UNIVERSITY OF CINCINNATI

May 21 19 76

I hereby recommend that the thesis prepared under my supervision by William E. Kline

entitled The Demonstration and Partial Characterization of T Substance in Body Fluids

be accepted as fulfilling this part of the requirements for the degree of Master of Science

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Form 668—Grad. School

THE DEMONSTRATION AND PARTIAL CHARACTERIZATION OF T SUBSTANCE IN BODY FLUIDS

A thesis submitted to the

Division of Graduate Studies
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in the Department of Surgery
Division of Hemotherapy
of the College of Medicine

1976

by

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B.S., Lebanon Valley College, 1970

AKNOWLEDGEMENT

I would like to express my gratitude to the entire staff of the Paul I. Hoxworth Blood Center for their generous assistance during my graduate study.

I would also like to express my thanks to Peter and Charla Issitt for their expert instruction and for their considerable efforts in teaching the subject of immunohematology. I have never met two people who are more totally dedicated to their work than Peter and Charla.

Finally, I want to express my sincere gratitude to my wife, Leslie, for her patience, understanding, and encouragement.

INTRODUCTION

When red cells are exposed to neuraminidase *in vivo* or *in vitro* they become agglutinable by a naturally occur- antibody found in all normal human sera. This phenomenon was described by Huebner in 1925 (17) and later by Thomsen in 1927 and by Friedenreich in 1930 (46, 7), and became known as the Huebner-Thomsen-Friedenreich phenomenon, which was later changed to T-activation.

Neuraminidase is a type of glycosidase (8) that cleaves N-acetylneuraminic acid derivatives (sialic acids) that are linked to other monosaccharides in the alpha-D configuration (9). N-acetylneuraminic acid is a monosaccharide that always occupies the terminal position at the non-reducing end of carbohydrate chains of glycoproteins or glycolipids (51). Neuraminidase is found in some mammalian tissue in relatively small amounts (9), but it is produced in large quantities by a wide range of common microorganisms including all viruses of the Myxo group, *Vibrio cholerae*, *Clostridium welchii*, *C. tertium*, *Pseudomonas flourescens*, *P. sturgeri*, *P. pyocyaneus*, *Lactobacillus bifidis*, all the Pneumococci, and all of the diptheroid bacilli (8). Neuraminidase is widely distributed in nature and exposure of cells and tissues to neuraminidase in varying amounts is probably a common event.

When red cells are exposed to neuraminidase, N-acetylneuraminic acid is cleaved from membrane bound oligosaccharide

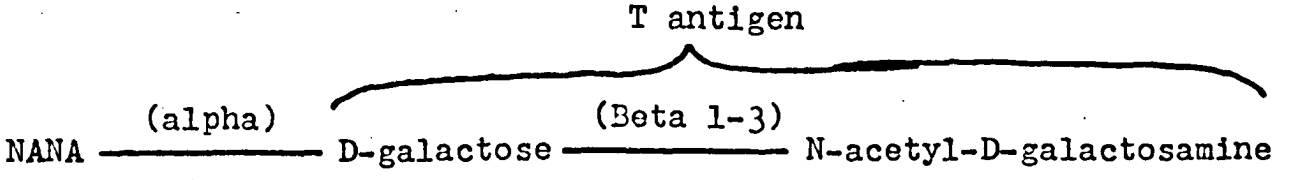
containing molecules (most likely a glycoprotein or glycolipid). The removal of N-acetylneuraminic acid (NANA) exposes a previously covered cryptantigen called T which then has the ability to react with anti-T found in all normal adult sera and with the anti-T lectin prepared from an extract of the peanut (Arachis hypogea) (3). The T antigen is present on virtually all human red cells.

The process of T-activation is an important process in transfusion therapy for three main resons. First, many typing reagents contain anti-T antibodies that can lead to false positive results due to the T-anti-T and the consequences of this error could be serious. Second, the plasma of whole blood as well as all plasma components contain varying amounts of anti-T. Consequently, when whole blood or plasma products are infused into a patient with T-activated red cells the anti-T in the plasma can react with the patient's red cells. There is one case of a sever transfusion reaction due to the infusion of whole blood that contained anti-T in a child whose cells were T-activated (27). Third, recently there have been two reports of patients that had hemolytic episodes due to the T-anti-T reaction when their cells became T-activated (4, 31). This can have obvious clinical importance.

It has been known for a considerable time that the immunodominant group of the T antigen is D-galactose (47). More recently, the D-galactose has been shown to have a

much higher inhibitory activity towards anti-T lectin if it is linked to N-acetyl-D-galactosamine, and further using a highly purified anti-T lectin that galactose linked in beta 1-3 linkage has a much higher inhibitory capacity than galactose in beta 1-4 linkage (28). Because the T antigen on red cells is never exposed unless the cells are first treated with neuraminidase which removes NANA molecules, the immunodominant galactose molecules must be covered by NANA. Since NANA is always in a terminal position and is only removed by neuraminidase when in alpha linkage, these facts suggest that the actual T antigen on red cells consists of the terminal two carbohydrates shown in Figure 1 plus a terminal NANA.

Figure 1: A diagramatic representation of the terminal carbohydrate structure of the T antigen on red cells.



Recently, a large amount of data has appeared dealing with the role of membrane bound oligosaccharide determinants, especially NANA, and their role in malignant processes. Hakamori and Maurikami (13) reported on the basis of studies with malignantly transformed tissue cells that the glycolipid-

oligosaccharide chains were incomplete in malignant cells. The malignant cells had very low levels of the glycolipid NANA - galactose- ceramide and very high levels of the glycolipid precursor galactose - ceramide. They hypothesized that in malignant cells terminal NANA is either lost from the membrane or that malignant cells were unable to add NANA to the subterminal galactose. Sanford and Codington (37) later showed that isogenic serum from strains of inbred mice could cause lysis of mouse adenocarcinoma mammary cells and lymphoid cells only if they were treated with neuraminidase. Keenan and Moore (22) found that in rat mammary carcinoma cells there was a marked decrease in the amount of sialyl transferase activity. This decrease caused the accumulation of a ganglioside with he oligosaccharide terminus galactose-N-acetyl-D-galactosamine. If the sialyl transferase of the cells had been normal NANA would have been added to the terminal galactose. Li et. al. (26) elucidated the structure of the major glucosamine containing ganglioside of human tissue and showed that it was similar to previously published data on the structure of gangliosides. The structure that they reported was NANA 2-3 galactose 1-4 N-acetyl-D-glucosamine 1-3 galactose 1-4 glucose ceramide. Simmon et. al. (39) showed that if neuraminidase was injected directly into malignant mammary carcinoma leisons it caused the carcinoma to regress. They theorized that the regression was immunospecific because injection of neuraminidase into carcionoma on one side

caused a parallel regression of the carcinoma on the opposite side. Springer (43) recently reported that the M and N antigens of red cells were found on human mammary cells both from benign and malignant leisons, however T antigen was found only on malignant cells. The anti-T levels in the serum ofthese women was severly depressed when compared to controls. The author stated that he planned to couple alkeran to anti-T antibodies as a means of localizing the treatment of breast malignancies at the actual site of the malignancy. Hakamori (12) has published a hypothesis that a fundamental aspect of malignant transformation of cells is an inability of the cells to complete cell surface oligosaccharide chains. This defect may possibly render the cells antigenic to the host and provoke an antibody response. Rogentine and Plocinick (35) showed that normal sera contain lymphocytotoxic antibodies to neuraminidase treated peripheral lymphocytes and human lymphoid cells. This antibody was inhibited by Beta-D-galactose and they hypothesized that NANA may be removed on damaged or infected cells and these cells are then removed by these antibodies. Rosenberg and Schwartz (36) showed that the serum of mice contained complement dependent cytotoxic activity towards lymphocytes treated with neuraminidase. They suggested that removing NANA revealed an antigen that caused the removal of these cells by the body. Hinklin and Beck (16) have reported that leukocytes and platelets also have the ability to become T-activated and absorb specific

anti-T reagents. Very recently, Novogrodsky et. al. (33) have reported that there is a receptor on lymphocytes that have the ability to bind a highly purified lectin of A. hypogea. This lectin is bound to the lymphocytes only after they have been treated with neuraminidase, and further treatment with Beta-galactosidase removes this receptor. This is excellent presumptive evidence that these membrane bound oligosaccharides may have T specificity.

The oligosaccharides with terminal NANA and subterminal galactose occur widely in human tissue. They have been reported in white cells (49), red cells (26), spleen (50), muscle (50), brain (50), and peripheral nerves (50).

The literature reviewed here shows that the structures of many of these membrane associated oligosaccharides show a very close similarity to the structure of the T antigen on red cells. This is further supported by studies that have demonstrated the T antigen on leukocytes and platelets (16). These oligosaccharides appear to be widely distributed in human tissue and body fluids and this distribution may be a reflection of their physiological importance. Many of the antigens detected by antibodies on red cells also occur in a water soluble form in many body fluids, such as cyst fluids, amniotic fluid, plasma, saliva, and serum (14,32,38,52). These water soluble antigens are easily recognized by their ability to inhibit the reactions of certain red cell antibodies when tested against appropriate red cells. Because

antigens from red cells are usually very difficult to isolate in pure form in sufficent quantities for chemical and immunochemical analysis, these water soluble antigens have proven of immense benefit in the process of determining the structure of the blood group antigens.

In view of the fact that the T antigen apparently occurs universally on red cells as well as being distributed on the membranes of many other cells (at least a structure similar to the T antigen of red cells) and many of the red cell antigens occur in a water soluble form in body fluids, a search was made for the T antigen in various body fluids. A hemagglutination inhibition assay (HIA) using a lectin prepared from A. hypogea was used to detect the presence of T antigen.

Before any activity that is demonstrated in body fluids by the HIA as a result of neuraminidase treatment can be attributed to the enzymatic activity of neuraminidase with certainity, two questions must be answered.

First, is theinhibitory activity produced by neuraminidase or is the inhibition due to the conditions used to treat the samples, process the samples, or measure the inhibition?

In order to insure that any T inhibitory activity that is revealed in body fluids by treating the fluids with neuraminidase actually results from the specific activity of neuraminidase very careful controls are necessary. It must be

clearly demonstrated that the inhibition of anti-T is not due to conformational changes in the molecules due to the heating process used to inactivate the enzymes, to simple neutralization of the test lectin by carbohydrate constituents in the enzyme molecules, or by the inactivation of the lectin used in the HIA by the activity of neuraminidase.

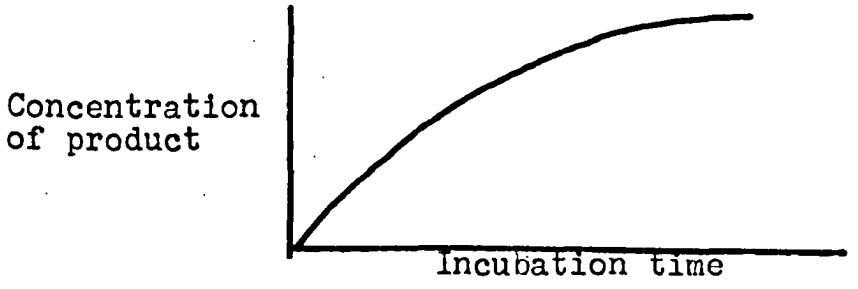
The other major question that must be answered is, does the production of the T antigen conform to the characteristics of an enzymatic reaction? To be certain that the acti-ty produced as a result of neuraminidase treatment is actually due to the enzymatic activity of neuraminidase, the characteristics of the reaction that produces the inhibition must conform to the characteristics of other enzymatic reactions.

One characteristic of enzymatic reactions is specificity, that is the activity must be produced by only one specific enzyme, and not by any enzyme used. These studies also show that the inhibition is not due to experimental manipulation, but rather to the actual activity of neuraminidase.

The effect of incubation time is another characteristic of enzymatic reactions. Figure 2 shows the graph of the generalized way in which a an enzymatic reaction forms product over a period of time, ifother factors such as enzyme concentration, substrate concentration, and temperature are constant. At the begining of enzyme mediated reactions the form-

ation of product occurs rapidly. Measurements taken at small time intervals will show large increases in the amount of product formed. Later even large time intervals will show only small additional increases in the amount of product formed. This is because the amount of product formed is now reach-

Figure 2: The effect of incubation time on the amount of product formed in an enzymatic reaction.

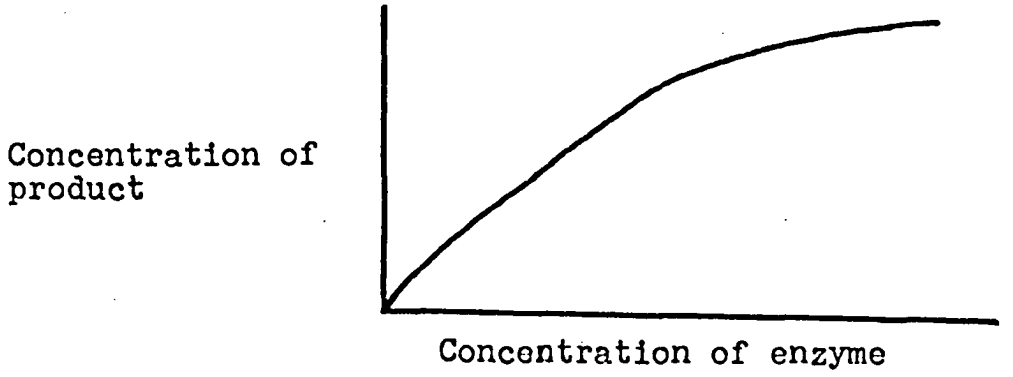


ing the maximum that can be formed by the enzyme under the conditions present (25).

Another characteristic of enzymatic reactions is how they vary in the amount of product formed in relationship to the amount of enzyme present. Figure 3 shows the characteristic pattern of this relationship. At low concentrations of enzyme small increases in the amount of enzyme added causes large increases in the amount of product formed. When higher concentrations are reached even relatively large increases in the amount of enzyme cause only small additional increases in the amount of product formed, because the maximum amount of product that can be formed under the conditions present in the system is being approached (25).

A lectin prepared from the seeds of Vicea graminea has

Figure 3: The effect of the concentration of enzyme in a system to the amount of product formed.



apparent anti-N specificity when tested against human red cells. However, the actual specificity of the lectin differs from the specificity of human anti-N antibodies. This is shown by the observation that when human red cells are treated with neuraminidase they then fail to react with human anti-N antibodies (29), but they still react with the V. graminea lectin. The immunodominant portion of the V.graminea receptor cannot be dependent upon NANA because removing NANA with neuraminidase does not alter the reactivity of the receptor with the Vicea lectin. The human receptor for anti-N is critically dependent upon NANA because removing them completely destroys the reactivity with human anti-N (40).

The Vicea lectin reacts with the antigenic determinants that are very similar to the T antigen. Springer and Desai (42) have published data that they feel shows that the T antigen may be a precursor to M and N antigens on red cells, although they point out that T and V. graminea active structures have been demonstrated in the abscence of M and N spec-

ificities as defined by human anti-M and anti-N (42). Because of these observations and the observations previously discussed concerning the almost universal distribution of glycolipids and glycoproteins with T-like oligosaccharide determinants, a search was made for substances in serum and saliva that inhibit V. graminea either in the native state or following neuraminidase treatment.

The immunodominant molecule of the antigen which reacts with A. hypogea is D-galactose and the immunodominant molecule of V. graminea receptor is either galactose or N-acetylgalactosamine (41). Beta galactosidase is an enzyme that has the ability to cleave the bonds of terminal galactose linked in beta linkage (48). The enzyme is produced in a wide variety of mammalian tissues and by many bacteria, notably E. coli (48). If inhibition of V. graminea or A. hypogea by body fluids is demonstrated this inhibiton should be destroyed by the action of this enzyme. The effects of this enzyme on the inhibiton of A. hypogea and V. graminea by neuraminidase treated body fluids were investigated.

Studies were also performed on the neuraminidase treated saliva and serum samples using gel filtration, immunodiffusion, dialysis, and protein free filtrates to try to demonstrate some of the properties of the molecules that cause the inhibiton of anti-T.

MATERIALS AND METHODS

Collection and processing of body fluids

Saliva: All saliva samples tested were collected from normal healthy adults. Either rubber bands or Parafilm were used as a sialogogue and saliva was collected into clean 20 mm x 100 mm glass tubes. The samples were then centrifuged for ten minutes to remove particulate matter and the tubes were then placed in a boiling water bath for ten minutes to inactivate any salivary enzymes. The interval between collection and boiling the sample was always less tham 30 minutes. The tubes were then centrifuged to remove any precipitate, and were either tested immediately, stored at 4C ifthe testing was to be done within eight hours, or frozen at - 20C until needed. These methods of collection, processing, and storage have no effect on the amount or characteristics of blood group substances present (45).

Serum: All serum samples tested were from normal, healthy adults, except for the ten cord sera tested which were routine post natal specimens. After allowing sufficent time to clot, the tubes were centrifuged and the serum was transfered to a clean glass tube. Because all normal sera contain anti-T in widely differing amounts (18) and this anti-T could interfere with assays using T-activated cells, the anti-T was removed by adsorption with T-activated cells at 4C for one hour. The tubes were then centrifuged and the serum was removed and

tested against T-activated cells to insure that all anti-T activity was removed. If the sera still reacted with T-activated cells, the absorption process was repeated until the serum showed no anti-T activity. None of the sera tested required more than three absorptions.

Urine: Urine samples were collected from normal, healthy adults. Samples were dialyzed overnight at 4C against normal saline to make the urine isotonic, and the samples were tested the next day. Three of the samples were concentrated to five and ten times after dialysis by covering the dialysis tubing with polyethylene glycol until the volume was one-fifth and one-tenth as great as the original volume. These samples were frozen at - 20C until tested.

Human milk and colostrum: One sample of human milk from a group B woman had been frozen at -20C for approximately 18 months. Another sample of colostrum and milk from a group A woman was frozen at - 20C until tested (approximately one month).

Enzyme solutions

Neuraminidase was obtained commercially from Grand Island Biologicals. The stock solution contained 500 units/cc. Beta galactosidase and galactose oxidase were obtained from Grand Island Biologicals and were made up into solutions containing 50 mg/cc (5%) in phosphate buffered saline, pH 7.2. Ficin, bromelin, and papain were made up into concentrations

of 10 mg/cc (1%) in phosphate buffered saline, pH 7.2. Trypsin was made up in Sorensons buffer pH 4.8 to a concentration of 10 mg/cc (1%) (21).

The amount of enzyme used for any experiment reported here is expressed as a volume, and the volume refers to the amount of the stock solution that is used.

Preparation of lectins

Arachis hypogea: Lectins from A. hypogea were prepared as described by Bird (3). The final lectin was tested against T-activated and normal cells, diluted, and stored at - 20C in small aliquots until used. When an aliquot was thawed it was used without refreezing.

