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FURTHER STUDIES OF A HERPES-LIKE VIRUS FROM CASES OF CAT SCRATCH DISEASE

DISSERTATION

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

By

WILLIE TURNER, B.S., M.Sc.

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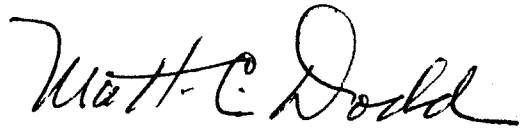
The Ohio State University
1961

Approved by



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INTRODUCTION

Evidence available to date indicates the probability of the viral etiology of cat scratch disease, although the actual causal agent has so far not been identified.

Following the demonstration by Dodd, Graber, and Anderson (1959) of a hemagglutinin in pus from persons with this disease, Turner, Bigley, Dodd and Anderson (1960) reported the isolation of a virus, antigenically related to herpes simplex, from chick embryos inoculated with such material, and which was demonstrable by hemagglutination of rabbit red blood cells.

The present work concerns the further examination of this virus to characterize it more definitely and to attempt to solve some of the questions suggested by the earlier study. For these reasons, careful study was made of the conditions required for optimal demonstration of hemagglutination, especially with reference to the chemical nature of the inhibitor present in allantoic fluid. Effort was also made to relate the virus to the cause of the disease, principally by attempts to show hemagglutination-inhibiting antibody in the sera of humans with the disease. This involved experiments with sera from rabbits immunized with the virus for

the purpose of determining the best conditions for such tests and methods for removing serum inhibitors.

The immune response of rabbits immunized with the virus and with the pus from human cases of the disease was compared by serologic and skin tests. Finally, further work on the relationship of the agent to herpes simplex revealed that hemagglutination methods utilized to demonstrate the former could also be applied to the demonstration of herpes virus which previously has been considered a non-hemagglutinating virus.

LITERATURE REVIEW

Cat scratch disease is a non-fatal systemic illness characterized by fever, malaise, and lymphadenitis. Little is known concerning this infection which was first described by Debre (1930) and Foshay (1932). The first description of this infection in American literature was recorded by Greer and Keefer (1951). Daesehner et al. stated that in 1951-1952 two hundred cases of this infection occurred throughout the world, twelve of which occurred in southern Texas. These authors felt that the incidence of this infection may be much greater than recorded because of the confusion of the symptoms of cat scratch disease with other types of infection.

The name, cat scratch disease, was given to this specific infection because the infected individuals usually have had some recent association with cats, although the patient may have had no definite knowledge of having been scratched, licked, or bitten by a cat. Actual animal contact was established in only two of four cases of cat scratch disease reported by Fox (1952). Greer and Keefer (1950) reported a case of cat scratch disease which had been contracted by a woman who merely sniffed a cushion on which feline urine was present. Numerous cases of cat scratch disease have been

reported in which the patients have had no actual contact with cats, but have been scratched by such objects as rose thorns and wood splinters. Thus, it is indicated that the presence of the cat, per se, may not be necessary for the occurrence of the infection. This concept led Mollaret et al. (1951) to designate this infection as benign inoculation lymphoreticulosis instead of cat scratch disease. Along with many others, these authors felt that the name, cat scratch disease, was a misnomer.

Even though cat scratches are not always part of the history of the infection, cats are associated with the disease far more than would seem likely by mere chance (Blattner, 1952). The study of various cases of cat scratch disease prompted Greer and Keefer (1951) to call attention to a large number of human diseases to which cats are subjected. These authors pointed out that the following human diseases are found in cats: tuberculosis (bovine type); tularemia; ringworm; amebiasis; diphtheria; salmonellosis (S. enteritidis, S. anatum); brucellosis; rabies; fauvus; trypanosomiasis (T. cruzi); leishmaniasis; creeping eruption; dog tapeworm; fish tapeworm; lung fluke (P. westermani); opisthorchiasis; strongyloidiasis; spirillosis; and scrub typhus. Those diseases transmitted by cats to man are listed

as follows: tularemia; rabies; rat-bite fever; feline pneumonitis (virus); diphtheria; tuberculosis; brucellosis; typhus; leptospirosis; ringworm; creeping eruption; fauvus; pasteurellosis; plague; and histoplasmosis.

From the preceding data, it is obvious that the recognition of transmission of diseases to man by cats is not new, even though the conditions are not well defined. Whether the cat is the reservoir of the organism or simply a vector of the agent causing cat scratch disease is unknown. Blattner (1952) stated that cat scratch disease could be transmitted to monkeys by inoculation with purulent material from cat scratch lesions in humans. This material produced no detectable ill effects on cats and thus indicated that cat scratch disease was not a disease of cats. Furthermore, Blattner (1952) suggested that birds might be the reservoir of the causative agent of this infection. However, attempts to infect birds with cat scratch disease have been fruitless. Consequently, this author has suggested that cats serve merely as vectors in the transmission of cat scratch disease, and that other vectors, both animate and inanimate, are significant in the transmission of this infection.

Cat scratch disease is a mild systemic illness charac-

terized by fever, malaise, and local adenopathy. The incubation period of this infection is variable, ranging from ten to thirty days. Daesehner et al. (1953) reported one case in which a patient developed adenopathy in eight days after contact. Twenty days later, the large central node was surrounded by smaller nodular masses and a skin papule appeared. The first symptom of the disease is usually a primary lesion which appears to heal slowly and resembles a bite, pustule, vesicle, or small scab. Exacerbation of redness and swelling occurs at this site. The lymph nodes in the area then become swollen and painful to the touch. The skin over the swollen node appears normal. The enlarged regional node may be hard or soft exhibiting a size of 1 x 2 to 5 x 5 centimeters. Purulent material may be aspirated from the infected node. All attempts to demonstrate bacteria in this aspirated fluid have been fruitless. The infected lymph node may later become abscessed and undergo necrosis, producing a pronounced necrotic lesion. This lesion usually heals in from four to twenty days. The healing process of the lesion is generally preceded by spontaneous drainage of the infected lymph node. Thus, cat scratch disease is, for the most part, a self-limiting localized infection. However, Stevens (1952)

reported a case of encephalitis during convalescence from cat scratch disease. The early symptoms of this infection were similar to those described for cat scratch disease, but the clinical picture changed at about the time cat scratch lesions usually heal and the symptoms of a mild type of encephalitis appeared. The patient exhibited a marked elevation in temperature, as well as convulsions, followed by coma of forty-eight hours duration. Upon examination, the spinal fluid of this patient exhibited neither bacteria nor viral agents. The serum of this patient was free of neutralizing antibodies for St. Louis (type C epidemic) or Eastern Equine encephalomyelitis viruses. Tests for Brucella as well as for Rickettsial organisms were all negative. Thus, the causative agent of this encephalitis was thought to be the etiological agent of cat scratch disease. Greer and Keefer (1951) reported a case of cat scratch disease in which the usual symptoms of the disease were accompanied by a mild skin eruption. These two cases indicated that cat scratch disease may not be strictly a localized infection under certain unknown conditions.

The symptoms that have been listed for cat scratch disease are non-specific symptoms which can be easily confused

with the symptoms exhibited by other infections. It has been observed that clinical aspects of cat scratch disease are quite variable. Lang (1951) described a case of cat scratch disease in which the patient showed pulmonary infiltration with a severe cough. Mollaret et al. (1951) and Debre et al. (1950) also observed cases of cat scratch disease probably contracted by inhalation. Cervical adenitis was the major symptom seen in two cases. de Lavergne (1951) described an oculoglandular form of cat scratch disease with conjunctivitis and cervical adenopathy, while Greer and Keefer (1951) reported a form of the disease in which the major symptom exhibited by the patient was an erythema-nodosum-like exanthem. It is evident from the foregoing examples that the clinical picture of cat scratch disease is non-specific and variable.

Even though the clinical course of cat scratch disease is extremely variable, there are certain pathological changes which are characteristic of this infection. Debre (1950), Mollaret (1950), and Fox (1952) observed that the pathological aspects of cat scratch disease occur in three stages. The first or elementary stage is characterized by simple hyperplasia of all node elements. The second pathological stage is the accentuated phase in which hyperplasia is

accompanied by the appearance of areas of early cellular necrosis. When sections of these necrotic nodes were stained, they took up the acid dye component and were called ophilic masses. The third stage in the development of lesions is referred to as the ultimate stage with displacement of the normal nodal structure by multiple areas of central necrosis; characteristic circumscribed foci of epitheloid cells; and scattered giant cells of the Laughans type. Fox (1952) referred to the lesions which developed in the third pathological stage as "pseudotubercle". This pathological phase corresponds in time to the drainage of the infected node with ensuing regression and healing.

The diagnosis of cat scratch disease is very difficult because of the variable clinical picture and the non-specific symptoms of the infection. However, there are several ways in which cat scratch disease may be distinguished from other diseases with similar symptoms. It can be distinguished from pyogenic adenitis by the fact that bacteria can be isolated from the pus from pyogenic adenitis and by improvement of the condition when the patient is given suitable antibiotic therapy. The positive tuberculin test distinguishes tuberculous adenitis from cat scratch disease even though symptoms of

some cases of the latter may be quite similar. Cat scratch disease may be differentiated from fungal infections such as sporotrichosis by differences in clinical aspects and laboratory tests. The cat scratch disease patient also exhibits a negative skin test with tularense antigen, and there are no agglutinins to Pasteurella tularensis demonstrable in the sera. Foshay (1950), Debre (1950), Greer and Keefer (1951), and Stevens (1952) reported that a positive skin test reaction occurs in patients with cat scratch disease injected intracutaneously with an antigen prepared by the Frei procedure with purulent material from infected nodes of known cases of the disease. The reaction is a delayed-type hypersensitive reaction which occurs about forty-eight hours after the intradermal injection of the antigen. The skin test is positive in most patients from one to fourteen months after the onset of lymphadenitis. The skin test is considered quite significant in the diagnosis of the disease.

Stevens (1952) demonstrated complement-fixing antibodies at a titer of 1:128 in the serum from a patient with cat scratch disease using lygranum antigen. The lygranum antigen is prepared from yolk sac of chick embryos moribund or recently dead from infection with the virus of lymphogranuloma

venereum. This has been confirmed by Greer and Keefer (1951) and Debre (1950). Since the titers of complement-fixing antibodies demonstrated by this procedure have been usually quite low, the diagnostic value of it is doubtful.

There are no specific therapeutic measures for this infection, but Lang (1951) states that patients receiving terramycin and/or chloromycetin showed rapid regression of the infected lymph node as well as reduction of the febrile stage of the disease. Mollaret (1950), Greer and Keefer (1951), Steven (1952), and Fox (1952) stated that aureomycin seemed to reduce the course in some cases, but none of the other antibiotics had any effect on the infection. Aureomycin is one of the broad spectrum antibiotics which are effective against large viruses like those of psittacosis-lymphogranuloma group. Thus, the response of the disease to aureomycin therapy supports the belief that the etiological agent of cat scratch disease is a virus belonging to the psittacosis-lymphogranuloma group of viruses.

The etiological agent of cat scratch disease is not known, but considerable evidence has been obtained which points to a virus as the causative agent of this infection. Mollaret *et al*. (1951) stated that the infection was due to an agent similar to the psittacosis-lymphogranuloma group

of viruses. This opinion is based on the fact that stained sections of primary skin lesions and nodes from cases of the disease showed intracellular and extra-cellular granules identical in appearance with elementary bodies characteristic of psittacosis. These granules were found particularly in reticulocytes bordering the zone of necrosis, but they may also be present in the lymphocytes.

Dodd and Graber (1957) detected the presence of a hemagglutinin in pus from three cases of cat scratch disease which agglutinated rabbit erythrocytes. Specific inhibition of this hemagglutination was demonstrated with rabbit antiserum to the pus and by the serum from human cases of the disease. Dodd and Bigley (1958) also demonstrated a hemagglutinin for rabbit erythrocytes in allantoic fluid from embryonated eggs inoculated with pus from a cat scratch lesion.

