CHARACTERIZATION

OF AN ADENOVIRUS INHIBITOR IN BOVINE SERUM

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ABSTRACT

A viral inhibitor present in calf sera was found to neutralize infectivity of Adenovirus type 3 and other representative Adenovirus serotypes. The methods for determining the presence of inhibitor are outlined. The inhibitory activity was not detected in foetal calf, horse, chick or rabbit sera. Seventeen of fifty individual calf sera tested displayed inhibitory properties although the degree of inhibition varied among different types of Adenoviruses.

The inhibitor is non-dialyzable, is inactivated slowly at 65°C, rapidly at 70°C but is stable at 56°C for thirty minutes. Treatment of serum with trypsin removes the activity of the inhibitor while extraction with ether or absorption with kaolin does not. The inhibitory activity is precipitated with the globulin fraction by half saturation with ammonium sulphate, but only incompletely by 33% ammonium sulphate saturation. Density gradient centrifugation indicates that the inhibitor is homogeneous and is primarily associated with a large molecular serum fraction.

The possibility of the inhibitory material being conglutinin is considered and rejected, although conglutinin may be involved to some degree.

The dynamics of virus inactivation by inhibitory serum are not characteristic of neutralizing antibody.

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INTRODUCTION

Animal sera obtained from many different species contain inhibitors for many kinds of viruses. The presence of these inhibitors in animal sera have been reported for Poliovirus, Influenza virus, Adenovirus, Arborviruses and many others (Pagano, 1965; Sabin and Feltsteel, 1953; Gold, 1962; Sabin and Buescher, 1950) Investigations into these inhibitors have tried to determine the nature, mode of action and possible stimulus of these factors. Many authors have termed these inhibitors non-specific in their action in contrast to others who are satisfied that the presence of these inhibitors is due to a discrete antigenic stimulus. This question is at the root of all studies concerning inhibitory substances in that a relationship between non-specificity and inhibition may lead to some prophylactic method of dealing with disease processes due to virus infection.

The importance of investigating inhibitors in animal and human sera can be approached from three directions.

The first and of most practical importance is that of the effect of inhibitors on in vitro laboratory diagnostic work. Since normal animal serum is widely used as a nutrient for cells in tissue culture for the isolation of viruses, the presence of non-specific viral inhibitors may reduce the sensitivity of virus isolation procedures. There is at the present time an increased awareness of this problem in diagnostic laboratories with resultant tests to determine the most efficient use of

sera (viz., concentration and origin) that will allow reliable isolation techniques (Schmidt, 1965). The fact that inhibitors may be demonstrated by one diagnostic technique, viz., neutralization but not by another (hemagglutination) further complicates this problem.

Inhibitory serum components may provide more than a laboratory nuisance as investigations into their nature and properties may enable one to learn more about the viruses that are susceptible to these inhibitors. Knowledge of their biological and chemical properties would, together with accumulated information about any one virus, allow greater breadth for classification and possible understanding of virus-cell interaction.

The third approach would be the possible roles of these inhibitors in natural host resistance. The neutralizing or protective effect seen in vitro may, if applied to animal studies, somehow lead to knowledge concerning mode of virus-host interaction at the in vivo level.

This project is concerned with the investigation of inhibitors in animal sera that affect the infectivity of Adenovirus when inoculated onto cultured human amnion cells. It had been noted by Gold (1962) and Carmichael (1963) that members of the Adenoviruses were sensitive to inhibitors in bovine sera. Using Adenovirus type 3, the effect of exposure of the virus to various animal and individual calf sera has been studied. The incidence of these inhibitors in calf sera as well as their nature and character have been explored. Preliminary attempts to determine the mode of action have been carried out. This thesis then, relates attempts

to detect, to determine the incidence, to characterize and to explore the possible mode of action of inhibitory substances found in individual calf sera.

LITERATURE REVIEW

LITERATURE REVIEW

Investigators have been aware of the presence of inhibitors in normal animal sera for a number of years. These inhibitors have been found both in the tissue fluids and sera of animals (Ginsberg, 1960). The inhibitors considered in this review will be those which have been demonstrated to be present in sera of animals and humans. In the early work in this area, inhibitory substances, while demonstrated frequently, were not characterized to any great degree and have generally been allotted the title of non-specific inhibitors. The advent of tissue culture techniques, both for viral research and diagnostic studies, has stressed the importance of sensitive assay methods, and the resultant errors that non-specific inhibitors may cause has led to various methods of removing or detecting any inhibitors that might be present.

Inhibitors in various sera have been found for almost all the viruses that are isolated by tissue culture methods. A general and very real concern about inhibitors is whether they are really non-specific in action, or are they in fact specific antibodies stimulated by some unknown agent. Klein (1958) has laid down some base lines to govern possible distinctions between these two phenomena. First, antibody will be destroyed if heated at 80^0C for twenty minutes. He feels that any inhibitory activity persisting after this treatment would definitely eliminate it from being antibody. In practice, this is sometimes difficult to assess, and generally experimenters have run parallel inactivation samples of specific neutral-

izing antiserum with inhibitory serum to determine at what temperature and time antibody activity is diminished. This is found to be within wide range of the conditions mentioned by Klein. Secondly, antibody should not be affected by exposure to receptor destroying enzyme (RDE), periodate, trypsin, or fat solvents and should not be removable by centrifugation at high speeds. Thirdly, if inhibition can be demonstrated by three different diagnostic techniques, viz., a) hemagglutination inhibition, b) virus neutralization and by c) complement fixation, this would lend credence to the presence of antibody. Other criteria that Klein mentions are the rise in titer shown in acute and post exposure serum and finally the most conclusive, that of isolation of provoking virus and proof that it provided the original stimulus.

It will be realized that isolation of any inhibitor that is not specific antibody is very difficult. Soluble inhibitors of viruses are of varying chemical composition, viz., the inhibitor known as the Francis inhibitor is a mucoprotein, while other inhibitors have been demonstrated to be proteins, glycoproteins, mucopolysaccharides and lipoproteins (Ginsberg, 1960). The possibility of having two or more non-specific inhibitors present further complicates attempts at characterization. Inhibitors are classified empirically into different categories by virtue of their chemical properties and their stability to heating at 56°C for 30 minutes.

The three main categories on this basis are; (1) inhibitors of

lipid nature extractable by fat solvents, (2) heat-labile proteins and protein complexes and (3) proteins, protein complexes, and mucopolysaccharides which are relatively stable to heat (Allen, 1958). In this review inhibitors which fall within these classes will be considered. Major emphasis will be placed on inhibitors that are relatively stable after heat treatment at 56°C for 30 minutes; the heat-labile inhibitors by our definition being those that are inactivated by such treatment. Because the effects of heat-labile serum components may be easily avoided, their practical importance in virus research and laboratory diagnosis of virus disease is negligible compared with heat stable factors. However, their presence and interactions with viruses are of great theoretical importance. Heat-labile inhibitors have been shown to be associated with the properdin system in the inactivation of viruses (Finklestein, 1958). Ginsberg (1960) concludes in his summary of heat-labile inhibitors that the properdin system is important in all observed inhibition phenomena with unheated sera. Other materials that have been associated with altered in vitro interactions are; interferon production (Isaac, 1960), lysozyme (Klein, 1965) and more recently conglutinin (Joshi & Ingram 1965).

I. LIPID INHIBITORS

It has been demonstrated by many authors that fatty acids, lipids, soaps, detergents and other surface active agents inactivate certain viruses (Burnet & Lush, 1940; Findlay, 1943; Theiler, 1957).

Casal and Olitsky (1947) first noted the virucidal effect of lipids in normal serum. By obtaining lipid fractions from normal animal sera by acetone-ether treatment they demonstrated reduction in titers when these fractions were tested by neutralization tests. Serum lipids from mice, hamsters, rabbits and horses were found to be active. The viruses that were tested were those of St. Louis, Russian Far East, and Japanese B Encephalitis. Inactivation of these viruses took place in the presence of serum protein at 2 - 4°C. The inactivating agent was neither dialyzable nor diminished by heating at 99°C for one hour. Fractionation of the serum by several solvents did not remove any of the inactivating material from the various fractions.

Confirmation of Casal's work came from Utz (1948) who tested the effect of serum lipids on three strains of Psittacosis virus. He found that exposure of the native whole serum to virus did not inactivate the virus or inhibit neutralization titers but, if this serum was fractionated by lipid solvents, the fractions themselves were able to demonstrate inactivation. The virus inactivating property appeared to be in the phospholipid fraction of the serum. In further work, Utz (1949) showed that a lecithin-like fraction of sera, obtained from seven different animals, inactivated four strains of Influenza virus and one strain of Newcastle Disease Virus (NDV) but did not affect the hemagglutinating property of either active or heat inactivated Influenza virus. It could

not be identified categorically as lecithin however, for although its properties were similar to those of lecithin, tests with egg yolk and brain material, (both rich in lecithin) failed to yield an inactivating material. There was no detectable "Francis inhibitor" (Francis, 1947) in the lecithin-like fraction.

Normal human, rabbit, monkey and mouse sera were found by Sabin and Buescher (1950) to be capable of inhibiting hemagglutination by the Japanese encephalitis virus. This heat stable inhibitor was not affected by treatment with periodate. However, chloroform extraction removed 95% of the inhibitory activity, and did not affect the antibody titer. As hemagglutinins were demonstrated among other Arboviruses, additional non-specific hemagglutinin inhibitors were found in normal sera. The inhibitor found by Sabin does not act on the erythrocytes but appears rather to combine with the hemagglutinin. Sera from rodents and marsupials have also been shown to contain an inhibitor which inactivates Yellow Fever, Japanese B, St. Louis and West Nile viruses (Koprowski, 1946). The St. Louis virus normal inhibitor is only partially removed from serum by extracting with chloroform, a method that removes practically all of the inhibitor for the Japanese B hemagglutinin (Chanock & Sabin, 1953). However, precipitation and extraction with appropriate amounts of acetone yields a fraction which is practically devoid of normal inhibitor, but contains all the specific hemagglutination-inhibition antibody. It was found that

the St. Louis encephalitis hemagglutinin combines with specific antibody in different proportions from normal inhibitor. All normal human and rabbit sera tested contained the normal inhibitor in much higher concentration than was found in mouse brain extracts which also showed inhibition of hemagglutination.

In working with Polyoma virus it was found that normal sera of most animal species were capable of modifying the behaviour of the virus. Bovine serum at high dilutions neutralizes virus infectivity and inhibits hemagglutination (Mori et al., 1961; Schieble & Ackermann, 1961). Halpern in 1963 investigated the inhibitor of Polyoma hemagglutinin. By treating bovine serum with bentonite, a material which selectively absorbs basic proteins, it was found that the inhibitor could be removed from the serum, purified and concentrated. Studies with purified inhibitor showed that the kinetics of virus-inhibitor interaction closely resembles the kinetics of virus-erythrocyte interaction, viz., the product in each case is stable at 4°C (but not at 22°C or 37°C). Like the erythrocyte receptor it is a mucoprotein. The inhibitor is not affected by heat and when dialyzed against water there is no observable effect on the ability of the purified preparation to inhibit Polyoma hemagglutination, provided phosphate buffered saline was used for dilution of virus and inhibitor before testing. The inhibitor was sensitive to treatment with periodate, trypsin and neuraminidase. Borecky (1962) extended these studies, and considered the action of this mucoprotein

isolated from bovine serum on neutralizing the infectivity of other hemagglutinating viruses. He found that besides Polyoma virus, Mumps virus and the PR8, A/57, Swine and Tokyo B strains of Influenza virus were inhibited by the mucoprotein. However, Sendai, NDV, A/53, C and Lee strains of Influenza, and ECHO (3, 9, 10, 11) viruses were not affected. Whole bovine serum was also examined for its spectrum of hemagglutination - inhibitory activity against the above viruses. Inhibitory activity was demonstrated in whole bovine serum to which the hemagglutinin of all of the viruses tested were sensitive. It was found that the inhibitory titers seemed to have no relationship to those found with purified Polyoma virus mucoprotein inhibitor. These facts suggest that bovine serum contains a complex of inhibitory substances, only one of which is the purified mucoprotein inhibitor. Further evidence for this complexity is the ability of the serum to inhibit Polyoma virus hemagglutin with pre-incubation at $4^{o}C$ and to neutralize infectivity, neither of which is exhibited by the isolated mucoprotein.

II. HEAT LABILE INHIBITORS

The realization that there were labile inhibitors was evident very soon after the advent of virus neutralization techniques. Early workers did not examine the nature of these inhibitors as diligently as they might because of their labile nature being easily removed by heating at $56^{o}C$ for 30 minutes. The ability of factors in serum to

enhance specific neutralization has been described by many scientists. Gordon (1925), in one experiment, was able to demonstrate a marked reduction in virus neutralizing power when a fresh anti-vaccinial rabbit serum was heated. He further demonstrated that the loss could be restored by the addition of fresh samples of guinea-pig serum. Meuller (1931) showed that the neutralizing capacity of fowl antibody to Rous Sarcoma could be intensified, and the loss of titer of the heated antiserum restored by the addition of fresh guinea-pig serum. Morgan (1945) and Whitman (1947) both working with Western Equine Encephalomyelitis (WEE) virus, showed that fresh immune human serum stored at -70°C possesses far greater neutralizing activity than either heated serum or serum kept at 4°C. By adding fresh guinea-pig serum Morgan could restore the lost activity of sera kept at 4°C but not that of heated sera. Whitman, however, could restore the lost activity to old and to heated sera by adding fresh normal serum from guinea-pigs, monkeys, or humans. It was also found that the neutralizing capacity of unheated immune serum drops more precipitously on dilution than that of heated serum. Throughout this early work the enhancing component of normal animal serum is spoken of as an "accessory substance" rather than complement.

In a study of the involvement of complement (C') in the neutralization of WEE, Dozois (1949) separated or inactivated the various

components of C'. Hemolytic titration for hemolytic complement showed more C' in guinea-pig and human serum than in that of rabbit. He demonstrated that the active neutralizing agent for WEE in fresh rabbit serum is complement. This potentiating factor in the serum involved the 2nd, 3rd, and 4th components of hemolytic complement while the 1st component did not seem to be necessary in the neutralization reaction. Leymaster and Ward (1949), using Mumps virus, showed that the chick embryo protective titer of a mumps antiserum was markedly reduced by heat inactivation or by absorption with an antigen-antibody precipitate, and that this lost activity could be restored by adding unheated normal human serum, but not heated. Working with Dengue virus, Sabin (1950) found that normal guinea-pig, rabbit or human sera could restore the loss incurred by heating an immune serum. This nonspecific, complement-like, heat labile accessory substance produced the maximum inactivation of sensitized virus only after incubation in vitro for two hours at 37°C.

The above data would seem to definitely correlate the effect of heat labile inhibitory factors with that of complement. However, some reports dealing with the effects of normal serum on viral neutralization fail to indicate enhancement by addition of fresh normal serum. Howitt (1934) found that fresh guinea-pig complement had no effect on the neutralization of WEE by horse antiserum, while Florman

and Enders (1942), reported that fresh normal rabbit serum did not enhance the neutralization of Vaccinia virus by rabbit antiserum. In an extensive study, Ginsberg and Horsfall (1949) found a labile component present in the sera of human beings, guinea-pigs and rabbits that neutralizes the infectivity of Mumps, NDV, and Influenza A and B viruses. The labile component of these sera and of mouse serum also inhibits hemagglutination of chicken erythrocytes by these viruses. The component is inactivated by heating at 56°C for 30 minutes and upon storage at 4°C for periods longer than two weeks. Its activity is diminished by treatment with crystalline trypsin and destroyed by ethyl-alcohol suggesting that it may be an unstable protein or protein complex. In order to confirm the relationship of the heat labile factor and complement, studies were carried out by three different procedures:

(a) the study of serum from which single components of complement had been removed by chemical treatment, (b) the study of serum from which complement had been removed by an antigen-antibody precipitate and, (c) the study of serum from which most of the heat labile component had been removed by combination with virus.

None of these studies indicated that these two factors were in fact one, and it was concluded that the labile component was distinct from hemolytic complement. Bang (1951) demonstrated a heat-labile virus neutralizing factor of NDV in the sera of humans, monkeys, rats

rabbits and in small amounts in chicken serum. It was not detected in mouse serum. Reduction of hemolytic activity of complement by absorption of serum with an antigen-antibody complex did not remove the inhibitor. He found a drop in the neutralization index after ether extraction, and that the neutralization activity was present in the globulin fraction. In 1951 Broidy et al., reported the presence in normal guinea-pig serum of a labile substance capable of neutralizing the egg infectivity of Vaccinia virus. This nonspecific inhibitor was inactive against Vaccinia hemagglutinin. Serum can be stored at -60°C with no loss of activity, but rapidly deteriorates at 4°C after a few hours. The relation of the inhibitor to complement was considered. Removal of the hemolytic activity of C' from the serum did not appreciably reduce the activity of the viral inhibitor. Binding of complement to an antigen-antibody complex, which did remove hemolytic activity of C', did not reduce the virucidal activity of the serum. It was found that the active factor was not able to be dialyzed and that the rapid decline of nonspecific virucidal activity on dilution contrasted with the gradual loss of specific antibody activity. This inhibitor of Vaccinia virus was not present in a detectable amount in the sera of mice.