Vicea graminea: Lectins from the seeds of V. graminea were prepared by thoroughly grinding one gram of the seeds into a fine powder in a mortar and pestal. This powder was suspended in 10 cc of normal saline, thoroughly mixed, and allowed to stand at 4C for 24 hours. This suspension was centrifuged to remove insoluble matter, titrated against cells of the phenotypes M, MN, and N, and diluted to an appropriate concentration and frozen at - 20C until used.

Ulex europaeus and Dolichos biflorus: Lectins from the seeds of U. europaeus and D. biflorus were prepared by grinding the seeds into a fine powder and adding three volumes of normal saline. These suspensions were allowed to remain at 4C for 24 hours with frequent mixing. They were

centrifuged to remove insoluble matter, titrated against group O, A_1, and A_2 red cells, appropriately diluted, and frozen at - 20C until used.

Lectins for immunodiffusion: Lectins to be used for immunodiffusion experiments were prepared according to the method of Grundbacher (10).

One batch of the A. hypogea concentrate was prepared exactly as described by Grundbacher (10). Another preparation was made as described above except that the two steps that call for precipitation with saturated ammonium sulphate were performed in an ice bath instead of at room temperature.

Preparation of rabbit and human anti-T reagents

Two rabbits were injected intravenously with one cc of autologous T-activated cells as a 50% saline suspension. Four weeks later they were given injections into four subcutaneous sites a total of two cc of autologous T-activated cells. Two weeks later they were bled and the serum was harvested and frozen at - 20C until appropriate absorptions and testing were done. The anti-species antibodies were removed by absorption at 4C with an equal volume of washed, packed human group O cells. This absorption process was repeated three times and the serum was then tested against the absorbing cells to insure that all activity had been removed. The same group O cells used for the absorptions were then T-activated and used for testing.

Human anti-T was obtained from normal adults. All such sera had been shown to lack blood group antibodies.

Eluates of human and rabbit anti-T were prepared by using a modification of the Kochwa-Rosenfield technique (24) as described by Jenkins et al. (20). This elution technique was prefered to the standard heat and ether elution techniques because the resulting eluates are not hemoglobin stained and are much easier to read for agglutination. The eluates were tested against T-activated cells and normal cells and frozen at - 20C.

2-Mercapto ethanol test

2-Mercapto ethanol inhibitions were performed according to the method cited in Issitt and Iasitt (19). 2-Mercapto ethanol will destroy the serological activity of IgM molecules by reducing the disulphide bonds, but it has no effect on IgG molecules.

T-activated red cells

Human red cells were T-activated as described in Issitt and Issitt (19). These cells were made up into a 4% saline suspension and used at this concentration throughout the experiments.

Hemagglutination inhibition assay

Hemagglutination inhibition assays (HIA) were performed by making a master, doubling dilution of appropriate antisera or lectin in saline, using a fresh pipette for each tube to avoid errors due to carryover. Two drops of each dilution

were put into a separate 10 mm x 75 mm tube and two drops of the substance to be tested for inhibiton were added to each of the tubes containing a different dilution of the antiserum. A control of two drops of saline added to each dilution was always run in parallel. In all experiments the mixture of the dilutions of antisera or lectins and the substance being tested for inhibition were incubated at room temperature (approximately 20C) for 30 minutes. Then one drop of a 4 % saline suspension of the appropriate indicator cells was added to each tube and incubated at room temperature for 30 minutes and the tests were read. The values for all titrations were expressed as a titer score.

Scoring of hemagglutination reactions

All titrations were scored according to the method described by Marsh (30).

Demonstration of T substances in normal and neuraminidase treated body fluids

Saliva, serum, urine, human milk, and colostrum were collected and processed as previously described. One cc of the fluid to be tested was treated with 20 λ of neuraminidase and one cc of the fluid was treated with 20 λ of saline as a control. Both tubes were incubated at 37C for one hour. The neuraminidase was inactivated in saliva and urine by placing the samples in a boiling water bath for ten minutes or by placing serum, human milk, and colostrum samples in a

56C water bath for 30 minutes. Saline controls were incubated and inactivated exactly the same as neuraminidase treated samples. After cooling, control and test samples were tested for T antigen by HIA as previously described using a serial dilution of anti-T lectin as the test antibody. The amount of T antigen produced as a result of neuraminidase treatment is expressed as percent inhibition. Percent inhbition is calculated by the following formula:

$$\text{Percent inhibition} = 100 - \left(\frac{\text{Titer score neuraminidase treated fluid}}{\text{Titer score untreated fluid}} \times 100 \right)$$

Test of heat stability of neuraminidase

In order to determine how to inactivate neuraminidase in these experiments the following experiment was performed. 20 λ of neuraminidase and nine cc of saline were thoroughly mixed and incubated at different temperatures for various time periods. Neuraminidase was incubated at 37C for periods up to eight hours, at 56C for periods up to 30 minutes, and at 100C for periods up to five minutes. At appropriate time intervals tubes were removed from the water bath and cooled to room temperature. Three cc of washed group O red cells were added to each tube, thoroughly mixed, and incubated at 37C for one hour. The cells were then washed five times with normal saline and diluted to a final concentration of 4%. A master dilution of A. hypogea lectin was prepared and two

drops of each dilution were mixed with one drop of the red cells and incubated at room temperature for 30 minutes. The tests were then graded for agglutination.

Test of the effect of neuraminidase on A. hypogea

Three different preparations of A. hypogea were treated with 0, 20, 50, 100, 200, or 400 λ of neuraminidase/ cc and 400, 380, 350, 300, 200, or 0 λ of saline respectively. These mixtures were incubated at room temperature for one hour and the neuraminidase was inactivated by placing the mixtures in a 56C water bath for 30 minutes. Each aliquot of A. hypogea treated with a different concentration of neuraminidase was titrated in saline and one drop of a 4% suspension of T-activated cells was added to 0.1 cc of each dilution. The tubes were allowed to incubate for 30 minutes at room temperature and centrifuged for 15 seconds and the agglutination graded.

Test of the effectof heating serum and saliva on the production of T antigen

To determine if the heating performed to inactivate neuraminidase in serum and saliva caused any production of T antigen, several serum and saliva samples were heated in a boiling water bath for periods of 5, 10, or 15 minutes for saliva and at 56C for periods of 10, 20, or 30 minutes for serum. Each aliquot was tested for T antigen by HIA employing anti-T lectin.

The effect of time and concentration of neuraminidase on T antigen production

To test the effect of incubation time on the amount of T substance produced by neuraminidase a saliva sample was divided into 24 one cc aliquots. 20λ of neuraminidase was added to eight aliquots, 50λ of neuraminidase was added to eight aliquots, and 50λ of saline was added to the remaining eight aliquots as a control. All aliquots were incubated at 37C. At intervals of 5, 15, 30, 45, 60, 90, 120, and 180 minutes one of the tubes containing 20λ of neuraminidase, one of the tubes containing 50λ of neuraminidase and one of the tubes containing salinewas removed from the water bath and placed in a boiling water bath for ten minutes and frozen at - 20C. After all tubes had been removed from the water bath, inactivated by boiling, and frozen they were thawed and tested by HIA for T substance.

The effect of different concentrations of neuraminidase on the amount of T substance produced in saliva was tested by dividing saliva samples into one cc aliquots and adding 10, 20, 50, 100, 200, 300, or 400λ of neuraminidase. To eliminate the effects of dilution 390, 380, 350, 300, 200, 100, or 0λ of saline respectively were added to the aliquots. All of the aliquots were incubated at 37C for one hour and then placed in a boiling water bath for ten minutes. Each aliquot was tested for T substance by HIA.

Effect of proteases on T substance production in saliva

Saliva samples, collected and processed as described previously, were divided into six one cc aliquots and treated with 50λ of neuraminidase, ficin, bromelin, papain, trypsin, or saline. Each aliquot was incubated at 37C for one hour and then inactivated in a boiling water bath for ten minutes. Each aliquot was tested for T substance by HIA using anti-T lectin.

The effect of increasing concentrations of each of the proteases was tested by dividing a saliva sample into twenty one one cc aliquots and adding 50, 100, 200, 300, or 400λ of ficin, bromelin, papain, or trypsin to the aliquots. To negate the effects of dilution 350, 300, 200, 100, or 0λ of saline respectively were added to the aliquots. A control of one cc of saliva plus 400λ of saline was run simultaneously. Each of the aliquots was incubated at 37C for one hour and then inactivated as was previously described. Each aliquot was tested for T substance by HIA.

The effect of various incubation times on the production of T substance by proteases was tested by dividing a sample of saliva into twenty five one cc aliquots. 50λ of the appropriate enzyme and 350λ of saline was sdded to four aliqouts. 400λ of the appropriate enzyme and no saline was added to four aliquots. A control of 400λ of saline plus one cc of saliva was run in parallel. Each tube was incubated at 37C and at time intervals of 60, 120, 180, or 240 minutes one tube containing 50λ of the enzyme znd one tube con-

taining 400λ of the enzyme were removed from the water bath and placed in a boiling water bath for ten minutes. These aliquots were frozen until all aliquots had been incubated and frozen. The aliquots were thawed and tested for T substance by HIA.

Comparison of T antigen production using different anti-T reagents in HIA

To determine if there is any difference in the detection of T antigen in body fluids when using different anti-T reagents in HIA, a series of saliva and serum samples were divided into two nine cc samples. One of the samples was treated with 20 λ/cc of neuraminidase and the other was treated with 20 λ/cc of saline. Both of the samples were incubated for one hour at 37C and the neuraminidase was inactivated as described. The neuraminidase and saline treated samples were each divided into nine one cc aliquots. Each of the neuraminidase and saline treated aliqouts were tested by HIA using on of the following antisera: A.hypogea, human anti-T serum (two examples), human anti-T eluates (two examples) rabbit anti-T serum (two examples), or rabbit anti-T eluates (two examples). The results were expressed as titration scores and the percent inhibition was calculated for each sample using each different reagent.

Test on the effect of neuraminidase on the anti-T in serum

To determine the effect of neuraminidase treatment on

naturally occuring anti-T present in normal adult sera, two cc samples of sera from 25 normal adults were collected and divided into two one cc aliquots. One cc of each sample was treated with 20 λ of neuraminidase and one cc of each sample was treated with 20 λ of saline. Both samples were incubated at 37C for one hour. The neuraminidase was inactivated as described and after cooling four drops of the neuraminidase treated sample and four drops of the saline control were incubated with one drop of a 4% suspension of T-activated cells for 30 minutes at room temperature. The tubes were centrifuged for 15 seconds and the agglutination graded.

Test of the effect of neuraminidase on IgG and IgM blood group antibodies

To determine the effect of neuraminidase on the activity of IgG and IgM blood group antibodies the following experiment was performed. Three examples each of anti-D and anti-M were treated with 2-Mercapto ethanol as described to determine whether the antibodies were IgG or IgM. The samples were divided into two aliquots. One aliquot was treated with 50 λ /cc of neuraminidase and the other with 50 λ /cc of saline. Both aliquots were incubated at 37C for one hour and the neuraminidase was inactivated by heating to 56C for 30 minutes. Each untreated and neuraminidase treated sample was then tested for antibody activity by serial titrations in saline and testing against appropriate cells. The anti-M titrations were read after 30 minutes at room temperature and the anti-D titrations were incubated at 37C for 30 min-

washed, and tested by the anti-human globulin technique.

Test of the ability of aged sera to inhibit anti-T

Ten samples of sera ranging in age from four to ten years that had been stored at 4C were tested for the presence of T substance. The sera were tested for anti-T by incubating four drops of the sera with one drop of a 4% suspension of T-activated cells at room temperature for 30 minutes. The tubes were centrifuged for 15 seconds and the agglutination graded. Each sample of aged sera was then tested by HIA using a serial dilution of A.hypogea to determine if there was any T substance present.

Immunodiffusion techniques

Various attempts were made at demonstrating the T antigen in serum and saliva that had been treated with neuraminidase using different preparations of lectins, human and rabbit anti-T. Commercially prepared immunodiffusion plates were obtained from Grand Island Biological Co.. Lectins of A. hypogea, U. europaeus, and D. biflorus were prepared as previously described both as raw saline preparations and as ammonium sulfate concentrates.

The raw lectins were used undiluted and diluted 1:2, 1:4, 1:8, 1:16, and 1:32. Preparations of U. europaeus and D. biflorus were concentrated four and eight times by covering dialysis tubing containing the lectin with polyethylene glycol until the volume of the lectin was reduced to one-fourth and one-eigth the original volume. Two different

preparations of A. hypogea were concentrated to two, four, and ten times their original concentration by the same method. Sera and eluates containing anti-T from human and rabbits were also tested against neuraminidase treated saliva. Sera from three rabbits immunized with saliva from a group O Le(a-b+) person and sera from three rabbits immunized with saliva from a group A Le(a+b-) person were also tested against various saliva samples.

Saliva, collected and processed as previously described, was tested against the lectins and antisera in various forms. They were tested undiluted, diluted 1:2, 1:4, 1:8, 1:16, and 1:32. Saliva was also concentrated by polyethylene glycol to two, ten, and one hundred times the original concentrations. Saliva sample volumes were altered by the number of times the wells of the immunodiffusion plates were filled. The plates containing the saliva samples were allowed to remain at room temperature until all of the saliva had diffused from the well and the well was refilled one, two or three times.

All of the immunodiffusion plates used in the experiments consisted of a central well with six equally spaced peripheral wells. The pattern of the wells was altered in two different ways, by increasing the diameter of the sample wells and by decreasing the distance between the peripheral wells and the central well. The spacing and sizes of the wells used are shown in Table 1.

Table 1: A chart showing the size and orientation of sample wells of the immunodiffusion plates.

Type of plate	Diameter central well	Diameter outer wells	Distance from outer to inner well
Commercial	5 mm	4 mm	8 mm
Prepared 1	8 mm	4 mm	8 mm
Prepared 2	8 mm	4 mm	4 mm

Various types and concentrations of solidifying media were used in preparing the gels. When plates were prepared the buffer that the gel was to be composed of was measured and an appropriate amount of solidifying media was added. The buffer was heated until all of the media went into solution. Enough of the heated solution was added to a 50 mm petri dish to make a base of media 4 mm deep. Plates were allowed to cool uncovered for one hour at room temperature, and covered in a refrigerator for at least one hour to allow complete solidification. Wells of appropriate size and orientation were cut in the media with cork borers using a template as a guide. The agar plugs were removed from the wells with an aspirator and the plates were stored in a plastic bag to prevent dessication at 4C until used. Agar was used in concentrations of 0.5, 1.0, and 1.5%. Agarose was used in concentrations of 0.5 and 1.0%.

Two different buffers were used to make the immunodiffusion plates. Phosphate buffered saline pH 7.2 was used most often. The other buffer that was used was a barbital buffer with added Ca^{++} and Mg^{++} (21). The pH of the buffer was var-

ied in different experiments. Phosphate buffered saline was used at pH 7.2, 5.5, and 8.5. Barbital buffer was always used at pH 7.2.

Test plates were incubated for a minimum of 14 days before being discarded. The plates were incubated in a moist chamber at room temperature until all of the sample had diffused from the wells and then they were incubated at 4C. Incubations carried out entirely at room temperature were also attempted.

Preparation of protein free filtrates

Protein free filtrates of serum and saliva were prepared by ultrafiltration using negative pressure. Three cc of the material to be tested were placed in a dialysis sac and subjected to a vacuum of 15 lbs/ sq. in. until all of the solution had passed through the membrane. The ultrafiltrates were frozen at - 20C until tested.

Dialysis studies

To determine if the T antigen present in native saliva or the T antigen produced by neuraminidase treatment of serum or saliva could be dialyzed, the following experiments were performed. A saliva or serum sample was divided into two parts. One part was treated with 20 λ / cc of neuraminidase and one part was treated with 20 λ /cc of saline. Both parts were incubated at 37C for one hour and the neuraminidase was inactivated as described. Each sample was placed

in a dialysis sac and dialyzed against normal saline at 4C for 24, 48, or 72 hours. A one cc aliquot was removed from the dialysis sac at the appropriate time interval and frozen at - 20C until it could be tested. One cc of the sample being tested was left at 4C for 72 hours as a control. After all of the samples were collected and frozen they were tested by HIA using anti-T lectin as the test antiserum.

Column chromatography

Saliva and serum samples were subjected to column chromatography on Sephadex G-200 by a modification of the method of Killander (23). The column used in these experiments was a model K-26 obtained from Pharmacia Fine Chemicals. The column had a diameter of 26 mm and a length of 1000 mm. The total bed volume was 530 cc. The gel was swollen for 72 hours in an excess of 0.1 M Tris-HCl, 1.0 M NaCl pH 8.0 and bubbles were removed from the slurry by placing in a boiling water bath under negative pressure for one hour. Saliva was concentrated by dialysis against polyethylene glycol until the volume was one-third of the original. Serum was used unconcentrated. Both types of samples were dialyzed for 48 hours against 0.1 M Tris-HCl, 1.0 M NaCl, pH 8.0. When neuraminidase treated samples were chromatographed the samples were treated with 50 λ of neuraminidase/ cc and then dialyzed for 48 hours. The sample size applied to the column 5 cc and the samples were eluted from the column with the same buffer used for dialysis. The flow rate was adjusted t

to approximately 0.3 cc/ minute and 4 cc fractions collected with a Gilson linear fractionator using a Gilson Aeolian Volumator. The optical density (O.D.) of the fractions were continuouosly scanned with a Gilson U.V. Spectrophotometer and recorded on a Gilson RP-1 recorder. Fractions were separated according to the protein peaks of the O.D. readings and were concentrated to the original volume of the sample by the polyethylene glycol method to insure that the separated components of the sample were present in the same concentration as in the original sample. The concentrated fractions were stored at -20C until used for testing. All fractions were tested for inhibition of anti-T by HIA using both untreated and neuraminidase treated fractions.

Tests with Beta-galactosidase and galactose oxidase

The effect of Beta-galactosidase and galactose oxidase on the T antigen in untreated saliva was tested by adding 20, 50, 100, 200, 300, 400, or 500 λ and adding 480, 450, 400, 300, 200, 100, or 0 λ respectively of saline to eliminate effects of dilution. A control of 500 λ of saline and one cc of saliva was run in parallel. All aliquots were incubated for one hour at 37C and the enzymes were inactivated as described. The tubes were cooled and tested for T antigen by HIA.

The effect of Beta-galactosidase and galactose oxidase on the T antigen in neuraminidase treated saliva was tested

by first treating saliva samples with 50λ/ cc of neuraminidase and incubating them for one hour at 37 C. The neuraminidase was inactivated by heating in a boiling water bath for ten minutes. The samples were divided into one cc aliquots and were treated with enzyme solutions of 20, 50, 100, 200, 300, 400, or 500λ per cc of sample. Amounts of 480, 450, 400, 300, 200, 100, or 0λ of saline respectively were added to the samples to eliminate the effects of dilution. The samples were incubated at 37C for one hour and the enzymes were inactivated as described. Each sample was tested for T antigen by HIA.