Mollaret et al. (1951) also succeeded in transmitting the infection to monkeys, but other attempts to transmit the disease to other animals and man have failed.

In the most recent review on cat scratch disease, Debre and Job (1954) expressed their belief in the viral etiology of this disease based on their observation of

inclusions in sections of lymph nodes from infected humans and monkeys. These authors were also of the opinion that the virus belongs to the psittacosis-lymphogranuloma venerum group because of the morphological similarity of the inclusions observed in tissue from lymph nodes in cases of cat scratch disease and the inclusions observed in tissue from cases of psittacosis and lymphogranuloma venerum. However, the failure of these authors to demonstrate growth of the cat scratch agent in the yolk sac of embryonated chick eggs has caused them to have some reservations on classifying it in the psittacosis-lymphogranuloma venerum group of viruses since the members of this group can be readily propagated in this manner.

All the work reviewed tends to lend support to the original contention of Mollaret; namely, that the etiological agent of cat scratch disease is a virus. However, the actual isolation and identification of the etiological agent of this infection have never been satisfactorily performed.

Turner, Bigley, Dodd, and Anderson (1960), as an extension of the work previously noted by Dodd and Graber (1959) and Dodd and Bigley (1958), found that a virus could be demonstrated by hemagglutination of rabbit and rat

erythrocytes in allantoic fluids from seven-day-old chick embryos inoculated with pus from three human cases of cat scratch disease. An inhibitor was present in these fluids which prevented the demonstration, but which was removed by treatment of the fluid with 0.28 m glucose and centrifugation, a procedure employed earlier by Sabin and Chanock (1953) to remove an inhibitor of hemagglutination by St. Louis and Japanese B encephalitis viruses found in mouse brain.

Inhibitor of hemagglutination by the CSD agent was also present in all rabbit sera examined and in most human sera which was inactivated by treatment with acetone and hydrochloric acid. This latter method was also employed by Sabin and Chanock (1953) for removing inhibitor of hemagglutination by the above-mentioned encephalitis viruses. Following the removal of serum inhibitor, Turner et al. (1960) were able to demonstrate hemagglutination-inhibition antibody in sera of rabbits immunized with inhibitor-free, infected allantoic fluid, but in serum from only a relatively few human cases of the disease and, surprisingly, in sera from several persons infected with herpes simplex virus. Although a marked antigenic relationship between the CSD

virus and herpes simplex was demonstrated, the former lacked the virulence for chick embryos, the cyto-pathogenic effect on cells in tissue culture, and the capacity to produce corneal lesions in rabbits so characteristic of herpes.

The results to be reported in the following sections of this dissertation are concerned with an attempt to investigate more thoroughly some of the findings reported by Turner, Bigley, Dodd, and Anderson (1960) noted previously. Factors affecting the optimal hemagglutination conditions for the cat scratch disease virus, as well as the chemical nature of the allantoic fluid and serum inhibitors, as reported by Turner et al. (1960), are extensively investigated.

Experiments to detect the presence of hemagglutination-inhibiting antibodies in the sera of patients with cat scratch disease, as well as the skin reactions demonstrated by rabbits immunized with both pus from cat scratch disease lesions and allantoic fluid from chick embryos inoculated with cat scratch disease virus, were carried out.

Since Hirst (1941) and, independently, McClelland and Hare first demonstrated hemagglutination of chick erythrocytes by influenza virus, numerous viruses have been shown to hemagglutinate erythrocytes. The following viruses were listed by Hirst (1959) as making up the three groups of the

so-called hemagglutinating viruses: 1) the influenza, Newcastle disease, mumps group; 2) vaccinia, variola, ectromelia, meningo-pneumonitis; 3) various encephalitis viruses (such as West Nile, St. Louis, Russian Spring-Summer); fowl plague virus and the virus of foot-and-mouth disease. Because of the previous report on the antigenic relationship of the CSD agent to herpes simplex virus (Turner et al.), the latter was tested for hemagglutinating activity by the same procedure used with the CSD agent. Surprisingly, since herpes has been considered previously as a non-hemagglutinating virus, data are presented indicating that it does have the capacity to hemagglutinate rat red cells.

MATERIALS AND METHODS

Preparation of CSD Virus Hemagglutinin

Pus from infected lymph nodes of cases of suspected cat scratch disease was obtained from Drs. George Anderson and William Murray of the Ohio State Department of Health Laboratories and Children's Hospital, Columbus, Ohio, respectively. One-tenth ml quantities of a 1:10 dilution of the pus were inoculated into the chorioallantoic cavities of seven-day-old chick embryos. Allantoic fluids from the infected embryos were harvested on successive days after inoculation. The harvested fluids were placed in sterile vaccine vials and stored at -20°C.

To obtain the various passage fluids for hemagglutination, the fluid from the preceding passage exhibiting the highest titer of hemagglutination was used as the inoculum. One-tenth ml of the undiluted fluid and 0.1 ml of a 1:10 dilution of the fluid were inoculated into the allantoic cavities of separate groups of seven-day-old chick embryos. The allantoic fluids from these embryos were harvested on successive days after inoculation. Freezing and storage of the subsequent passage fluids were identical to the methods employed with the original passage material.

Preparation of Herpes Virus Hemagglutinin

Tissue culture adapted strains of herpes virus, HF and CS strains, obtained from Dr. George Anderson, Ohio State Department of Health Laboratories, Columbus, Ohio, and an egg adapted strain of herpes virus obtained from the Communicable Disease Center, Chamblee, Georgia, were employed. Five-tenth ml of the tissue culture adapted and egg adapted strains of the herpes virus were inoculated into the yolk sacs of seven-day-old embryos. The inoculated eggs were candled once every 24 hours and those in which the embryos died in less than 48 hours were discarded since death during this time was considered to be due to non-specific causes. Allantoic fluid from inoculated eggs in which the embryos died after 72 hours was harvested and placed in sterile vaccine vials and stored at -20°C.

To obtain the various passage fluids showing high titer of virus as measured by hemagglutination, the fluid from preceding passage exhibiting the highest titer of virus hemagglutinin was used as the inoculum. Five-tenth ml of the undiluted fluid was inoculated into the yolk sacs of seven-day-old chick embryos. The allantoic fluid was harvested from the embryos dying after 72 hours. Freezing and

storage of the subsequent passage fluids were identical to the methods employed with the original passage material.

Preparation of the Influenza Virus (PR-8) Hemagglutinin

Stock influenza virus (PR-8) was prepared by inoculating 0.1 ml of virus into the allantoic cavity of 10-11-day-old fertile hen's eggs. Following a 48-hour incubation at 37°C, the inoculated eggs were refrigerated for 3 to 12 hours and infected allantoic fluid was removed. The virus-containing fluid was then tested for sterility, titrated for hemagglutinin, ampuled, and frozen at -50°C.

Influenza Virus Hemagglutination Titration

Virus hemagglutination titrations were performed in round-bottom tubes measuring 7 x 75 mm. Doubling dilutions of virus suspensions were prepared in 0.5 ml amounts of phosphate buffered saline (pH 7.4) and 0.5 ml of 0.5 per cent human O cells were added to each virus dilution. Titrations were incubated one hour at room temperature and read on the basis of the pattern of sedimented cells.

CSD Virus Hemagglutination Titrations

Round-bottom tubes measuring 7 x 75 mm were used for the titrations. Doubling dilutions of the treated hemagglutinating allantoic fluid were made in 0.85 per cent

sodium chloride solution in 0.5 ml volumes. An equal volume of a 2.0 per cent suspension of rabbit erythrocytes was added to each tube. After a 30-minute incubation at room temperature, the tubes were centrifuged in the Servall Angle Head Centrifuge at 3500-4000 rpm. The centrifuge was allowed merely to reach maximum speed before the centrifuging procedure was stopped. The tubes were then read for the presence of hemagglutination.

When CSD passage fluids are treated with cysteine (0.1 m) and sodium sulfite (.06 m), the centrifugation and titrations of the treated fluids were carried out in tightly stoppered centrifuge and hemagglutination tubes to prevent oxidization of the cysteine and sodium sulfite in the fluids by atmosphere oxygen.

Titrations of the CSD virus hemagglutinin were also carried out in 0.25 ml volumes using an equal amount of a 1.0 per cent suspension of rat erythrocytes. After a 30-minute incubation at room temperature, the tubes were observed for pattern-type hemagglutination of rat erythrocytes. The method of treatment of CSD passage fluid titrated with rat erythrocytes was the same as that used to treat passage fluids titrated with rabbit erythrocytes.

Herpes Virus Hemagglutination Titrations

Round-bottom tubes measuring 7 x 75 mm were used for the titrations. Doubling dilutions of the hemagglutinating allantoic fluid were made in .016 molar borate-potassium-chloride in 0.25 ml volumes. An equal volume of a 1.0 per cent suspension of rat erythrocytes was added to each tube. After 30-minute incubation at room temperature, the tubes were observed for pattern-type hemagglutination of the rat erythrocytes.

Preparation of Suspension of Normal Bovine, Equine, Human "O" Rh^+, Sheep, and Chicken Erythrocytes

The bovine, equine, sheep, chicken, and human "O" Rh^+ erythrocytes were obtained from blood from normal individuals of these species by cardiac or venous puncture. The blood was collected in a five per cent citrate solution. It was then centrifuged and the supernatant fluid including the buffy coat removed. The red cells were then washed three times in 0.85 per cent salt solution and used as a 2.0 per cent suspension in physiological salt solution.

Preparation of Suspension of Normal Rat Erythrocytes

The rat erythrocytes were obtained from normal hooded rats. Blood was collected by cardiac puncture in one per

cent citrate solution and heparin (10,000 units/cc) and was freed of plasma and white cells by centrifugation and subsequent removal of supernatant fluid and buffy coat. The erythrocytes were then washed three times in 0.85 per cent saline and used as 1.0 per cent suspension.

Preparation of Fractions of Normal Human Serum

The fractions of human serum were provided by Dr. Nancy Bigley and Mrs. Virginia Geyer, Department of Microbiology, The Ohio State University. The fractions were collected by means of a continuous flow paper electrophoresis cell, Spinco model P, separated at 40 milliamperes constant current. Nine liters of Veronal buffer pH 8.6, 0.02 ionic strength, were used in the electrophoresis. The buffer consisted of 1.65 grams of sodium barbital, 2.51 grams barbituric acid, and 0.40 grams sodium hydroxide per liter.

Other serum fractions were purchased from the Pentex Incorporated, Kankakee, Illinois, and prepared by the methods of Cohn (1946) and Mehl (1949).

Precipitation Titration of Serum Glycoprotein and Urinary Mucoprotein

Doubling dilutions of the glycoprotein and urinary mucoprotein were made in 0.85 per cent NaCl. Twenty-five hundreth ml of each of these dilutions were added to

precipitation tubes and layered with undiluted urinary mucoprotein antiserum. These tubes were then incubated at refrigeration temperature for four to seven days. At the end of this period of time the tubes were read for the presence of a precipitate in the bottom of the tubes.

Preparation of Urinary Inhibitor (Mucoprotein) and Antiserum to the Urinary Inhibitor (Mucoprotein)

The urinary mucoprotein and rabbit antiserum to it were supplied by Mr. Howard Johnson, Department of Microbiology, The Ohio State University, Columbus, Ohio. The urinary mucoprotein was prepared according to the method of Tamm and Horsfall (1952).

Antiserum to the urinary mucoprotein was prepared in rabbit by method of Johnson (1949).

