8)	5.52	14.1	15.3
Lymphocytes	58.00 (8)	50.20 (5)	55.75 (8)	5.07	11.5	18.9
Monocytes	4.50 (8)	6.60 (5)	2.25 (8)	1.20	1.34	1.67
Eosinophils	3.50 (8)	2.40 (5)	5.00 (8)	1.85	2.19	2.39
Basophils	0.88 (8)	0.60 (5)	0.75 (8)	0.835	0.894	0.707
SDH	2.64 (8)	5.36 (5)	3.34 (9)	2.24	4.48	3.32
Cl	117.10 (10)	112.50 (3)	117.31 (8)	1.90	9.10	2.22
Na	150.35 (10)	154.40 (5)	150.60 (10)	1.45	3.58	3.34
K	4.92 (10)	4.86 (5)	4.89 (10)	0.257	0.241	0.160
CO_2	22.10 (10)	23.40 (5)	18.47 (10)	3.12	3.36	3.20

TABLE 2 (continued)

Variable	Arithmetic Mean			Estimated Standard Deviation		
	$f(n_i)$	$m(n_i)$	$cm(n_i)$	f	m	cm
BUN	33.30 (10)	22.00 (5)	28.10 (10)	7.57	6.32	7.58
Alk. P.	44.30 (10)	52.40 (5)	63.25 (10)	11.3	10.6	29.2
SGPT	150.25 (10)	157.60 (5)	201.30 (10)	49.3	79.9	142.
T. P.	6.00 (10)	5.86 (5)	5.82 (10)	0.467	0.336	0.266
Albumin	3.78 (10)	3.70 (5)	3.68 (10)	0.230	0.141	0.225
Ca	9.02 (10)	9.54 (5)	9.28 (10)	0.253	0.615	0.492
P	6.69 (10)	6.70 (5)	6.10 (9)	0.601	1.21	0.583
Glucose	104.90 (10)	104.00 (5)	95.40 (10)	16.4	15.0	17.0
Creatinine	0.36 (10)	0.40 (5)	0.30 (10)	0.0567	0.141	0.0438

f: females
m: male
cm: castrated male
n_i: sample size

TABLE 3

HEMATOLOGICAL AND SERUM PROFILES IN FERRETS

Variable	90% Confidence Intervals on Population Means		
	f	m	cm
PCV	47.20 – 49.56	37.23 – 49.56	46.79 – 51.45
Hb	15.44 – 16.32	12.29 – 16.29	15.49 – 16.63
WBC	4.69 – 7.07	8.52 – 14.12	5.98 – 8.60
Bands	0.84 – 2.92	0.05 – 1.95	0.81 – 4.95
Neutrophils	27.55 – 34.95	26.41 – 51.99	23.11 – 43.65
Lymphocytes	54.60 – 61.40	39.20 – 61.20	43.09 – 68.41
Monocytes	3.70 – 5.30	5.32 – 7.88	1.13 – 3.37
Eosinophils	2.26 – 4.74	0.13 – 4.67	3.40 – 6.60
Basophils	0.32 – 1.44	0 – 1.48	0.28 – 1.22
SDH	1.14 – 4.14	1.09 – 9.63	1.29 – 5.41
Cl	116.00 – 118.20	97.16 – 127.84	115.82 – 118.80
Na	149.51 – 151.19	150.00 – 157.81	148.66 – 152.54
K	4.77 – 5.07	4.63 – 5.09	4.80 – 4.98
CO_2	20.29 – 23.91	20.20 – 26.60	16.62 – 20.32
BUN	28.91 – 37.69	15.97 – 28.03	23.70 – 32.50
Alk. P.	37.75 – 50.85	42.29 – 62.51	46.31 – 80.19
SGPT	121.66 – 178.84	81.40 – 233.80	118.76 – 283.84
T. P.	5.73 – 6.27	5.54 – 6.18	5.67 – 5.97
Albumin	3.65 – 3.91	3.57 – 3.84	3.55 – 3.81
Ca	8.87 – 9.17	8.95 – 10.13	9.00 – 9.56

TABLE 3 (continued)

Variable	90% Confidence Intervals on Population Means		
	f	m	cm
P	5.34 – 7.04	5.55 – 7.85	5.74 – 6.46
Glucose	95.40 – 114.40	89.74 – 118.26	85.56 – 105.24
Creatinine	0.33 – 0.39	0.27 – 0.53	0.27 – 0.32

f: females

m: male

cm: castrated male

n_i: sample size

TABLE 4

90% TOLERANCE LIMITS ON 90% OF POPULATION OF FERRET MEASUREMENTS

Variable	f	m	cm
PCV	43.53 – 53.23	20.79 – 66.01	39.57 – 58.67
Hb	14.10 – 17.66	6.84 – 21.86	13.73 – 18.39
WBC	1.01 – 10.75	0.98 – 21.46	1.94 – 12.64
Bands	0+ – 6.14	0+ – 4.49	0+ – 11.35
Neutrophils	16.10 – 46.40	0+ – 88.64	0+ – 75.45
Lymphocytes	44.09 – 81.91	9.88 – 90.52	3.91 – 107.59
Monocytes	1.22 – 7.78	1.92 – 11.28	0+ – 6.83
Eosinophils	0+ – 8.58	0+ – 10.05	0+ – 11.56
Basophils	0+ – 3.17	0+ – 3.72	0+ – 2.69
SDH	0+ – 8.80	0+ – 21.01	0+ – 12.12
Cl	112.30 – 121.90	59.29 – 165.71	111.22 – 123.40
Na	146.66 – 154.04	141.89 – 166.91	142.13 – 159.07
K	4.27 – 5.57	4.02 – 5.70	4.48 – 5.30
CO_2	14.18 – 30.02	11.66 – 35.14	10.59 – 26.81
BUN	14.10 – 52.50	0+ – 44.08	8.88 – 47.32
Alk. P.	15.64 – 72.96	15.36 – 89.44	0+ – 137.57
SGPT	25.22 – 275.28	0+ – 436.81	0+ – 562.23
T. P.	4.82 – 7.18	4.69 – 7.03	5.15 – 6.49
Albumin	3.20 – 4.36	3.21 – 4.19	3.11 – 4.25
Ca	8.38 – 9.66	9.39 – 11.69	6.95 – 11.61

TABLE 4 (continued)

Variable	f	m	cm
P	5.17 - 8.21	2.47 - 10.93	4.47 - 7.53
Glucose	63.35 - 146.45	51.69 - 156.31	52.33 - 138.73
Creatine	0.22 - 0.50	0 - 0.89	0+ - 1.41

If n=8, K=2.743 in $\bar{x} \mp Ks$; if n=10, K=2.535; if n=5, K=3.494; if n=3, K=5.847; and if n=9, K=2.626.

TABLE 5

HEMATOLOGICAL AND SERUM PROFILES IN FERRETS
SIMPLE LINEAR CORRELATION COEFFICIENTS

Variables with	Age (ni) or	Weight (nil)
PCV	+0.050NS (19)	+0.210NS (5)
Hemoglobin	-0.179NS (19)	+0.272NS (5)
White Blood Cell (WBC)	+0.002NS (19)	+0.487NS (5)
Bands	+0.226NS (19)	+0.105NS (5)
Neutrophils	+0.409† (19), P=.07	+0.024NS (5)
Lymphocytes	-0.448* (19), P=.04	-0.097NS (5)
Monocytes	+0.096NS (19)	-0.421NS (5)
Eosinophils	-0.007NS (19)	+0.611NS (5)
Basophils	+0.341NS (19)	+0.171NS (5)
SDH	-0.403† (20), P=.06	+0.511NS (8)
Cl	-0.110NS (19)	-0.012NS (7)
Na	+0.157NS (23)	+0.561† (8), P=.09
K	+0.275NS (23)	+0.228NS (8)
CO_2	+0.324NS (23)	-0.807** (8), P=.005
BUN	+0.371† (23), P=.07	-0.125NS (8)
Alkaline Phosphatase	-0.729** (23), P=.0001	+0.402NS (8)
SGPT	-0.585** (23), P=.002	+0.007NS (8)

TABLE 5 (continued)

Variables with	Age (ni) or	Weight (ni)
T. P.	$+0.169^{NS}$ (23), NS	-0.338^{NS} (8)
Albumin	$+0.338^{\dagger}$ (23), P=.098	$+0.148^{NS}$ (8)
Ca	$+0.292^{NS}$ (23), NS	$+0.684^{*}$ (8), P=.03
P	$+0.217^{NS}$ (22), NS	$-0.627^{\dagger}$ (7), P=.07
Glucose	$+0.476^{*}$ (23), P=.02	-0.678^{*} (8), P=.03
Creatinine	$+0.072^{NS}$ (23)	-0.507^{NS} (8)

$\dagger$,*,** indicate statistical significance at the 10, 5, 1% levels, respectively.

P = significance probability.

NS = Nonsignificant.

SECTION IV

IMMUNOLOGICAL STUDIES IN FERRETS INOCULATED WITH

INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

I. Humoral and Cell Mediated Immune Responses

Abstract

Ferrets inoculated with inactivated or modified live
(ML) infectious bovine rhinotracheitis virus (IBRV) did not
manifest clinical signs of IBRV infection. Statistical
analyses were performed on the hematological and serum
chemistry profiles for ferrets inoculated with formalin in-
activated and modified live IBRV. Three days post intranasal
(IN) vaccination with modified live virus (MLV), IBRV was
isolated from the nasal washings of one of the three ferrets;
however, it was not detected in the subsequent washings.
Specific and nonspecific virus neutralizing factors were
detected in the nasal washings only at the fourth week
post-IN inoculation with inactivated IBRV. Serum neutralizing
antibodies were detected three weeks following intramuscular
(IM) injection of inactivated IBRV with Freund's incomplete
adjuvant. Humoral antibodies increased following a second
inoculation given IM. Maximum virus neutralization was ob-
served between the 12th - 14th week. The MLV elicited low
levels of serum antibodies that persisted from the third to
seventh week post inoculation. The degree of cell mediated
immunity was low and variable as observed by specific lympho-
cyte stimulation (^{3}H-TdR incorporation) in whole blood cul-
tures from ferrets inoculated with inactivated IBRV. Control
and MLV inoculated ferrets did not demonstrate cell mediated
immune responses to the IBRV antigen.

Introduction.   The ubiquitous nature and the variety of clin-
ical manifestations observed in infectious bovine rhino-
tracheitis, such as conjunctivitis (Abinanti and Plumer,
1961), rhinotracheitis, (Madin, York and McKercher, 1956),
meningoencephalitis (French, 1962), vaginitis (McKercher,
et al., 1959), abortion (Owens, Chows and Mollelo, 1964) and
balanoposthitis (Kahrs, 1977), have resulted in the increas-
ing economic concern to manage this disease in the United
States and other parts of the world.   The detrimental effects
of IBRV infections have a direct bearing on the reproductive
health and the milk and meat production of cattle.   Inevit-
ably this disease affects cattle of all ages.

Investigations evaluating the various types of IBRV
vaccines available and the different routes of inoculation
have provided much information on the levels of immunity
conferred; however, the complexity of the immune system has
also led to considerable obscurities.   It has been reported
that IBRV infections occurred in the presence of humoral anti-
bodies (Snowden, 1965) implicating the importance of the
cell mediated immune (CMI) system.

The ferret, Mustela putorius furo, has been found to
simulate a pathological response with influenza virus
similar to man and has been used to evaluate the efficacy of
the virus vaccines.   Porter, Larsen and Cox (1975) reported
the isolation of IBRV in ferrets.   In more recent studies,
(NADC, 1977) ferrets have been under investigation to

evaluate the immune response to IBRV inoculations.

The main objective of this investigation is to study
the immune response of ferrets inoculated with IBRV vaccines.

Materials and Methods.

Animals. Ferrets four to eight months old were used in these
studies. Rectal temperatures and body weights were recorded
prior to and at specific intervals post inoculation with IBRV.
Anesthesia was induced with ketamine hydrochloride, (Bristol
Laboratories, Syracuse, NY), administered intramuscularly
(IM) at a dose of 12-15 mg/lb. of body weight. Blood was
drawn by cardiac or jugular vein puncture using a 22 guage,
one inch needle.

Cell cultures. Monolayer cultures of Madin Darbin bovine
kidney cells, obtained from the American Type Culture Col-
lection (ATCC) Laboratories (Rockville, MD) were grown in
prescription bottles (32 ounces) or tissue culture dishes
(60 x 15 mm; Falcon, Oxnard, CA) and incubated at 37° C in
a humidified incubator. The cells were maintained in minimal
essential medium (MEM), Eagles (Grand Island Biological Co.,
Grand Island, NY), supplemented with 10% heat inactivated
horse serum (KC Biological Inc., Lenexa, KS), 0.1% sodium
bicarbonate ($NaHCO_3$) and an antibiotic mixture of penicillin,
100 units/ml., streptomycin, 1 ng/ml and kanamycin, 5 ng/ml
(PSK).

Propagation and assay of viruses. Infectious bovine

rhinotracheitis virus, the Los Angeles (LA) strain and bovine virus diarrhea virus (BVDV), the National Animal Disease Laboratory (NADL) strain used in these studies were obtained from ATCC Laboratories. Monolayer cultures (70-90% confluent) of MDBK cells were infected with IBRV or BVDV virus with a multiplicity of 3-5 plaque forming units (PFU)/cell and maintained in Medium 199, (Grand Island Biological Co.) at 37° C. The cultures were harvested by scraping with a rubber policeman when maximum cytopathic effect (CPE) was observed (approximately 48-72 hours). The cell-virus suspension was frozen and thawed twice to release virus from the cells, then centrifuged at 2,000 g for 30 minutes, at 5° C. The supernatant (IBRV) was stored in aliquots at -80° C. The supernatant containing BVDV was ultracentrifuged (Beckman L2-65B, Beckman Instruments, Inc., Palo Alto, CA), at 100,000 g for five hours at 5° C. The pellet was resuspended in Medium 199 and stored in aliquots at -80° C. All viruses were assayed by the procedure for animal viruses, described by Dulbecco, (1952) with some modifications. Briefly, serial log dilutions of virus were adsorbed for one hour onto MDBK cell monolayers in dishes (0.2 ml/dish). Seven mls of an agar (2% purified) and media (2 X MEM) mixture supplemented with 5% HS were overlaid onto the cultures and incubated (three days for IBRV and four days for BVDV). Plaques were observed 24 hours after a second overlay of agar and media mixture containing 0.015% neutral red stain.

Ultraviolet inactivation of viruses. One ml of IBRV or BVDV (1×10^7 PFU/ml) was UV irradiated for 4.5 and five minutes, respectively, in a petri dish (60 x 15 mm) at a distance of 10 cm from a UV germicidal sterilamp (630T8, 30 watt, Westinghouse Electric Co., Bloomfield, NJ). The virus was completely inactivated as determined by the plaque assay method.

Preparation of inactivated IBRV vaccine. One ml of IBRV (5×10^8 PFU/ml) was treated with formalin at a final concentration of 1:1000 (40% formaldehyde solution, Fisher Scientific Co., Fairlawn, NJ) for six hours at 25° C, then overnight at 4° C. The virus was dialyzed in 1/4" dialyzing tubing against three changes of cold PBS at 4° C over a 24 hour period. Inactivated virus as determined by the plaque assay method was stored in aliquots at -80° C.

Serum and hematological profiles. Serum profiles were determined in the Sequential Multiple Analyzer (SMA-12, Technicon Instruments Corp., Tarrytown, NY) using one ml serum samples. Complete blood counts (CBC) were determined using one ml of blood. Both analyses were performed prior to and at specific intervals post inoculation of IBRV at the Clinical Pathology Laboratory, Department of Infectious Diseases, Kansas State University, Manhattan, KS.

Inoculations of ferrets. The absence of IBRV serum neutralizing antibodies was confirmed by the plaque reduction method prior to inoculation with IBRV vaccines.