The effect of incubation time on the T antigen in saliva when treated with Beta-galactosidase or galactose oxidase was tested by dividing a saliva sample into 17 aliquots. 50λ of the stock solution of Beta-galactosidase was added to seven of these aliquots, 50λ of galactose oxidase was added to seven other aliquots, and 50λ of saline was added to the remaining three aliquots. All of the samples were incubated at 37C and at time intervals of 5, 15, 30, 45, 60, 90, and 120 minutes one tube with Beta galactosidase and one tube with galactose oxidase were removed from the 37C water bath and the enzyme activated as described. The samples were frozen at - 20C. Control tubes containing saline plus saliva were removed from the water bath at 0, 60, and 120 minutes, heated to 100C for ten minutes and frozen. After all of the samples were incubated, inactivated, and frozen they were tested for

the presence of T antigen by HIA.

The effect of Beta-galactosidase on the T antigen in neuraminidase treated serum was tested by the following experiment. Serum was incubated with 50 λ/cc of neuraminidase at 37C for one hour and the enzyme was inactivated by heating to 56C for 30 minutes. Varying amounts of the stock solution of Beta-galactosidase were added to aliquots of neuraminidase treated serum. The serum sample was divided into six one cc aliquots and 0, 20, 50, 100, 200, or 400 λ of Beta-galactosidase was added to an aliquot. To overcome the effects of dilution 400, 380, 350, 300, 200, or 0 λ of saline was added to the aliquots. All aliquots were incubated at 37C for one hour and the enzyme was inactivated at 56C for 30 minutes. Each tube containing a different amount of enzyme was assayed for the presence of T antigen by HIA.

Studies on the inhibition of Vicea graminea by body fluids

Samples of saliva or serum were divided into two cc aliquots. One aliquot was treated with 20 λ of neuraminidase and the other was treated with 20 λ of saline. Both aliquots were incubated at 37C for one hour and the enzyme was inactivated by heating the sample to 100C for ten minutes for saliva and to 56C for 30 minutes for serum. The aliquots were divided into two parts. One of the neuraminidase treated aliquots and one of the saline treated aliquots was tested for T antigen by HIA using A. hypogea as the antiserum and T-activated cells as the indicator cells. The other neur-

aminidase treated aliquot and saline treated aliquot were tested by HIA using V. graminea as the test antiserum and group O N red cells as the indicator cells.

To determine if the group O N indicator cells with neuraminidase would effect the results of HIA using V. graminea the following experiment was performed. A saliva sample was divided into two parts and one part was treated with 20λ/ cc of neuraminidase and one part was treated with 20λ /cc of saline. Both were incubated at 37C for one hour and the enzyme was inactivated by heating to 100C for ten minutes. Both samples were divided into two parts. One part of the neuraminidase treated sample and one part of the saline treated sample by HIA using V. graminea and normal group O N red cells and the other part of the neuraminidase treated and saline treated sample was tested by HIA using V. graminea and T-activated group O N red cells.

To determine the effect of various concentrations of neuraminidase on theinhibition of V. graminea the following experiment was performed. Saliva samples were collected and divided into seven one cc aliquots. To these aliquots 20, 50, 100, 200, or 400λ of neuraminidase were added along with 380, 350, 300, 200, or 0λ respectively of normal saline. A control of 400λ of saline was run in parallel. All of the samples were incubated at 37C for one hour and the neuraminidase was inactivated as described. Each sample was tested by HIA using V. graminea as the test antiserum and group O

N cells as the indicator cells.

To test the effects of Beta-galactosidase on the inhibition of V. graminea the following experiment was performed. Three cc samples of of saliva or serum were divided into three one cc aliquots. 50λ of neuraminidase was added to two of these aliquots and 50λ of saline was added to the other. All of the samples were incubated at 37C for one hour and the neuraminidase was inactivated as described. 50λ of the Beta-galactosidase solution was added to one of the neuraminidase treated samples and 50λ of saline was added to the other two. All three samples were incubated at 37C for one hour and the enzyme was inactivated as described. All of the samples were tested by HIA using V. graminea as the antiserum and group O N red cells as theindicator cells.

RESULTS

Inhibition of anti-T with saliva

A total of 54 saliva samples was treated with neuraminidase and tested for the ability to inhibit anti-T by HIA. Results of HIA using neuraminidase treated and untreated saliva are shown in Table 2. All samples tested except one (sample 14) inhibited the *A. hypogea* lectin more after treatment with neuraminidase. The titer score before and after neuraminidase treatment were used to calculate the percent inhbition and those values ranged from 4 to 88.3%. The mean percent inhibition for the series is 55.9% with a median score of 58.1 and a standard deviation of 18.1.

Another feature of the series seen in Table 2 is that prior to treatment with neuraminidase all saliva samples tested showed some ability to inhibit anti-T lectin. The *A. hypogea* lectin when tested with saline gave a titer score of 94. Using this value as the baseline and calculating the percent inhibition values for the untreated samples the values in Table 2 were obtained. The percent inhibition values for the untreated saliva samples when compared to the titer score of the saline control ranged from 2.1 to 93.6%. The overall series of untreated saliva samples had a mean percent inhibition of 54.2% with a median score of 54.2 and a standard deviation of 22.1.

The mean titration score using untreated saliva samples

Table 2: The results of testing 54 saliva samples before and after neuraminidase treatment with the HIA.

Sample number	Score untreated	Percent inhibition	Score neuraminidase treated	Percent inhibition
1	80	13.0	38	52.5
2	71	21.1	53	25.4
3	92	2.1	38	58.7
4	54	42.5	13	75.9
5	66	29.8	39	29.8
6	43	36.1	43	28.3
7	51	45.7	51	58.8
8	39	45.7	19	51.3
9	35	62.8	16	54.2
10	52	44.6	18	65.4
11	54	42.6	20	63.0
12	52	44.6	16	69.2
13	27	71.3	26	4.0
14	16	82.9	16	0.0
15	55	41.5	25	54.5
16	33	64.9	25	24.2
17	83	11.7	46	44.5
18	56	40.4	29	48.2
19	74	21.3	31	58.1
20	50	46.8	9	82.0
21	39	58.5	26	33.0
22	26	72.3	17	34.6
23	15	84.0	6	60.0
24	29	69.1	16	44.8

Table 2: (Continued)

Sample number	Score untreated	Percent inhibition	Score neuraminidase treated	Percent inhibition
25	23	75.5	6	73.9
26	16	82.9	7	56.3
27	54	42.6	11	79.6
28	16	82.9	11	31.3
29	59	88.1	7	37.3
30	6	93.6	3	50.0
31	47	50.0	12	74.5
32	35	62.8	7	80.0
33	15	84.0	3	80.0
34	60	36.2	7	36.2
35	16	82.9	6	62.5
36	36	66.7	7	80.5
37	43	54.2	12	72.1
38	68	27.6	11	83.8
39	72	23.4	26	63.9
40	48	48.9	35	27.1
41	41	56.4	23	43.9
42	30	68.1	9	70.0
43	24	74.5	10	58.3
44	55	41.5	11	80.0
45	16	82.9	8	50.0
46	61	35.1	34	43.3

Table 2: (Continued)

Sample number	Score untreated	Percent inhibition	Score neuraminidase treated	Percent inhibition
47	14	85.1	10	28.6
48	16	82.9	3	81.3
49	24	74.5	16	33.3
50	21	77.7	11	47.6
51	37	60.6	8	78.4
52	37	60.6	18	51.4
53	50	46.8	15	70.0
54	70	25.5	24	65.7

Table 3: Distribution of the percent inhibition values for 54 saliva samples treated with neuraminidase.

Percent inhibition interval	Number of samples in interval	Percent of samples in interval
0 -10	2	3.7
10.1-20	0	0.0
20.1-30	5	9.3
30.1-40	4	7.4
40.1-50	7	12.9
50.1-60	13	24.1
60.1-70	6	11.1
70.1-80	8	14.8
80.1-90	8	14.8
90.1-100	1	1.9

was 42.9 with a median score of 48, and the mean of the titration scores using the same saliva samples after neuraminidase treatment was 18.7 with a median score of 20. The t test for calculating the signifigance of the difference between two mean values gives a value for t of 23.1 which shows that the observed difference is signifigant at the P = 1.0 and P = 0.1 level.

The distribution for the percent inhibition values for the 54 saliva samples tested before and after neuraminidase treatment are shown in Graph 1 and Table 3. Only two samples (3.7%) had a percent inhbition value of 20% or less and only one (1.9%) sample had a percent inhibition of 90% or more. The other 51 samples (94.4%) fell within the 20 to 90% inhbition range. In the lower half of the 20 to 90 percent inhibition range (20.1 to 55%) there was a total of 23 samples (42.6%) while in the upper half of the range (55.1 to 90%) there was a total of 28 samples (51.9%). These data suggest that the distribution of the percent inhbition is in the form of a normal distribution curve that peaks in the 50 to 60% range and is slightly skewed toward the higher values. This is supported by the mean value being slightly less than the median value and by the general shape of the graph in Graph 1.

The distribution of the percent inhbition exhibited by the 54 untreated saliva samples is shown in Graph 2 and Table

4. The distribution is similar to the one shown previously in Graph 1 for the neuraminidase treated samples. Only three samples (5.6%) fell in the range of 0 to 20 percent inhbition while only one sample (1.9%) fell in the 90 to 100 percent inhbition range. 25 (48.1%) had values that fell in the lower half of the 20 to 90 percent inhibition range (20.1 to 55%) and 25 samples (48.1%) of the samples had values that fell in the upper half of the 20 to 90 percent inhibition range (55.1 to 90%). This data and the fact that the median and mean score for the series are identical tends to show that the amount of inhbition produced by untreated saliva samples of anti-T follows a normal distribution pattern. The slight departure from a normal distribution curve seen in Graph 2 can be assumed to the relatively small number of samples tested.

Table 5 shows the results of testing saliva of 14 donors at intervals of one week and two weeks. As seen from the data in Table 5 the amount of inhibition produced by a person's saliva in not constant over a period of time. These dats show that the amount of inhibition produced by neuraminidase treatment of saliva is not constant over a period of time.

The data shown in Table 6 indicate that the percent inhibition produced by saliva samples from individuals of different ABO groups and secretor status shows no signifigant difference in the amoun of inhbition produced. The only

Table 4: The distribution of percent inhibition values of anti-T for 54 untreated saliva samples.

Percent inhibition internal	Number of samples in interval	Percent of samples in interval
0-10	1	1.9
10.1-20	2	3.7
20.1-30	5	9.3
30.1-40	5	9.3
40.1-50	13	24.1
50.1-60	4	7.4
60.1-70	8	14.8
70.1-80	7	12.9
80.1-90	8	14.8
90.1-100	1	1.9

Table 5: The results of testing the saliva samples of 14 donors at intervals of one week.

Donor number	Day 1	Percent inhibition	Day 8	Perdent inhibition	Day 15	Percent inhibition
1 Pre	14		27		26	
1 Post	11	21.4	26	3.0	11	57.7
2 Pre	16		49		28	
2 Post	16	0.0	14	71.4	3	89.2
3 Pre	25		33		17	
3 Post	13	48.0	25	24.2	10	41.2
4 Pre	74		83		67	
4 Post	17	77.0	46	44.5	16	76.1
5 Pre	72		73		NT	
5 Post	28	61.1	31	57.5	NT	
6Pre	72		52		35	
6Post	13	81.9	18	65.4	7	80.0

Table 5: (Continued)

Donor number	Day 1	Percent inhibition	Day 8	Percent inhibition	Day 15	Percent inhibition
7 Pre	29		50		38	
7 Post	8	72.4	9	82.0	11	71.0
8 Pre	63		56		50	
8 Post	19	69.8	42	23.2	20	66.1
9 Pre	48		55		60	
9 Post	21	56.3	23	58.2	28	53.3
10 Pre	62		51		55	
10 Post	16	74.2	21	58.8	15	72.2
11 Pre	29		52		38	
11 Post	14	51.7	16	69.2	8	78.9
12 Pre	23		37		23	
12 Post	8	65.2	16	56.8	15	34.8
13 Pre	50		54		27	
13 Post	7	86.0	20	63.0	7	74.0
14 Pre	39		NT		25	
14 Post	26	33.3	NT		12	52.0

Pre = titer score from HIA before neuraminidase treatment
Post= titer score from HIA after neuraminidase treatment
NT = not tested

Table 6: The percent inhibition value of saliva after neuraminidase treatment arranged according to ABO group and secretor status of donor.

		Secretors			
	NonSecretors	Group O	Group A	Group B	Group AB
Number of donors	11	12	21	7	2
Mean percent inhibition value	56.5%	56.3%	54.0%	59.6%	71.5%

group that is different in the percent inhbitition mean is the AB secretors. The mean percent inhibition for the AB secretors differs signifigantly from the other mean percent inhibition values, but because only two AB secretors were tested this difference could be attributed to the small number tested rather than to an actual difference in the composition of their saliva samples.

Inhibibion of anti-T by neuraminidase treated serum

The data presented in Table 7 shows the results of treating serum samples from 50 healthy donors (40 adults and 10 cord sera) with neuraminidase and then assaying the serum for the presence of T substance by HIA. All sera tested inhibited anti-T after treatment with neuraminidase. The percent inhibition after treatment with neuraminidase ranged from 21.7 to 67.6%. The series had a mean percent inhibition of 50.6% with a median value of 51.9 and a standard deviation 7.8. If the results from the adult sera and the cord sera tested are examined individually and compared to the values for the overall series it is seen that the mean of the adult sera tested was 52.0% with a median value of 50.5 and a standard deviation of 7.8 and the cord sera tested had a mean percent inhbition value of 52.0% witha median value of 51.1 and a standard deviation of 8.0. These values are in very good agreement and if the t value is calculated to show the signifigance of the difference between the mean of the cord and adult sera the t value is 0.1821 which shows

Table 7: The results of treating 50 serum samples with neuraminidase and assaying for T substance with the HIA.

Sample number	Score before neuraminidase treatment	Score after neuraminidase treatment	Percent inhibition
1	94	42	55.3
2	93	41	56.4
3	94	31	67.1
4	94	57	39.8
5	91	48	48.7
6	94	45	52.6
7	92	38	59.8
8	94	49	47.6
9	93	47	50.3
10	94	45	52.2
11	94	47	50.0
12	94	74	21.7
13	91	50	45.3
14	94	51	45.6
15	94	56	40.6
16	93	44	52.8
17	93	44	52.8
18	94	45	51.9
19	94	44	52.4
20	94	45	51.9
21	93	44	52.4
22	91	48	47.2
23	95	56	40.7
24	94	35	62.3
25	90	48	46.1

Table 7: (continued)

Sample number	Score before neuraminidase treatment	Score after neuraminidase treatment	Percent inhibition
26	92	44	51.9
27	94	44	52.8
28	94	48	47.6
29	94	56	39.8
30	94	35	62.9
31	92	43	53.4
32	90	45	50.0
33	95	47	50.1
34	94	56	40.6
35	93	44	52.2
36	94	42	54.9
37	93	45	51.7
38	92	52	43.6
39	94	42	55.8
40	91	37	59.6
41	94	30	67.6
42	94	37	60.8
43	93	44	52.7
44	94	55	41.6
45	92	51	44.7
46	91	49	45.9
47	94	45	52.3
48	92	45	51.1
49	93	41	56.3
50	94	50	47.2

Samples 1 - 40 are from adults; 41 - 50 are cord sera.

that the difference is not statistically signifigant. This comparison is summarized in Table 8.

Table 8: A comparison ofthe mean, median, and standard deviation for the overall series, the cord sera, and the adult sera inhibition of anti-T after neuraminidase treatment.

Value	Overall series	Adult sera	Cord sera
Mean percent inhibition	50.6	50.6	52.0
Median score	51.9	50.5	51.1
Standard deviation	7.8	7.8	8.0

In contrast to saliva where all of the samples tested showed some degree of inhibition prior to neuraminidase treatment, untreated serum samples demonstrated no inhibitory effect.

Table 9 compares the mean, median, and standard deviation of titration scores from inhibition of A. hypogea by neuraminidase treated and untreated serum. Table 9 also presents the values of t calculated for the differences between the means of the untreated and neuraminidase treated cord sera, adult sera, and the overall series. In all cases the t value shows that the difference is statistically signifigant at the P = 1.0 and P = 0.1 level. The data in Table 9 also demonstrates that the mean titer scores after neuraminidase treatment are very close for the overall series, the cord sera, and the adult sera tested.

Table 9: A comparison of the mean, median and standard deviation for the titer scores obtained from HIA for sera samples before and after neuraminidase treatment.

Before neuraminidase treatment:

Value	Overall series	Adult sera	Cord sera
Mean titer score	93.1	93.1	93.1
Median score	93	93	93
Standard deviation	1.3	1.4	1.1

After neuraminidase treatment:

Value	Overall series	Adult sera	Cord sera
Mean titer score	46.0	45.4	48.4
Median score	45.5	45.5	45
Standard deviation	7.3	7.4	7.5
t value	44.3	34.1	7.9

Graph 3 and Table 10 show the results of tests from the serum samples when they are grouped according to the number of samples in each percentage interval. There are only 3 sera (6%) that gave values for percent inhbition in the range of 0 to 40% and only 5 serum samples (10%) gave values of percent inhbition in the range of 60 to 100%. The other 42 samples tested (84%) gave values that fell in the range of 40 to 60%.

Table 10: The number and percent of sera tested giving values for percent inhbition after neuraminidase treatment in each percentile range.

Percent interval	Number of sera in interval	Percent of sera in interval
0 - 10	0	0.0
10.1 - 20	0	0.0
20.1 - 30	1	2.0
30.1 - 40	2	4.0
40.1 - 50	16	32.0
50.1 - 60	26	52.0
60.1 - 70	5	10.0
70.1 - 80	0	0.0
80.1 - 90	0	0.0
90.1 -100	0	0.0

The calculated values and Graph 3 and Table 10 show that the amount of inhbition of A. hypogea by sera show a normal distribution pattern with most of the values falling in the 40 to 60% range and with a minority of values falling outside of this range. The peak number of values within this range are concentrated very close to the mean and median values which also indicates a normal distribution with little or no skewing. As shown in Graph 4 and Table 11 if the intervals between 20 and 70% are broken down into 5% intervals instead of 10% intervals as shown in Graph 3 the distribution appears as a perfectly normal distribution. These dist-

GRAPH 4: DISTRIBUTION OF PERCENT INHIBITION FOR 50 SERUM SAMPLES TREATED WITH 20 I NEURAMINIDASE / cc

NUMBER OF SAMPLES

PERCENT INHIBITION

ribution results are very close to the results seen for the series of saliva samples that were tested.

Table 11: The number and percent of sera tested giving values for percent inhbition after neuraminidase treatment in each percentile range.