Preparation of Chemical Solutions Used to Treat CSD Passage Fluid to Remove Inhibitor

The various molar concentrations of the chemicals used were prepared in 50 ml volumetric flasks. The amounts of the various chemicals added to 50 ml of distilled water to produce the given molar concentrations is shown below:

Chemical	Amount in Grams	Molar Concentration
Glucose, fructose, mannose	2.52	0.28 m
Lactose, maltose, sucrose	4.79	0.28 m
NaCNS	1.05	0.26 m
Na_2SO_4	2.13	0.28 m
Cysteine	0.61	0.1 m
Sodium sulfite	0.38	.06 m
Calcium gluconate	0.23	.01 m
Potassium periodate	0.57	.05 m

The flasks containing the above solutions were kept tightly sealed and refrigerated.

Preparation of Diethylaminoethyl and Carboxymethyl Cellulose Resins

The resins were first washed with 1.0 N sodium hydroxide followed by several washings with distilled water. The pH of the final distilled water wash was measured and if the pH was above 7.0, then the resins were washed with KH_2PO_4 (pH 6.3) buffer until the pH of the discarded wash was 7. If the pH of the final distilled water wash was below 7, then the resins were washed with 1.0 N sodium hydroxide until the pH of the discarded wash was 7.

Treatment of Passage Fluids with Glucose, Mannose, Fructose, Lactose, Sucrose, Maltose, Sodium Sulfate (Na_2SO_4), Sodium Thiocyanate, Sodium Sulfite and Cysteine

A 1:5 dilution of the fluid to be treated was made with the various concentrations of each of the substances listed above. This mixture was centrifuged at 14,360 rpm for 60 minutes at 4°C.

When sodium sulfite and cysteine were used, the solutions were protected at all times from oxidation by atmospheric oxygen by keeping them in tightly stoppered flasks and tubes.

Treatment of Passage Fluid with Diethylaminoethyl (DEAE) and Carboxymethyl (CM) Cellulose Resins

Five ml of the passage fluid to be treated were added to a concentrated slough of DEAE and CM resins and allowed to stand at room temperature for 15 minutes. The mixtures were then centrifuged at low speed. The supernatant fluids were then removed and tested in the usual manner for the presence of the CSD virus hemagglutinin.

Treatment of Sera with Acetone for Removal of Inhibitor

One-tenth ml of the serum was diluted to 1.0 ml by the addition of 0.9 ml of physiological saline. Twelve ml of

acetone were then added to the diluted serum and centrifuged at 2,000 rpm in the Servall centrifuge for 3 minutes. The supernatant acetone was decanted. An additional 12 ml of fresh acetone was added to the precipitate which was stirred and shaken for one minute prior to a 10-minute centrifugation at 2,000 rpm. After decanting the acetone and allowing the precipitate to dry for one hour at room temperature, 0.92 ml of a mixture consisting of 7.6 parts of 0.02 molar phosphate saline, pH 6.5, and 1.6 parts of 0.02 normal hydrochloric acid (HCl) was added yielding a final volume of 1.0 ml of solution at a pH of approximately 6.5. This reconstituted mixture corresponded to a serum dilution of 1:10.

Treatment of Serum with Periodate for Removal of Inhibitor

One volume each of serum and 0.05 molar potassium periodate (KIO_4) were thoroughly mixed and incubated at room temperature for two hours. After this period of time, two volumes of five per cent glucose were added. Glucose stops the action of periodate by reducing the remaining periodate to a radical which is not biologically active.

Titrations of Antisera to CSD or Herpes Simplex Viruses for Hemagglutination-Inhibition Antibody

Doubling dilutions of the treated anti-CSD sera were made in 0.25 ml volumes of saline. Twenty-five hundredth ml of a dilution of CSD infected allantoic fluid, previously treated with glucose, was added to each tube. This dilution was selected as the highest dilution exhibiting maximum hemagglutination and was kept in an ice bath until used. The addition of this fluid was immediately followed by the addition of 0.25 ml of a two per cent suspension of normal rabbit erythrocytes. The tubes were incubated at room temperature for 60 minutes, centrifuged at 2,000 rpm for one minute, and observed for hemagglutination.

Titration of the herpes antiserum was carried out in the same manner as given above with the exception that rat erythrocytes (one per cent) were used and the titrations were observed for pattern-type hemagglutination of the rat erythrocytes. Cross titration of herpes and CSD antiserum against the herpes and CSD virus agents were carried out in much the same manner as given above. Both the rat and rabbit erythrocytes were used in these cross titrations of the herpes and CSD antisera. When rabbit erythrocytes were

used the tubes were observed for hemagglutination of the cells after centrifugation, while the tubes were observed for pattern-type hemagglutination of the red blood cells when rat erythrocytes were used in the titrations.

Hemagglutination-Inhibition Titration of Antiserum for Influenza Virus (PR-8)

Doubling dilutions of periodate-treated serum were made in 0.25 ml volumes of phosphate buffered saline (pH 7.4). Four hemagglutinating units of virus contained in a 0.25 ml volume were added to each tube. Following a 10-minute incubation period, 0.5 ml of a 0.5 per cent suspension of human erythrocytes was added to each tube. The tests were then incubated at room temperature for one hour and observed for the pattern of sedimented cells.

Adsorption of Human Sera with Rabbit Erythrocytes

Human sera were adsorbed at room temperature with packed rabbit erythrocytes in the following manner:

Adsorption	Ratio of Serum to Cells		Time
1st	1	2	60 min
2nd	1	2	60 min

Rabbit Immunizations

Four injections (0.2 ml each) of pus diluted in saline from CSD lesions were given intravenously at two-day

intervals. One week after the last of these injections a fifth injection of 0.2 ml of pus was given. Seven days later, the animals were skin-tested and subsequently bled and the serum collected, inactivated, treated, and titrated.

Two other rabbits were immunized to CSD virus by injections of a strongly hemagglutinating passage fluid which had previously been filtered through a selas filter. The immunization procedure was the same as above with the exception that each of the four injections of passage fluid was 0.5 ml, while the fifth injection consisted of 1.0 ml.

Skin Testing Procedure

Hair was removed from the backs of the immunized rabbits by means of hair clippers. One-tenth ml of pus, CSD infected allantoic fluid, and normal allantoic fluid were inoculated intradermally in separate areas of the skin of rabbits immunized with pus and passage fluid. The rabbits were then observed over a period of 5 days for the appearance of redness, swelling, or necrosis at the sites of injections of the pus and fluids.

RESULTS

The purpose of this work was to extend the earlier observations on the properties of the CSD virus, including (1) optimal conditions for hemagglutination; (2) nature of the inhibitor; (3) possible relationship of virus as cause of cat scratch disease by serologic and skin tests; and (4) further evidence on the relationship to herpes virus including the fact that the latter will also hemagglutinate with method similar to those used with the CSD virus. It was shown previously (Turner *et al.*) that 0.28 m glucose effectively removed or inactivated the inhibitor of the CSD hemagglutinin. In the present work, both the quantitative aspects of this effect, as well as the effects of other similar sugars, were examined as a possible means of increasing the effectiveness of methods for inhibitor removal, as well as to obtain information on the mechanism of the action of glucose and on the nature of the inhibitor.

The effects of various concentrations of glucose and other sugars on the allantoic fluid inhibitor of the CSD hemagglutinin are shown in Table 1. In all instances, the infected fluid was originally diluted 1:5 with the concentrations of the sugars listed and then centrifuged at

TABLE 1

The Effect of Various Concentrations of Sugars on the Allantoic Fluid Inhibitor of CSD Virus Hemagglutinin

Sugars	Conc. in moles	Reciprocal of Fluid Dilution						
		10	20	40	80	160	normal fluid control	cell control
glucose	0.18	-	-	-	-	-	-	-
	0.28-0.48	4+	4+	4+	4+	4+	-	-
	0.58-0.68	4+	4+	-	-	-	-	-
fructose	0.18-0.68	4+	4+	4+	4+	4+	-	-
mannose	0.18	3+	3+	3+	4+	4+	-	-
	0.28-0.68	4+	4+	4+	4+	4+	-	-
sucrose	0.18-0.48	4+	4+	4+	4+	4+	-	-
	0.58-0.68	4+	4+	3+	2+	2+	-	-
lactose	0.18-0.28	4+	4+	4+	4+	4+	-	-
	0.48	4+	4+	-	-	-	-	-
maltose	0.18-0.48	4+	4+	4+	4+	4+	-	-
CSD	0.58-0.68	4+	4+	4+	-	-		
fluid	untreated	-	-	-	-	-	-	-

TABLE 2

The Effect of Various Concentrations of Calcium Gluconate on the Allantoic Fluid Inhibitor of the CSD Virus Hemagglutinin

Conc. in moles	Reciprocal of Fluid Dilutions						
	10	20	40	80	160	normal fluid control	cell control
.01	+	+	+	+	-	-	-
.03	-	4+	4+	4+	4+	-	-
.05	4+	4+	4+	4+	4+	-	-
untreated CSD fluid	-	-	-	-	-	-	-
CSD-glucose fluid	4+	4+	4+	4+	4+	-	-

TABLE 3

The Effect of Various Amounts of 0.28 Molar Glucose on the Allantoic Fluid Inhibitor of the CSD Virus Hemagglutinin

Dilutions of 0.28 m Glucose	Reciprocal of Fluid Dilutions 10	20	40	80	160	normal fluid control	cell control
undiluted	4+	4+	4+	4+	4+	-	-
1:5	4+	4+	4+	4+	4+	-	-
1:20	4+	4+	4+	4+	4+	-	-
1:40	4+	4+	4+	4+	4+	-	-
1:80	+	+	-	-	-	-	-
1:160	+	±	-	-	-	-	-
1:320	+	-	-	-	-	-	-
1:640	±	-	-	-	-	-	-
CSD fluid	-	-	-	-	-	-	-

14,000 rpm. Subsequent dilutions were made in 0.85 per cent NaCl. The data on glucose show that concentrations slightly below this range (0.18 m) and above (0.58 m) are less effective or completely ineffective. It is quite possible from the results with 0.58 and 0.68 m that the inhibitor may have been removed but that the hemagglutinin was inactivated in these and the 0.18 m concentration. The results with the other sugars listed show that they are also effective over a very similar range of concentration. In fact, fructose and mannose are practically equally active over a range from 0.18 m to 0.68 m. However, sucrose and maltose, which unlike glucose are active at 0.18 m, are like the latter less effective at concentrations above 0.48 m, while lactose was ineffective at 0.48 m.

Calcium gluconate was also used to treat passage fluid to remove the inhibitor. As seen in Table 2, 0.1 molar concentration of calcium gluconate partially removed the inhibitor while .03 molar and .05 molar concentrations of calcium gluconate effectively removed or inactivated the fluid inhibitor of the CSD hemagglutinin. Optimal removal or inactivations of the fluid inhibitor was obtained with a .05 molar concentration of calcium gluconate, but increasing

the concentration of calcium gluconate further brought about enhancement of the capacity of this substance to remove inhibitor.

As noted in the previous work, the effects of these agents apparently occur in the original 1:5 dilution since subsequent dilutions for titrations were in 0.85 per cent NaCl. In order to examine this original dilution effect further, the effect of various dilutions of 0.28 molar glucose, as listed in Table 3, on allantoic fluid inhibitor was tested. These results show that a dilution as high as 1:40 completely removed or inactivated the fluid inhibitor, while higher dilutions were ineffective. The small amount of glucose necessary to remove or inactivate the inhibitor of hemagglutination by the CSD virus, in addition to the partial removal or inactivation of this inhibitor by various concentrations of sodium chloride reported by Turner et al. (1960), suggested that the mode of action of glucose and other sugars in removing or inactivating the fluid inhibitor possibly could be due to their effects on the ionization constant of the fluid inhibitor. If this hypothesis were valid, then the inhibitor might be more effectively removed or inactivated by highly ionizing substances since glucose is a comparatively non-ionizing substance.