Group A: Five ferrets were inoculated intranasally (IN) with 2.5×10^7 PFU/0.1 ml of formalin inactivated IBRV. Five control ferrets were sham inoculated with formalized cell lysate (from MDBK cells). Formalin inactivated IBRV, 5×10^7 PFU/0.2 ml was homogenized (Multi-Churn Homogenizer) with an equal volume of Freund's Incomplete Adjuvant (Grand Island Biological Co.) and administered IM into animals three weeks post IN inoculation. A booster of the homogenate was given into the opposite quarter three weeks later. Controls were inoculated with a homogenous mixture of the cell lysate and Freund's incomplete adjuvant.

Group B: Three ferrets were inoculated IM with 1.6×10^4 PFU/0.2 ml of modified live IBRV (Delvine[R], Dellen Laboratories, Omaha, NB). One control animal was inoculated with the diluent provided for rehydration of the antigen.

Group C: Three ferrets were etherized and inoculated intranasally with 0.1 of ML IBRV vaccine containing 2×10^5 PFU, (Nasalgen I, Monovalent, Serial 511) kindly provided by Dr. J. D. Todd, Jensen-Salsbery Laboratories, Kansas City, MO. One control animal was inoculated with the diluent provided for rehydration of the antigen.

Collection of nasal washings. Ferrets were tranquilized with Ketamine hydrochloride (5 mg/lb of body weight). Three mls of PBS were introduced intranasally with a 3 cc. syringe (without a needle). Sneezing reflexes resulted and the contents of each nostril were collected in a sterile petri dish

(100 x 20 mm, Falcon). Approximately 2.5 ml was recovered
and immediately transferred into tubes in an ice bath con-
taining antibiotics supplemented with 0.5 ug/ml of Fungizone[R]
(E. R. Squibb and Sons, NY).

The nasal washes, 0.3 ml from animals in group C were
passaged thrice on MDBK monolayers and observed for CPE.

Detection of serum neutralizing antibodies by the plaque
reduction method. The presence of serum neutralizing anti-
bodies against IBRV in heat inactivated ferret serum (56° C
for 30 minutes) was determined by the plaque reduction
method. Briefly, the serum samples were mixed with equal
volumes (0.3 ml) of IBRV, 5×10^2-1×10^3 PFU and incubated
for one hour at 25° C. The samples were plaque assayed to
determine the percent recovery of the virus.

Detection of specific and nonspecific factors against viruses
in nasal washings. The absence of specific and nonspecific
neutralizing factors in ferret nasal washings was confirmed
prior to inoculations with IBRV vaccines. The presence of
specific and nonspecific virus neutralizing factors was
determined by the plaque reduction method post virus inocul-
ation. The samples were mixed with equal volumes (0.3 ml)
of either IBRV or BVDV, 5×10^2 - 1×10^3 PFU and incubated
for one hour at 25° C. The samples were plaque assayed to
determine the percent recovery of the virus.

Blastogenesis studies in whole blood cultures from control
and vaccinated ferrets. The method of whole blood cultures
used by Gerber and Brown (1974) was adopted with modifications.
Heparinized whole blood, 0.1 ml (5 units/ml of blood, Pan-
heprin, Abbott Laboratories, North Chicago, IL) was added to
duplicate tubes (17 x 100 mm, Falcon) containing RPMI 1640
(Roswell Park Memorial Institute, Grand Island Biological Co.,
Grand Island, NY) and 10% heat inactivated fetal calf serum,
free of IBRV and BVDV antibodies (KC Biological Co.). UV
inactivated virus (2.0 x 10^5 PFU/0.2 ml) was added to the
cultures. The cultures were incubated for 108 hours then
exposed to 0.5 uCi/ml of ^{3}H-TdR (specific activity 5 Ci/mmole,
New England Nuclear). Cultures were removed from the CO_2 in-
cubator after 12 hours of exposure to label and placed in
an ice bath to terminate cellular activity. The tubes were
centrifuged at 500 g for 15 minutes in a refrigerated centri-
fuge. The supernatant was carefully aspirated, cultures
gently vortexed and treated two to three times with six ml
of cold distilled (not deionized) water for 30 seconds each
time to lyse erythrocytes. Isotonicity was restored with
six ml of cold 1.7% NaCl solution. The remaining cells
(mixture of WBC's in a one ml volume) were precipitated with
five mls of cold 5% trichloroacetic acid (TCA) and kept
in an ice bath for 30 minutes. Acid insoluble, precipitated
macromolecules were collected on glass filters (GF/C, 2.4 cm.
Whatman, England) using a sampling manifold (Millipore
Corporation, Bedford, MA). The tubes were washed twice with

five ml of cold 5% TCA and the contents poured onto the filters. The filters were finally placed into glass scintillation vials containing 10 mls of scintillation fluid. Samples were counted for 20 minutes by a Scintillation Spectrometer (Packard Tri-Carb, Model 3002, Downers Grove, IL).

Results.

Clinical observations. Clinical signs of IBRV infection were not observed in ferrets inoculated with inactivated or modified live IBRV; however, local inflammation persisted for approximately two weeks at the site of inoculation of the adjuvated vaccine. The mean rectal temperatures of each group remained within the normal range (101.0-104.0° F) as reported for ferrets by Pyle (1940). The behavior and appetities appeared normal as compared to pre-inoculation observations. Control and inactivated IBRV inoculated ferrets demonstrated a plateau in weight between 28 and 44 weeks of age, followed by a decrease in weight for four to five weeks (Figure 1). Although the females experienced a greater weight loss than the males, both reached a plateau by 50 weeks of age. The maximum weight observed for females and males was 1.2 Kg and 1.55 Kg, respectively.

Hematological and serum profiles. Table 1 presents the statistical significance of changes in the hematological and serum chemistry profiles between pre- and post-inoculation values for four groups of ferrets. Ferrets inoculated with formalin inactivated IBRV with Freund's Incomplete Adjuvant

demonstrated significant changes (P = 0.05 or 0.01) in
chloride (Cl), potassium (K), carbon dioxide (CO_2), albumin,
calcium (Ca), phosphorus (P), and bands. Animals inoculated
with modified-live IBRV, given IM had significant differences
(P = 0.05 or 0.01) between pre- and post-inoculation means
in Cl , sodium (Na), blood urea nitrogen (BUN), creatinine,
packed cell volume (PCV) and hemoglobin (Hb). Statistically
significant differences (P = 0.05 or 0.01) in pre- and post-
inoculation means from ferrets inoculated with modified live
IBRV given IN were detected in Cl , BUN, alkaline phosphatase,
glucose, and creatinine. The control ferret group, inoculated
with incomplete adjuvant only, demonstrated significant pre-
and post-inoculation changes (P = 0.05, 0.01) in total pro-
tein, P, white blood cell (WBC) and monocytes. Albumin,
BUN, SDH, creatinine, PCV and bands were significant among
the treatments (Table 2).

Virus isolation from nasal washings. Two of the three ferrets
inoculated with modified live IN IBRV vaccine had a red hue
in the nasal washings three days post-inoculation; however,
subsequent samples were similar to the control washings.
Virus was not detected in the nasal washings from ferrets
inoculated IN with inactivated IBRV.

Virus (3 x 10' PFU/ml) was recovered on day three post-
inoculation in one of the three nasal washings from ferrets
inoculated IN with MLV. This sample produced CPE in the
first passage onto MDBK cells. Virus was not demonstrated

in subsequent samples.

Detection of nasal antibodies. The presence of specific and nonspecific virus neutralizing factors was detected four weeks post IN inoculation in samples taken from ferrets vaccinated with inactivated IBRV (Figure 2). These factors were not found in following samples.

Detection of humoral antibodies. Serum neutralizing antibodies were detected three weeks following IM injection of inactivated IBRV vaccine (six weeks post IN inoculation). Humoral antibodies were detected as indicated by a 40% recovery of IBRV and increased following a second IM booster (Figure 3). Maximum antibody production (0% virus recovery) occurred between the 12th and 14th week and declined thereafter to an 80% virus recovery rate by the 20th week at which time the experiment was terminated.

Low and variable levels of serum neutralizing antibodies were detected in ferrets following the inoculation of IN and IM modified live vaccines (Figure 4). The IM vaccine elicited antibodies two weeks post inoculation and persisted at low levels until week seven. The IN vaccine produced a weak immune response at week one and three post-inoculation.

The stimulation of lymphocytes by specific antigen. The synthesis of DNA as measured by ^{3}H-TdR incorporation in lymphocytes from ferrets vaccinated with inactivated adjuvated IBR vaccine was detected at variable levels three weeks

post-inoculation (Table 3). A maximum stimulation index of 27.7 was observed 11 weeks post-inoculation.

Ferrets inoculated with modified live virus did not show the incorporation of ^{3}H-TdR in lymphocyte cultures (Table 4).

Lymphocytes from the control animals did not incorporate appreciable amounts of ^{3}H-TdR (SI = 0-4.5). Relatively low levels of non-specific stimulation by lymphocytes from both control and vaccinated animals was observed (S.I. = 1.3-6.2) in cultures supplemented with UV inactivated IBRV beginning the eighth week post IN vaccination of inactivated IBRV. This was observed till the termination of the experiment. Whole blood cultures from ferrets inoculated with MLV vaccine were not tested for non-specific stimulation with UV-BVDV.

Discussion. In this study, the absence of clinical IBRV infection in ferrets, confirms the observations made by Porter, Larsen and Cox, (1975). They isolated IBRV from apparently healthy ferrets that were not manifesting signs of IBRV infection. It is not clear from their studies, however, that IBRV once introduced can infect and replicate in ferret tissues. In studies by Shump and Shump (1978), the weights of ferrets plateaued at approximately 20 weeks of age as opposed to 28 weeks observed in our studies. The decrease in weight observed at 44 weeks (after inoculations) may have been due to numerous blood samplings with greater stress and weight loss occurring in the females. Ferrets

reached higher weight levels, 1.55 Kg in males and 1.2 Kg in females as compared to 1.5 Kg in males and 0.9 Kg in females reported by Shump and Shump (1978).

The pre- and post-inoculation mean differences observed in the hematological serum chemistry profiles between the ferrets inoculated with formalin inactivated IBRV with Freund's Incomplete Adjuvant and the controls inoculated only with incomplete adjuvant are signigicantly different (P = 0.10) for albumin, creatinine and bands (Table 1 and 2). Ferrets inoculated with MLV were significantly different (P = 0.10) for albumin and PCV with no significant changes detected in the other 21 profiles, suggesting little differences between the routes of inoculation.

The absence of significance for the other 17 variables among the treatments (Table 1 and 2), indicates that the treatments A,B,C and D did not have appreciable effects on those variables during the pre-inoculation to the post-inoculation period.

The isolation of virus three days post IN vaccination with MLV from one of the three ferrets was probably due to the presence of residual virus from the inoculum, rather than replicated virus particles in the nasal mucosa. Subsequent nasal washings did not yield any virus. The red coloration in two of the three nasal washings may have been caused by a local inflammatory reaction to the antigen.

Inflammation resulting in the production of local secretory antibodies and interferon in the nasal mucosa, as demonstrated in cattle vaccinated IN with MLV (Todd, Volenec and Paton, 1972) would prevent virus replication. The reason CPE was not observed beyond the first passage of the sample on MDBK cells is obscure. It is likely that the ferret nasal washings containing the products of an inflammatory response were cytotoxic to MDBK cells in the initial passage and was diluted with subsequent passages. The inability of IBRV to persist in the ferret nasal mucosa may have resulted in the low virus recovery and the subsequent loss of the virus from following samples.

The specific and non-specific virus inactivating factors detected in the nasal washings (Figure 2) from ferrets inoculated IN with inactivated IBRV may demonstrate the presence of local immunity.

The duration and levels of serum neutralizing antibodies in ferrets inoculated with inactivated IBRV with Freund's Incomplete Adjuvant exceeded the duration and levels of antibody detected in ferrets inoculated IN or IM with MLV vaccines (Figures 3 and 4). Since it is likely that the MLV did not replicate in the ferret, the titer of the antigen present in vivo may not have been sufficient to induce a comparable humoral response as observed in the ferrets inoculated with inactivated adjuvated vaccine. In studies by Porter, Larsen and Cox, (1975), the presence of serum antibodies was detected three weeks post-inoculation of IBRV

(10^6 PFU), given intraperitoneally in mink. Inactivated
influenza virus vaccines with Freund's Incomplete Adjuvant
have been found to elicit serum antibodies in ferrets (Potter,
Jennings and McLaren, 1973). It is possible that the activity
of the vaccine with adjuvant involved one or a combination of
the following phenomena: (1) the establishment of the anti-
gen in a persistent form at the site of injection, allowing
the gradual but continuous release of antigen for stimu-
lating antibody production, (2) the provision of a vehicle
for the transport of emulsified antigen to the lymphatic
system, and (3) the formation of an inflammatory response
and the accumulation of mononuclear cells which elicit anti-
body production at local and distant sites (Hilleman, 1967).
The later explains the inflammatory response observed in the
ferrets post IM vaccination of inactivated IBRV with adjuvant
(Figure 2). It is probable that the presence of adjuvant in
the inactivated vaccine was an important factor for eliciting
an immune response in ferrets. Hobson, (1973) and Woodhour,
et al. (1969), demonstrated that vaccine preparations with
adjuvant produced higher levels of antibody than equal doses
of aqueous vaccine. They also reported that a single dose of
vaccine containing adjuvated virus, produced higher levels
of antibody than two doses containing a greater amount of
antigen in an aqueous vaccine preparation. In influenza
virus studies, only ferrets inoculated with inactivated virus
vaccine in Freund's Incomplete Adjuvant produced serum anti-
bodies, as compared to ferrets inoculated with an equal dose

of virus without adjuvant (Potter, Jennings and McLaren, 1973). Future studies using increasing doses of IBRV with a constant amount of adjuvant would provide more conclusive evidence for comparing the degrees of immunity conferred by an adjuvated virus preparation.

Cell mediated immune responses as indicated by lymphocyte transformation and the incorporation of ^{3}H-TdR in the presence of a specific antigen were only detected in ferrets inoculated with inactivated IBRV with adjuvant (Table 3). The degree of specific stimulation demonstrated by the lymphocytes from inactivated IBRV vaccinated ferrets was higher than the stimulation observed in cultures from control ferrets. However, both control and inactivated IBRV vaccinated ferret cultures demonstrated low degrees of non-specific stimulation. The reasons for this type of stimulation are not clear. It is known that adjuvants can modify the expression of antigenic determinants, alter the ratio of various antibody classes and change the balance between antibody mediated and cell mediated immunity (White, 1967). From these observations the mechanisms resulting in the superior levels of immunity elicited by the inactivated vaccine with adjuvant as compared to the aqueous MLV can be explained.

