1967

Characterization of receptors and lysosomes of cells susceptible to infectious bronchitis virus

Phil Dean Lukert
Iowa State University

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LUKERT, D.V.M., Phil Dean, 1931-
CHARACTERIZATION OF RECEPTORS AND LYSOSOMES
OF CELLS SUSCEPTIBLE TO INFECTIOUS BRONCHITIS
VIRUS.

Iowa State University, Ph.D., 1967
Microbiology

University Microfilms, Inc., Ann Arbor, Michigan
CHARACTERIZATION OF RECEPTORS AND LYSOSOMES OF CELLS SUSCEPTIBLE TO INFECTIONOUS BRONCHITIS VIRUS

by

Phil Dean Lukert, D.V.M.

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major Subject: Veterinary Microbiology

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1967
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INTRODUCTION

The virus-cell interaction between avian infectious bronchitis virus (IBV) and a number of susceptible cell types originating from various tissues of the chicken embryo is interesting in that each virus-cell system responds differently to infection with this virus. Previous work has shown that primary cells cultured from chicken embryo kidney, liver, lung, and whole embryos differ as to sensitivity, length of eclipse period, cytopathology, and distribution of antigen as detected by immunofluorescence (Lukert, 1965; Lukert, 1966b). These observations aroused an interest in determining some of the reasons for the varied responses of the various cell types to IBV.

Two aspects of IBV-cell interactions were selected for study. The first was that of attachment of virus to cells. A thorough study of this event might explain the previously observed differences in sensitivity of various cell types to IBV and would also serve to define the chemical nature of IBV receptor sites. The second aspect for study was the distribution and fate of lysosomes in infected cultures of cells from chicken embryo kidney and liver. It was thought that these cellular organelles might be implicated in susceptibility to IBV and may play a role in viral-induced cytopathic alterations. To elucidate differences in the above mentioned virus-cell interactions, cells from chicken embryo kidney and liver were
chosen for study because of their observed differences in both susceptibility to IBV and length of eclipse period. Information derived from a study of this nature will provide us with greater knowledge of the basic characteristics of IBV as well as a better understanding of the virus-cell relationship as it pertains to cell, tissue, and host susceptibility.
Virus Attachment and Virus Receptors

Attachment

Virus attachment\(^1\) to host cells is usually considered to be divided into 2 stages. The initial stage is probably a rather nonspecific but reversible stage requiring electrolyte and is electrostatic in nature (Levine and Sagik, 1956; Tolmach, 1957). The second stage of attachment is considered irreversible and appears to be quite specific (Tolmach, 1957; Hirst, 1965). In general, animal viruses appear to attach to cells less specifically and much more inefficiently than do bacterial viruses (Hirst, 1965). Studies on the attachment of animal viruses are complicated by the fact that not all viral particles can express themselves as infective units (Taylor and Graham, 1961; Joklik and Darnell, 1961) and the possibility of subsequent elution of attached virus as in the case of vaccinia virus (Smith and Sharp, 1961) and poliovirus (Joklik and Darnell, 1961; Fenwick and Cooper, 1962).

The effect of temperature on attachment has not been definitely resolved. The attachment of fowl plague and vac-

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\(^1\)The term "attachment" was selected in preference to "adsorption" in accordance with the view of Tolmach, 1957, as the term adsorption has the connotation of physicochemical nonspecificity while viral attachment is notably a specific reaction with the cell.
cinia viruses (Allison and Valentine, 1960), Newcastle disease virus (NDV) (Levine and Sagik, 1956) and poliovirus (Bachtold et al., 1957) was reported to occur independent of temperature. Youngner (1956) and Holland and McLaren (1959) found that poliovirus attachment was temperature dependent being favored by increased temperature. Philipson and Bengtsson (1962) observed that the initial attachment of poliovirus was independent of temperature while irreversible attachment was temperature dependent. Bachrach et al. (1957) showed that the plating efficiency of foot-and-mouth disease virus was much greater, about 10-fold, at 37° and 43° as compared to 29° and 26°.

The presence of electrolyte is essential for virus attachment as shown by Levine and Sagik (1956) for NDV. Bachtold et al. (1957) demonstrated that the divalent cations, Ca or Mg, in concentrations from 10⁻¹ to 10⁻⁵ molar were required for poliovirus attachment with 10⁻³ molar being optimal. Allison and Valentine (1960) found that fowl plague attached to chicken embryo cell monolayers independent of the valency of cations and surprisingly found attachment was depressed with high concentrations of divalent and trivalent cations. They postulated that the cations reduce the thickness of the layer of positively charged ions surrounding viruses and cells which normally carry a net negative charge, thus allowing the viruses to approach the cell sufficiently close so that short range attractive forces can bind them together.
The pH of the environment during attachment has a wide optimal range and has been shown to have little effect except at a pH of 4.0, where attachment is depressed for NDV and fowl plague virus (Levine and Sagik, 1956; Allison and Valentine, 1960). At pH 4 the carboxyl groups of virus and cells would be ionized and increase the net negative charge, thus reducing attachment while at pH 5.5 and above ionization of these groups are suppressed and attachment can occur.

The early findings that attachment is independent of temperature, enhanced by electrolyte, especially in the presence of cations (Allison and Valentine, 1960), and depressed at pH 4.0 has lead to the hypothesis that initial attachment is electrostatic. Allison and Valentine (1960) speculated that the main interacting groups were likely the amino groups of the virus and phosphate groups of the cell.

While the initial attachment of virus may be electrostatic in nature and somewhat nonspecific, the evidence is that the irreversible second stage is probably quite specific with definite receptor substances involved for each virus type. Hirst (1965) and Green (1966) pointed to the fact that very little is actually known about the mechanism of animal virus attachment and the receptors involved except in the case of myxoviruses which have mucoprotein receptors. Philipson (1963) indicated that even though the myxovirus receptors on erythrocytes have been rather well characterized, the nature of receptors on host cells are much more complex and less well defined.
Myxovirus receptors

The biochemical evidence establishing mucoproteins as the receptor substance for myxoviruses was first presented by Gottschalk (1958) when he showed that the influenza virus enzyme was a neuraminidase that split off neuraminic acid sidechains from mucoproteins. Using neuraminidase from Vibrio cholerae, Stone (1948a) showed that infection of chick embryos with a number of myxoviruses could be prevented. A similar finding (Stone, 1948b) was observed for influenza viruses in mice. Johnson et al. (1964) found a similar reduction of infectivity of influenza virus in chicken embryos using neuraminidase derived from Asian influenza virus, Vibrio cholerae, and Clostridium perfringens. They observed that it required more enzyme to protect against the PR-8 strain of influenza A virus than the Lee-B strain. Haff and Stewart (1965) found a 2.5- to 4.7-fold inhibition of plaques with influenza viruses when chicken embryo cell monolayers were treated with neuraminidase. In contrast, neuraminidase 800 times less concentrated than that required to alter infectivity modified receptors on erythrocytes. They concluded that the receptors on host cells were either not fully identical with those of erythrocytes or were refractory to neuraminidase treatment.

Mori et al. (1962) demonstrated that the receptors on erythrocytes for polyoma virus and influenza viruses were similar as they mutually excluded one another and influenza virus neuraminidase destroyed polyoma receptors on the erythro-
cytes. The erythrocyte receptors for both viruses could be removed by treatment with neuraminidase and periodate, however, these treatments, while destroying host-cell receptors for influenza, had no effect on polyoma host-cell receptors for infection. Ackermann et al. (1955) demonstrated that α-aminop-methoxyphenylmethane sulfonic acid (AMPS) impaired attachment of influenza virus to the chorioallantoic membrane. When the chorioallantoic membrane was first treated with neuraminidase and then AMPS, attachment of virus was completely prevented. The results of Haff and Stewart (1965) and Mori et al. (1962) and Ackermann et al. (1955) show the possible discrepancies encountered when trying to draw analogies between receptors on erythrocytes and host cells. Marcus et al. (1965) found that neuraminidase-treated HeLa cells and isolated nuclei failed to adsorb virus. They quantitated the molecules of N-acetylmuramidase on the surfaces of the cell and nuclei and found that both surfaces contained approximately $10^5$ molecule/μ2.

Further proof that myxovirus receptors are mucoproteins with an N-acetylmuramidase acid residue in the sugar moiety are the mucoprotein inhibitors of hemagglutination as reviewed by Gottschalk (1959). Mucoproteins with sialic acid residues other than N-acetylmuramidase acid are ineffective as inhibitors of hemagglutination.
Picornavirus receptors

While little is known about the biochemistry of receptors for picornaviruses, the need for specific receptors was irrevocably proven when Holland et al. (1959) showed that infectious ribonucleic acid extracted from poliovirus could infect naturally insusceptible nonprimate cells. McLaren et al. (1959) had earlier reported that poliovirus did not attach to insusceptible cells thus showing a lack of specific receptors for the virus. Holland and McLaren (1959) demonstrated that disrupted HeLa cells inactivated poliovirus irreversible and had the same kinetics as did irreversible attachment of virus to whole HeLa cells. Disrupted insusceptible cells did not inactivate poliovirus. Quersin-Thiry (1961) confirmed these findings and extended the study to NDV, WEE, and vaccinia viruses and found that these were also inactivated by susceptible disrupted cells. They found that NDV and WEE inactivation kinetic curves were interrupted by phases of reactivation. The NDV peaks of reactivation could be prevented by metaperiodate treatment, while the same treatment had no effect on poliovirus and WEE virus. Quersin-Thiry and Nihoul (1961) extended this study and found that disrupted HeLa cells contained three different inactivating substances. One substance combines with poliovirus, is heat labile, and is destroyed by trypsin and sonic waves. The second substance combines with WEE virus, is resistant to trypsin and sonic waves but is destroyed by lipase and heat. The third substance inactivates
NDV and Coxsackie B1 and B4 viruses. It is inactivated by trypsin and living influenza PR8 viruses, modified by metaperiodate treatment, and is heat stable. Both groups of workers (Holland and McLaren, 1959) (Quersin-Thiry and Nihoul, 1961) implicate receptor substances as being the inactivating substance and feel the receptors for poliovirus are lipoprotein in nature. The work of Quersin-Thiry and Nihoul (1961) however implicates receptors for Coxsackie B1 and B4 viruses as being similar to myxovirus receptors which are mucoprotein substances.

Holland (1961) examined the role that receptors might play in tissue tropisms of poliovirus. He found that cell-free homogenates of brain, spinal cord, and intestine of human fetuses and adult rhesus monkeys would bind virulent poliovirus, while homogenates of tissues which do not normally propagate poliovirus did not bind the virus. He also demonstrated that tissue cells, normally refractory to poliovirus in vivo and whose homogenates failed to bind virus, gained receptors upon cultivation in vitro and supported poliovirus replication. Six molar LiCl and 8M urea, hydrogen bond-disrupting agents, prevented virus binding to susceptible whole cells and cell homogenates. Several ECHO and Coxsackie viruses also showed correlation between virus tissue tropism and ability of tissue homogenates to bind virus in vitro. Holland (1962) also found that treatment of disrupted HeLa cell-poliovirus complexes with 6M LiCl and 8M urea would release infective virus, showing
That these agents not only prevented attachment but also could reverse attachment of virus.

While the consensus of opinion is that poliovirus does not attach to nonsusceptible cells, Warren and Cutchins (1957) showed that poliovirus was rapidly removed from the inoculum by bovine embryonic cell cultures but did not replicate in them. The idea that poliovirus only attaches and replicates in primate cells was challenged by the results of Sheffield and Churcher (1957), who grew poliovirus in an embryonic rabbit kidney (ERK) line, and Mascoli et al. (1959), who grew poliovirus in 2 different guinea pig spleen cell lines. Antiserum prepared against the ERK cell line was later shown by Brand and Syverton (1960) to hemagglutinate human but not rabbit erythrocytes indicating that the ERK line might have been contaminated with a human cell type. Hsuing and Melnick (1958) also demonstrated that poliovirus would attach to primate cells that were insusceptible.

The possible role of thiol groups in virus attachment has been shown by a number of workers. Much of the work pointing to thiol groups has been done with picornaviruses. Philipson and Choppin (1960) and Buckland (1960) independently found that the hemagglutinating capacity of ECHO and Coxsackie viruses were inactivated using the sulfhydryl compound p-hydroxymercuribenzoate (PHMB). φ Philipson and Choppin (1960) demonstrated

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φThis compound is referred to in most literature as p-chloromercuribenzoate but is now more correctly referred to as p-hydroxymercuribenzoate (Boyer, 1954).
that the reduced capacity to hemagglutinate erythrocytes was due to failure of virus attachment. This was shown by reactivating the PHMB treated virus with reduced glutathione after the first exposure to erythrocytes and finding that all the virus remained in the supernate.

Choppin and Philipson (1961) subsequently reported on the effect of PHMB on the infectivity of ECHO, Coxsackie, and poliovirus. They found all three virus types to be sensitive to PHMB but some were more sensitive to PHMB than others. They also showed that PHMB inactivated picornaviruses did not form a stable attachment to host cells. Allison et al. (1962) studied the effect of PHMB and iodoacetamide on the infectivity of a wide range of viruses. They showed that 6 of 9 picornavirus types were inactivated by these sulfhydryl compounds. The infectivity and hemagglutinating activity of simian enterovirus SV2 is also destroyed by PHMB (Heberling and Cheever, 1965).

Hirst (1961) and Philipson and Choppin (1962) reported that poliovirus was inactivated by polysulfides and this inactivation was reversed by cysteine, reduced glutathione and mercaptoethanol. Their results differed in that Hirst (1961) observed that poliovirus attachment to HeLa cells was not inhibited, while Philipson and Choppin (1962) found that attachment to HeLa cells was inhibited. Hirst (1961) indicated that virus could be reactivated with cysteine even after the virus had penetrated sufficiently to be insusceptible to anti-
The finding by Pohjanpelto (1959) that polio virus was stabilized against heat inactivation by cystine is also indicative of the importance of thiol and disulfide groups in the capsid structure of picornaviruses.

Although inactivation of picornaviruses with sulfhydryl reagents apparently inhibits virus attachment to cells, it is probably not true for other viruses so inactivated. Vaccinia, fowl plague, influenza A, and influenza B viruses inactivated with PHMB were found to attach normally to host cells and the hemagglutinating activity of the latter 3 was not altered (Buckland, 1960; Choppin and Philipson, 1961; and Allison, 1962).

Another group of picornaviruses, the encephalomyocarditis (EMC) viruses seem to have myxovirus type receptors for attachment, and infection of HeLa cells was prevented with neuraminidase (Kodza and Jungeblut, 1958). Buckland (1960) also showed that the hemagglutinating activity of these viruses was abolished with PHMB.

Receptors of other virus groups

Very little is known about the nature of receptors for groups of viruses other than the myxoviruses and picornaviruses. Since these two groups have been used as the model systems for studying virus attachment, the other virus groups have been studied very little and usually only in comparison.
with myxoviruses or picornaviruses.

While many of the adenoviruses hemagglutinate erythrocytes from a number of species, nothing is known of the nature of these receptors (Tamm and Eggers, 1965). Adenovirus type 7 hemagglutinating activity was prevented with PHMB but Adenovirus type 9 and SV17 were unaffected (Buckland, 1960). The infectivity of 2 adenoviruses tested was reduced by PHMB as shown by Allison et al. (1962).

Quersin-Thiry and Nihoul (1961) found that possible receptor substances of cellular extracts specific for WEE virus, an arbovirus, was susceptible to lipase but resisted trypsin and sonic waves. This plus the fact that inhibitors of arbovirus hemagglutination found in serums is removed by ether extraction (Hirst, 1965) leads to the conclusion that lipid substances may act as receptors for arboviruses. A number of arboviruses were treated with PHMB by Allison et al. (1962) and they found the arbovirus A group resisted the treatment while those of the B group and some miscellaneous arboviruses were inactivated.

The reovirus receptor substances seem to be varied. Reovirus types 1, 2, and 3 all agglutinate human erythrocytes but the receptors are unaltered by neuraminidase treatment (Tamm and Eggers, 1965). Reovirus type 3, however, hemagglutinates bovine erythrocytes and these receptors are destroyed by neuraminidase (Gomatos and Tamm, 1962). The hemagglutinating activity and infectivity of all 3 serotypes of reovirus
are inactivated by PHMB (Buckland, 1960; Allison et al., 1962; Gomatos and Tamm, 1962; and Lerner et al., 1963).

As mentioned under the discussion of myxovirus receptors, the polyoma virus erythrocyte receptors are similar to those of the myxoviruses and mutually exclude one another (Hartley et al., 1959; Mori et al., 1962). The receptors on host cells, however, were also shown to resist neuraminidase, thus, they are not identical to those on the erythrocyte. The hemagglutinating activity and the infectivity of polyoma virus is unaltered by sulfhydryl compounds (Tamm and Eggers, 1965). Another papovavirus, Shope papilloma virus, was also reported to be resistant to PHMB (Schmidt, 1957).

Rous sarcoma virus is inactivated by PHMB (Schmidt, 1957) and iodoacetamide (Allison et al., 1962). As this property may or may not implicate thiol groups as a functional part of the virus receptor more work must be done to determine how the sulfhydryl compounds inactivate the virus.

Vaccinia virus is inactivated by mercury (Kaplan, 1959) and PHMB (Allison et al., 1962) and can be reactivated by thiol compounds indicating that thiol groups are probably involved in the inactivating process. Allison (1962) however showed that purified preparations of vaccinia virus inactivated by PHMB attached to chicken embryo cell monolayers normally. This indicates that the inactivation is probably not due to alteration of receptor sites on the virus.
Attachment of avian infectious bronchitis virus (IBV)

Cunningham and Spring (1965) and Lukert (1965) studied the effect of time on IBV attachment to primary chicken embryo kidney cell monolayers. Both reports showed that maximum plaque counts were achieved after a 90-minute attachment period. In both instances, the conditions were essentially the same except the temperature was 37° in the former study and 25° in the latter study. Powers (1965) found that IBV was inactivated by PHMB but did not determine if attachment of the virus was hindered by this reagent. Biswal et al. (1966) demonstrated that both the hemagglutination of erythrocytes by trypsin-treated IBV and the infectivity of IBV in chicken embryos were inhibited by neuraminidase treatment.

Lysosomes with Relation to Viral Infection

Structure and function of lysosomes

de Duve et al. (1955) first described a cell fraction that contained a number of hydrolytic enzymes, all operating at an optimum pH of 5. These workers called this cell fraction "lysosomes" before the morphologists had observed their structure. Novikoff et al. (1956) subsequently examined this cell fraction with the electron microscope and found dense bodies surrounded by a single membrane along with a high proportion of mitochondria. He suspected that the dense bodies were the structures that contained the acid hydrolases described by de Duve et al. (1955).
Since this initial observation a large number of acid hydrolases have been associated with the lysosome. The sizes range from 0.2μ to 0.8μ and they seem to be involved in digestion of material taken into the cell, the removal of intracellular material, and autolysis of dead cells. The varied functional aspects of these particles account for the polymorphism shown by the particles in different cells (de Robertis, E.D.P. et al., 1965). These structures are characterized by the simultaneous release of their enzymes in a soluble, fully active form following injury to the membrane by a number of agents. Acid phosphatase activity is the usual marker enzyme in most cytochemical studies of the lysosome (de Duve, C., 1959).

Lysosomes and viral induced cytopathology

Allison and Sandelin (1963) were the first to study lysosomes in virus-infected cells. They measured the activity of 5 lysosomal enzymes in lysosomal and supernatant fractions of mouse liver and monkey kidney cells infected with mouse hepatitis virus and vaccinia virus, respectively. There was a release of these enzymes into the supernatant fraction of infected cells while no release occurred in uninfected cells. They further suggested that release of the lysosomal enzyme may contribute to the cytopathic effect caused by these viruses.

Allison and Mallucci (1965), using a histochemical technique for acid phosphatase and esterase activity, found that
infection of cells with 5 cytopathogenic viruses; influenza A, NDV, mouse hepatitis, Adenovirus type 5, and vaccinia; caused activation and release of lysosomal enzymes. They described 3 stages of activation. The first was a reversible stage characterized by increased permeability of the lysosomal membrane without release of enzymes. In the second stage, which was irreversible, there was release of lysosomal enzymes into the cytoplasm. In the third stage, not usually seen in cell cultures, the lysosomal enzymes were no longer detectable, either being released from or inactivated in the cells. They postulated that the lysosomal enzymes could be responsible for cytopathic effects, polykaryocytosis, and malignant cell transformation. An in vivo study reported by Allison and Sandelin (1963) demonstrated an increased acid phosphatase activity in liver foci infected with mouse hepatitis virus. This enzyme alteration preceded the appearance of histologically demonstrable liver cell degeneration. Mallucci and Allison (1965) then compared a noncytopathogenic virus, the MEL strain of influenza A, with a cytopathogenic virus, fowl plague, and found no lysosomal enzyme release with MEL strain virus and a release of these enzymes within 6 hours of infection with fowl plague virus.

Beeuwkes (1964), using histochemical methods, studied the distribution of a number of enzymes in virus infected monkey kidney cells. The viruses used were poliovirus type 1, ECHO 4 and 9, and Coxsackie B3. One of the enzymes he chose was
acid phosphatase, a lysosomal enzyme. He was unable to detect any differences between the acid phosphatase activity of infected and uninfected cells with any of the viruses. Wolff and Bubel (1964), using poliovirus type 1, vesicular stomatitis virus (VSV) and vaccinia virus found that poliovirus type 1 infection produced a release of lysosomal enzymes while the other two cytopathogenic viruses did not. They suggested that lysosomal enzymes were not released in response to all the types of cytopathogenic virus infections. Flanagan (1966) supported these findings using KB cells infected with poliovirus and herpes simplex virus. He found that 5 hydrolytic lysosomal enzymes were released 6 hours after poliovirus infection but no such release was observed with herpes simplex virus. McAuslan (1965) studied the deoxyribonuclease (DNAase) activity in herpesvirus, adenovirus type 2, and vaccinia virus infected cells. In the case of the first 2 viruses, no increase of acid DNAase was seen but there was an increase of this enzyme in vaccinia infected cells. The increase in acid DNAase could be prevented by inhibiting protein synthesis with puromycin and actinomycin D, indicating that this increase was due to release of existing acid DNAase.

**Lysosomes with relation to viral penetration and uncoating**

Joklik (1964) and Dales and Kajioka (1964) studied, by electron microscopy, the penetration and uncoating of vaccinia virus by cells. They both found that the virus was taken into
phagocytic vacuoles after initial attachment and there the virus was reduced to viral cores by removal of its viral phospholipid and protein coat. This was followed by a rapid transfer of the viral core to the cytoplasmic matrix where there was release of the naked viral DNA. Joklik (1964) found that in the second stage release of naked viral DNA could be prevented by inhibiting cellular protein synthesis. This indicates that new enzymes are probably induced by some viral protein to perform this task. Both groups of investigators found acid phosphatase activity associated with the virus containing phagocytic vacuoles. Dales and Kajioka (1964) felt that lysosomal enzymes were not involved in the active uncoating of virus but might perhaps be responsible for the slow degradation of heat inactivated virus and virus neutralized with antibody. These inactivated particles were shown to be phagocytosed but not uncoated normally.

Dales and Gomatos (1965) showed that reovirus uncoating differed from that of vaccinia virus. After the virus became incorporated into the phagocytic vacuole it then became packed into dense, membrane bound inclusions, possibly lysosomes, where they were digested and the RNA probably freed into the cytoplasmic matrix. They felt that in the case of reovirus, with its RNAase resistant double stranded RNA, the proteolytic enzymes of the lysosome might play a role in the uncoating process.
Mallucci (1966) treated mouse macrophage cultures with chloroquine, a stabilizer of lysosomal membranes, and found that these cells produced a much lower yield of mouse hepatitis virus than untreated cells. He felt that variations in enzymes escaping from the lysosomes were responsible for differences in virus uncoating and consequent virus yield. David-Ferreira and Manaker (1965) reported that mouse hepatitis virus, after being phagocytosed, was observed in dense bodies thought to be lysosomes. These findings were similar to those reported for vaccinia virus (Dales and Kajioka, 1964).
MATERIALS AND METHODS

Viruses

Infectious bronchitis virus

The Beaudette chicken embryo-adapted strain of IBV was used in this study and was kindly supplied by Dr. C. H. Cunningham. After receipt of this virus it was serially passed 33 times in chicken embryo kidney (CEK) cells. This 33rd passage was frozen and maintained at -60° and was used as stock virus throughout the study. Under the usual conditions of virus titration in CEK cells, this stock contained approximately $10^{7.5}$ plaque-forming units (PFU)/ml.

Newcastle disease virus

The B-1 strain of NDV was kindly supplied by Dr. W. A. Boney. The virus was then serially passed in CEK cells and the 3rd passage used as stock for the entire study. The virus stock was maintained at -60° and under the usual condition of virus titration in CEK cells contained approximately $10^{6.8}$ PFU/ml.

---

1 Dr. C. H. Cunningham, Michigan State University, East Lansing, Michigan.

2 All temperatures will be in degrees centigrade.

3 Dr. W. A. Boney, National Animal Disease Laboratory, Ames, Iowa.
Cell Culture Media and Solutions

Cultural medium

Medium 199 was used for the growth and maintenance of cell cultures used in this study. The complete growth medium was prepared by combining the following to make 1 liter of medium.

- Medium 199\(^1\) 800 ml
- Tryptose phosphate broth (TPB)\(^2\) 100 ml
- Calf serum 80 ml
- 2.8\% NaHCO\(_3\) 20 ml

Serum-free maintenance medium was prepared by substituting an additional amount of Medium 199 in place of the calf serum. All media contained 100 units penicillin G potassium, 100 \(\mu\)g dihydrostreptomycin, and 50 units Mycostatin per ml.\(^3\)

Phosphate buffered saline (PBS)

The following solutions were combined to prepare PBS:

**Solution A:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>16.0 gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4 gm</td>
</tr>
<tr>
<td>(\text{Na}_2\text{HPO}_4)</td>
<td>2.3 gm</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>0.4 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1500 ml</td>
</tr>
</tbody>
</table>

\(^1\)Medium 199, Hyland Laboratories, Los Angeles, California.

\(^2\)Tryptose Phosphate Broth, Difco Laboratories, Detroit, Michigan.

\(^3\)Antibiotics, E. H. Squibb & Sons, New York, N. Y.
Solution B: MgCl₂·6H₂O 0.2 gm
Distilled water 250 ml

Solution C: CaCl₂ 0.2 gm
Distilled water 250 ml

All chemicals used were analytical reagent grade. After the three solutions were made up they were combined and sterilized by filtration through a Millipore¹ filter pad with a pore size of 0.33 μ. The sterilized PBS was distributed into 1 liter volumes and stored at 4°. The final pH of this solution was 7.4 to 7.5.