TABLE 4

The Effect of Various Concentrations of Highly Ionizing Substances on the Allantoic Fluid Inhibitor of the CSD Virus Hemagglutination

Substances	Conc. in moles	Reciprocal of Fluid Dilutions						
		10	20	40	80	160	normal fluid control	cell control
NaCNS	0.17-1.7	-	-	-	-	-	-	-
Na_2SO_4	0.1 -0.78	-	-	-	-	-	-	-
	1.0	-	-	-	±	3+	-	-
CSD fluid	untreated	-	-	-	-	-	-	-
"	0.28 m glucose	4+	4+	4+	4+	4+	-	-

TABLE 5

The Effects of Cellulose Resins on the Allantoic Fluid Inhibitor of the CSD Virus Hemagglutinin

Resins	pH of Resins	Charge on Resins	Reciprocal of Fluid Dilutions							
			4	8	16	32	64	128	normal fluid control	cell control
Carboxymethyl (CM)	7.0	cationic	-	-	-	-	-	-	-	-
Diethylaminoethyl (DEAE)	7.7	anionic	±	±	3+	2+	2+	+	-	-
CSD-glucose fluid			4+	4+	4+	4+	4+	4+	-	-

In order to test this hypothesis, infected fluids were treated with strongly ionizing substances. As indicated in Table 4, the various concentrations of sodium thiocyanate (NaCNS) had no effect on the fluid inhibitor, while sodium sulfate (Na_2SO_4) at 1.0 molar concentration effected partial removal or inactivation of the fluid inhibitor as indicated by the presence of a "zone" type hemagglutination titration. Both sodium sulfate (Na_2SO_4) and sodium thiocyanate (NaCNS) are strong ionizing agents and, thus, should have effectively removed or inactivated the fluid inhibitor if the above hypothesis of alteration of the ionization constant of the inhibitor is valid. Although this was not proved, the partial removal or inactivation of the fluid inhibitor by 1.0 molar sodium sulfate (Na_2SO_4) indicated that this hypothesis warranted further investigation.

Certain cellulose resins have been used successfully to separate serum antibodies from inhibitors. Since it was postulated by Chanock and Sabin (1953) that the mouse brain inhibitor of St. Louis and Japanese B virus hemagglutinins was a lipoprotein which could be removed or inactivated by treatment of the mouse brain with glucose (0.28 m), it was postulated that the CSD hemagglutinin inhibitor might be

TABLE 6

The Effect of Sodium Sulfite and Cysteine on the Allantoic Fluid Inhibitor of CSD Virus Hemagglutination

Substances used to treat CSD fluid	Reciprocal of Fluid Dilutions					normal fluid control	sodium sulfite control	cysteine control	cell control
	10	20	40	80	160				
0.1 m cysteine	4+	4+	4+	4+	4+	-		-	-
.06 m sodium sulfite	4+	4+	4+	4+	4+	-	-		-
untreated CSD fluid	-	-	-	-	-	-			-
CSD-glucose fluid	4+	4+	4+	4+	4+	-			

TABLE 7

The Effect of Heating CSD Fluid at 60° for One Hour on the Fluid Inhibitor of the CSD Virus Hemagglutinin

Substance	Reciprocal of Fluid Dilution									
	2	4	8	16	32	64	128	256	normal fluid control	cell control
Heated CSD fluid	-	-	-	-	2+	4+	4+	4+	-	-
Non-heated CSD fluid	-	-	-	-	-	-	-	-	-	-

some form of conjugated protein which could be absorbed to cellulose resins. The data in Table 5 show that the cationic resin, Carboxymethyl (CM), had no effect on the fluid inhibitor. The absence of hemagglutination in the infected fluid treated with Carboxymethyl cellulose resin may be due to the adsorption of the particles from virus-containing solutions. The anionic cellulose resin, diethylaminoethyl (DEAE), did effect the removal of some of the fluid inhibitor, but the results were not impressive enough to warrant its further use, especially in view of the superior action of glucose and the simplicity of the method.

All of the substances tested up to this point most effective in removing inhibitor were either glucose or contained glucose. The one property of glucose which had not been considered was its ability to act as a reducing agent. If the mode of action of glucose on the fluid inhibitor of the CSD virus hemagglutinin was due to its reducing ability, then other reducing agents should also be effective.

This conclusion was shown to be valid by the results shown in Table 6. Cysteine (0.1 m) and sodium sulfite (.06 m), two strong reducing agents, were very effective in

removing or inactivating the CSD virus fluid inhibitor. Strong oxidizing agents such as potassium periodate (.05 m) had no effect on this fluid inhibitor.

The effects of heat on the CSD virus fluid inhibitor were examined because of the possible protein nature of this inhibitor. As shown in Table 7, it is seen that CSD fluid heated at 60°C for one hour showed the presence of the CSD virus hemagglutinin at a dilution of 1:32 and maximum hemagglutination at a dilution of 1:64, while non-heated CSD fluid showed the absence of the CSD virus hemagglutinin. These results suggest that the fluid inhibitor is inactivated by heat and that treatment of the fluid at temperature higher than 60°C would bring about complete inactivation of the CSD virus fluid inhibitor. However, inactivation of CSD fluid inhibitor by more severe heat treatment is biologically impractical. It is thus concluded from these results that the fluid inhibitor of the CSD virus is possibly heat labile, but from a biological point of view this substance is considered heat stable in this work.

These results and the results of previous experiments led to the belief that the allantoic fluid inhibitor of the

TABLE 8

Hemagglutination Tests with Red Cells of Various Animal Species Utilizing Both Sedimentation and Centrifugation Methods

Red Cells	% Suspension	Agg	Reciprocal of Fluid Dilutions 10	20	40	80	160	normal fluid control	cell control
Human "O" Rh^+	0.75-2	C*	-	-	-	-	-	-	-
		S*	-	-	-	-	-	-	-
Bovine	0.5 -2	C	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-
Equine	0.5 -2	C	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-
Chicken	0.25-3	C	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-
Sheep	0.5 -2	C	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-
Mouse	1-2	C	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-
Rat	1-2	C	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-

*C - clumps

*S - sedimented

CSD virus hemagglutinin is a highly oxidized heat-stable chemical substance of a protein nature.

The hemagglutination titrations of the CSD virus using rabbit erythrocytes were observed for direct clumping of the red cells. The general method of observing viral hemagglutination is on the basis of the pattern of sedimented red blood cells. In an attempt to find if the red cells of some other species might be agglutinated more readily and perhaps without centrifugation, red cells of different species of animals were tested for agglutination by CSD virus in this manner. As shown in Table 8, 1-2 per cent suspensions of rat erythrocytes were the only cells tested which agglutinated in pattern form by the CSD virus. Hemagglutination occurred both after centrifugation and by sedimentation of cells into pattern in mixtures allowed to stand at room temperature.

The specificity of the hemagglutination of rat red cells was shown to be due to the CSD virus by the fact that rabbit antiserum to this agent, previously shown to inhibit the hemagglutination of rabbit erythrocytes to a titer of 1:64, inhibited the agglutination of rat red cells to a titer of 1:2560. The higher titer in the latter case

may be due to the type of test employed, although it may also be that fewer CSD viral particles are necessary to agglutinate rat erythrocytes than rabbit erythrocytes, thus a smaller concentration of antibodies would cause inhibition of the hemagglutination of rat erythrocytes than rabbit erythrocytes.

It should be noted that before the above test for hemagglutination-inhibiting antibody could be performed, methods had to be developed to remove the non-specific serum inhibitor previously demonstrated in both normal human and rabbit sera. Chanock and Sabin (1953) reported the presence in both human and rabbit sera of an inhibitor of the hemagglutinin of both the St. Louis and Japanese B encephalitis viruses. This inhibitor was inactivated by treating the serum with acetone and hydrochloric acid. Turner, Bigley, Dodd, and Anderson (1960) reported the presence of an inhibitor of the CSD virus hemagglutinin in both human and rabbit sera which could be inactivated by essentially the same method described by Chanock and Sabin (1953). Neither of the above reports contained any experiments designed to determine the nature of the inhibitor, although Chanock and Sabin suggested it was a lipoprotein. Since

TABLE 9

Inhibitory Capacity of Fractions of Normal Human Serum Obtained by Continuous Flow Electrophoresis

Number of the fraction	Serum fraction	Reciprocal of Serum Dilution								
		2	4	8	16	32	64	128	serum control	virus control
1-9	Gamma	4+	4+	4+	4+	4+	4+	4+	-	4+
10	Globulin	2+	3+	3+	4+	4+	4+	4+	-	
11		2+	2+	3+	4+	4+	4+	4+	-	
12	Beta	+	4+	4+	4+	4+	4+	4+	-	
13	Globulin	±	±	3+	4+	4+	4+	4+	-	
14	Alpha	-	-	4+	4+	4+	4+	4+	-	
15	Globulin	-	-	4+	4+	4+	4+	4+	-	
16		±	4+	4+	4+	4+	4+	4+	-	
17		-	-	4+	4+	4+	4+	4+	-	
18		2+	4+	4+	4+	4+	4+	4+	-	
19		±	4+	4+	4+	4+	4+	4+	-	
20		4+	4+	4+	4+	4+	4+	4+	-	
21		3+	4+	4+	4+	4+	4+	4+	-	
22		4+	4+	4+	4+	4+	4+	4+	-	
23		-	-	3+	4+	4+	4+	4+	-	
24		3+	3+	4+	4+	4+	4+	4+	-	

TABLE 9 (contd.)

Number of the fraction	Serum fraction	Reciprocal of Serum Dilution								
		2	4	8	16	32	64	128	serum control	virus control
25		2+	3+	4+	4+	4+	4+	4+	-	
26	Albumin	3+	4+	4+	4+	4+	4+	4+	-	
27		4+	4+	4+	4+	4+	4+	4+	-	

serum inhibitor was not affected by glucose as was the inhibitor in allantoic fluid, Turner et al. (1960) suggested that the two were not the same substance. Table 9 shows the results of an experiment in which an attempt was made to determine the fraction of human serum in which the inhibitor of the CSD virus hemagglutinin exists. The various samples listed by numbers in Table 9 represent fractions obtained by continuous flow electrophoresis described in the previous section.

From these data, the CSD virus serum inhibitor occurs mainly in the fractions which are primarily alpha and beta globulin. However, it must be borne in mind that this type of protein separation does not produce strictly pure fractions of the particular proteins by which they are labelled. Thus, some slight inhibiting activity can be noted in gamma globulin fractions adjacent to the beta fractions probably due to contamination by the latter. Likewise, a fraction of albumin (#26) showed very slight inhibition which probably can be related to the presence of some alpha or beta globulin. Obviously, none is present in the majority of the gamma fractions which decreases the possibility that the normal serum inhibition is due to specific antibody. The fact

TABLE 10

Inhibition Tests with Cohn Fractions of
Normal Human and Rabbit Serum

Serum fractions*	Reciprocal of Serum Fraction Dilution									
	2	4	8	16	32	64	128	Serum Control	Virus Control	Cell Control
AGR F-IV-I	-	-	-	-	3+	4+	4+	-	4+	-
AGR F-VI	-	-	3+	3+	3+	3+	3+	-		-
AGR F-IV	-	-	-	3+	3+	3+	3+	-		-
BGR F-IV-I	-	3+	3+	3+	4+	4+	4+	-		-
HG F-II & III	4+	4+	4+	4+	4+	4+	4+	4+		-
GGH F-II	4+	4+	4+	4+	4+	4+	4+	4+		-

TABLE 10 (Contd.)