In this investigation the humoral and cell mediated immune responses of ferrets to inactivated and ML IBRV was studied. Since ferrets do not simulate bovines with regard to IBRV pathogenesis (Kahrs, 1977; Porter, Larsen and Cox, 1975) and immunity to inactivated and ML IBRV vaccines

(present study) it is questionable whether these animals may be satisfactory models for studying IBRV infections or evaluating IBRV vaccines.
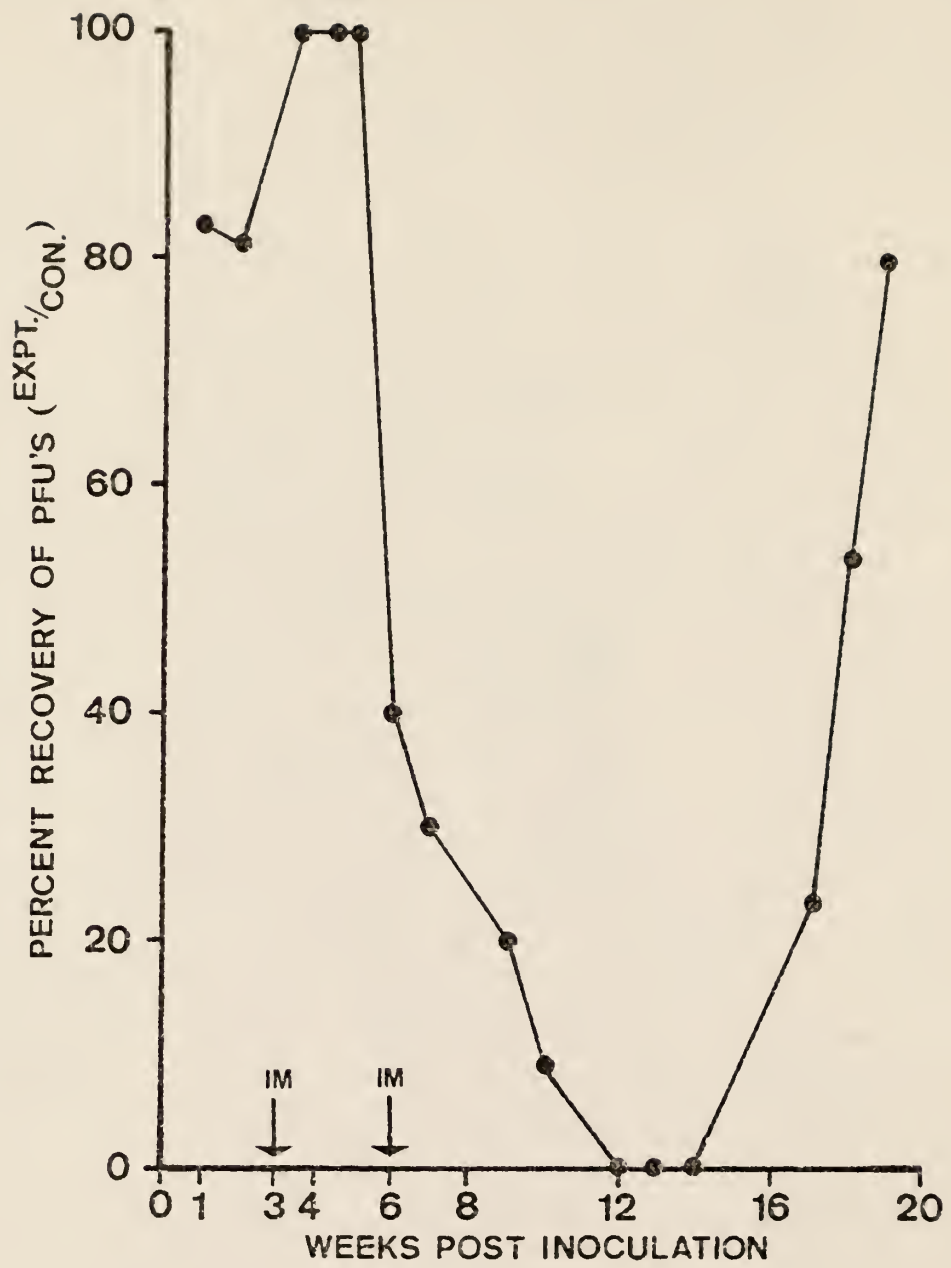
Figure 3. Serum neutralizing antibodies in ferrets inoculated with inactivated virus. Two samples, one each from a control and an experimental ferret, were tested at specified intervals. The serum samples were incubated for one hour at 25° C with an equal volume of IBRV, 5×10^{2} - 1×10^{3} PFU/0.3 ml. The samples were plaque assayed to determine the percent recovery of the virus.
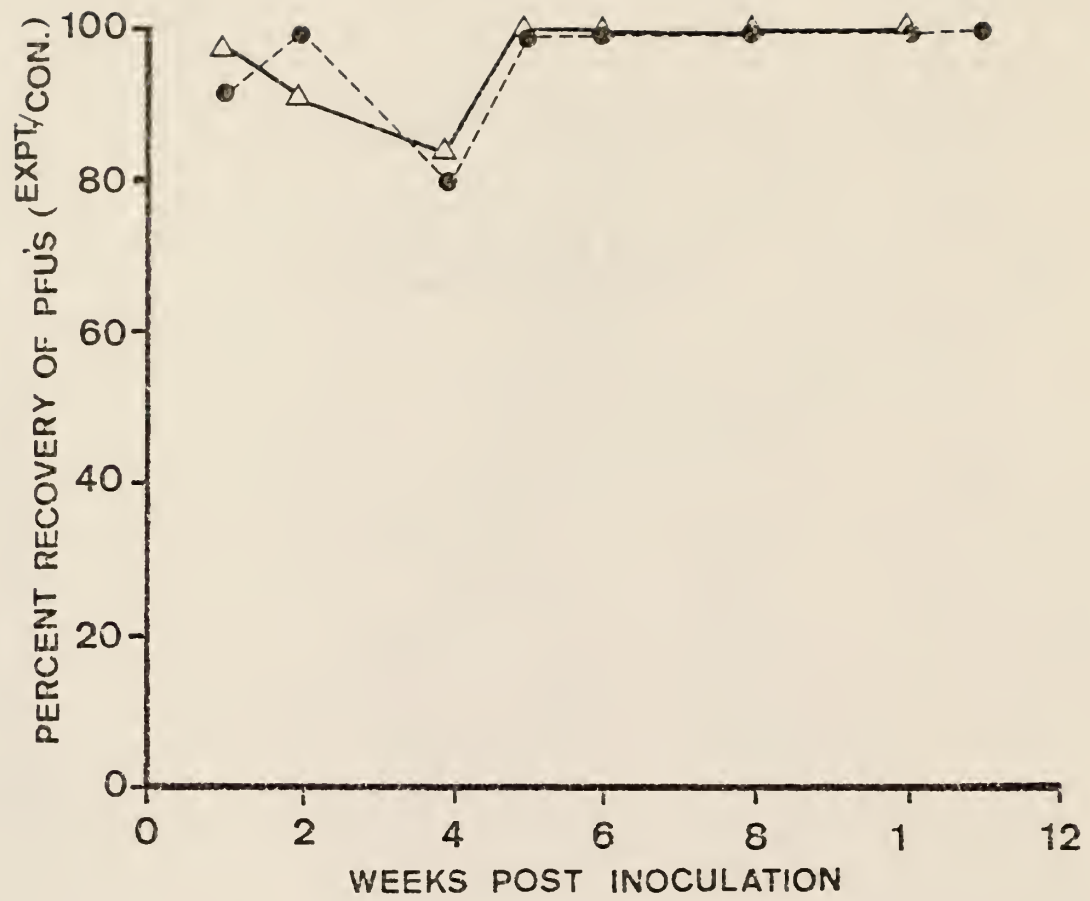
Figure 2. <u>Specific and nonspecific virus neutralizing</u>
<u>factors in nasal washings</u>. Two samples, one each from
a control and an experimental ferret, were collected
at specified intervals. The nasal washings from
ferrets inoculated IN with inactivated virus were in-
cubated for one hour at 25° C with an equal volume of
IBR and BVD virus, $5 \times 10^2 - 1 \times 10^3$ PFU/0.3 ml. The
samples were plaque assayed to determine the percent
recovery of virus: ●----● Specific Factors;
 △——△ Non-specific Factors.
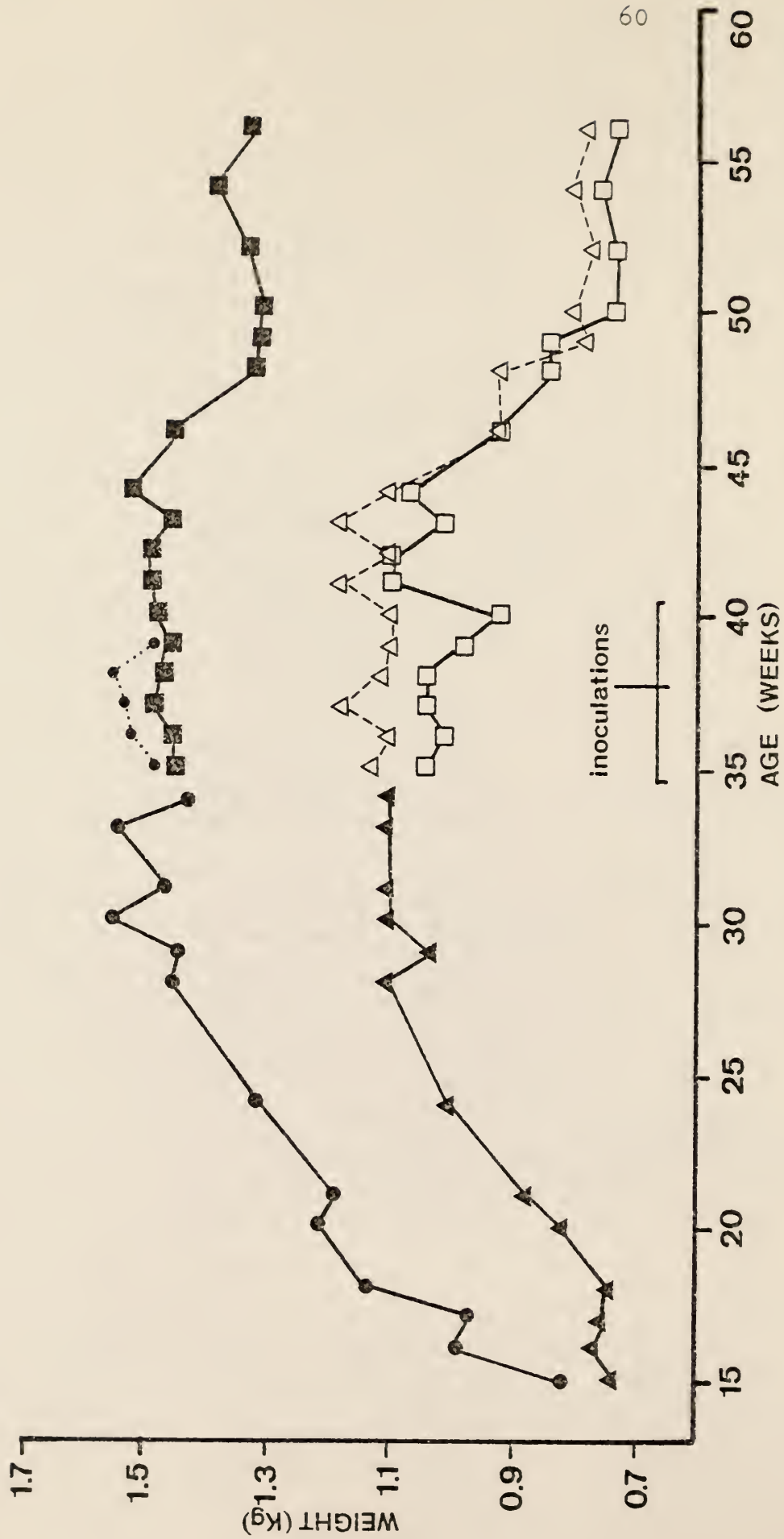
Figure 1. Weights of male and female ferrets. Weights

of animals before inoculation from five castrated males

and five females were recorded. At 35 weeks of age, four

males and one female were inoculated with inactivated

IBRV given IN, while one male and two females were sham

inoculated. At 38 and 41 weeks of age, the experimental

animals were inoculated IM with a homogenous mixture

of inactivated IBRV and Freund's Incomplete Adjuvant.

The controls received the mixture without virus:

●——● Castrated Males Pre-inoculation; ●····● Castrated

Male Control; ■——■ Castrated Male Experimental;

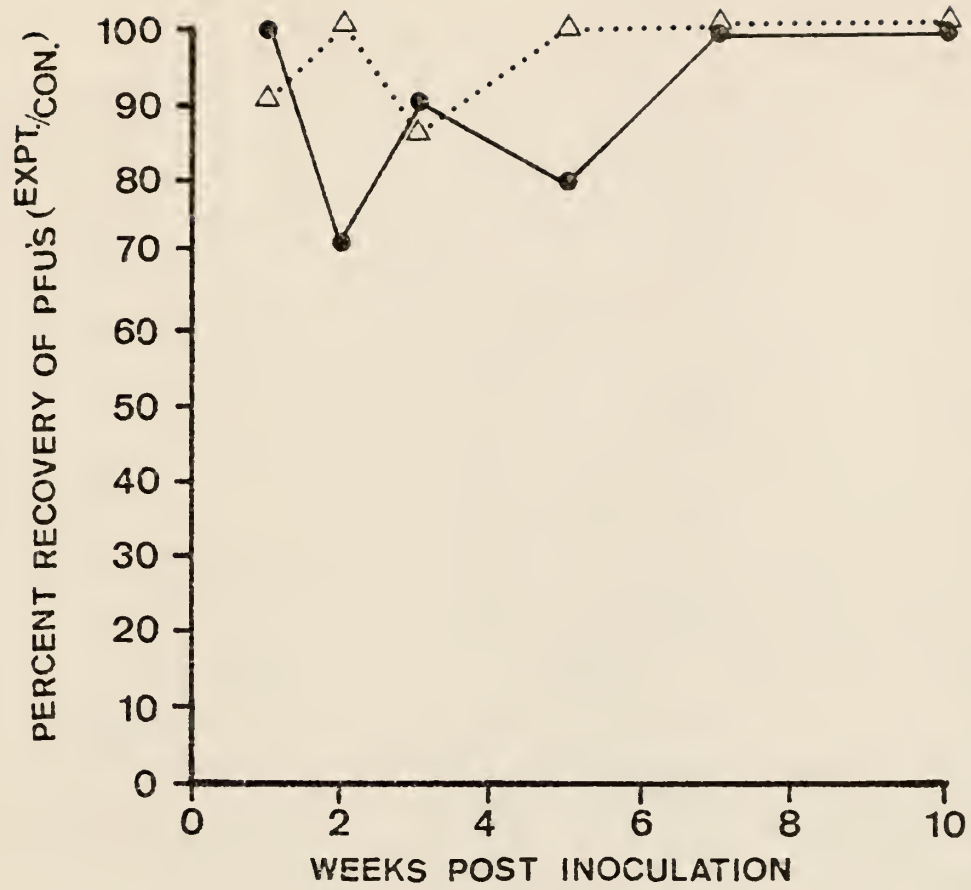▲——▲ Female Pre-inoculation; △···△ Female Control;

□——□ Female Experimental.

TABLE 1

STATISTICALLY SIGNIFICANT CHANGES BETWEEN PRE- AND POST-INOCULATION
MEANS IN FERRET HEMATOLOGICAL AND SERUM CHEMISTRY PROFILES

Treatment

Variables Measured	A Inactivated IBRV and Freund's Incomplete Adjuvant n = 10-13	B Modified Live IBRV n = 3-5	C Modified Live IBRV n = 3-5	D Incomplete Adjuvant Only n = 3-8	Among Treatments
Packed Cell Volume (PVC) %	2.36 +	-3.33 **	0.333 NS	1.19 NS	**
Hemoglobin gm %	-0.0818 NS	-1.13 *	0 NS	-1.10 +	
White Blood Cell (WBC) Cu mm	0.755 NS	-2.63 +	-3.63 +	-2.61 *	**
Bands/Cu mm	2.55 *	0.333 NS	-1.67 +	0.125 NS	
Neutrophil/Cu mm	0.818 NS	10.33 NS	-0.667 NS	-4.50 +	
Lymphocyte/Cu mm	-6.64 NS	-6.67 NS	6.33 +	-0.750 NS	
Monocyte/Cu mm	-1.60 +	-2.67 NS	-3.33 +	-3.75 **	
Eosinophil/Cu mm	0.455 NS	-1.00 NS	-1.00 NS	1.88 +	
Basophil/Cu mm	-0.0909 NS	-0.333 NS	0.333 NS	0.375 NS	
Sorbital Dehydrogenase (SDH) I.U.	0.342 NS	2.36 NS	2.67 +	-0.700 NS	*
Chloride (Cl) meq/L	-4.82 **	-6.50 *	-6.29 **	-3.00 NS	
Sodium (Na) meq/L	1.29 NS	-4.60 *	-2.00 NS	-2.29 NS	
Potassium (K) meq/L	0.569 ***	0.00 NS	0.114 NS	-0.129 NS	
Carbon Dioxide (CO2) meq/L	-4.22 **	0.840 NS	0.857 NS	-3.40 NS	
Blood Urea Nitrogen (BUN) mg %	-2.16 NS	-24.60 **	-20.14 **	1.14 NS	**
Alkaline Phosphatase mu/ml	3.23 NS	26.00 +	25.29 *	13.00 +	
Serum Glutamate Pyruvate Transaminase (SGPT) mu/ml	16.75 NS	-59.80 NS	-4.00 NS	61.33 NS	
Total Protein (T.P.) gm %	-0.100 NS	-0.160 NS	0.186 +	-0.343 *	**
Albumin gm %	0.315 **	-0.200 NS	0.114 NS	-0.100 NS	

TABLE 1 (continued)

Treatment

Variables Measured	A Inactivated IBRV and Freund's Incomplete Adjuvant n = 10-13	B Modified Life IBRV n = 3-5	C Modified Live IBRV n = 3-5	D Incomplete Adjuvant Only n = 3-8	Among Treatments
Calcium (Ca) mg %	0.923 **	-0.160 NS	0.0857 NS	0.943 NS	
Phosphorus (P) mg %	0.854 **	0.460 NS	-0.171 NS	0.871 *	
Glucose mg %	-10.92 +	-8.00 NS	-25.29 *	8.86 NS	
Creatinine mg %	-0.0154 NS	-0.260 *	-0.229 **	0.0571 +	**

NS, +, * and ** = statistically significant at nonsignificant, 10%, 5% and 1% levels, respectively.