Trypsin

A 0.25% trypsin solution used for dispersing cells was prepared by dissolving 2.5 gm of 1-300 trypsin² in 1 liter of PBS without Ca and Mg. This solution was filter sterilized with a 0.33 μ Millipore filter pad.

Agar-overlay medium

Agar-overlay medium for plaque assays was prepared as follows:

2% agar³ in distilled water 100 ml
2 x concentrated medium 199 100 ml

¹Millipore Filter Corporation, Bedford, Massachusetts.
²1-300 trypsin, Nutrition Biochemical Corp., Cleveland, Ohio.
Tryptose Phosphate Broth (TPB) 30 ml
2.8% NaHCO₃ 5.2 ml
Calf serum 6 ml

The Medium 199, TPB, NaHCO₃, and serum were mixed and put into a 43° water bath. The agar was melted at 100° and then put into the 43° water bath. When both solutions had equilibrated to 43°, they were mixed and the medium was ready to be distributed onto the cell culture monolayers.

Preparation of Cell Cultures

Chicken embryo kidney (CEK) cell cultures

Monolayers of CEK cells were prepared from the kidneys of 18-day-old chicken embryos. The kidneys were minced and washed in PBS. The cells were dispersed with trypsin with trypsinization being carried out at ambient temperature for a period of 60 minutes. Approximately 3 to 4 ml of trypsin were used per pair of kidneys. A sterile Teflon-covered magnetic bar and magnetic stirrer were used to agitate the tissue-trypsin mixture for facilitation of the digestive process. Cells were washed free of trypsin by centrifuging at 224 x g at ambient temperature for 3 to 5 minutes then decanting and resuspending the cells in PBS. After 2 cycles of centrifugation with PBS, the supernatant fluid was decanted and the packed cells resuspended in cultural growth medium. A ratio of 1 ml of packed cells to 250 ml of medium was used for implanting cultures. Five ml of the cell suspension was implanted on 60 x-15 mm plastic
tissue culture dishes. The cultures were incubated at 37° in an atmosphere of 3% CO₂ and air at 85% relative humidity. After 24 hours, the cell sheets were 85% to 100% confluent and were ready for use. If the cell cultures were not used at 24 hours, they could be held at 4° for as long as 24 hours. This prevented cultures from getting overly dense and fibroblastic in appearance. Three to 4 hours before the use of refrigerated cultures they were returned to the usual 37° incubation. All cultures were routinely washed once or twice with PBS to remove unattached cells and cell debris before being used. Upon microscopic examination, the CEK cultures contained many islands of cuboidal epithelial-like cells surrounded by swirls of spindle-shaped fibroblastic cells.

Chicken embryo liver (CELi) cell cultures

Monolayers of CELi cells were prepared using either 15- or 16-day-old embryos. The cultures were prepared and handled the same as CEK cultures except they were implanted at a ratio of 1 ml of packed cells to 200 ml growth medium. At 24 hours the cell sheets were 80% to 100% confluent and were ready for use. Microscopic examination of CELi cultures revealed hexagonal cells which resembled liver parenchymal cells, while few fibroblastic cells were in evidence. Cultures of CELi cells

1Falcon Plastics, 5500 West 83rd Street, Los Angeles, California.
could be used either at 24 or 48 hours without loss of epithelial appearance or growth of fibroblastic cells.

Whole embryo chicken embryo fibroblast (WCEF1) cell cultures

Cultures of CEF1 cells were prepared using 9-day-old embryos. The embryos were decapitated and the appendages removed. The remainder of the embryo was minced, trypsinized, and washed similar to the CEK cells. The cultures were implanted at a ratio of 1 ml packed cells to 300 ml growth medium. At 24 hours these cultures were 100% confluent and ready for use. These cultures contained predominantly spindle-shape fibroblastic cells with many small islands of epithelial cells throughout the culture. For some of the experiments the above procedure was modified by removing the abdominal viscera from the embryos. The resulting cultures contained practically none of the islands of epithelial cells and were almost entirely of the spindle-shaped fibroblastic type cells. These culture types will be referred to as chicken embryo fibroblast (CEF1) cells.

PK-15 swine kidney cell line

This cell line was grown and maintained in medium 199 with 0.15 gm of L-glutamine/liter added just before use.

1 Supplied by hog cholera project, NADL, Ames, Iowa.
2 L-glutamine, Difco Laboratories, Detroit, Michigan.
The medium for growth contained 5% calf serum. Cells were serially passed in 250 ml plastic tissue culture bottles.\(^1\) Trypsin (0.25%) was used to disperse the cells and the cells from 1 bottle were implanted into 5 bottles for routine passage. For use in experiments cells from 1 bottle were implanted onto 10 of the usual 60 x 15 mm plastic tissue culture dishes. Cultures were usually confluent and ready for use in 3 days.

**Plaque assays**

Virus was routinely diluted in serum-free maintenance medium 199. Monolayers of CEK cells were inoculated with 0.5 ml of the selected dilutions of IBV, unless otherwise stated. The temperature and time allowed for attachment will be described with each experimental procedure. After the attachment period the inoculum was removed and the monolayers overlayed with 5 ml of the agar medium. The plates were incubated under the usual temperature and atmospheric conditions. At 44 to 48 hours postinoculation the plates were removed and stained with a solution of neutral red (0.01% neutral red in 0.15M NaCl) by allowing the stain to diffuse through the agar for 3 to 4 hours and then removing the stain solution. The plaques were then readily visible and could be easily counted. NDV plaques were enumerated at 72 hours.

\(^{1}\)Falcon Plastics, Los Angeles, California.
Preparation of Fluorescent Antibody

Anti-IBV serum

Five 11-day-old chickens were exposed to an aerosol of 0.5 ml of Massachusetts strain\(^1\) of IBV with a DeVilbiss No. 40 nebulizer. Three weeks following recovery from signs of infectious bronchitis, the chickens were given 1.0 ml IV injections of virus each week for 4 weeks. The virus suspension used contained approximately $10^8.5$ chicken embryo infecting doses per ml. Two weeks following the last injection the birds were exsanguinated. The whole blood was allowed to clot in petri dishes and the serum collected. The serum was clarified by centrifugation for 30 minutes at 1400 x g and stored at -20\(^{\circ}\) until used.

Serum fractionation

Globulins were obtained from the hyperimmune chicken serum by dropwise addition of 1 volume of a saturated solution of ammonium sulfate at room temperature to 2 volumes of the serum at 4\(^{\circ}\). The serum was kept in an ice bath and constantly agitated with a magnetic stirrer during the addition of the ammonium sulfate. Globulins were then sedimented by centrifugation for 15 minutes at 2000 x g. The supernatant fluid was carefully decanted from the sedimented globulins and

\(^1\)Obtained from Dr. C. H. Cunningham, Michigan State University, East Lansing, Michigan.
the precipitate restored to the original serum volume by adding cold 0.85% NaCl solution.

This fractionation procedure was repeated two additional times but on the last time the precipitate was only restored to 1/2 the original serum volume. The redissolved globulins were then freed of residual ammonium sulfate by dialysis against 0.85% NaCl (100 volumes saline/volume globulin solution). Dialysis tubing\(^1\), average pore diameter of 4.8 m\(\mu\), was used for the dialyzing process. To increase agitation of the fluid outside the dialysis tubing, a magnetic stirrer was used. The sample was dialyzed for 24 hours at 4\(^\circ\)C during which time the saline was changed three times. Insoluble material present in the globulin solution after the dialysis was removed by centrifugation for 15 minutes at 2000 \(x\) g.

**Protein determination**

The concentration of protein contained in the globulin fraction was determined by the biuret spectrophotometric method (Gornall et al., 1949) at a wavelength of 540 m\(\mu\) utilizing a B&L Spectronic 20 spectrophotometer.\(^2\) Two ml of a 1:2 and 1:4 dilution of the globulin solution in 0.85% NaCl was mixed with 8 ml of the biuret reagent and incubated for 30 minutes at ambient temperature before determining the optical density.

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\(^1\)Visking Company.

\(^2\)Bausch and Lomb, Incorporated, Rochester, New York.
A blank was prepared with 2 ml 0.85% NaCl and 8 ml of the biuret reagent. The optical density of the unknown was compared to that of known quantities of purified bovine albumin\(^1\) for which a standard curve had previously been determined.

**Conjugation of globulins**

The protein concentration of the globulin solution was adjusted to 10 mg/ml by dilution with 0.85% NaCl. A carbonate-bicarbonate buffer, pH 9, was made up by adding 4.4 ml of a solution of Na\(_2\)CO\(_3\) (5.3 gm/100 ml water) to 100 ml of a solution of NaHCO\(_3\) (4.2 gm/100 ml water). The pH was checked on a meter and it was sometimes necessary to add some additional Na\(_2\)CO\(_3\) solution to arrive at the proper pH. Twenty-five thousandths mg of fluorescein isothiocyanate isomer B\(^2\) (FITC) mg of protein was dissolved in a sufficient volume of the carbonate-bicarbonate buffer to comprise 10% of the combined volume of the reactants. The FITC solution was slowly added to the globulin solution and the mixture allowed to react for 16-20 hours while being constantly stirred at 4\(^\circ\) using a magnetic stirrer (Cherry et al., 1960).

**Gel filtration of conjugated globulin**

Separation of free FITC from the FITC-globulin complex was accomplished by gravity passage of the mixture through a

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\(^1\)Armour Pharmaceutical Co., Kankakee, Illinois

\(^2\)Baltimore Biological Laboratory, Baltimore, Maryland
Sephadex gel column. The Sephadex (G-25, coarse) was washed with PBS and maintained as a stock preparation 1:4 in PBS. A column was poured measuring 2 by 20 cm and was used for the separation of 25-30 ml volumes of the conjugated globulin solution. After the gel had settled in the column, the fluid level was adjusted to the top surface of the column and the sample carefully added. When the last of the sample entered the gel, more PBS was carefully added to the column in a sufficient volume to elute the FITC-globulin complex. The unconjugated FITC was left in the upper portion of the column. Progress of the conjugated globulin through was readily followed by its yellow-brown color.

**Adsorption of FITC conjugated globulin**

Portions of the FITC conjugated antibody preparation responsible for nonspecific staining were essentially removed by adsorption with 20-30 mg of rabbit liver powder (RLP) /mg of protein. A slurry of the liver powder in PBS was prepared by adding 2.5 ml PBS for each gm of RLP. This allowed for hydration of the RLP, thus preventing a marked reduction in the volume of the conjugated antibody. The adsorption was performed for 16-20 hours at 4° with constant stirring with a magnetic stirrer. The bulk of the RLP was removed from the conjugated antibody solution by centrifugation at 2,000 x g

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1Pharmacia, Ltd., Uppsala, Sweden.
2Kindly supplied by Dr. W. L. Mengeling, NADL, Ames, Iowa.
for 30 minutes. The remaining RLP was sedimented by centrifugation at 78,000 x g for 1 hour using a #30 rotor in a Spinco Model L ultracentrifuge. After clarification, the fluorescent antibody solution was distributed into 1 dram screw-cap vials in aliquots of 0.5 ml. These were stored at -20° until needed. These aliquots were diluted 1:4 with PBS before use without loss of intensity of bright specific staining.

Staining and examination of cell cultures

For fluorescent antibody studies and other histochemical techniques, coverslips were added to the petri dishes before implanting the cells. At prescribed times following infection of the cultures with virus, the cells on coverslips were removed and rinsed in PBS. The cells were then fixed in acetone at ambient temperature for 3 to 5 minutes. After fixation, the coverslips were air dried and then stained with fluorescent antibody. The cells were kept in a humidity chamber at 37° for 30-45 minutes while staining. They were then washed in running water, dried, and mounted in PBS-glycerine (1:1) on a microscope slide. The cells were examined for specific fluorescence using a Leitz ortholux microscope equipped with a dark-field condenser, BG-12 primary filter, OG-1 barrier filter and illuminated by an Osram HBO-200 mercury vapor burner.

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1Beckman Instruments, Inc., Spinco Division, Palo Alto, California.

2E. Leitz, 468 Park Avenue South, New York, New York.
Attachment of IBV to Cell Monolayers

Effect of temperature, time and pH on attachment

Since two previous studies had shown maximum attachment was achieved in 90 minutes at both ambient temperature and 37°C (Cunningham and Spring, 1965; Lukert, 1965), it was felt that the effect of temperature on IBV attachment should be examined more carefully.

To determine the effect of temperature on attachment, a simple experiment was performed in which 2 dilutions of the virus were adsorbed onto monolayers at 3 different temperatures, i.e., 4°C, ambient, and 37°C. Triplicate counts were made for each temperature and dilution. Each plate was inoculated with 0.5 ml of either a 10^{-4.3} or 10^{-5} dilution of virus and a 90-minute attachment period was allowed. This experiment was repeated with the exception that the 4°C attachment temperature was omitted and 5 replicate plates instead of 3 were inoculated for each temperature.

Since both previous reports had limited their longest attachment period to 2 hours, 2 experiments were conducted using extended periods of attachment up to 4 hours. In the first experiment 24 plates were inoculated with 0.5 ml of a 10^{-5.6} dilution of virus and allowed to attach at 37°C. At 30-minute intervals 3 plates were removed from the incubator, the inoculum removed, and then overlayed with agar medium. This experiment was repeated using 5 plates per time interval rather than 3.
The possibility that pH as well as temperature might influence the attachment of virus was considered. The maintenance medium 199 used as virus diluent having a carbonate-bicarbonate buffer increases in pH when standing at ambient temperature due to loss of CO₂ to the air. Conversely, the pH decreases when the cell-virus attachment was allowed to progress at 37° in an atmosphere of 3.0% CO₂ and air. A phosphate buffer and a glycine buffer were made up at pH 6, 7, 8, and 9 and used as diluent for the virus. These buffers were not influenced greatly by the either addition or loss of CO₂.

Standard phosphate buffers were made by first preparing stock solutions of M/15 Na₂HPO₄ and M/15 NaH₂PO₄. The pH 6 buffer was made by adding 1 part Na₂HPO₄ to 9 parts NaH₂PO₄ and the pH was checked with a meter. Minor pH adjustments were made by addition of either of the appropriate stock solutions. For pH's 7, 8, and 9, the ratio of Na₂HPO₄ to NaH₂PO₄ was 6:4, 9.5:0.5, and 9.9:0.1, respectively.

Sorensen's glycine buffers were made by first preparing a stock solution containing 7.505 gm glycine and 5.85 gm NaCl per liter of distilled water. The pH of this stock solution was adjusted by the addition of either 0.1N HCl or 0.1N NaOH and the pH determined on a meter. Solutions of the glycine buffer were made at pH's 6, 7, 8 and 9.

The effect of pH on IBV attachment was determined by placing 0.5 ml of a 10⁻⁵ dilution of virus in each buffer at pH's 6, 7, 8, and 9 onto 4 replicate CEK cell cultures. A
90-minute attachment at 37° was allowed, the inoculum removed and the cells overlayed with agar medium.

**Efficiency of virus attachment**

Since the previous studies on IBV attachment had disregarded unattached virus remaining in the inoculum, experiments were designed to assay for this unattached virus. A preliminary experiment showed that a great deal of infective virus remained unattached after 90 minutes. A series of experiments were designed to determine the efficiency of IBV attachment by performing successive 90-minute attachments on monolayers. In the first 2 experiments 4 successive 90-minute attachment periods were carried out in the following manner:

1. Two sets of plates were inoculated, one with 0.5 ml of a 10⁻³ and the other with 0.5 ml of a 10⁻⁴ dilution of stock virus. The virus was allowed to attach to the cells for 90 minutes at ambient temperature.

2. The inocula from each set of plates were removed and pooled separately. A sample from each pool was retained at 4° while the remainder was reinoculated, in 0.5 ml amounts, onto another set of plates and a second attachment period of 90 minutes at ambient temperature was allowed.

3. After 3 additional cycles of attachment, those samples that had been retained from each successive attachment period were assayed.
The second series of experiments were conducted in a manner similar to the first with the exception that only 3 successive attachment periods were accomplished. In these experiments, successive attachment was compared at 3 temperatures, at 37\(^{\circ}\), ambient, and 4\(^{\circ}\). A \(10^{-4.3}\) dilution of the virus was used as inoculum in the first experiment and a \(10^{-5}\) dilution used in the second. As a control to determine the degree of inactivation of unattached virus at 37\(^{\circ}\), the following experiment was run. Five plates without cells were inoculated with 0.5 ml of a \(10^{-5}\) dilution of virus and held at 37\(^{\circ}\) for 4.5 hours, which is equivalent to the time involved in 3 successive attachment periods. The inocula were then removed and assayed for surviving virus. Similarly, virus held at 4\(^{\circ}\) for 4.5 hours was assayed.

**Comparison of virus attachment to different cell types**

The following experiment was designed to determine if one of the reasons for the differences in sensitivity observed with various cell types might be attributed to differences in virus attachment. Five cell types were chosen for this study. Four cell types were derived from chicken embryo, and were, in order of their sensitivity, the CEK, CELi, WCEF1, and CEFi cell types. The fifth cell type was a nonsusceptible cell type, the PK-15 cell line. Three successive 90-minute attachment periods were conducted at 37\(^{\circ}\) with each of these cell types. The dilution of the stock virus was \(10^{-5}\) and samples
from each successive attachment period were inoculated onto either 3 or 4 CEK cell cultures to determine the amount of unattached virus. This experiment was repeated 2 times.

**Attachment interference**

From the results of the previous experiments, the attachment of IBV appears to cease after 90 minutes while a large amount of infective virus remains unattached. A series of experiments were run to determine if either some mechanism of interference was present or was being established in cell cultures that would limit further viral attachment. The first series of experiments was conducted to determine how efficiently a superinfecting dose of virus would attach to monolayer and they were conducted as follows:

1. Monolayers of CEK cells were infected with an appropriate dilution of the stock virus and a 90-minute attachment period allowed.

2. The inoculum was removed, the plates washed once with PBS, and half of them were reinoculated with the same dilution of virus while the other half were covered with 0.5 ml of diluent.

3. After a second 90-minute attachment period, the inoculum was removed and the plates overlayed with agar medium.

In one series of experiments, the attachment period was carried out at ambient temperature and in the other series at 37°.
The results of the above series of experiments indicated that there was an interference present at 90 minutes which limited attachment of a superinfecting dose of virus. To determine at what time this interference became evident, a series of experiments were designed as follows:

1. CEK cultures were inoculated with approximately 50 PFU's of IBV.

2. At prescribed time intervals the inoculum was removed from 8 of the plates and washed once with PBS. Half of the plates were inoculated with a second dose of virus, equal to the first, while the other half received only diluent. A second period of time, equivalent to the first, was allowed after which the inoculum was removed and the plates overlayed with agar.

3. The time at which interference begins should then be reflected by the prescribed time intervals beyond which plaque numbers in the dually exposed cultures failed to be twice those of the singly exposed cultures.

In some experiments these prescribed time intervals were 15 minutes while in others they were 30 minutes. This type of experiment was conducted at both ambient temperature and 37°.

**Ability of ultraviolet-irradiated virus to produce attachment interference**

Ultraviolet (UV) inactivated IBV was used in an attempt to establish attachment interference. The virus was irradiated
by first placing 1 ml of undiluted stock virus in a 60x15 mm plastic petri dish and exposing it to UV irradiation from a G15T8 Sylvania germicidal lamp at a distance of 30 mm. A preliminary experiment had shown that a 15-second exposure inactivated the virus completely. Since complete inactivation was not desired in this experiment, 1 ml of virus was irradiated for 5 seconds and another for 10 seconds. A 0.5 ml aliquot of each was diluted 1:10 and 0.5 ml inoculated onto 5 CEK cell cultures while control cultures were inoculated with 0.5 ml of diluent. After 90 minutes at 37° the cultures were washed once with PBS, inoculated with 0.5 ml of a 10⁻⁵.3 dilution of stock virus and were allowed to attach for 90 minutes at 37°. The remainder of the undiluted and 1:10 diluted UV-treated virus was assayed to determine the amount of virus surviving irradiation.

**Ability of NDV to establish IBV attachment interference**

An experiment designed to determine if NDV could interfere with IBV attachment or if IBV could interfere with NDV attachment was conducted as follows. One set of 6 CEK monolayers were inoculated with approximately 50 PFU's of NDV and a similar set with approximately 50 PFU's of IBV. After a 90-minute attachment period at 37°, the inocula were removed and the plates washed once with PBS. Two plates from each set received 0.5 ml of diluent alone, while the other 4 plates from each set were then reinoculated with doses of heterologous
virus that were similar to the first dose, i.e., plates infected initially with IBV were reinfeeted with NDV and vice versa. This experiment was repeated a second time to confirm the results and since IBV and NDV plaques are readily distinguishable, the number of each was separately determined in the dually infected cultures.

Thiol Inhibitors of IBV Attachment

Substances inhibitory to IBV attachment were studied and evaluated by their incorporation into the virus diluent and allowing the virus to attach in the presence of the inhibitor. The degree of attachment in the presence of inhibitor was compared to a control in which virus was allowed to attach with no inhibitor present.

Before a study of substances that might interfere with virus attachment could be evaluated, it was necessary to establish the normal variation in average plaque counts which might be expected.

Normal variation in average plaque counts

These experiments were designed to compare the normal variation encountered in a number of equivalent dilutions of virus made with varying volumes of virus and diluent. In an initial trial, 4 separate $10^{-5}$ dilutions of virus were prepared in the following manner:
1. First a stock $10^{-4}$ dilution of virus was prepared.

2. Four separate $10^{-5}$ dilutions were made, 3 by adding 0.5 ml of the $10^{-4}$ dilution to 4.5 ml of diluent and the other by adding 2.0 ml of the $10^{-4}$ dilution to 18 ml of diluent.

3. Each $10^{-5}$ dilution was assayed on 10 CEK cell cultures after an attachment period of 90 minutes at ambient temperature.

A second experiment, using the same procedures as just described, was performed except 8 separate $10^{-5}$ dilutions were prepared as follows: 1 with 5 ml virus:45 ml diluent, 4 with 2 ml virus:18 ml diluent, and 3 with 0.5 ml virus:4.5 ml diluent. Seven replicate counts were made on each of the 8 dilutions of virus.

Thiols\(^1\) and sulfhydryl (SH) reagents\(^2\) and their effect on IBV attachment

The work of Powers (1965) which demonstrated that IBV was inactivated by PHMB suggests that SH groups are possibly involved in the attachment of IBV virus to host cells. To investigate this possibility, experiments were conducted using thiols and various SH reagents to find what effect they might have on IBV attachment.

\(^1\)Compounds containing sulfhydryl groups will be designated as thiols.

\(^2\)A substance which can react with sulfhydryl groups will be designated as a sulfhydryl reagent.
The following group of experiments were conducted using the thiol, L-cysteine. In these experiments L-cysteine was dissolved in the maintenance medium 199 used as virus diluent and the virus was allowed to attach in the presence of various molarities of L-cysteine. A typical example of an experiment to determine the effect of L-cysteine on virus attachment would be as follows:

1. A fresh stock solution of 0.1M L-cysteine in virus diluent was made before each experiment.

2. Subsequent dilutions of the L-cysteine were made in the diluent until the desired concentrations was reached. The pH was adjusted to 7.2 with 0.1N NaOH when required.

3. The virus was then diluted to approximately 100 PFU/0.5 ml either in L-cysteine or diluent alone. Monolayers of CEK cells were inoculated with 0.5 ml of virus and the difference between plaque counts in the presence and absence of L-cysteine were compared.

4. Viral attachment to the monolayers was carried out at 37° for 90 minutes in all experiments with thiols and SH reagents.

In the first trial to determine the effect of L-cysteine on IBV attachment approximately 100 PFU were inoculated onto each plate in the presence of concentrations of 10^{-2}, 10^{-2.3},

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1L-cysteine, Calbiochem, Los Angeles, California.
10^{-3}, 10^{-3.3}, and 10^{-4} M L-cysteine. Five plates were inoculated per concentration of L-cysteine and the plaque counts compared to 8 control plates inoculated without L-cysteine in the diluent. A second trial using L-cysteine concentrations of 10^{-2}, 10^{-3}, and 10^{-4} was done using 5 plates for each concentration and 5 plates for the control.

To determine if there was a linear response of plaque inhibition to increased concentration of L-cysteine the following experiment was run. Approximately 100 PFU's of IBV were adsorbed in the presence of 2-fold dilutions of L-cysteine ranging from 10^{-3} to 10^{-5.5}. Two experiments were run using 6 CEK monolayers per concentration in the first and 5 in the second.

The effect of 3 other thiols were checked to determine if they had a significant effect on IBV plaque counts. Concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M reduced glutathione{superscript 1}, and 10^{-2}, 10^{-3}, and 10^{-4} M 2-mercaptoethanol{superscript 2} and 1,4-dithiothreitol{superscript 1} (Cleland's reagent) were used in these experiments. These solutions were prepared fresh before each experiment and the pH of each concentration was adjusted to pH 7.2 with 0.1N NaOH where necessary. These experiments were run in the same manner as those described for studying

{superscript 1}Reduced glutathione and 1,4-dithiothreitol, Calbiochem, Los Angeles, California.

{superscript 2}2-mercaptoethanol, Eastman Organic Chemicals, Rochester, New York.
The effect of L-cysteine on IBV attachment. Replicate plaque counts were made on either 4 or 5 CEK monolayers per concentration of thiol and compared to plaque counts on 5 thiol-free virus control CEK monolayers.

Since the thiols are reducing agents, it seemed necessary to use another non-thiol reducing agent to confirm that plaque inhibition was due to the SH groups and not a general property of reducing agents. Ascorbic acid was selected as the non-SH reducing agent and 100 PFU of IBV was allowed to attach to CEK monolayers in the presence of the ascorbic acid. A range of $10^{-1}$M to $10^{-5}$M concentration of the ascorbic acid was used and 3 CEK monolayers per concentration were inoculated. The counts were compared to a control in which attachment was carried out in the absence of ascorbic acid.

To show that cysteine was interfering with attachment and not inactivating the virus or interfering with subsequent replication of virus, 2 experiments were conducted.

The first experiment was designed to demonstrate whether virus is inactivated by cysteine and was conducted as follows. Three 5-ml samples of IBV containing approximately 100 PFU's/0.5 ml were prepared, one containing no cysteine and the other 2 containing .001M cysteine. One of the cysteine-containing samples and the sample containing no cysteine were dialyzed against one liter of medium 199 overnight at $4^\circ$ with constant agitation. The third sample, containing cysteine, was held at $4^\circ$ overnight. The number of plaques per 0.5 ml of inoculum
was then determined as previously described and 5 plates per sample were used. This experiment was repeated a second time except 4 replicate plates per sample were used rather than 5.