McCarty and Germer (1952) demonstrated the presence in guinea-pig, rabbit and human sera of two heat labile factors against Variola virus, one which enhanced specific neutralization by heated

human immune serum and another which acted like natural antibody in that it could itself neutralize Variola virus. They found that inhibitory activity was greatest in guinea-pig serum and least in human serum. It was found that if fresh unheated normal human serum, chosen because it lacked any significant inhibiting power when tested by itself, was added to a mixture of virus and heated immune serum, the extent to which the virus was neutralized was greatly increased. If, however, the normal serum was first inactivated by heat, this neutralizing power was lost. It was shown that this potentiating factor was also present in sera possessing a natural inhibiting factor. The neutralization of 50% or more of the virus was noted with these sera in tests with Herpes Simplex and Cowpox viruses as well as Variola virus. These factors appeared to be independent of each other and were not correlated directly with the hemolytic complement titer.

Much of the investigation into heat lability of nonspecific serum inhibitors evolved around the problems inherent in hemagglutination and neutralization tests with the Influenza viruses. At the present time there are four factors that have been described in various types of inhibition of Influenza viruses. The heat labile factors will be discussed in this section and the heat stable inhibitors in the following section.

Smith and Westwood (1948) found that the immune sera devoid of nonspecific inhibitor substances of heat stable α or Francis type

gave lower hemagglutinin inhibition titers against heat killed Influenza A viruses than against living virus. This heat labile inhibitor of hemagglutination of live Influenza virus was found in sera of mice, rabbits and guinea-pigs. Guinea-pig serum, which was particularly effective, was also shown to neutralize infective virus. Similar inhibitors were found in human sera. Chu (1951) described a heat labile factor in normal mouse serum which inhibited hemagglutination and neutralized the infectivity of Type A Influenza virus. This nonspecific substance inhibited live and heated virus to approximately the same extent. The inhibitor could be destroyed by treatment of mouse serum with crude filtrates of Vibrio comma, crude trypsin or kaolin but not by treatment with purified RDE, crystalline trypsin or periodate. In the presence of calcium ions, the inhibitor formed a stable complex with active virus but was not destroyed by it. This inhibitor had greater activity against unadapted virus than against mouse-adapted strains of virus. Adaptation of virus strains to mice or passage of virus in chick embryos in the presence of normal mouse serum led to the appearance of variants which were resistant to the inhibitor. This normal mouse serum factor has been termed "Chu inhibitor" or β inhibitor. In a study for the presence of this factor in serum (Sampaio, 1952) it was found that mouse serum is rich in Chu inhibitor but devoid of Francis inhibitor. Ferret and fowl sera, which contain high titers of Francis inhibitor, show negligible Chu

activity. Rabbit and guinea-pig sera, however, apparently contain both inhibitors. Broidy et al. (1955) reported that ox serum also contains a heat labile inhibitor similar to Chu inhibitor.

The influence of normal serum inhibitors on Types A, A' and B Influenza viruses in monkey kidney cell cultures was studied by Henry and Youngner (1957). A thermolabile substance, capable of neutralizing the cytopathogenic effects of Influenza A' strains, was demonstrated in rabbit, guinea-pig and calf sera. This factor was not active against Type B or mouse-pathogenic A strains and was believed to be identical to Chu inhibitor. Studies were carried out on a heat-labile virus-inactivating substance (VIS) found in human and mammalian sera against NDV (Karzon, 1956). This substance was inactivated by treatment with trypsin, streptokinase, ethylenediamine-tetra-acetate and tissue homogenates. After the union of virus and inhibitor, with subsequent destruction by any one of the above treatments, the investigators failed to observe the release of infectious virus. This indicates an irreversible inactivation by the inhibitor. This substance differed from Chu inhibitor in that the Chu inhibitor showed greater stability at $4^{o}C$ while ox serum with a high titer of Chu inhibitor failed to inactivate NDV infectivity.

In 1956, Wedgewood, Ginsberg and Pillemer reported on the inactivation of Newcastle Disease Virus of normal sera by a system of substances called the properdin system. The discovery and

isolation of properdin were the direct result of experiments carried out by Pillemer and his colleagues in an attempt to purify C'3, the third component of complement (Pillemer et al., 1954). Properdin, the four components of complement, and magnesium ions were collectively termed the properdin system. The absence of any single component of this system resulted in complete lack of activity. Properdin is a protein which has been demonstrated by investigation of its electrophoretic characteristics and physical properties to be an γ-1 globulin (Ginsberg, 1960). It sediments in the 19S fraction and consists of only a small fraction (less than 0.02%) of the total serum proteins. Whether properdin is a single substance or a number of substances is a point of contention but it is known that the components of complement and the divalent cation, magnesium, have to be present to demonstrate the activity of the properdin system. The exposure of serum to zymosan destroys the biological activity of properdin and material treated in this way is termed (RP) material (Wedgewood, 1956).

In referring to an earlier paper by Ginsberg (1949) concerning heat-labile inhibition of viruses, Wedgewood noted that a labile component present in normal serum inhibited NDV, Influenza A and B, and Mumps viruses as discussed earlier. The requirements and kinetics of the interaction of the properdin system with zymosan and certain erythrocytes bore a resemblance to the requirements indicated for virus-serum interaction as noted by Ginsberg. Wedgewood set out to relate the possible

effect of the properdin system on NDV. By treating serum with:

(1) heat, (2) exposure to zymosan, (3) depleted properdin serum (RP) with added active properdin and with (4) properdin alone, his results indicated that indeed properdin was necessary for the action of the heat-labile viral inhibitor in normal serum.

Properdin, however, was not effective alone as additional factors present in the serum were needed. Viral inhibition required all known components of the properdin system, with the removal of any one constituent resulting in a loss of inhibition. By replacing, after removal, any one constituent he was able to restore the inhibitory effect. The process of inhibition by serum resulted in a decrease in the amount of properdin available in the serum without any measurable effect on the components of complement. Generally, dissociation of the serum-virus complex did not occur as assayed by infectivity techniques, but upon prolonged incubation of inactive serum-virus mixtures with cation-exchange resin he did recover some, but not all, of the hemagglutinating activity of the virus. He interprets this finding as indicating the part played by magnesium as a possible integral part of the virus-properdin complex. The complex is probably accomplished through a magnesium bridge and chelation of magnesium at least partially dissociates the complex with resultant reactivation of the virus.

The inactivation of Herpes Simplex virus by the properdin system in normal rat serum has also been reported (Finkelstein, et al., 1958).

The inhibitory activity was removed by treatment with zymosan, was temperature dependent and required the presence of Mg^{++}. Karson (1960), extending his studies on VIS (virus inhibitor substance) in relation to NDV failed to reactivate virus after association with VIS. He found four different activities inactivated by treatment with VIS, viz., infectivity, interference, neurotoxicity for animals and loss of hemagglutination activity. By studying the kinetics of inactivation for heated and unheated serum he found presence of heat-stable inactivating substances in human and animal sera. The inactivation values by plaque assay (chick fibroblast monolayer) were found to be higher than those obtained for NDV inactivation by zymosan or plaque assay methods. There was no obvious relationship between C' values for human, rabbit and guinea-pig sera and VIS activity. RP treated serum when compared to its whole untreated serum control showed only moderate reduction in the degree of inhibition. This evidence of negative relationship to properdin was, however, contradicted by the finding that rabbit antiserum to human properdin absorbed with untreated human serum markedly reduced NDV VIS activity. He concluded that the inactivation of NDV by factors in serum was due in part to the properdin system with the presence of other factors in the serum that may play a role in inactivation of virus.

In studying the thermolabile virus neutralizing fractions of different sera for Influenza virus, Polyak (1959) discounted the association of properdin in the inactivation reaction. Polyak fractioned the sera of normal

rabbits, guinea-pigs, rats and mice by the Cohn ethanol method. He tested and found the labile inhibitor in fraction III of rabbit, guinea-pig and white mice sera. The remaining protein fractions had no effect on Influenza virus. The original whole sera and the 3 fractions from these animals were inactive against virulent strains of Influenza, but neutralized the infectivity and hemagglutinating activity of six avirulent strains that were tested. He found that both properdin and the labile inhibitors were in the III fraction and both were inactivated after treatment of serum with zymosan. In determining the blood levels of properdin and heat-labile substances, he found that in the white rat there were high levels of properdin but low incidences of heat-labile inhibitor. In contrast, heat-labile substances were high and properdin levels low in guinea-pig serum. Other differences were the sensitivity of the inhibitor to trypsin while properdin is not, and the fact that the literature reported that properdin quickly disappears from the sera of animals subjected to intensive X-irradiation (Wardlow and Pillemer, 1956) while this was not the case with thermolabile serum substances in the sera of mice with irradiation disease.

The lack of effect of the properdin system on different viruses has been reported by other authors. Southham (1960) found that the properdin system did not inactivate West Nile or Mengo virus nor did it enhance the virus neutralizing effect of specific antisera. In vivo alteration of serum properdin levels in mice had no detectable difference

on resultant susceptability to infection by these viruses. Polio, Coxackie, Echo and Adenoviruses are known not to be sensitive to inactivation by the properdin system (Ginsberg 1960, ibid 1956).

III. HEAT STABLE INHIBITORS

Inhibitors in Sera for Poliovirus. Many of the earliest observations concerning nonspecific inhibitors in serum that had been heated at 56°C for 30 minutes was in work concerned first with in vivo and later in vitro studies of Poliovirus. Flexner and Lewis (1910) were among the first to report the presence of nonspecific virus inhibitors in animal sera. While attempting to produce a therapeutic immune serum against poliomyelitis, they found that normal sheep serum possessed a slight neutralizing activity for Poliovirus. Jungeblut and Engle (1932) reported on the capacity of certain normal animal sera to neutralize the virus of poliomyelitis. Although sera of immature rhesus monkeys were inactive, sera from four of nine adult monkeys neutralized Poliovirus. However, sera from four sheep and one cock failed to inactivate virus. The authors concluded that certain normal animal sera possess neutralizing substances for Poliovirus which behave identically with antibodies but may occur under conditions which preclude previous contact with specific antigen. A comparison was made (Hammon, 1947) of sera from domestic cows, horses, chickens and birds and similar animals held on desert ranges or in isolation. Many sera from domestic

animals were found capable of neutralizing Poliovirus while few were positive among the isolated groups or wild species. The finding of Poliovirus neutralizing substances in the sera of a large number of domestic animals suggested the possibility of extrahuman reservoirs of poliomyelitis virus. Gordon (1945) reported the neutralization of Poliovirus by dog serum. Three out of thirty-seven canine sera collected following the 1943 epidemic of poliomyelitis in Chicago were found to neutralize the Lansing mouse-adapted Poliovirus. Hammon felt there was no adequate basis for assuming that anti-Lansing virus substances in the sera of certain domestic mammals resulted from infection with Poliovirus and such virus neutralizing substances may be the result of infection with an agent antigenically related to Poliovirus.

In looking for a possible relationship between a naturally occurring animal virus infection and the incidence of neutralization found in different animal sera, Chang and Wenner (1951) described reciprocal neutralization between an isolate from bovine encephalomyelitis and the Poliovirus Type 2 (Lansing) using monkey immune sera. This, the authors felt, would explain the perplexing observations regarding the occurrence of neutralizing antibodies for Lansing Poliovirus in body fluids of cattle and other mammals. Wenner (1953) extended this study further by inoculating calves with Type 2 Poliovirus. He was able to demonstrate neither infection, overt or occult, nor an antibody response in the animals. It therefore seemed unlikely that the occurrence of sub-

stances neutralizing the Type 2 Poliovirus in serum from cattle was the result of poliomyelitis infection, and probably resulted from non-specific inhibitor substances. Sabin and Fieldsteel (1953) found that colostrum from each of sixteen cows tested neutralized Type 2 (Lansing) Poliovirus. A smaller proportion of calf sera showed inhibitory properties. Neutralizing substances for Types 1 and 3 Polioviruses were also demonstrated in bovine sera. The antipoliomyelitic activity of bovine serum was found to resemble the activity of antibody in human serum, in that incubation of the mixtures was not necessary to demonstrate neutralization, and heating at $60^{\circ}C$ for 30 minutes did not remove the activity which was associated with the globulin fraction of the serum. Attempts were made to infect calves with a virulent strain of Poliovirus at a time when their serum was devoid of neutralizing activity against the virus, but it was impossible to produce even inapparent infection in the calves. These authors concluded that cattle were possibly infected with an agent or agents antigenically related to the Poliovirus.

The presence of neutralizing substances in sera of horses, hogs, chicks and cattle led Bartell and Klein (1955) to conclude that these factors were indeed true antibodies. Attempts to isolate Poliovirus from cattle were, however, unsuccessful. No neutralizing activity was found in the sera of dogs, cats, calves or lambs. These inhibitory factors in the sera of cattle neutralized all three types of Poliovirus, but to different degrees for any one virus type and any one serum. They

considered that the variation in level of inhibitor found in individual cattle sera indicated antibody rather than a non-specific substance. Further, the activity was precipitated in the globulin fraction and was not inactivated by heating at 56°C for 30 minutes.

The ability of normal bovine serum to neutralize all three types of Poliovirus was substantiated by Takemori in 1958. By using plaque overlay techniques, the authors isolated a mutant Poliovirus that was not inhibited by the bovine serum used in the overlay. This mutation was not accompanied by any change of antigenic type, rate of growth in Hela cells, or change in virulence for mice. Evidence that the inhibitor may differ both qualitatively and quantitatively was illustrated by the differing sensitivity of resistant mutants to sera from individual animals. Despite the ease with which inhibitor resistant mutants were obtained, the authors were unable to isolate Poliovirus particles genetically resistant to homologous antibody. Experimental evidence was obtained for the existence of several qualitatively distinct inhibitors. This survey would seem to indicate that Poliovirus neutralizing substances in normal bovine serum are not antibodies but nonspecific serum inhibitors.

An inhibitor of Type 1 Poliovirus was found in horse serum by Takemori and Habel (1959) that had no effect on the other types of Polio virus. The addition of 2.5% horse serum to the overlay medium caused a reduction in plaque size and a delay of 20 - 48 hours in plaque development. An increase of serum to 5% in the medium reduced by 50% both the plaque

size and plaque count. The inhibitor had no effect on Coxsackie, Vaccinia or ECM (encephalomyocarditis) virus. There was no evidence to indicate that the inhibitor was a virus stimulated antibody, and it differed from previous described bovine inhibitors in that it did not have neutralizing activity per se but rather reduced the rate of adsorption of virus to susceptible cells. Growth curve experiments revealed this reduced rate of adsorption to the cells and also the delayed release of virus from infected cells. This inhibitor was found in only two of eleven horse sera tested, and retention of this capacity to inhibit remained when tested over intervals of two months. The activity was in the gamma globulin fraction and was lost when heated for fifteen minutes at 80°C but not at 70°C for this time. Neutralization tests at various time periods when the mixture was held at 37°C showed no loss in virus activity which is in contrast to results obtained by Takemori (1958) for bovine inhibitor.

Arguments for the neutralizing substances in bovine sera being antibody were discussed in a paper by McFerran (1962) and his observations and conclusions parallel those of Bartell and Klein (1955). He found that the majority of adult cattle sera displayed inhibition against Poliovirus Type 2 rather than Type 3. This activity developed in calves between 6 and 16 months in age and was inactivated by trypsin but showed less heat stability than that of antibodies to Poliovirus in human sera.

Treatment with kaolin, bentonite and periodate did not alter the capacity to inhibit. The wide range of titers, transfer in colostrum, higher titers in older animals, and the fact that the inhibitor protects infected mice, substantiates the author's belief that the inhibitor is antibody. He does mention that resistance of calves to Poliovirus infection, the failure to recover virus from feces and the lack of support of viral growth in cultured bovine cells are strong points in favor of some type of antigenic stimulus being responsible for this neutralizing effect. Adult horse, sheep and monkey sera were tested for inhibitors to Poliovirus by Plummer (1963). He found that for each, the Type 2 inhibitor titers were highest and those of Type 3 inhibitor least. There was a statistically significant association between the presence in equine sera of inhibitors to Polioviruses Types 1 and 2, and between 1 and 3, but no such marked correlation between Types 2 and 3. The activity of the inhibitor was that of neutralizing the virus as revealed by experiments designed so as to take samples of the mixture held at 37°C over a period of time. The heat sensitivity of the equine inhibitors is much the same as that of Poliovirus antibodies prepared by hyperimmunization of horses. Gamma globulin precipitation brought a complete removal of inhibitory activity from the supernatant. The dissolved gamma globulin neutralized Poliovirus, although to a titer about two-fold lower than the original serum. The fact that the inhibitors neutralize the virus is contrary to the results reported by Takemoto and Habel (1959), (vide supra). Poliovirus was not

isolated from any of the horses and they seemed to be insusceptible to Poliovirus infection. The authors feel that because of the evidence accumulated for antigenic relationships between the three types of Poliovirus, and between Polioviruses and the various enteroviruses, that the inhibitors are stimulated by the more deeply situated antigens common to enteroviruses. Thus they are stimulated by various entero-virus infections experienced by the host during its life.