TABLE 2

LEAST SIGNIFICANT DIFFERENCES (LSD)
BETWEEN PRE- AND POST-TREATMENT MEANS

Variable Measured	Significant Treatments	
SDH	C&B, B&A, B&D, C&A	+ *
BUN	A&C, C&D, A&B, D&B	** **
Albumin	A&C, C&D, C&B A&D, A&B	+ * **
Creatinine	A&C A&C, D&C A&B, D&B	* ** **
PVC	C&B D&B A&B	+ * **
Bands	A&B A&D A&C	+ * **

1. Group A: Inactivated Virus and Adjuvant
   Group B: Modified Virus (IM)
   Group C: Modified Virus (IN)
   Group D: Adjuvant Only

2. +, *, ** = statistically significant at 10%, 5% and 1% levels, respectively.

TABLE 3

STIMULATION INDICES[A]

FERRETS INOCULATED WITH FORMALIN INACTIVATED IBRV

WEEKS POST-INOCULATION	CONTROL[B] S.I.	EXPERIMENTAL[B] S.I.
3	0.0	0.0
6	0.0	6.0
8	0.0	1.1
10	0.0	1.3
11	1.3	27.7
12	1.9	2.9
13	0.0	10.4
14	3.6	1.1

[A]Stimulation Index (S.I.) = $\frac{\text{DPM (Cells + Antigen)}}{\text{DPM Cells}}$

[B]Two ferrets, one control and one experimental, were bled each time.

TABLE 4

STIMULATION INDICES[A]

FERRETS INOCULATED WITH MODIFIED LIVE IBRV

Modified Live IBRV	Control S.I.	Experimental S.I.
I.N.	4.5	0
10 Weeks		0
Post-Inoculation		<1
I.M.	0.0	0
8 Weeks		0
Post-Inoculation		0

[A]Stimulation Index (S.I.) = $\dfrac{\text{DPM (Cells + Antigen)}}{\text{DPM (Cells)}}$

SECTION V

IMMUNOLOGICAL STUDIES IN FERRETS INOCULATED WITH

INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

II. Effect of Phytohemagglutinin and Cortisol on Leukocytes

Abstract

Phytohemagglutinin (PHA) at a concentration of 5 ug/ml demonstrated maximum stimulation of ferret lymphocytes in whole blood cultures. The optimal effect of PHA on deoxyribonucleic acid (DNA) synthesis was observed between 72 and 168 hours after incubation of the cultures. Cultures stimulated with PHA incorporated maximum label when they were exposed to tritiated thymidine (^{3}H-TdR) for 12 hours prior to harvest.

Viability of leukocytes after incubation for 96-144 hours was approximately 10 fold greater in cultures with PHA as compared to cultures without PHA or containing ultraviolet irradiated infectious bovine rhinotracheitis virus (UV-IBRV).

Cortisol in physiological concentrations inhibited the viability of PHA stimulated lymphocytes as indicated by an appreciable decrease in cell count and incorporation of ^{3}H-TdR. The cell mediated immune response was also depressed in UV-IBR stimulated cultures. The presence of increasing concentration of cortisol (0-5000 mg/ml) did not affect the adsorption of ^{3}H-IBRV to ferret leukocytes.

Introduction. Infectious bovine rhinotracheitis is capable
of producing a latent infection and a variety of clinical
manifestations (Kahrs, 1977; Owens, Chow and Mollelo, 1964;
French, 1962; Abinanti and Plumer, 1961; McKercher, et al.,
1959; Madin, York and McKercher, 1956). Recrudescence or
activation of latent IBR infections can occur spontaneously
during periods of stress when physiological hormone levels
are altered or with the administration of corticosteroids
(Davies and Duncan, 1974; Davies and Carmicheal, 1973).

It has been demonstrated that IBRV latency and spontan-
eous recrudescence can occur in the presence of humoral
antibodies (Sheffy and Rodman, 1973). As a result there
has been increasing interest in the role of the cell
mediated immune responses in an attempt to understand and
control IBRV infections. The primary factors of the cellular
immune system are the thymus (T) dependent lymphocytes.
There are various techniques for measuring T cell stimula-
tion or blastogenesis to a mitogen or specific antigen. The
method reported by Sorensen, Anderson, and Giese (1969) measures
the incorporation of radioactively labelled DNA precursors
as an indication of T-lymphocyte blastogenesis and is used
as an index for measuring CMI responses.

It has been found that adrenal cortical hormones by
causing cytotoxic effects and altering cellular metabolism
and activity in the cellular components of the immune system,
suppress the CMI responses to either mitogen or specific

antigen. In vitro studies have illustrated the inhibition of blastogenesis in lymphocyte cultures supplemented with cortisol before or simultaneously with the stimulus (Fauci and Dale, 1974; Tormey, Fudenberg and Kamin, 1967; Stevens and Dougherty, 1964; Nowell, 1961).

A better understanding of the complex mechanisms of the immune response in an animal to an antigen or a mitogen and the suppression observed in lymphocytes with cortisol would provide insight for controlling latency and the recrudescence of IBRV infections. The objective of this investigation is to study the effect of cortisol on lymphocytes in phytohemaglutinin and antigen supplemented ferret whole blood cultures.

Materials and Methods.

Animals. Fitch and English ferrets, Mustela putorius furo (Marshall Research Animals, North Rose, NY) were used in these studies. The animals were vaccinated with infectious bovine rhinotracheitis virus (LA strain) as previously described in Section IV.

Preparation of ultraviolet inactivated virus for lymphocyte stimulation. Virus (IBRV) for in vitro stimulation of whole blood cultures was prepared as described in Section IV.

Determining the time course of DNA synthesis. The method of whole blood cultures used by Gerber and Brown (1974) was adopted with modification as previously described. A stock

solution of phytohemagglutinin (PHA-V; Sigma Chemical Co.,
St. Louis, MO) was prepared in RPMi-1640 (Grand Island
Biological Co., Grand Island, NY). Whole blood cultures
containing 5 ug/ml of PHA were incubated for varying time
periods up to 168 and 240 hours. The cultures were exposed
to 0.5 uCi/ml of tridiated thymidine (^{3}H-TdR; specific
activity 5 Ci/mmole, New England, Boston, MA) in medium, 12
hours prior to harvest each time. Samples were harvested
at various intervals. The cultures were treated with equal
volumes of distilled water and a 1.7% NaCl solution, pro-
cessed and counted on glass filters as previously described.
A set of unlabelled samples were treated in a similar manner
and the leukocyte viability determined by the trypan blue
stain (0.04%, in normal saline, Grand Island Biological Co.)
exclusion method. The fold increase over the zero hour
control was calculated.

The effect of PHA concentration on DNA synthesis. Whole
blood cultures supplemented with different concentrations
of PHA were incubated and labelled with ^{3}H-TdR for 24 hours
prior to harvest at 120 hours. The cultures were harvested,
processed and DPM's recorded as previously described.

Determining the time of exposure for maximum ^{3}H-TdR incor-
poration into lymphocytes. Whole blood cultures containing
5 ug/ml of PHA were incubated. Beginning at 96 hours of
incubation, ^{3}H-TdR was added to different sets of cultures
at four hour intervals. All cultures were harvested at 120

hours, processed and DPM's determined.

Effect of UV inactivated IBRV and PHA on leukocyte viability.
Whole blood cultures from control and experimental ferrets
were supplemented with UV-IBRV (2.0×10^5 PFU/0.2 ml), PHA
(5 ug/ml or UV-IBRV and PHA and incubated between 0 and 240
hours. Percent cell viability over the zero hour control
was determined at harvest by the method as described.

Preparation of ^{3}H-thymidine labelled IBRV. Monolayers of
MDBK cells (ATCC Laboratories, Rockville, MD) grown in
tissue culture dishes (100 x 20 mm, Falcon), were infected
with IBRV at a multiplicity of 10 PFU/cell and maintained
in medium 199. Radioactive medium containing 0.25 uCi/ml
of ^{3}H-thymidine (^{3}H-TdR, specific activity 90 Ci/mmole,
New England Nuclear, Boston, MA) was added to monolayers
after virus adsorption. Cultures were harvested when 90%
of the cells showed CPE (approximately 40 hours). The
cells were scraped from the dishes and the cell-virus sus-
pension was collected and partially purified as follows.
After low speed centrifugation, the supernatant containing
virus was ultracentrifuged at 100,000 g for five hours. The
supernatant was discarded and the virus pellet was resus-
pended in cold medium 199 and treated with DNase at a final
concentration of 10 ug/ml. The mixture was incubated for
30 minutes at 25° C, and ultracentrifuged for 1.5 hours at
200,000 g. The pellet was washed in cold media, resuspended
and dialyzed (1/4" tubing, size one, Fisher Scientific,

Pittsburgh, PA) against MEM in an ice bath for three hours and stored at -80° C.

Effect of Cortisol on Lymphocytes from Control and IBRV Vaccinated Ferrets.

(a) Inhibition of ^{3}H-TdR incorporation in ferret lymphocytes.

A stock solution of cortisol (Calbiochem, San Diego, CA) was prepared in an acetone-methanol (1:1) mixture and diluted in RPMI 1640 to various concentrations (0 ng-50,000 ng). Different concentrations of cortisol were added to whole blood cultures containing either 5 ug PHA or UV inactivated IBR (2.0 x 10^5 PFU/0.2 ml). The cultures were exposed to 0.5 uCi/ml of ^{3}HTdR at 108 hours. Samples were removed from a CO_2 incubator after 12 hours of exposure to the label and placed in an ice bath. The cultures were processed and counted as previously described. The percent DPM's was calculated from the PHA control.

(b) The effect of cortisol on the viability of lymphocytes.

The viability of lymphocytes isolated from whole blood cultures incubated 120 hours, containing or lacking 5 ug PHA or containing PHA and varying concentrations of cortisol (0 ng-50,000 ng) was determined by the method of trypan blue stain exclusion.

(c) Competition of cortisol and ^{3}H-IBR for leukocyte receptor sites.

A suspension of leukocytes (0.1 ml) isolated from heparinized blood, was added to duplicate sets of tubes containing a mixture of varying concentrations of cortisol (0 ng-5,000 ng) and a constant amount of ^{3}H-IBRV (2.0 x 10^4 PFU containing 3800 DPM. The mixture was incubated for four hours, gently mixing every hour. The cultures were placed in an ice bath and washed thrice with cold sterile PBS to remove the unadsorbed virus. Cells were then pelleted at 900 g for 10 minutes. The final pellet was resuspended in two ml of cold PBS and 0.2 ml of the sample was removed for plaquing. The remaining suspension was collected on glass filters and counted as previously described.

Results.

The effect of PHA concentrations on DNA synthesis.

Phytohemagglutinin at a concentration of 5 ug/ml stimulated lymphocytes maximally as indicated by the incorporation of ^{3}H-TdR in cultures incubated for 120 hours (Figure 1). Concentrations greater than 5 ug/ml demonstrated relatively decreased incorporation of the label.

Time course of DNA synthesis.

Figure 2A and 2B present results of the time course of DNA synthesis in whole blood culture each from two different control and vaccinated ferrets. Cultures lacking PHA did not incorporate ^{3}H-TdR, whereas those containing PHA demonstrated maximum DNA synthesis as indicated by the incorporation of the label between 72 and 120 hours.

Time of exposure of cells for maximum ^{3}H-TdR incorporation.
Incorporation of ^{3}H-TdR at variable but high levels was
demonstrated in cultures incubated for 120 hours with PHA,
when the label was added between four and 24 hours prior
to harvest (Figure 3). Maximum incorporation, however, was
observed 12 hours prior to harvest.

Leukocyte viability in PHA supplemented whole blood cultures.
Figure 4 represents the composite results from five control
and four experimental ferrets. Cultures containing PHA
showed a decrease in cell viability up to 48 hours, followed
by an increase (approximately seven fold) at 144 and 168
hours in the control and experimental ferrets, respectively.
A substantial decline in viability was subsequently observed
until 240 hours of incubation. The viability of leukocytes
from control ferrets containing PHA was relatively greater
as compared to leukocytes from vaccinated ferrets.

Viability of leukocytes in the presence of PHA and UV-IBRV.
Leukocytes from both the control and experimental animal
lacking PHA showed a decline in the percent viability over-
time (Figure 5A, 5B). Cultures containing PHA demonstrated
a decrease in percent viability up to 48 hours, followed by
a five to eight fold increase in viable cells at 96 and 144
hours in control (Figure 5A) and vaccinated (Figure 5B)
animals, respectively. The number of viable cells from
control and vaccinated ferrets was greater in cultures con-
taining either PHA or PHA and UV-IBRV as compared to cultures

lacking them. The cells from the vaccinated ferret, containing both PHA and UV-IBRV demonstrated greater viability at the peak hour (Figure 5B) as compared to control (Figure 5A).

The effect of cortisol on ^{3}H-TdR incorporation and viability of PHA stimulated lymphocytes. Figure 6A illustrates the composite results of DNA synthesis in PHA stimulated cultures from three control and three experimental ferrets. A decrease in the ^{3}H-TdR incorporation was evident with increasing concentrations of cortisol. The degree of inhibition of DNA synthesis was greater in the cultures from controls as compared to the vaccinated animals and was minimum at a cortisol concentration of 5,000 ng/ml.

Figure 6B represents the percent viability of lymphocytes from control and experimental ferrets. Viability was greatly reduced as the concentration of cortisol was increased. The highest concentrations (5,000 ng/ml) of cortisol demonstrated only three to six percent viability of the cells.

The effect of cortisol on cell mediated immunity. Lymphocytes from vaccinated animals were specifically stimulated with UV-IBRV in cultures without cortisol as demonstrated by ^{3}H-TdR incorporation (Figure 7). Cortisol (5 ng/ml) inhibited label incorporation 50-75% in cultures from the vaccinated animals. Cells from the control animal did not incorporate label in the presence of UV-IBRV suggesting the

lack of cell mediated immunity to IBRV in the control.

Effect of cortisol on ^{3}H-IBRV adsorption. Table 1 demonstrates that the presence of different concentrations of cortisol (0-5,000 ng/ml) did not affect the adsorption of a constant amount of labelled IBRV onto ferret leukocytes. This was indicated by relatively similar radioactive counts and PFU's recovered from the leukocyte suspensions. It appears that the amount of labelled virus adsorbed to experimental ferret leukocytes was somewhat higher as compared to the adsorption to control animals.