Serum fractions*	Reciprocal of Serum Fraction Dilution									
	2	4	8	16	32	64	128	Serum Control	Virus Control	Cell Control
GPH F-VI	-	-	-	-	-	-	4+	-		-
AGH F-IV-4	4+	4+	4+	4+	4+	4+	4+	4+		-
AGH F-IV-I	4+	4+	4+	4+	4+	4+	4+	4+		-
AGH F-IV	4+	4+	4+	4+	4+	4+	4+	4+		-
BGH F-III	4+	4+	4+	4+	4+	4+	4+	4+		-

*AGR - Rabbit alpha globulin
BGR - Rabbit beta globulin
GGH - Human gamma globulin
HG - Human globulin
BGH - Human beta globulin
AGH - Human alpha globulin
GPH - Human glycoprotein

TABLE 11

The Effect of .05 M KIO_4 on the Titer of the Glycoprotein Inhibitor of CSD Virus Hemagglutination

Substances	Reciprocal of Glycoprotein Dilutions								viral control	glycoprotein control
	4	8	16	32	64	128	256	512		
*GPH - KIO_4	-	-	+	+	4+	4+	4+	4+	4+	-
Untreated GPH	-	-	-	-	-	-	4+	4+	4+	-

*GPH - human glycoprotein

that all fractions of alpha and beta globulins did not demonstrate inhibition is again related to the nature of the process of separation since, as noted before, the fractions are not all pure globulin of the type indicated and, although not indicated here, protein nitrogen determination indicates a considerable variation in the amount of protein in various fractions. However, the results emphasize the fact that this inhibitor is associated principally with alpha globulins and possibly some beta globulin.

To further identify the serum inhibitor of the CSD virus hemagglutination involved, various Cohn fractions (1946) of human and rabbit serum obtained from the Pentex Incorporated, Kankakee, Illinois, were examined. The results in Table 10 indicate that the alpha globulin fraction (AGR-F-IV-I) of rabbit serum inhibited the CSD virus hemagglutinin at a titer of 1:16 and partial inhibition at a titer of 1:32, while inhibition with alpha globulin fractions (AGR-F-IV) and (AGR-F-VI) of rabbit serum was complete at titers of 1:8 and 1:4 respectively and was partial at a titer of 1:128. The beta globulin fraction (BGR-F-IV-I) inhibited completely at a titer of 1:2 and partially at a titer of 1:16. In contrast to the data in the previous

TABLE 12

The Effect of .05 M KIO_4 on the Inhibitor in Normal Rabbit Serum and Immune Serum Against the CSD Virus

Serum	Treatment	Reciprocal of Serum Dilutions								
		8	16	32	64	128	256	512	viral control	serum control
*NRS	KIO_4	2+	4+	4+	4+	4+	4+	4+	4+	-
NRS	None	-	-	-	-	-	-	-		-
Anti-CSD	KIO_4	-	±	±	4+	4+	4+	4+		-
Anti-CSD	Acetone	-	-	-	4+	4+	4+	4+		-

*NRS - Normal rabbit serum

experiment (Table 9), no inhibition occurred in tests with the alpha and beta fractions of human serum. This might be attributed to the different fractionation procedures, but inspection of the serum controls revealed that the rabbit red cells employed in the test were agglutinated in the absence of virus by alpha, beta, and gamma fractions brought about probably by the well-known, naturally occurring hemagglutinins for red cells found in practically all human sera which may have masked the inhibition. However, the glycoprotein fraction of human serum (GPH-F-VI) completely inhibited CSD virus hemagglutination at a titer of 1:64, better than any other fraction tested, and, in this instance, the control was also not agglutinated. Thus, it is evident that the inhibitor of CSD virus hemagglutination in human serum is a glycoprotein. This type of protein occurs mainly in the alpha fraction when serum is separated electrophoretically (Haurowitz, 1950) which, along with the presence of the naturally occurring hemagglutinins for rabbit cells referred to above, explains the seeming contradiction between Table 9 and Table 10. The presence of glycoprotein in the Cohn-type alpha and beta fractions of rabbit serum (Haurowitz, 1950) undoubtedly accounts for the inhibition shown by these fractions.

TABLE 13

The Effect of .05% KIO_4 on the Inhibitory Action of Alpha and Beta Globulin Fractions of Rabbit Serum and Anti-CSD Rabbit Serum

Serum Fractions	Reciprocal of Serum Fraction Dilutions										
	2	4	8	16	32	64	128	256	512	viral control	serum control
BGR F-IV-I			-	+	+	2+	3+	3+	3+	3+	-
AGR F-IV-I			3+	3+	3+	3+	3+	3+	3+	3+	-
Non-treated BGR F-IV-I	-	3+	3+	3+	3+	4+	4+	4+	4+	4+	-
Non-treated AGR F-IV-I	-	-	-	-	3+	4+	4+	4+	4+	4+	-

AGR - Rabbit alpha globulin

BGR - Rabbit beta globulin

TABLE 14

The Effect of Glycoprotein and Mucoprotein Inhibitors on Hemagglutination by CSD and Influenza Viruses

Substance	Virus	Reciprocal of Inhibitor Dilution									
		2	4	8	16	32	64	128	viral control	muco-protein control	glyco-protein control
*GPH	CSD	-	-	-	-	-	-	4+	4+		-
*UMP	CSD	4+	4+	4+	4+	4+	4+	4+	4+	-	
GPH	Influenza	+	+	+	+	+	+	+	+		-
UMP	Influenza	-	-	-	±	+	+	+	+	-	

*UMP - Urinary mucoprotein

*GPH - Human glycoprotein

TABLE 15

Specificity of Hemagglutination-inhibiting Antibody in Anti-CSD and Influenza Serum

Serum	Virus	Reciprocal of Serum Dilutions								
		8	16	32	64	128	256	512	viral control	serum control
Anti-CSD	CSD	-	-	-	-	3+	3+	3+	3+	-
	Influenza	+	+	+	+	+	+	+	+	-
Anti-influenza	CSD	4+	4+	4+	4+	4+	4+	4+	4+	-
	Influenza	-	-	±	+	+	+	+	+	-

The presence in serum of a mucoprotein inhibitor of hemagglutination by influenza virus has been known for a long time, and since glycoprotein is a form of conjugated protein similar to mucoprotein, it was possible that the serum glycoprotein that inhibited the CSD virus hemagglutinin was the same substance. One of the most effective methods used in removing it is periodate. The effect of periodate treatment on the serum glycoprotein inhibitor of the CSD virus hemagglutinin is shown in Table 11. It is seen that the inhibitory titer of the serum glycoprotein was reduced from a titer of 1:64 to a titer of 1:8 by the treatment with .05 molar periodate (KIO_4). These results suggested that periodate could be used to remove the inhibitor found in rabbit and human serum for the CSD virus hemagglutinin. As shown in Table 12, periodate treatment of normal rabbit serum effectively inactivated the serum inhibitor and allowed the determination of antibody in the immune serum at the same titer as immune serum treated by the previously reported method of acetone. This experiment thus indicates that periodate treatment of serum could be substituted for the acetone method of treatment described by Turner, Bigley, Dodd, and Anderson (1960), since there

is no reduction of antibodies on treatment of the serum with periodate, plus the fact that periodate treatment of serum is less time-consuming.

The effect of periodate on the inhibitory action of beta globulin (F-IV-I) and alpha globulin (F-IV-I) fractions of rabbit serum on the CSD virus hemagglutinin is shown in Table 13. The inhibitory activity of the alpha globulin (F-IV-I) fraction was completely destroyed by the periodate treatment while the inhibitory activity of the beta globulin (F-IV-I) fraction was markedly reduced. This might be explained by the fact that although glycoprotein is found in the beta globulin fraction of serum, the concentration of this substance in the beta globulin fraction is reduced (Haurowitz, 1950). However, lipoprotein is present in high concentrations in this fraction of globulin (Haurowitz, 1950). The results obtained with beta globulin thus suggest that lipoprotein, as well as glycoprotein, acts as an inhibitor of the CSD virus hemagglutinin since a high concentration of the periodate resistant lipoprotein is present in the beta globulin fractions.

Turner et al. (1960) noted the absence of hemagglutination-inhibiting antibody in anti-influenza serum. However, the

similarity of the nature of the inhibitors for these two viruses prompted a further examination of the specificity of both inhibitors and antiserum. Table 14 shows the effects of glycoprotein and urinary mucoprotein on the hemagglutination of influenza and CSD viruses. These results show, as seen in Table 10, that glycoprotein inhibited the CSD virus hemagglutination at a titer of 1:64, while urinary mucoprotein showed no inhibitory activity on this virus. The influenza virus hemagglutination was inhibited completely at a titer of 1:8 and partially at a titer of 1:16 by urinary mucoprotein, while no inhibitory action was shown by glycoprotein to the hemagglutination of the latter. These results suggest that the glycoprotein, which inhibits CSD virus hemagglutination, is different from the mucoprotein inhibitor of the influenza virus hemagglutination.

Turner et al. (1960) reported the absence of antigenic relationship between the CSD and influenza viruses. The absence of cross-reaction between anti-CSD and anti-influenza against influenza and CSD viruses respectively, as shown in Table 15, confirmed the original report by the above authors.

Further evidence of the difference between the inhibitor of the CSD virus hemagglutinin and the urinary

TABLE 16

Precipitin Titrations of Antiserum to Influenza Mucoprotein Inhibitor with Homologous Mucoprotein and Serum Glycoprotein

Substances	Reciprocal of Urinary Inhibitor and Glycoprotein Dilutions						
	2	4	8	16	32	64	Saline control
Urinary mucoprotein	+	+	+	+	+	±	-
Serum glycoprotein	+	-	-	-	-	-	-

mucoprotein inhibitor of the influenza virus hemagglutinin is provided by comparing the antigenic specificity of the two substances. As noted in Table 16, there was only a very minimal cross-reaction in the precipitin test with anti-mucoprotein serum and glycoprotein, using dilutions of a one per cent solution of the glycoprotein, as compared to the homologous system in which dilutions of a solution containing 0.4 mg per cubic centimeter of mucoprotein, was employed. These results indicate that, at best, there is only a slight chemical relationship between these.

Turner, Bigley, Dodd, and Anderson (1960) reported the immunization of rabbits with strongly hemagglutinating CSD virus passage fluid. Serum from these rabbits demonstrated a hemagglutinin titer of 1:32. In contrast to this antigenicity of the virus in rabbits, the demonstration of hemagglutination-inhibiting antibody in human serum from cases of the disease has been unsuccessful. Attempts were made to determine a possible relationship of the so-called CSD virus as the cause of cat scratch disease in humans by demonstration of hemagglutination-inhibiting antibodies in the paired sera of persons with the disease. However, normal human sera contain a normal or naturally-occurring

antibody for the rabbit red cells used in the test which must be removed by two-hour-long absorption of human serum with packed rabbit erythrocytes. This method was described in a previous section of this work. In addition, sera must also be treated to remove viral hemagglutination inhibitor.

Sera were tested from ten patients showing a positive skin test to pus from lesions of cat scratch disease. As shown by the results in Table 17, the serum of only one of the patients (G.F.) contained hemagglutination-inhibiting antibodies to the CSD virus. The samples were obtained 32 days apart and the second shows a slight increase in titer. Since the first sample was obtained two weeks after the first symptoms occurred, in the early acute stage of the disease, this slight rise in titer may be significant. In many of the others, serum was not obtained early since there is frequently some delay in the diagnosis of the disease, either because of the mildness of the usual case or, in some cases, because the disease is not suspected and the individual is treated with antibiotics which are not effective and time is lost before skin testing is performed.