In a series of papers, Pagano (1964 a, b; 1965 a, b,) brought a new dimension to the matter of non-specific inhibitors by showing that they may display a degree of specificity. He found that certain bovine and equine sera inhibit some, but not all strains of Type 1 Poliovirus. By employing these inhibitory sera in conjunction with dextran sulfate he was able to use this difference in sensitivity as virus genetic markers, enabling him, for example, to discriminate reliably between the Type1 attenuated Poliovirus strains, CHAT and LS_c 2ab. An inhibitory horse serum was found that acted on CHAT strain with reduction of both the number and size of CHAT plaques. Plaque formation of the LS_c 2ab virus is even more strongly retarded by the inhibitory serum. He has also obtained a bovine serum that specifically inhibits the CHAT virus without affecting LS_c 2ab. The equine inhibitor can combine directly with the virus and inactivate it. Exposure of neutralized complexes of virus and equine serum to an acid environment (pH 2.5) causes dissociation of the complexes with recovery of the original quantity of infectious virus. Both

the virus and the inhibitor are unaltered after going through this treatment. Fractionation of the equine serum by gel filtration with Sephadex G-200 resulted in the CHAT plaques being significantly reduced in size only when treated with material from the region of the 19S globulins. In contrast, the LS_c 2ab virus is affected by material in the region of the 7S and possibly also the 10S globulins.

In the latest paper (1965, b) he considered some of the characteristics of the inhibitory bovine serum. Eight of the fifty-six bovine sera produced striking plaque reduction of CHAT strain of Poliovirus. Any one serum showed a high degree of specificity in that it inhibited CHAT plaque formation, but had little effect on the twenty-four other Type 1 strains tested, whether attenuated or virulent. The inhibitor was again a globulin located in the 19S peak by serum fractionation on Sephadex G-200. Intracellular virus production in the presence of the bovine serum inhibitor was unimpaired when assayed up to nineteen hours after infection. The inhibitory bovine sera neutralized infectious Poliovirus according to kinetics similar to those observed with dilute type specific antiserum. The complex was susceptible to dissociation by acid exposure. In experiments with sequential neutralization tests, the addition of fresh bovine serum to mixtures containing virus previously exposed to bovine serum did not cause neutralization of the remaining infectious virus. However, Type 1 antiserum did neutralize additional virus. Although bovine serum added to complexes of virus and Type 1

antiserum did not neutralize the residual virus, these results indicate that the inhibitors were not specific antibody.

Inhibitors in Sera for Influenza Viruses. The discovery that Influenza viruses agglutinate red blood cells (Hirst, 1941) made possible the development of a simple and rapid in vitro method for measuring virus in the pre-tissue culture era. This simple method, however, was soon complicated by the demonstration of heat stable nonspecific inhibitors of viral hemagglutination and later of virus infectivity in normal animal serum. The presence of these inhibitors in ferret, guina pig, fowl and human sera was noted by many investigators: (Burnet and McCrea, 1946; Smith and Westwood, 1948). Francis (1947) while investigating the properties of Type B Influenza virus found that after heating the virus for 30 minutes at 56°C the virus retained the capacity to agglutinate red blood cells but could no longer measure specific antigen when used as antigen in titrations of serum antibody. He found that by heating virus material, he was able to demonstrate hemagglutinin inhibitor which was more active against heated virus than live virus. The type of inhibitor has become known as the Francis or α inhibitor, and is present in normal tissues, secretions and sera of man and animals. In the section on heat-labile inhibitors (vide supra), reference was made to an inhibitor of Influenza viruses generally known as the Chu or β inhibitor. A subsequent study by Chu (1951) characterized the Francis inhibitor. He found that it is heat stable, is destroyed by receptor-

destroying enzyme (RDE) and periodate, and inhibits heated virus to much higher titer than live virus. It is active against both mouse-adapted and unadapted strains of flu virus, forming transient combination with active virus although it is subsequently destroyed by the virus. The α inhibitor does not, as a rule, neutralize Influenza virus in vivo. It is thought to be a mucopolysaccharide. By contrast, the Chu inhibitor is heat labile and is highly active against unadapted strains of Influenza A but only weakly so against mouse-adapted strains (Sampaio, 1953). Crude V. cholerae extracts, but not purified RDE, inactivate the inhibitor in normal mouse and rabbit sera.

The Francis phenomenon was originally described in relation to Influenza B (Lee), and a number of sera both normal and immune. The same phenomenon occurs with strains of Influenza A, namely, an increase in the inhibitory action of many normal sera against a standard agglutinating dose of virus when the virus is heated. McCrae and Burnet (1946) and McCrea (1946) demonstrated the differences between α and β inhibitors. Referring to the Chu inhibitor as a nonspecific inhibitor of living virus, they found that it is a gamma globulin completely recoverable in the precipitate at 33% saturation with ammonium sulfate. It is rapidly destroyed at 62°C and is relatively unaffected by periodate. The Francis inhibitor of heated virus is a mucoprotein, not completely recovered at 33% saturation and is rapidly inactivated by periodate and RDE. In studying the mechanism of the Francis phenomenon, Smith and West-

wood (1950) mention that prolonged heating of virus results in progressively increased Francis effect as long as any hemagglutinating activity remains. The inhibitor is active at room temperature and at 2°C. Crystalline trypsin inactivated α and β inhibitors in normal ferret, fowl, rabbit, guinea pig and mouse sera under conditions where no significant effect on specific antibody was observed (Sampaio and Isaacs, 1953).

The sera of many different animals were found to contain non-specific inhibitory substances (Smith, 1949). In addition to quantitative differences, species differ in respect to the qualitative behaviour of their serum inhibitors. Different strains of Influenza virus may vary widely in their sensitivity to these inhibitors (Hilleman and Werner, 1953). Smith, (1951), observed the presence of two differing heat stable inhibitors in serum. The first is inactivated by RDE and is presumed to be inhibitor. The enzyme is thought to destroy the receptors on the cell surface which are the points of attachment for the virus in the hemagglutinin reaction. The second inhibitor was not destroyed by RDE, and is most effective in inhibiting the hemagglutination caused by certain of the unheated A' viruses isolated in 1951. Investigation of the kind and amount of non-specific inhibitor in human sera for Influenza A, B, and C was made by Hilleman and Werner, (1953). They found a heat-stable, non-specific inhibitor for Influenza A and B hemagglutinins, but not for C. The nature of the inhibitor was of the α type (i.e., destroyed by RDE) and it showed a high degree of inhibition of PR8, FM, and Lee strains.

Electrophoretic studies of serum inhibitors of Influenza viruses was done by Levy (1959). Heat-stable inhibitors normally present in serum of man and certain animals were separated by starch gel electrophoresis. The hemagglutination inhibitors of a strain of Influenza A virus were segregated into two components in human, rabbit, horse and guinea pig sera. Heating of these fractions at 65°C for 30 minutes increased the hemagglutination inhibition titers. It was found that the Asian strain was neutralized by whole rabbit, horse and heated fractions of these sera, while the strain Lee virus was not affected.

By passage of Influenza A strains at limiting dilutions in normal horse serum two sub-strains of the original New York 1957 strain were isolated, (Choppin and Tamm, 1959). One strain was found to be sensitive to the inhibitor in horse serum, the other insensitive. The authors suggested that the flu A virus in 1957 consisted of two kinds of particles whose proportions varied in natural passage, depending on encounters with inhibitory substances.

Takatsy (1959) noted that non-avid (Q phase) strains of Influenza virus are not inhibited by normal sera, while avid (R phase) strains are. He found a factor in horse serum that was different from the heat stabile α inhibitor described. This new inhibitor is destroyed by boiling, is partially inactivated by periodate and is resistant to crude RDE. Purified material prepared by ammonium sulphate precipitation contains no carbohydrate although the Francis (α) inhibitor does. This new inhibitory effect

was noted by other investigators (Shimojo, 1959; Zhdanor, 1959; Cohen and Belyavin, 1959) and called inhibitor by Cohen. The inhibitor is heat-stable and inhibits hemagglutination of both living and heat-killed virus. It is not inactivated by treatment with RDE, but is partially affected by trypsin, while periodate abolishes the inhibitory capacity. The inhibitory activity is reduced by RDE and purified neuraminidase in human serum, but is not affected in horse serum (Chappin and Tamm, 1960). The inhibitor is present in horse serum in very high titer and is also found in guinea pig, rabbit, ferret and human sera (Cohen et al., 1963). Mouse adaptation of the highly sensitive A_2 Influenza strain resulted in a loss of sensitivity of the virus to horse serum inhibitor, (Cohen, 1960). The data suggests that particles of varying sensitivity are produced by mutation, and this may result in heterogeneity of an apparently stable passage strain of virus. These changes in inhibitor sensitivity may depend upon the relative proportion of two or more types of virus particles with subsequent selection by the host. However, in vivo protection of mice when infected with flu virus by intra-nasal instillation confirmed the inhibitory nature of the serum (Cohen, 1960 b; Link, 1965). This protection is of long duration, probably due to the large particle size which renders adsorption difficult and is of greater efficacy when administered before virus challenge, but does protect when given up to six hours after virus exposure. This protective effect was confirmed in tests using a non-adapted mouse strain of virus (Link et al, 1964).

Purification of γ inhibitor was accomplished by Krizanova (1961) from horse serum with hot phenol extraction of lyophilized serum filtrate and was identified as a homogeneous glycoprotein. This material showed capacity for both hemagglutination inhibition and virus neutralization for A_2 Influenza. The glycoprotein did not exhibit any inhibition reaction with the indicator B Lee and A PR8 Influenza viruses, but hemagglutination by these viruses is inhibited by human serum glycoprotein (Stulber et al, 1951). The authors suggest that there are differences, not only between various Influenza virus strains in respect to their reactivity with serum inhibitors, but also between glyco proteins from different sera. Treatment of the horse serum glycoprotein with RDE split off 40 - 50% of the sialic acid without loss of inhibitor activity for avid A_2 Influenza viruses, but treatment with trypsin resulted in the loss of its activity.

A paper by Levy (1959) referred to earlier, pointed out the possibility for the existence of two inhibitors in horse serum. The glycoprotein inhibitor (Krizanova, 1961) moved faster in an electric field when separated by electro-phoresis. On further investigation of horse serum, Krizanova (1961) isolated a slow-moving inhibitor which he designated as gamma globulin inhibitor. This gamma globulin inhibitor was isolated from horse serum by precipitation with a butylnaphthalene sulphonate (neokal). This purified material possessed both hemagglutination inhibiting and virus neutralizing properties. At pH 8.6 it migrated between the beta and gamma

globulin fractions of serum. To determine if the two substances were in fact singular complete entities, the effect of the HI and virus neutralization activities were tested by exposure to heat, sulphosalicylic acid, phenol, neokal and ethanol-ether. The results were found to be different for each inhibitor. There were also differences in the electrophoretic and sedimentation properties of the gamma globulin and glycoprotein inhibitor. This inhibitor was distinguished from β inhibitor by tests on characteristic sensitive strains and did not show any HI or virus neutralization with non-avid strains, illustrating that they are not identical with specific antibodies which do show a cross reaction with both avid and non-avid A_2 Influenza viruses. In an active preparation of gamma globulin inhibitor, no properdin was detectable by the zymosan method and the inhibitor did not adsorb onto the zymosan. The fact that γ inhibitor displays a virus neutralizing activity resembling specific antibodies as distinct from β inhibitors that do not neutralize virus, stimulated Szanto (1962) to investigate the interaction of γ Inhibitor (the glyco-protein inhibitor of Krizanova) with A_2 flu virus. It was found that γ inhibitor reacts with the virus in a manner similar to specific antibody. The virus-gamma-inhibitor complex disassociated following dilution, with the released virus still retaining sensitivity to inhibitor. The virus-inhibitor complex disassociated more rapidly than that of virus-antibody.

Another heat stable inhibitor for Influenza virus was discovered

by Hana in 1959. He noticed that the serum of white rats contained a relatively large amount of Influenza C virus hemagglutination inhibitor, and was earlier designated "C-inhibitor" by Styk (1955). This inhibitor is not destroyed by the action of RDE but it is partially inactivated by pneumococcal filtrate. It was found that the maximal activity of C-inhibitor corresponds to α fraction of rat serum. The mobility of C-inhibitor in acid solution, and the distribution of inhibitory activity estimated from electro-phorograms dyed for glycoproteins indicate that the C-inhibitor is a glycoprotein. Removal of lipids from rat serum by shaking with ether at -25°C or by treatment with chloroform at room temperature, as well as heating at 56°C for 30 minutes resulted in increased HI titres against C Influenza viruses, (Hana and Styk, 1960). On testing the effect of inhibitor-destroying substances of the non-specific inhibitor of C Influenza virus (Styke, 1963) C-inhibitor proved to be sensitive only against the action of trypsin. Periodate, RDE, crude V. cholerae filtrate or CO_2 did not substantially affect its titre. The author feels that this evidence is enough to differentiate this C-inhibitor from other known inhibitors of Influenza virus such as α, β and δ nonspecific viral inhibitors.

In summary, the identification of serum inhibitors for Influenza viruses that are regarded as distinct and separable from each other can be placed in four classifications. The first is that of the heat-labile substance first reported by Chu and subsequently referred to as the β inhi-

bitor. The remaining inhibitors are heat stable by the definition of retaining their inhibitory properties after heating at 56°C for 30 minutes. These can be divided into the Francis or α inhibitor, the γ inhibitor of Cohen and Shimojo found in horse serum, and the C-inhibitor characterized by Styk. It must be realized that these divisions may be in fact artificial with the possibility of one or more inhibitors being involved in hemagglutination inhibition or virus neutralization.

Serum Inhibitor for Other Viruses. Serum inhibitors have been reported for other viruses and have been accredited with altering both in vivo and in vitro tests. With respect to Yellow Fever virus, Stode (1951) stated that one of the most important sources of variability in mouse protection tests in Yellow Fever depends on the inherent properties of the serum of different animal species. Nonspecific inhibitors have been reported for Yellow Fever by Bugher et al., (1944) and Koprowski (1946). They found inhibitors in the serum of animals that had not been exposed to the virus as well as from animals from endemic areas. Koprowski found that the sera of certain species of marsupials and rodents captured in Brazil neutralized Yellow Fever virus, with the same sera neutralizing Japanese, St.Louis and West Nile encephalitis viruses. These viruses are not known to occur in Brazil.

Specific and nonspecific substances from serum derived from monkeys inoculated with Mumps and NDV as well as from human serum

was noted by Wenner et al., (1952). The component present in the sera of healthy human beings and rhesus monkey inhibits hemagglutination of chick erthyrocytes by Mumps and NDV. He also noted a similar non-specific component, which undergoes fluctuation, occured in sera of human beings convalescing from Mumps parotitis and in monkeys infected with Mumps or NDV. This non-specific inhibitor is not antibody, is partially inactivated at 62°C for 30 minutes, and may be destroyed by acetone, chloroform, trypsin, papain and periodate. The specific serum inhibitor substance is relatively stable to these treatments.

In a report (Goldfield et al., 1957) of hemagglutinins associated with certain human enteric viruses, nonspecific inhibition was encountered with both human and animal sera. A bovine enterovirus neutralizing substance was found in the sera of man, monkeys, pigs, sheep, guinea pig, rabbit and fowl, (McFerran, 1962). In man and cattle this inhibitor was found in all age groups and in every individual tested. The neutralizing substance acted directly on the virus. The heat stability was similar to Poliovirus antibody in human sera and treatment with bentonite, kaolin, and periodate did not affect the inhibitor. Trypsin had no effect on human sera but did destroy all inhibitor to Poliovirus in cattle sera. The inhibitor in human sera was not associated with the gamma globulin fraction.