Discussion. Studies on the maximum incorporation of ^{3}H-TdR in ferret whole blood cultures, supplemented with PHA, was regarded as an important part of these studies, since PHA induced stimulation of lymphocytes provided an index of functional competence of the CMI response (Good, 1972). Based on these results, the effect of cortisol on CMI responses to either specific or non-specific stimulation could be determined adequately. Phytohemagglutinin, in ferret whole blood cultures (incubated 120 hours) at a concentration of 5 ug/ml demonstrated maximum DNA synthesis as measured by the incorporation of ^{3}H-TdR (Figure 1). Higher levels of PHA appeared to depress ^{3}H-TdR incorporation, while concentrations of three and four ug/ml produced intermediate degrees of stimulation. Concentrations of PHA ranging from 2.5-50 ug/ml have been used in studies with bovine and human lymphocytes (Unsgaard and Lamvik, 1977;

Muscoplat, et al., 1974a, 1974b; Rouse and Babiuk, 1973).
The differences in concentrations reported are probably due
to the species differences and the various type of PHA used.
In the present study, it is not known that the low ^{3}H-TdR
incorporation in cultures with concentration of PHA higher
than 5 ug/ml was due to the decrease in viability or meta-
bolic activity of the cells.

Maximum DNA synthesis, in PHA stimulated ferret cultures,
occurred between 72 and 120 hours of incubation (Figure 2A,
2B). It was reported by Fernald and Metzgar, (1971), that
in a given experiment, the cell concentration influenced the
timing of optimal DNA synthesis induced by PHA. Furthermore,
increased cell concentrations tended to shorten the time
for the maximal response to occur. Despite the differences
observed for individual animals in these studies, whole
blood cultures for subsequent studies were incubated for
120 hours. Ferret cultures containing PHA and exceeding 120
hours of incubation became clumped and the lysis of erythro-
cytes by the distilled water treatment, to obtain lympho-
cytes was increasingly difficult over time. Some investi-
gators (Muscoplat, et al., 1974a, 1974b; Pospisil, et al.,
1973; Davies and Duncan, 1973; Phillips and Zweiman, 1970)
using bovine and guinea pig cultures containing PHA observed
optimum incorporation of the label between 48-72 hours of
incubation. In other studies, bovine and canine whole
blood cultures were similarly incubated for 120 hours (Gerber
and Brown, 1974; Rouse and Babiuk, 1974). Incorporation of

^{3}H-TdR decreased rapidly in all cases following the peak of DNA synthesis. This was probably due to the depletion of nutrients in the media and decreased cell viability. Whole blood cultures lacking PHA essentially did not incorporate ^{3}H-TdR and the viability counts of these cultures revealed few viable cells (Figure 4). It is known that PHA is a T cell mitogen (Janossy and Greaves, 1971; Sorensen, Anderson and Giese, 1969) suggesting that in these studies, PHA maintained the lymphocytes in whole blood cultures by stimulating mitosis and increasing lymphocyte numbers. Electron microscopic examination of PHA stimulated mink cell cultures demonstrated the blast transformation of lymphocytes at 45 hours of incubation (Marsh, Miller and Hanson, 1973).

Uptake of ^{3}H-TdR was optimum in 120 hour PHA stimulated cultures, labelled 12 hours prior to harvest (Figure 3). This indicated that the maximum uptake of the label, a DNA precursor, occurred between 108-120 hours of PHA exposure. Rouse and Babiuk (1974) and Muscoplat, et al. (1974a, 1974b) reported the addition of ^{3}H-TdR into bovine lymphocyte cultures 24 hours prior to harvest. In our studies, high levels of ^{3}H-TdR incorporation was also observed when label was added four to 24 hours prior to harvest, suggesting relatively active DNA synthesis between these time intervals.

Whole blood cultures from control and vaccinated ferrets (lacking PHA) had low cell viabilities as measured by the trypan blue stain exclusion method (Figure 5A, 5B). It is apparent that the mitotic effects of PHA are not manifested

until 48 hours after supplementation, as illustrated by the decrease in viability (Figure 5A and 5B). In the control animal cultures, the viability of PHA stimulated cultures exceeded the viability of cells containing both PHA and UV-IBRV, indicating that the full mitotic activity of PHA was not realized in the presence of UV-IBRV (Figure 5A). The viability of vaccinated ferret lymphocytes containing both UV-IBRV and PHA or PHA alone, demonstrated similar peaks. This may be due to the added effect by specific antigen (UV-IBRV) on the stimulation of vaccinated ferret lymphocytes (Figure 5B) as compared to the control (Figure 5A). Cell mediated immunity was also demonstrated with specific antigen in cultures of vaccinated animals as indicated by ^{3}H-TdR incorporation.

Decreased incorporation of ^{3}H-TdR (Figure 6A) and low viability (Figure 6B) in PHA stimulated lymphocytes were demonstrated with increasing concentrations of cortisol. It is probable that cortisol in these studies may have resulted in a cytotoxic effect (Claman, 1972) or the inhibition of PHA stimulation (Zeman, et al., 1973; Roath and Cuppari, 1965). The mechanism of suppression may involve the presence of steroid-receptor complexes in the cell nucleus which are capable of combining with the chromatin. The nuclear complexes then influence DNA transcription, leading to specific changes in cellular function (Feldman, Funder and Edelman, 1972). Lymphocytes from IBRV vaccinated ferrets stimulated with specific antigen (UV-IBRV) required lower concentrations

of cortisol to depress ^{3}H-TdR incorporation (Figure 7) than PHA stimulated lymphocytes (Figure 6A). This effect may have been due to the presence of relatively fewer cells in cultures containing UV-IBRV as illustrated in Figures 5A and 5B. Control ferret lymphocytes did not demonstrate specific stimulation with the IBRV antigen; therefore the effect of cortisol on cell mediated immunity was not demonstrated (Figure 7).

It is apparent from the studies in Table 1 that increasing concentrations of cortisol did not affect the adsorption of ^{3}H-IBRV as indicated by recovery of DPM's and PFU's adsorbed to cells. It is also illustrated that a relatively greater number of virus particles (DPM and PFU) were adsorbed to leukocytes from vaccinated ferrets as compared to controls. This may indicate higher affinity of primed leukocytes for the adsorption of specific antigen. These studies suggest that IBRV and the hormone do not compete for the same receptor sites on ferret leukocytes. Furthermore, it indicates that the mechanism of inhibition of the blastogenic response (^{3}H-TdR incorporation by UV-IBRV stimulation) by cortisol is possibly independent of the sites of adsorption of IBRV on ferret leukocytes.

The use of both whole blood and short-term ferret lymphocyte cultures in stimulation and immune suppression studies has immense potential. The high levels of response by ferret lymphocytes to PHA has been demonstrated and would be invaluable in studying the mechanisms of immune suppression.

Figure 1.   Effect of PHA concentrations on DNA synthesis:
Whole blood cultures supplemented with varying concentra-
tions of PHA were labelled with ^{3}H-TdR 24 hours prior
to harvest.   At 120 hours the samples were harvested,
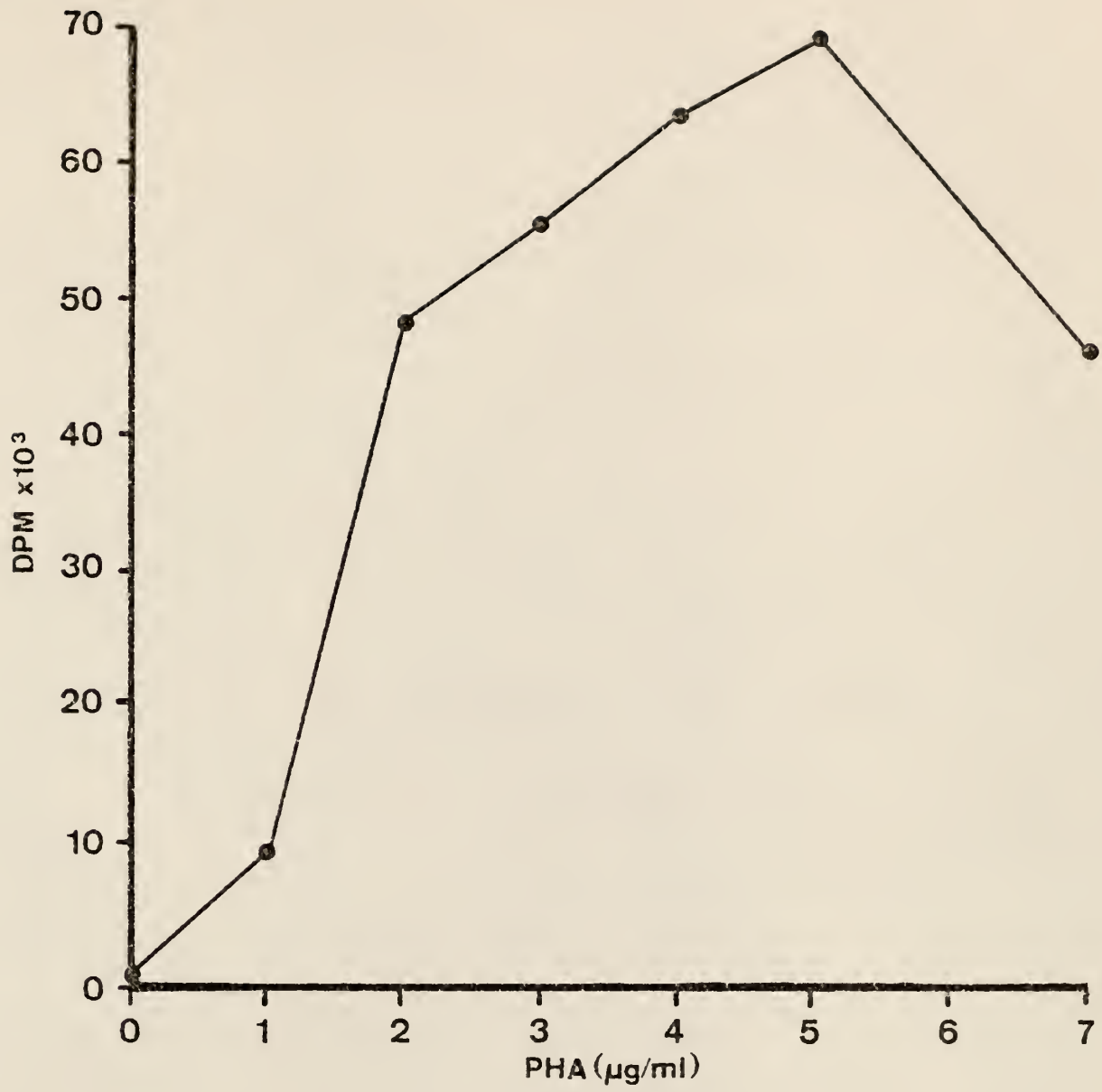processed and DPM's recorded.