Probably the most significant diagnostic procedure for cat scratch disease is the skin test using a saline suspen-

TABLE 17

Hemagglutination-inhibition Antibody Titrations of Sera from Patients with Positive Skin Test to Pus from CSD Lesions

Patients	Date serum obtained	Reciprocal Serum Dilutions 8	16	32	64	128	256	virus control	serum control
E.S.	2/10/61	3+	3+	4+	4+	4+	4+	4+	-
	3/25/61	4+	4+	4+	4+	4+	4+		-
G.F.	2/17/61	-	-	4+	4+	4+	4+		-
	3/15/61	-	-	2+	2+	4+	4+		-
B.R.	2/25/61	4+	4+	4+	4+	4+	4+		-
L.V.	9/ 1/54	4+	4+	4+	4+	4+	4+		-
	3/ 2/61	4+	4+	4+	4+	4+	4+		-
D.D.	1/ 6/61	4+	4+	4+	4+	4+	4+		-
	1/24/61	4+	4+	4+	4+	4+	4+		-
H.M.	3/ 4/61	4+	4+	4+	4+	4+	4+		-
M.M.	2/ 9/61	4+	4+	4+	4+	4+	4+		-
	3/ 2/61	4+	4+	4+	4+	4+	4+		-
J.B.	1/ 3/61	4+	4+	4+	4+	4+	4+		-
	1/24/61	4+	4+	4+	4+	4+	4+		-
J.S.	1/ 9/61	4+	4+	4+	4+	4+	4+		-
	1/24/61	4+	4+	4+	4+	4+	4+		-
M.C.	12/16/60 12/29/60	4+	4+	4+	4+	4+	4+		-

sion of pus from known lesions which has been treated to destroy living virus. Patients with the disease develop a delayed hypersensitivity to the antigen. This type of response is known not to be associated with circulating antibody. However, it is considered to be a specific immune type response which develops during many infectious diseases and may be present in addition to circulating antibodies to which it bears no relation. An attempt to show an antigenic relationship between the CSD virus and cat scratch disease was performed utilizing skin test for delayed hypersensitivity in rabbits immunized with the virus or with pus from lesions of known human cases of the disease.

Two rabbits were immunized with both pus from cat scratch lesions and strongly hemagglutinating virus passage fluid. Each rabbit was then skin tested with normal allantoic fluid, CSD pus, and glucose treated CSD passage fluid. The rabbits immunized with the CSD pus showed no skin reaction with any of the skin test antigens. The rabbits immunized with the virus showed redness and swelling at the sites inoculated with both pus and CSD virus passage fluid. No reaction occurred at the sites inoculated with normal

allantoic fluid. The reaction at the positive reacting sites appeared about 20 hours after inoculation and obtained maximum severity, 9 millimeter in area, in about 40 hours. The reaction persisted for about 48 hours and then began to disappear. There was no necrosis. The rabbits giving positive skin tests were given a sixth 1 ml intravenous injection of the CSD virus passage fluid after the disappearance of the skin reaction. This caused a reappearance of the reaction just described at the original sites of injection in the skin. Both the original and subsequent reactions of the rabbits to the pus (CSD) and CSD virus passage fluid were good examples of a delayed hypersensitive reaction. The absence of positive skin reaction in the rabbit immunized with CSD pus is most likely due to the failure to immunize adequately with the small amount of material available and probably a low concentration of virus in the pus.

Turner, Bigley, Dodd, and Anderson (1960) reported the existence of an antigenic relationship between herpes virus and the CSD virus. This antigenic relationship was determined by the cross-reaction of CSD virus with antiserum to herpes virus. This cross-reactivity of herpes and CSD virus antiserum was determined by means of the hemagglutination-

TABLE 18

Hemagglutination of Rat Erythrocytes by Herpes Virus

Substance Used to Dilute Saline-treated Virus 1:5	Diluent Used in Titration	Reciprocal of Fluid Dilutions 1000	2000	4000	8000	16,000	32,000	normal fluid control	cell control
(.016 m) Borate-KCl	saline	-	-	-	-	-	-	-	-
	borate-KCl	+	+	+	+	+	+	-	-
(0.28 m) Glucose	saline	-	-	-	-	-	-	-	-
	borate-KCl	-	-	-	-	-	-	-	-
Untreated herpes virus	saline	-	-	-	-	-	-	-	-
	borate-KCl	-	-	-	-	-	-	-	-

inhibiting titration method. These authors reported that herpes virus, previously thought of as a non-hemagglutinating virus, hemagglutinated rabbit erythrocytes much in the same way as the CSD virus. The possibility that herpes virus could be made to hemagglutinate rat erythrocytes, in much the same way as the CSD virus, was investigated in this work because of the antigenic relationship between the two viruses and the ability of each of the viruses to hemagglutinate rabbit erythrocytes.

The results of the original experiments suggested that glucose treated and untreated herpes virus did not have the capacity to hemagglutinate rat erythrocytes. A series of experiments were then performed in which temperature of incubation of the test; length of the incubation; concentration of rat erythrocytes; concentration of glucose; concentrations of sucrose, lactose, and fructose; and speed of centrifugation were varied. The results of these experiments were also fruitless. However, hemagglutination of rat red cells by herpes virus was observed if allantoic fluid was first diluted in saline and borate-potassium chloride buffer employed for all further dilutions in the titration (Table 18). The actual procedure involved the

use of 1:100 saline suspension of virus to which was added buffer to make a further 1:5 dilution (or 1:500 of the original suspension) which was then titrated in the buffer. If this 1:500 suspension was titrated in saline, no hemagglutination resulted. The data also show that glucose treated fluids titrated in saline or buffer did not produce hemagglutination. These latter results indicate that it was not just a matter of dilution removing inhibitor, but that when rat red cells were employed the physiological saline-borate-potassium chloride buffer was required to inactivate the inhibiting substance. It is rather curious, but so far unexplainable, that different conditions were required by herpes virus to agglutinate rat red cells in view of the fact that both CSD virus and herpes agglutinated rabbit cells under similar conditions which also allowed the hemagglutination of rat cells by the CSD virus. No hemagglutination was observed when herpes virus was diluted in this way and titrated in saline, and no hemagglutination resulted with treated herpes virus passage fluid titrated in both saline and .016 molar borate-potassium chloride buffer. As indicated in Table 19, the hemagglutination of rat red cells by herpes virus was inhibited by homologous antiserum

TABLE 19

Demonstration of Hemagglutination-inhibiting Antibody in Anti-herpes and Anti-CSD Sera

Sera	Reciprocal of Serum Dilutions								
	20	40	80	160	320	640	1280	viral control	serum control
Anti-herpes	-	-	-	-	-	-	+	+	-
Anti-CSD	-	-	-	±	+	+	+	+	-

to the virus at a titer of 1:640 and by anti-CSD serum at a titer of 1:80. These results confirm the report of an antigenic relationship between herpes virus and CSD virus reported by Turner, Bigley, Dodd, and Anderson (1960). The specificity of the hemagglutination of rat erythrocytes by herpes virus is also confirmed by these results.

Perhaps the greatest difficulty encountered in establishing the conditions necessary for the hemagglutination of rat erythrocytes by herpes virus was the preparation of suitable red cell suspensions. Originally Turner et al. (1960) reported no difficulty with cells from rat blood in one per cent citrate according to the method of Keeler (1952). However, when attempting to repeat the procedure non-specific agglutination was observed. This non-specific agglutination was thought at first to be due to the diluents used, but experiments varying diluents and temperature of incubation were fruitless. In addition, the possibility that age of the rats might affect the stability of the cells in suspension was suspected. However, experiments with red cells from both young and old rats suspended in saline or borate-potassium-chloride buffer showed that non-specific agglutination occurred in these diluents after the

cells had been stored in saline for 72 hours at 4-8°C. Considerable effort was then directed to the problem of the storage of rat red cells from blood drawn in one per cent citrate in order to achieve stable suspensions for use with the borate-potassium-chloride buffer. However, none was satisfactory. Red cells from citrated blood stored as packed cells were agglutinated non-specifically in 72 hours. When stored as whole blood, the cells were unsuitable in 48 hours, while those stored at 25 per cent in merthiolate-saline agglutinated spontaneously as early as 24 hours. Stored as a 25 per cent suspension in Alsever's solution, the cells underwent lysis. Rat cells treated with 40 per cent formaldehyde agglutinated as soon as they were suspended in the buffer.

The fact that rat red cells from blood drawn in one per cent citrate did not form stable suspension in the borate-potassium-chloride buffer after storage under such a variety of conditions suggested that citrate was not a suitable anti-coagulant for this purpose. This was emphasized when it was found that cells from defibrinated rat blood formed stable suspensions in the diluent for as long as 96 hours. The problem was solved by collecting the blood in heparin

(10,000 units), removing the red cells and storing them as a saline suspension. Such cells formed stable suspension in the buffer for as long as five days which was considered adequate.

The instability of rat erythrocyte suspension made from blood drawn in citrate is not explainable from the observation made here, especially since this anti-coagulant was recommended in a book on the laboratory rat. It seems likely that the best assumption is that citrate modified the cell surface in some manner which affected the suspension stability.

In addition to the skin test mentioned previously, another significant factor in the diagnosis of cat scratch disease is the usually frequent history of association with cats. It seems curious then that no evidence of a virus related as the cause of the disease has ever been produced, in spite of considerable effort to do so. During the course of this work, pus from lymph nodes of a child with typical symptoms of cat scratch disease, who when skin-tested produced a strongly positive reaction, was provided by Dr. Walter Murray of the Children's Hospital, Columbus, Ohio. A virus was isolated from seven-day-old chick embryos

inoculated with this pus, which hemagglutinated rabbit red cells under conditions previously described and which was specifically inhibited by antiserum to previously isolated strains of the CSD virus and to anti-herpes serum. In addition, the serum samples from this child were shown to be lacking hemagglutinating-inhibiting antibodies to the CSD virus. The virus has been passed seven times and still has all of the properties of the strains described by Turner et al. (1960).

In a matter of a few months, several other cases of the disease were observed by Dr. Murray in individuals in the same neighborhood, all having some contact with several neighborhood cats. Again through the cooperation of Dr. Murray, samples of tissues, hair, claws, saliva, and blood from these cats were obtained. Extracts of tissue, hair, and claws and the blood and saliva were inoculated into seven-day-old chick embryos, which were inoculated and tested for the CSD virus described here. Interestingly, the virus was isolated only from the saliva and claws of one of the cats. Both isolates have been passed in chick embryos and have maintained their properties.

DISCUSSION

Although the viral etiology of cat scratch disease is as yet not proved, the probability that a virus is involved is accepted by practically all who have studied this disease. Thus, the previous report from this laboratory indicating the presence of a hemagglutinin in pus from persons with the disease and the subsequent isolation of a hemagglutinating virus from chick embryos inoculated with such material, which was antigenically related to herpes simplex virus, prompted a more extensive study of this so-called CSD virus. While the earlier data demonstrated that the virus could be isolated from cases of the disease, providing that an inhibitor of the viral hemagglutination was removed from infected allantoic fluid, very little success was obtained in establishing any other relationship of the virus to the disease. For example, unlike herpes virus, the CSD agent did not have the capacity to kill chick embryos or to produce corneal lesions in rabbits. More importantly, the detection of hemagglutination-inhibiting antibody in paired or successive samples of sera obtained from patients during the course of the disease was accomplished in only a few instances. However, such antibody was demonstrable readily

in antisera of rabbits immunized with infected allantoic fluid. In order to demonstrate antibody in human and rabbit serum, it was necessary again to remove a hemagglutination inhibitor which was accomplished originally by treatment of sera with acetone and hydrochloric acid. This procedure, while removing inhibitor, required at least a 1:10 dilution of serum and possibly affected the serological results either by modifying or removing antibody protein. The primary purpose of the present work was, therefore, to attempt to increase the available information on the demonstration of the hemagglutinin and antibody to it, and also to study the nature of both the allantoic fluid and serum inhibitors and to improve the methods for their removal. By such studies, it was hoped that the relationship of the virus to cat scratch disease and its antigenic relationship to herpes could be established more definitely.