Percent interval	Number of sera in interval	Percent of sera in interval
20.1 -25	1	2.0
25.1 -30	0	0.0
30.1 -35	0	0.0
35.1 -40	2	4.0
40.1 -45	6	12.0
45.1 -50	10	20.0
50.1 -55	20	40.0
55.1 -60	6	12.0
60.1 -65	3	6.0
65.1 -70	2	4.0

Tests on neuraminidase treated urine, human milk, and colostrum

Table 12 shows the results of tests performed on untreated neuraminidase treated urines to determine if there are substances in urine that could cause the inhbition of anti-T reagents. 18 samples of urine were tested unconcentrated and three samples were concentrated five and ten times. The amount of inhbition produced by neuraminidase treatment ranged from -6.4 to 4.8%. The mean percent inhibition of the series was 0.9%. This value is very low, even when some of

the samples were concentrated, and when considered with the fact that some of the samples showed a negative percent inhibition value suggests that there is no true inhbiition of the lectin caused by treating samples with neuraminidase, and that the small amount of inhibition exhibited by some of the samples is due to variables inherent in the HIA. The mean value of the titer score from HIA on untreated urine samples was 82.9 with a standard deviation of 1.9. The mean value of the titer scores obtained for neuraminidase treated urine is 81.4 with a standard deviation of 2.2. The t value for determining the signifigance of the difference between the means is 2.42 which shows that the difference is not statisticaly signifigant at the P = 1I0 or P = 0.1 level.

Table 12: The results of testing urine samples for T substances before and after neuraminidase treatment.

Sample number	Score before neuraminidase treatment	Score after neuraminidase treatment	Percent inhibition
1	84	82	1.6
2	84	84	0.0
3	82	76	7.3
4	83	83	0.0
5	84	84	0.0
6	83	81	2.1
7	82	84	-2.4
8	84	82	1.2
9	83	79	4.8
10	83	81	2.4
11	84	81	3.5

Table 12: (Continued)

Sample number	Score before neuraminidase treatment	Score after neuraminidase treatment	Percent inhibition
12	82	80	2.4
13	84	83	1.2
14	84	86	-2.4
15	84	81	3.5
16 (x1)	81	81	0.0
16 (x5)	78	79	-1.3
16 (x10)	78	81	-3.8
17 (x1)	82	79	3.6
17 (x5)	82	81	1.2
17 (x10)	81	81	0.0
18 (x1)	78	83	-6.4
18 (x5)	81	81	0.0
18 (10)	81	79	2.5

Table 13 shows tests on human milk from two donors and on colostrum from a single donor. The percent inhibition values are very low and probably reflect slight variations in HIA rather that actual T substance.

Table 13: Tests on human milk and colostrum before and after neuraminidase treatment for T substance.

Sample	Score before neuraminidase treatment	Score after neuraminidase treatment	Percent inhibition
Milk 1	78	80	-6.4
Milk 2	83	82	1.2
Colostrum	81	76	6.7

Tests on the effect of neuraminidase on the anti-T in serum

Table 14 shows the results of tests to determine the effect of neuraminidase treatment on the anti-T in serum. As seen in Table 14 of the 25 sera tested all reacted strongly with the T-activated cells. After treatment with neuraminidase 22 of the samples failed to react with the T-activated cells, and the three that did react reacted considerably weaker than they did before neuraminidase treatment. When the reactions were scored the sera had an average reaction strength of 11.2 prior to neuraminidase treatment and an average score of .36 after neuraminidase treatment. When the t test is applied to the two means the t value is 101.5 which shows that the difference between the means is signifigant at the P = 0.1 and P = 1.0 level.

Tests on the effect of neuraminidase on blood group antibodies

Three examples of IgG anti-D and three examples of IgM anti-M antibodies were tested in titration with the appropriate cells and then treated with neuraminidase and retested against appropriate cells. Table 15 shows the results of titrations with these antisera before and after treatment with neuraminidase. These results clearly show th that neuraminidase treatment of IgG or IgM immunoglobulins has no effect on their reaction strength or characteristics.

Results of tests on the inhbition of anti-T by aged sera

Sera that had been stored for four to nine years were

Table 14: The reaction of 25 srum samples with T-activated red cells before and after neuraminidase treatment.

Serum number	Reaction with T-activated red cells before neuraminidase treatment (score)	Reaction with T-activated red cells before neuraminidase treatment (score)
1	4+ (12)	0
2	2+ (8)	0
3	3+ (10)	0
4	3+ (10)	0
5	4+ (12)	0
6	4+ (12)	± (3)
7	$3+^{s}$(11)	0
8	3+ (10)	0
9	3+ (10)	0
10	4+ (12)	0
11	4+ (12)	± (3)
12	3+ (10)	0
13	4+ (12)	0
14	4+ (12)	0
15	3+ (10)	0
16	4+ (12)	0
17	4+ (12)	0
18	3+ (10)	0
19	$2+^{s}$(9)	0
20	3+ (10)	0
21	4+ (12)	0
22	4+ (12)	0
23	4+ (12)	± (3)
24	$2+^{s}$(9)	0
25	3+ (10)	0

Table 15: Titration scores of three examples of anti-M and three examples of anti-D before and after neuraminidase treatment.

Serum	Titer score before neuraminidase	Titer score after neuraminidase
Anti-M(1)	25	26
Anti-M(2)	31	31
Anti-M(3)	26	24
Anti-D(1)	56	56
Anti-D(2)	49	52
Anti-D(3)	45	43

tested to determine if they had any increased ability to inhibit anti-T. Table 16 shows the results of these tests.

Table 16: The results of tests on the inhbition of anti-T by aged serum samples.

Sample number	Reaction with T-act. cells	Score from HIA	Sample number	Reaction with T-act. cells	Score from HIA
1	0	71	7	0	71
2	0	69	8	0	69
3	0	69	9	0	72
4	0	69	10	0	69
5	0	69	Fresh 1	4+	69
6	0	69	Fresh 2	$3+^{W}$	69
			Saline	0	71

This data demonstrates that the titer scores obtained from HIA using aged serum samples caused no increased inhbition when compared to the fresh serum controls. Thus there is no

demonstrable exposure of the T antigen in aged serum samples.

Effect of incubation time and concentration of neuraminidase on the amount of inhibition of anti-T

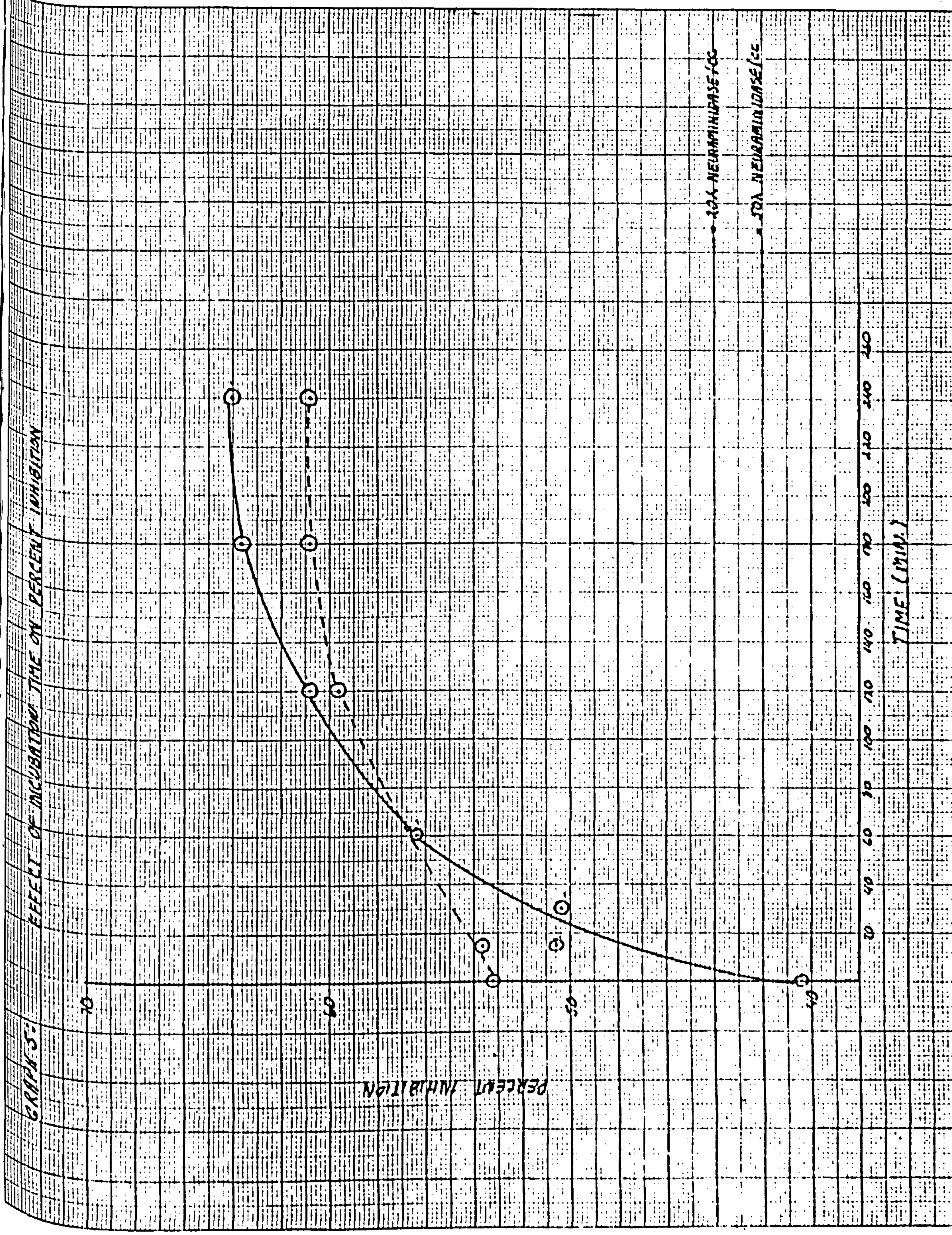
Table 17 presents data from the results of a study to determine the effect of incubation time on the amount of inhibition of anti-T produced by neuraminidase treatment of saliva. Graph 5 presents the data from Table 17 expressed as percent inhibition plotted against incubation time. The trend of the graph for both concentrations of neuraminidase is a curve that increases rapidly at first and then tends to level off at greater time intervals. The amount of inhibition of anti-T increases with incubation time until a point is reached where further increases in time produces little or no increase in the amount of T antigen. Some of the slight variations in the curve on the graph are due to the fact that the percent inhibition values are calculated from untreated controls which give values that vary slightly due to experimental variables of HIA and due to the fact that neuraminidase treated samples are subject to the same experimental variables. One of these variables could be overcome by plotting the reciprocal of the titer score of the neuraminidase treated sample against time. This method disregards the slight variations in the untreated sample titer score. Graph 6 shows this plot and also clearly shows the curve as seen in Graph 5. This curve is not as apparent in the samples treated with 50 of neuraminidase because there

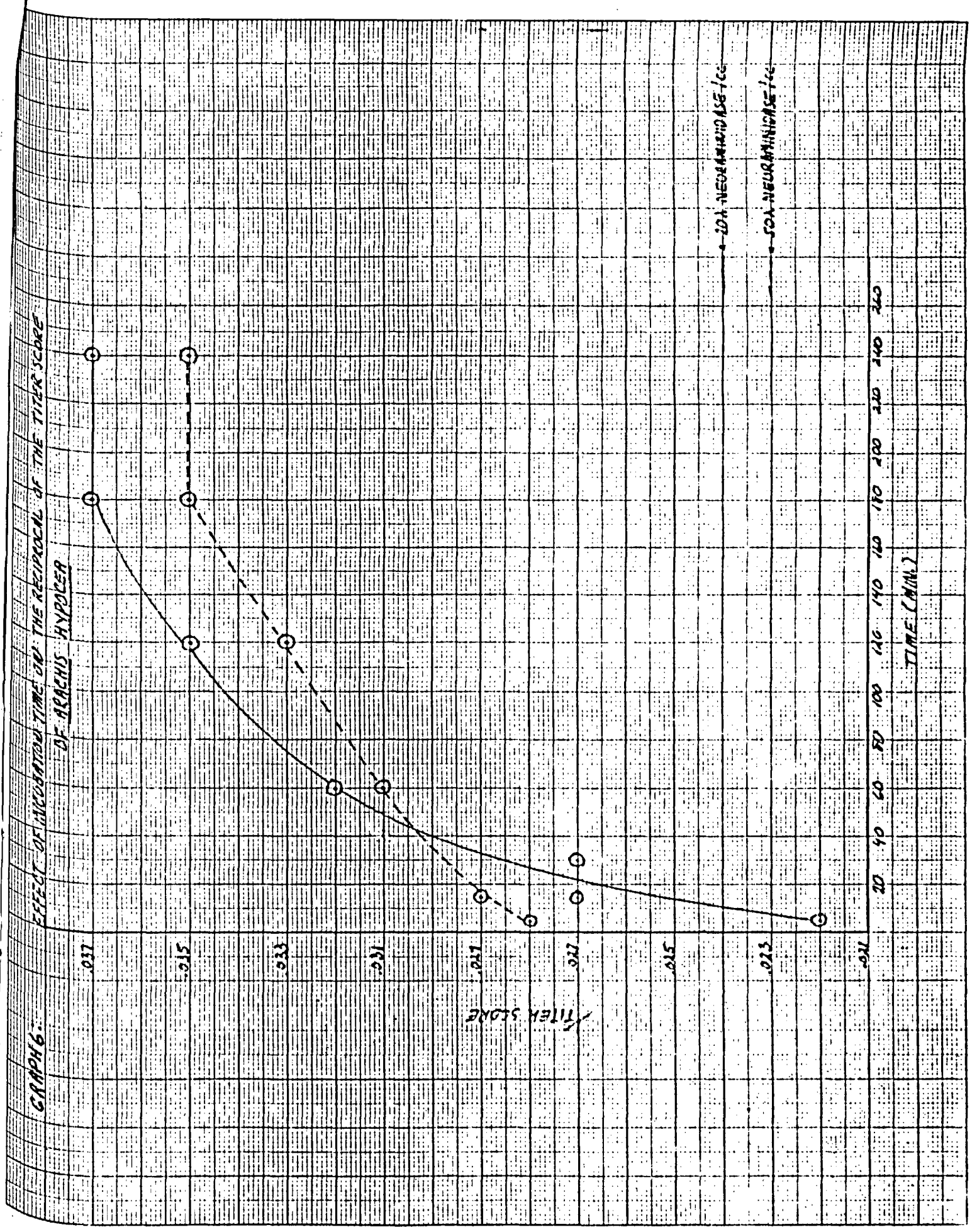
Table 17: The effect of incubation time on the amount of inhibition of anti-T produced by neuraminidase.

Time (min)	Amount of neuraminidase (λ)	Titer score	Percent inhibition	1/titer score
5	0	77	0.0	
	20	46	40.3	.022
	50	36	53.2	.028
15	0	75	0.0	
	20	37	50.7	.027
	50	35	53.3	.029
30	0	74	0.0	
	20	37	50.0	.027
	50	37	50.0	.027
60	0	73	0.0	
	20	32	56.2	.032
	50	32	56.2	.031
120	0	74	0.0	
	20	29	60.8	.035
	50	30	59.5	.033
180	0	74	0.0	
	20	27	63.5	.037
	50	29	60.8	.035

Table 18: The effect of different concentrations of neuraminidase on the amount of inhibition produced.

Amount of neuraminidase (λ)	Amount of saline (λ)	Sample 1	Sample 2	Sample 3
10	390	50	27	42
20	380	40	23	35
50	350	37	19	32
100	300	32	20	31
200	200	32	19	31
300	100	30	19	31
400	0	30	19	30





is more initial inhibition at the first sampling point and this tends to flatten the curve.

Table 18 presents data from experiments in which three different saliva samples were treated with gradually increas- amounts of neuraminidase and assayed by HIA. This data is presented graphically in Graph 7 as a plot of the titer score against the amount of neuraminidase added to the saliva. From the table and graph it is seen that at first increasing the amounts of neuraminidase causes a substantial increase in the amount of inhibition produced (reflected as lower titer scores). This large increase in inhibition is not apparent after a level of 50λ to 100λ/cc of neuraminidase is reached. After these levels the addition of more neuraminidase does not cause any further increase in the amoont of inhbiition produced.

Tests on the heat stability of neuraminidase

To insure that neuraminidase was actually inactivated by heating to 100C for ten minutes or to 56C for 30 minutes heat stability studies were performed. Table 19 presents the results of these experiments. The residual activity of neuraminidase is expressed as the titer score of A. hypogea tested against red cells that were treated by the neuraminidase being tested, and as the percent decrease in this titer score as compared to the control value of red cells T-activated by unheated neuraminidase.

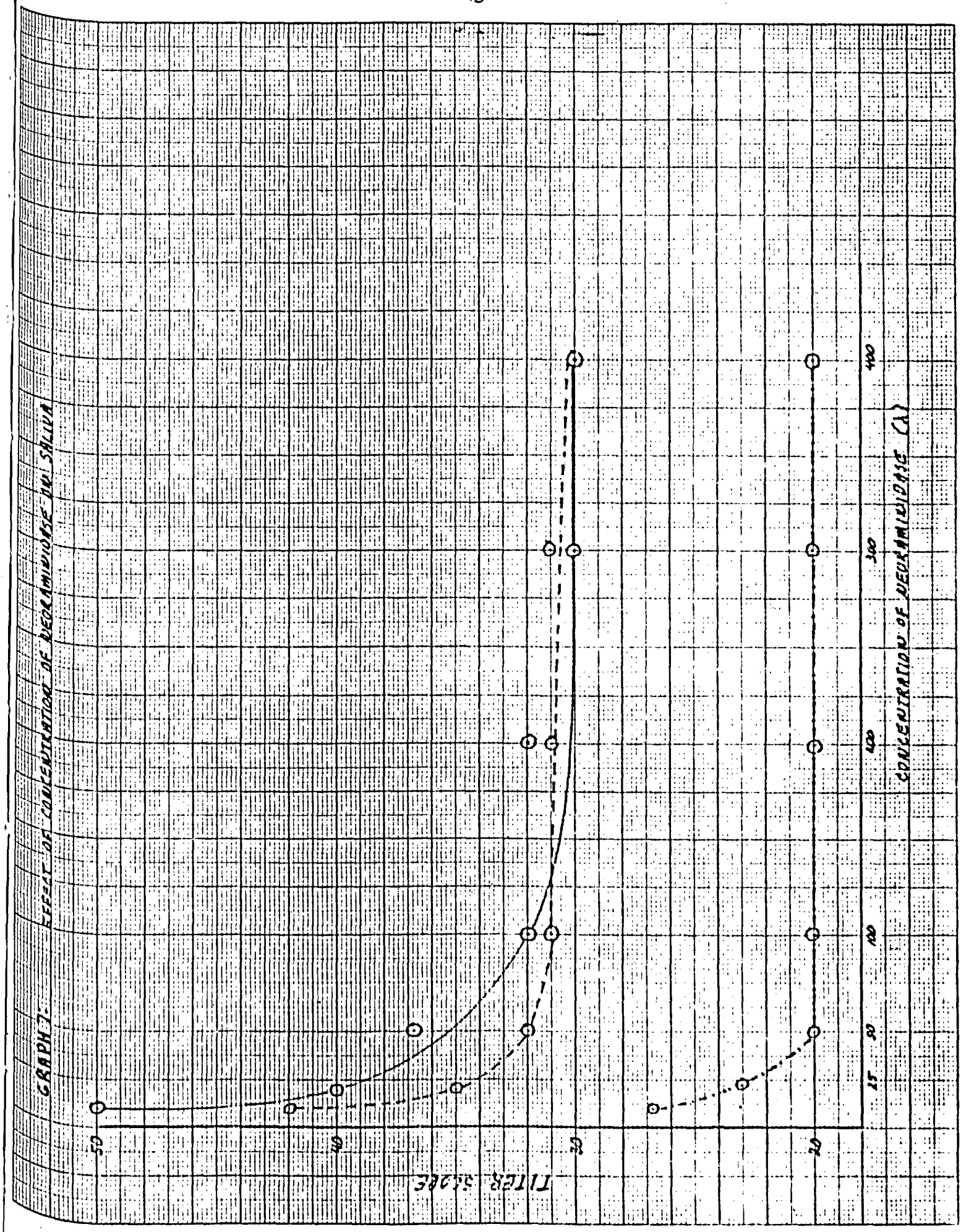


Table 19: Tests on the heat stability of neuraminidase

Temperature	Time (min)	Titer score	Percent decrease
37°	0	87	0.0
	15	87	0.0
	30	87	0.0
	60	87	0.0
	120	87	0.0
	180	84	3.4
	240	86	1.1
	300	87	0.0
	360	87	0.0
	420	87	0.0
	480	84	3.4
56°	0	88	0.0
	5	69	21.6
	10	17	80.7
	15	11	88.0
	20	3	97.0
	25	0	100.0
	30	0	100.0
100°	0	88	0.0
	1	8	90.9
	2	0	100.0
	5	0	100.0

These results are presented graphically in Graphs 8, 9, and 10. From the data in Table 19 and Graph 8 it is evident that incubation of neuraminidase at temperatures of 37C for periods of up to eight hours has no effect on the ability of the neuraminidase to T-activate red cells and therefore no effect on its enzymatic activity.