Figure 2A and 2B. Time course of DNA synthesis: Whole
blood cultures from control and vaccinated ferrets lacking
or containing 5 ug/ml of PHA were incubated for varying
time intervals up to 168 hours. Samples were labelled
with ^{3}H-TdR 12 hours prior to harvest, processed and
counted: △···△ Control With PHA; ○···○ Control Without
PHA; ■——■ Experimental With PHA; □——□ Experimental
Without PHA.
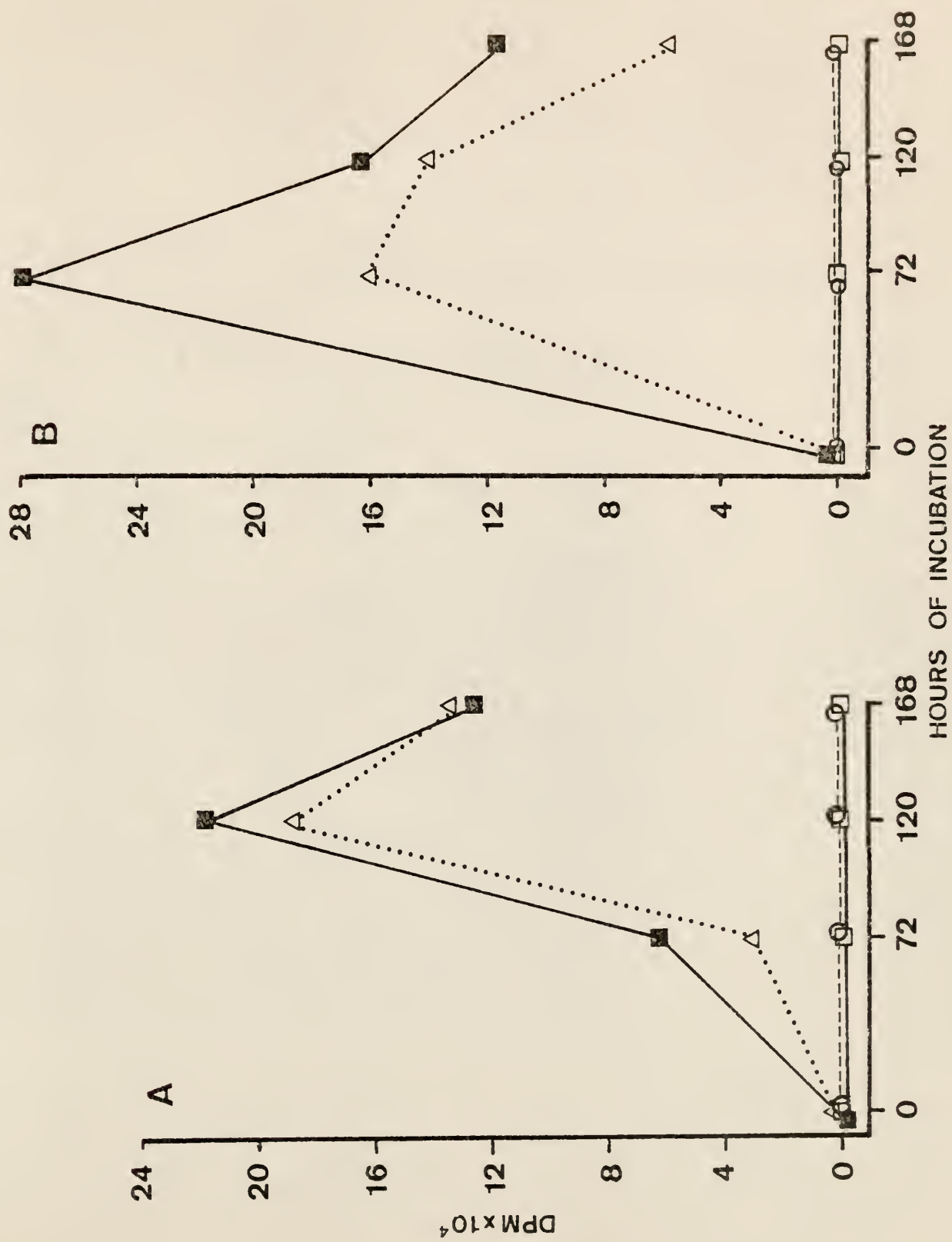
Figure 3. Time of exposure of cells to ^{3}H-TdR for maximum label incorporation: Whole blood cultures containing 5 ug/ml of PHA were incubated. Beginning at 96 hours of incubation, ^{3}H-TdR was added to sets of cultures at four hour intervals. All cultures were harvested at 120 hours, processed and DPM's determined.
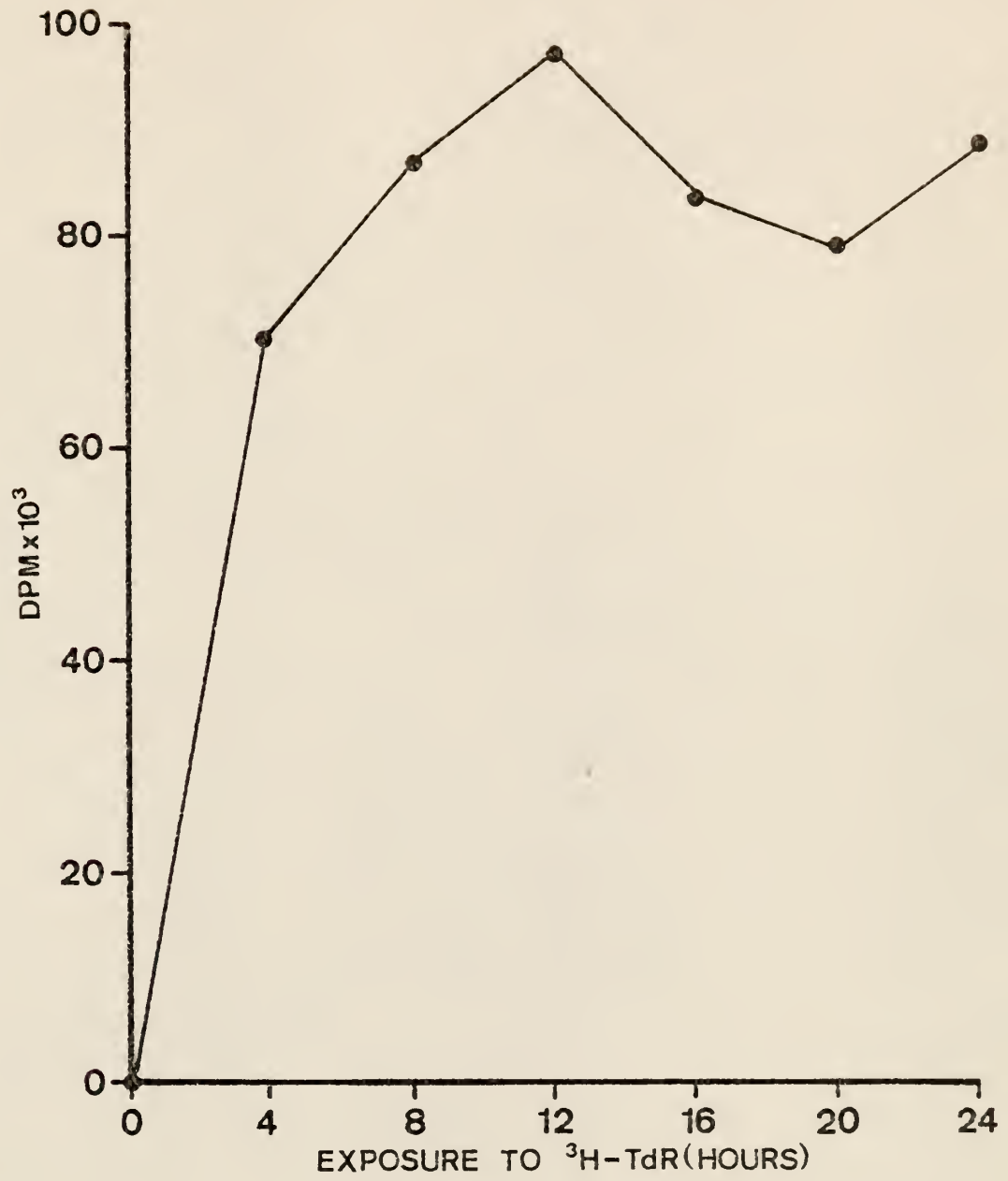
Figure 4. Leukocyte viability in PHA supplemented whole
blood cultures: Whole blood cultures from control and
vaccinated ferrets lacking or containing 5 ug/ml of PHA,
were incubated for varying time intervals up to 240 hours.
The cultures were harvested every 24 hours. The fold
increase of viable cells was determined by the trypan
blue stain exclusion method: ●——● Control Without PHA;
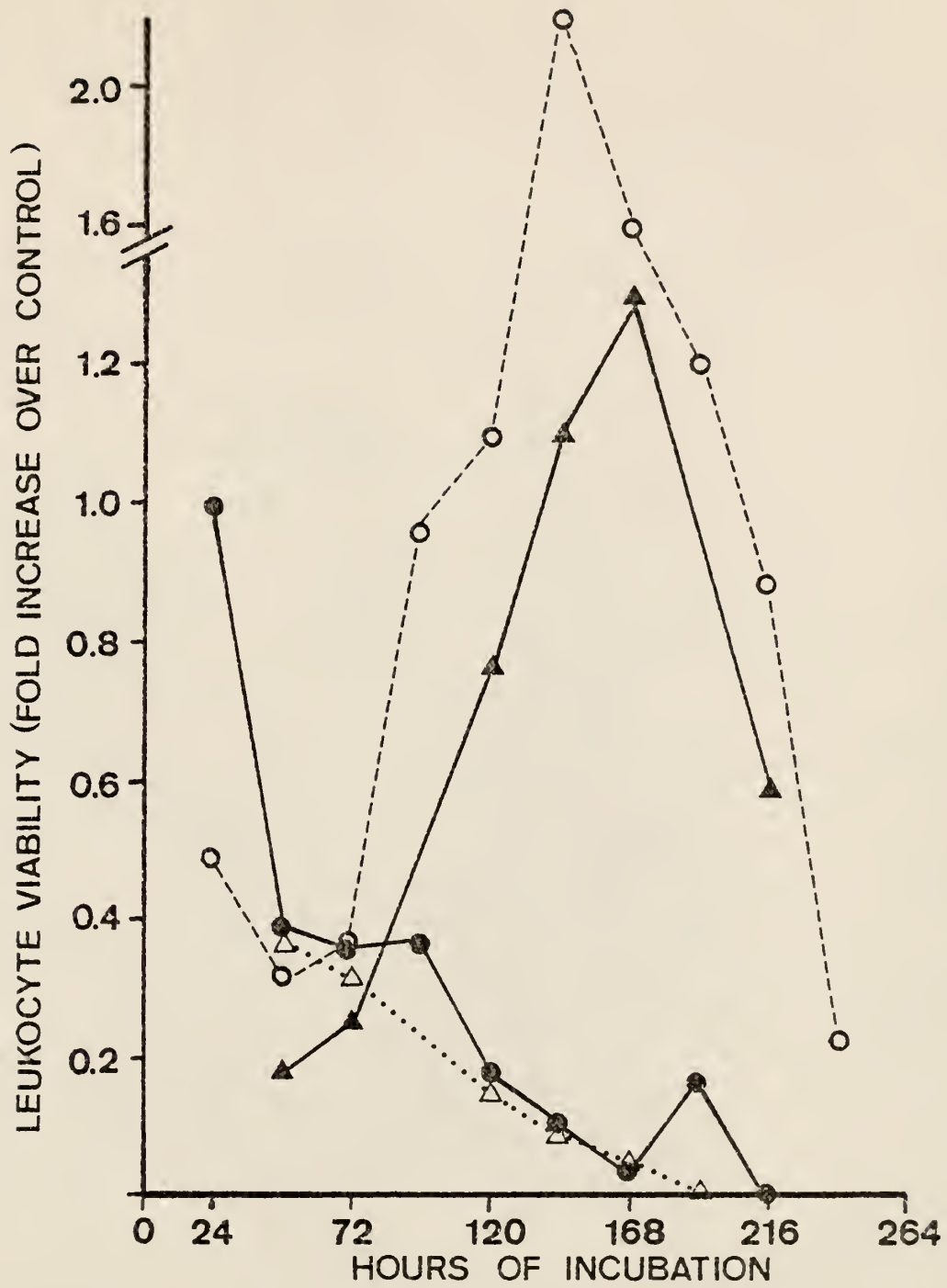○---○ Control With PHA; △....△ Experimental Without PHA;
▲——▲ Experimental With PHA.

Figure 5A and 5B. Effect of UV inactivated IBRV and PHA on leukocyte viability: Whole blood cultures from control and vaccinated ferrets were supplemented with UV-IBRV (2.0×10^5, PFU/0.2 ml), PHA (5 ug/ml) or both. Leukocyte viability was determined by the trypan blue stain exclusion method. The percent viability was calculated from the zero hour control: △—△ Media; O⋯O PHA; ▲--▲ UV-IBRV; ●—● PHA and UV-IBRV.
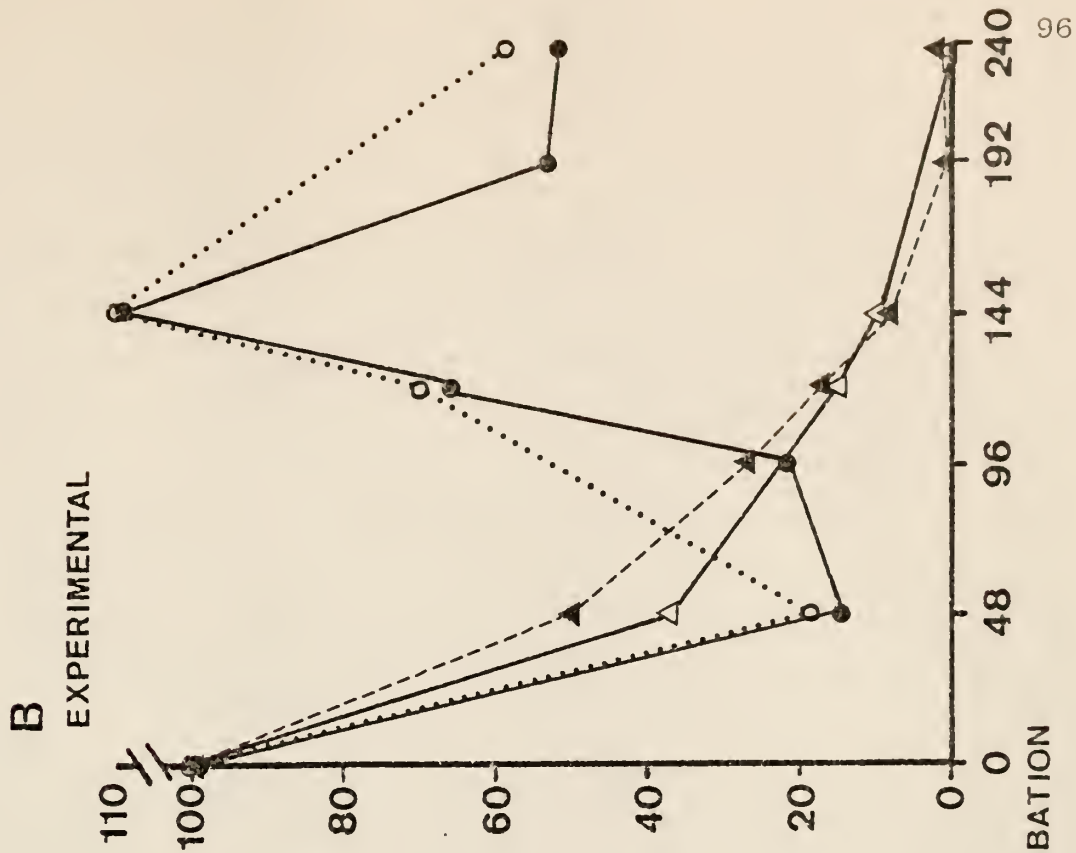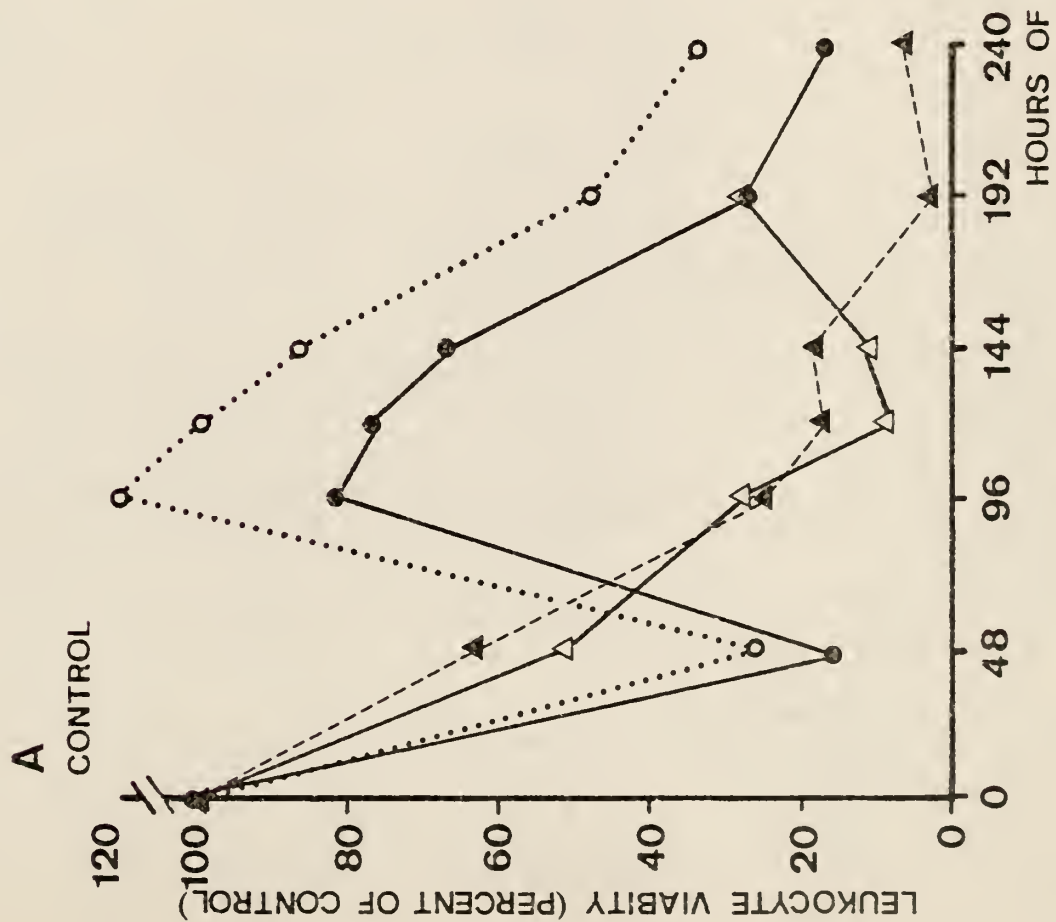
Figure 6A. Effect of cortisol on PHA stimulated lymphocytes: Whole blood cultures from control and vaccinated ferrets were stimulated with 5 ug/ml of PHA in the presence of varying concentrations of cortisol. ^{3}H-TdR was added 12 hours prior to harvest. The cultures were harvested at 120 hours, processed and counted. The DPM's were calculated as percent of the control with PHA: ●—● Control;   △-·-△ Experimental.