The second experiment was conducted to demonstrate whether more virus remained unattached to cells in the presence of cysteine than in its absence. A $10^{-4.3}$ dilution of stock virus was made in $10^{-3}$M cysteine and another similar dilution in the absence of cysteine. Five plates were inoculated with 0.5 ml each of the cysteine containing virus dilution and 5 plates with 0.5 ml of virus dilution without cysteine. A 90-minute attachment period at $37^\circ$ was allowed and then the inocula from each group of plates were removed and pooled separately. Each pool was dialyzed overnight against one liter of medium 199 at $4^\circ$ with constant agitation. Three CEK monolayers were then inoculated with each pool and plaque counts made as previously described. This experiment was repeated a second time using a $10^{-4.6}$ dilution of the virus.

To further verify the need for active thiol groups to prevent IBV attachment, the oxidized products of L-cysteine and reduced glutathione, L-cystine and oxidized glutathione, were tested. Approximately 100 PFU's of IBV were allowed to attach to CEK monolayers in the presence of concentrations ranging from $10^{-2}$M to $10^{-5}$M of both L-cystine and oxidized glutathione. Plaque counts were made on 5 CEK cultures and

\footnote{L-cystine and oxidized glutathione, Calbiochem, Los Angeles, California.}
compared to plaque counts made in the absence of the compound tested.

The SH reagents studied in relation to their effect on IBV plaque numbers were PHMB\(^1\), Iodoacetamide\(^2\), and HgCl\(_2\). A 0.01M stock solution of PHMB was made by dissolving of the compound in pH 7.5 glycine buffer. This stock was stored at 4\(^\circ\) and was used throughout the study to make various concentrations of PHMB. Iodoacetamide and HgCl\(_2\) were made up as fresh 0.1M solutions in medium 199 before each experiment. These 3 reagents were incorporated into the diluent, as were the thiols, so that virus attachment occurred in the presence of the compound and could be compared to a similar dilution of virus which contained no SH reagent and thus served as a control.

The virus control contained approximately 100 PFU's per 0.5 ml. The concentration levels of the 3 SH reagents were varied from 10\(^{-2}\)M to 10\(^{-6}\)M, and plaque counts performed as previously described using either 4 or 5 CEK monolayers per concentration of reagent. Two trials with each SH reagent were conducted.

To demonstrate whether the virus is inactivated by the PHMB, the following experiment was run. Three samples of IBV, containing approximately 100 PFU's/0.5 ml, were made; 2 con-

\(^{1}\text{p-Hydroxymercuribenzoate, Sigma Chemical Co., 3500 DeKalb St., St. Louis, Missouri.}\)

\(^{2}\text{Iodoacetamide, K&K Laboratories, Inc., 177-10 93rd Avenue, Jamaica, New York.}\)
tained 10^{-4} M PHMB in medium 199 and 1 contained only medium 199. One PHMB-containing sample and the non-PHMB-containing sample were dialyzed overnight against 1 liter of medium 199 at 4° with constant agitation. The other PHMB-containing sample was held at 4° for the same period of time. After dialysis each sample was assayed on 4 CEK monolayers. This experiment was repeated a second time using 5 plates to assay each sample rather than 4.

The kinetics of IBV inactivation by PHMB was determined using 3 concentrations of the SH reagent and the inactivation was conducted as follows:

1. Preparations of virus-PHMB were made up containing approximately 10^{7.2} PFU's/ml of IBV and either 10^{-2}, 10^{-3}, or 10^{-4} M concentrations of PHMB. The 10^{-2}M virus-PHMB was made up in glycine buffer, pH 7.5. The other virus-PHMB preparations were made up in medium 199.

2. The virus-PHMB mixtures were held at ambient temperature and at 30-minute intervals a sample was removed and the number of virus survivors determined by the plaque method. The first 10-fold dilution made for the plaque titrations was made in medium 199 containing 10^{-3} M L-cysteine in order to stop the action of the PHMB. Two CEK monolayers per dilution were inoculated.

3. A virus control preparation without PHMB was held at ambient temperature and a sample titrated at 30-minute intervals. One CEK monolayer per dilution was used.
4. The inactivation experiments employing \(10^{-3}\)M concentrations of PHMB were repeated 3 times while those with \(10^{-2}\) and \(10^{-4}\)M PHMB were performed only once.

An experiment designed to reactivate PHMB inactivated virus using L-cysteine was conducted in the following manner.

1. Four virus-PHMB mixtures containing approximately 100 PFU's/0.5 ml and \(10^{-4}\)M PHMB were made up and held for 1 hour at 4°. A control virus preparation without PHMB was handled in the same manner.

2. A fresh solution of .1M L-cysteine was made up and appropriate quantities added to 3 of the virus-PHMB mixtures to make .02M, .01M, and .005M L-cysteine. The virus control and the remaining virus-PHMB mixture received only an equal quantity of virus diluent.

3. All preparations were dialyzed against 1 liter of medium 199 overnight at 4° with constant agitation to remove L-cysteine and free PHMB.

4. Each preparation was then titrated on 3 CEK monolayers to determine the degree of virus reactivation.

5. The experiment was repeated 2 times.

An experiment was conducted to determine if the sensitivity of cells was altered if treated with PHMB and L-cysteine prior to inoculation with IBV monolayer of CEK cells were treated for 2 hours at 37° with 1 ml of either \(10^{-3}\), \(10^{-4}\) or \(10^{-5}\)M PHMB or \(10^{-2}\), \(10^{-3}\), or \(10^{-4}\)M L-cysteine. The cells were then washed twice with PBS, infected with approximately
100 PFU's of IBV, and plaque determinations made in the usual manner. Plaque counts were made on either 4 or 5 CEK monolayers per concentration of PHMB or L-cysteine. Plaque counts were made on 5 CEK control cultures handled in a similar manner with the exception that they were not treated with either compound. This experiment was repeated 2 times.

In one experiment, the effect of L-cysteine incorporated into the agar overlay was determined. Ten CEK monolayers were inoculated with approximately 100 PFU's of IBV and one-half of them were overlayed with agar medium containing $10^{-2}$M L-cysteine while the remainder served as controls.

Since the results of the previous work show that IBV attachment seems to be altered by thiols and SH reagents, the following experiment was conducted to see what effect L-cysteine and PHMB have on NDV attachment. The two compounds were tested similarly to the method used to evaluate their effect on IBV. Three trials with each compound were made using $10^{-2}$ and $10^{-3}$M L-cysteine and $10^{-3}$ and $10^{-4}$M PHMB. In 2 trials approximately 50 PFU's/0.5 ml of NDV were used in each concentration of compound while in the third trial about 200 PFU's/0.5 ml were used. Dithiothreitol was tested in one of the trials at a concentration of $10^{-3}$M to determine its effect on NDV plaque counts. Plaque counts were at 3 days postinoculation with NDV rather than at 2 days in the case of IBV.
Determination of cell-associated thiol groups

Because of the finding that thiol groups on the cell membrane were apparently active in IBV attachment, it seemed plausible that differences in thiol activity might be discerned on the membranes of the cells with different abilities to attach IBV. Cytochemical staining for thiol group activity was done on CEK and CELi cells. Quantitative determinations of thiol groups were done on CEK, CELi, WCEFp, CEPi, and PK-15 cells.

Qualitative determination  The cytochemical reagent chosen for staining thiol groups of the CEK and CELi cells was mercury orange\textsuperscript{1} $\left[1-(4\text{-}\text{chloromercuriphenylazo})\text{-naphthol-2}\right]$ as described by Bennett (1951). The stain was prepared by making a saturated solution of mercury orange in 80% ethanol. To make this saturated solution, 0.01 gm of mercury orange was added to 1 liter of 80% ethanol and allowed to dissolve with agitation for 48 hours. There was considerable mercury orange that remained undissolved in this solution but it settles to the bottom and the mercury orange solution can be decanted off just before use. The procedure for fixing and staining the CEK and CELi cells was as follows:

1. Cover glasses with monolayers of either CEK or CELi cells were rinsed in PBS and then fixed for 5 minutes in acetone.

\textsuperscript{1}Mercury orange obtained from Sigma Chemical Co., 3500 DeKalb St., St. Louis 18, Missouri.
2. The monolayers were then air dried, hydrated from absolute ethanol to 80% ethanol and placed in the mercury orange for staining. The specificity of the staining was checked by placing some of the cells in PBS containing 0.001M PHMB for 2 hours prior to staining. The PHMB blocks specific staining due to thiol groups.

3. Monolayers were removed from the stain at 0.5, 1, 2 and 3 hours. In one trial, some monolayers were stained for 5 hours.

4. After removal of the monolayers from the stain they were rinsed in 2 changes of 80% ethanol and dehydrated through 95% and absolute ethanol. They were then cleared in xylene and mounted on slides in Permount(R).1

5. After drying, the cells of the monolayer were examined for the intensity of staining and for specificity by comparing them with PHMB blocked cells.

**Quantitative determination** The quantitative method used for determining thiol group activity was the method of Ellman (1959) employing the reagent 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB)2 which reacts with thiol groups causing yellow color to develop. The intensity of this color reaction

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1Permount(R) obtained from Fisher Scientific Co., Fairlawn, New Jersey.

2DTNB obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin.
can be measured by absorbance in a spectrophotometer\(^1\) at 412 \(\text{m} \mu\). The molar concentration of thiol groups can then be calculated by the formula:

\[
C = \frac{A}{\epsilon D}
\]

where \(C\) = molar concentration
\(A\) = absorbance at 412 \(\text{m} \mu\)
\(\epsilon\) = extinction coefficient
\(= 13,600/\text{M/cm}\)
\(D\) = dilution

The general procedure for the quantitation was as follows:

1. The DTNB reagent was prepared by dissolving 39.6 mg of DTNB in 10 ml phosphate buffer (pH 7.0).

2. The determination of the thiol groups in an unknown cell preparation was made by mixing:

- Unknown cell preparation \(3 \text{ ml}\)
- Phosphate buffer, pH 8.0 \(2 \text{ ml}\)
- Distilled water \(5 \text{ ml}\)
- DTNB reagent \(0.070 \text{ ml}\)

3. The color was allowed to develop for 2 hours and absorbance at 412 \(\text{m} \mu\) determined spectrophotometrically.

Two methods of cell preparation were utilized for measuring thiol group activity of the various cell culture types.

\(^1\)All spectrophotometric determinations were performed on a B & L Spectronic 20, Bausch & Lomb, Inc., Rochester, New York.
The first method involved rupturing the cells and measuring thiol activity of a sedimented portion and a nonsedimented portion. The thiol group activity of CEK and CELi cells in culture were compared in this manner. The second method involved the incubation of intact cells with the DTNB reagent in physiological saline and following the development of the color reaction in the different cell culture types. The theory behind this second procedure was that the early color reaction that developed should represent the activity of surface thiol groups and any later color development should indicate reactions of the DTNB with thiol groups within the cell as the permeability of the cell membrane was altered. The thiol activity of CEK, CELi, WCEF1, CEFI, and PK-15 cell cultures were studied and compared in this manner.

The procedure followed for the first method was as follows:

1. Cells from a number of CEK and CELi cultures were scraped from the surface of the culture vessel with a rubber policeman and pooled separately. The cells were sedimented at 322 x g for 5 minutes, resuspended in 10 ml of distilled water and allowed to lyse overnight (16-22 hours) at ambient temperature. The approximate number of cells/culture were determined by trypsinizing two cultures and counting the number of cells in a hemocytometer.

2. The lysed preparation was then centrifuged at
1400 x g for 10 minutes and the lysate decanted. The pellet was then resuspended in 3 ml of distilled water.

3. The thiol activity of both the lysate and pellet were determined as described in the general procedure and the molar concentration of thiol groups/10^8 cells determined. After the color had developed the preparations were routinely classified by passing them through a 0.33μ Millipore filter.

Two trials were made with the above procedure. In the first trial, cells from 20 CEK and 30 CELi cell cultures were used for the determinations. In the second trial, cells from 28 CEK and 30 CELi cell cultures were used.

A third trial was made with a modification in the preparation of the cell lysate and pellet. A trypsin-versene solution was used to remove the cells from the cultures and the cells were disrupted with a Thomas tissue grinder^1 with a Teflon pestle. The trypsin-versene solution was prepared as follows:

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>NaCl</td>
<td>8.0 gm</td>
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<tr>
<td>KCl</td>
<td>0.4 gm</td>
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<tr>
<td>Dextrose</td>
<td>1.0 gm</td>
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<tr>
<td>NaHCO_3</td>
<td>0.58 gm</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.5 gm</td>
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<tr>
<td>Versene</td>
<td>0.2 gm</td>
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<tr>
<td>H_2O</td>
<td>1000 ml</td>
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</table>

^1 Thomas tissue grinder, size B, 0.005-.007 inch clearance obtained from Arthur H. Thomas Co., Philadelphia, Pennsylvania.
This solution was filtered sterilized with a 0.33μ Millipore filter. The cells were separated from the trypsin-versene by centrifugation at 322 x g and resuspended in 10 ml distilled water. This suspension was put into the homogenizer and the cells ground until the majority of the cells were disrupted as detected by microscopic examination. In this trial, the cells from 28 cultures of CEK cells and 29 cultures of CEL1 cells were used for thiol group determinations.

The procedure for the second method of cell preparation for cellular thiol group determination was used on CEK, CEL1, WCEF1, CEP1 and PK-15 cells and was performed as follows:

1. A working solution of DTNB was prepared by adding 7.0 ml of the stock DTNB reagent to 800 ml of 0.85% saline and 200 ml of phosphate buffer, pH 8.0.

2. Two and one-half ml of this solution was added to cell cultures previously washed 2 times with PBS. At various time intervals, the solution from 2 cultures was removed, filtered through a 0.33μ Millipore filter, and the absorbance at 412 μ determined spectrophotometrically.

3. This procedure was followed in 3 separate trials. The reagent was allowed to be in contact with the cells at ambient temperature in the first trial, 37° in the second, and in the third trial a parallel study was made at both temperatures. The first time intervals were at 0.5 and 1 hour in all trials and at either 1 or 2-hour intervals up to 5 or
6 hours. In the third trial a 20-hour time interval was included as well.

Inhibitors of IBV Attachment Found in Serum

Demonstration and quantitation of IBV-inhibitors in serum

In an experiment in which medium 199 with 8% bovine serum was used as virus diluent, it was observed that the plaque counts fell far short of the expected value. To investigate whether this plaque reduction was due to the presence of serum the following experiment was conducted:

1. A virus preparation containing approximately 100 PFU's/0.5 ml was made in either serum-free virus diluent or virus diluent containing 5%, 10% or 20% bovine serum.

2. Each preparation was inoculated onto 4 or 5 CEK monolayers and plaque determinations performed as previously described.

3. A preliminary trial was run to test the inhibitory effect of bovine serum #681 at the 20% level. In a second trial the same serum was tested at the 5%, 10% and 20% level. In a similar manner bovine serum pool #951 and a pool of fetal calf serum was tested for the presence of inhibitors.

With the finding of IBV-inhibitors in serum of bovine origin it seemed desirable to test serums from other species for inhibitory activity. For this purpose serums from swine, chickens and rabbits were tested for activity at the 10% level.
The occurrence of inhibitory activity amongst individuals from different species was also determined. Serums from 20 cattle, 10 rabbits and 4 chickens were tested. The determination of activity was performed as previously described.

A method for the quantitation of inhibitory capacity of different serums was investigated. Since little difference in inhibition was observed between 10% and 20% levels of bovine serum #681, it appeared that an inhibition plateau was reached at the 10% level. The procedure involved attachment of 100 PFU's of IBV in the presence of serum varying from 1% to 10% and determining the degree of inhibition at each level to a serum-free virus control. Four separate serums were titrated in this manner, either 2 or 3 plates were inoculated with each concentration of serum and plaque determinations made.

Effect of bovine serum on NDV plaques

An experiment was conducted to determine if bovine serum inhibited NDV as it did IBV. Three trials were conducted to establish whether bovine serum inhibited NDV attachment.

Effect of serum inhibitors applied to cell cultures prior to or following IBV attachment

To test the effect of IBV-inhibitors in bovine serum when applied to cells prior to attachment of IBV the following 3 experiments were conducted.
1. In the first experiment, CEK cells were implanted with the usual medium 199 containing either 8% or 20% bovine serum #681. These cultures were then inoculated with approximately 50 PFU's of IBV and comparisons of plaque counts made between cells implanted in 8% serum with those implanted in 20% serum. Two replicate trials were conducted to make this comparison.

2. In the second experiment, CEK cell cultures were implanted with the usual medium but were treated with medium containing 40% serum for 2 hours at 37° just prior to inoculation. Plates were washed 2 times with PBS before inoculation. Five CEK cultures were treated in this manner and were compared to those of 5 untreated CEK cultures.

3. The third experiment consisted of implanting CEK cell cultures with a non-inhibitory bovine serum #5533 and comparing plaque counts in such cultures with those implanted with an inhibitory bovine serum pool #1181. Plaque counts were made on 4 CEK cultures per treatment.

The effect of serum on plaque numbers following virus attachment was investigated by incorporating bovine serum #681 into the agar-overlay medium at varied concentrations and comparing plaque counts. Since serum was routinely incorporated into the agar-overlay medium at a concentration of 2.5%, the first 2 trials compared serum-free agar medium to a 2.5% serum containing agar medium. Further trials compared serum-free, 2.5%, 5.0%, and 20% serum-containing agar medium.
Characterization of the IBV inhibitor in bovine serum

Inhibitory fractions of bovine serum Commercial preparations of bovine albumin\(^1\), gamma globulin\(^2\), and fetuin\(^3\) were tested for their inhibitory capacity. The albumin and gamma globulin solutions were prepared by dissolving 1 gm of the powder in 5 ml of medium 199. Fetuin purified by 2 different procedures was tested, one had been purified by \((\text{NH}_4)_2\text{SO}_4\) precipitation and the other by alcohol precipitation. The fetuin stock solutions were prepared by dissolving 0.5 gm fetuin in 5 ml 0.85% NaCl. For the test, 1 ml of the stock solution of fetuin was added to 4 ml medium 199. Virus preparations containing approximately 50 PFU's/0.5 ml were prepared in the above solutions and plaque counts compared to a control in which the virus inoculum contained no serum components. Plaque counts on 8 CEK cultures per solution were made in the usual manner.

A gamma globulin preparation was also prepared from bovine serum pool \#681 by precipitation with \((\text{NH}_4)_2\text{SO}_4\). The precipitation procedures used was the same as that previously described for obtaining gamma globulin for fluorescent antibody


\(^2\)Bovine gamma globulin fraction II, Nutritional Biochemical Corp., Cleveland, Ohio.

\(^3\)Fetuin, Grand Island Biologics Co., Inc., Grand Island, New York.
preparation. After the gamma globulin solution was prepared and the volume adjusted to the original volume of serum, its plaque inhibitory activity was determined by allowing 50 PFU's of IBV to attach in the presence of 10% of the gamma globulin solution and comparing plaque counts with those allowed to attach in the absence of gamma globulin.

The activity of the (NH₄)₂SO₄ precipitated bovine gamma globulin #681 was compared to the activity of non-fractionated whole bovine serum #681. The method of quantitation of the inhibitory activity of the 2 preparations was the same as previously described.

To determine if all of the inhibitory activity of serum was removed by precipitation with 1/3 volume saturated (NH₄)₂SO₄ additional sequential precipitations with 1/2 and then 3/5 volume saturated (NH₄)₂SO₄ were conducted. The supernatant fluid from the final precipitation was also tested. The 3 sequentially precipitated serum fractions were tested for IBV inhibitory activity at the level of 20% and 30% while the supernatant fluid fraction was tested at the 40% level.

**Destruction and removal of IBV inhibitors in bovine serum** The susceptibility of viral inhibitors subjected to various physical and chemical treatments can sometimes aid in determining their nature. Bovine serum #681 was studied as to its heat stability at various temperatures as well as its susceptibility to neuraminidase, acetone, kaolin adsorption, and 1,4-dithiothreitol.
Heat stability The heat stability of the inhibitory activity was first determined at 60° and 65°. Since serum was routinely inactivated at 56° for 30 minutes for routine cell culture, it was evident that this treatment did not destroy the inhibitory activity. The effect of heat on the inhibitor was determined as follows:

1. A 10% preparation of bovine serum #681 in medium 199 was prepared and distributed in 10 ml amounts in Pyrex test tubes.

2. These serum preparations were then placed in either a 60° or 65° water bath and tubes were removed at prescribed time intervals and placed in a 4° water bath. Time intervals were 15, 30, 60, 90, and 120 minutes for the 60° temperature and 10, 20, and 30 minutes for the 65° temperature. A period of time was allowed for temperature equilibration and was 3 minutes in the 60° study and 4 minutes in the 65° study.

3. After heat treatment the 10% serum samples were tested for their IBV inhibitory activity in the usual manner. In the case of the 60° temperature study, 2 samples were removed at each time interval and the activity of each determined separately. Only 1 sample per time interval was used in the 65° temperature study.

To determine if any inhibitory activity was destroyed by the routine 56° treatment of whole serum for 30 minutes, 3 samples of bovine serum #1181 were treated as follows: One
was left unheated, the second was heated at 56° for 30 minutes and the third was heated at 65° for 20 minutes. Quantitation of their activity was then determined as previously described.

Neuraminidase treatment

Treatment of bovine serum #681 with neuraminidase was performed as follows:

1. A 20% serum solution in medium 199 was prepared and 5 ml samples were treated with 5, .5, .05, and .005 units of neuraminidase, respectively, for 40 minutes at 37°. A control sample was left untreated.

2. Each sample was checked for inhibitory activity in the usual manner.

Acetone treatment

The acetone extraction procedure described by Clarke and Casals (1958) was used. The procedure was as follows:

1. The serum was diluted 10-fold with 0.85% NaCl and chilled in a 4° water bath.

2. Cold acetone (4°) was added to the serum at the rate of 12 volumes acetone: 1 volume diluted serum.

3. Extraction was carried out for 5 minutes with periodic shaking and then centrifuged for 5 minutes at 1,400 x g.

4. The acetone was aspirated and the precipitate reextracted with the same volume of acetone. Vigorous shaking

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1Neuraminidase (vibrio cholera), B grade, Calbiochem, Los Angeles, California.
was necessary to resuspend the pellet.

5. After centrifugation and removal of the acetone, the precipitate was dried with a stream of nitrogen gas.

6. The dried precipitate was then restored to the original serum volume by dissolving in PBS.

Two samples of bovine serum #681 were extracted in parallel by the above procedure and were tested for inhibitory activity in the usual manner.

**Kaolin adsorption** The adsorption of serum with kaolin was carried out as follows:

1. One gm of acid-washed kaolin\(^1\) was added to 5 ml of serum.

2. This mixture was held at room temperature for 5 hours with occasional shaking to resuspend the kaolin.

3. The mixture was centrifuged at 1,400 \(x \ g\) and the supernatant serum withdrawn and filtered through a 0.45 \(\mu\) Millipore filter.

One sample of bovine serum #681 was treated in this manner and then tested for inhibitory activity.

**1,4-Dithiothreitol (DTT) treatment** To test the effect of DTT on the IBV inhibitor of bovine serum, the \((NH_4)_2SO_4\) precipitated gamma globulin fraction was used. A stock solution of 10\(^{-1}\)M DTT was prepared in phosphate buffer, pH 8.0.

\(^1\)Kaolin (acid-washed) obtained from Fisher Scientific Co., Fair Lawn, New Jersey.
The procedure followed was essentially that of Gunewardena and Cooke (1966) and was as follows:

1. One volume of phosphate buffer, pH 9.0 was added to 9 volumes gamma globulin solution. DTT was added and allowed to react for 1 hour. The final concentration of DTT was $10^{-3} M$ in the first trial and $10^{-2} M$ in the second.

2. The DTT-treated solution was then alkylated with iodoacetamide (final concentration of $10^{-3} M$ in both trials) and then dialyzed against PBS overnight at $4^\circ$ with constant stirring. In the second trial alkylation was eliminated in one sample.

3. The IBV-inhibitory activity of the DTT-treated gamma globulin fractions was tested in the usual manner.

Physical characterization of IBV-inhibitors in bovine serum  
To gain more knowledge of the physical size of the IBV inhibitory substance, serum was examined by ultrafiltration, rate zonal ultracentrifugation and gel filtration methods.

Ultrafiltration  
Serum was filtered through an S&S collodion bag\textsuperscript{1} by applying 15 lb of vacuum to the suction vessel. Bovine serum #681 was concentrated to 10 times its original volume. The concentrate was restored to the original serum and was tested for its inhibitory activity. The filtrate was also tested for any inhibitory activity.

\textsuperscript{1}S&S collodion bag and collodion bag apparatus obtained from Carl Schleicher and Schuell Co., Keene, New Hampshire.
Rate-zonal centrifugation\(^1\) Ammonium sulfate precipitated gamma globulin of bovine serum #681 was subjected to rate zonal centrifugation as follows:

1. The gamma globulin was first concentrated 6 times using the ultrafiltration apparatus and the amount of protein determined by the biuret method.

2. A sucrose gradient was formed by layering various concentrations of sucrose in a 5-ml cellulose nitrate centrifuge tube. Sucrose concentration percentages are expressed on a weight/volume basis. One-half ml of 59% sucrose was put on the bottom of the tube and then 1 ml each of 30%, 25%, 20%, and 15% sucrose, respectively, were layered in the tube. One-half ml of the gamma globulin solution was layered on top of the sucrose.

3. The preparation was centrifuged in a Spinco Model L preparative ultracentrifuge\(^2\) using an SW39L rotor. The maximum centrifugal force was 127,000 x g and the time of centrifugation was 16 hours.

4. Fractions were removed from the top of the tube in 1/2 ml amounts, diluted 1:10 in medium 199, and dialyzed against 1 liter of medium 199 overnight to remove the sucrose.

\(^1\)Rate zonal centrifugation kindly performed by Mrs. Judith Patterson, NADL, Ames, Iowa.

\(^2\)Spinco Model L preparative ultracentrifuge, Spinco Division of Beckman Instruments, Inc., Palo Alto, California.
Each 1:10 dialyzed sample was tested for IBV inhibitory activity at 2 different concentrations. The concentrations tested represented 10% and 5.55%, respectively, of the original fraction removed from the gradient.

**Gel filtration**

The $(\text{NH}_4)_2\text{SO}_4$ precipitated gamma globulin of bovine serum #681 was chromatographed on a Sephadex G200\(^1\) column using the method described by Thomssen et al. (1966). A 4 x 40 cm column of Sephadex G200 was poured and equilibrated with a 1.0M NaCl-0.2M Tris [Tris (hydroxymethyl) aminomethane] buffer, pH 8.0. The gamma globulin solution was dialyzed against this same buffer for 20 hours at 4\(^\circ\). Before the sample was applied 6.0 ml of a 0.1% solution of blue dextran was passed through the column to determine the hold-up volume of the column and the eluate volume of the sample. Six ml of the gamma globulin was applied to the column and 6-ml samples of eluate were collected. Forty samples were collected and each sample was checked for protein by absorbance at a wavelength of 280 m\(\mu\) on a Beckman DK2A spectrophotometer.\(^2\) The first 26 samples were dialyzed against PBS overnight at 4\(^\circ\) and the IBV inhibitory activity of each fraction tested at the 20% level. One ml of samples 5-10 and 0.5 ml of samples 1-26 were pooled and concentrated with an S&S Sephadex G200 obtained from Pharmacia, Uppsala, Sweden.