The results in table 19 and Graph 9 show that incubation of neuraminidase at 56C rapidly destroys its activity. After as little as five minutes there is a 21.6% decrease in activity, after ten minutes there is an 80.7% decrease, after 20 minutes there is a 97% decrease. After 25 minutes incubation there is no demonstrable activity remaining.

The results from Table 19 and Graph 10 show that heating neuraminidase at 100C causes a very rapid decrease in neuraminidase activity. After one minute the activity is reduced 90.9% and after two minutes there is no activity demonstrable.

These results show that the methods used to inactivate neuraminidase in these experiments were adequate.

The effect of neuraminidase on A. hypogea

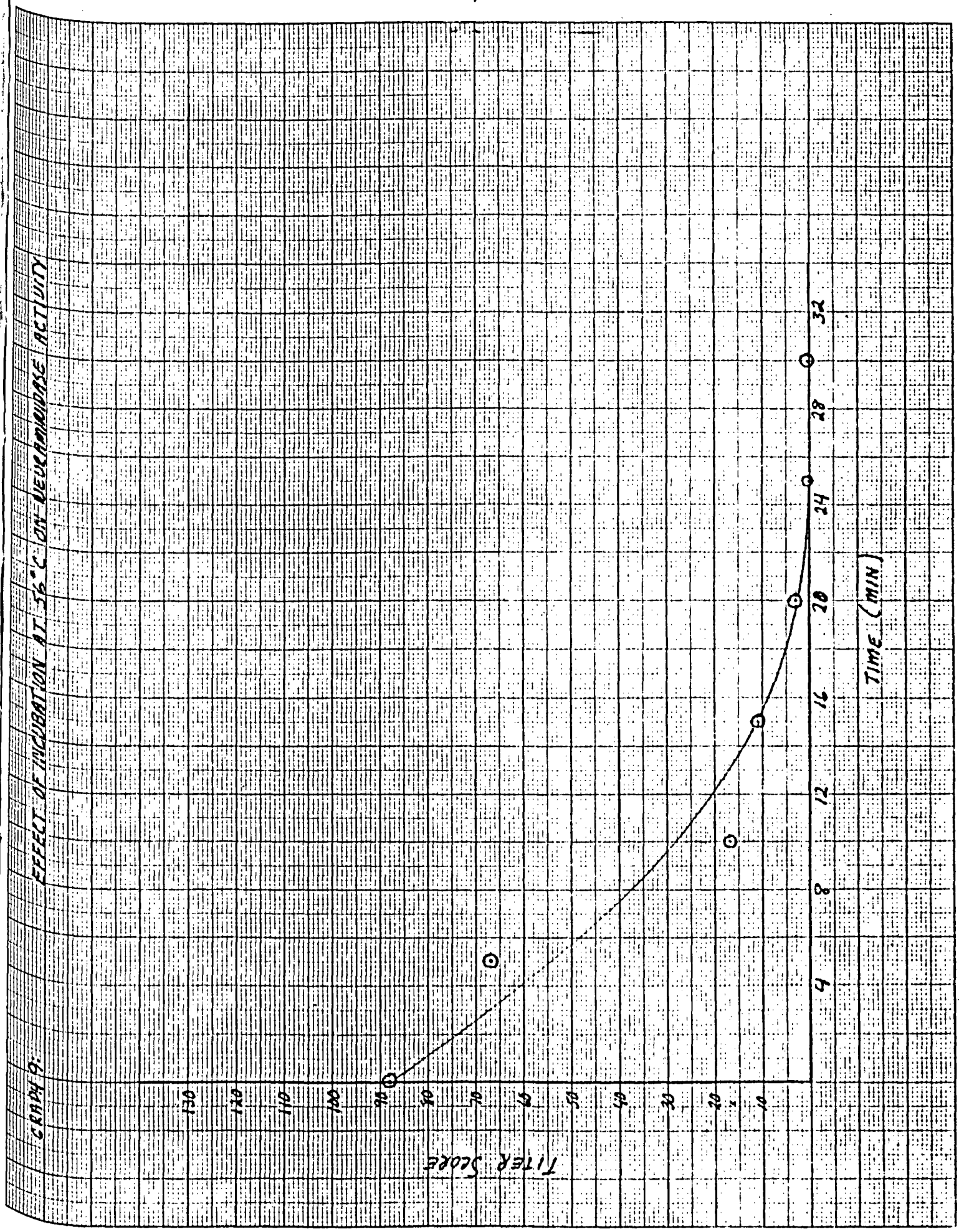
Table 20 shows the data demonstrating the effect of neuraminidase an A. hypogea lectin used in these experiments. The data is recorded as the titer score of the lectin when tested against a suspension of strongly T-activated cells.

As seen from the data in Table 20 and Graph 11 neuraminidase added to A. hypogea even in amounts as high as 400 λ

GRAPH 8: EFFECT OF ETHANOL ON NEUTRALIZATION OF ACETYLCHOLINE

TIMER SCALE

TIME (MIN)



GRAPH 10: EFFECT OF INCUBATION AT 100°C ON NEUTRAMINIDASE ACTIVITY

/cc of lectin had no effect on the activity of the lectin.

Table 20: The effect of neuraminidase on the titer score of three preparation of A. hypogea

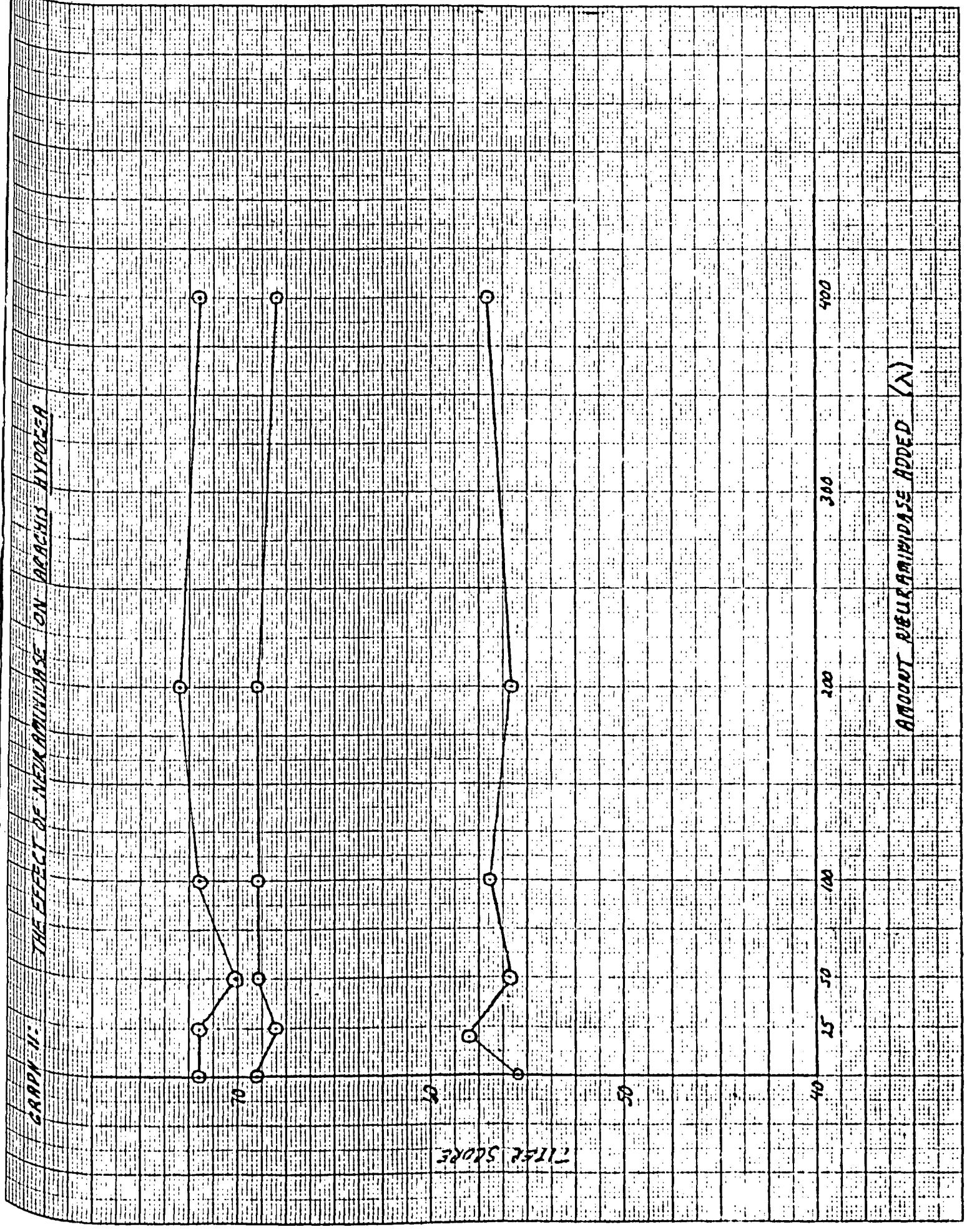
Amount neuraminidase (λ)	Amount saline (λ)	Arachis 1	Arachis 2	Arachis 3
0	400	71	56	69
20	380	71	59	68
50	350	70	56	69
100	300	71	57	69
200	200	72	56	69
400	0	71	57	68

This is shown by the almost identical titer scores obtained by testing the neuraminidase treated A. hypogea against a suspension of T-activated cells. This is good evidence that neuraminidase causes no loss of serological activity of A. hypogea.

The effect of heating serum and saliva samples on the inhibition of anti-T

Because all serum and saliva samples tested for inhibition of anti-T were heated to inactivate neuraminidase it was necessary to show that simple heating of these samples did not cause the inhibition of anti-T. The results of these experiments are shown in Table 21 as the titer score obtained by HIA using A. hypogea as the test lectin. This data is presented graphically in Graphs 12 and 13.

As seen in Table 21 and Graph 12 the saliva samples



tested all showed different amounts of inhibition of anti-T and heating them to 100C for periods up to 15 minutes neither destroyed or enhanced this inhibition. Similarly, heating serum samples to 56C for up to 30 minutes resulted in no increase in T inhibitory effect. These results are given in Table 21 and Graph 13.

Table 21: The effect of heating saliva and serum samples on th inhbition of anti-T

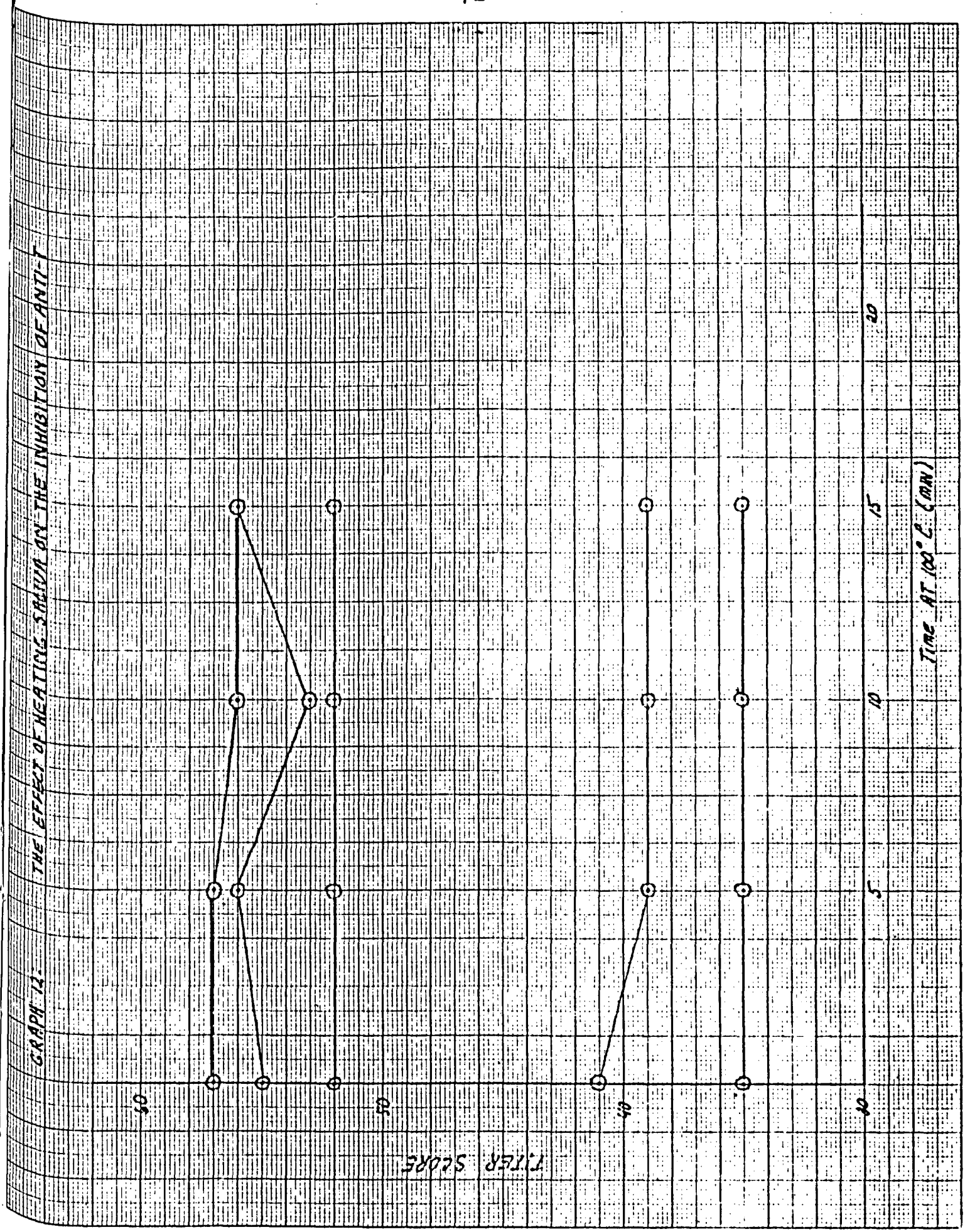
Time at 100C (min)	Saliva 1	Saliva 2	Saliva 3	Saliva 4	Saliva 5
0	57	41	52	35	55
5	57	39	52	35	56
10	56	39	52	35	56
15	56	39	52	35	53
Time at 56C (min)	**Serum 1**	**Serum 2**	**Serum 3**	**Serum 4**	**Serum 5**
0	82	83	84	83	82
10	83	82	83	84	82
20	82	82	83	83	82
30	82	82	83	83	82

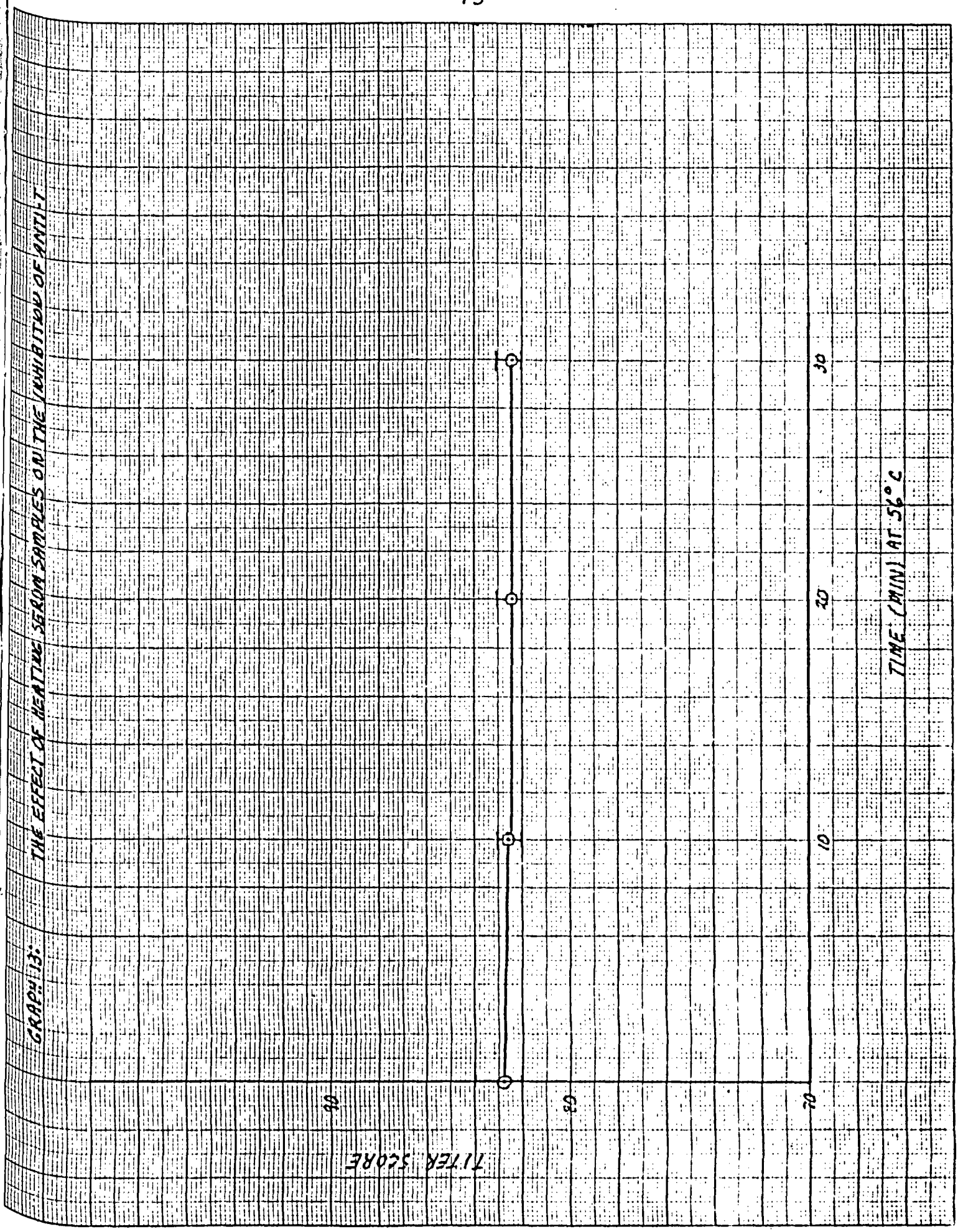
These results demonstrate that the amount of heating used in these experiments to inactivate neuraminidase was not responsiblo for causing th inhibition of anti-T that was observed in neuraminidase treated serum and saliva samples.