Turner et al. (1960) reported the isolation of a viral agent from pus from lesions found in cases of cat scratch disease. The viral agent isolated by inoculation of pus from CSD lesions into chick embryos was demonstrable by hemagglutination of rabbit erythrocytes. The hemagglutination of rabbit red cells by the CSD virus was only

demonstrable by direct clumping of the erythrocytes following centrifugation at 2000 rpm for one minute rather than by the presence of "pattern" type sedimentation of the red cells which is the usual manner of demonstrating viral hemagglutination. However, the presence of a hemagglutinin in the infected allantoic fluid could only be demonstrated when a normally occurring fluid inhibitor was removed or inactivated by treatment of the fluid with 0.28 molar glucose.

Since the demonstration of pattern type sedimentation of red blood cells is the usual and convenient manner of demonstrating viral hemagglutination, and since the nature of the fluid inhibitor of the CSD virus hemagglutination had not been determined, two of the primary goals of this work were (1) to determine the nature of the fluid inhibitor, and (2) to find, if possible, suitable conditions for the demonstration of hemagglutination without the use of centrifugation.

In order to determine the nature of the allantoic fluid inhibitor of the CSD virus hemagglutination, an indirect approach consisted of determining the mode of action of glucose in removing or inactivating the fluid inhibitor, hoping that the acquisition of this information would subsequently

lead to the determination of the nature of the fluid inhibitor. Earlier studies had shown that the inhibitor was partly removed by dilution of infected CSD fluid with varying concentrations of NaCl and with water. Complete removal of inhibitor, however, was accomplished by first diluting the fluid with concentrations of glucose ranging from 0.18 m to 0.45 m, while concentrations above and below this range had no effect. At the same time, it was found that a concentration of glucose within this optimal range, such as 0.28 m, was effective in dilutions as high as 1:40. It should be emphasized that the effect of glucose seemed to occur after the original addition or dilution of the infected fluid in this substance, since subsequent dilutions, for example for titration, could be made with 0.85 per cent NaCl.

Since the above-mentioned earlier work had indicated that inhibitor was partially affected if the original dilution of allantoic fluid was made in water or various concentrations of NaCl, it was thought, at first, that these substances somehow affect the ionization constant of the inhibitor. With the discovery that glucose inactivated inhibitor completely, the validity of this hypothesis was

somewhat questionable since glucose is a notoriously non-ionizing substance. Nevertheless, this hypothesis was tested by treating infected fluid with varying concentrations of highly ionizing substances such as sodium sulfate (Na_2SO_4) and sodium thiocyanate (NaCNS). Experiments in which these substances were used indicated that they were ineffective as inactivators of inhibitor. Thus, as might have been expected, the action of glucose depends on some property of this sugar to inactivate inhibitor rather rapidly in certain concentrations, since the mixture may then be diluted in NaCl without reversal of the inhibition.

Further experiments performed to determine the effect of other sugars on the fluid inhibitor led to the rather interesting finding that all sugars which are isomers of glucose, or contained glucose, were effective in inactivating or removing it. All the sugars tested, maltose, lactose, fructose, sucrose and mannose, were effective over the same concentration range as glucose, while fructose and mannose, isomers of glucose, were also effective at slightly higher and lower concentrations. The fact that a variety of substances containing glucose were effective inhibitor removers was confirmed when it was shown that a .05 molar concentra-

tion of calcium gluconate was also active. The presence of glucose or its isomers in all the substances effective in inactivating the fluid inhibitor, (in addition to the fact that the original dilution of the infected fluid to 1:5 with the sugar seemed to be necessary for effective removal or inactivation of the inhibitor), suggests that inactivation was related to the reducing property of glucose, since all of those tested, with the exception of sucrose, are reducing sugars. This hypothesis was supported further by the fact that inhibitor was readily removed from allantoic fluid by other reducing agents such as cysteine and sodium sulfite (Na_2SO_3).

Thus, in spite of the fact that the inhibitor was affected by sucrose, a non-reducing sugar, and calcium gluconate, a very weak reducing agent, it is still apparent that reducing substances of quite different chemical constitution and properties were the most effective inactivators, making it extremely difficult to assign the activity to any other property. Even if these exceptions indicate that further investigation may reveal some other property than reducing capacity may also be involved in their action on the inhibitor, the prominence of this type of agent in the

list of inhibitors seems to justify the characterization of the inhibitor as being an oxidized substance, or at least containing such a component. In this respect, it was found that inhibitor in normal allantoic fluid was also affected somewhat by heating the fluid at 60°C for an hour, although it was not completely removed. This suggests that the inhibitor is also at least partially protein.

These speculations on the nature of the inhibitor in allantoic fluid are further complicated by the findings on the nature of the inhibitor existing in human and rabbit sera. In their earlier work on this factor, Turner et al. (1960) reported that it was removed by the addition of acetone and hydrochloric acid to the serum. They also pointed out that Sabin and Chanock (1953) had used the same method to remove inhibitor in sera for hemagglutination by St. Louis and Japanese B encephalitis virus. Although no further data in support of their contention was provided, the latter authors stated their belief that the inhibitor was a lipoprotein. However, the data reported in the present work indicate that the inhibitor of CSD virus hemagglutination in human serum was found primarily in fractions obtained by continuous flow paper electrophoresis

containing principally alpha globulins and a small amount in fractions containing beta globulin. The slight amount noted in any gamma fraction was associated with those fractions known to contain some beta globulin since separation by this method is not capable of producing a sharp separation, but occurs gradually. This concentration of inhibitor mainly in the alpha fractions (with some in beta fraction) was corroborated by the fact that when Cohn fractions of rabbit sera were tested, inhibitor was mainly in the alpha and beta globulin fractions and in very high titer in the glycoprotein fraction of human serum. Since glycoproteins are usually associated with alpha and beta globulins (markedly in alpha fractions) Haurowitz (1950), it was assumed that the primary inhibitor in sera was a glycoprotein. This was further attested to by the complete success obtained in removing inhibitor by treating both kinds of sera, as well as markedly reducing the action of inhibitory fractions, with periodate (KIO_4), which has a selective oxidizing effect on the glycol bonds of carbohydrates. The single exception to this was noted with the beta fraction of rabbit serum which was not affected. However, the concentration of this fraction in serum must be insufficient

to interfere with hemagglutination since periodate freed serum from inhibitor completely.

These results are in contrast to the claim of Sabin and Chanock (1953) that the serum inhibitor of hemagglutination by encephalitis viruses removed by acetone was lipoprotein. They are also in sharp contrast to the findings mentioned earlier in which it was shown that the inhibitor of CSD virus hemagglutination in allantoic fluid was probably an oxidized substance existing in connection with a protein. If it is assumed that viral hemagglutination inhibitors represent soluble cell receptors, and thus the one in allantoic fluid should correspond to the one in serum, the only possible solution to the data obtained with CSD virus is that the inhibitor, supposedly a glycoprotein, contains a carbohydrate in an oxidized form in allantoic fluid and a reduced form in serum. The alternative is the probability that more than one inhibitor exists, a situation applicable to some other viruses such as the virus of Newcastle disease (Howitt, 1950, and Winner et al., 1952) for which heat stable and heat labile inhibitors have been described. In either event, glucose, which is so effective on the fluid inhibitor, is without effect on serum inhibitor

and, in turn, periodate, which has no effect on the inhibition of allantoic fluid, is quite effective with serum inhibitor. It is quite possible from the data to identify serum inhibitor as glycoprotein, but the possibility that the inhibitor in allantoic fluid is related to a glycoprotein can only be speculated upon.

Although it had been shown (Turner et al. 1960) that anti-influenza sera did not contain hemagglutination-inhibiting antibody for the CSD virus, the similarity of the glycoprotein inhibitor in serum for the latter virus and the well-known mucoprotein inhibitor in serum of influenza virus hemagglutination suggested the further investigation of any relationship between both the inhibitors and the two viruses. No such relationship was found. The influenza mucoprotein inhibitor isolated from human urine failed to inhibit hemagglutination by CSD virus and the glycoprotein serum fraction inhibitory for the latter was completely ineffective against influenza hemagglutination. Likewise, precipitation tests with anti-mucoprotein sera demonstrated only a very slight cross-reaction with the serum glycoprotein indicating a marked antigenic difference in the two substances. Finally, antiserum to the CSD virus,

previously treated with periodate to remove both inhibitors, contained no hemagglutination-inhibiting antibody for influenza, but inhibited hemagglutination by the CSD virus in a dilution of 1:64.

While it was not possible to compare the serum inhibitor of CSD virus hemagglutination with the inhibitor of encephalitis virus hemagglutination described by Sabin and Chanock (1953), it seems likely from the methods used to remove inhibitors for these viruses that their inhibitors may also be a glycoprotein instead of a lipoprotein as they suggested. When the data described in the previous section of this paper on the removal from serum of an inhibitor of hemagglutination by herpes simplex virus by identical procedures as those used with CSD virus inhibitor are considered, it is quite possible that inhibitors for encephalitis virus, CSD virus, and herpes virus, as well as for the group of myxoviruses, are all glycoprotein in nature. If so, the role of this serum fraction in virus invasion and in resistance to virus infection becomes of considerable interest. Even though evidence to date has not demonstrated any effect of serum inhibitors on virus multiplication, nor any role in the body defenses, the previous work (Turner _et al_.

1960) on CSD virus showed that allantoic fluid inhibitor interfered with the antigenicity of the virus *in vivo*. This has not been demonstrated with serum inhibitor, but considering the failure of various procedures to be described later, to demonstrate serum antibody in individuals with cat scratch disease, further investigation of inhibitor in the defense reactions seems warranted. Furthermore, the seemingly anomalous role of antibody in immunity to infection with herpes simplex virus may very well be explained if the inhibitor of hemagglutination by this virus to be described later can be shown to be involved with resistance to the virus or even to a similar interference with antibody production. It is interesting to note that hemagglutination by pus from cases of cat scratch disease was demonstrated by Dodd, Graber, and Anderson (1959) without previous treatment for the removal of inhibitor.

One of the principal objectives of this work was to attempt to obtain evidence of a direct relationship of the so-called CSD virus to cat scratch disease other than the fact that the virus was isolated from persons having the clinical symptoms, history of contact with cats and positive

skin tests following the injection of heated pus from known cases. The expectations expressed by Turner et al. (1960), that hemagglutination-inhibiting antibody might be demonstrable in the sera of persons with the disease when a better procedure than acetone treatment of sera was devised, were not realized. Even though the discovery that sera treated with periodate were devoid of inhibitor provided a seemingly ideal method lacking the objectionable aspects of acetone treatment, and very similar to the method used daily in laboratories throughout the world to detect antibody to influenza virus, all attempts to detect hemagglutination-inhibiting antibody for the CSD virus in sera of 9 persons having exhibited positive skin reactions failed with one exception. Several reasons may be advanced to explain this failure. One of these concerns the possibility that periodate may actually destroy CSD viral antibody as well as inhibitor. This is unlikely, since as stated above, it is used successfully for treatment of sera containing influenza antibody. However, various reports have definitely implicated periodate as a destructive agent for this antibody, and it may be that CSD antibody is even more susceptible. Another more likely possibility is that owing to

the localized nature of the lesions in cat scratch disease, together with the fact that usually the systemic symptoms are mild and the disease uncomplicated, serum antibody may be a transient phenomenon easily missed unless frequent serum samples are examined. This possibility may account for the fact that antibody was detected in two samples of sera from the one individual mentioned above, both of which were obtained during the late acute or early convalescence stage of the disease.