Figure 6B. Effect of cortisol on the viability of PHA stimulated lymphocytes: Whole blood cultures from control and vaccinated ferrets were stimulated with 5 ug/ml of PHA in the presence of varying concentrations of cortisol. The cultures were incubated for 120 hours. Viability of cells was calculated as percent of the control with PHA: ●—● Control;   △-·-△ Experimental.
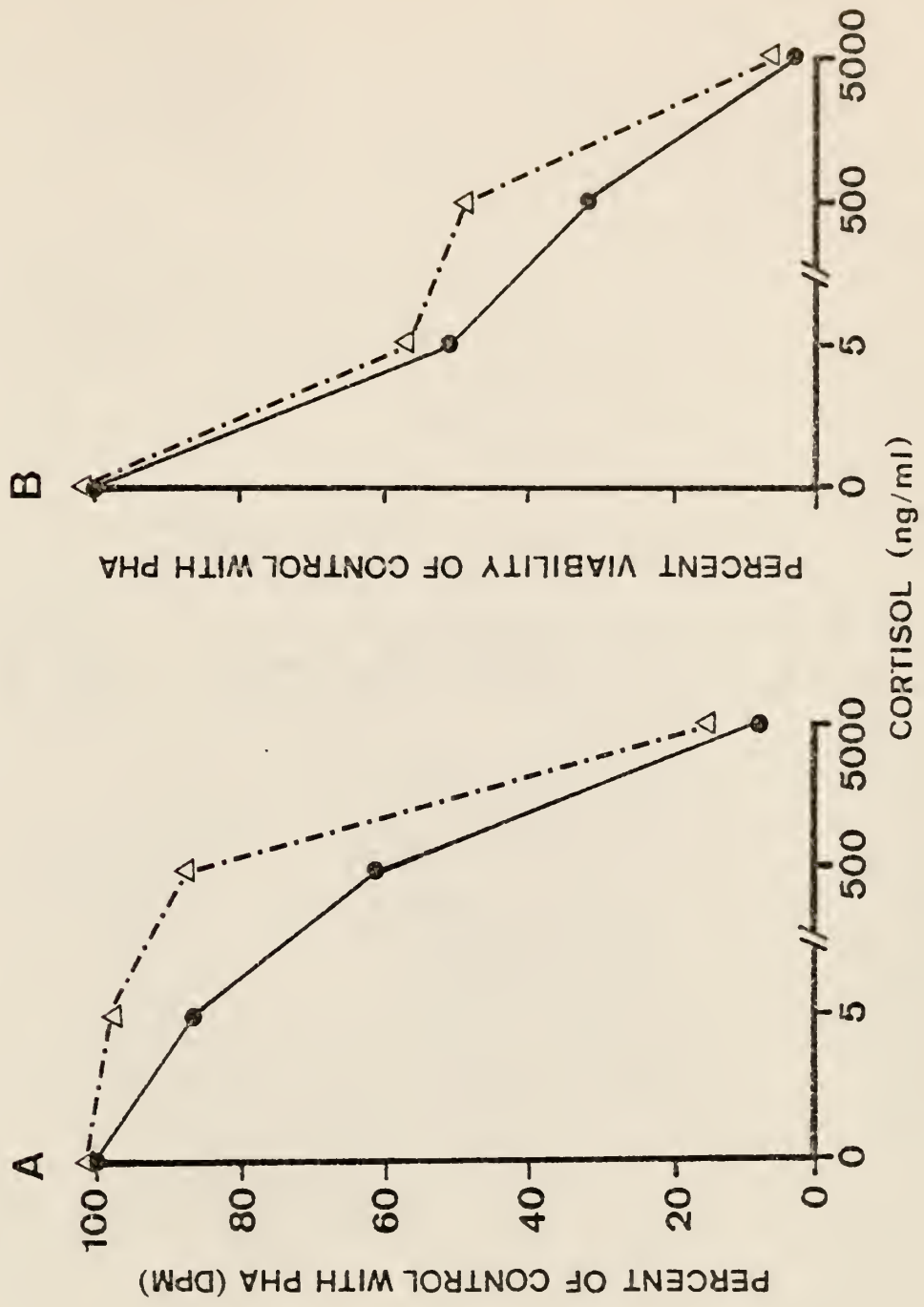
Figure 7. <u>Effect of cortisol on cell mediated immunity:</u>
Lymphocytes from vaccinated and control ferrets were
inoculated with UV-IBRV in the presence of varying
concentrations of cortisol. ^{3}H-TdR was added 12 hours
prior to harvest. The cultures were harvested and
processed at 120 hours and DPM's determined:

○—○
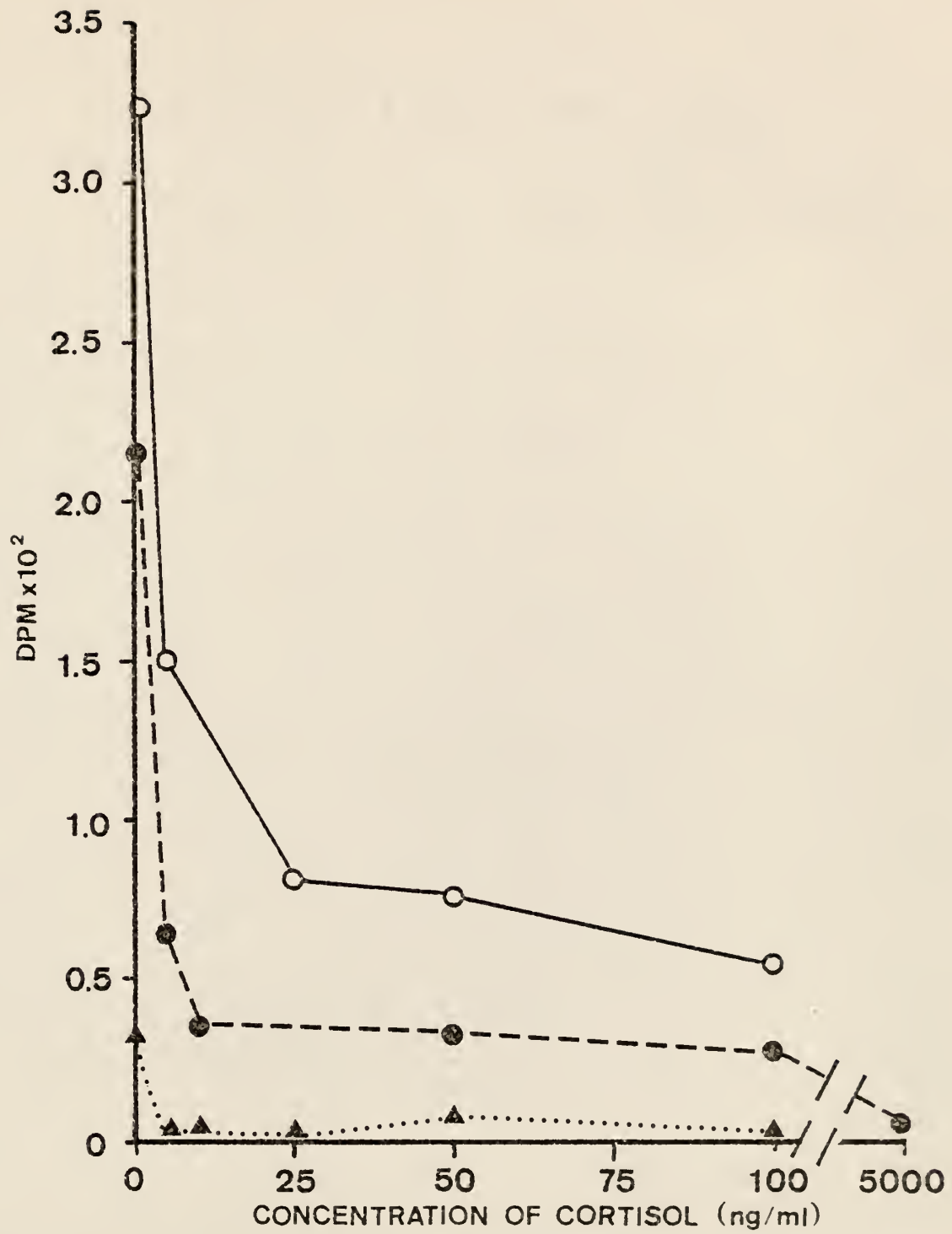●--● } Experimental;   ▲···▲ Control.

TABLE 1

EFFECT OF CORTISOL ON ^{3}H-IBRV ADSORPTION TO FERRET LEUKOCYTES

Concentration of Cortisol	Control				Experimental			
	DPM	Total PFU	DPM	Total PFU	DPM	Total PFU	DPM	Total PFU
0 ng	3028.5	8.0×10^3	2309.5	8.0×10^3	3259.1	–	3359.8	1.8×10^4
5 ng	2731.3	7.9×10^3	2776.5	8.4×10^3	3149.0	1.42×10^4	3159.4	8.4×10^3
50	2831.3	7.8×10^3	2144.3	8.3×10^3	3248.5	1.7×10^4	3031.7	2.01×10^4
500 ng	3061.1	–	2525.6	1.18×10^4	3381.4	1.64×10^4	3577.8	1.03×10^4
5000 ng	2853.9	1.1×10^4	2835.0	7.6×10^3	3240.5	–	3162.1	–

SECTION IV

APPENDIX

Materials and Methods.

UV inactivation of viruses. One ml of BVDV or IBRV (1×10^7 PFU/ml) was UV irradiated at one minute intervals for 0-6 minutes, in a petri dish (60 x 15 mm) at a distance of 10 cm from a UV germicidal sterilamp (G30T8, 30 watt, Westinghouse Electric Co., Bloomfield, NJ) (Figure 1). The virus was collected and plaque assayed to determine the percent inactivation for each interval of exposure.

Formalin inactivation of IBRV. For the preparation of inactivated vaccine, 5×10^8 PFU/ml of IBRV was treated with formalin at final concentrations of 1:1000, 1:2000, 1:4000, 1:8000, and 1:16000 (40% formaldehyde solution, Fisher Scientific Co., Fairlawn, NJ) for six hours at 25° C, then overnight at 4° C (Figure 2). The virus was dialyzed in 1/4" dialyzing tubing against three changes of cold PBS at 4° C over a 24 hour period. The virus was collected and plaque assayed to determine the percent inactivation for each concentration.

Butylated hydroxytoluene inactivation of IBRV. A stock solution of butylated hydroxytoluene (BHT) (Sigma Chemical Co., St. Louis, MO) 0.5 ug/10 ml ethanol was prepared. One ml of IBRV, 1×10^6 PFU/ml was treated with 0, 5, 50, 100, 250 and 500 ug BHT, agitated every five minutes, for 30 minutes at 37° C (Figure 3). The preparation was stored at -80°C then plaque assayed to determine the percent

inactivation of virus.

Determining the time course of DNA synthesis. The method
of whole cultures used by Gerber and Brown (1974) was
adopted with modification as previously described. Whole
blood cultures containing 5 ug/ml of PHA were incubated for
varying time periods up to 240 hours. The cultures were
exposed to 0.5 uCi/ml of tritiated thymidine (^{3}H-TdR) in
medium 24 hours prior to harvest each time. The samples
were harvested at various intervals. The cultures were
treated with equal volumes of distilled water and 1.7%
NaCl solution, processed and counted on glass filters as
previously described (Figure 4).

Figure 1. Inactivation of IBRV and BVDV by UV irradiation. One ml of virus (1×10^7 PFU) was UV irradiated for 0-6 minutes. The virus was collected and plaque assayed to determine the percent inactivation for each interval of exposure: ●——● IBRV;   ○——○ BVDV.
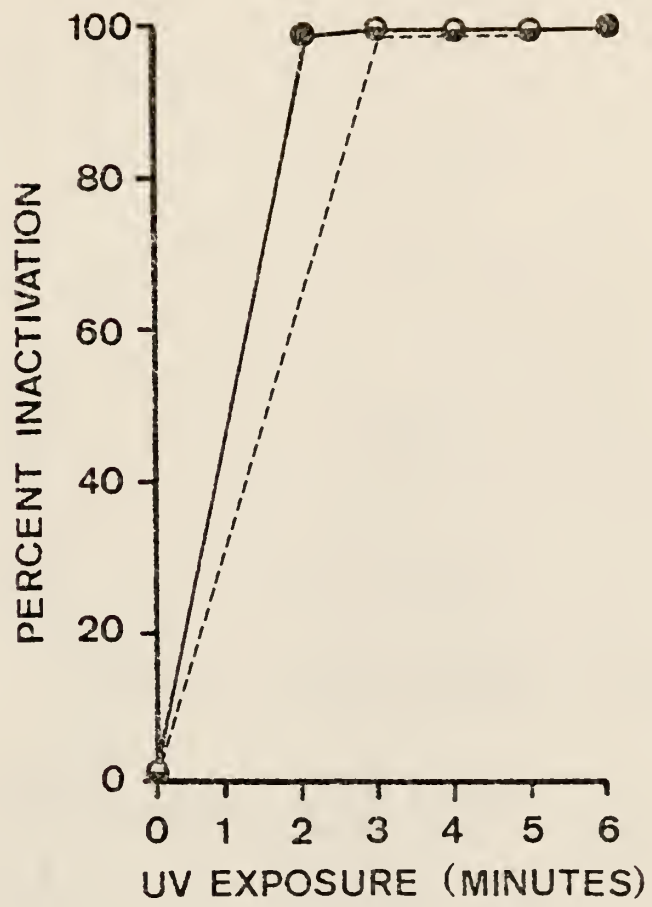
Figure 2. Formalin inactivation of IBRV. One ml of IBRV (5×10^8 PFU) was treated with formalin at final concentrations of 1:1000, 1:2000, 1:4000, 1:8000, 1:16,000 and 1:32,000. The virus was plaque assayed to determine the percent inactivation for each concentration.
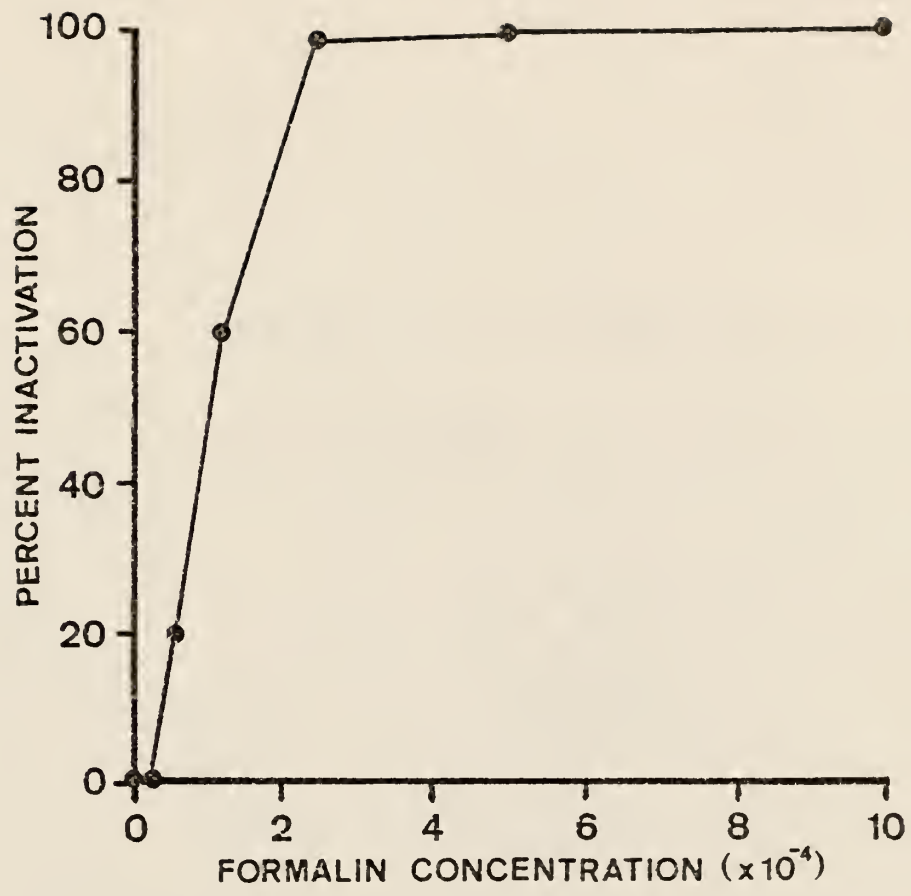
Figure 3. <u>Inactivation of IBRV by butylated hydroxytoluene</u>.
One ml of IBRV (1×10^6 PFU) was treated with 0-500 ug BHT.
The virus preparation was plaque assayed to determine the
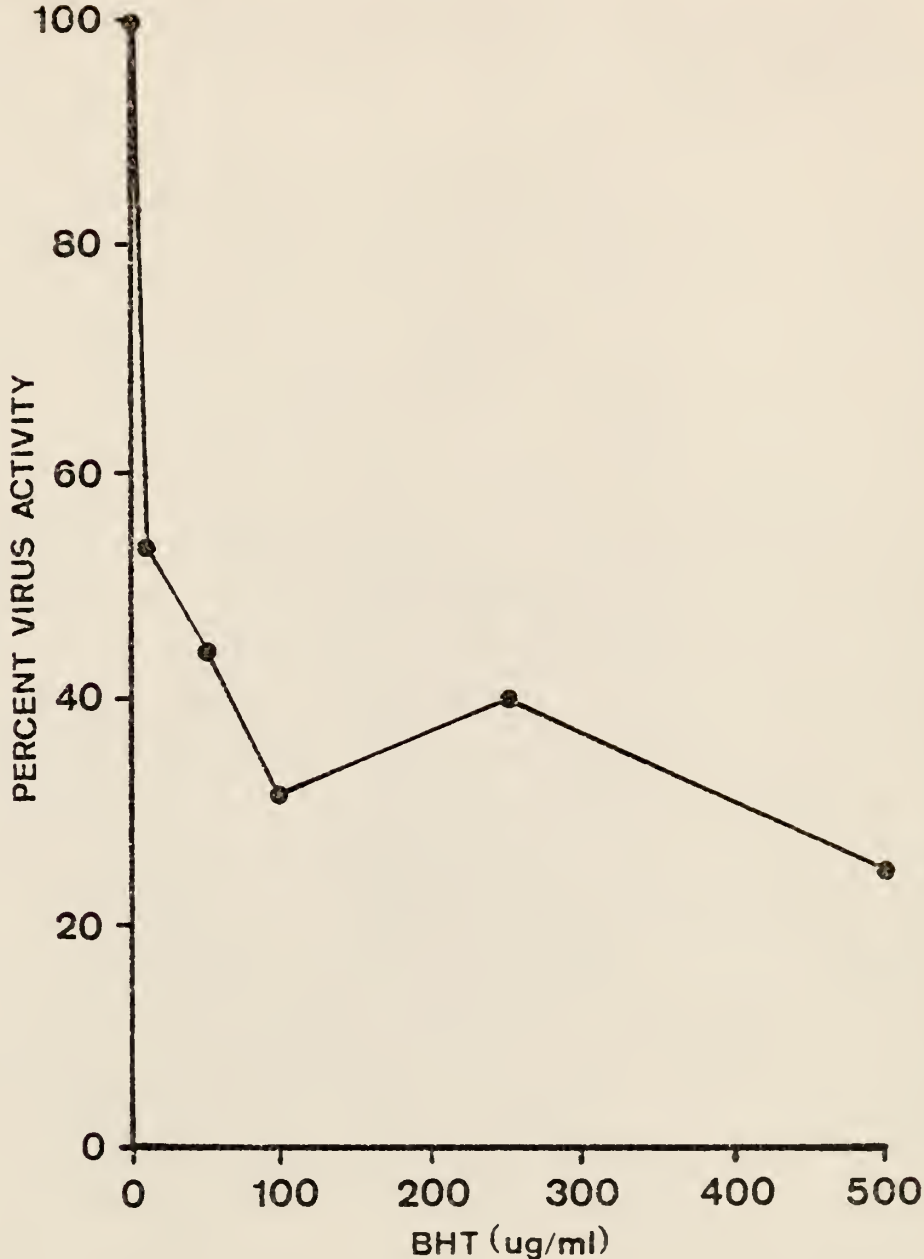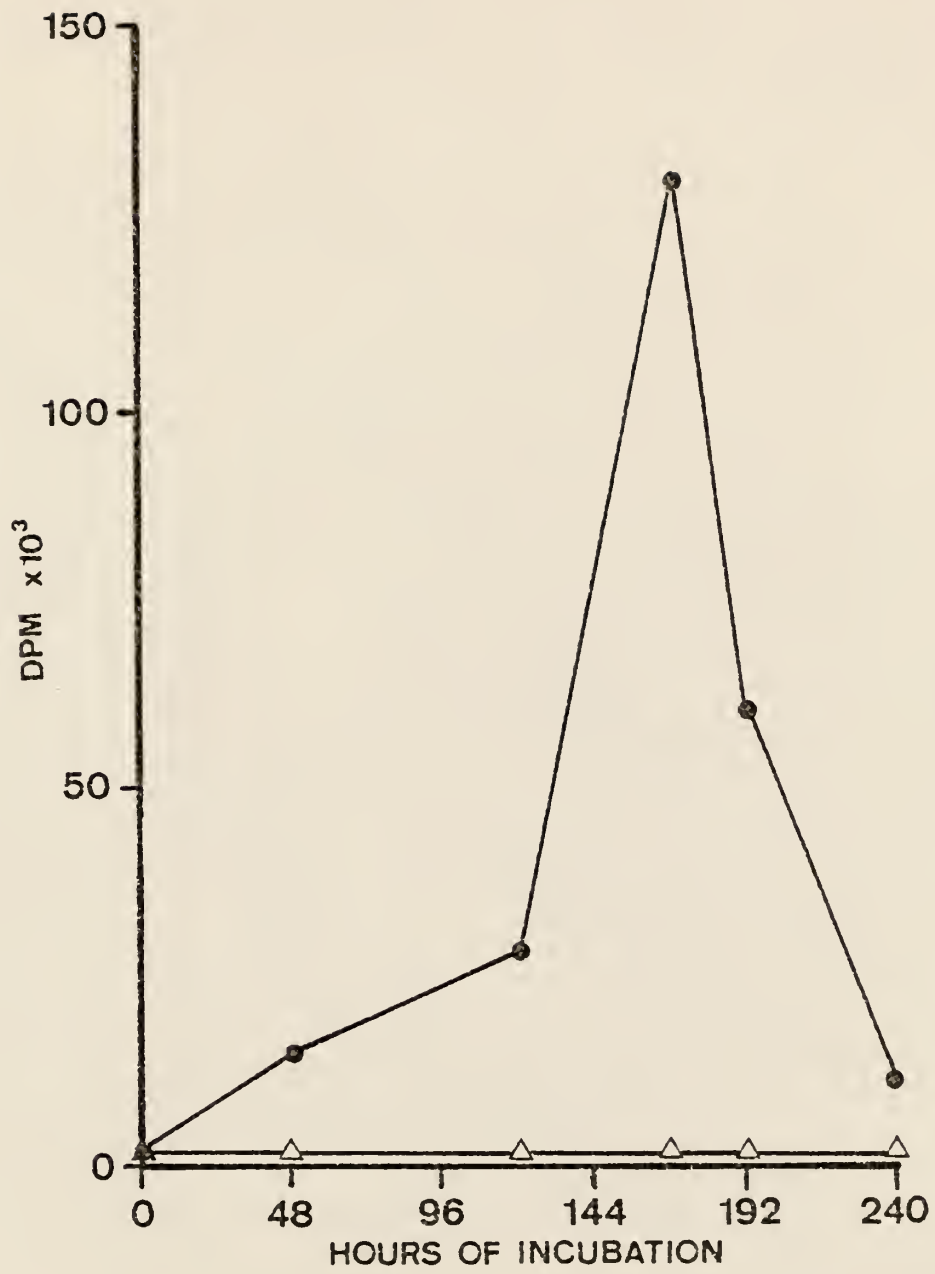percent inactivation for each concentration.