The effect of various proteases on T antigen production

The results of experiments in which saliva samples were treated with different proteases are given in Table 22 and in Table 23 the results of experiments in which saliva

GRAPH 14: THE EFFECT OF HEATING SALIVA ON THE INHIBITION OF ANTI-T





were treated with varying amounts of the proteases. Table 24 presents the results of experiments in which saliva samples were treated with proteases for varying time periods.

The data indicate that none of the proteases tested produced inhibition of anti-T. For all of the saliva samples tested the control value for the untreated Samples were essentially the same as the values for the samples that were treated with 50 to 300 Å of the proteases. Neuraminidase treatment of the samples caused inhibition of anti-T and this demonstrated that the T-antigen could be produced in these samples.

Table 22: The effect of treatment of saliva samples with proteases on the amount of inhibition of anti-T present in the sample.

Sample number	Untreated	Neuraminidase	Ficin	Bromelin	Papain	Trypsin
1	81	40	81	81	81	77
2	66	32	66	66	68	64
3	76	43	83	78	81	83
4	72	42	77	74	77	79
5	69	32	65	69	68	69
6	74	28	71	76	75	71
7	66	33	62	67	67	61
8	74	35	71	72	74	69
9	60	33	62	59	61	59
10	61	23	65	66	61	66

Table 23: The effect of various concentration of protease on saliva on the inhibition ov anti-T

Sample	Amount of protease (λ)	Amount of saline (λ)	Ficin	Papain	Bromelin	Trypsin
1.	50	350	60	55	56	64
	100	300	56	54	54	59
	200	200	57	54	53	57
	300	100	57	54	53	57
	400	0	59	54	54	58
2	50	350	62	62	62	61
	100	300	63	63	64	63
	200	200	61	62	62	64
	300	100	63	64	58	62
	400	0	58	62	62	58

Table 24: The effect of incubation time on the amount of inhibition of anti-T caused by two concentrations of proteases.

Time (min)	amount of protease (λ)	amount of saline (λ)	Papain	Ficin	Trypsin	Bromelin
60	50	350	62	62	63	60
120	50	350	62	63	58	62
180	50	350	64	61	62	61
240	50	350	62	62	65	62
60	400	0	62	62	62	63
120	400	0	63	62	64	56
180	400	0	61	60	61	62
240	400	0	62	61	63	61

Tests on protein free filtrates

Neuraminidase treated and untreated protein free filtrates and serum and saliva were tested for their ability to inhibit anti-T. Those results are given in Tables 25 and 26. The data indicated that neuraminidase treated and untreated protein free filtrates of saliva and serum had little if any ability to inhibit anti-T.

The small amounts of inhbition caused by treating protein free filtrates with neuraminidase as well as the small amount of inhibition caused by the protein free filtrates of untreated saliva samples are probably due to the variables involved in HIA used to measure the amount of inhibition produced. These values were usually calculated from differences of only one or two in the titer scores of the untreated and neuraminidase treated samples.

These results show that the inhibition of A. hypogea produced by treating serum or saliva with neuraminidase is is some way dependent upon the presence of a large molecule, probably a protein or lipid molecule. If these large molecules are removed then the treatment with neuraminidase no longer has the ability to produce inhibition of anti-T.

Results of Immunodiffusion studies

The T antigen in saliva could never be demonstrated using immunodiffusion techniques. The only precipitin lines that were ever produced were when sera from rabbits immunized with human saliva were used as the antisera. Whatever

Table 25: Percent inhibition values for saliva samples prior to and after neuraminidase treatment of raw saliva samples and protein free filtrates.

Sample no.	Raw		Protein free filtrates	
	percent inhibition untreated	percent inhibition neuraminidase	percent inhibition untreated	percent inhibition neuraminidase
1	25.7	78.8	-1.4	-1.4
2	28.5	54.0	8.5	1.6
3	35.7	48.9	-1.4	-1.4
4	35.7	48.9	-1.4	0.0
5	2.9	55.9	0.0	1.4

Table 26: Titer scores from the HIA using untreated and neuraminidase treated serum samples as raw samples and as protein free filtrates.

Sample no.	RAW	PROTEIN FREE FILTRATE
	Percent inhibition after neuraminidase	Percent inhibition after neuraminidase
1	48.2	-1.2
2	43.2	-1.2
3	51.8	-1.3
4	71.1	-3.7
5	83.1	3.5
6	54.2	2.4
7	53.0	-2.5
8	50.6	1.2
9	51.8	2.2
10	48.2	0.0
11	31.7	1.2
12	51.8	0.0
13	57.8	0.0
14	53.0	-1.2
15	45.7	1.2

these precipitin lines resulted from they were independent of T antigen, because there was a line of identity formed between neuraminidase treated saliva and untreated saliva and there was no difference in the thickness of the lines. All of the experimental variations that were previously described failed to produce any demonstration of soluble T antigen.

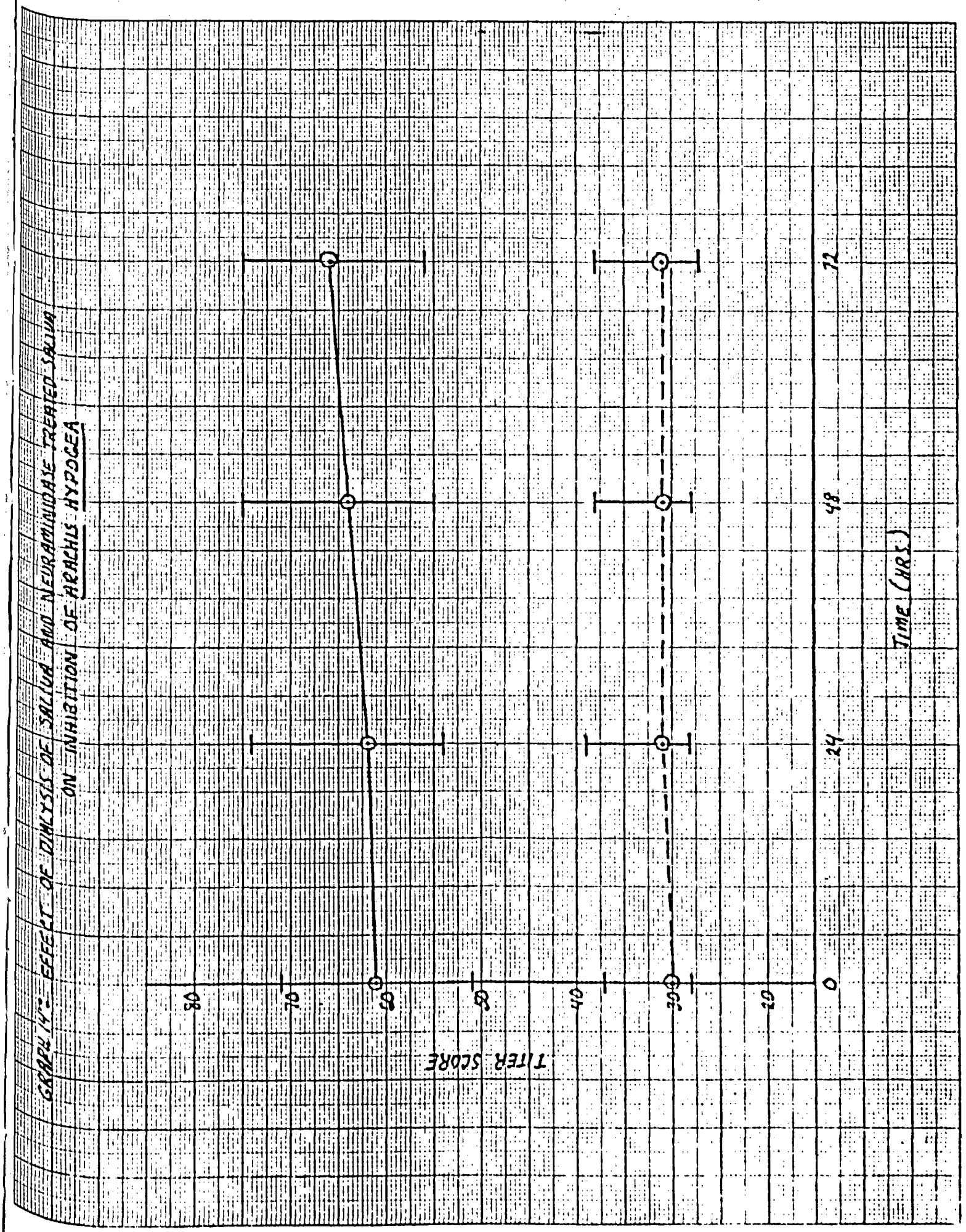
Dialysis studies

Samples of serum and saliva, both neuraminidase treated and untreated, were dialyzed for varying time periods and assayed for inhibitory activity against anti-T. The results of these experiments are shown in Table 27 where they are expressed as the titer score of A. hypogea when tested by the HIA. This data is presented graphically in Graphs 14 and 15.

No signifigant increase or decrease in titer score was obtained following dialysis which indicates that the molecules that are responsible for the inhibition of anti-T cannot be dialyzed. This data also demonstrates that the molecules that cause the inhibition in untreated saliva samples are not dialyzable.

Table 27: The effect of dialysis on the inhibition of anti-T by untreated and neuraminidase treated serum and saliva.

	Normal				Neuraminidase treated				
Sample	0	24	48	72	0	24	48	72	(hours)
Saliva 1	62	61	62	62	28	28	30	28	
Saliva 2	51	54	55	56	37	39	38	38	



GRAPH IV: EFFECT OF DIALYSIS OF SALIVA AND NEURAMINIDASE TREATED SALIVA ON INHIBITION OF ARACHIS HYPOGEA

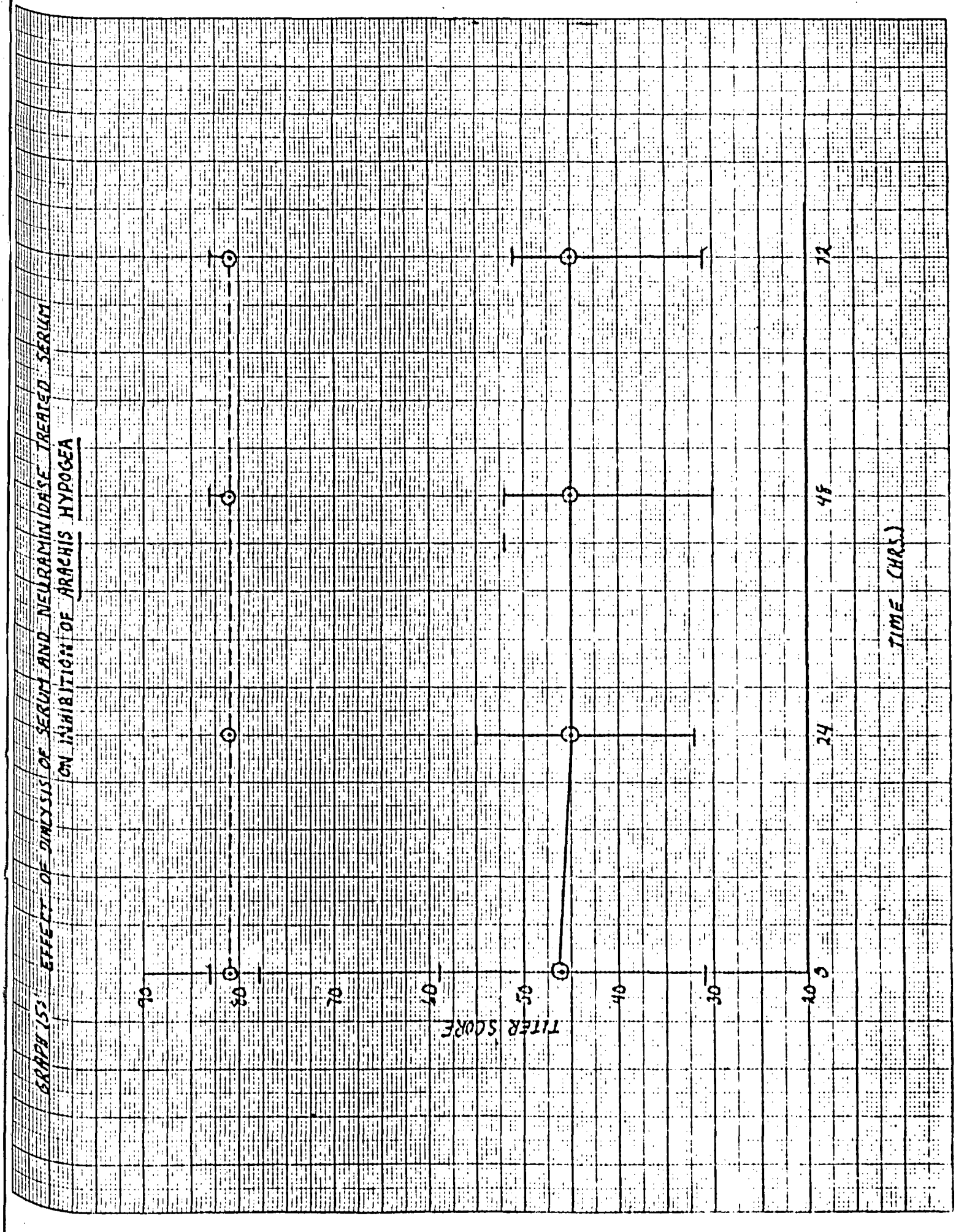


Table 27: (Continued)

Sample	Normal 0	24	48	72	Neuraminidase treated 0	24	48	72 (hours)
Saliva 3	54	54	58	63	30	31	30	30
Saliva 4	66	67	70	74	28	28	28	27
Saliva 5	71	74	75	75	27	28	30	32
Serum 1	81	81	81	82	50	51	52	49
Serum 2	81	81	81	81	31	30	32	31
Serum 3	82	81	81	81	42	43	41	41
Serum 4	83	81	81	83	54	55	52	52
Serum 5	78	81	81	83	54	55	52	52

Inhibition of other anti-T reagents by neuraminidase treated serum and saliva samples

Table 28 shows the results of testing serum and saliva samples, both neuraminidase treated and untreated, against five different anti-T reagents. These results are expressed as the percent inhbition value calculated from HIA. These results show that the inhibition caused by untreated saliva and neuraminidase treated serum and saliva samples can also be demonstrated by other anti-T reagents. The values calculated for the percent inhibition differ markedly from the values calculated from tests using A. hypogea, but this is expected because of the different strengths of the antisera and because of the possibility that they have slightly different specificities and affinities

Table 28: Percent inhibition values for saliva and serum samples after neuraminidase treatment as measured by various anti-T reagents

Saliva sample no. Reagent	1	2	3	4	5
Arachis hypogea	62.9	62.7	68.3	45.3	45.2
Human serum 1	66.7	100.0	76.9	100.0	69.2
Human serum 2	78.3	85.0	81.3	78.3	100.0
Human eluate 1	83.3	80.0	80.0	100.0	61.5
Human eluate 2	71.4	78.3	85.0	68.9	78.9
Rabbit serum 1	80.9	77.1	80.0	90.7	88.0
Rabbit serum 2	80.9	92.5	73.3	75.0	86.7
Rabbit eluate 1	87.9	88.5	100.0	75.0	82.6
Rabbit eluate 2	82.1	88.9	60.9	76.3	69.6

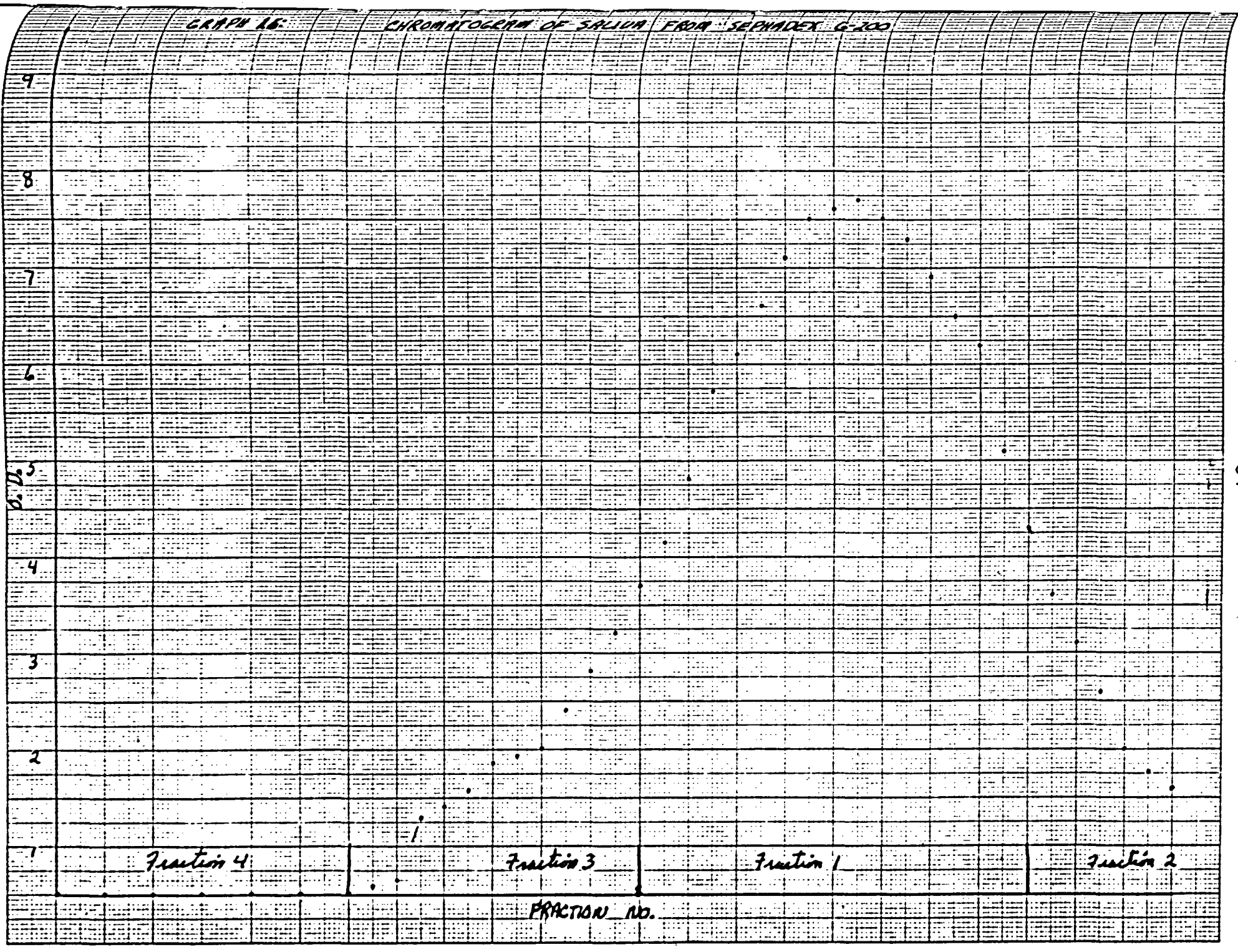
Serum sample no. Reagent	1	2	3	4	5
Arachis hypogea	72.3	39.0	46.4	32.5	65.9
Human serum 1	65.2	78.3	83.3	77.3	86.9
Human serum 2	70.4	100.0	75.0	39.2	100.0
Human eluate 1	89.2	72.4	82.1	83.3	89.6
Human eluate 2	79.5	79.5	86.5	81.5	71.1
Rabbit serum 1	90.1	85.2	83.0	80.4	84.6
Rabbit serum 2	81.6	85.4	85.0	77.0	88.7
Rabbit eluate 1	74.4	78.5	83.7	82.2	76.7
Rabbit eluate 2	65.9	78.7	81.3	72.0	81.6