Work on a serum inhibitor for bovine enteroviruses was also

carried out by Klein et al., (1964). This serum factor that neutralized the virus was inactivated by trypsin, periodate and neuraminidase. Part of the activity is associated with serum albumin (Cohen fraction 5). The inhibitor was fairly heat stable at 65°C for 30 minutes but was completely inactivated when held at 70°C for 30 minutes. In contrast, antibody demonstrated only moderate loss at 70°C for 30 minutes. The nature of the inhibitor is not clearly established. However, the reduction of serum activity occasioned by treatment with neuraminidase, periodate and trypsin indicates a glycoprotein substance containing sialic acid. The attempts to establish a protective role for this inhibitor were unsuccessful (Klein and Defaret, 1962).

Serum Inhibitor for Adenoviruses. The Adenoviruses, first identified in 1953 (Rowe et al., Hilleman et al.) are associated with a variety of respiratory and other clinical syndromes. The members of this large group of medium-sized, ether-resistant, DNA viruses are found in a number of animal species. There are 45 different types based on serologic tests and these are divided into six sub-groups; human (types 1 to 28), simian, bovine, canine, murine and avian Adenoviruses. The Adenoviruses share several chemical, morphologic and biologic properties including

(1) a unique fine structure of the virion, (2) the possession of a common group-specific antigen, (3) the production of a characteristic cytopathogenic effect in cell culture, and (4) the formation of the "Adenovirus type nuclear inclusion bodies" (Green, 1965).

The capsid is of icosahedral cubic symmetry and its diameter has been estimated by various techniques as being between 60 and 85 mu. The capsid is composed of 252 capsomeres with a centre-to-centre spacing of approximately 7 mu. A pericapsidal membrane has not been demonstrated. These viruses are very stable at low temperatures and over a wide range of pH values, but are readily inactivated at 56°C for 30 minutes (Pereira et al., 1963).

The exciting findings that preparations of Adenoviruses types 12 and 18 induce tumor formation in newborn hamsters (Trentin et al., 1962; Huebner et al., 1962) have generated a great deal of interest in the group. These are the first 'human' viruses reported to possess carcinogenic activity. Other members of the group that have been implicated in oncogenic activity are Adenovirus type 7 (Girardi et al., 1964), type 31 (Pereira, 1965) and type 3 (Koprowski, 1965).

Clinical manifestations of infection include the common illnesses of the respiratory tract, conjunctivitis, and rarely, aseptic meningitis and other central nervous system diseases. Identification of an isolate as an Adenovirus can be made promptly by means of the CF test. For determining the specific type, neutralization or HI tests are required. Infections induce CF, neutralizing and HI antibodies.

Studies on in vitro neutralization of Adenoviruses by Gold (1957) also reported the inhibition of Adenoviruses by normal animal

sera. They observed during investigation of fluids for propagation of Hela cells that ox, calf and horse sera markedly inhibited the CPE of Adenoviruses. The serum inhibitor not only reduced the titer by two logs, but also combined with and neutralized the virus. Types 4 and 7 were inhibited to the greatest extent, while types 2 and 6 were least affected by the inhibitor. Of the three sera tested the inhibitor was presented in highest concentration in ox serum. The inhibitor studied was heat stable and formed a non-dissociable complex with strains of types 1 and 7. It was not destroyed by RDE, trypsin, or incubation with infectious virus, nor was it absorbed by kaolin or removed by dialysis against water. The addition of ammonium sulfate to half saturation resulted in precipition of inhibitory substance with the globulin fraction. Further work by Gold and Ginsberg (1962) characterized the range of action of the heat stable globulin inhibitor found in ox serum. The presence of pooled heated ox serum in media inhibited to the greatest degree the infectivity titer of types 4, 5 and 7 Adenoviruses. Although this inhibitor has many characteristics of neutralizing antibody, the observation that type 3 Adenovirus removed the inhibitor against both types 3 and 4 to a similar extent indicated that the inhibitor is not a neutralizing antibody. Inhibitors have recently been found against type 12 in various sera, including calf, horse and human serum (Hoosier et al., 1964). This observation is in disagreement with

work done by Klein (1959, 1962) in which he isolated and identified bovine Adenovirus. These viruses identified as bovine Adenoviruses 10 and 19 share a common complement fixing antigen with the Adenovirus group. The authors concluded that this was an Adenovirus stimulation as, upon injection into test animals (guinea pig, hamster) the post immunization sera showed a rise in complement fixation titer, (with human Adenovirus as antigen) indicating infection with an Adenovirus type virus. These bovine Adenoviruses however, are not neutralized by antiserum to any of the twenty-eight human Adenoviruses and, therefore, would not likely provoke the development of antibody in cattle that would have inhibitory capacity for human Adenoviruses. Klein does stipulate that these bovine Adenoviruses are related to human Adenoviruses and inhibitory substances in bovine sera may be considered as antibodies. Confirmatory work of this nature (Darbyshire and Pereira, 1964) was obtained by use of gel diffusion techniques in which increases in Adenovirus antibody precipitating potency is observed with sera from cattle concerned in outbreaks of respiratory disease. This suggests that Adenoviruses are in some way involved. The Adenovirus group reactive antigen which is detectable by complement fixation and gel diffusion precipitation has reacted with the sera of six different animal species, viz., horse, ox, sheep, goat, pig and deer. This finding suggests that either Adenoviruses or Infectious Canine Hepatitus (ICH) virus infect each species or, alternately, that other Adenoviruses

are involved which possess a common antigenic component. ICH virus, another member of the Adenovirus group, has been shown to give rise to precipitating antigen in the livers of infected dogs. Darbyshire has validated this conclusion by observing a two-way relationship between the Adenovirus type 5 group antigenic component which was in identity with the ICH antigenic material.

The relationship between ICH virus and antibody using neutralization tests was studied by Carmichael et al. (1963). He reports variation in neutralization titers when different animal sera are incorporated in common balanced saline solutions. On preliminary experiments, he found that none of the balanced saline solutions inactivated ICH virus, nor were any inhibitory effects observed with lactalbumin hydrolysate, TC 199, neonatal calf serum, or normal canine serum. In contrast, addition to the medium of serum from certain horses, sheep, rabbits and especially adult cattle caused marked viral inhibition. In studying the nature of the inhibitor in cattle sera, results showed that the inhibitor was in the globulin fraction of bovine serum, but not in albumin fractions, and was not destroyed by treatment with heat, periodate, trypsin, ether, or dialysis. However, treatment with both trypsin and chymotrypsin destroyed the globulin inhibitor. The inhibitor has characteristics suggestive of neutralizing antibody, but is not considered specific by the author because of the above reports of nonspecific inhibitors for bovine and human Adenoviruses.

Working with human sera, Swartz-Malmber (1964) found that heat inactivation brought about a decrease in neutralizing capacity against type 7 Adenovirus. On collecting animal sera at different time intervals after immunization with type 7, then inactivating with heat and recording by a neutralization test, the author observed that heat inactivated pre-immune sera of rabbits and guinea pigs had little or no inhibitory capacity against type 7. The unheated sera of rabbits had considerable neutralizing effect while the unheated guinea pig sera did not. By testing serum taken at time intervals, half inactivated, half containing labile inhibitors and doing neutralization tests, the author concluded that on injection of Adenovirus type 7 the host gives rise not only to thermostable antibodies, but also to inhibitory factors dependent on or consisting of thermolabile components.

MATERIALS AND METHODS

MATERIALS AND METHODS

Virus strains.

Four different types of Adenoviruses were used. The Adenovirus type 3 strain used was the prototype GB strain (Rowe et al., 1955) originally obtained from the American Type Culture Collection (ATCC) where it had been passed seven times in Hela cells. Three pools of this type were used; pool 19 (fifth passage in primary human amnion cell (HAM) since receipt in this laboratory), pool 25 (seventh passage in HAM) and pool 26 (sixth passage in HAM). The type 5 Adenovirus was an isolate obtained from the Provincial Virus Laboratory of Manitoba on the 24/6/64, and had been passaged three times on HAM. The prototype Gomen strain of Adenovirus type 7 (Berge et al., 1955) was also obtained from ATCC where it had been passed ten times in Hela cells. The material used was pool A' derived from HAm and had been passed approximately 8 times on HAM. The type 8 Adenovirus was also isolated and typed by the Provincial Laboratory (14/12/65) and had been passaged twice on HAM.

Cell Cultures.

Primary human amnion cells were used in all experiments. The cells were collected and seeded in this laboratory by the techniques described by Wilt et al., (1964), and were grown out in roller tubes in growth medium consisting of 60% Hank's balanced salts solution, 20%

tryptose phosphate broth (Difco), and 20% inactivated calf serum. The medium was initially adjusted to pH 7.4 with 1.4% sodium bicarbonate and contained 200 IU of penicillin, 100 γ streptomycin, 100 γ chloramphenicol and 50 γ mycostatin per milliliter. Complete monolayers were formed six to eight days after seeding.

The growth medium was removed if the cells were to be used immediately, and maintenance medium (Syverton-Scherer's medium) supplemented either with selected serum as outlined in Procedure A or with 5% horse serum as outlined in Procedure B was added. In some cases where cells were not used on the same day as received, maintenance medium containing antibiotics and bicarbonate, but no serum, was added to carry over the cells until use. Maintenance medium was Syverton-Scherer's supplemented with 5% of the specific serum required and penicillin-streptomycin, buffered to pH 7.2 with sodium bicarbonate.

Inhibitor Assay

Two different methods of measuring presence of viral inhibitors in calf serum were used. These two techniques have been designated procedure A and procedure B.

Procedure A: Upon receiving cells the growth medium was removed and replaced with 1.5 ml. of maintenance medium containing 5% of the specific serum to be tested. Serial tenfold dilutions of virus

were prepared in Hank's BSS. Each dilution of virus was inoculated into four to six roller tube cultures. The volume of inoculum used was 0.2 ml. Final readings were taken after 21 to 23 days. $TCID_{50}$ was calculated by the method of Karber, (Finney, 1962).

Procedure B: In this technique the cells were maintained in Syverton-Scherer's medium supplemented with 5% horse serum (which was found to have a low index of inhibitory activity). Antibiotics and buffer were added as described above.

After the appropriate chemical or enzymic treatment, serial doubling dilutions of each test serum were made in 0.5 ml. of Hank's BSS. 0.5 ml. of virus material containing 50-100 $TCID_{50}$ was added to each Kahn tube containing the diluted serum. These tubes were incubated in a water bath at 37°C for three hours. At the end of this time 0.2 ml. of each virus-serum mixture was inoculated into two tubes of HAM. Readings were taken at two to three day intervals. The end point was calculated when the control tubes containing virus and non-inhibitory foetal calf serum read 1.0 or less as determined by the Karber method of reading the 50% neutralizing dose (ND_{50}). The titer was expressed as $10g_2ND_{50}$ per 0.2 ml. of virus inoculum.

Sera.

Fifty-one individual calf blood samples were collected from a local slaughter house over a period of eighteen months. The serum from each sample was poured off 24 hours after collection

and filtered through a HA 0.45m millipore membrance. All sera were inactivated at 56°C for 30 minutes to remove thermolabile inhibitors unless otherwise stated. Serum was stored at 4°C in most cases.

Samples of specific species sera were obtained from Microbiological Associates, Connaught Medical Research Laboratories and from animals maintained in this laboratory. Horse, foetal calf, calf, and newborn calf were obtained from the commercial sources. Upon receipt of these sera they were tested for sterility, heat inactivated and stored either at 4°C or at -20°C. Some of the calf sera samples collected locally were pooled, others were kept separately.

In conducting the experiments it had been observed that commercial foetal calf serum has consistently demonstrated lower inhibition titers than other sera. Consequently, foetal calf serum is used as a non-inhibitory reference standard in all experiments, allowing calculation of end-points as outlined in Inhibitor Assay.

Chemicals and Enzymes.

In order to determine the influence of different chemicals and enzymes on serum inhibitors, the following experiments were done.

Treatment With Kaolin. Kaolin was made up to a 25% concentration by volume in distilled water. The suspension was centrifuged at 3000 rpm for ten minutes, the supernantant decanted and the kaolin pellet was

resuspended in a volume of serum that would give a concentration equal to that of the original kaolin suspension.

This mixture was allowed to absorb for twenty minutes and was then centrifuged at 3000 rpm for 10 minutes. A portion of the once absorbed serum was pippetted off for testing, and the process was repeated to obtain twice absorbed serum.

Treatment with Trypsin. Crude trypsin[2] was made up in Phosphate Buffered Saline (PBS) to give a 0.8% solution of trypsin (8 mgms/ml. of PBS). The trypsin was sterilized by filtration through a grade HA 0.45m millipore filter and stored at -20°C. One part of test serum was mixed with three parts of crude trypsin to give a final concentration of 0.6% trypsin.

Crystalline trypsin[3] was made up to a concentration of 10 mgms. of trypsin per ml. of PBS. One part of test serum was mixed with four parts of crystalline trypsin solution to give a final concentration of 8 mgms. of crystalline trypsin per ml. of serum (Sampaio, 1958).

Treatment with KIO_4. Potassium periodate[4] was made up to a strength of M/90. The periodate was sterilized by filtration using a

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[2]Trypsin 1-3000, Nutritional Biochemicals Corp., Cleveland, Ohio.

[3]Trypsin, 2x crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio.

[4]British Drug Houses, Toronto, Ontario.

0.45m millipore membrane and stored at 4°C. One volume of serum was added to three volumes of periodate to give a final concentration of 0.0083M periodate.

Ether Extraction of Serum. The test serum was diluted in an equal volume of diethyl-ether[5]. The mixture was shaken for five minutes, allowed to stand, and the serum was then drawn off from below the interphase. This process was then repeated. Residual ether was allowed to evaporate by placing the recovered serum in a water bath at 37°C in a loosely stoppered Kahn tube.

Treatment With Crude RDE. A vial of Lyophilized crude V. cholerae filtrate[6] was reconstituted in 10 ml. of sterile distilled water. This material was diluted 1:5 for use, with one ml. of the diluted RDE added to 0.5 ml. of serum to be tested.

Treatment With Chymotrypsin. Chymotrypsin[7] was made up to contain 900 units/mgm. The chymotrypsin (45 units per mem.) was dissolved in PBS to give a concentration of 180 units/ml. This was sterilized by filtration through a HA 0.45m millipore membrane.

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[5]Diethyl-ether, British Drug Houses, Toronto, Ontario.

[6]Crude V. cholerae filtrate, Sigma Chemicals, St. Louis, Mo. 4 Mmolar units neuraminidase per vial

[7]Chymotrypsin, 3x crystalline, Worthington Biochemicals Corp., Freehold, New Jersey.

One part serum was added to three parts chymotrypsin for a final enzyme concentration of 3 mgm/ml.

Dialysis

To determine if serum inhibitor was dialyzable the following procedures were carried out. Three ml. of each serum sample to be tested was dialyzed against: (1) running tap water. The material was placed in a dialysis membrane that had been sterilized by boiling for ten minutes. This membrane containing the serum was put into a flask in which runing tap water was allowed to flow overnight. (2) A duplicate sample of material was dialyzed against one litre of 0.85% NaCl. This saline solution was changed three times over a period of eighteen hours. The dialyzed material was collected aseptically and tested.

Ammonium Sulphate Precipitation

Saturated ammonium sulphate solution was added to either an equal volume of serum (50% saturation) or to two volumes of serum (33% saturation) contained in a centrifuge tube. The samples were left overnight at 4°C. They were then centrifuged at 2000 rpm for ten minutes. The supernatant was drawn off, and the precipitate was redissolved in a volume of PBS equal to that of the initial volume of serum. The supernatant and the redissolved precipitate were dialyzed against 0.85% NaCl solution to remove excess $(NH_4)_2SO_4$. Tests were

then carried out with these separated fluids, and with the untreated serum.

Rate Zonal Density Gradient Centrifugation

These experiments were carried out using a Spinco Model L ultracentrifuge with a SW 39 rotor. A Sucrose gradient was prepared as outlined by Shulman et al., (1964). 4.0 ml. volumes of sucrose were prepared in lusteroid tubes with 1.0 ml. volumes of 40%, 30%, 20% and 10% of sucrose in PBS, layered consecutively. The tubes were placed in the cold room for three hours before use to allow partial equilibration. One ml. of a 1:2 dilution of test serum in 5% sucrose was layered on top of the gradient and about 0.5 ml. of sterile liquid paraffin was layered over the sample. The material was spun at 100,000g. for approximately sixteen hours. Fractions were collected dropwise from a pinhole made in the bottom of the centrifuge tube. Five fractions were collected and the volume of each fraction recorded. The technique was carried out aseptically with sterilization of tubes, centrifuge buckets and caps by ultra violet exposure for periods over an hour prior to use.

Assay of Conglutinin

Conglutinin is the name given to the substance in bovine sera which reacted to a antibody-antigen complex, after the complex had absorbed complement, bringing about a marked clumping or conglutination of the reactants (Coombs, 1961). The test involved the use of sheep erthyrocytes, serum to be tested, sensitized sheep erthyrocytes and fresh horse serum containing C^4 component of complement. The treatment of these materials and the technique of the test is described in the Appendix.