\(^1\)Sephadex G200 obtained from Pharmacia, Uppsala, Sweden.

\(^2\)Beckman DK2A spectrophotometer, Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, California.
collodion bag to 1 ml and 0.5 ml, respectively. These concentrated samples were tested for IBV inhibitory activity at the 10% level.

Reversal of IBV-inhibitory activity in bovine serum A preliminary test before the sucrose rate zonal centrifugation study was run to see if sucrose had any deleterious effect on either the virus or cell cultures. The result of this test showed that much of the inhibitory effect of 10% serum was lost when virus-serum-sucrose mixtures were allowed to attach to cells. When this mixture was dialyzed against PBS to remove the sucrose, the inhibitory activity of the serum was regained. This finding led to a survey of a number of di- and monosaccharides to determine the variety of sugars that could reverse the effect of IBV inhibitor.

The survey consisted of allowing approximately 100 PFU's of IBV to attach in the presence of 10% bovine serum #681 and 1.5% of the various sugars. Controls consisted of virus attached in 10% serum alone and serum-free virus attachment. The sugars checked were as follows:

1. Disaccharides - sucrose, lactose, and maltose.
2. Monosaccharides
   a. Hexoses - D-glucose, L-glucose, D-galactose, D-mannose, and D-fructose.
3. Derivatives of glucose - 2-deoxy-D-glucose, D-glucosamine, alpha-methyl-D-glucoside, and beta-methyl-D-
The effect of pretreatment of CEK cells with D-glucose and alpha-methyl-D-glucoside was determined by incubating cells in 1.5% solutions of each sugar for 2 hours previous to inoculation with 100 PFU's of virus.

To determine if reversal of IBV-inhibitor was possible after the virus and inhibitor were allowed to react together, the effect of glucose, alpha-methyl-D-glucoside and galactose on IBV-inhibitor were checked in the following way. About 200 PFU's of IBV were allowed to attach for 90 minutes at 37° in the presence of 10% serum. After this period, 0.1 ml of a 9% solution of the above sugars was added to the inoculum and another period of 90 minutes at 37° allowed. The inoculum was withdrawn and plates overlayed in the usual manner. Plaque counts on virus control and virus-serum control CEK cultures were also done.

To determine if the IBV-inhibitors found in swine, rabbit, and chicken serums was similar to those in bovine serums with respect to reversal by sugars the following experiment was conducted. Virus was allowed to attach in the presence of 10% serum plus 1.5% alpha-methyl-D-glucoside. Serums from 5 rabbits, 4 chickens, and 1 swine serum pool were tested in this manner. Since the inhibitory activity of the rabbit serums was very high at the 10% level, 2 rabbit serums were selected and the effect of 1.5% alpha-methyl-D-glucoside was determined on 1%, 3%, 5% and 10% of each serum.
Mode of action by IBV-inhibitors in serum  While it had been felt during the entire study that IBV-inhibitors in serum acted by preventing viral attachment, it was difficult to prove. With the finding that the IBV-inhibitor could be reversed with sugars, it was possible to confirm that viral attachment was interfered with. To do this, approximately 100 PFU's of IBV was allowed to attach to CEK cell monolayers for 90 minutes at 37° in the presence of 0% and 10% bovine serum #681. The inocula were removed from each group and pooled separately. Glucose was added to each pool to make a final concentration of 1.5%. Five CEK cell cultures were inoculated per pool to determine the amount of unattached IBV.

To determine if thiol groups of the IBV-inhibitors were the active groups in preventing IBV attachment, the following experiment was conducted. About 100 PFU's of IBV allowed to attach in the presence of 0%, 5%, 10%, and 20% bovine serum #681. The same amount of virus was also allowed to attach in the same concentrations of serum except PHMB was added at the level of 10^{-3}M and 10^{-4}M concentrations.

Effect of bovine serum and filtrate of bovine serum on IBV cytopathological changes and plaque size When bovine serum was incorporated into fluid medium of IBV infected cells at the level of 5% and 10%, it was observed that cells were "spared" from the cytopathic effect (CPE) of IBV compared to IBV infected cells in medium free of serum. It was also observed that a filtrate of bovine serum filtered through an
S&S collodion bag had the opposite effect and caused an enhancement of the CPE of IBV. To demonstrate these observations CEK cell monolayers on cover glasses were infected with either $10^{6.5}$ or $10^{2.5}$ PFU's of IBV. Whole bovine serum and the filtrate were incorporated in the medium at the levels of 1%, 10%, and 20% and cover slips of each treatment were stained with fluorescent antibody at 24 and 48 hours.

The effect of whole bovine serum and the filtrate on plaque size was determined by incorporating 5% serum and 20% filtrate into the agar medium and observing the plaque size at 40 hours in comparison to plaques formed under agar medium alone.

**Effect of some selected compounds on IBV attachment**

In addition to the thiol inhibitors and inhibitors found in serum, a number of other compounds which reportedly affect viruses other than IBV were tested for their effect on IBV.

**Salicylates** The report of Inglot et al. (1966) demonstrating that encephalomyocarditis virus attachment was inhibited by salicylates led to an investigation of the effect of these compounds on IBV attachment. Approximately 100 PFU's of IBV were allowed to attach in the presence of acetylsalicylate, p-aminosalicylate, and sodium salicylate in concentrations of $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-3.3}$M of each compound. The pH of these solutions was adjusted to pH 7.2 using .1N NaOH or .1N HCl when required.
Amantadine hydrochloride\(^1\) While Davies et al. (1964) reported that the attachment of Influenza A to chicken embryo fibroblasts and red blood cells was not interfered with, this compound was tested for its effect on IBV attachment. The effect of the presence of amantadine hydrochloride on IBV plaque numbers during the attachment period was checked at concentrations of 100, 50, 25, and 10 \(\mu g/ml\).

Pancreatin The reports (Wallis et al., 1966a, and Wallis et al., 1966b) that pancreatin enhanced enterovirus and reovirus plaque numbers led to the study of the effect on this enzyme preparation on IBV. A stock solution of pancreatin was prepared by dissolving 1 buffered pancreatin tablet\(^2\) in 50 ml of distilled water. Several preliminary trials indicated that there was a significant increase in plaque numbers when IBV was allowed to attach in the presence of a 1:20 to 1:40 dilution of the pancreatin stock solution in medium 199. To demonstrate this effect and to also investigate the possible relationship of pancreatin with enhancement of plaque numbers by alpha-methyl-D-glucoside and inhibition by bovine serum the following experiment was conducted. One hundred PFU's of IBV were allowed to attach to CEK monolayers in the presence of 1:20, 1:30, and 1:40 dilutions of stock pancreatin. The same

\(^1\)Amantadine hydrochloride obtained from E. I. DuPont de Nemours & Co., Inc., Wilmington, Delaware.

\(^2\)Buffered pancreatin tablets, Oxoid, Colab Laboratories, Inc., Chicago Heights, Illinois.
amount of virus was also allowed to attach in those same concentrations of pancreatin plus either 1.5% alpha-methyl-D-glucoside or 5% bovine serum. As controls, virus was allowed to attach without any treatment, with 5% bovine serum alone, and with 1.5% alpha-methyl-D-glucoside alone.

Neuraminidase and N-acetylneuraminic acid (NANA)
The work of Biswal et al. (1966) showed that neuraminidase destroyed IBV hemagglutinin receptors on chicken erythrocytes and inhibited IBV infection in the embryonating chicken's egg. An experiment was designed to test the effect of neuraminidase on IBV-receptors of CEK cell monolayers. Cell monolayers were treated for 1 hour at 37° with 10, 5, and 2.5 units of neuraminidase. A unit of activity is equivalent to 1 µg NANA in 15 minutes at 37° from a glycoprotein substrate obtained from human serum. The effect of the neuraminidase treatment of CEK cells on IBV and NDV receptors was determined by inoculating the monolayers with 50 PFU's of IBV and 100 PFU's of NDV and comparing plaque counts with those on untreated cultures.

The effect of NANA was tested by allowing 50 PFU's of IBV and 100 PFU's of NDV to attach to CEK cell monolayers in the presence of 1.5% and 0.75% NANA.

1Neuraminidase (vibrio cholera), B grade, Calbiochem, Los Angeles, California.
Lysosomal Changes in IBV Infected Cells

Acid phosphatase activity was chosen as the marker enzyme for lysosomes in this study. The activity of this enzyme during the course of IBV infection in CEK and CELi cell cultures was determined qualitatively by a histochemical procedure. A quantitative procedure to determine the activity of this same enzyme was used on IBV-infected CEK cell cultures.

**Histochemical procedure**

The Gomori technique (Duspiva, 1963) modified as suggested by Bitensky, 1963, was used for the detection of acid phosphatase activity. This technique involved the staining of unfixed monolayers of cell cultures since preliminary experiments using formalin fixed cells had shown a high degree of inconsistent and non-specific staining of the cytoplasm and nuclei. The staining of unfixed cells allowed for subsequent acetone fixation as required for fluorescent antibody staining.

The entire procedure for the detection of acid phosphatase activity fluorescent antibody staining was as follows:

1. Cell monolayers were washed briefly in 0.85% NaCl and then incubated at 37° in a beta-glycerophosphate substrate solution made by mixing:

   \[ 1M \text{ acetate buffer pH 5.0} \]  
   \[ 10 \text{ ml} \]

\[ 1M \text{ acetate buffer pH 5.0 prepared by mixing 70.5 ml of a solution of 136.1 gm sodium acetate/1000 ml with 29.5 ml 1N acetic acid.} \]
5% aqueous lead nitrate 3 ml
Distilled water 20 ml
2% aqueous sodium-beta-glycerophosphate 10 ml

A control on the specificity of staining was determined by incubating cell monolayers in the above substrate solution containing 2 mg NaF, which inhibits acid phosphatase.

2. After the incubation in the substrate, the cell monolayers were removed and rinsed for 10 seconds in 1% acetic acid and then transferred to 0.85% NaCl saturated with hydrogen sulfide. This solution was prepared by bubbling hydrogen sulfide generated from one 5 gm Aitch-Tu-Ess^R^ cartridge through 500 ml of 0.85% NaCl. The cell monolayers were left in this solution for 5 minutes.

3. The cell monolayers were then washed briefly in 0.85% NaCl and fixed for 5 minutes in acetone.

4. After allowing the cell monolayer to air dry, they were stained with the fluorescein conjugated anti-IBV serum for 45 minutes at 37° in a high humidity atmosphere.

5. The cell monolayers were then washed in distilled water, allowed to air dry and mounted in a solution of 1 part PBS and 1 part glycerol.

6. Cells stained in the above manner were then examined by light, phase, and darkfield microscopy as well as UV darkfield microscopy.

^1Aitch-Tu-Ess cartridges obtained from Hengar Company, Philadelphia, Pennsylvania.
The effect of IBV infection on the lysosomes of infected CEK and CEL1 cells was determined by infecting cultures of each cell type with $10^{6.5}$ PFU's of IBV. Cell monolayers were removed immediately after infection and at 4, 6, 8, 12 and 18 hours post-infection. They were stained as described above with cell monolayers being incubated in the substrate for 15, 30, 60, 120, and 180 minutes. Two controls were run at each time interval; one was a control on specificity of acid phosphatase staining and the other was the staining of uninfected cell monolayers.

**Qualitative procedure**

The quantitation of acid phosphatase was performed on CEK cellular fractions using a Sigma acid phosphatase kit.\(^1\) Two fractions of cells were prepared, a mitochondrial-lysosomal (ML) fraction and a supernatant (soluble) fraction. They were prepared as described by Allison and Sandelin, 1963, and the procedure was as follows:

1. Cells from 20 CEK cell cultures were scraped from the surface of the culture vessel with a rubber policeman. The cells were separated from the culture medium with centrifugation at $224 \times g$ for 5 minutes. They were resuspended in 10 ml of cold ($4^\circ$) 0.25M sucrose, centrifuged, and resuspended in 10 ml of cold hypotonic 0.125M sucrose.

---

\(^1\)Sigma acid phosphatase kit, Sigma Chemical Co., 3500 DeKalb St., St. Louis, Missouri.
2. The 0.125M sucrose-cell suspension was then transferred to a Thomas tissue grinder with a Teflon pestle. The cells were homogenized at 4° with 20 strokes of the Teflon pestle rotating at 2,000 RPM.

3. The homogenate was centrifuged at 2,000 x g for 15 minutes at 4° to remove nuclei and cell debris. The remainder of the homogenate was then centrifuged at 12,000 x g for 1 hour at 4°. The soluble fraction was decanted and saved and the pellet (ML fraction) suspended in 10 ml of cold 0.125M sucrose. Both fractions were then assayed for acid phosphatase activity.

The assay procedure utilizing para-nitrophenylphosphate as the substrate was conducted as follows:

1. Tubes containing 0.5 ml of Sigma stock substrate solution and 0.5 ml Sigma acid buffer were warmed in a 37° water bath.

2. To each tube 0.2 ml of the cell fraction to be assayed was added and then incubated for 30 minutes at 37°. A reagent blank was prepared in which 0.2 ml of water was added rather than the cell fraction.

3. After incubation, 5 ml of 0.1N NaOH was added to each tube to stop the reaction and develop the color. The optical density (O.D.) of each sample was read on a spectrophotometer at a wavelength of 410 mμ using the reagent blank as a reference to adjust the O.D. 410 to zero.
4. A blank containing 0.2 ml of each cell fraction and 6 ml of 0.1N NaOH was also prepared and the O.D. at 410 μm determined. A corrected O.D. reading for each sample was obtained by subtracting the O.D. value of the sample in step 4 from the O.D. value of the blank in step 3.

The quantitation of acid phosphatase activity of the 2 cell fractions of IBV infected and uninfected control CEK cell cultures was performed at 8 and 16 hours post-infection. The cultures were infected with $10^6.5$ PFU's of virus.
RESULTS

Attachment of IBV to Cell Monolayers

Effect of temperature, time, and pH on attachment

The effect of temperature on attachment of IBV to CEK cell monolayers is shown in Table 1. There was no difference between attachment of IBV at 4°C and ambient temperature but at 37°C there was a substantial increase in attachment. Titers of the virus were increased approximately 5 times thus effectively increasing the log_{10} PFU/ml by 0.7 of a log.

While extended periods of virus attachment for longer than 90 minutes did increase the number of PFU's, it was considered to be slight (Fig. 1 and Table 40). It should be pointed out that the number of PFU's at 90 and 120 minutes was approximately the same in both trials indicating a plateau with another slight increase in PFU's at 150 to 180 minutes.

There was little effect on attachment of IBV when the pH of the environment was varied from 6 to 9 (Table 2). The low mean (82 PFU's) of the plaque counts at pH 6 in the first trial is 30% lower than the highest mean plaque count (117 PFU's) at pH 7 and this amount of variation can normally be expected as will be shown later, and was considered to be insignificant especially since there was no depression of plaques at pH 6 in trial 2.
Fig. 1. Effect of time on the attachment of IBV to CEK cell monolayers.
79a

![Graph showing the Log10 fraction of PFU attached over time for TRIAL 1 and TRIAL 2.](image)

**TIME OF ATTACHMENT (MINUTES)**

- **TRIAL 1**
- **TRIAL 2**
Table 1. Results of the effect of temperature on attachment of IBV to CEK cell monolayers for 90 minutes

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Attachment temperature</th>
<th>Dilution of virus</th>
<th>Number of plaques</th>
<th>Mean number of plaques</th>
<th>Log_{10} PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40°</td>
<td>5 x 10^{-4}</td>
<td>115, 102, 75</td>
<td>97 ± 18(^{a})</td>
<td>6.6</td>
</tr>
<tr>
<td>1</td>
<td>40°</td>
<td>1 x 10^{-5}</td>
<td>17, 19, 25</td>
<td>20 ± 5</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>5 x 10^{-4}</td>
<td>102, 87, 105</td>
<td>98 ± 11</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>1 x 10^{-5}</td>
<td>22, 13, 23</td>
<td>19 ± 6</td>
<td>6.6</td>
</tr>
<tr>
<td>2</td>
<td>37°</td>
<td>5 x 10^{-4}</td>
<td>TNTC(^{b})</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>37°</td>
<td>1 x 10^{-5}</td>
<td>94, 85, 104</td>
<td>94 ± 11</td>
<td>7.3</td>
</tr>
<tr>
<td>2</td>
<td>Ambient</td>
<td>5 x 10^{-4}</td>
<td>75, 72, 62, 66, 73</td>
<td>70 ± 5</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>37°</td>
<td>1 x 10^{-5}</td>
<td>40, 65, 60, 61, 57</td>
<td>57 ± 9</td>
<td>7.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± 2 standard deviations of the mean (2S\(_{X}\)).

\(^{b}\) TNTC - Plaques too numerous to count.

Efficiency of attachment

The experiments conducted to determine the efficiency of IBV attachment by performing successive attachments of a virus inoculum to CEK cell monolayers showed that attachment was very inefficient. Each successive period for attachment revealed that a large amount of virus remained unattached. The results in Table 3 show that the inoculum removed from each
attachment period still contained a large relative amount of unattached virus. While it is impossible to demonstrate just how much of the total virus inoculum is removed with each attachment, an indication of the efficiency of attachment can be depicted by expressing as a percentage the ratio of the virus (PFU's) detected after the first attachment: PFU's detected after each of the attachment periods. Thus, the ratio would be 1 after the first attachment and would represent 100% of the virus. The results of the 2 experiments in Table 3 show that this ratio expressed as a percentage was 46% and 45% in the 2 experiments after the 4th successive attach-
Table 3. Results of attachment of IBV inocula to primary CEK cell monolayers in 4 successive attachment periods of 90 minutes at ambient temperatures

<table>
<thead>
<tr>
<th>Number of successive attachment</th>
<th>Mean number of unattached PFU's detected in inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio of: PFU's detected after 1st attachment to PFU's detected after each attachment x 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Exp. 1</td>
</tr>
<tr>
<td>1st</td>
<td>87 ± 14</td>
<td>100%</td>
</tr>
<tr>
<td>2nd</td>
<td>62 ± 12</td>
<td>71%</td>
</tr>
<tr>
<td>3rd</td>
<td>49 ± 16</td>
<td>56%</td>
</tr>
<tr>
<td>4th</td>
<td>40 ± 14</td>
<td>46%</td>
</tr>
</tbody>
</table>

<sup>a</sup>The plaque counts represent the unattached virus in an inoculum of a 10<sup>-4</sup> dilution of IBV.

<sup>b</sup>Means in Exp. 1 are for 3 counts ± 2 standard deviations of the mean (2S<sub>x</sub>).

<sup>c</sup>Means in Exp. 2 are for 5-7 counts ± 2.8S<sub>x</sub>.

Table 4 gives the results when the efficiency of IBV attachment was compared at 37°, ambient and 4° by performing 3 successive attachments at each temperature. It appears from these data that the efficiency of attachment is greater at 37° than at ambient temperature and 4°. Only 20% and 30% of the virus PFU's detected in the first attachment remained after the third attachment while at ambient temperature, 57%
Table 4. Results of attachment of IBV inocula to primary CEK cell monolayers in 3 successive attachment periods of 90 minutes at 4°, ambient, and 37°.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Number of successive attachment</th>
<th>Mean number of unattached PFU's detected in inoculum</th>
<th>Ratio of: PFU's detected after 1st attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exp. 1(^a)</td>
<td>Exp. 2(^b)</td>
</tr>
<tr>
<td>37°</td>
<td>1st</td>
<td>470 ± 54</td>
<td>117 ± 22</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>243 ± 5</td>
<td>65 ± 15</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>92 ± 7</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>ambient</td>
<td>1st</td>
<td>98 ± 11</td>
<td>25 ± 3</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>60 ± 6</td>
<td>20 ± 5</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>56 ± 5</td>
<td>10 ± 4</td>
</tr>
</tbody>
</table>

\(^a\)Means are from 2 or 3 counts ± 2S\(^x\) and represent unattached virus in an inoculum of a 10\(^{-4.3}\) dilution of IBV.

\(^b\)Means are from 3 counts ± 2S\(^x\) and represent unattached virus in an inoculum of a 10\(^{-5}\) dilution of IBV.
Table 4. (Continued)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Number of successive attachment</th>
<th>Mean number of unattached PFU's detected in inoculum</th>
<th>Ratio of: PFU's detected after 1st attachment x 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exp. 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Exp. 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4°</td>
<td>1st</td>
<td>97 ± 24</td>
<td>21 ± 5</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>42 ± 6</td>
<td>15 ± 1</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>79 ± 3</td>
<td>12 ± 1</td>
</tr>
</tbody>
</table>
Table 5. Results of exposure of IBV virus for 4.5 hours (equivalent to three 90-minute attachment periods) to 37° and 4°.

<table>
<thead>
<tr>
<th>Exposure temperature</th>
<th>Mean number of PFU's remaining ± 2SE</th>
<th>Ratio of: PFU's after 4° exposure / PFU's after each exposure x 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°</td>
<td>71 ± 7^a</td>
<td>100%</td>
</tr>
<tr>
<td>37°</td>
<td>14 ± 3^b</td>
<td>20%</td>
</tr>
</tbody>
</table>

^a Mean of 5 plaque counts.  
^b Mean of 4 plaque counts.

and 40% still remained, and at 4°, 82% and 57% still remained. These data may, however, be misleading, as shown in Table 5, because the virus is not only being removed by attachment but is also being inactivated by the increased temperature. The inactivation at 37° appears to account for the removal of as much virus as does the attachment; thus it would be difficult to conclude that IBV attaches more efficiently at 37° than at lower temperatures.

Comparison of virus attachment to different cell types

The results of 3 successive attachments to 5 different cell types are shown in Table 6. The CEK cells are the most efficient at removal of IBV from an inoculum and have been shown to be the most sensitive cell type (Lukert, 1965). The PK-15 is insensitive to IBV, while the sensitivities of
Table 6. Results of successive attachment of IBV to CEK, CELi, WCEFi, CEFi, and

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Number of successive attachment</th>
<th>Mean PFU's detected $\pm 2S_x^a$</th>
<th>Per cent PFU's detected$^b$</th>
<th>Mean PFU's detected $\pm 2S_x^a$</th>
<th>Per cent PFU's detected$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CEK</td>
<td></td>
<td>CELi</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1st</td>
<td>19 ± 4</td>
<td>100</td>
<td>25 ± 5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>3 ± 2</td>
<td>16</td>
<td>11 ± 4</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>3 ± 2</td>
<td>16</td>
<td>4 ± 2</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>1st</td>
<td>49 ± 9</td>
<td>100</td>
<td>84 ± 7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>14 ± 2</td>
<td>14</td>
<td>34 ± 5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>6 ± 1</td>
<td>6</td>
<td>14 ± 5</td>
<td>17</td>
</tr>
</tbody>
</table>

$^a$ Means is from 4 plaque counts $\pm 2$ standard deviations of the mean ($2S_x$).

$^b$ Per cent PFU's detected is the ratio of: PFU's detected after 1st attachment PFU's detected after each attachment.
Fi, and PK-15 cells. Each attachment was for 90 minutes at 37°

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>WCEF\textsubscript{i}</th>
<th>CEF\textsubscript{i}</th>
<th>PK-15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean PFU's detected $\pm 2S_x$</td>
<td>Per cent PFU's detected</td>
<td>Mean PFU's detected $\pm 2S_x$</td>
</tr>
<tr>
<td>0</td>
<td>42 ± 2</td>
<td>100</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>19 ± 5</td>
<td>45</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>9 ± 3</td>
<td>21</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>0</td>
<td>82 ± 4</td>
<td>100</td>
<td>109 ± 10</td>
</tr>
<tr>
<td>0</td>
<td>48 ± 6</td>
<td>58</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>7</td>
<td>20 ± 6</td>
<td>24</td>
<td>27 ± 5</td>
</tr>
</tbody>
</table>
CELi, WCEF1, and CEF1 lie intermediate between the CEK and PK-15 cell types. When the percentage expression of the ratio of PFU's detected in the first attachment: PFU's detected in each successive attachment is expressed graphically (Fig. 2) it can be readily seen that the CEK cells attached IBV more efficiently in both experiments while PK-15 cells were the least efficient in both cases. Thus, there is a direct correlation between the sensitivity of a cell type to IBV and its ability for attachment of the virus.

Attachment interference

Table 7 shows that in 7 trials less than 50% (7% to 46%) of a second dose of virus attached to CEK cell monolayers. This is strong indication that the first dose of virus established an interference preventing the equivalent attachment of a second dose of virus. In trial 2, Table 7, the unattached virus remaining in the 1st and 2nd dose was determined as being $100 \pm 34$ PFU's in the first dose and $161 \pm$ PFU's in the second dose, thus indicating that the attachment of the second dose was being prevented.

The time of appearance of attachment interference seems to be somewhere around 60 minutes after exposure to the first dose of virus as shown in Fig. 3 and Table 41. While it was difficult to establish a specific time at which attachment interference begins, there was a pattern in all trials in which there was a time at which the plaque numbers were doubled.
Fig. 2. Per cent of virus detected as unattached PFU's after each of 3 successive attachment periods to CEK CELi, WCEF1, CEF1 and PK-15 cell monolayers.
SUCCESSIVE ATTACHMENT

EXP. 1
CEK CELLS
WCEF1 CELLS
Celi CELLS

EXP. 2
CEF1 CELLS
PK-15 CELLS

RELATIVE % OF PFU'S ATTACHED

1st 2nd 3rd

1st 2nd 3rd
Table 7. Results of the comparison of PFU's on singly and doubly infected CEK cell monolayers

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Number of doses of virus allowed to attach to cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean plaque count ± 2S&lt;sub&gt;x&lt;/sub&gt;</th>
<th>Percent increase in plaques in doubly infected cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>148 ± 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>158 ± 10</td>
<td>7%</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>168 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>215 ± 14</td>
<td>22%</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>25 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34 ± 1</td>
<td>36%</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>29 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33 ± 3</td>
<td>7%</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>97 ± 16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>142 ± 16</td>
<td>46%</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>20 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26 ± 3</td>
<td>30%</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>88 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>112 ± 3</td>
<td>27%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Attachment was at ambient temperature in trials 1-4 and 37° in trials 5-7.