Results of column chromatography

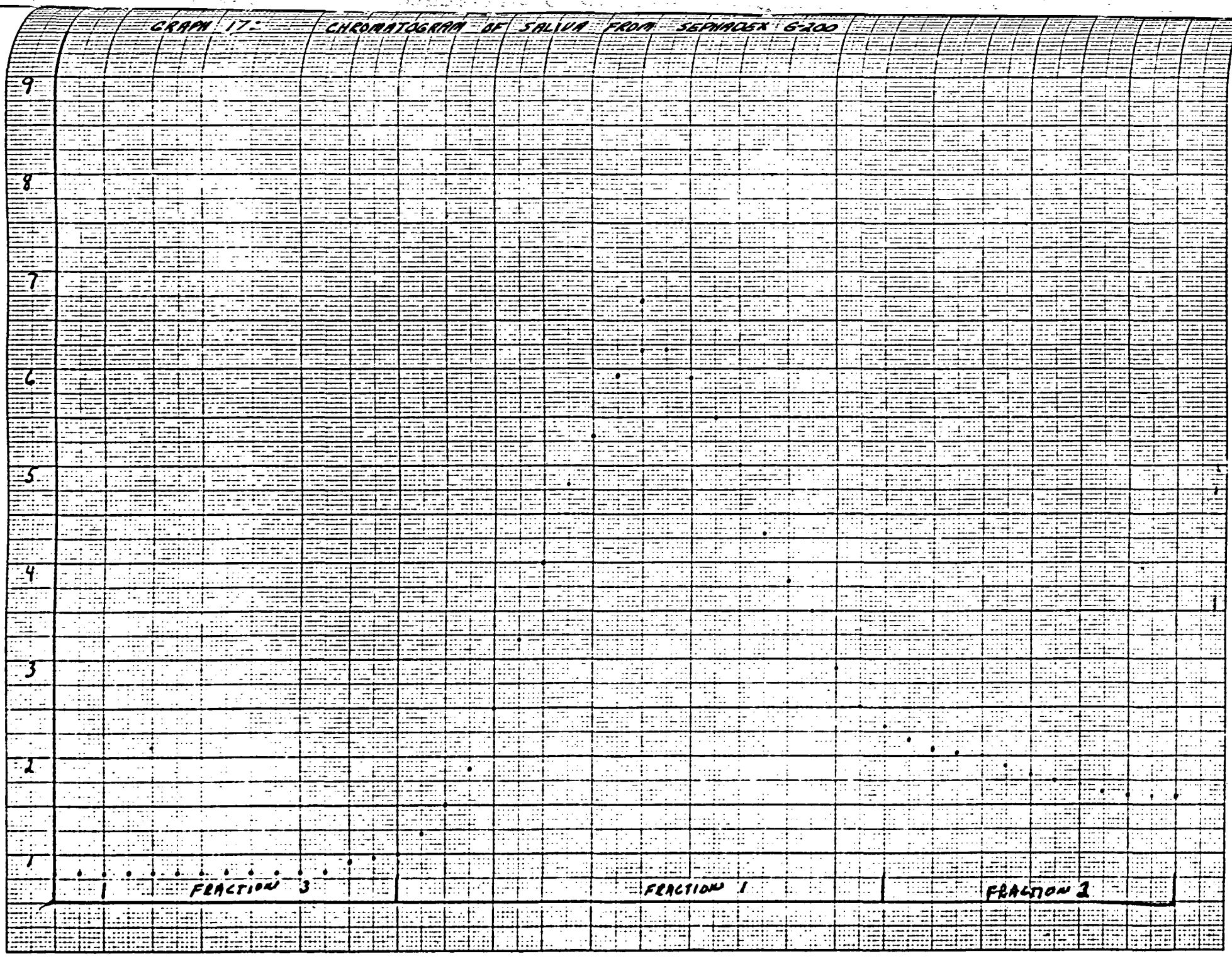
Graphs 16, 17, and 18 show the chromatograms that are obtained when normal saliva is subjected to chromatography on Sephadex G-200. Graph 19 shows the chromatogram obtained when neuraminidase treated saliva is eluted from Sephadex G-200. The chromatograms are obtained by plotting the optical density (O.D.) of the fractions at 280 m against the fraction number. In all cases the saliva samples were eluted as a single protein peak. Table 29 shows the results of HIA performed on various fractions from the column, both untreated and neuraminidase treated. The various designations for the fractions are shown in Graph 16, 17, 18, and 19.

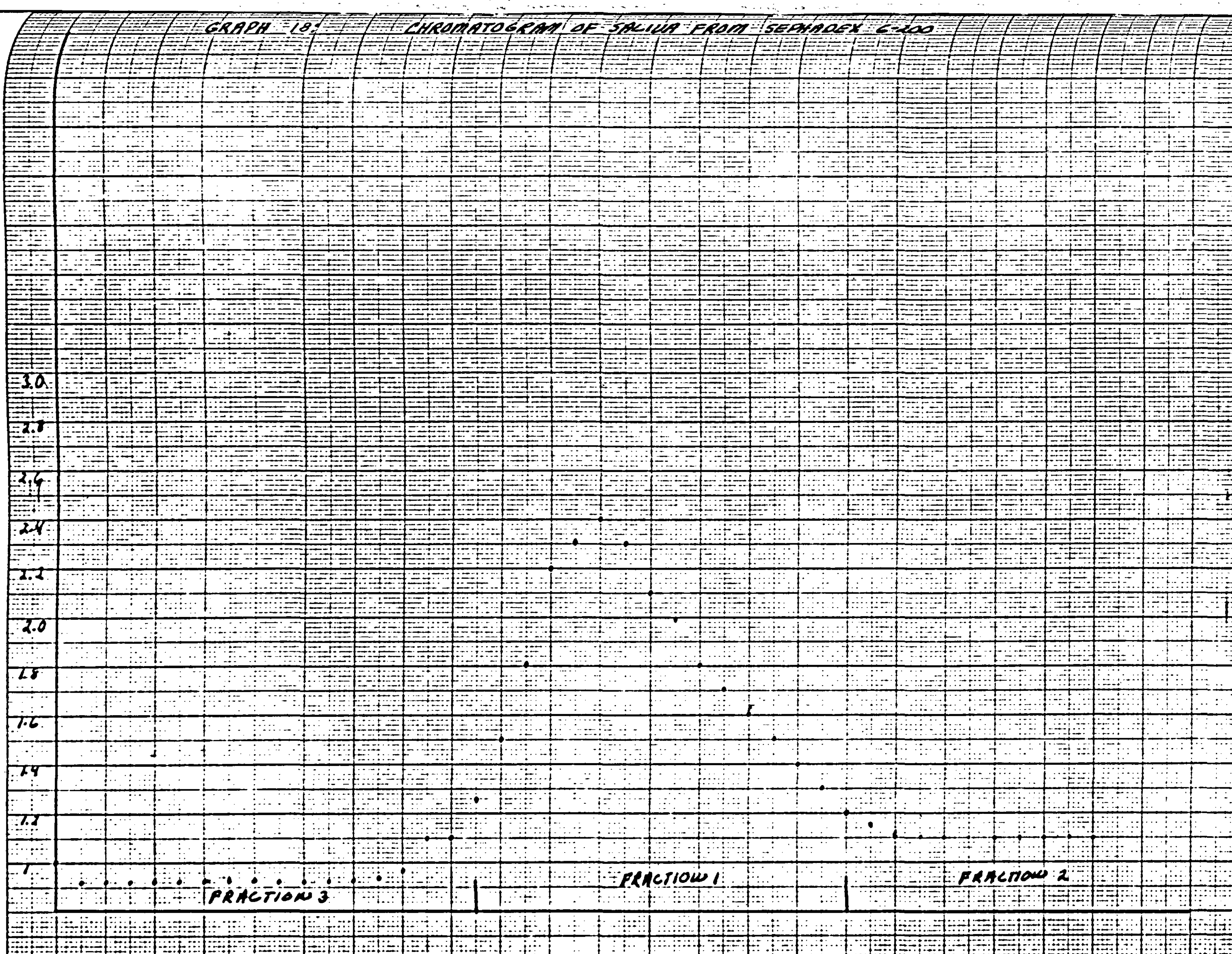
This data shows that the molecules that cause the inhibition of anti-T in untreated saliva are eluted only with the protein peak labeled fraction 1. Fractions collected immediately before and after the protein peak reacted the same in the HIA as the saline controls. The run shown in Graph 19 shows that when neuraminidase treated saliva is separated by Sephadex all inhibitory activity of the sample is found in the protein peak (fraction 1) and not in fractions collected immediately before or after fraction 1. This data also shows that only fractions from the protein peak (fraction 1) will inhibit anti-T after neuraminidase treatment. Neuraminidase treatment of other fractions never resulted in inhibition of anti-T.

Graphs 20 and 21 show the chromatograms of serum ob-



GRAPH 17: CHROMATOGRAM OF SALIVA FROM SEPHADEX G-200





GRAPH 19: CHROMATOGRAM OF NEURAMINIDASE TREATED SALIVA FROM SEPHADEX G-200

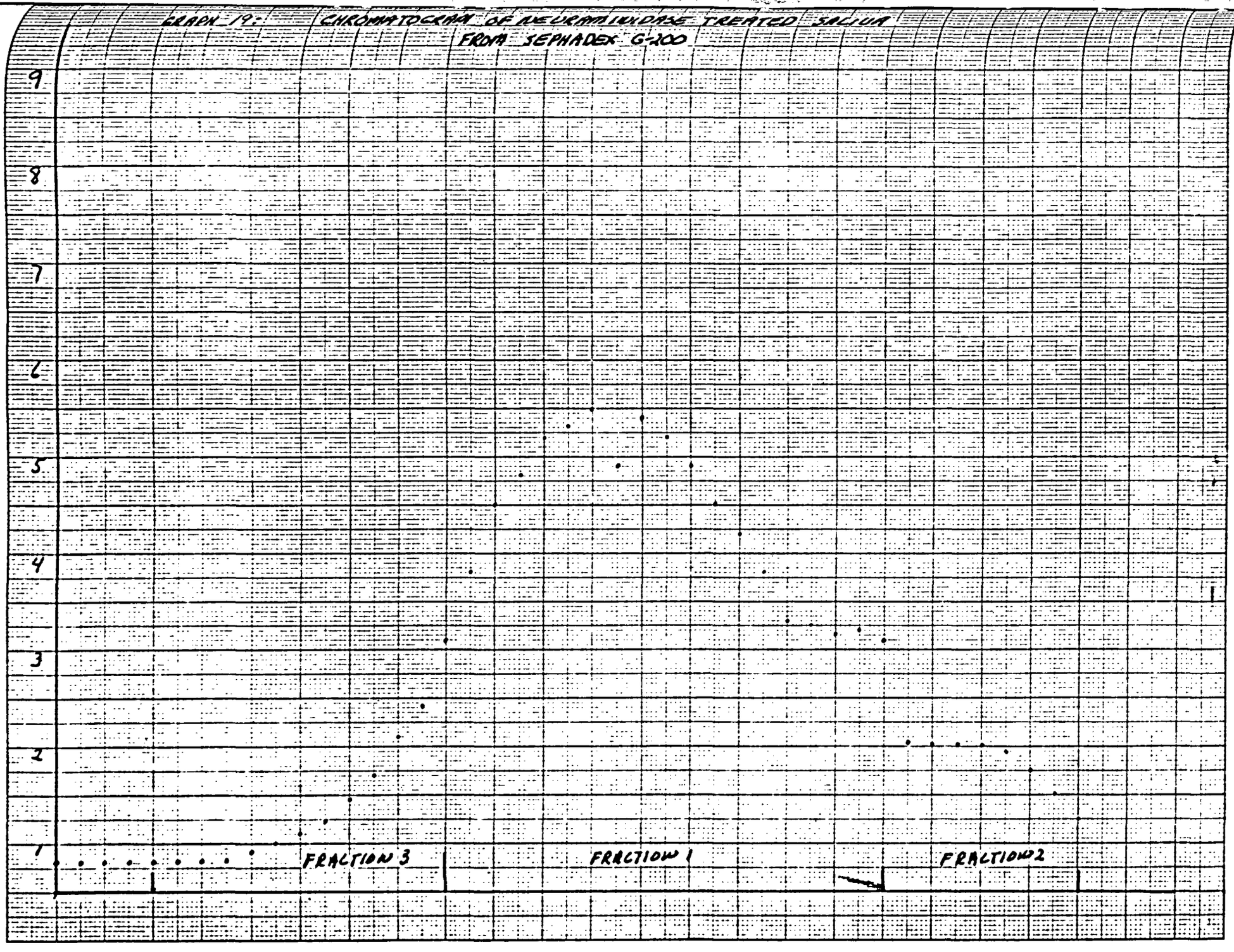


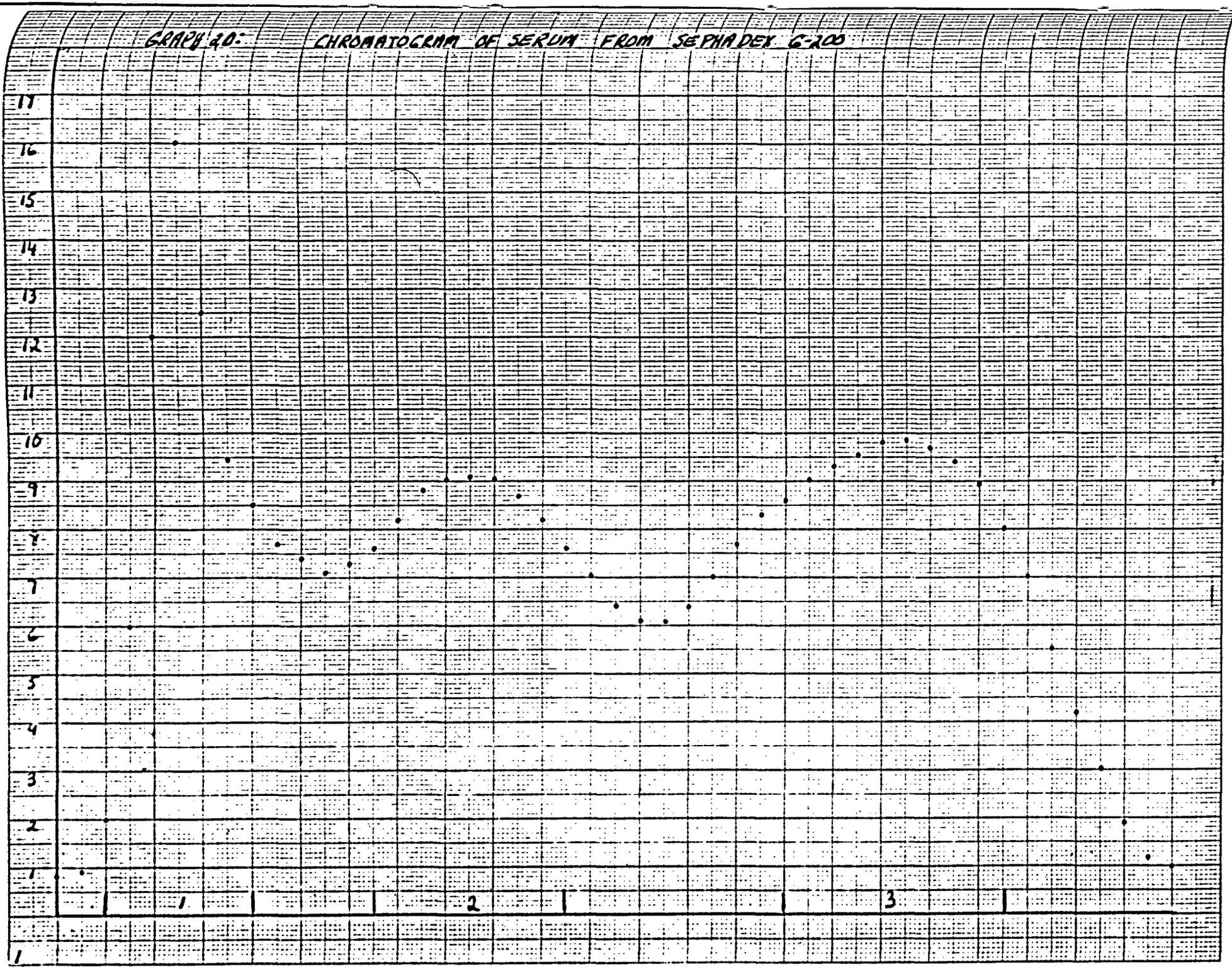
Table 29: The results of HIA performed on the fractions of saliva subjected to column chromatography on Sephadex G-200.