EXPERIMENTAL PROCEDURES AND RESULTS

I. DETECTION AND MEASUREMENT OF INHIBITOR

As described in the introduction, the suspicion that calf serum contained an Adenovirus inhibitor arose from the observation that an apparent reduction in the sensitivity of the infectivity assay occurred when calf serum was substituted for horse serum in the tissue culture maintenance medium.

This section describes experiments which confirm that calf serum does indeed contain an Adenovirus inhibitor and also describes the development of two methods for the detection and measurement of such an inhibitor.

Detection of inhibitor by procedure A

Confirmatory results on the ability of different animal sera to inhibit virus growth were obtained by testing batches of local calf serum, commercial calf serum and other species of sera by a technique designated procedure A (vide supra, materials and methods). This method differed from that of a routine infectivity assay only in that selected test sera were incorporated in the maintenance medium at a 5% concentration after the propagating material had been removed. It was reasoned that if, in fact, the serum was responsible for the variation in readings, the exposure of a common dose of virus to cells containing different sera in the maintenance media would allow comparative measurements of the sera employed. The virus stock pool used was Adenovirus type 3. Final readings were taken after incubation for 21 to 22 days. The inhibition as measured by

the reduction index is significantly higher with local and commercial calf sera than with rabbit or the reference horse serum. Comparative results were measured by calculation of the reduction index for each serum, viz., Reduction index= Log_{10} $TCID_{50}$ of horse serum - Log_{10} $TCID_{50}$ test serum. The serum that showed the highest $TCID_{50}$ (horse serum) was used as a reference for calculation of the inhibitory activity of other sera as illustrated in Table I.

The consistent inhibitory qualities of calf sera as measured by the reduction index is seen in Table II. Three different batches of local calf as well as the commercial calf demonstrate higher readings than rabbit, chick or foetal calf sera. The local calf sample in test #3 is of low order. Although these results are not extensive they do illustrate the consistent range of inhibition displayed by pooled local calf sera when compared to other animal species. Horse serum was used as a standard for calculation of the reduction index. The inhibition displayed by calf sera was not a local phenomena as the bought serum also displayed good inhibition levels.

These results were convincing enough to warrant acceptance of the postulate that different animal sera, particularly those of individual calf serum, contain a substance(s) that does inhibit the cytopathic effects of virus.

TABLE I

INFLUENCE OF SERUM IN CULTURE MEDIUM

SERUM 5% conc.	LOG_{10} $TCID_{50}$/0.2 ml	LOG_{10} $TCID_{50}$ REDUCTION INDEX
HORSE (Connaught)	6.5	0
RABBIT (local)	6.0	0.5
CALF (MBA)	5.5	1.0
LOCAL CALF	5.4	1.1

REDUCTION INDEX = LOG_{10} $TCID_{50}$ HORSE (6.5) -

LOG_{10} $TCID_{50}$ RABBIT (6.0)

= 0.5

MBA - Microbiological Associates, Bethesda, Md.

Connaught Laboratories, Toronto, Ontario

TABLE II

COMPARATIVE INHIBITION OF ANIMAL SERA

SERUM 5% conc.	LOG_{10} $TCID_{50}$ REDUCTION INDEX*			
	TEST #1	#2	#3	#4
LOCAL CALF**	1.1	1.8	0.6	1.4
CALF (MBA)	1.0	-	-	-
RABBIT (local)	0.5	-	-	-
CHICK (local)***	-	0	0.3	0
FOETAL CALF (MBA)	-	-	-	0.4

** four separate batches of pooled calf serum

*** two separate batches of pooled chick serum

* relative to horse serum

Detection of inhibitor by procedure B

It was decided that a different technique using another measure of the parameters of the virus-cell-serum relationship should be attempted in order to obtain verification of the apparent different inhibitor capacities in sera. A modified neutralization test with altered incubation time and temperature was established. This technique, referred to as procedure B, (vide supra) included the use of a reference non-inhibitory serum (foetal calf serum), as a control serum with which to measure differences between other test sera included in the experiment. In any one experiment where the reference serum was included along with several test sera the end point readings for the test sera were taken when all tubes inoculated with mixtures of virus and dilutions of reference serum showed a cytopathic effect. The higher the ND_{50} reading for any serum (viz., the greater difference between it and foetal calf serum) the greater the inhibitory qualities in this serum.

The inclusion of a reference serum, foetal calf serum in every experiment, made it possible to compare, validly, results from different experiments. This technique allowed another method for measurement of inhibitors in calf sera. Because of the lack of inhibition demonstrated by horse serum it was used in a 5% concentration in the maintenance medium (MM) in all experiments.

In earlier studies concerning the inhibitory effects of calf serum on

Adenovirus it was observed that there was considerable variation in degree of inhibition by any one sera when the time period in a water bath at 37°C was altered. It was noted by Klein (1964) that full inhibitory effects of normal human sera for Bovine Enteroviruses varied when the incubation time and temperature were altered. Maximal neutralization of Enterovirus was realized after an incubation period of three hours at a temperature of 37°C. Shorter time periods of incubation and lower temperature gave significantly lower neutralization titers. In order to ascertain the optimal conditions for neutralization by procedure B two experiments were performed and their results are illustrated in Table III.

Experiment I was designed to determine the optimal incubation at a temperature of 37°C for measurement of inhibitory activity. Approximately 100 $TCID_{50}$ of Adenovirus type 3 was incubated with dilutions of inhibitory serum for various time periods at 37°C. Samples were removed at the times shown in the table and assayed by procedure B. Three hours was found to be the optimal time for measure of Log_2 ND_{50}. In subsequent tests by procedure B the time period of three hours for incubation was adopted as standard technique.

In the second experiment two temperatures were used to confirm the previous finding that inhibitory serum did not neutralize virus significantly at room temperatures. Three samples were taken over a 24 hour period from material kept at room temperature (21°C). The results

TABLE III

INFLUENCE OF TIME AND TEMPERATURE OF INCUBATION ON VIRUS NEUTRALIZING ACTIVITY OF INHIBITORY CALF SERUM

	$-LOG_2$ ND_{50}			
	EXPERIMENT 1		EXPERIMENT 2	
	37°C		37°C	21°C
1 1/2hours	3.0	3 hours	6.0	2.5
3 hours	5.6	6 hours	-	4.6
4 1/2hours	5.5	24 hours	-	4.7

confirmed previous studies in that the inhibitory substances were not fully effective at 21°C and although neutralization titer increases after three hours it still does not reach after 24 hours the activity seen after three hours at 37°C. This dependence upon temperature for full expression of inhibitory potential is not characteristic of antibody.

From these results the logical choice of a temperature of 37°C and an incubation period of three hours in the neutralization technique (procedure B) would allow the demonstration and measurement of any inhibitory substances in calf serum.

To decide the dosage of virus to be used in procedure B three different dilutions of virus were examined. The optimal dosage of virus would be the dilution that allowed the greater difference in ND_{50} between test and reference serum within a reasonable period of time. This virus material should be enough so as to allow any inhibitory differences to be readily distinguishable but not enough to overpower the amount of inhibitor present in calf sera. In Table IV the results show that, as expected, the test is most sensitive with small doses of virus but a practical limit to the use of small amounts of virus is imposed by the much greater time period which must elapse before final readings can be taken. This along with results from similar experiments indicate that a 100 $TCID_{50}$/0.2 ml if used in a standard neutralization test gave the most satisfactory readings for procedure B when sensitivity and time were considered. As a measure

TABLE IV

INFLUENCE OF VIRUS DOSE ON $-LOG_2ND_{50}$

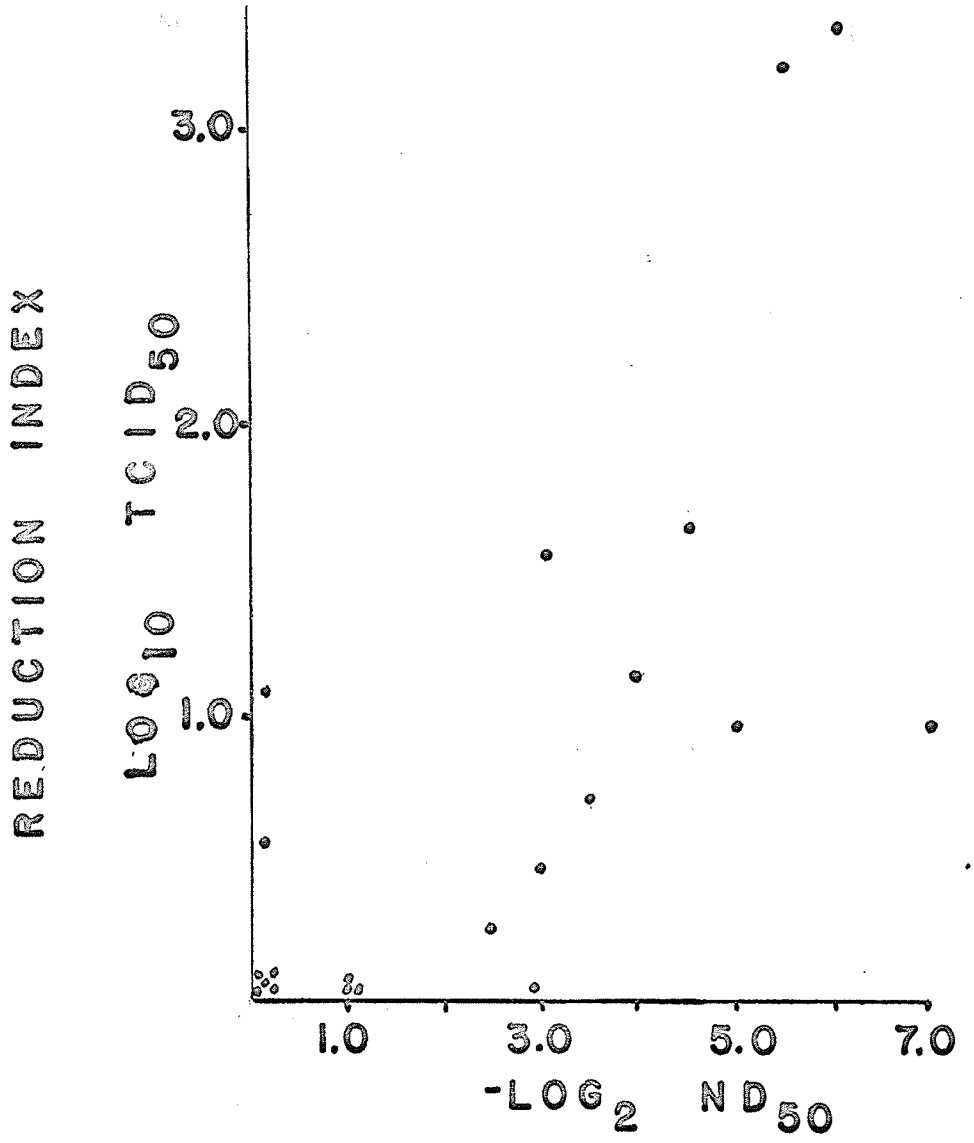
VIRUS DOSE $TCID_{50}/0.2$ ml	$-LOG_2$ ND_{50}	TIME
70	6.0	14 DAYS
140	5.5	12 DAYS
280	2.5	12 DAYS

of the reproducibility of the procedure, the variation in the ND_{50} of serum #36 classified as inhibitory in ten different experiments showed a range of 4.5 to 6.0 and a mean of 4.9 in the inhibitory levels.

Correlation of procedure A and procedure B.

Results obtained from procedure A and B were compared to see if there was any correlation between the two methods of measuring inhibitor. Twenty-one sera have been examined by both methods and the collected results are illustrated in Figure 1, which shows that although there is not a clear cut correlation, yet generally speaking, a serum found to have high levels of inhibitor when tested by one method, is also highly inhibitory when tested by the other.

Although some discrepancies are apparent, it is pointed out that all sera with $Log_2 ND_{50} > 3.0$ display a $TCID_{50}$ reduction index of at least 0.8 log. Because of this observation only sera having $Log_2 ND_{50}$ equal to or greater than 3.0 were considered to contain significant levels of inhibitor, when tested by procedure B. Since procedure B is the simpler test from the technical point of view, it was used in measuring inhibitor levels in most of the remaining part of this work.



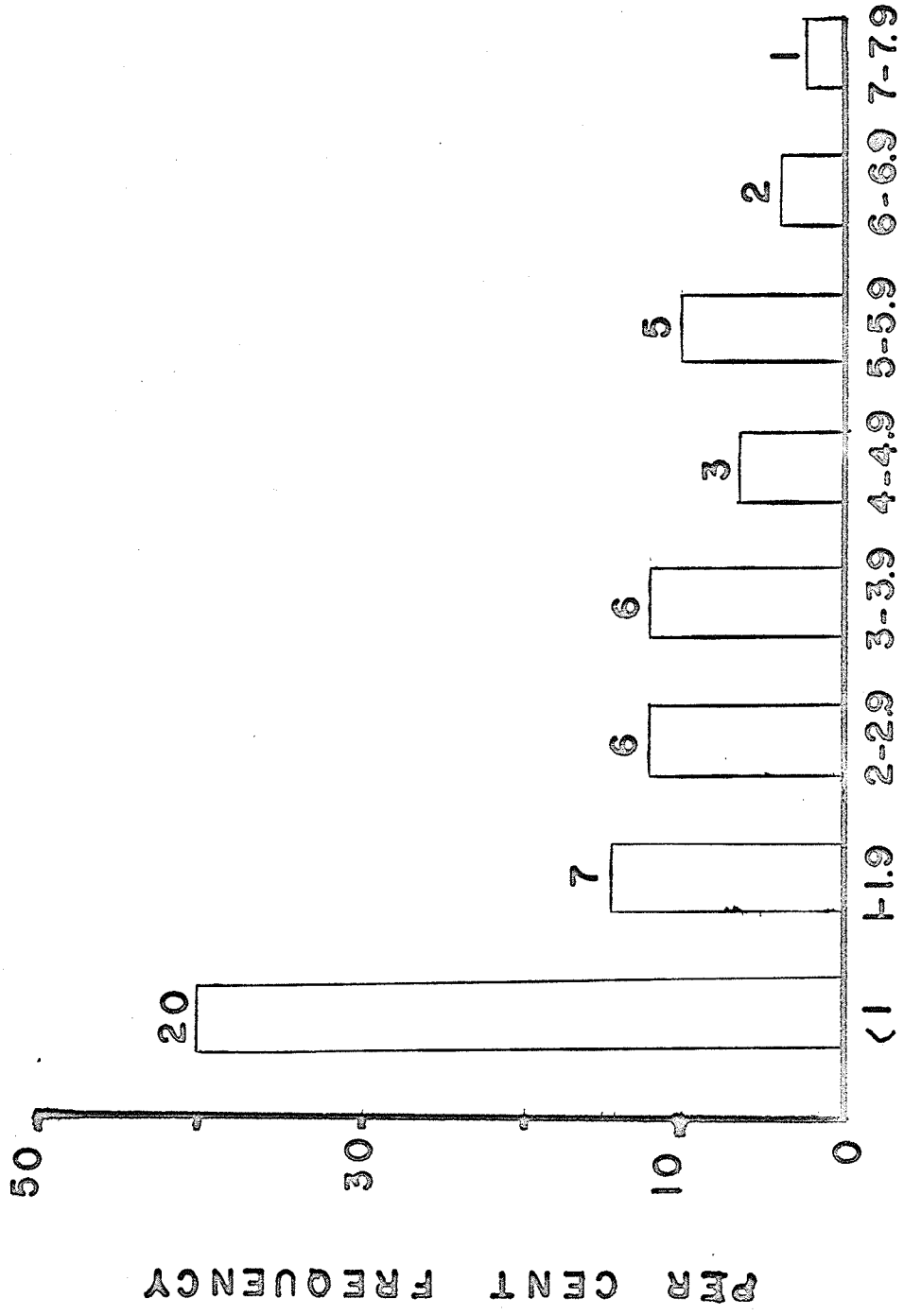
CONTRAST OF PROCEDURE A & B

FIGURE 1

II. DISTRIBUTION OF INHIBITOR IN CATTLE POPULATION

To arrive at some idea of the proportion of sera that could be classified as inhibitory, the ND_{50} (procedure B) of 50 individual calf sera, collected over a period of 15 months, were sampled. Figure 2 illustrates the percent frequency of distribution of inhibitory and non-inhibitory sera. Serum that exhibited ND_{50} of 3 or greater were classified as inhibitory (vide supra). The range of inhibition demonstrated by the sera measured from Log_2 ND_{50} of <1 (non-inhibitory) to 7.9 (greatest inhibition). Seventeen of the 50, or approximately 33% of the sera, were found to display a marked degree of inhibition when tested by procedure B.