<sup>b</sup>Means were from 2, 2, 3, 8, 5, 7 and 10 counts, respectively, in trials 1-7.
Fig. 3. Time of development of IBV attachment interference in CEK cell monolayers. Shaded areas indicate the interference observed in each trial.
MEAN COUNT OF SINGLY INFECTED CULTURES X2

OBSERVED MEAN COUNT IN DUALLY INFECTED CULTURES

TRIAL 4

TRIAL 5

AMBIENT

37°

MEAN NUMBER OF PLAQUES

MEAN NUMBER OF PLAQUES

0 30 60 90 0 30 60 90

TIME (MINUTES)

TIME (MINUTES)
Table 8. Effect on attachment of an initial exposure of NDV and IBV on a second exposure to the heterologous virus

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Initial virus exposure</th>
<th>Second virus exposure</th>
<th>Mean plaque count ± 2S&lt;sub&gt;Y&lt;/sub&gt;</th>
<th>Mean plaque count expected in dually exposed cultures&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IBV</td>
<td>-</td>
<td>22 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>-</td>
<td>36 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBV</td>
<td>NDV</td>
<td>57 ± 5</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>IBV</td>
<td>61 ± 5</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>IBV</td>
<td>-</td>
<td>44 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>-</td>
<td>20 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBV</td>
<td>NDV</td>
<td>26 ± 2 (NDV)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>42 ± 5 (IBV)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>NDV</td>
<td>IBV</td>
<td>28 ± 7 (NDV)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 ± 9 (IBV)</td>
<td>44</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total of mean counts of singly infected IBV and NDV cultures.

or nearly doubled in dually infected cultures and this was followed by a decrease in the percent of attachment of a second dose of virus.

**Ability of UV-irradiated virus to produce attachment interference**

Cultures of CEK cells treated for 90 minutes with 10<sup>6.2</sup> UV-inactivated PFU's of IBV were able to attach virus as
readily as untreated cultures. Five cultures previously treated with 10-second UV-irradiated IBV had a mean plaque count of $134 \pm 8$ while 5 untreated cultures had a mean count of $140 \pm 16$. The UV-irradiated virus used for treatment had 10 PFU's/ml, thus, only 1 PFU per count on UV-virus-treated plates was attributable to the treatment.

**Ability of NDV to establish IBV attachment interference**

The results in Table 8 show that cultures receiving NDV first did not interfere with IBV attachment nor did IBV interfere with NDV attachment.

**Normal variation of mean plaque counts**

Table 9 shows the variation of mean plaque counts encountered in 2 trials. All the means in the first trial overlap when 2 standard deviations of the mean are considered and all but 1 of the means in trial 2 overlap. Only the means of $22 \pm 2.8$ and $15 \pm 3.2$ do not overlap. The lowest mean represented a reduction of plaques by $31.8\%$ when compared to the highest mean, i.e., $\frac{22 - 15}{22} \times 100\% = 31.8\%$. For purposes of subsequent evaluation of inhibitors of IBV a change in plaque counts of $40\%$ was considered to be significant and changes of $30\%$ to $40\%$ were evaluated more closely before rejecting or classifying the substance as non-inhibitory.
Table 9. Results of experiment determining the variation in mean plaque counts that can be expected normally in an IBV-CEK cell system

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Dilution number</th>
<th>Parts $10^{-4}$ virus: parts diluent</th>
<th>Mean plaque count $\pm 2S_\bar{x}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.5: 4.5</td>
<td>25 $\pm$ 4.2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.5: 4.5</td>
<td>27 $\pm$ 3.8</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.5: 4.5</td>
<td>21 $\pm$ 5.4</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2.0:18.0</td>
<td>22 $\pm$ 3.4</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>5.0:45.0</td>
<td>18 $\pm$ 4.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2.0:18.0</td>
<td>21 $\pm$ 4.6</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2.0:18.0</td>
<td>16 $\pm$ 4.0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2.0:18.0</td>
<td>21 $\pm$ 4.6</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>2.0:18.0</td>
<td>15 $\pm$ 3.2</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.5: 4.5</td>
<td>17 $\pm$ 2.8</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.5: 4.5</td>
<td>19 $\pm$ 4.0</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.5: 4.5</td>
<td>22 $\pm$ 2.8</td>
</tr>
</tbody>
</table>

Thiols and SH reagents and their effect on IBV attachment

The results in Table 10 demonstrate the inhibitory activity of L-cysteine on IBV plaque counts when the substance is present at the time of attachment. Fig. 4 and Table 42 show the inhibitory dose response to L-cysteine and doses of about $10^{-2.3}$M to $10^{-3}$M seem to be optimal and there is a linear response of inhibition over an 8-fold decrease in L-cysteine.
Table 10. Effects of the presence of thiols on the attachment of IBV

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Trial number</th>
<th>Molar concentration of thiol</th>
<th>Mean plaque count ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine</td>
<td>1</td>
<td>10^{-2}_M</td>
<td>23 ± 5</td>
<td>-75%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-23}_M</td>
<td>26 ± 5</td>
<td>-72%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-3}_M</td>
<td>41 ± 10</td>
<td>-56%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-33}_M</td>
<td>43 ± 6</td>
<td>-54%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-4}_M</td>
<td>51 ± 5</td>
<td>-45%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>93 ± 7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10^{-2}_M</td>
<td>12 ± 2</td>
<td>-77%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-3}_M</td>
<td>9 ± 1</td>
<td>-83%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-4}_M</td>
<td>25 ± 3</td>
<td>-53%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>1</td>
<td>10^{-2}_M</td>
<td>30 ± 8</td>
<td>-64%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-3}_M</td>
<td>42 ± 11</td>
<td>-49%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-4}_M</td>
<td>69 ± 9</td>
<td>-17%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-5}_M</td>
<td>86 ± 4</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>1</td>
<td>10^{-2}_M</td>
<td>30 ± 5</td>
<td>-85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-3}_M</td>
<td>58 ± 5</td>
<td>-65%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-4}_M</td>
<td>151 ± 19</td>
<td>-23%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>197 ± 7</td>
</tr>
</tbody>
</table>
Table 10. (Continued)

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Trial number</th>
<th>Molar concentration of thiol</th>
<th>Mean plaque count $\pm 2S_e$</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-mercaptoethanol</td>
<td>2</td>
<td>$10^{-2}M$</td>
<td>$4 \pm 1$</td>
<td>-82%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}M$</td>
<td>$6 \pm 6$</td>
<td>-73%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-4}M$</td>
<td>$17 \pm 4$</td>
<td>-23%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-5}M$</td>
<td>$24 \pm 4$</td>
<td>+ 9%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td></td>
<td>$22 \pm 3$</td>
<td>---</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1</td>
<td>$10^{-2}M$</td>
<td>$4 \pm 1$</td>
<td>-99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}M$</td>
<td>$41 \pm 5$</td>
<td>-80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-4}M$</td>
<td>$119 \pm 7$</td>
<td>-40%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td></td>
<td>$197 \pm 7$</td>
<td>---</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>2</td>
<td>$10^{-2}M$</td>
<td>$1 \pm 1$</td>
<td>-99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}M$</td>
<td>$19 \pm 3$</td>
<td>-81%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-4}M$</td>
<td>$89 \pm 7$</td>
<td>-12%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-5}M$</td>
<td>$110 \pm 3$</td>
<td>+ 9%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td></td>
<td>$101 \pm 10$</td>
<td>---</td>
</tr>
</tbody>
</table>
Fig. 4. Dose response to L-cysteine as detected by the inhibition of IBV attachment to CEK cell monolayers.
% INHIBITION OF IBV PLAQUES

MOLAR CONCENTRATION OF L-CYSTEINE (X10^-4)

TRIAL 1
TRIAL 2
EXPECTED RESPONSE CURVE
concentration. The variation in dose response between the 2 trials probably comes from the fact that a new stock solution of L-cysteine must be made with each experiment, thus, standardization was rather difficult.

It was also shown that the thiols, reduced glutathione, 2-mercaptoethanol, and DTT, were equally as effective as L-cysteine in reducing the attachment of IBV (Table 10). A non-thiol reducing agent (ascorbic acid) was ineffective as an inhibitor of IBV (Table 43). The necessity of active thiol groups for inhibition of IBV is also shown (Table 43) by the fact that L-cystine and oxidized glutathione (disulfide compounds) are ineffective as inhibitors.

L-cysteine does not inactivate IBV as shown in Table 11 because the removal of L-cysteine by dialysis restores the activity of virus as demonstrated by its plaque-forming ability. The relatively low activity of the L-cysteine in the control on L-cysteine inhibition (33% and 35%) is probably due to the oxidation of L-cysteine during the overnight holding period, because in all other experiments in which 10^{-3}M L-cysteine is used as a fresh solution, its inhibitory activity was greater than 60%.

The results of the experiment to confirm that L-cysteine interfered with IBV attachment are shown in Table 12. In trials 1, 2, and 3, there was 54%, 71%, and 31%, respectively, more virus remaining unattached in inocula containing 10^{-3}M L-cysteine than in its absence.
Table 11. Results of experiments to determine if IBV is inactivated by L-cysteine

<table>
<thead>
<tr>
<th>Virus treatment</th>
<th>Trial number</th>
<th>Mean plaque count ± 2Sd</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus + 10^{-3}M L-cysteine</td>
<td>1</td>
<td>61 ± 7</td>
<td>-16%</td>
</tr>
<tr>
<td>(dialyzed overnight)</td>
<td>2</td>
<td>52 ± 10</td>
<td>+ 6%</td>
</tr>
<tr>
<td>Virus + 10^{-3}M L-cysteine</td>
<td>1</td>
<td>49 ± 7</td>
<td>-33%</td>
</tr>
<tr>
<td>(not dialyzed)</td>
<td>2</td>
<td>32 ± 6</td>
<td>-35%</td>
</tr>
<tr>
<td>Virus alone</td>
<td>1</td>
<td>73 ± 12</td>
<td>---</td>
</tr>
<tr>
<td>(dialyzed overnight)</td>
<td>2</td>
<td>49 ± 4</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 12. Effect of L-cysteine on IBV attachment

<table>
<thead>
<tr>
<th>Treatment of virus during first attachment</th>
<th>Trial number</th>
<th>Mean PFU's detected in inoculum containing L-cysteine</th>
<th>% increase of virus detectable in inoculum containing L-cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus + 10^{-3}M L-cysteine</td>
<td>1</td>
<td>194 ± 22</td>
<td>54%</td>
</tr>
<tr>
<td>(dialyzed overnight after removal from culture)</td>
<td>2</td>
<td>156 ± 6</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>134 ± 18</td>
<td>31%</td>
</tr>
<tr>
<td>Virus alone</td>
<td>1</td>
<td>126 ± 5</td>
<td>---</td>
</tr>
<tr>
<td>(dialyzed overnight after removal from culture)</td>
<td>2</td>
<td>40 ± 5</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>102 ± 6</td>
<td>---</td>
</tr>
</tbody>
</table>
All of the SH-reagents (PHMB, IA, and HgCl₂) were effective in reducing plaques as shown in Table 13. These compounds are very toxic for cells and there is a narrow range of concentration in which their activity on the virus can be detected and not also affect the cells. Table 14 shows that virus is inactivated by PHMB as the removal of unbound PHMB by dialysis does not restore virus infectivity.

The reversal of PHMB-inactivated IBV is shown in Table 15. At the concentration of $5 \times 10^{-3}$ M L-cysteine 43% and 44% of the PHMB-inactivated virus could be reactivated in the 2 trials. There was no increase in the reactivation of virus by increasing the concentration of L-cysteine 2-fold and in one trial this increase was much less effective than the lesser concentration.

The kinetics of IBV inactivation by PHMB at concentrations of $10^{-2}$, $10^{-3}$ and $10^{-4}$ are shown in Fig. 5 and Table 44. At concentrations of $10^{-2}$ and $10^{-3}$ M, the inactivation appears to proceed as a first order reaction during the first 60 minutes after which there is a "tailing" effect observed. There was little difference in the rate of inactivation between concentrations of $10^{-2}$ and $10^{-3}$ M PHMB, but there was a proportionately larger amount of virus inactivated with the higher concentration. There appeared to be a delay of 30 minutes before inactivation of IBV with $10^{-4}$ M PHMB became apparent.

Treatment of cells with PHMB prior to inoculation effectively reduced the sensitivity of CEK cell cultures for IBV.
Table 13. Effects of p-hydroxymercuribenzoate (PHMB), Iodoacetamide (IA) and HgCl₂ on IBV

<table>
<thead>
<tr>
<th>SH-reagent</th>
<th>Molar concentration</th>
<th>Mean plaque count ± 2Sₓ¹</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
</tr>
<tr>
<td>PHMB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻³M</td>
<td>Toxic</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10⁻³.3M</td>
<td>---</td>
<td>Toxic</td>
<td>---</td>
</tr>
<tr>
<td>10⁻⁴M</td>
<td>29 ± 3</td>
<td>30 ± 7</td>
<td>-65%</td>
</tr>
<tr>
<td>10⁻⁴.4M</td>
<td>---</td>
<td>63 ± 9</td>
<td>---</td>
</tr>
<tr>
<td>10⁻⁵M</td>
<td>75 ± 5</td>
<td>75 ± 5</td>
<td>-10%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>83 ± 9</td>
<td>105 ± 3</td>
</tr>
</tbody>
</table>

| IA         |         |            |         |            |
| 10⁻³M      | Toxic   | ---        | ---      | ---        |
| 10⁻³.3M    | ---      | Toxic      | ---      | ---        |
| 10⁻³.6M    | ---      | Toxic      | ---      | ---        |
| 10⁻⁴M      | 36 ± 5  | 21 ± 5     | -65%     | -50%       |
| 10⁻⁴.3M    | ---      | 30 ± 4     | ---      | -29%       |
| 10⁻⁵M      | ---      | 30 ± 4     | ---      | -29%       |
| Virus control | ---  | 105 ± 3   | 42 ± 12 | ---        |

¹Means determined from 5 counts.

²Concentration of SH-reagent toxic for the cell culture.
Table 13. (Continued)

<table>
<thead>
<tr>
<th>SH-reagent</th>
<th>Molar concentration</th>
<th>Mean plaque count $\pm 2S_{\bar{x}}$</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>HgCl$_2$</td>
<td>$10^{-4}\text{M}$</td>
<td>Toxic</td>
<td>Toxic</td>
</tr>
<tr>
<td></td>
<td>$10^{-4.3}\text{M}$</td>
<td>---</td>
<td>Toxic</td>
</tr>
<tr>
<td></td>
<td>$10^{-4.6}\text{M}$</td>
<td>---</td>
<td>2 $\pm$ 1</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}\text{M}$</td>
<td>75 $\pm$ 12</td>
<td>33 $\pm$ 6</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}\text{M}$</td>
<td>102 $\pm$ 3</td>
<td>39 $\pm$ 9</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>108 $\pm$ 6</td>
<td>42 $\pm$ 12</td>
</tr>
</tbody>
</table>
Table 14. Results of experiment demonstrating that PHMB inactivates IBV

<table>
<thead>
<tr>
<th>Treatment of virus</th>
<th>Mean plaque counts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% inactivation of IBV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt;M PHMB for 1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(dialyzed 19 hours)</td>
<td>7 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt;M PHMB for 20 hours</td>
<td>0</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(dialyzed 19 hours)</td>
<td>49 ± 4</td>
<td>73 ± 13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means determined from 5 counts.

When cells were treated with L-cysteine, there was no effect observed. The results in Table 16 show that cell cultures treated prior to virus inoculation with 10<sup>-4</sup>M PHMB were 55% and 69% less sensitive to IBV than untreated cell cultures.

It was also found that incorporation of L-cysteine into the agar overlay medium had no significant effect on IBV plaque numbers. Plaque counts on 5 CEK cell cultures overlayed with 10<sup>-2</sup>M L-cysteine containing agar medium had a mean of 86 ± 9 while counts on 5 other CEK cell cultures without L-cysteine in the agar medium had a mean of 105 ± 4.
Table 15. Reactivation of PHMB inactivated IBV

<table>
<thead>
<tr>
<th>Treatment of virus</th>
<th>Mean plaque count ± 2Sx</th>
<th>Mean number of PFU's inactivated</th>
<th>% virus reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
</tr>
<tr>
<td>10⁻⁴M PHMB + 10⁻²L-cysteine</td>
<td>79 ± 12</td>
<td>48 ± 7</td>
<td>44</td>
</tr>
<tr>
<td>10⁻⁴M PHMB + 10⁻².3L-cysteine</td>
<td>81 ± 9</td>
<td>80 ± 5</td>
<td>42</td>
</tr>
<tr>
<td>10⁻⁴M PHMB</td>
<td>48 ± 5</td>
<td>24 ± 3</td>
<td>75</td>
</tr>
<tr>
<td>Virus control</td>
<td>123 ± 8</td>
<td>153 ± 23</td>
<td>0</td>
</tr>
</tbody>
</table>

a All virus treatment and the virus control were dialyzed overnight.

b Percent virus reactivation determined by the ratio:
\[
\frac{PFU's \text{ inactivated by PHMB} - PFU's \text{ inactivated by PHMB + L-cysteine}}{PFU's \text{ inactivated by PHMB}} \times 100\%
\]
Fig. 5. Kinetics of IBV inactivation using $10^{-2}$, $10^{-3}$, and $10^{-4}$M PHMB.
Table 16. Effect of L-cysteine and PHMB on the sensitivity of CEK cell cultures to IBV when treated with these compounds for 2 hours prior to inoculation of virus

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar concentration</th>
<th>Mean plaque count ± 2SE</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>10^-2 M</td>
<td>90 ± 19</td>
<td>167 ± 25</td>
</tr>
<tr>
<td></td>
<td>10^-3 M</td>
<td>92 ± 8</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>10^-4 M</td>
<td>100 ± 10</td>
<td>---</td>
</tr>
<tr>
<td>PHMB</td>
<td>10^-3 M</td>
<td>Toxic</td>
<td>Toxic</td>
</tr>
<tr>
<td></td>
<td>10^-4 M</td>
<td>62 ± 13</td>
<td>56 ± 7</td>
</tr>
<tr>
<td></td>
<td>10^-5 M</td>
<td>118 ± 12</td>
<td>114 ± 11</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>137 ± 8</td>
<td>181 ± 10</td>
</tr>
</tbody>
</table>

*aMeans determined from 5 counts.

The effect of L-cysteine, DTT and PHMB on the infectivity of NDV are shown in Table 17. The thiols, L-cysteine and DTT, did not reduce the attachment of NDV nor did PHMB at a concentration of 10^-4 M inactivate the virus.

**Determination of cell associated thiol groups**

**Qualitative determination** When the staining of CEK and CEL1 cells by mercury orange were compared, the following was observed. After 1 hour of staining the CEK cells were stained while the CEL1 cells showed very little visible stain
Table 17. Effect of L-cysteine, DTT and PHMB on NDV

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar concentration</th>
<th>Mean Plaque count ± 2S̄</th>
<th>% change in mean plaque count in trials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>10⁻²</td>
<td>---</td>
<td>26 ± 4</td>
</tr>
<tr>
<td></td>
<td>10⁻³</td>
<td>51 ± 10</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>DTT</td>
<td>10⁻³</td>
<td>49 ± 6</td>
<td>---</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>57 ± 11</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>PHMB</td>
<td>10⁻³</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>53 ± 7</td>
<td>250 ± 17</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>57 ± 11</td>
<td>276 ± 25</td>
</tr>
</tbody>
</table>
(Fig. 6). After 2 hours both cell types were stained and the intensity of staining was considered to be equal (Fig. 7). Both cell types were more intensely stained at 3 hours but further staining for up to 5 hours had no observable effect. The staining was almost completely blocked when the cells were treated with .001M PHMB prior to staining.

Quantitative determination When the lysate and nuclear-cell membrane fractions of CEK and CELi cells were compared (Table 18), there was little difference in the content of thiol groups in trials 1 and 2. In trial 3, however, the nuclear-cell membrane and lysate fractions of CELi cells had 2 and 4 times more thiol activity than did CEK cells. The observed difference in trial 3 may only reflect the difference in preparation of the cell fractions.

When the second procedure was applied for the quantitation of thiol groups of 5 different cell types, the results in Table 19 were obtained. The thiol group activity of each cell type is illustrated graphically in Fig. 8. While the cultures were incubating in the DTNB reagent they were examined microscopically and all cell culture types appeared to remain intact and attached to the culture vessel during the 6-hour incubation. At 20 hours, however, the cells had detached from the vessel and all cells appeared to be lysed. Because of this complete lysis, the thiol activity detected at this time was regarded as the total thiol activity of the culture. The thiol activity of CEK and CELi cultures was essentially equal
Fig. 6. Appearance of CELi (left) and CEK (right) cells after 1 hour staining with mercury orange.

Fig. 7. Appearance of CELi (left) and CEK (right) cells after 2 hours staining with mercury orange.
Table 18. Quantitative determination of thiol groups in a lysate fraction and a nuclear-cell membrane fraction of CEK and CELi cells in culture

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Cell type</th>
<th>Lysate fraction</th>
<th>Nuclear-cell membrane fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CEK</td>
<td>$1.19 \times 10^{-4}$ M</td>
<td>$0.286 \times 10^{-4}$ M</td>
</tr>
<tr>
<td></td>
<td>CELi</td>
<td>$1.63 \times 10^{-4}$ M</td>
<td>$0.167 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>2</td>
<td>CEK</td>
<td>$1.46 \times 10^{-4}$ M</td>
<td>$0.385 \times 10^{-4}$ M</td>
</tr>
<tr>
<td></td>
<td>CELi</td>
<td>$1.6 \times 10^{-4}$ M</td>
<td>$0.49 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>3</td>
<td>CEK</td>
<td>$1.12 \times 10^{-4}$ M</td>
<td>$0.475 \times 10^{-4}$ M</td>
</tr>
<tr>
<td></td>
<td>CELi</td>
<td>$4.42 \times 10^{-4}$ M</td>
<td>$0.96 \times 10^{-4}$ M</td>
</tr>
</tbody>
</table>

with this procedure. The thiol activity of the two fibroblastic chicken cell types were 2 to 3 times less than the epithelial CEK and CELi cell types during the first 6 hours of cell-DTNB reagent incubation. At 20 hours, the total activity of the fibroblastic cultures was 1.2 to 1.4 times less active. The PK-15 cultures, which are epithelial in appearance, had thiol group activity similar to fibroblastic cultures during the first 6 hours but there total activity at 20 hours slightly exceeded that of the CEK and CELi cultures.
Table 19. Quantitation of thiol group activity of cultures of CEK, CEL1, WCEF1, CEF1, and PK-15 by reacting the CTNB reagent with intact cells in culture

<table>
<thead>
<tr>
<th>Reaction time of reagent with cells (hours)</th>
<th>Molar concentration of SH groups ( \times 10^{-4} )/cell culture of type:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEK 37(^\circ)</td>
</tr>
<tr>
<td></td>
<td>0.27 0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.32 0.23</td>
</tr>
<tr>
<td>2</td>
<td>1.08 0.45</td>
</tr>
<tr>
<td>3</td>
<td>1.65 0.58</td>
</tr>
<tr>
<td>4</td>
<td>1.73 0.71</td>
</tr>
<tr>
<td>5</td>
<td>1.74 0.74</td>
</tr>
<tr>
<td>6</td>
<td>4.74 3.84</td>
</tr>
</tbody>
</table>
Fig. 8. Thiol activity of CEK, CELi, WCEF1, CEF1 and PK-15 cells after incubation in DTNB reagent for different time intervals.
Inhibitors of IBV Attachment Found in Serum

Demonstration and quantitation of IBV-inhibitors in serum

Two bovine serum pools and a fetal calf serum pool were shown to have high IBV-inhibitory activity when present during the viral attachment period (Table 20). Activity was also demonstrated in serum pools of swine, chickens and rabbits (Table 20) with the highest apparent activity in the rabbit serum pool.

When the IBV-inhibitory activity of individual serums from 3 different species of animals were tested, it was found that 4 of 20 cattle, 10 of 10 rabbits, and 3 of 4 chickens had a high level of activity (Table 21). Again the rabbit serums were the most active with 94% or greater inhibitory activity with all 10 serums.