A third possibility is that although antibody appeared in the sera of rabbits immunized with the virus, due to the nature of the disease in humans, as just described, the only antibody produced in humans is the type found only in cells and associated with the delayed hypersensitivity detected only by skin tests. The fact that this state was also produced, in addition to circulating antibody, in rabbits immunized with the virus was shown and provided one bit of indirect evidence of an antigenic relationship between the virus and pus from cat scratch disease. This was evidenced by the production of a typical delayed skin reaction by rabbits previously immunized with virus, when injected intracutaneously with pus from known cases of cat

scratch disease and with allantoic fluid containing CSD virus. Rather interestingly, these rabbits were given a "booster" intravenous injection a few days after the skin reactions had subsided, and the areas of skin corresponding to the sites of the original skin test again reacted typically although no reinjections in those areas were performed. Unfortunately, animals immunized with human pus did not react to intracutaneous injections of either virus or homologous antigen. This can be explained either by a lack of sufficient immunization or that the establishment of delayed type hypersensitivity with the material requires some other route of immunization or perhaps an adjuvant. In any event, the reactions noted were typical of the delayed type reactions seen in humans injected with CSD pus and indicate that a common antigen is shared by this material and the so-called CSD virus.

Another bit of indirect evidence of the relationship of the CSD virus to cat scratch disease was obtained when a virus similar in antigenicity and ability to grow in chick embryos was isolated from the claws and saliva of a cat located in an area in which several cases of CSD had occurred. The frequency with which patients with CSD show a

history of association with cats is accepted by most authorities as suggesting more than mere chance association of the cat with this disease. Thus, cats are considered as a vital link in the transmission of the virus or probably the reservoir of the CSD agent. However, as far as is known, no one has ever observed a disease in cats with the symptoms of CSD, nor has the disease been produced in human volunteers by inoculation of these individuals with extracts of cat tissues or secretions. Neither has it been possible to isolate a virus from cats which would produce the symptoms of CSD by subsequent inoculation into a susceptible monkey or human. However, the virus isolated from both the saliva and claws of a cat in the present work behaved in the same way as the virus isolated from human CSD pus when the former was inoculated into eggs. This virus hemagglutinated both rat and rabbit erthrocytes in the same manner as the virus isolated from the CSD pus. Antiserum previously prepared against the virus isolated from human CSD pus inhibited hemagglutination by the cat strains in the identical titer found with the human isolates. Like the human strains, hemagglutination by the cat strain was also inhibited by herpes antiserum. These findings suggest that the virus

isolated from the saliva and claws of the cat is a strain of, or the same virus isolated from CSD pus. Attempts to isolate this virus from other tissue and secretions of cats, as well as other animals, failed. The presence of the virus in the saliva of cats suggests that there may be a localization of the virus in the lower respiratory tract, or salivary gland of the cat. The presence of the virus in the saliva may be due to an earlier viremic stage of the CSD virus in the cat. The isolation of the virus from the cat claws is probably due to a habit of cats of cleaning their claws by licking. It is suggested that further attempts to isolate this virus from the cat should be done using glandular extracts, serum, or tissue richly supplied with blood.

In their original report on the isolation of CSD virus, Turner et al. (1960) noted an antigenic relationship between the virus and herpes simplex virus. This prompted them to examine the capacity of herpes virus to cause hemagglutination under the same conditions which were optimal for CSD virus hemagglutination. They noted that an egg-adapted strain of herpes virus, previously considered a non-hemagglutination virus, caused the hemagglutination of rabbit erythrocytes if the allantoic fluid was first treated

with glucose as described for the CSD virus.

As noted earlier, one of the purposes of the present work was the establishment of conditions which would allow hemagglutination by CSD virus without centrifugation, that is, by mixing virus and red cells, allowing the mixture to stand and observing hemagglutination by the so-called "pattern" of the sedimented cells formed, in contrast to the so-called smooth "button" of cells in controls containing no virus. This was not possible with rabbit red cells which required about 4 hours to sediment when mixed with dilutions of infected allantoic fluid previously treated with glucose. Since this appeared to be associated with the manner in which rabbit cells sediment, as opposed to red cells of other species, hemagglutination tests using both CSD and herpes virus previously treated with glucose were performed with suspension of red cells of other species. Surprisingly, red cells of hooded rats settled out in a definite "pattern", easily discernible from control cells, in the presence of CSD virus. Suspensions of bovine, equine, sheep, human, and chicken red cells were not agglutinated by either virus. However, glucose-herpes virus allantoic fluid did not agglutinate rat red cells which seemed curious

insofar as both viruses reacted identically with rabbit cells. An extensive investigation of the various factors which might be involved in this failure of herpes also to hemagglutinate rat cells was performed. Without going into detail, factors such as concentration of glucose and other sugars; time and temperature of incubation; and concentration of cell suspensions were varied without success, until during an examination of the effect of different buffers, borate potassium chloride (pH 8.6) was found to produce the desired conditions. Interestingly, it was found necessary first to dilute the allantoic fluid in physiological NaCl 1:100, then in the borate buffer 1:5, (final dilution being 1:500), and then to use the buffer as diluent for further dilution for titrations. No hemagglutination resulted if the same dilutions were performed with sodium chloride alone, nor with buffer, indicating that the effect of the NaCl-buffer combination was not merely due to dilution.

This finding creates a rather interesting and additional aspect to the previous discussion of the nature of the allantoic fluid inhibitor described for the CSD virus. Since the same reducing agents removed the inhibitor of hemagglutination by herpes virus when rabbit red cells were

used, and glucose-treated CSD fluid caused hemagglutination of rat cells as well, it is curious that the same situation did not apply when herpes and rat cells were utilized. Furthermore, the borate potassium chloride buffer has no reducing capacity, yet it must be concluded that the effect of the buffer was to remove inhibitor. As noted in the earlier discussion of the allantoic fluid inhibition of CSD virus, a non-reducing sugar, sucrose, was also effective when rabbit red cells were used. The fact that herpes virus agglutinated rabbit cells when the allantoic fluid was first treated with glucose, seems to have established the fact that the inhibitor of this virus is the same, or at least very similar to the one for the CSD virus. It also seems logical to assume that the receptor on rabbit erythrocytes is similar for both viruses. However, since the addition of glucose to allantoic fluid permitted the demonstration of hemagglutination of rat erythrocytes by CSD virus, the assumption seems warranted, on the basis of the conclusion that inhibition for both viruses is the same, or similar, that perhaps the failure of herpes viruses to agglutinate rat cells when the fluid was treated with glucose was due to some effect of these conditions on the rat cell

receptor. This, in turn, points to the interesting possibility that herpes receptor on rat cells, like allantoic fluid inhibitor is susceptible to reducing agents, while the receptors for the CSD virus on both types of red cells, and for herpes virus on rabbit cells, like serum inhibitor, are not. If the previously postulated explanations for the difference between allantoic fluid and serum inhibitor of CSD virus is valid, namely, that the former is in an oxidized form and the latter not, it can also be applied to the cell receptors. Thus, the receptor on rat cells is comparable to the postulated oxidized inhibitor, but on rabbit cells it exists in a more reduced form like serum inhibitor as do the CSD virus receptors on both types of cells.

Such considerations may be supported by the experience encountered in attempting to find a method of obtaining rat blood so that the red cells did not agglutinate non-specifically when suspensions were made in the borate potassium chloride buffer required for hemagglutination of these cells by herpes virus. As noted by Turner et al. (1960), no such difficulty had been encountered in the early work on CSD virus hemagglutination using cells from rat blood collected in one per cent sodium citrate by the method

recommended by Keeler (1952). Extensive experiments established the fact that rat red cells from blood containing citrate as an anti-coagulant did not form stable suspensions in the presence of borate buffer, although this recommendation of Keeler's is in a book on the laboratory rat, whereas defibrinated blood, or the use of heparin as an anti-coagulant provided cells which were completely satisfactory. The reason for this incompatibility cannot now be explained, but it is assumed that this combination of chemicals in some way modified the surface of the erythrocytes making them anti-agglutinable. It is possible that changes were also produced on the surface of red cells from blood drawn in citrate and placed in the glucose-treated allantoic fluid which did not produce non-specific agglutination but altered the herpes virus receptor.

The fact that such specified conditions are necessary for the hemagglutination of rat erythrocytes by herpes virus, suggests that the reason herpes and other viruses not included in the list of hemagglutinating viruses, (Hirst, 1959), is that extensive investigations have not been undertaken to determine the proper conditions under which hemagglutination by these viruses might occur. Further

investigations of non-hemagglutinating viruses along such lines might remove some other viruses from this list or establish a new group of hemagglutinating viruses.

The present work also confirms the antigenic relationship between herpes simplex virus and the CSD virus previously noted by Turner et al. (1960) who showed that CSD virus hemagglutination was specifically inhibited by both antisera to the CSD virus and to herpes virus. The data given here show that, in turn, hemagglutination by herpes virus was inhibited by homologous antiserum at a titer of 1:640, and by anti-CSD virus serum at a titer of 1:80. Although it is now an established fact that an antigenic relationship exists between herpes and CSD viruses, no other similarities in the properties of the two viruses have been found. The lack of pathogenicity of the CSD virus for chick embryos, cells maintained in tissue culture, mice, etc., in addition to difference in titer between CSD virus inhibited with homologous anti-CSD serum and herpes antiserum, have supported the assumption that the two viruses are different. However, it has been observed by Gard (1958) that herpes virus produces swelling of lymph nodes in monkeys characterized by spontaneous draining of

the nodes in the same manner as seen in cases of cat scratch disease. This observation suggests that the CSD virus may be a mutated form of herpes virus. The demonstration of pathogenicity of the CSD virus for suckling mice; cortisonized or irradiated mice; established and primary tissue culture cell lines; and chick embryos, as well as the demonstration of the interference phenomenon between the two viruses, would further confirm the above assumption, since one of the major differences between the viruses is the complete lack of pathogenicity of the CSD virus for hosts which are readily susceptible to the herpes virus. Young kittens, as well as tissue cultures of cells from various cat organs, should be tested for their susceptibility to the CSD virus since the cat is thought to be the reservoir of this virus.

SUMMARY

1. The conditions for hemagglutination by the so-called CSD virus were investigated.

2. Hemagglutination of rat erythrocytes was demonstrated by the usual method of observing the "pattern" of sedimented cells in contrast to the demonstration with rabbit red cells which require centrifugation.

3. The inhibitor in CSD virus infected allantoic fluid, previously reported to be removed by glucose, was also inactivated by other reducing sugars and by other reducing agents. It was also removed by the non-reducing sugar, sucrose, and by calcium gluconate, which has a very slight reducing capacity.

4. The nature of the inhibitor of hemagglutination in human and rabbit sera was shown to be a glycoprotein associated with the alpha and beta globulins of serum. It was inactivated by the use of periodate, and was shown also to be distinct from the mucoprotein inhibitor of influenza virus.

5. Hemagglutinin-inhibiting antibody to the virus was demonstrable in only one of ten persons giving positive skin

tests to cat scratch disease pus. However, rabbits immunized with the virus produced hemagglutination-inhibiting antibody and demonstrated a delayed type hypersensitivity when skin-tested with either the virus or pus from human cases of the disease.

6. A virus having the same hemagglutinating properties, and which was inhibited by rabbit antiserum to the strains isolated from humans, was isolated from the saliva and claws of a cat.

7. The previously reported antigenic relationship between CSD virus and herpes simplex virus was confirmed. In addition, using the same procedure employed for CSD virus hemagglutination, hemagglutination of rabbit red cells by herpes virus was demonstrated. By a slight modification of the procedure, hemagglutination of rat erythrocytes by this virus was also accomplished. These results are original findings since herpes virus is considered to be a non-hemagglutinating virus.

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I, Willie Turner, was born in Suffolk, Virginia, on February 1, 1935. I received my secondary school education in the public schools of Bellaire, Ohio. My undergraduate training was obtained at Maryland State College, where I received the Bachelor of Science degree in 1957. In 1957, I entered The Ohio State University, which granted me the Master of Science degree in 1959. While in residence there, I served as Graduate Assistant in the Department of Bacteriology from September, 1957, to August, 1959. In September, 1959, I was appointed a predoctoral fellow of the National Institutes of Health. I held this position while completing the requirements for the degree of Doctor of Philosophy.