Four sera samples collected from slaughtered adult cattle were shown to be inhibitory as measured by above limits. There were not enough samples considered to ascertain if inhibitory sera appeared more frequently in adult cattle than in calves (viz; below six months of age).



DISTRIBUTION OF INHIBITOR IN 50 CALF SERUM SAMPLES

FIGURE 2

III. CHARACTERIZATION OF INHIBITOR

Spectrum of inhibitory activity for Adenoviruses

The bovine serum inhibitor investigated by Gold & Ginsberg (1962) was shown to affect several Adenovirus serotypes, but to varying degree; thus, types 3, 4, 5 and 7 were more sensitive than types 2 and 6. Experiments were designed to determine the differential effects, if any, of the inhibitors in individual calf serum on virus serotypes representative of each of the four Adenovirus sub-groups as defined by Rosen (1958) according to hemagglutination activity with Rhesus monkey and rat erythrocytes. The technique used to measure inhibitory activity was procedure A. Two inhibitory and two non-inhibitory calf sera samples, previously tested with type 3 virus, were selected and added as supplement to the maintenance medium to a concentration of 5%. Readings were taken for at least 21 days. The Adenoviruses tested were types 5, 8 and 12 representative of sub-groups 3, 2 and 4 respectively. Before use, the individual serum samples were coded by another individual, so as to avoid bias in reading the results, and the code was not broken until after the titration end points were calculated. Three experiments of this nature were done, using foetal calf serum as reference, and the results are shown in Table V. No results were obtained for Adenovirus type 12, although type 7 results are included.

The results illustrated in Table V leave little doubt of the inhibitory potential of sera #40 and #30 for all four types of Adenoviruses tested.

TABLE V

ADENOVIRUS SENSITIVITY TO INHIBITOR IN CALF SERA

VIRUS TYPE	$-LOG_{10}$ $TCID_{50}$ REDUCTION INDEX			
	SERUM			
	#40	#30	#35	#27
8	2.2	2.4	1.8	1.8
3	3.5	1.2	0	0.2
7	2.2	-	0.4	-
5	1.2	1.0	0	0.4

Sera #27 and #35 are non-inhibitory for types 3, 7 and 5 but, by contrast, are markedly inhibitory for type 8 virus. This indicates that the inhibitor or inhibitors common to these individual calf serum display a greater inhibitory capacity for Adenovirus type 8 than for other types tested. Further experiments of this nature would have to be carried out to determine if this difference in sensitivity was paralleled by other viruses (viz., types 9, 10, 13, 15 etc.) within sub-group 2 or is specific for type 8. The results may also indicate that there is an additional inhibitory substance present in sera #27 and #35, but one to which types 3, 5 and 7 viruses are not sensitive.

Physico-chemical properties of serum inhibitor

Dialysis of serum. An inhibitory serum (#28), and a non-inhibitory serum (#27) and the control foetal calf serum were dialyzed against (1) running tap water, and (2) o.85% NaCl overnight at 4°C, with two changes of 500 volumes of dialyzing fluid. The dialyzing membrane was sterilized by boiling in water for ten minutes before introduction of the sample. There was a slight turbidity in both sera after dialysis against tap water but there was no turbid material found in foetal serum. No attempt was made to separate the small amount of insoluble material found in the treated sera. After treatment the material was removed aseptically from the membrane and assayed by procedure B. Adenovirus type 3 was used in this experiment and in all subsequent experiments con-

cerning the nature of the inhibitor. The results in Table VI show that the level of inhibitor is not altered as a result of dialysis and therefore it is concluded that the inhibitor is likely associated with a large molecular fraction of the serum.

Heat-labile inhibitors. The presence of non-specific, heat-labile inhibitors in serum for other viruses has been noted in the literature (Chu, 1950; Bang, 1951; Broidy, 1951). An experiment was carried out to find out if any heat-labile Adenovirus inhibitors were present in individual calf serum. Three samples of individual sera were taken, a portion of each received no treatment after processing, while the balance was heat inactivated at a temperature of 56°C for 30 minutes. Measurement of inhibitory activity for all samples was made by procedure B using Adenovirus type 3. The readings obtained after 13 days are illustrated in Table VII. Only one of the three sera tested demonstrated a large enough difference between heat inactivated and non-heat inactivated material to warrant the assumption that heat-labile inhibitory factors were present. Other sera collected were not tested for the presence of labile inhibitors but it would seem from this one experiment and from previous citations (vide supra) with other viruses in the literature that their occurrence would not be unexpected. For the purpose of our work these labile inhibitors are of little significance because of the practice of heat-inactivating all sera used in the reported experiments. The work outlined in this thesis is concerned with inhibitors that are not removed

TABLE VI

INFLUENCE OF DIALYSIS ON SERUM INHIBITORY ACTIVITY

SERUM	$-LOG_2$ ND_{50}		
	CONTROL	RUNNING WATER	SALINE
FOETAL CALF	<1	<1	<1
#27	2.0	2.0	1.7
#28	5.5	5.2	4.0

TABLE VII

INFLUENCE OF HEATING AT 56°C FOR 30 MINUTES ON SERUM INHIBITOR LEVEL

SERUM	LOG_2 ND_{50}	
	HEAT INACTIVATED	NOT HEAT INACTIVATED
#29	3.0	6.0
#30	4.0	3.6
#31	1.0	1.0

by heating the serum at 56°C for 30 minutes.

Thermal stability of serum inhibitor. Most of the characterization of heat stable inhibitors reported in the literature have included data pertaining to the sensitivity of the inhibitors to heat. These studies have compared the kinetics of heat inactivation of antibody and of the particular inhibitor under investigation in order to determine the possible relation of inhibitor to antibody.

In these studies of the thermal stability of the inhibitor in calf serum an initial experiment was carried out to determine the temperature sensitivity and this was followed by an experiment to measure the rate of inactivation at a specific temperature. In the first experiment duplicate samples of inhibitory serum were placed in different water baths at controlled temperatures of 60°, 70° and 80°C after the serum had previously been inactivated by heating at 56°C for 30 minutes. After 20 minutes incubation the sera were removed and assayed by procedure B. The results are shown in Table VIII. It is observed from the mean readings that there is rapid inactivation of the inhibitory capacity of this serum with exposure for 20 minutes at higher temperatures. The loss of inhibitory quality begins at a temperature of 65°C and continues at a rapid rate to 80°C. The material incubated at 80°C coagulated forming particulate material. This rapid heat inactivation is interesting when compared with other heat inactivation experiments by other investigators. Gold (1962), while investigating an inhibitor in ox sera, found that heating the sera at 60°C for 20

TABLE VIII

INACTIVATION OF INHIBITOR BY HEAT

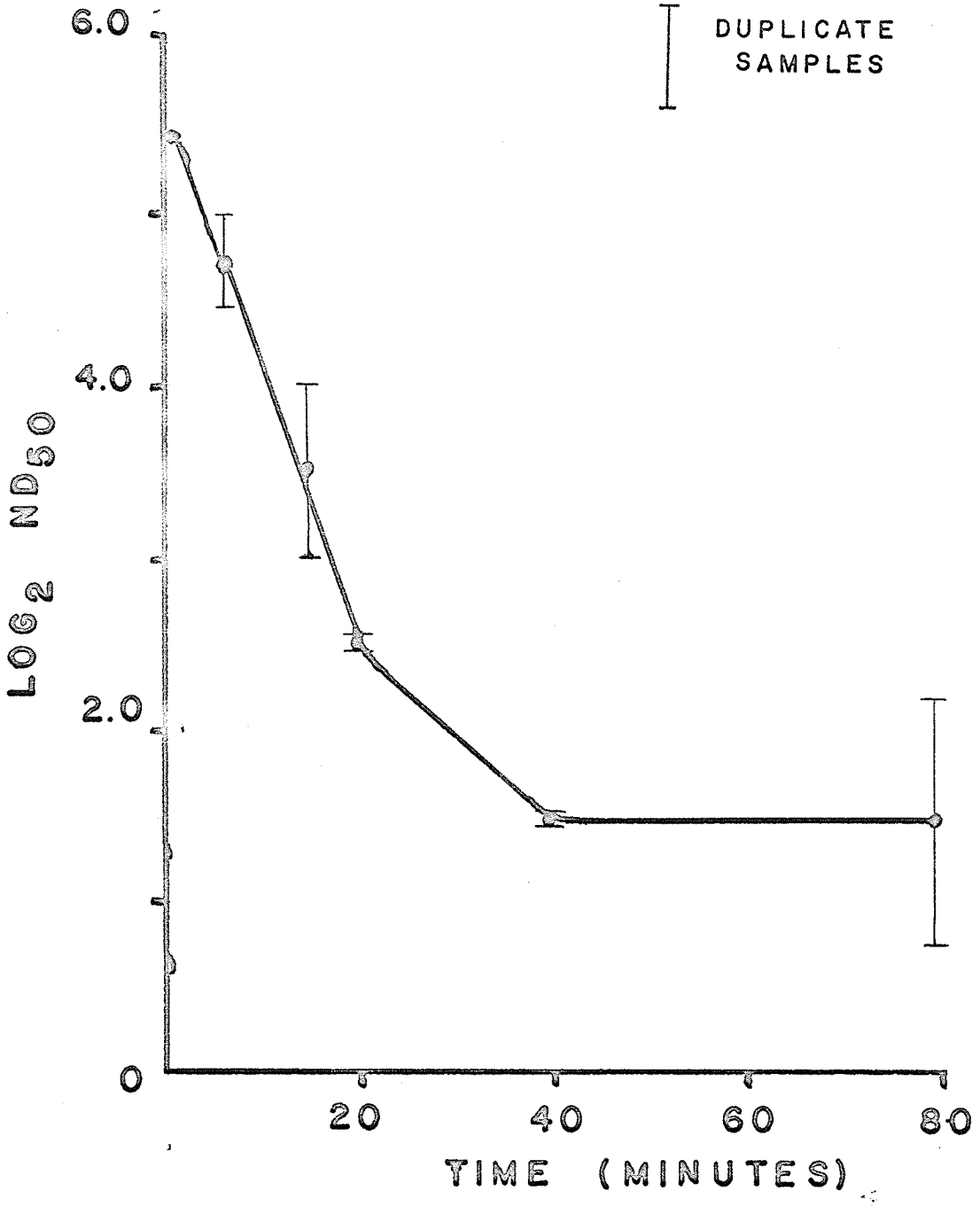
TREATMENT FOR 20'	$-LOG_2$ ND_{50}		
	SAMPLE A	SAMPLE B	MEAN
60°C	5.0	6.0	5.5
65°C	3.5	3.5	3.5
70°C	1.5	1.0	1.0
75°C	1.0	1.0	1.0
80°C	1.0	1.0	1.0
test serum control*			5.5

*previously heated to 56°C for 30 minutes

minutes resulted in no reduction in the activity of the inhibitory substance. Heating at 65°C for 20 minutes reduced the inhibitory titer 33%, while complete inactivation of the inhibitor occurred at 75°C for 20 minutes. Investigation by Klein (1964) of the Bovine Enterovirus inhibitory substance in human and rabbit sera demonstrated similar rates of heat inactivation. He found that heating sera at 65°C for 30 minutes resulted in a significant loss in inhibitory titer while complete loss of activity was observed in serum heated at 70°C for 30 minutes. However, Klein reports that specific antibody prepared in rabbits and tested under the same conditions showed only a moderate loss in titer after heating at 70°C for 30 minutes.

These results are similar to ours, although our tests did not include study of inactivation of antibody. We can therefore not draw any conclusions as to the relationship of the inhibitor and antibody in calf serum, but can only refer to the literature and note that the heat sensitivity of the inhibitor is not altogether different from that of antibody, but the nature is difficult to assess when confronted with information such as reported by Klein.

The second experiment dealing with thermal stability represents an attempt to measure the rate of inactivation at a temperature which is known to affect the inhibitor. This experiment again employed duplicate samples and the mean results are plotted graphically in Figure 3. The material was placed in a water bath at 65°C and samples were drawn off



INACTIVATION OF INHIBITOR BY HEAT (65° C)

FIGURE 3

at 5, 15, 20, 40 and 80 minutes. Neutralization titer was established by procedure B. The temperature of 65°C was chosen because the previous experiment indicated the initial inactivation of inhibitor occurred at this temperature. The first sample collected at five minutes shows about one log lower titer than the initial level, and for up to 20 minutes, inactivation occurs at the same rate. Afterwards the rate of inactivation apparently decreases. Thus, it seems likely, though not unequivocal, that the inhibitory activity is due to a single substance and not to more than one substance. In addition, the results serve to characterize the inhibitor for purposes of reference to other inhibitors.

Chemical and enzymic treatments of inhibitory sera. It has been established that treatment of inhibitory sera with various chemical reagents end enzymes can, in some cases and not in others, remove inhibitors to various viruses (Chu, 1950; Frances, 1947; Klein, 1965). It is a logical step to expose the serum to some of these treatments, not only to remove inhibitors, but also by their removal to gain some insight into their chemical nature, and possibly into their mode of action. With this in mind experiments were designed to determine if treatment of serum with RDE (receptor destroying enzyme), ether, periodate, kaolin, crystalline trypsin and chymotrypsin had any effect on the inhibitory qualities of calf sera. Each treatment is reviewed and selected results from no less than two different experiments are illustrated in Table IX.

TABLE IX

CHEMICAL TREATMENT OF INHIBITORY SERUM

REAGENT	TREATMENT	LOG_2 ND_{50}	
		Treated	Untreated Control
RDE	3 hrs, 37°C	3.0	4.0
Ether	2x extracted	4.0	3.5
Periodate	0.0083M, 4°C, 18 hrs	6.0	6.5
Kaolin	2x absorbed	5.5	5.5
Crystalline trypsin	10 mgm/ml, pH 7.0	2.0	6.0
Chymotrypsin	3 mgm/ml, pH 7.0	2.5	6.0

Crude receptor destroying enzyme (RDE). Lyophilized Cholera vibrio filtrate was reconstituted in ten ml of sterile distilled water. A 1:5 dilution of this material was made up to a total volume of one ml to which 0.5 ml. of inhibitory serum was added. This mixture was then incubated at 37°C in a water bath for three hours after which the enzyme was inactivated by placing in a water bath at 56° for 30 minutes. There was no detectable difference between RDE treated serum and untreated serum controls. The test serum control in this and subsequent experiments dealing with enzymic treatments underwent the same conditions of treatment as the sample exposed to the enzyme with sterile phosphate buffered saline (PBS) substituted for the particular enzyme used.

Ether extraction. Diethyl-ether was mixed with an equal volume of inhibitory serum. This material was shaken for five minutes at room temperature, the serum layer was drawn off and again exposed to ether for five minutes. The serum was then removed and placed in a water bath at 37°C for one hour to allow residual ether to evaporate. Control samples were also included in this treatment. It was found that after this treatment the aroma of ether was still present and the serum was left in a 1:2 dilution in Hank's balanced solution for another two hours at room temperature before being tested by procedure B. The results indicate that ether extraction has no effect on the inhibitory substance in individual calf serum.

Periodate. Three volumes of potassium periodate, sterilized by

millipore filtration, were mixed with one volume of inhibitory serum and one volume of foetal serum. The final concentration of periodate was 0.0083M. The mixture was allowed to stand overnight at 4°C wrapped in aluminum foil to prevent reduction by light (Kabat & Mayer, 1961). The excess periodate was neutralized by the addition of 0.5 ml of 2.7% glucose (one volume of 5.4% glucose and one volume of PBS, pH7.2) to 2.0 ml of the periodate serum mixture. Samples were then placed in the water bath at 37°C for fifteen minutes and tested by procedure B. Exposure of the serum to periodate did not influence the ND_{50} levels of treated material when compared to untreated samples which had undergone the same conditions.

Kaolin. An inhibitory serum was absorbed twice with kaolin and the treated material tested by procedure B. A 25% suspension of kaolin in sterile water was centrifuged and the supernatant discarded. Four ml of the inhibitory serum were added to two ml of the packed kaolin. Foetal control serum underwent similar treatment. This material was allowed to absorb for 20 minutes after which the samples were centrifuged again. A sample of once absorbed kaolin serum was removed at this stage for testing. The remaining supernatant was then again suspended in kaolin and re-absorbed for 20 minutes. The twice absorbed material was collected from the supernatant after centrifugation and tested. There was no loss or removal of inhibition for either once or twice absorbed serum after these treatments.