Figure 4. Time course of DNA synthesis: Whole blood cultures lacking or containing 5 ug/ml of PHA were incubated for varying time intervals up to 240 hours. Samples were labelled with ^{3}H-TdR 24 hours prior to harvest, processed and counted: ●——● With PHA; △——△ Without PHA.

MEAN RECTAL TEMPERATURES[A] FROM FERRETS INOCULATED WITH IBRV

		Control		Experimental	
		Sample Size	Mean (+S.D.)	Sample Size	Mean (+S.D.)
Inactivated Vaccine Day 1-65 Post- Vaccination	Pre:[B] Post:	10 126	102.1 (+0.2) 102.1 (+0.5)	10 158	101.9 (+0.3) 102.1 (+0.4)
MLV Vaccine (IN) Day 1-49 Post- Vaccination	Pre: Post:	1 7	102.0 101.4 (+0.5)	3 19	101.5 (÷0.4) 102.5 (÷0.4)
MLV Vaccine (IM) Day 1-49 Post- Vaccination	Pre: Post:	1 8	102.4 101.3 (+0.5)	3 22	102.0 (÷0.5) 101.8 (÷0.5)

[A]Normal Ferret Temperatures: 101-104° F (Pyle, 1940).

[B]Pre-Inoculation
Post-Inoculation

WEIGHTS[A] OF CONTROL FERRETS[B]
INOCULATED WITH FREUNDS INCOMPLETE ADJUVANT

Age (Weeks)	Weight Mean ± (S.D.)
13	0.85 (±0.03)
14	0.92 (±0.07)
15	1.0 (±0.15)
16	1.04 (±0.13)
17	0.99 (±0.14)
18	1.07 (±0.13)
21	1.04 (±0.18)
22	1.21 (±0.16)
23	1.15 (±0.13)
24	1.24 (±0.10)
28	1.42 (±0.11)
30	1.45 (±0.09)

A. Weights (kg)

B. Two castrated male ferrets also served as controls for the inactivated IBRV inoculated ferrets, but were of a different age.

WEIGHTS[A] OF FERRETS INOCULATED WITH MLV

Age (Weeks)	Nasalgen (IN)		Delvine[R] (IM)	
	Control	Experimental Mean (±S.D.)	Control	Experimental Mean (±S.D.)
16	0.91	0.94 (±0.08)	0.94	.90 (±0.11)
17	0.99	0.92 (±0.05)	0.91	0.92 (±0.09)
18[B]	0.94	0.91 (±0.0)	0.94	0.91 (±0.12)
21	0.97	1.17 (±0.09)	0.80	1.01 (±0.16)
22	1.02	1.16 (±0.06)	0.77	1.10 (±0.19)
23	1.02	1.33 (±0.05)	0.71	1.24 (±0.14)
24	1.05	1.36 (±0.05)	0.77	1.31 (±0.18)
26	1.08	1.49 (±0.08)	0.99	1.47 (±0.2)
28	-	-	1.19	1.51 (±0.19)
30	1.42	1.57 (±0.13)	1.36	1.51 (±0.18)

A. Weight (kg)

B. Ferrets were vaccinated with MLV at 20 weeks of age.

SECTION VII

DETAILED METHODOLOGY AND REAGENTS

Reagents

<u>Agar</u>: 2% purified agar dissolved in deionized water and
autoclaved for use. A mixture of 50% agar - 50% (2X)
MEM is used for plaquing.

<u>Antibiotics</u>:

Penicillin (1,000,000 units)

Streptomycin (1 g)

Kanamycin (50 mg) is dissolved in 100 ml of sterile
deionized water. One ml of PSK stock solution is
added to give a final concentration of: 100 units
penicillin, 1 mg streptomycin and 5 ng kanamycin/ml
media.

<u>Dimethyl Sulfoxide</u> $(CH_3)_2SO_4$:

For freezing cells, this is made to an appropriate
concentration (10%) and autoclaved 10 minutes at
15 minutes.

<u>DNase</u>: 2 mg/ml, stock. A final concentration of 10 ug/ml
is used.

<u>Fungizone</u>: 50 mg (one vial) plus 20 ml sterile deionized
water. Add 0.2 ml/l. media.

<u>Neutral Red</u>: A solution (0.015%) for plaquing is prepared
from powder with deionized water and dissolved in a
37° C water bath. Whatman #2 filter paper is used
for filtration. Sterilize by autoclaving and store
at 4° C, protected from light. 1.5 ml stock/100 ml
agar and media.

Phytohemagglutinin: 50 ug/ml in RPMI 1640 stock. A final
concentration of 5 ug/ml is used.

Phosphate-Buffered Saline (PBS):

Solution 1

$Na_2HPO_4 \cdot 7H_2O$ 134 g/l deionized H_2O

Solution 2

$NaH_2PO_4 \cdot H_2O$ 69.1 g/l deionized H_2O

Buffer Stock

$Na_2HPO_4 \cdot 7H_2O$ 386 ml Solution 1

$NaH_2PO_4 \cdot H_2O$ 114 ml Solution 2

Deionized Water   500 ml

pH 7.15

PBS:

80 ml buffer stock

17 g NaCl

1920 ml deionized water

pH 7.2

Autoclave at 15 lbs. for 15 minutes

Scintillation Fluid:

360 ml	Toluene
360 ml	Dioxane
80 g	Napthalene
216 ml	Absolute alcohol
4 g	Ominifluor

Reagents and Addresses

Absolute Alcohol
(analytical grade)

Agar   BBL, Cockeysville, MD

Cortisol   Calbiochem, Los Angeles, CA

Dimethyl Sulfoxide   Mallinckrodt Chemical Co.,
                                  St. Louis, MO

Dioxane   Scientific Products, IL

DNase (pancreatic)   Sigma Chemical Co., St. Louis,
                                  MO

EDTA   Mallinckrodt Chemical Co.,
                                  St. Louis, MO

Fetal Calf Serum   Kansas City Biological Co.,
                                  Kansas City, KS

Fungizone   E. R. Squibb and Sons, NY

Horse Serum   Kansas City Biological Co.,
                                  Kansas City, KS

Kanamycin (Kantrex)   Bristol Laboratories,
                                  Syracuse, NY

Ketamine Hydrochloride   Bristol Laboratories,
                                  Syracuse, NY

Medium 199   Grand Island Biological Co.,
                                  Grand Island, NY

Minimal Essential Media   Grand Island Biological Co.,
                                  Grand Island, NY

Napthalene (recrystalized)   Mallinckrodt Chemical Co.,
                                  St. Louis, MO

Neutral Red   Fisher Scientific Co.,
                                  Fairlawn, NJ

Omnifluor   New England Nuclear, Boston, MA

Penicillin G   E. R. Squibb and Sons, NY

Phytohemagglutinin	Sigma Chemical Co., St. Louis, MO
RPMI-1640	Grand Island Biological Co., Grand Island, NY
Streptomycin Sulfate	Eli Lily, Indianapolis, IN
Toluene	Mallinckrodt Chemical Co., St. Louis, MO
Trichloroacetic Acid	Mallinckrodt Chemical Co., St. Louis, MO
Tritiated Thymidine	New England Nuclear, Boston, MA
Trypsin	Difco, Detroit, MI

SECTION VIII

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IMMUNOLOGICAL RESPONSES IN FERRETS INOCULATED WITH
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by

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

The hematological and serum chemistry profiles and
the immunological responses of ferrets (<u>Mustela putorius</u>
<u>furo</u>) to Infectious bovine rhinotracheitis virus (IBRV) vac-
cines were studied.

Statistical analyses were performed on the hematologi-
cal and serum profiles of normal female, male and castrated
male ferrets. No appreciable differences in the mean values
were observed among the groups. Simple linear correlation
coefficients were calculated for these parameters with
respect to age or body weight. Significant age related
changes were found in the neutrophils, lymphocytes, sorbital
dehydrogenase, blood urea nitrogen, alkaline phosphatase,
serum glutamate pyruvate transaminase, albumin and glucose.
Significant weight related changes were associated with
sodium, carbon dioxide, calcium, phosphorus and glucose.

Ferrets inoculated with different IBRV vaccines
demonstrated significant differences in treatment means for
sorbital dehydrogenase, blood urea nitrogen, albumin,
creatinine, packed cell volume and bands. Statistical
significance was not found among the other hematological
and serum chemistry profiles.

Although all ferrets did not manifest clinical signs of
infection, IBRV was isolated three days post vaccination in
the nasal washings from one of the three ferrets inoculated
with modified live virus (MLV); however, virus was not

detected in subsequent samples. Low degrees of specific and non-specific virus neutralizing factors were detected by the plaque reduction method in the nasal washings only at the fourth week post IN inoculation with inactivated IBRV.

Serum neutralizing antibodies were demonstated in ferrets three weeks following an intramuscular (IM) injection of inactivated IBRV with Freund's Incomplete Adjuvant. and increased after a second IM inoculation. Complete virus neutralization was observed between the 12th and 14th week. The MLV vaccines (given IM or IN) elicited low levels of serum antibodies that persisted from the third to the seventh week post inoculation. The degree of cell mediated immune response was variable (stimulation index: 1.1-27.7) as observed by specific (UV-IBRV) lymphocyte stimulation and the incorporation of tritiated thymidine in whole blood cultures from ferrets inoculated with inactivated IBRV. Controls and MLV vaccinated ferrets did not demonstrate cell mediated immune responses to the IBRV antigen.

Phytohemagglutinin (PHA) at a concentration of 5 ug/ml demonstrated maximum stimulation of lymphocytes in ferret whole blood cultures. The optimal effect of PHA on DNA synthesis was observed between 72 and 168 hours after incubation and when the cultures were exposed to the label 12 hours prior to harvest.

Following an initial decline in viability at 48 hours, the number of viable lymphocytes in PHA supplemented cultures increased approximately 10 fold between 96 and 144 hours of incubation. Cultures lacking PHA or containing UV-IBRV demonstrated a time related decline in the viability of lymphocytes.

Cortisol at physiological concentrations inhibited the viability of PHA stimulated lymphocytes as indicated by an appreciable decrease in cell count and the incorporation of ^{3}H-TdR. Cortisol also depressed the cell mediated immune response in UV-IBRV stimulated cultures. The presence of increasing concentrations of cortisol (0-5,000 ng/ml) did not affect the adsorption of ^{3}H-IBRV to ferret leukocytes. This suggests that cortisol and IBRV did not compete for the same receptor sites.