The quantitation of the inhibitory activity of serums could be accomplished by allowing 100 PFU's of IBV to attach in the presence of varying concentrations of serum. The results of the quantitation of 4 different serums (Fig. 9 and Table 45) showed that the 2 serum pools, #681 and #1181, reached a maximum inhibition at the 6% level and were considered to be comparable in inhibitory activity. Serum #98 had a moderate inhibitory activity at the 10% level and serum #5533 had no real significant activity up to the 10% level.
Table 20. Effect of pools of serum from cattle, swine, chickens, and rabbits on the attachment of IBV to CEK cell monolayers

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Serum type</th>
<th>Concentration of serum</th>
<th>Mean plaque count ± 2SE</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bovine #681</td>
<td>20%</td>
<td>5 ± 3</td>
<td>-93%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>72 ± 9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Bovine #681</td>
<td>5%</td>
<td>40 ± 8</td>
<td>-67%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>28 ± 9</td>
<td>-77%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20%</td>
<td>13 ± 7</td>
<td>-90%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>121 ± 19</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Bovine #951</td>
<td>5%</td>
<td>22 ± 3</td>
<td>-88%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>12 ± 3</td>
<td>-90%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>115 ± 16</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fetal calf</td>
<td>10%</td>
<td>10 ± 5</td>
<td>-95%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20%</td>
<td>5 ± 3</td>
<td>-99%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>198 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Swine</td>
<td>10%</td>
<td>20 ± 4</td>
<td>-78%</td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td>10%</td>
<td>20 ± 2</td>
<td>-78%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>92 ± 6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Rabbit</td>
<td>10%</td>
<td>6 ± 2</td>
<td>-96%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>156 ± 11</td>
<td></td>
</tr>
</tbody>
</table>
Table 21. Effect of some individual serums from cattle, chickens, and rabbits on the inhibition of IBV attachment. All serums tested at 10% concentration

<table>
<thead>
<tr>
<th>Species tested</th>
<th>Identification</th>
<th>Mean plaque count ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>4698</td>
<td>123 ± 30</td>
<td>+ 8%</td>
</tr>
<tr>
<td></td>
<td>4940</td>
<td>4 ± 2</td>
<td>-96%</td>
</tr>
<tr>
<td></td>
<td>4958</td>
<td>96 ± 10</td>
<td>-16%</td>
</tr>
<tr>
<td></td>
<td>5095</td>
<td>88 ± 24</td>
<td>-23%</td>
</tr>
<tr>
<td></td>
<td>5131</td>
<td>99 ± 16</td>
<td>-14%</td>
</tr>
<tr>
<td></td>
<td>5394</td>
<td>87 ± 9</td>
<td>-23%</td>
</tr>
<tr>
<td></td>
<td>5397</td>
<td>99 ± 12</td>
<td>-14%</td>
</tr>
<tr>
<td></td>
<td>5400</td>
<td>83 ± 7</td>
<td>-28%</td>
</tr>
<tr>
<td></td>
<td>5409</td>
<td>9 ± 6</td>
<td>-92%</td>
</tr>
<tr>
<td></td>
<td>5412</td>
<td>117 ± 6</td>
<td>+ 2%</td>
</tr>
<tr>
<td></td>
<td>5478</td>
<td>85 ± 9</td>
<td>-26%</td>
</tr>
<tr>
<td></td>
<td>5479</td>
<td>6 ± 3</td>
<td>-95%</td>
</tr>
<tr>
<td></td>
<td>5506</td>
<td>83 ± 16</td>
<td>-28%</td>
</tr>
<tr>
<td></td>
<td>5571</td>
<td>99 ± 24</td>
<td>-14%</td>
</tr>
<tr>
<td></td>
<td>5666</td>
<td>74 ± 20</td>
<td>-36%</td>
</tr>
<tr>
<td></td>
<td>5672</td>
<td>73 ± 5</td>
<td>-37%</td>
</tr>
<tr>
<td></td>
<td>5675</td>
<td>91 ± 14</td>
<td>-21%</td>
</tr>
<tr>
<td></td>
<td>5677</td>
<td>23 ± 10</td>
<td>-80%</td>
</tr>
<tr>
<td></td>
<td>5679</td>
<td>80 ± 20</td>
<td>-30%</td>
</tr>
<tr>
<td>Virus control</td>
<td>----</td>
<td>116 ± 16</td>
<td>----</td>
</tr>
</tbody>
</table>
Table 21. (Continued)

<table>
<thead>
<tr>
<th>Species tested</th>
<th>Identification</th>
<th>Mean plaque count ± 2Sx</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>1</td>
<td>2 ± 3</td>
<td>-98%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1 ± 2</td>
<td>-99%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2 ± 2</td>
<td>-98%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1 ± 2</td>
<td>-99%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1 ± 2</td>
<td>-98%</td>
</tr>
<tr>
<td>Virus control</td>
<td>--</td>
<td>100 ± 14</td>
<td>----</td>
</tr>
<tr>
<td>Rabbit</td>
<td>13</td>
<td>4 ± 0</td>
<td>-97%</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3 ± 2</td>
<td>-99%</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6 ± 2</td>
<td>-96%</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>4 ± 2</td>
<td>-97%</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>10 ± 3</td>
<td>-94%</td>
</tr>
<tr>
<td>Virus control</td>
<td>--</td>
<td>156 ± 12</td>
<td>----</td>
</tr>
<tr>
<td>Chicken</td>
<td>1</td>
<td>33 ± 2</td>
<td>-67%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17 ± 4</td>
<td>-83%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31 ± 12</td>
<td>-69%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>87 ± 9</td>
<td>-13%</td>
</tr>
<tr>
<td>Virus control</td>
<td>--</td>
<td>100 ± 14</td>
<td>----</td>
</tr>
</tbody>
</table>
Fig. 9. Quantitation of IBV-inhibitory activity of 2 bovine serum pools (#681 and #1181) and 2 individual bovine serums (#98 and #5533).
Table 22. Effect of bovine serum on the attachment of NDV to CEK cell monolayers

<table>
<thead>
<tr>
<th>Serum concentration</th>
<th>Mean plaque count ± 2SE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>2</td>
</tr>
<tr>
<td>20%</td>
<td>51 ± 12</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>10%</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Virus control</td>
<td>38 ± 4</td>
<td>57 ± 12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean determined from 4 counts.

**Effect of bovine serum on NDV plaques**

Three trials with bovine serum #681 showed that NDV was unaffected by the presence of serum at the level of 10% and 20% during viral attachment (Table 22).

**Effect of serum inhibitors applied to cell cultures prior to or following IBV attachment**

Table 23 shows the results of 4 experiments conducted to show the effect of IBV-inhibitory serum on the ability of CEK cells to attach IBV. When cell cultures were either implanted in a high concentration of serum or were treated with a high concentration of serum for 2 hours, there was a significant decrease in the attachment of IBV to such cultures. Also,
Table 23. Effect of exposure of cells to high concentrations of IBV-inhibitory serum upon their ability to attach IBV

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Treatment of cells</th>
<th>Mean plaque count $\pm 2S_{x}$</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a. Cells implanted in 20% serum</td>
<td>$29 \pm 7$</td>
<td>-31%</td>
</tr>
<tr>
<td></td>
<td>b. Cells implanted in 8% serum (control)</td>
<td>$42 \pm 6$</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>a. Cells implanted in 20% serum</td>
<td>$30 \pm 7$</td>
<td>-50%</td>
</tr>
<tr>
<td></td>
<td>b. Cells implanted in 8% serum (control)</td>
<td>$60 \pm 6$</td>
<td>----</td>
</tr>
<tr>
<td>3</td>
<td>a. Cells treated with 40% serum for 2 hours prior to virus inoculation</td>
<td>$96 \pm 12$</td>
<td>-30%</td>
</tr>
<tr>
<td></td>
<td>b. Cells treated with serum free medium for 2 hours prior to virus inoculation (control)</td>
<td>$137 \pm 8$</td>
<td>----</td>
</tr>
<tr>
<td>4</td>
<td>a. Cells implanted in 8% IBV-inhibitor free serum</td>
<td>$77 \pm 7$</td>
<td>+47%</td>
</tr>
<tr>
<td></td>
<td>b. Cells implanted in 8% IBV-inhibitor containing serum</td>
<td>$51 \pm 8$</td>
<td>----</td>
</tr>
</tbody>
</table>
attachment of IBV was increased by 47% when CEK cells were implanted in a non-inhibitory rather than an inhibitory bovine serum.

There was little or no effect on mean plaque counts when serum was incorporated into the agar-overlay medium at 2.5%, 5.0% and 20% concentrations as shown in Table 24. There was a reduction in the plaque size when serum was present and plaque sizes of 0.75 to 1.5 mm in diameter at 40 hours were observed with 5% serum-agar medium while they were 1.5-2.25 mm in diameter without serum in the agar. When 20% serum was used in trial 4 (Table 24) the plaques were very minute and required an additional 24 hours incubation before they were large enough to count with any accuracy.

Characterization of the IBV-inhibitor in bovine serum

Inhibitory fractions of bovine serum There was no inhibitory activity against IBV found in commercial preparations of bovine albumin, gamma globulin, or fetuin (Table 25). There was, however, high activity observed in a gamma globulin preparation of bovine serum #681 (Y681) obtained by precipitation with 1/3 volume saturated (NH₄)₂SO₄ (Table 25).

When the inhibitory activity of Y681 was compared to that of whole bovine serum #681, it was found that they were essentially equal (Fig. 10 and Table 46). The results of sequential precipitation of bovine serum #681 with 1/3, 1/2 and then 3/5 volume saturated (NH₄)₂SO₄ are given in Table 26. Essen-
Table 24. Effect of bovine serum #681 on mean plaque counts when incorporated into the agar-overlay medium

<table>
<thead>
<tr>
<th>Concentration of serum in agar medium</th>
<th>Mean plaque counts $\pm 2S_x$ in trial:</th>
<th>% change in mean plaque count in trial:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control (0%)</td>
<td>28 $\pm$ 4</td>
<td>88 $\pm$ 13</td>
</tr>
<tr>
<td>2.5%</td>
<td>20 $\pm$ 6</td>
<td>99 $\pm$ 9</td>
</tr>
<tr>
<td>5.0%</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>20.0%</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

$^a$Means were determined from 5 counts.
Table 25. Effect of some selected serum fractions on the attachment of IBV

<table>
<thead>
<tr>
<th>Serum fraction</th>
<th>Concentration tested</th>
<th>Mean plaque count ± 2Sx</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine albumin</td>
<td>0.2 gm/ml</td>
<td>25 ± 6</td>
<td>+13%</td>
</tr>
<tr>
<td>Bovine gamma globulin (commercial)</td>
<td>0.2 gm/ml</td>
<td>34 ± 4</td>
<td>+55%</td>
</tr>
<tr>
<td>Fetuin [(NH₄)₂SO₄ precipitated]</td>
<td>0.02 gm/ml</td>
<td>21 ± 3</td>
<td>- 4%</td>
</tr>
<tr>
<td>Fetuin (alcohol precipitated)</td>
<td>0.02 gm/ml</td>
<td>20 ± 3</td>
<td>- 9%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>22 ± 3</td>
<td>---</td>
</tr>
<tr>
<td>Bovine gamma globulin #681 [(NH₄)₂SO₄ precipitated]</td>
<td>10%</td>
<td>2 ± 1</td>
<td>-97%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>70 ± 8</td>
<td>---</td>
</tr>
</tbody>
</table>

tially all of the inhibitor is contained in the first precipitate. There was moderate activity in the second precipitate at levels of 20 and 30% while the third precipitate and the non-precipitated fraction had no significant inhibitory activity.

Destruction and removal of IBV-inhibitors in bovine serum

Heat stability Results in Table 27 show that the inhibitory activity of bovine serum #681 was essentially destroyed at 60° for 90 minutes and at 65° for 10 minutes. When unheated serum activity was compared with that of serum heated
Fig. 10. Quantitation of whole serum #681 and the \((\text{NH}_4)_2\text{SO}_4\) precipitated globulin fraction of serum #681.
% INHIBITION OF IBV PLAQUES

- O - O WHOLE SERUM No.681
- • - • Y GLOBULIN No.681

% SERUM
Table 26. Effect of serum fractions from bovine serum #681 sequentially precipitated with \((NH_4)_2SO_4\)

<table>
<thead>
<tr>
<th>Serum fraction of bovine serum #681</th>
<th>Concentration tested</th>
<th>Mean plaque count ± 2Sₓ</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3 volume ((NH_4)_2SO_4) precipitate</td>
<td>20%</td>
<td>9 ± 4</td>
<td>-91%</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>8 ± 3</td>
<td>-92%</td>
</tr>
<tr>
<td>1/2 volume ((NH_4)_2SO_4) precipitate</td>
<td>20%</td>
<td>61 ± 7</td>
<td>-42%</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>40 ± 3</td>
<td>-62%</td>
</tr>
<tr>
<td>3/5 volume ((NH_4)_2SO_4) precipitate</td>
<td>20%</td>
<td>108 ± 5</td>
<td>+ 3%</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>77 ± 7</td>
<td>-27%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>105 ± 4</td>
<td>---</td>
</tr>
<tr>
<td>Non-precipitated fraction</td>
<td>40%</td>
<td>110 ± 8</td>
<td>- 8%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>120 ± 6</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 27. Effect of $60^\circ$ and $65^\circ$ on IBV-inhibitory activity of bovine serum #681. A concentration of 10% serum was heated and then tested for IBV inhibition.

<table>
<thead>
<tr>
<th>Length of time serum heated in minutes</th>
<th>Temperature</th>
<th>Mean plaque count $\pm 2S_x$</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>0</td>
<td>---</td>
<td>$12 \pm 1$</td>
<td>$10 \pm 4$</td>
</tr>
<tr>
<td>15</td>
<td>$60^\circ$</td>
<td>$18 \pm 3$</td>
<td>$21 \pm 6$</td>
</tr>
<tr>
<td>30</td>
<td>$60^\circ$</td>
<td>$27 \pm 4$</td>
<td>$25 \pm 5$</td>
</tr>
<tr>
<td>60</td>
<td>$60^\circ$</td>
<td>$33 \pm 4$</td>
<td>$28 \pm 5$</td>
</tr>
<tr>
<td>90</td>
<td>$60^\circ$</td>
<td>$38 \pm 4$</td>
<td>$41 \pm 5$</td>
</tr>
<tr>
<td>120</td>
<td>$60^\circ$</td>
<td>$45 \pm 7$</td>
<td>$43 \pm 11$</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>$49 \pm 7$</td>
<td>$49 \pm 7$</td>
</tr>
<tr>
<td>0</td>
<td>---</td>
<td>$7 \pm 5$</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>$65^\circ$</td>
<td>$98 \pm 13$</td>
<td>---</td>
</tr>
<tr>
<td>20</td>
<td>$65^\circ$</td>
<td>$74 \pm 16$</td>
<td>---</td>
</tr>
<tr>
<td>30</td>
<td>$65^\circ$</td>
<td>$76 \pm 5$</td>
<td>---</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>$112 \pm 10$</td>
<td>---</td>
</tr>
</tbody>
</table>

at $56^\circ$ for 30 minutes and $65^\circ$ for 20 minutes, it was found that the $65^\circ$ treatment destroyed all activity and $56^\circ$ reduces the inhibitory only slightly (Fig. 11 and Table 47).

**Neuraminidase treatment**: Treatment of a 20% solution of bovine serum #681 with as high as 1 unit/ml of neur-
Fig. 11. Comparison of IBV-inhibitory activity of unheated serum #681 with that of the same serum heated at 56° for 30 minutes and 65° for 20 minutes.
aminidase had no effect on reducing its inhibitory activity (Table 28).

**Acetone treatment** Extraction of serum with acetone did not destroy the serum's inhibitory activity (Table 28).

**Kaolin adsorption** The inhibitory activity of serum could not be removed on kaolin as shown in Table 28.

**DTT treatment** Treatment of serum with either .001M or .01M DTT destroyed the majority of the IBV-inhibitor in bovine serum #681 (Table 28). It was also shown that alkylation with iodoacetamide was not necessary after DTT treatment to prevent restoration of the inhibitory activity after the DTT was removed by dialysis.

**Physical characterization of IBV-inhibitors in bovine serum**

**Ultrafiltration** When serum was passed through a collodion filter with pores less than 5 μm in diameter the IBV-inhibitory activity was completely retained by the filter (Table 29). These filters allow passage of molecules of 30,000 molecular weight or less to pass, thus, the IBV-inhibitor must be a relatively large molecule.

**Rate-zonal centrifugation** When a 6-fold concentrate of Y681 was subjected to a sucrose rate-zonal centrifugation, the results in Fig. 12 and Table 48 were found. The IBV-inhibitory activity was evenly distributed from the 4th fraction to the 8th fraction. It had been previously estab-
Table 28. Results of treatment of IBV-inhibitory bovine serum #681 with neuraminidase, acetone, kaolin and dithiothreitol (DTT)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration of:</th>
<th>Mean plaque count ± 2SE</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reagent</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>5.0 units</td>
<td>20%</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>.5 units</td>
<td>20%</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>.05 units</td>
<td>20%</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>.005 units</td>
<td>20%</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Untreated serum</td>
<td>---</td>
<td>20%</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>---</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Acetone extraction 1</td>
<td>---</td>
<td>10%</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>Acetone extraction 2</td>
<td>---</td>
<td>10%</td>
<td>67 ± 13</td>
</tr>
<tr>
<td>Untreated serum</td>
<td>---</td>
<td>10%</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>---</td>
<td>198 ± 9</td>
</tr>
<tr>
<td>Kaolin adsorbed</td>
<td>1 gm/5 ml</td>
<td>10%</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Untreated serum</td>
<td>---</td>
<td>10%</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>---</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>DTT treated Y681 (alkylated)</td>
<td>.001M</td>
<td>10%</td>
<td>123 ± 7</td>
</tr>
<tr>
<td>Untreated Y681</td>
<td>---</td>
<td>10%</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>---</td>
<td>166 ± 11</td>
</tr>
</tbody>
</table>
Table 28. (Continued)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration of:</th>
<th>Mean plaque count ± 2SE</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reagent Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTT treated γ681 (alkylated)</td>
<td>.01M 10%</td>
<td>84 ± 12</td>
<td>-12%</td>
</tr>
<tr>
<td>DTT treated γ681 (not alkylated)</td>
<td>.01M 10%</td>
<td>77 ± 4</td>
<td>-20%</td>
</tr>
<tr>
<td>Untreated γ681</td>
<td>----- 10%</td>
<td>7 ± 2</td>
<td>-93%</td>
</tr>
<tr>
<td>Virus control</td>
<td>----- ---</td>
<td>96 ± 11</td>
<td>----</td>
</tr>
</tbody>
</table>
Table 29. IBV-inhibitory activity of the retained portion and the filtrate of bovine serum #681 when filtered through a collodion ultrafilter

<table>
<thead>
<tr>
<th>Portion of ultrafiltered serum</th>
<th>Concentration tested</th>
<th>Mean plaque count ± 2Sx</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter-retained portion</td>
<td>20%</td>
<td>1 ± 0.6</td>
<td>-92%</td>
</tr>
<tr>
<td>Whole serum</td>
<td>20%</td>
<td>2 ± 0.8</td>
<td>-96%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>25 ± 3</td>
<td>----</td>
</tr>
<tr>
<td>Filtrate portion</td>
<td>20%</td>
<td>61 ± 6</td>
<td>+11%</td>
</tr>
<tr>
<td>Whole serum</td>
<td>20%</td>
<td>2 ± 0.7</td>
<td>-96%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>55 ± 7</td>
<td>----</td>
</tr>
</tbody>
</table>

lished that under these conditions of centrifugation the 75 globulins were primarily in fractions 3 to 5 and the 195 globulins in fractions 7 to 9 (Page et al., 1967).

Gel filtration The IBV-inhibitory activity of each fraction collected from the Sephadex G-200 column is shown in Table 49. When this activity is plotted graphically in relation to UV-absorbance at 280 m (Fig. 13), it can be seen that only samples 5-10 and sample 17 appeared to show significant levels of IBV inhibition and these samples lie near but just off of the peaks of O.D. 280. When samples 1-26 and 5-10 were pooled and concentrated, their inhibitory activities were almost equivalent and both had only about 50% of
Fig. 12. Distribution of IBV-inhibitory activity in fractions collected from a sucrose rate-zonal centrifugation.
INHIBITION BY 10% FRACTION CONCENTRATION

INHIBITION BY 5.55% FRACTION CONCENTRATION

% INHIBITION OF IBV PLAQUES

FRACTION COLLECTED FROM RATE-ZONAL CENTRIFUGATION
Fig. 13. Fractionation of $\gamma$ globulin #681 by gel filtration through Sephadex G-200 and the IBV-inhibitory activity associated with each fraction.
Table 30. IBV-inhibitory activity of concentrated pooled samples 1-26 and 5-10 from the Sephadex G-200 column

<table>
<thead>
<tr>
<th>Samples tested</th>
<th>Concentration</th>
<th>Mean plaque count $\pm 2S_x$</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled samples 1-26</td>
<td>10%</td>
<td>$30 \pm 5$</td>
<td>-57%</td>
</tr>
<tr>
<td>Pooled samples 5-10</td>
<td>10%</td>
<td>$49 \pm 8$</td>
<td>-43%</td>
</tr>
<tr>
<td>Pooled samples 5-10</td>
<td>20%</td>
<td>$23 \pm 3$</td>
<td>-67%</td>
</tr>
<tr>
<td>Y681</td>
<td>10%</td>
<td>$2 \pm 1$</td>
<td>-97%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>$70 \pm 8$</td>
<td>---</td>
</tr>
</tbody>
</table>

the activity of the original Y681 (Table 30). Thus, it appears that most of the activity in the first 26 samples from the column is found in samples 5-10.

Reversal of IBV-inhibitor activity in bovine serum

The results of the reversal effect of 15 sugars on the IBV-inhibitor of bovine serum are presented in Table 31. There were 4 sugars that did not significantly reverse the inhibitor, i.e., lactose, D-galactose, L-arabinose, and D-glucosamine. Eleven of the sugars reversed the inhibitory effect by 60% to 176% with the disaccharides being less active than the monosaccharides. The effects of some of the sugars alone on virus attachment are shown in Table 32. While the sucrose and glucose had no significant effect on mean plaque counts, there was a marked increase in plaque counts when the methylated glucosides were used.
Table 31. Effects of several simple sugars on the IBV-inhibitory effect of serum. Virus was allowed to attach in the presence of 1.5% sugar and 10% serum.

<table>
<thead>
<tr>
<th>Sugar tested</th>
<th>Mean plaque count ± 2Sx</th>
<th>% of IBV-inhibitory activity reversed by sugara</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>79 ± 20</td>
<td>---</td>
</tr>
<tr>
<td>Lactose</td>
<td>16 ± 5</td>
<td>---</td>
</tr>
<tr>
<td>Maltose</td>
<td>105 ± 10</td>
<td>---</td>
</tr>
<tr>
<td>D-glucose</td>
<td>130 ± 24</td>
<td>134 ± 17</td>
</tr>
<tr>
<td>L-glucose</td>
<td>---</td>
<td>90 ± 20</td>
</tr>
<tr>
<td>D-galactose</td>
<td>45 ± 20</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>D-mannose</td>
<td>---</td>
<td>120 ± 12</td>
</tr>
<tr>
<td>D-fructose</td>
<td>107 ± 8</td>
<td>122 ± 3</td>
</tr>
<tr>
<td>D-xylose</td>
<td>116 ± 3</td>
<td>71 ± 11</td>
</tr>
<tr>
<td>D-ribose</td>
<td>---</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>56 ± 11</td>
<td>28 ± 12</td>
</tr>
<tr>
<td>2-deoxy-D-glucose</td>
<td>---</td>
<td>120 ± 5</td>
</tr>
<tr>
<td>D-glucosamine</td>
<td>---</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>d-methyl-D-glucoside</td>
<td>---</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>/β-methyl-D-glucoside</td>
<td>---</td>
<td>76 ± 16</td>
</tr>
<tr>
<td>10% serum control</td>
<td>9 ± 1</td>
<td>8 ± 8</td>
</tr>
<tr>
<td>Virus control</td>
<td>143 ± 12</td>
<td>84 ± 8</td>
</tr>
</tbody>
</table>

aPercentage determined by the ratio of:

\[
\frac{\text{Mean count with sugar} - \text{Mean count serum control}}{\text{Mean count virus control} - \text{Mean count serum control}} \times 100\%
\]
Table 32. Effect of sucrose, glucose, and α and β methylated glucosides on attachment of IBV

<table>
<thead>
<tr>
<th>Sugar tested</th>
<th>Concentration</th>
<th>Mean plaque count ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>1.0%</td>
<td>138 ± 7</td>
<td>-3%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.5%</td>
<td>115 ± 6</td>
<td>-20%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>143 ± 12</td>
<td>---</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.5%</td>
<td>95 ± 15</td>
<td>+13%</td>
</tr>
<tr>
<td>α-methyl-D-glucoside</td>
<td>1.5%</td>
<td>166 ± 6</td>
<td>+98%</td>
</tr>
<tr>
<td>β-methyl-D-glucoside</td>
<td>1.5%</td>
<td>122 ± 10</td>
<td>+45%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>84 ± 8</td>
<td>---</td>
</tr>
<tr>
<td>α-methyl-D-glucoside</td>
<td>1.5%</td>
<td>109 ± 10</td>
<td>+60%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>68 ± 9</td>
<td>---</td>
</tr>
</tbody>
</table>

When CEK cells were treated with either 1.5% D-glucose or alpha-methyl-D-glucoside for 2 hours prior to inoculation with IBV, there was a 30% increase in plaque counts in the alpha-methyl-D-glucoside treated culture and no increase in the glucose treated cells (Table 33). This was considered significant since the inoculum came from a single dilution, thus reducing the possible variation due to dilution.

When virus was allowed to attach for 90 minutes in the presence of 10% serum and then either D-glucose, D-galactose
Table 33. Effect on IBV attachment by treating CEK cell monolayers with either D-glucose or alpha-methyl-D-glucoside for 2 hours prior to virus inoculation

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Concentration</th>
<th>Mean plaque count ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>1.5%</td>
<td>72 ± 4</td>
<td>+3%</td>
</tr>
<tr>
<td>α-methyl-D-glucoside</td>
<td>1.5%</td>
<td>93 ± 6</td>
<td>+30%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>70 ± 5</td>
<td>---</td>
</tr>
</tbody>
</table>

or alpha-methyl-D-glucoside was added and another 90-minute attachment period allowed, there was an effective reversal of the inhibitor by D-glucose and the methylated glucose but not by D-galactose (Table 34). The reversal was not as complete as when virus-serum-sugar mixtures are allowed to attach at the same time.

The IBV-inhibitors of swine, chicken, and rabbit serums were similar to those in bovine serums with respect to their reversal by alpha-methyl-D-glucoside. The results of their reversal are shown in Table 35. In trial 1, it can be seen that the levels of inhibitor were so high in rabbit serum that 1.5% alpha-methyl-D-glucoside only reversed the effect of 10% serum slightly. In Trial 2, it can be readily seen that 2 rabbit serums were reversed significantly only when 1% serum was used.
Table 34. Effect of adding sugars to inocula of IBV containing 10% inhibitory bovine serum after an initial 90-minute attachment

<table>
<thead>
<tr>
<th>Sugar added</th>
<th>Concentration</th>
<th>Mean plaque count ± 2S_x</th>
<th>% of IBV-inhibitory activity reversed by sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-methyl-D-glucoside</td>
<td>1.5%</td>
<td>122 ± 2</td>
<td>55 %</td>
</tr>
<tr>
<td>D-glucose</td>
<td>1.5%</td>
<td>89 ± 16</td>
<td>39 %</td>
</tr>
<tr>
<td>D-galactose</td>
<td>1.5%</td>
<td>18 ± 4</td>
<td>2.5%</td>
</tr>
<tr>
<td>10% serum control</td>
<td>----</td>
<td>13 ± 4</td>
<td>----</td>
</tr>
<tr>
<td>Virus control</td>
<td>----</td>
<td>210 ± 16</td>
<td>----</td>
</tr>
</tbody>
</table>

*Percentage determined by ratio of:*
\[
\frac{\text{Mean count with sugar} - \text{Mean count serum control}}{\text{Mean count of virus control} - \text{Mean count of serum control}} \times 100\%
\]

**Mode of action by IBV-inhibitors in serum**
When virus attachment was performed in the presence and absence of serum and the unattached virus assay assayed in the presence of 1.5% glucose, which reverse the inhibitor, there was 43 ± 7 PFU's remaining in inocula containing serum and 18 ± 4 PFU's in inocula without serum. This indicated that 2.5 times more virus remained unattached in the presence than in the absence of serum.