Crystalline trypsin. Initial experiments using crude trypsin made up to 0.8% concentration in PBS indicated that a loss of inhibition resulted when inhibitory serum was treated with this material. After these results with crude trypsin, crystalline trypsin was used to determine if the action depended on crude trypsin since it is known that crude trypsin preparation contains other enzymes (Parker, 1950). Crystalline trypsin was made up in PBS to a concentration of 10 mgm of trypsin/ml (Sampaio, 1953). Excess material was made and if not completely used up the trypsin was frozen and used in verifying experiments. Four parts of trypsin were mixed with one part of inhibitory serum (8 mgm/ml) and one part of controls. These mixtures were placed in a water bath at 37°C for both one hour and two hour intervals. At the end of this treatment the enzyme was inactivated at 56°C for 30 minutes. Procedure B was then carried out with the test material. Four experiments using crystalline trypsin were completed with all results confirming to a very close approximation the representative figures shown in Table IX. It is concluded that calf serum inhibitor is destroyed by exposure to crystalline trypsin.

Chymotrypsin. The chymotrypsin used was made so as to contain 900 units/ml with the final concentration of 20 mgm/ml of chymotrypsin in PBS. This material was filtered by millipore filtration and stored in the deep freeze. The chymotrypsin was further diluted 1:5 and 1:7 and these were used to mix with test serum. A 1:3 dilution of serum was made in

the chymotrypsin material giving a final concentration of 3 mgm/ml of chymotrypsin. Tests were placed in water bath at 37°C for periods of one and two hours. After withdrawal from the water bath the enzyme was inactivated by heating at 56°C for 30 minutes.

Chymotrypsin reduced the inhibitory activity of inhibitory sera to the same degree as did trypsin. The reduction by these enzymic treatments are interesting as they are the only ones of the above chemical treatments that have any effect on the inhibitory substance. These results would indicate that the inhibitor is protein in nature and its insensitivity to RDE, ether and periodate indicate that there are no polysaccharide or lipid moieties involved.

Fractionation of inhibitory serum by ammonium sulphate. Previous investigators have reported the presence of non-specific heat stable inhibitors in the globulin fraction after precipitation with ammonium sulphate for Adenoviruses and Bovine Enteroviruses (Klein, 1964; Carmichael, 1963; Gold, 1962). If the inhibitor is antibody it should therefore be precipitated by 50% $(NH_4)_2SO_4$, although if found in this fraction it is not necessarily antibody. Experiments were carried out to determine if the inhibitor in calf serum was precipitated by this treatment. Two ml. of a saturated solution of $(NH_4)_2SO_4$ was added to two ml of the serum samples to be tested. The mixture was allowed to stand overnight in the cold room (4°C). The precipitate was then packed by

centrifugation, the supernatant removed and the precipitate dissolved in a volume of PBS equal to the volume of supernatant withdrawn. The supernatant and the fluid containing the dissolved precipitate were placed in sterilized dialysis membranes and dialyzed against 0.85% NaCl solution for approximately two days with frequent changes of saline until a test of the dialyzing fluid for sulphate ion using $BaCl_2$ solution proved negative.

The dialyzed material was removed from the membrane aseptically and tested along with whole inhibitory and foetal serum controls by procedure B. The results in Table X and those from previous experiments indicate that the inhibitor is precipitated with the globulin fraction when serum is treated by half saturation with ammonium sulphate.

Inquiries into the possible nature of the inhibitor in calf serum led us to entertain the idea that this might be conglutinin, a substance found in normal bovine serum (Coombs, 1961). It has been reported by Coombs that conglutinin is precipitated by 33% saturation with ammonium sulphate and is also precipitated when unheated serum is dialyzed against distilled water. The experiment using 33% saturation with ammonium sulphate was carried out in a manner identical to that described above. The unheated serum was placed in a sterilized membrane and dialyzed against distilled water at a temperature of 4°C for two days, by which time a heavy white precipitate had developed. At this time the material within the membrane was centrifuged and the precipitate re-dissolved in

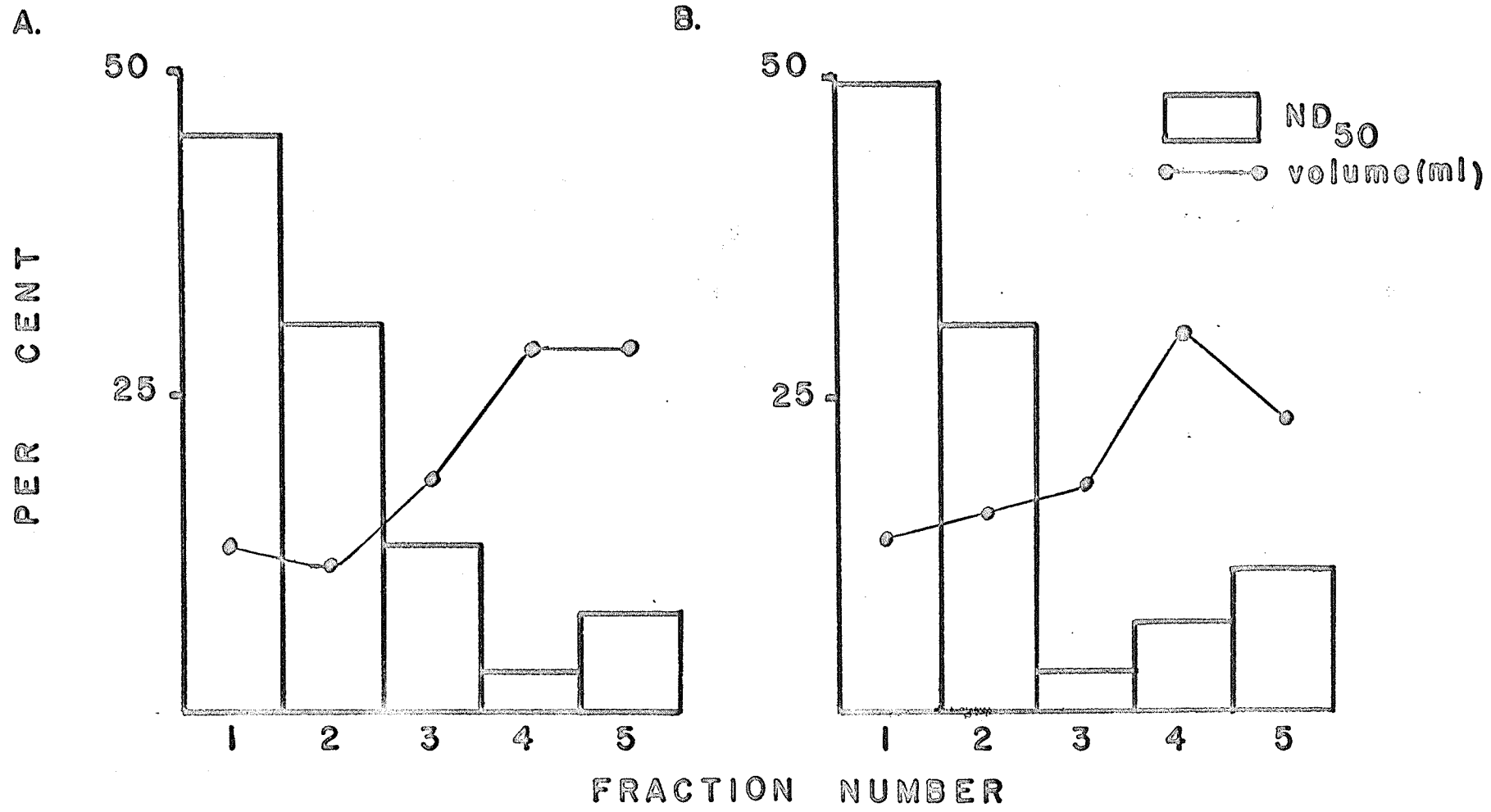
TABLE X

FRACTIONATION OF INHIBITORY CALF SERUM

	LOG_2 ND_{50}		
	Precipitate	Supernatant	Untreated Control
50% saturation $(NH_4)_2SO_4$	6.0	2.0	5.0
33% saturation $(NH_4)_2SO_4$	4.5	5.5	5.0
Dialysis of unheated serum against distilled water (4°C)	6.0	4.0	6.0

PBS so as to make a volume equal to the initial volume used. This material and the supernatant were then heat inactivated at 56°C for 30 minutes and tested for ND_{50}. The results show that inhibitor is about equally distributed between precipitate and supernatant when either 33% saturation with $(NH_4)_2SO_4$, or dialysis against distilled water, is used for serum treatment. These do not indicate that conglutinin is the inhibitor although they do not necessarily indicate that it is not involved. The levels of conglutinin will be referred to in a later section.

Study of inhibitory serum by rate zonal density centrifugation. The inhibitory serum was layered on a sucrose gradient viz., five ml layers of 40%, 30%, 20%, 10% of sucrose in PBS, pH 7.2 that had been sterilized and left to stand at 4°C for three hours. Five ml of a 1:2 dilution of serum in 5% sucrose was layered on top of the gradient and covered with a thin layer of sterile liquid paraffin. This material was placed in the Spinco ultra-centrifuge with the SW39 swinging bucket (horizontal) rotor, held at a temperature of 4°C and centrifuged for sixteen hours at 100,000 G. Five fractions were collected in a dropwise manner from a pin hole made in the bottom of the lusteroid tubes. These fractions were assayed by procedure B. The readings obtained were corrected for activity of the total volume of fraction collected and the results are illustrated in Figure 4 for two separate samples. Five fractions were collected from each sample. It can be seen that the greatest degree of



FRACTIONATION OF INHIBITORY CALF SERUM BY DENSITY GRADIENT CENTRIFUGATION

FIGURE 4

neutralizing activity has collected in the first two bottom fractions viz., that from 45 - 49% of the total inhibitory activity is found in the bottom fraction which is only about 12% of the total volume. It has been confirmed that centrifugation of serum under these conditions separates 19S and 7S globulins (Shulman et al., 1964), with the 19S globulins found in the bottom 20% of the gradient. Thus, it appears that the bulk of the Adenovirus inhibitor in calf serum is associated with a large molecular serum factor, of the same order of size as 19S globulin.

Correlation of conglutinin levels and serum inhibition titers.

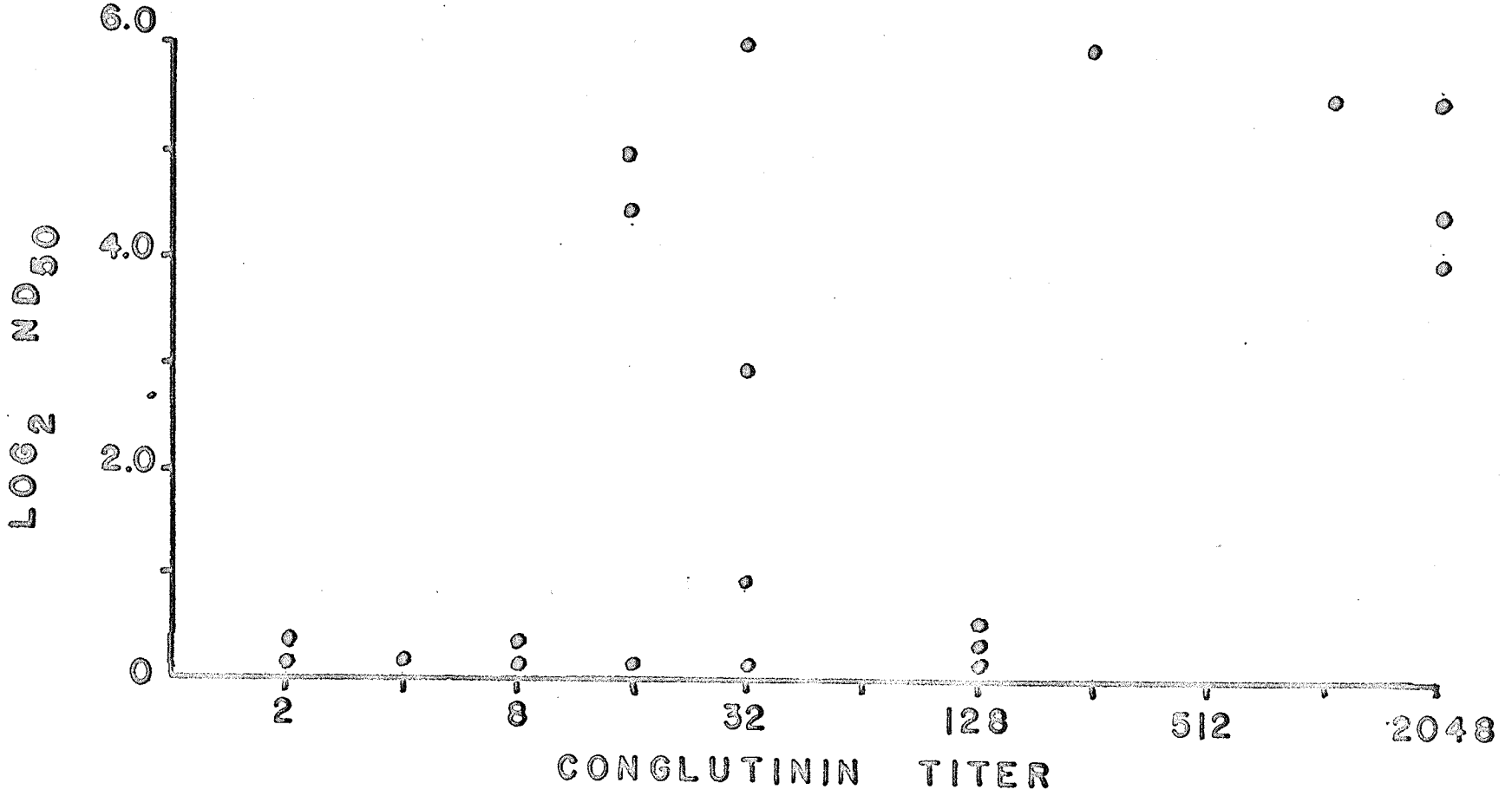
The possibility that naturally occurring conglutinin in the sera of normal cattle may be in some way involved prompted a study of the conglutinin levels in the serum of collected calf sera. Conglutinin is material found in normal calf and adult serum that is known to be heat stable, and is an euglobulin that reacts with complement that is adsorbed to a antigen-antibody complex. It is apparently of a 19S size (Ingram, personal communication, 1966) and may in addition possess anti-viral activity (Joshi, 1965). Earlier results (vide supra) indicate that conglutinin is precipitated by 33% ammonium sulphate or when dialyzed against distilled water might be only partially involved in any inhibition that is observed. Recent work by Joshi concerning the application of rabbit serum with high immuno-conglutinin titers to tissue culture cells with subsequent delay of cytopathic effect upon exposure to virus,

suggested investigation into the levels of conglutinin in our calf samples. Bovine conglutinin is a globulin which has the property of reacting with complement once the latter has been absorbed to antigen-antibody complexes viz., it will conglutinate sheep erythrocytes sensitized with non-agglutinating Forssman antibody and horse or bovine C'. The conglutinin levels were assayed in the stored (4°C) individual calf sera by procedure IIa described by Coombs (1961). Of the twenty sera tested for conglutinin levels (Figure 5), eight sera had been declared inhibitory by procedure B, while twelve had been measured as non-inhibitory. Of these, five showed high levels of conglutinin as well as high ND_{50}. Three of the sera were intermediate in that they showed moderate inhibitory qualities but diminished conglutinin levels. The balance were of both low inhibition and conglutinin titers. Eight of the twenty have high ND_{50} viz., > 3.0, while five have conglutinin levels > 256.

These preliminary results with conglutinin would lead one to decide that conglutinin itself is not the sole factor responsible for the inhibitory qualities although it may be involved to some degree.

Mode of action of inhibitor in calf serum.