The effect of serum and serum-PHMB mixtures on the attachment of IBV showed that PHMB enhanced the reduction of plaques
Table 35. Reversal of the IBV-inhibitor of swine, chicken, and rabbit serums by 1.5% alpha-methyl-D-glucoside

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Type of serum</th>
<th>Concentration of serum</th>
<th>Mean plaque count ± 2S- ( \bar{x} )</th>
<th>% of IBV-inhibitory activity reversed[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Swine</td>
<td>10%</td>
<td>5 ± 3 96 ± 16</td>
<td>210%</td>
</tr>
<tr>
<td></td>
<td>Chicken #124</td>
<td>10%</td>
<td>24 ± 8 95 ± 16</td>
<td>300%</td>
</tr>
<tr>
<td></td>
<td>Chicken #240</td>
<td>10%</td>
<td>17 ± 2 95 ± 13</td>
<td>250%</td>
</tr>
<tr>
<td></td>
<td>Chicken #281</td>
<td>10%</td>
<td>27 ± 7 95 ± 9</td>
<td>320%</td>
</tr>
<tr>
<td></td>
<td>Rabbit #14</td>
<td>10%</td>
<td>1 ± 1 15 ± 2</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>Rabbit #15</td>
<td>10%</td>
<td>1 ± 1 17 ± 6</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>Rabbit #16</td>
<td>10%</td>
<td>2 ± 2 17 ± 10</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>Rabbit #17</td>
<td>10%</td>
<td>1 ± 1 16 ± 8</td>
<td>32%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>48 ± 6 48 ± 6</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Rabbit #16</td>
<td>1%</td>
<td>13 ± 3 56 ± 3</td>
<td>78%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3%</td>
<td>8 ± 2 29 ± 5</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%</td>
<td>2 ± 2 19 ± 3</td>
<td>26%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>2 ± 2 13 ± 5</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>Rabbit #17</td>
<td>1%</td>
<td>6 ± 3 50 ± 5</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3%</td>
<td>4 ± 2 26 ± 10</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%</td>
<td>4 ± 2 21 ± 5</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>1 ± 1 15 ± 3</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>Virus control</td>
<td>---</td>
<td>68 ± 9 68 ± 9</td>
<td>---</td>
</tr>
</tbody>
</table>

[^a]: Percentage expressed as a ratio of:
\[
\frac{\text{Mean count (serum and sugar)} - \text{Mean count (serum alone)}}{\text{Mean count (virus control) - Mean count (serum alone)}} \times 100\%
\]
Table 36. Effect of PHMB on the IBV-inhibitor of bovine serum when virus attachment in the presence of bovine serum and PHMB-bovine serum mixtures are compared

<table>
<thead>
<tr>
<th>Treatment of virus</th>
<th>Mean plaque count ± 2S_\bar{x}</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>PHMB</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>121 ± 18</td>
</tr>
<tr>
<td>0</td>
<td>10^{-4} M</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>5%</td>
<td>0</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>5%</td>
<td>10^{-4} M</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>10%</td>
<td>0</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>10%</td>
<td>10^{-4} M</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>20%</td>
<td>0</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>20%</td>
<td>10^{-4} M</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

when added to serum (Table 36). This is not true of cysteine-PHMB mixtures in which the inhibitory effect of both is reduced; thus, the active inhibitory groups of bovine serum IBV-inhibitors are probably not thiol groups.

Effect of bovine serum and filtrate of bovine serum on IBV cytopathological changes and plaque size When 10% and 20% whole bovine serum was used in culture medium of cells inoculated with 10^6.5 PFU's of virus, there was a sparing effect on the infected cells exhibited by the fact that they remained attached to the culture vessel for about 24 to 48 hours longer than infected cells with 1% serum (Figs. 14, 16,
Fig. 14. (Upper left) Fluorescent antibody staining of CEK cells 24 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 1% bovine serum.

Fig. 15. (Upper right) Fluorescent antibody staining of CEK cells 24 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 20% bovine serum filtrate.

Fig. 16. (Lower left) Fluorescent antibody staining of CEK cells 24 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 10% bovine serum.

Fig. 17. (Lower right) Fluorescent antibody staining of CEK cells 24 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 1% bovine serum filtrate.
17, 18 and 19). The spread of viral infection in cultures inoculated with $10^{2.5}$ PFU's was greatly curtailed in the presence of 10 and 20% serum as shown at 48 hours in Fig. 24. The serum filtrate exhibited neither the "cell sparing" property nor the "anti-spreading" property of whole serum. Figs. 14 and 15 show that when cell cultures were inoculated with $10^{6.5}$ PFU's of virus and contained 20% filtrate, the destruction of the monolayer was much more complete at 24 hours. Virus spread and cell destruction in cultures infected with $10^{2.5}$ PFU's of virus was much greater at 24 and 48 hours as shown in Figs. 20, 21, 22 and 23.

Fig. 25 shows 40-hour IBV plaques formed under agar medium containing serum filtrate, whole serum, and serum-free medium. The plaques range from 2-4 mm with 20% filtrate, 0.75-2 mm with 5% whole serum, and 1-3 mm with only agar medium.

**Effect of some selected compounds on IBV attachment**

**Salicylates** No effect on IBV attachment was observed by the presence of 3 different salicylates as shown in Table 50.

**Amantadine hydrochloride** When IBV was allowed to attach in the presence of concentrations of amantadine hydrochloride up to 100 µg/ml, there was no significant decrease in attachment (Table 50).

**Pancreatin** The results in Table 37 show that pancreatin alone increased plaque numbers significantly and
Fig. 18. (Upper left) Fluorescent antibody staining of CEK cells 48 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 10% bovine serum.

Fig. 19. (Upper right) Fluorescent antibody staining of CEK cells 48 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 20% bovine serum.

Fig. 20. (Lower left) Fluorescent antibody staining of CEK cells 24 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 1% bovine serum filtrate.

Fig. 21. (Lower right) Fluorescent antibody staining of CEK cells 24 hours postinfection with $10^6.5$ PFU's of IBV. The medium contained 20% bovine serum filtrate.
Fig. 22. (Upper left) Fluorescent antibody staining of CEK cells 48 hours postinfection with $10^{2.5}$ PFU's of IBV. The medium contained 1% bovine serum filtrate.

Fig. 23. (Upper right) Fluorescent antibody staining of CEK cells 48 hours postinfection with $10^{2.5}$ PFU's of IBV. The medium contained 20% bovine serum filtrate.

Fig. 24. (Lower left) Fluorescent antibody staining of CEK cells 48 hours postinfection with $10^{2.5}$ PFU's of IBV. The medium contained 10% bovine serum.
Fig. 25. Appearance of IBV plaques at 40 hours postinfection with agar overlay containing 5% bovine serum, serum-free medium, and 20% bovine serum filtrate. Top row - 5% serum overlay; middle row - serum-free overlay; and bottom row - 20% serum filtrate overlay.
Table 37. Effect on IBV attachment by pancreatin and combinations of pancreatin with either alpha-methyl-D-glucoside (AMG) or bovine serum

<table>
<thead>
<tr>
<th>Virus attachment in presence of:</th>
<th>Mean plaque count ± 2Sx</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatin (1:20)</td>
<td>172 ± 12</td>
<td>+ 90%</td>
</tr>
<tr>
<td>Pancreatin (1:30)</td>
<td>161 ± 23</td>
<td>+ 78%</td>
</tr>
<tr>
<td>Pancreatin (1:40)</td>
<td>148 ± 14</td>
<td>+ 54%</td>
</tr>
<tr>
<td>Pancreatin (1:20) + 1.5% AMG</td>
<td>184 ± 15</td>
<td>+102%</td>
</tr>
<tr>
<td>Pancreatin (1:30) + 1.5% AMG</td>
<td>170 ± 11</td>
<td>+ 88%</td>
</tr>
<tr>
<td>Pancreatin (1:40) + 1.5% AMG</td>
<td>142 ± 8</td>
<td>+ 48%</td>
</tr>
<tr>
<td>Pancreatin (1:20) + 5% serum</td>
<td>16 ± 3</td>
<td>- 83%</td>
</tr>
<tr>
<td>Pancreatin (1:30) + 5% serum</td>
<td>16 ± 3</td>
<td>- 83%</td>
</tr>
<tr>
<td>Pancreatin (1:40) + 5% serum</td>
<td>15 ± 4</td>
<td>- 84%</td>
</tr>
<tr>
<td>1.5% AMG</td>
<td>136 ± 3</td>
<td>+ 42%</td>
</tr>
<tr>
<td>5% serum</td>
<td>9 ± 3</td>
<td>- 91%</td>
</tr>
<tr>
<td>Virus control</td>
<td>96 ± 11</td>
<td>-----</td>
</tr>
</tbody>
</table>

When both pancreatin and alpha-methyl-D-glucoside were present, there seemed to be an even greater increase. When pancreatin and serum IBV-inhibitor were present, the inhibitor appeared to be only slightly less effective.

Neuraminidase and NANA. Table 38 shows that cell monolayers treated with 5 and 10 units of neuraminidase were less sensitive to IBV while these same concentrations did not
Table 38. Effect on IBV and NDV attachment by either neuraminidase treatment of CEK cell monolayers or presence of N-acetylneuraminic acid (NANA) during virus attachment period

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>IBV</th>
<th>NDV</th>
<th>IBV % change</th>
<th>NDV % change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidasea</td>
<td>25 units</td>
<td>22 ± 4</td>
<td>97 ± 3</td>
<td>-86%</td>
<td>-63%</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>153 ± 14</td>
<td>264 ± 1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Neuraminidasea</td>
<td>10 units</td>
<td>6 ± 2</td>
<td>79 ± 13</td>
<td>-83%</td>
<td>-18%</td>
</tr>
<tr>
<td></td>
<td>5 units</td>
<td>14 ± 4</td>
<td>103 ± 15</td>
<td>-60%</td>
<td>+ 7%</td>
</tr>
<tr>
<td></td>
<td>2.5 units</td>
<td>22 ± 4</td>
<td>115 ± 8</td>
<td>-37%</td>
<td>+20%</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>35 ± 7</td>
<td>96 ± 13</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NANA b</td>
<td>1.5%</td>
<td>9 ± 3</td>
<td>---</td>
<td>-96%</td>
<td>---</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>152 ± 15</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NANA b</td>
<td>1.5%</td>
<td>0.25 ± 0.5</td>
<td>31 ± 6</td>
<td>-98%</td>
<td>-67%</td>
</tr>
<tr>
<td></td>
<td>0.75%</td>
<td>1.0 ± 1</td>
<td>45 ± 11</td>
<td>-96%</td>
<td>-53%</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>27 ± 5</td>
<td>95 ± 3</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

a Cells were treated for 1 hour at 37° just prior to inoculation.

b Virus was allowed to attach in the presence of NANA.
significantly decrease NDV plaques; however, treatment with 25 units was effective against both viruses. When NANA was present during attachment of both IBV and NDV, there was a significant decrease in attachment of both viruses with IBV apparently being inhibited to the greatest extent (Table 38).

Lysosomal Changes in IBV Infected Cells

Histochemical procedure

When unfixed uninfected CEK and CELi cells were stained for acid phosphatase activity, the staining was confined to cytoplasmic foci with no diffuse staining observed. In CEK cells, lysosomes began to stain at 1 hour with more and more becoming evident until at 3 hours maximum staining was observed with all cells showing specific acid phosphatase staining. Figs. 26, 27, and 28 show uninfected CEK cells stained for 1, 2, and 3 hours. The photographs were taken with dark-field illumination, thus, the stained lysosomes appear as bright foci. The uninfected CELi cells exhibited a different staining pattern in that it required 2 hours of staining to detect lysosomes and by 3 hours the lysosomal staining was maximal (Figs. 29, 30, 31 and 32). The CELi cells also differed in that not all cells exhibited the same degree of acid phosphatase activity as did the CEK cells when stained maximally.

Infected cells of both CEK and CELi cell types exhibited the same acid phosphatase staining pattern for up to 12 hours
Fig. 26. (Upper left) Uninfected CEK cells incubated for 1 hour in $\beta$-glycerophosphate substrate. Acid phosphatase activity is detected as bright granules when observed by dark field illumination.

Fig. 27. (Upper right) Uninfected CEK cells incubated for 2 hours in $\beta$-glycerophosphate substrate. Dark field illumination.

Fig. 28. (Lower left) Uninfected CEK cells incubated for 3 hours in $\beta$-glycerophosphate substrate. Dark field illumination.
Fig. 29. (Upper left) Uninfected CELi cells incubated for 2 hours in α-glycerophosphate substrate. Dark field illumination and 72X magnification.

Fig. 30. (Upper right) Uninfected CELi cells incubated for 2 hours in α-glycerophosphate substrate. Bright field illumination and 480X magnification.

Fig. 31. (Lower left) Uninfected CELi cells incubated for 3 hours in α-glycerophosphate substrate. Dark field illumination and 72X magnification.

Fig. 32. (Lower right) Uninfected CELi cells incubated for 3 hours in α-glycerophosphate substrate. Bright field illumination and 480X magnification.
postinfection that was observed in uninfected cells. Figs. 33, 34, 35, and 36 show CEK cell 8 and 12 hours after IBV infection that were stained for acid phosphatase for 2 hours and then stained with fluorescent antibody. There is no direct relationship between infected cells as detected by fluorescence and cells showing acid phosphatase activity. Not until 18 hours postinfection was there observable deviation from normal acid phosphatase staining and these deviations were detectable in infected cells showing very advanced cytopathological changes. These dying cells were stained after shorter incubation in the substrate and there was no diffuse cytoplasmic staining, indicating release of the enzyme from lysosomes. This type of staining was especially evident in the large virus induced syncytia as shown in Figs. 37 and 38. The same staining pattern was observed with the infected CELi cells. The cytopathic effect of IBV in CELi cells differs in that cells do not die and release from the vessel as rapidly and viral induced syncytia are much smaller than in CEK cells. Figs. 39 and 40 show a small CELi syncytium 18 hours postinfection stained for acid phosphatase for 2 hours and then stained with fluorescent antibody. It can be readily seen that this syncytium does not exhibit any marked acid phosphatase activity.
Fig. 33. (Upper left) Infected CEK cells 8 hours postinfection. The cells were incubated for 2 hours in $\beta$-glycerophosphate substrate and stained with fluorescent antibody to show infected cells (300X magnification).

Fig. 34. (Upper right) Same field as Fig. 33 with dark field illumination to show acid phosphatase activity.

Fig. 35. (Lower left) Infected CEK cells 12 hours postinfection. The cells were incubated for 3 hours in $\beta$-glycerophosphate substrate and stained with fluorescent antibody to show infected cells (300X magnification).

Fig. 36. (Lower right) Same field as Fig. 34 with dark field illumination to show acid phosphatase activity.
Fig. 37. (Upper left) Infected CEK cells 18 hours postinfection. The cells were incubated for 2 hours in \( \beta \)-glycerophosphate substrate. Bright field illumination and 120X magnification.

Fig. 38. (Upper right) Syncytium observed in Fig. 37 with 480X magnification showing both localized and diffuse acid phosphatase staining of the cytoplasm.

Fig. 39. (Lower left) Infected CELi syncytium 18 hours post-infection. The cells were incubated for 2 hours in \( \beta \)-glycerophosphate substrate and stained with fluorescent antibody (480X magnification).

Fig. 40. (Lower right) The same syncytium as Fig. 39 with bright field illumination showing absence of acid phosphatase activity.
Table 39. Acid phosphatase activity of soluble (S) and mitochondrial-lysosomal (ML) fractions of CEK cells infected with IBV as compared to uninfected cells

<table>
<thead>
<tr>
<th>Hours after infection of CEK cell with $10^6.5$ PFU's of IBV</th>
<th>Acid phosphatase activity of:</th>
<th>O.D.</th>
<th>Sigma units activity$^a$/ 10 mg protein$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>ML</td>
</tr>
<tr>
<td>8 hour (uninfected)</td>
<td></td>
<td>.18</td>
<td>.12</td>
</tr>
<tr>
<td>8 hour (infected)</td>
<td></td>
<td>.15</td>
<td>.11</td>
</tr>
<tr>
<td>16 hour (uninfected)</td>
<td></td>
<td>.09</td>
<td>.12</td>
</tr>
<tr>
<td>16 hour (infected)</td>
<td></td>
<td>.07</td>
<td>.10</td>
</tr>
</tbody>
</table>

$^a$One Sigma unit of acid phosphatase liberates 1 μM of p-Nitrophenol per hour at pH 5 and 38°.

$^b$Protein determinations of each fraction were made by the biuret procedure (Gornall et al., 1949).

Qualitative procedure

The results of acid phosphatase assays of the soluble and ML fractions of IBV infected CEK cells confirmed the histochemical results and are shown in Table 39. There was no significant release of acid phosphatase activity from the ML fraction of CEK cells infected for either 8 or 16 hours.
DISCUSSION

The results of this study concerning the attachment of IBV to susceptible cell monolayers clearly reveals the complexity of this initial step of viral infection and replication. At least two factors affecting viral attachment, attachment interference and serum IBV-inhibitors, were shown to be present in the virus-cell systems studied. These, and possibly other factors, make the attachment of IBV to cells a very inefficient process. A third factor that might well be considered is that the cell systems as prepared in this study of IBV-cell interactions are difficult to standardize. The CEK cell cultures, the most IBV-sensitive cell type, do not contain a completely homogeneous cell population and some nonsusceptible cells are present in each culture. It was also shown that the chemical nature of IBV receptors is quite complex. At least 3 separate, seemingly unrelated, chemical groups may be involved: (1) thiol groups, (2) neuraminidase sensitive groups, and (3) serum IBV-inhibitor sensitive groups. The prevention of IBV attachment with relatively simple compounds such as cysteine and NANA was of great interest and surely substantiates the role of thiol groups and carbohydrates as a part of the cellular IBV receptor moieties. The reversal of the serum IBV-inhibitor with many simple sugars may eventually shed some light on which chemical groups function as attachment inhibitors. Receptors for IBV were shown to be similar to those of
NDV only in their sensitivity to neuraminidase and inhibition by NANA, while thiols, SH reagents, and serum did not alter NDV attachment.

In regards to virus attachment, two previous reports (Cunningham and Spring, 1965, and Lukert, 1965) had determined that maximum IBV attachment to CEK monolayers occurred by 90 minutes. In light of the findings of this study, it can be more correctly said that for a given IBV-CEK cell culture system essentially all of the virus that will attach to a particular culture has attached by 90 minutes but by no means has all of the infective virus been removed from the inoculum.

The attachment interference of a 2nd dose of IBV by a 1st dose of homologous virus appears to resemble that described for enteroviruses by Crowell and Syverton (1961). They initially found that HeLa cells continually carrying and producing coxsackievirus B3 interfered with coxsackievirus B1 and B5. Later reports by Crowell (1963 and 1966) showed that exposure of HeLa cells to high levels of virus (200 PFU's or more/cell) for 1 hour could also induce this interference and it was felt that the mechanism was either destruction or blockade of cell receptors.

The attachment interference stimulated in CEK cell monolayers by IBV was shown to become evident at around 1 hour at both ambient temperature and 37°C. Two facts strongly indicating that the homologous interference observed in this study is, indeed, an attachment interference are: (1) interference
develops at ambient temperature as rapidly and to the same extent as at $37^\circ$, and (2) a high portion of any inoculum remains as unattached virus, readily detectable in a fresh virus-cell system. Probably neither interferon production nor competition for intercellular sites essential for viral synthesis plays a role in this interference because these 2 mechanisms would require viral penetration and metabolically active cells. Since the penetration of IBV does not occur during a 90-minute attachment period as shown by its susceptibility to antibody neutralization (Lukert, 1966a), the latter 2 interference mechanisms are probably not a factor.

There were 2 striking differences between the attachment interference described here and that for coxsackieviruses. One difference was that dosages required to establish interference with IBV were much lower. Crowell (1966) found that 20 PFU's/cell did not cause interference, while in this study as few as 50 to 100 PFU's of IBV/culture were sufficient to induce interference. The second difference was that UV-irradiated virus could establish interference in the coxsackievirus system but could not in the case of IBV. If saturation or blockade of receptors was the mechanism of attachment interference for IBV, then at least 3 possibilities exist to explain why such low dosages of virus are effective and they are as follows: (1) There is a high ratio of non-infective virions or viral subunits to infective virus which are capable of saturating receptors. (2) There exists only a few receptors per cell,
thus, they are readily saturated. (3) The presence of IBV-inhibitors in the serum used for implantation of the cells may block most of the receptors thus making it possible to establish interference with low dosages of virus. It may well be that a combination of any 2 or all 3 of the above factors are responsible.

The fact that UV-irradiated virus did not establish interference is disturbing and does not support the receptor blockade theory. While this study did not establish whether UV-irradiated IBV is capable of attaching to cells, care was taken to not irradiate the virus excessively so that denaturation of the viral envelope material would be minimal. Baluda (1957) showed that UV-treated NDV could attach to erythrocytes and lung epithelial cells equally as well as active virus.

It appears that many types of interference other than that mediated by interferon exists. Interference between enteroviruses has been described that is not attributed to interferon, nor to attachment interference but it does involve very early events of viral replication (Drake, 1958; Ledinko, 1963; Cords and Holland 1964; and Pohjanpelto and Cooper, 1965). Baluda (1957) demonstrated that homologous interference with NDV was not attributable to the prevention of attachment of a second virus exposure but did probably occur at the cell surface to block the eclipse of the second virus.

Future studies on the mechanism of attachment interference with IBV should be done with cells implanted with
inhibitor-free serum. Virus should also be labelled with radioactive compounds to determine if UV-treated IBV will attach to cells. The attachment receptors of IBV may well be more susceptible to UV-irradiation than other virus types.

While attachment of IBV to cell monolayers was shown to be relatively inefficient, it was the most efficient in monolayer cultures of CEK cells which are about 30-60 times more sensitive than CELi cells (Lukert, 1965). Less IBV was shown to attach to CELi, WCEF1 and CEF1 cells which are susceptible but less sensitive cell types. The virus attached least effectively, or not at all, to the non-susceptible PK-15 cell type, thus it appears that the susceptibility and sensitivity of different cell types can be explained in part by differences in the cell's ability to attach virus. The WCEF1 and CEF1 cell cultures only differ from one another in respect to the relative number of epithelial cells present. The WCEF1 cells were prepared without removal of the viscera, thus, more epithelial type cells were evident than in CEF1 cells. The requirement of epithelial cells for initial virus infection has been shown for IBV (Lukert, 1966). These findings are similar to those of Holland et al. (1959) in which poliovirus did not attach to non-susceptible cells because they lacked specific receptors for the virus. Holland (1961) also showed that tissues refractory to polio in vivo became susceptible in vitro and evidently gained receptors upon cultivation. It appears that the opposite may be true with IBV, because upon
subculture of CEK cells they become less sensitive thus indicating a loss of receptors. Another possibility for the loss of sensitivity is that the susceptible epithelial cells fail to proliferate and cultures become predominately fibroblastic. Of great interest was the finding that the highest thiol group activity is found on the surface of primary CEK and CELi cell cultures as compared to primary fibroblastic cell cultures especially since thiol groups are a part of IBV-receptors. While these differences in thiol activity could not be directly related to the sensitivity of the cell types, there may be some correlation with the susceptibility of cells to IBV and also reflect basic differences between epithelial and fibroblastic cells. The epithelial PK-15 cell had surface thiol activity similar to fibroblasts and total thiol activity similar to primary chicken embryo epithelial cells. Whether primary swine kidney cells would demonstrate high surface thiol activity was not determined. It is interesting to speculate that surface thiol activity may be lost upon continued cultivation \textit{in vitro}.

Even though there was no correlation between the degree of sensitivity of cells to IBV and the relative amount of thiol activity associated with cell surface, it was clearly shown that thiol groups are intimately associated with IBV receptors on both the virus and cell. Thiol groups are active in many cellular components especially enzymes of all types and non-enzyme proteins of the cytoplasm, plasma membrane, and those
involved in movement such as ciliary proteins (Webb, 1966, pp. 635-653). The recent reports of Hoorn and Tyrrell (1966) and McIntosh et al., 1967, have described the isolation from humans of a new group of viruses, morphologically resembling IBV, using ciliated tracheal organ cultures. These viruses seem to have a fastidious requirement for ciliated epithelial cells and thus there may be a correlation between highly thiol active ciliary proteins and receptor substances for IBV-like viruses.

The inhibition of IBV attachment by cysteine and other thiols is probably one of competitive inhibition in which the compound acts as a cellular receptor to prevent cell-virus binding. The forces involved are evidently weak because the cysteine does not form a stable complex with the virus and is readily removed by dialysis. Lerner et al. (1963) found the receptors of erythrocytes agglutinated by reoviruses were not altered by PHMB, thus thiol groups were not a part of the cell receptor. The IBV-receptors of CEK cells were altered by PHMB, indicating that thiol groups are an integral part of the cell receptor, thus it is probable that only viruses having thiol-containing cell receptors would be inhibited by thiols such as cysteine.

An inhibitor of poliovirus type 1 found in horse serum was described by Takemoto and Habel (1959) and was associated with the gamma globulin fraction. It, like the IBV-inhibitor of bovine serum, prevented poliovirus attachment and reduced
plaque size when incorporated into agar-overlay medium. Pagano (1965) showed that the poliovirus inhibitor was found in bovine as well as horse serum and was associated with the 19S macro-

globulins. Thomssen et al. (1966) confirmed that the polio-
virus inhibitor of horse serum was 19S macroglobulin and they proposed that the inhibitor formed antibody-like complexes with the viral surface. Ackermann and Dinka (1965) found that the poliovirus inhibitory activity of bovine serum was distrib-
uted over a variety of sizes of macroglobulins. The IBV-
inhibitor was found in sera from at least 4 species and all rabbit serums seem to contain high quantities of this inhibitor. While the bovine serum IBV-inhibitor resembles the poliovirus inhibitors, it differs from those characterized by Pagano (1965) and Thomssen et al. (1966) in respect to size. The IBV-inhibitor is not solely a 19S macroglobulin and by both gel filtration and sucrose rate-zonal centrifugation was found to be associated with macroglobulins ranging from 7S to 19S in size with most of the activity between these two extremes. The poliovirus inhibitor characterized by Ackermann and Dinka (1965) more closely resembles the heterogeneous size of the IBV-inhibitor. Takemoto and Habel (1959) found the horse serum poliovirus inhibitor to resist 15-minute heating at 70°, and the bovine serum IBV-inhibitor was almost completely de-
stroyed in 10 minutes at 65°. Because the inhibitor of IBV was not affected by neuraminidase, kaolin adsorption, and acetone treatment, it is probably not similar to the serum
mucoprotein inhibitors of myxoviruses (Cohen et al., 1965) or the serum lipoprotein inhibitors of arboviruses (Clarke and Casals, 1958). It is probably a polymer of 7S gamma globulin because of its large molecular size as shown by gel filtration and its susceptibility to DTT.

The mode of action of serum IBV-inhibitor may well be from an ability to complex with the virus, however, it may also inhibit IBV by reacting with cellular receptors. The fact that its action can be prevented by the presence of simple sugars clearly differentiates this substance from antibody. Pagano (1965) found that dextran sulfate reversed the action of poliovirus inhibitor in horse serum and thus may be similar to the IBV-inhibitor in respect to reversal by saccharides. It would seem very plausible that the reversal of IBV-inhibitor by sugars may indicate that sugars, like thiol groups, are an integral part of viral and cellular receptors and may compete with virus and cells for the inhibitor substance. It is interesting to speculate that the spatial configuration around one carbon atom may be the determining factor as to whether a sugar will reverse the IBV-inhibitor. With the exception of D-glucosamine the non-reversing sugars, lactose, D-galactose, and L-arabinose, differ from the reversing sugars by the configuration around the 4th carbon atom.

Many of the sugars seemingly reversed the serum inhibitors completely (Table 31) and plaque counts were even significantly higher than in serum and sugar-free controls. These same
sugars alone, however, would not cause an increase in plaque counts. The explanation for this may be that the serum, no longer inhibitory due to the presence of sugar, protects virus from inactivation at $37^\circ$ and more viable virus is able to attach to and infect cells. It is not known what the mode of action is for the methylated glucose derivatives which produced significantly higher plaque counts when virus was allowed to attach in its presence. One possible explanation might be that these compounds have a greater affinity for the serum IBV-inhibitor than other sugars and is capable of removing the inhibitor from the cell surface, thereby exposing more cellular IBV-receptors.

The recent findings that simple sugars inhibited the hemagglutination of a number of enteroviruses (Lerner et al., 1965; Lerner et al., 1966; and Kunin, 1967) would seem to clearly show that these compounds are active in the virus-cell union. Lerner et al. (1966) proposed that the carbonyl groups of the sugars were the active groups involved in the inhibition of virus-erythrocyte attachment. Faust et al. (1967) reported that D-glucose binding to brush borders of intestinal epithelial cells is blocked by HgCl$_2$ and proposed that thiol groups are involved in the binding and transport of sugars. This would indicate that the mechanism of some virus-cell unions may be similar to that of a thiol-sugar interaction. The enterovirus group and IBV would be good candidates for an attachment mechanism such as this.
The inhibition of IBV attachment by NANA and removal of cellular receptors by neuraminidase presented the only similarity of IBV receptors with those of NDV. In fact, the IBV receptors appeared to be more sensitive to neuraminidase treatment and attachment of IBV was inhibited to a greater extent by NANA than was NDV attachment. This could be indicative of the fact that there are fewer IBV than NDV receptors/cell or that more cells, thus more receptors, are susceptible to NDV than IBV.

The separation of a serum portion that enhances the cytopathic effect and spread of IBV only illustrates the complexity of a biological fluid such as serum. This enhancing effect of the serum filtrate is evidently normally masked by the IBV-inhibitor substances and other large molecular weight substances that tend to spare cells from the cytopathic alterations caused by IBV. Further studies are needed as to the chemical nature of this enhancing substance, its mode of action, and to determine the range of viruses that are affected by it. The more rapid spread of virus and the increased plaque size in the presence of filtrate may be explained simply by the fact that virus is released more rapidly from infected cells.

The increased plaquing efficiency in the presence of pancreatin is of great interest but the mechanism of its action can only be speculative because of the complex nature of a crude enzymatic preparation of this kind. Zajac and Crowell (1965) reported that HeLa cells treated with pancreatin and
chymotrypsin were unable to attach group B coxsackieviruses and cells treated with trypsin failed to attach type 1 poliovirus. From this evidence, they postulated that the receptors for these two enteroviruses differed. Wallis et al. (1966a) reported that pancreatin and cysteine enhanced plaque numbers in monkey kidney cultures of many types of echoviruses and group A and B coxsackieviruses. The enzyme and cysteine were added to the agar overlay in the latter study and thus the results of the two studies may not be in conflict. Wallis et al. (1966b) also found that pancreatin enhanced the plaqueing efficiency of reovirus by incorporation of the enzyme into the agar overlay. From these results, it can be seen that the effect of crude enzymes may be variable and in each case a different component may be acting and the action probably depends upon the time at which the virus or cell is exposed to the enzyme preparation. In this study, the pancreatin may act to enhance IBV in at least two ways. It may remove the serum IBV-inhibitor from cellular receptor sites so that more receptors are available or the possibility exists that it is inhibiting the attachment interference mechanism, thus allowing a more efficient attachment. Further study would be required to determine what specific enzymes are active and their mode of action.

While the attachment receptors of IBV resemble those of enteroviruses and reoviruses in many respects, there are several basic differences. One is the fact that thiol groups
of the reovirus erythrocyte receptors are not active in attachment as in IBV (Lerner et al., 1963). Another difference is that enterovirus receptors are not altered by neuraminidase treatment (Zajac and Crowell, 1965). Finally, it appears that the attachment of enteroviruses, at least their attachment to erythrocytes, can be inhibited by simple sugars (Lerner et al., 1965; and Kunin, 1967) while IBV attachment is not.

The evidence presented in this study showed that conditions or substances which altered IBV attachment seemed to have an effect on both cells and virus. One explanation for this could be that IBV has a lipoprotein envelope (Berry et al., 1964) which is probably derived from the cell membrane. This fact may account for the complimentary nature of both the viral and cellular receptors as they may be similar chemically. This would not be the case with the enterovirus system where the nucleocapsid is probably made de novo.

The finding that lysosomes apparently play no role in IBV cytopathology confirms the results of Wolff and Bubel (1964) and Flanagan (1966) in which they demonstrated that not all viral cytopathology was a result of lysosome activation. Allison and Sandelin (1963) and Allison and Mallucci (1965) had proposed lysosome activation as a mechanism for all viral cytopathology. IBV infection did not alter the distribution of the enzyme nor the permeability of the lysosomal membrane of infected cells until very late in infection and these
alterations were considered to be secondary rather than primary responses to viral infection.

The observation that all liver cells in culture did not show the same acid phosphatase activity was in agreement with the in vitro studies of Novikoff (1959). He found that centrolobular hepatic cells exhibited much less acid phosphatase and acid esterase activity than did peripherolobular cells. Since both cells with high and low acid phosphatase were susceptible to IBV, it would appear that this particular enzyme has no bearing on the susceptibility of these cells to IBV.
SUMMARY

The attachment of IBV to cell monolayers was found to be very inefficient even to CEK cells, the most sensitive of the cell culture systems studied. While the kinetics of attachment were similar at 4°, 22°, and 37°, there was about 6 times more virus attached at 37° than at either 4° or 22°. IBV attached very poorly or not at all to non-susceptible cells. IBV attachment was also less efficient to the cell culture types that were less sensitive than CEK cultures. During the period of attachment there was an interference that developed, starting at around 60 minutes, which prevented further attachment of virus after 90 minutes. There was also a significant inhibition of attachment of a second dose of homologous virus. This attachment interference developed at ambient temperature and 37° and could not be stimulated with either UV-irradiated IBV or heterologous NDV.

Attachment receptors of both the virus and cell were shown to be sensitive to sulfhydryl reagents and attachment could be prevented with thiols such as cysteine and reduced glutathione. An IBV-inhibitor, which prevented viral attachment was demonstrated in bovine, swine, chicken, and rabbit serums. The inhibitor was proteinaceous in nature and was associated with gamma globulins ranging from 7S to 19S in molecular size. This IBV-inhibitor was susceptible to DTT, 65° for 10 minutes and its effect could be reversed using a
number of simple sugars. The inhibitor was shown to adsorb to cells and prevent virus attachment and when it was incorporated into the agar overlay, it reduced plaque size but not plaque numbers. The receptors for IBV were also shown to be neuraminidase-sensitive and the attachment of both IBV and NDV could be inhibited with NANA. This was the only similarity between NDV and IBV receptors as NDV was not sensitive to sulfhydryl reagents nor to the serum inhibitors.

Alpha and beta methylated glucose not only reversed the action of IBV-inhibitors in serum but also effectively increased plaque numbers when used alone. Pancreatin was also effective in increasing plaque counts significantly; however, neither the specific enzyme responsible nor the mode of action were determined.

It was shown both histochemically and quantitatively that the distribution of acid phosphatase activity of IBV infected cells during the early hours of infection was the same as that observed in uninfected cells and release of this enzyme from lysosomes did not occur until very late in viral infection. The conclusion of this study was that lysosomes play no part in the cytopathology induced by IBV and that the late changes were secondary to infection rather than primary.


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ACKNOWLEDGMENTS

This study was conducted under the United States Government Employees Training Act Public Law 85-507.

The author wishes to thank Dr. R. A. Packer for his suggestions during this study and for his editorial suggestions in preparing this manuscript.

The author also wishes to thank Mr. Jerold Peterson for his excellent technical assistance throughout the study and Mr. Ralph Glazier for his assistance in the preparation of the photographic work required for this manuscript.
Table 40. Effect of time on attachment of IBV

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Time of attachment (minutes)</th>
<th>Number of plaques</th>
<th>Mean (-) number of plaques $\pm 2S_x$</th>
<th>Ratio of: PFU's at 240 min. $\times 100%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>46,40</td>
<td>$43 \pm 6$</td>
<td>56%</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>48,41,36</td>
<td>$42 \pm 7$</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>44,44,36</td>
<td>$41 \pm 5$</td>
<td>53%</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>55,61,48</td>
<td>$51 \pm 9$</td>
<td>66%</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>69,52,78</td>
<td>$66 \pm 15$</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>78,74,73</td>
<td>$75 \pm 3$</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>71,77,83</td>
<td>$77 \pm 7$</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>5,13,10,7,10</td>
<td>$9 \pm 3$</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>16,30,16,24,16</td>
<td>$22 \pm 6$</td>
<td>49%</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>39,25,34,30,31</td>
<td>$32 \pm 5$</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>28,44,38,30,33</td>
<td>$36 \pm 6$</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>25,44,46,61,40</td>
<td>$43 \pm 12$</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>35,21,29,30,34</td>
<td>$30 \pm 5$</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>49,38,35,37,40</td>
<td>$42 \pm 5$</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>58,43,36,50,37</td>
<td>$45 \pm 8$</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 41. Results of trials comparing plaque counts on singly and dually infected CEK cell monolayers at progressively increasing attachment periods

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Time at which culture was dually infected</th>
<th>Number of doses of virus</th>
<th>Mean plaque count $\pm 2\sigma$</th>
<th>Percent increase in plaques in dually infected cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>1</td>
<td>$46 \pm 3$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$74 \pm 6$</td>
<td>61%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$75 \pm 12$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1</td>
<td>$119 \pm 14$</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$140 \pm 8$</td>
<td>105%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>1</td>
<td>$68 \pm 5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$95 \pm 14$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$194 \pm 8$</td>
<td>104%</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1</td>
<td>$100 \pm 5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$169 \pm 7$</td>
<td>69%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$111 \pm 8$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>1</td>
<td>$175 \pm 12$</td>
<td>53%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$22 \pm 5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$41 \pm 3$</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>1</td>
<td>$27 \pm 4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$38 \pm 4$</td>
<td>41%</td>
</tr>
</tbody>
</table>

$^a$Attachment temperature was 37° in the 1st 3 trials and ambient temperature in the 4th and 5th.

$^b$Mean was determined from 5 counts.
<table>
<thead>
<tr>
<th>Trial number</th>
<th>Time at which culture was dually infected</th>
<th>Number of doses of virus</th>
<th>Mean plaque count $\pm 2S_r$</th>
<th>Percent increase in plaques in dually infected cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>30</td>
<td>1</td>
<td>$44 \pm 4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$71 \pm 10$</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>$56 \pm 6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$79 \pm 8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>1</td>
<td>$70 \pm 5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$114 \pm 12$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>$54 \pm 6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$115 \pm 9$</td>
<td></td>
<td>110%</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>$86 \pm 9$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$180 \pm 7$</td>
<td></td>
<td>110%</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>$120 \pm 8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$200 \pm 10$</td>
<td></td>
<td>67%</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>$29 \pm 7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$63 \pm 7$</td>
<td></td>
<td>117%</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>$37 \pm 9$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$54 \pm 12$</td>
<td></td>
<td>45%</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>$57 \pm 9$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$69 \pm 7$</td>
<td></td>
<td>21%</td>
</tr>
</tbody>
</table>
Table 42. Results of dose response of L-cysteine to inhibition of IBV attachment

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Molar concentration of L-cysteine x 10^{-4}</th>
<th>Mean plaque count ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1^a</td>
<td>10.0</td>
<td>22 ± 3</td>
<td>-80%</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>28 ± 5</td>
<td>-74%</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>54 ± 9</td>
<td>-50%</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>70 ± 10</td>
<td>-36%</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>80 ± 10</td>
<td>-24%</td>
</tr>
<tr>
<td></td>
<td>0.3125</td>
<td>97 ± 11</td>
<td>-11%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>109 ± 5</td>
<td>---</td>
</tr>
<tr>
<td>2^b</td>
<td>10.0</td>
<td>38 ± 8</td>
<td>-62%</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>63 ± 12</td>
<td>-38%</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>88 ± 9</td>
<td>-23%</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>94 ± 6</td>
<td>- 7%</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>119 ± 12</td>
<td>+18%</td>
</tr>
<tr>
<td></td>
<td>0.3125</td>
<td>111 ± 7</td>
<td>+10%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>101 ± 9</td>
<td>---</td>
</tr>
</tbody>
</table>

^a Means determined from 7 counts.

^b Means determined from 5 counts.
Table 43. Effect of a non-thiol reducing agent (ascorbic acid) and two disulfide compounds (L-cystine and oxidized glutathione) on IBV attachment

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Mean plaque count</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ascorbic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-1}$M</td>
<td>129 ± 7</td>
<td>+7%</td>
<td></td>
</tr>
<tr>
<td>$10^{-2}$M</td>
<td>139 ± 26</td>
<td>+16%</td>
<td></td>
</tr>
<tr>
<td>$10^{-3}$M</td>
<td>140 ± 15</td>
<td>+17%</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>126 ± 14</td>
<td>+5%</td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$M</td>
<td>123 ± 10</td>
<td>+2%</td>
<td></td>
</tr>
<tr>
<td><strong>Virus control</strong></td>
<td></td>
<td>120 ± 7</td>
<td>----</td>
</tr>
<tr>
<td><strong>L-cystine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-2}$M</td>
<td>78 ± 12</td>
<td>-16%</td>
<td></td>
</tr>
<tr>
<td>$10^{-3}$M</td>
<td>80 ± 5</td>
<td>-14%</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>93 ± 7</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$M</td>
<td>91 ± 13</td>
<td>-2%</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$M</td>
<td>88 ± 10</td>
<td>-5%</td>
<td></td>
</tr>
<tr>
<td><strong>Virus control</strong></td>
<td></td>
<td>93 ± 10</td>
<td>----</td>
</tr>
<tr>
<td><strong>Oxidized glutathione</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-2}$M</td>
<td>96 ± 13</td>
<td>+16%</td>
<td></td>
</tr>
<tr>
<td>$10^{-3}$M</td>
<td>104 ± 9</td>
<td>+24%</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>84 ± 6</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$M</td>
<td>63 ± 6</td>
<td>-24%</td>
<td></td>
</tr>
<tr>
<td><strong>Virus control</strong></td>
<td></td>
<td>83 ± 9</td>
<td>----</td>
</tr>
</tbody>
</table>
Table 44. Kinetics of IBV inactivation with concentrations of \(10^{-2}\), \(10^{-3}\), \(10^{-4}\) M PHMB

<table>
<thead>
<tr>
<th>Time of reaction in minutes</th>
<th>(10^{-2}) M</th>
<th>(10^{-3}) M</th>
<th>(10^{-4}) M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 3</td>
</tr>
<tr>
<td>0</td>
<td>6.9</td>
<td>6.5</td>
<td>6.9</td>
</tr>
<tr>
<td>30</td>
<td>5.9</td>
<td>5.5</td>
<td>6.1</td>
</tr>
<tr>
<td>60</td>
<td>5.2</td>
<td>5.0</td>
<td>5.8</td>
</tr>
<tr>
<td>90</td>
<td>4.9</td>
<td>5.1</td>
<td>5.7</td>
</tr>
<tr>
<td>120</td>
<td>4.8</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>150</td>
<td>4.6</td>
<td>---</td>
<td>5.4</td>
</tr>
<tr>
<td>180</td>
<td>4.4</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 45. Results of quantitation of 4 bovine serums for their IBV-inhibitory activity

<table>
<thead>
<tr>
<th>Serum and identification</th>
<th>Concentration of serum</th>
<th>Mean plaque count ± 2S_σ</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine #681</td>
<td>0% (control)</td>
<td>138 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>93 ± 8</td>
<td>-33%</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>60 ± 1</td>
<td>-56%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>38 ± 5</td>
<td>-72%</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>35 ± 2</td>
<td>-75%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>24 ± 3</td>
<td>-83%</td>
</tr>
<tr>
<td></td>
<td>6%</td>
<td>17 ± 3</td>
<td>-88%</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>18 ± 3</td>
<td>-87%</td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>19 ± 2</td>
<td>-86%</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>15 ± 3</td>
<td>-89%</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>13 ± 3</td>
<td>-91%</td>
</tr>
<tr>
<td>Bovine #1181</td>
<td>0% (control)</td>
<td>70 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>39 ± 3</td>
<td>-44%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>19 ± 1</td>
<td>-73%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>20 ± 1</td>
<td>-72%</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>9 ± 1</td>
<td>-87%</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>11 ± 6</td>
<td>-84%</td>
</tr>
<tr>
<td>Bovine #98</td>
<td>0% (control)</td>
<td>48 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>51 ± 12</td>
<td>+ 6%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>44 ± 10</td>
<td>- 8%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>32 ± 9</td>
<td>-33%</td>
</tr>
</tbody>
</table>
Table 45. (Continued)

<table>
<thead>
<tr>
<th>Serum and identification</th>
<th>Concentration of serum</th>
<th>Mean plaque count ± 2Sx</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine #5533</td>
<td>10%</td>
<td>22 ± 9</td>
<td>-54%</td>
</tr>
<tr>
<td></td>
<td>0% (control)</td>
<td>48 ± 6</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>56 ± 13</td>
<td>+16%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>53 ± 5</td>
<td>+10%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>47 ± 17</td>
<td>- 1%</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>35 ± 9</td>
<td>-28%</td>
</tr>
</tbody>
</table>
Table 4-6. Results of comparative titration of (NH₄)₂SO₄ precipitated Y globulin and whole serum of bovine serum pool #681

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Concentration of material</th>
<th>Mean plaque count ± 2S_x</th>
<th>% inhibition of mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y681</td>
<td>2%</td>
<td>22 ± 11</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>13 ± 3</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>6%</td>
<td>10 ± 5</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>10 ± 3</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>8 ± 5</td>
<td>95%</td>
</tr>
<tr>
<td>Whole serum</td>
<td>2%</td>
<td>18 ± 13</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>12 ± 8</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>6%</td>
<td>10 ± 5</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>9 ± 5</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>5 ± 1</td>
<td>97%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>156 ± 12</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 47. Titration of bovine serum #681 heated at 56° for 36 minutes and 65° for 20 minutes in comparison to unheated serum

<table>
<thead>
<tr>
<th>Serum treatment</th>
<th>Concentration of serum</th>
<th>Mean plaque counts ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>56° for 30 min.</td>
<td>1%</td>
<td>39 ± 3</td>
<td>-44%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>19 ± 1</td>
<td>-73%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>20 ± 1</td>
<td>-72%</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>9 ± 1</td>
<td>-87%</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>11 ± 6</td>
<td>-84%</td>
</tr>
<tr>
<td>65° for 20 min.</td>
<td>1%</td>
<td>67 ± 2</td>
<td>-4%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>63 ± 13</td>
<td>-10%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>70 ± 9</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>61 ± 10</td>
<td>-13%</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>90 ± 15</td>
<td>+29%</td>
</tr>
<tr>
<td>Unheated serum</td>
<td>1%</td>
<td>20 ± 8</td>
<td>-68%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>8 ± 3</td>
<td>-89%</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>7 ± 1</td>
<td>-90%</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>6 ± 0</td>
<td>-91%</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>2 ± 2</td>
<td>-97%</td>
</tr>
<tr>
<td>Virus control</td>
<td>--</td>
<td>70 ± 5</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 48. Inhibitory activity of the 10 fractions obtained from a sucrose rate-zonal centrifugation of a gamma globulin fraction (125 mg protein/ml) of bovine serum #681. Each fraction was tested at a concentration of 10% and 5.55%.

<table>
<thead>
<tr>
<th>Fraction and % sucrose represented by each fraction before centrifugation</th>
<th>Mean plaque count $\pm 2S_X$</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10%$</td>
<td>$5.55%$</td>
</tr>
<tr>
<td>1 (sample)</td>
<td>$37 \pm 5$</td>
<td>$54 \pm 11$</td>
</tr>
<tr>
<td>2 (15%)</td>
<td>$40 \pm 6$</td>
<td>$58 \pm 14$</td>
</tr>
<tr>
<td>3 (15%)</td>
<td>$30 \pm 11$</td>
<td>$41 \pm 13$</td>
</tr>
<tr>
<td>4 (20%)</td>
<td>$15 \pm 7$</td>
<td>$38 \pm 5$</td>
</tr>
<tr>
<td>5 (20%)</td>
<td>$18 \pm 6$</td>
<td>$30 \pm 7$</td>
</tr>
<tr>
<td>6 (25%)</td>
<td>$19 \pm 5$</td>
<td>$32 \pm 5$</td>
</tr>
<tr>
<td>7 (25%)</td>
<td>$15 \pm 4$</td>
<td>$30 \pm 6$</td>
</tr>
<tr>
<td>8 (30%)</td>
<td>$17 \pm 3$</td>
<td>$35 \pm 5$</td>
</tr>
<tr>
<td>9 (30%)</td>
<td>$27 \pm 6$</td>
<td>$39 \pm 13$</td>
</tr>
<tr>
<td>10 (59%)</td>
<td>$56 \pm 9$</td>
<td>$47 \pm 6$</td>
</tr>
<tr>
<td>Virus control</td>
<td>$52 \pm 6$</td>
<td>$50 \pm 6$</td>
</tr>
</tbody>
</table>
Table 49. IBV-inhibitory effect of samples collected from Sephadex G200 column. Each sample was tested at a level of 10%

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Number of plaques detected</th>
<th>Mean plaques</th>
<th>% change in mean count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.36</td>
<td>43</td>
<td>-10%</td>
</tr>
<tr>
<td>2</td>
<td>34.47</td>
<td>41</td>
<td>-14%</td>
</tr>
<tr>
<td>3</td>
<td>33.38</td>
<td>36</td>
<td>-25%</td>
</tr>
<tr>
<td>4</td>
<td>42.48</td>
<td>45</td>
<td>-6%</td>
</tr>
<tr>
<td>5</td>
<td>26.29</td>
<td>28</td>
<td>-42%</td>
</tr>
<tr>
<td>6</td>
<td>30.22</td>
<td>26</td>
<td>-46%</td>
</tr>
<tr>
<td>7</td>
<td>41.41</td>
<td>41</td>
<td>-15%</td>
</tr>
<tr>
<td>8</td>
<td>30.31</td>
<td>31</td>
<td>-35%</td>
</tr>
<tr>
<td>9</td>
<td>31.33</td>
<td>32</td>
<td>-33%</td>
</tr>
<tr>
<td>10</td>
<td>29.34</td>
<td>32</td>
<td>-33%</td>
</tr>
<tr>
<td>11</td>
<td>43.33</td>
<td>38</td>
<td>-21%</td>
</tr>
<tr>
<td>12</td>
<td>37.35</td>
<td>36</td>
<td>-25%</td>
</tr>
<tr>
<td>13</td>
<td>39.39</td>
<td>39</td>
<td>-19%</td>
</tr>
<tr>
<td>14</td>
<td>40.37</td>
<td>39</td>
<td>-19%</td>
</tr>
<tr>
<td>15</td>
<td>41.35</td>
<td>38</td>
<td>-21%</td>
</tr>
<tr>
<td>16</td>
<td>44.49</td>
<td>47</td>
<td>-2%</td>
</tr>
<tr>
<td>17</td>
<td>28.29</td>
<td>29</td>
<td>-40%</td>
</tr>
<tr>
<td>18</td>
<td>38.52</td>
<td>46</td>
<td>-17%</td>
</tr>
<tr>
<td>19</td>
<td>43</td>
<td>43</td>
<td>-10%</td>
</tr>
<tr>
<td>20</td>
<td>48.49</td>
<td>49</td>
<td>+2%</td>
</tr>
<tr>
<td>21</td>
<td>47.44</td>
<td>46</td>
<td>-4%</td>
</tr>
</tbody>
</table>
Table 49. (Continued)

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Number of plaques detected</th>
<th>Mean plaques</th>
<th>% change in mean count</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>37,36</td>
<td>37</td>
<td>-23%</td>
</tr>
<tr>
<td>23</td>
<td>47,45</td>
<td>46</td>
<td>-4%</td>
</tr>
<tr>
<td>24</td>
<td>41,47</td>
<td>44</td>
<td>-8%</td>
</tr>
<tr>
<td>25</td>
<td>41,46</td>
<td>44</td>
<td>-8%</td>
</tr>
<tr>
<td>26</td>
<td>33,39</td>
<td>36</td>
<td>-25%</td>
</tr>
<tr>
<td>Virus control</td>
<td>42,39,47,54,55,42,43,62</td>
<td>48 ± 6</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 50. Effect of acetyl salicylate, para-amino salicylate, sodium salicylate, and amantadine hydrochloride on attachment of IBV to CEK cell monolayers

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Concentration</th>
<th>Mean plaque count ± 2S_x</th>
<th>% change in mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl salicylate</td>
<td>10^-1 M</td>
<td>Toxic to cells</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>10^-2 M</td>
<td>116 ± 12</td>
<td>+ 7%</td>
</tr>
<tr>
<td></td>
<td>10^-3 M</td>
<td>130 ± 17</td>
<td>+20%</td>
</tr>
<tr>
<td></td>
<td>10^-3.3 M</td>
<td>128 ± 14</td>
<td>+18%</td>
</tr>
<tr>
<td>p-amino salicylate</td>
<td>10^-1 M</td>
<td>86 ± 15</td>
<td>-20%</td>
</tr>
<tr>
<td></td>
<td>10^-2 M</td>
<td>112 ± 17</td>
<td>+ 4%</td>
</tr>
<tr>
<td></td>
<td>10^-3 M</td>
<td>112 ± 5</td>
<td>+ 4%</td>
</tr>
<tr>
<td></td>
<td>10^-3.3 M</td>
<td>117 ±</td>
<td>+ 8%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>108 ± 10</td>
<td>---</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>10^-1 M</td>
<td>Toxic to cells</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>10^-2 M</td>
<td>59 ± 8</td>
<td>- 2%</td>
</tr>
<tr>
<td></td>
<td>10^-3 M</td>
<td>57 ± 11</td>
<td>-5%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>60 ± 11</td>
<td>---</td>
</tr>
<tr>
<td>Amantadine HCl</td>
<td>100 μg</td>
<td>45 ± 13</td>
<td>-16%</td>
</tr>
<tr>
<td></td>
<td>50 μg</td>
<td>49 ± 14</td>
<td>- 9%</td>
</tr>
<tr>
<td></td>
<td>25 μg</td>
<td>46 ± 6</td>
<td>-15%</td>
</tr>
<tr>
<td></td>
<td>10 μg</td>
<td>55 ± 7</td>
<td>+ 2%</td>
</tr>
<tr>
<td>Virus control</td>
<td>---</td>
<td>54 ± 7</td>
<td>---</td>
</tr>
</tbody>
</table>