These results obtained by procedure B suggest that the inhibitor inactivates virus. Experiments were carried out to confirm this fact and also to obtain a measure of the rate of virus inactivation by the inhibitor. The serum samples to be tested, (viz., one inhibitory serum and reference foetal serum) were diluted 1:2 in PBS and an equal volume

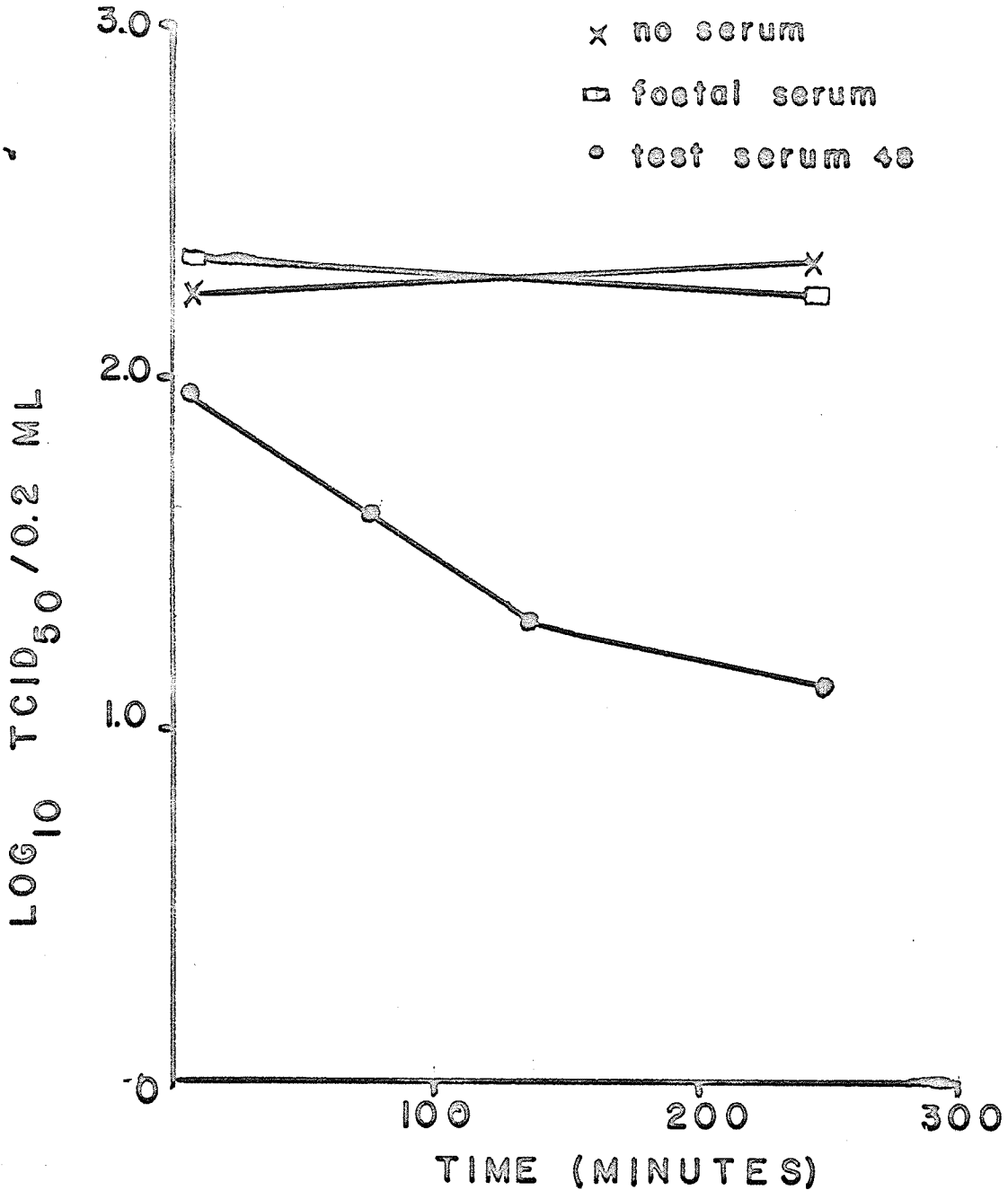


LEVELS OF CONGLUTININ AND OF VIRUS INHIBITOR IN 20 CALF SERA

FIGURE 5

(one ml to one ml) of virus suspension containing approximately 100 $TCID_{50}$ of Adenovirus type 3. This material along with a second control containing virus, but no serum, were placed in water baths at temperatures of 37°C and 21°C. The samples were removed at time intervals, a volume (0.2 ml) of mixture removed and samples replaced in the water baths. The material removed from the reaction tube was cooled in an ice water bath until inoculated onto tissue cultures. Infectivity assays were carried out as soon as possible after removal from heated water baths. Assays were done on samples taken at zero time, 1, 2 and 4 hours for the inhibitory serum, while zero and 4 hour samples were titrated for the foetal and control without serum. The results after 22 days are shown in Figure 6. This figure illustrates readings obtained from samples collected at 37°C. The inactivation observed at 21°C occurred to the same degree although this was unexpected in light of earlier work.

The $TCID_{50}$ of virus material mixed either with foetal serum or with PBS is maintained over the time period tested. Conversely the virus material mixed with the inhibitory test serum shows a decrease in infectivity. The loss in titer is not extremely large (one log) over four hours but the loss considered along with the rate is not at all characteristic of antibody, which is reported in the literature as showing a much more rapid loss of activity over the time periods considered here.



KINETICS OF VIRUS

INACTIVATION BY INHIBITORY SERUM

FIGURE 6

DISCUSSION

DISCUSSION

The initial observation that pooled batches of local calf sera affected the sensitivity of infectivity assays for Adenovirus was not a surprising phenomenon. The report of inhibitory substances in bovine sera by Klein (1959, 1963) which he considered antibodies to bovine Adenoviruses, could account for the presence of these inhibitory substances. Preliminary investigations were carried out to establish if the inhibition observed was due to antibody. These results did not confirm the nature of the inhibitor as being similar to that of antibody and further experiments to elucidate its nature were made.

Results were obtained from the use of the two techniques, Procedure A and Procedure B. Other investigators (Gold & Ginsberg 1961; Carmichael et al., 1963) of inhibitors in serum have employed the standard neutralization test (procedure B) as a measure of inhibitory quantities with others (Klein 1964) altering the test and varying the time and temperature as I have done. The use of foetal and horse serum as low inhibitory serum with which to establish standards of measure is an arbitrary choice and it may well be that both horse and foetal sera are inhibitory to a slight degree.

The experiments describing the choice of 37°C and three hours as standard in experiments using procedure B are interesting in their revelation of the dependency upon temperature for full neutralizing effect. If the inhibitor is antibody there would be no variation expected

in neutralizing ability (Kjellen, 1962) at the two temperatures cited. A later experiment studying the effect of temperature on the rate of neutralization of Adenovirus by inhibitory sera is in conflict with the above observation as the loss of infective virus at room temperature is similar to the loss seen at the temperature of 37°C. These conflicting results may be attributed to the use of different inhibitory serum with different action of the inhibitory substances within these sera.

The slight discrepancies in the results obtained when procedure A and procedure B were compared indicate either that; 1) more than one kind of inhibitor is present in the serum or, 2) that there is one inhibitor in some sera but it expresses its activity in more than one way. For example, in procedure A, if inhibitor is present at the time of the primary interaction between virus and host cell, the inhibitor in addition to directly inactivating the virus may also interfere with virus adsorption. In procedure B, on the other hand, the inhibitory activity is the direct action on the virus, as the amount of inhibitory serum present during the time virus is adsorbing to the cells is very small, even when cultures are inoculated with the lower dilutions of the virus-serum mixtures.

The variation of inhibitory serum in the cattle population by the criteria used can lend credence to arguments both for and against the substance being antibody. Klein (1955) felt that the inhibitor found in cattle sera that neutralized all three types of Poliovirus to different

degrees indicated that this variation was due to antibody rather than that of non-specific inhibitors. There is, of course, no valid reason why a variation as observed in the 50 bovine samples could not represent non-specific stimulation, say of an agent or agents antigenically related to Adenovirus or even individual inhibitors of a metabolic nature. All of the adult bovine sera tested were classified as inhibitory. Other workers with Adenovirus inhibitors (viz., Gold, 1961; Carmichael, 1963) used adult sera in their investigations. Both demonstrated inhibitors in this sera. The results obtained cannot allow any conclusions as to the incidence between calf and adult sera but from these limited results and the literature it is interesting to speculate on the possibility of adult ox serum proving to be more inhibitory. Is this increased inhibition due to repeated stimuli of bovine or possible human Adenoviruses or the greater exposure of animals to possible non-specific stimuli due to age? Again this observation could be interpreted from either point of view.

The sensitivity of the Adenoviruses tested, sub-groups representatives, does not fully agree with the results obtained by Gold (1951). He noted that the types 4, 5 and 7 Adenoviruses were more sensitive than types 2, 6 and 3. Types 3 and 7 belong to a different sub-group than do 2, 4, 5 and 6. This indicates that classification by hemagglutination does not prove a valid basis on which to differentiate inhibitory ability. The results seen in Table V substantiate the crossing of virus sensitivity

within sub-groups although type 8 of sub-group 2 indicates greater sensitivity to the inhibitor in these four test sera. Although Adenovirus type 12 was included in these experiments conclusive results were not forthcoming. However, other observations made in this laboratory indicate that type 12 is highly sensitive. The appearance of this inhibitor of Adenovirus in calf sera stimulates one to look for other inhibitors of a similar nature. Gold (1961) and Carmichael (1963) have investigated the sensitivity of Adenovirus and Infectious Canine Hepatitis, respectively, to bovine serum inhibitors and in both cases some similar and dissimilar features to the inhibitor investigated here were found. Gold's studies showed that his inhibitor had the same thermal lability as the inhibitor observed in the present investigation. It was precipitated with half saturated ammonium sulphate with the globulins, but was not inactivated by treatment with RDE or trypsin.

Results from Carmichael showed that the inhibitor was in the globulin fraction and was not destroyed by treatments with heat, periodate, trypsin, ether or dialysis. When both trypsin and chymotrypsin were used the globulin inhibitor was destroyed.

The characteristics of the inhibitor investigated in this thesis does not appear to be similar to any other inhibitors found either for Adenoviruses or other viruses. Whether the inhibitory substance found in bovine sera has a distinct identity or is of similar composition to

other recognized inhibitors but varys in its ability to neutralize (inhibit), is fundamental to the question of its identity being antibody or not.

Investigations into the physical and chemical nature of the inhibitor reveals information with which to classify and compare the inhibitor to others that have been demonstrated. The significant points brought out are the ability to reduce inhibitory action by treatment with crude, crystalline trypsin and chymotrypsin. The insensitivity to the other chemical treatments permits the postulate that there are no polysaccharides or lipid moieties involved. It seems highly significant that antibody has been shown not to be sensitive to these enzymic treatments (Sampaio, 1952; Klein, 1960).

The thermal inactivation is not altogether different than that noted by Gold (1961) and Klein (1965). Klein says that antibody to Bovine Enterovirus prepared in rabbits in only moderately affected by treatment at 70°C for 30 minutes. Both the inhibitor studied by Klein and the inhibitor found in calf sera show greater loss in inhibitory titer at this temperature. Literature dealing with thermal inactivation of Poliovirus antibody is inconsistent in the results relating thermal inactivation falling within the range of 65° to 75°C.

The observation by Klein would lend credence to the

substance being other than antibody but specific thermal inactivation studies with Adenovirus antiserum, preferably bovine antiserum, would have to be done to validate any conclusions drawn from Klein's work.

The possibility of there being more than one inhibitor has been referred to before. The rate of inactivation of the inhibitor at 65°C gives some indication that this may be the case. The graph (Figure 3) shows that a rapid loss of activity occurs in fifteen minutes with a decrease in the rate thereafter. This rapid loss would indicate the presence of only one inhibitor, at least until the curve is altered at fifteen minutes when a second inhibitor may be exerting an influence. However, the level remaining at this time (2 ND_{50}) is so low as to be almost negligible. The rest of the experiments do not completely rule out the possibility of more than one inhibitor being present.

The results from the second working hypothesis, that of the inhibitor being conglutinin, have not indicated any consistent significant correlation between the presence of conglutinin and serum inhibition levels. The partial precipitation of the inhibitory substance by both 33% saturated ammonium sulphate when dialyzed against distilled water, when the literature mentions that all of the conglutinin is precipitated by these techniques, leaves doubt as to the actual involvment of conglutinin in inhibition. However, as we did not actually measure conglutinin levels in supernatants and precipitates we can't actually confirm that conglutinin was precipitated and rely completely on the literature (Coombs, 1961)

concerning conglutinin precipitation. Further studies would have to be done to ascertain the place of conglutinin in calf serum inhibition.

The virus-cell-serum relationship has been investigated in order to determine the mode of action of inhibitor sera on Adenovirus. The presence of inhibitor could affect the resultant virus titers in many ways. Experiments concerning the kinetics of virus inactivation by serum have demonstrated the loss of virus titer over various time periods. The greater the time of exposure of virus to serum, the greater the loss of infectivity. This demonstrates that virus can be inactivated by serum before being incorporated in tissue culture as well as afterwards, viz., before virus adsorbs to and penetrates the host cell. This extent and rate of inactivation is not characteristic of antibody. Antibody under these conditions demonstrates a faster rate than seen with inhibitory serum. The inhibitory substances have, however, been shown to be a large molecule in the 19S range, and could be an antibody that is not as avid in combination with antigen as the later 7S antibody, which could account for this moderate rate of inactivation. The other aspects of the cell-virus-serum relationship such as inhibition of adsorption of virus to cells and altered host reaction to virus have not been considered although there is evidence that inhibitory serum can interfere with virus attachment to host cells (Hannan, unpublished results).

The evidence favouring the identity of the inhibitor as that of antibody is fairly strong viz., gamma globulin, stimulus and incidence, heat

heat inactivation, although the treatment with trypsin and chymotrypsin is not consistent with the substance being antibody.

SUMMARY

SUMMARY

Inhibitory substances have been found for Adenovirus type 3 in 33% of individual calf serum samples collected from a local packing-house. This inhibitory factor has been assayed by two techniques, that called procedure A, an infectivity assay with test sera included in maintenance medium, and procedure B, a modified neutralization test. A temperature of 37°C for three hours provided optimal conditions for obtaining readings by procedure B.

There appears to be a slight difference of sensitivity among the serotypes of Adenoviruses to this inhibitor, with type 8 demonstrating greater susceptibility than types 3, 7 and 5.

The inhibitor was not removed when sera was dialyzed against running tap water of o.85% Na Cl. It is not inactivated at temperatures up to 56°C for 30 minutes or 60°C for 30 minutes, but is rapidly inactivated at 70°C. At 65°C a rapid loss of activity occurs in fifteen minutes with a decrease in the rate thereafter.

The inhibitor was found to be sensitive to trypsin and chymotrypsin. It is not affected by treatment with RDE, ether extraction, periodate, or absorption with kaolin. It is precipitated in the globulin fraction by 50% saturated ammonium sulphate and is partly precipitated by either 33% saturated $(NH_4)_2SO_4$ or by dialysis against distilled water. These results were interpreted to indicate that the inhibitor is protein only and contains no lipid or polysaccharide moieties.

Density gradient centrifugation studies indicate that the inhibitor is a large molecule of the order of size of 19S serum globulins.

Attempts to determine a relationship between the inhibitor in calf sera and conglutinin indicate they are not related although conglutinin may be responsible for some of the inhibitory action.

The inhibitor is found to inactivate Adenovirus type 3 virus but to an extent and rate not at all characteristic of antibody.

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APPENDIX

APPENDIX.

CONGLUTININ ASSAY.

1. Absorption of test sera with sheep ertyrocytes.

   a) Centrifuge 10 ml of 10%, 3x washed sheep erythrocytes at 2000 rpm for 10 minutes and discard supernatant.

   b) To each packed cell sediment add 2.0 ml of test serum. Resuspend erythrocytes, incubate 20 minutes at room temperature.

   c) Centrifuge at 200 rpm for 10 minutes, withdraw serum and transfer to clean, sterile container.

2. Preparation of sensitized sheep erythrocytes.

   a) In a 14 ml centrifuge tube mix 4.0 ml of 5x washed sheep erythrocytes and 4.0 ml of heat inactivated bovine serum #48.

   b) Incubate mixture at 37°C for 15 minutes.

   c) Centrifuge 1500 rpm for 10 minutes, discard supernatant.

   d) Add 8 ml PBS, resuspend cells.

   e) Centrifuge 1500 rpm 10 minutes, discard supernatant.

   f) Add PBS to total volume of 4.0 ml and suspend cells.

3. Preparation of alexinated sheep erythrocytes.

   a) Prepare a mixture of the following materials in a 15 ml centrifuge adding in the order shown:

      1. 7 ml PBS
      2. 1 ml horse C'
      3. 1 ml horse serum inactivated at 56°C for 30 minutes
      4. 1 ml of 15% sheep erythrocytes.

   b) Incubate at 37°C. for 15 minutes.

   c) Centrifuge 1500 rpm 10 minutes and discard supernatant.

   d) Add 10 ml of PBS, resuspend cells, centrifuge 1500 rpm for 10 minutes, discard supernatant.

   e) Add PBS to total volume of 10 ml and resuspend cells.

APPENDIX (cont.)

4. Preparation of control sheep erythrocytes.

   a) Procedure is exactly the same as for #3 but the mixture is as follows:

      1. 7 ml PBS
      2. 2 ml horse serum inactivator 56°C
      3. 1 ml 5% sheep erythrocytes

5. Test.

   a) Prepare, for each test serum, 2 sets of serial 2-fold dilutions in PBS in volumes of 0.1 ml. Range of 1:2 to 1:1024.

   b) To every tube, add 0.1 ml of 1:10 heat inactivated horse serum.

   c) To one set, labelled A, add 0.1 ml of 0.5% A sheep erythrocytes.

   d) To the second set, labelled C, add 0.1 ml of 0.5% C sheep erythrocytes.

   e) Incubate 37°C for 30 minutes.

   f) Read after centrifuging tubes at 1500 rpm on one minute. Shake and read.