Sample	Neuraminidase treated?	Graph 22	Graph 23	Graph 24	Graph 25
Raw	no	55	63	59	57
	yes	21	41	43	31
Fraction 1	no	43	54	75	25
	yes	19	28	54	25
Fraction 2	no	79	80	80	82
	yes	78	77	79	80
Fraction 3	no	80	82	83	81
	yes	81	83	80	80

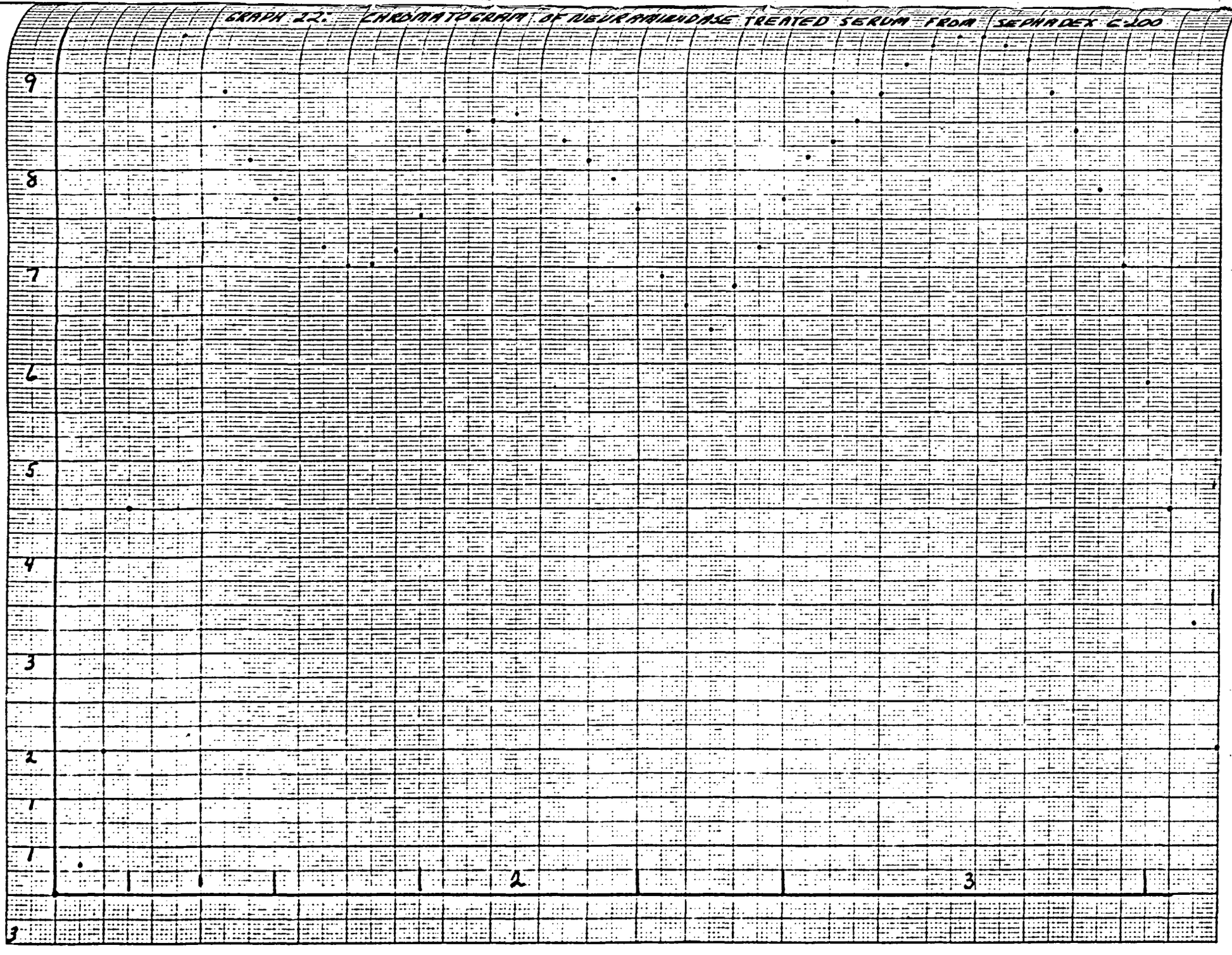
Table 30: The results of HIA performed on the fractions of serum subjected to column chromatography on Sephadex G-200.

Sample	Neuraminidase treated?	Graph 26	Graph 27	Graph 28
Buffer	no	81	82	81
	yes	82	81	83
Fraction 1	no	81	83	31
	yes	22	38	27
Fraction 2	no	81	81	84
	yes	81	82	83
Fraction 3	no	75	81	81
	yes	74	78	80

GRAPH 20: CHROMATOGRAM OF SERUM FROM SEPHADEX G-200



GRAPH 4.1: CHROMATOGRAM OF SERUM FROM SEAMEN 2-200



tained when the serum is subjected to chromatography on Sephadex G-200. Graph 22 shows the results of chromatography of serum that was previously treated with neuraminidase. The results of HIA performed on the various fractions are presented in Table 30.

The data demonstrates that the only fraction of serum that demonstrates any signifigant inhibition of anti-T after neuraminidase treatment is in the fraction labeled fraction 1. The buffer control and the fractions labeled fraction 2 and 3 never produced signifigant inhibition of anti-T after neuraminidase treatment. When neuraminidase treated serum was run (Graph 22) all of the inhibitory activity for anti-T was found in fraction 1. These results indicate that the molecules that cause the inhibition of anti-T are of a homogeneous class with respect to molecular weight and conformation and do not represent a large spectrum of carrier molecules with similar immunodominant carbohydrate groups.

The results of experiments with Beta-galactosidase and galactose oxidase

Saliva samples were treated with Beta-galactosidase and galactose oxidase and tested for their ability to inhibit anti-I. These results are illustrated in Table 31 and Graph 23. As can be seen, increasing the amount of Beta-galactosidase used decreases the amount of inhibition of anti-T caused by the saliva. The data points in the curve show that at first there is a large increase in the titer score for

small additional amounts of enzyme added. Later increases in the concentration of enzyme do not produce a large increase in the titer score and the curve levels off. These data also show that galactose oxidase has no effect in the amount of inhibition of anti-T that is present in the saliva.

The effect of varying concentrations of Beta-galactosidase on the degree of inhibition of anti-T by neuraminidase treated saliva is given in Table 32 and Graph 24. It is seen from this data that the amount of Beta-galactosidase used tho treat the neuraminidase treated saliva at first results in large increases in the titer score and later the amount of increase in titer score/ unit of enzyme added decreases. This data also shows that galactose oxidase has no effect on the T antigen in neuraminidase treated saliva.

The data shown in Table 33 and Graph 25 show the effect of incubation time with Beta-galactosidase and galactose oxidase on the T substance in neuramimidase treated saliva. The titer score of A.hypogea obtained from HIA increases sharply for the first 30 minutes of incubation and then levels off at incubation times greater than 30 minutes. These data demonstrate again that galactose oxidase has no effect on the amount of inhibition of anti-T.

Table 34 and graph 26 demonstrate the effect of different amounts of Beta-galactosidase on neuraminidase treated serum. The results are essentially the same as those observed with neuraminidase treated and untreated saliva

Table 31: The effect of various concentrations of Beta-galactosidase and galactose oxidase on the inhibition of anti-T in untreated samples of saliva.

Enzyme	amount of enzyme (λ)	amount of saline (λ)	titer score
Beta-galactosidase	20	480	57
	50	450	66
	100	400	71
	200	300	73
	300	200	75
	400	100	82
	500	0	84
Galactose oxidase	20	480	59
	50	450	55
	100	400	57
	200	300	56
	300	200	54
	400	100	57
	500	0	56

Table 32: The effect of various concentrations of beta-galactosidase and galactose oxidase on neuraminidase treat- saliva.

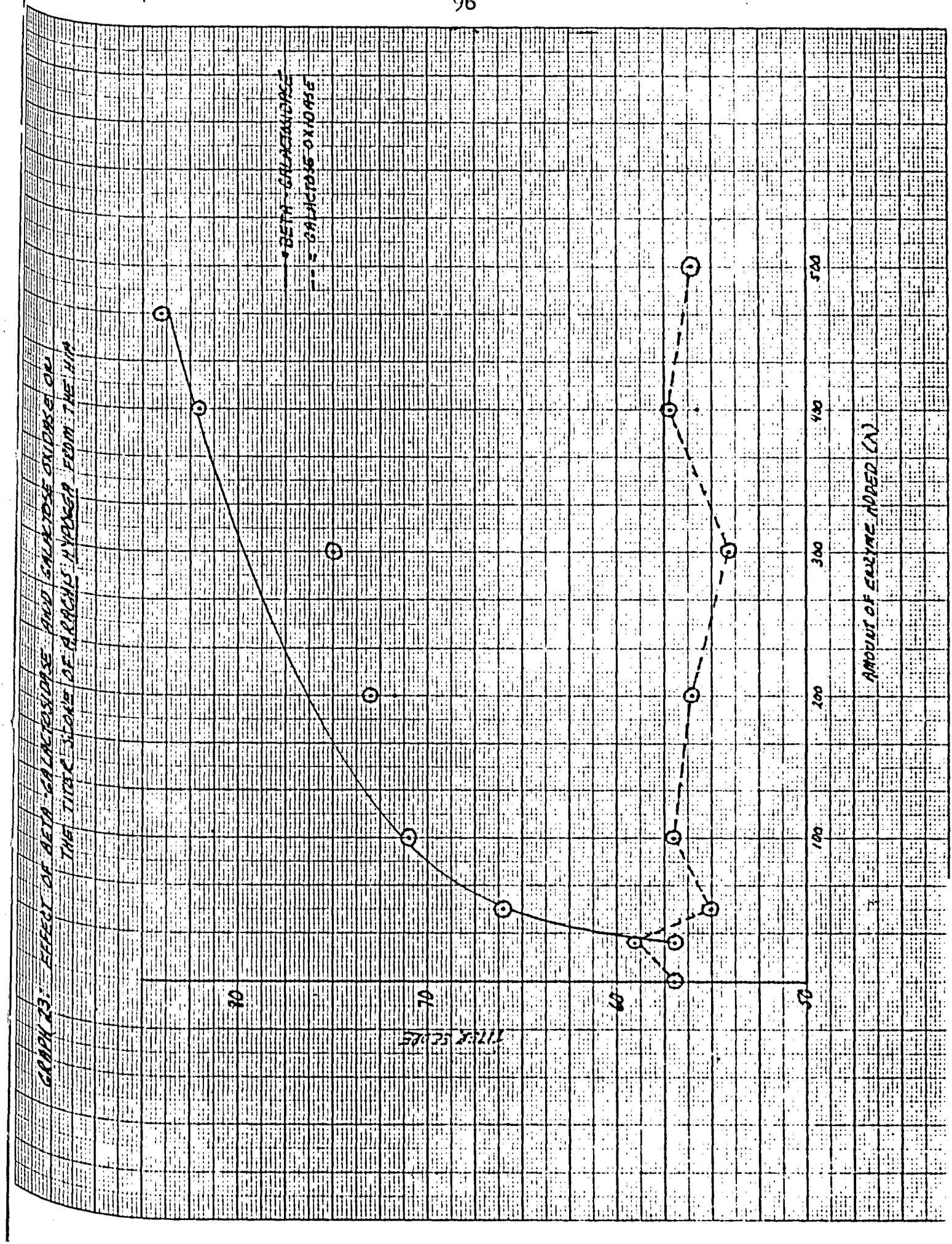
Enzyme	Amount enzyme (λ)	Amount saline (λ)	Sample 1	Sample 2	Sample 3	Sample 4
Beta-galactos-idase	20	380	40	38	28	40
	50	350	44	52	30	48
	100	300	52	61	39	54
	200	200	54	67	49	60
	400	0	62	78	69	73

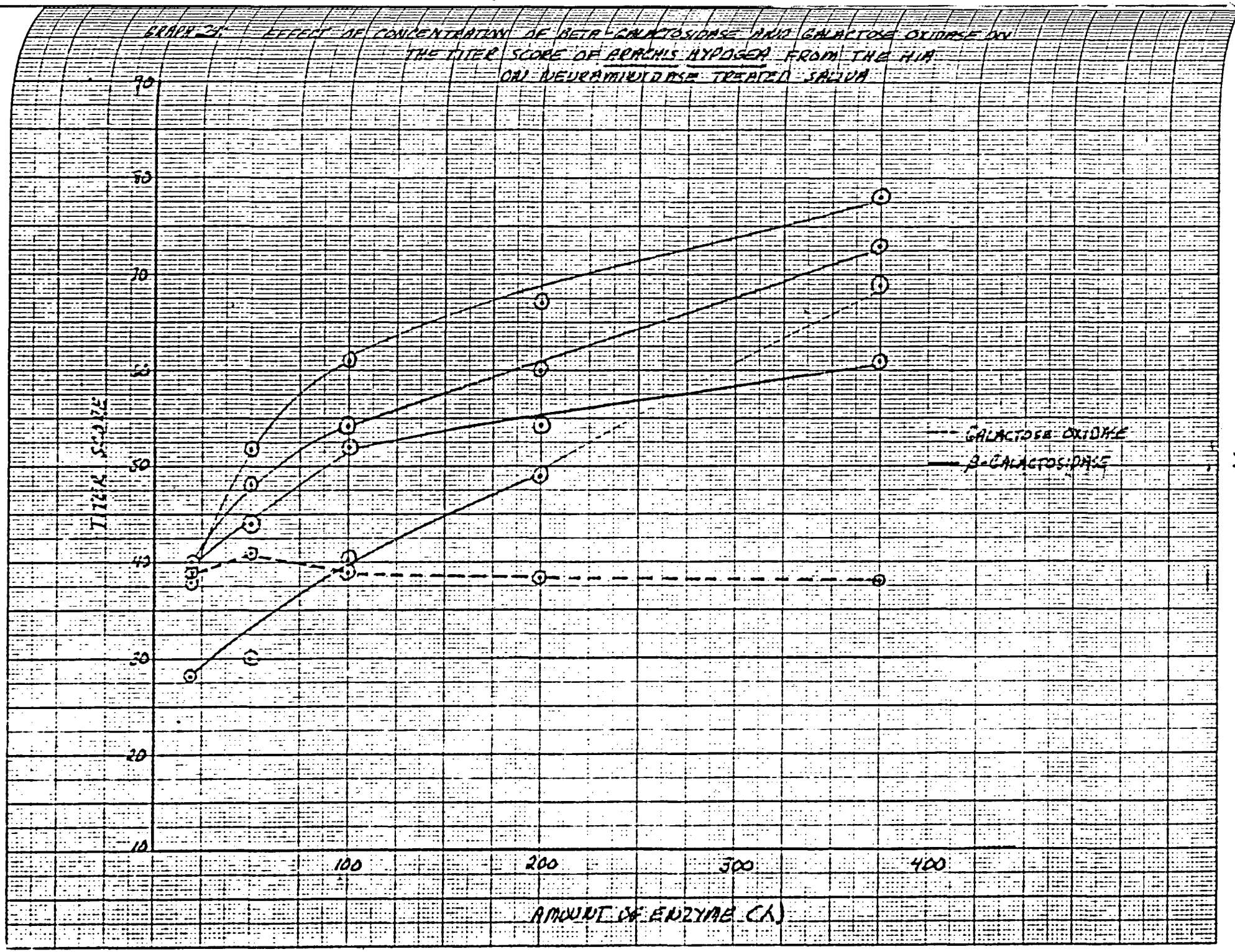
Table 32: (Continued)

Enzyme	Amount of Enzyme (λ)	Amount of saline (λ)	Titer score
Galactose oxidase	20	480	39
	50	450	41
	100	400	40
	200	300	38
	300	200	38
	400	100	37
	500	0	41

Table 33: The effect of various incubation times on the action of galactose oxidase and beta-galactosidase, as reflected by the titer score from HIA using Arachis hypogea.

Incubation time (min)	Beta-galactosidase	Galactose oxidase
5	37	38
15	40	39
30	49	42
45	51	38
60	54	40
90	54	37
120	56	40





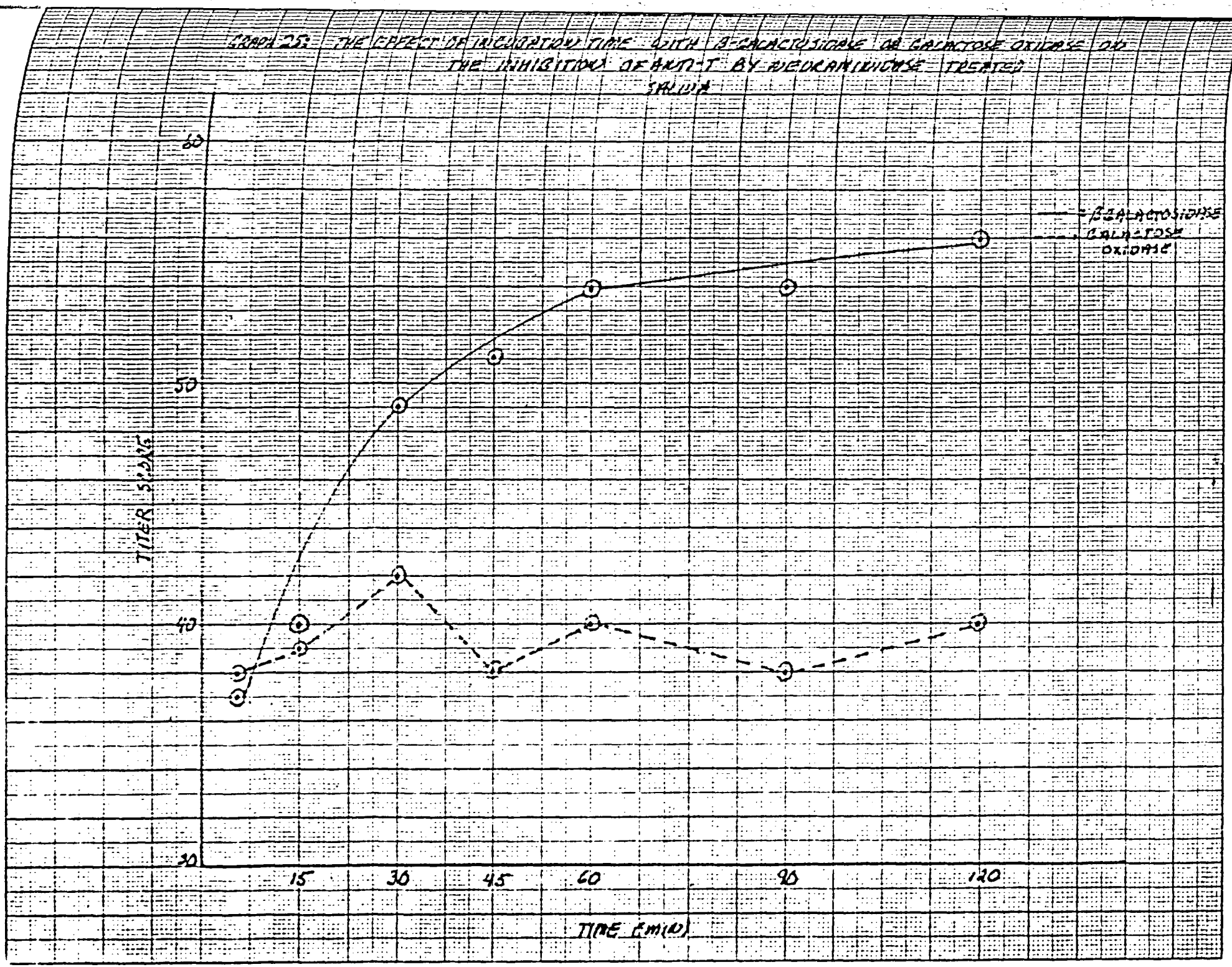
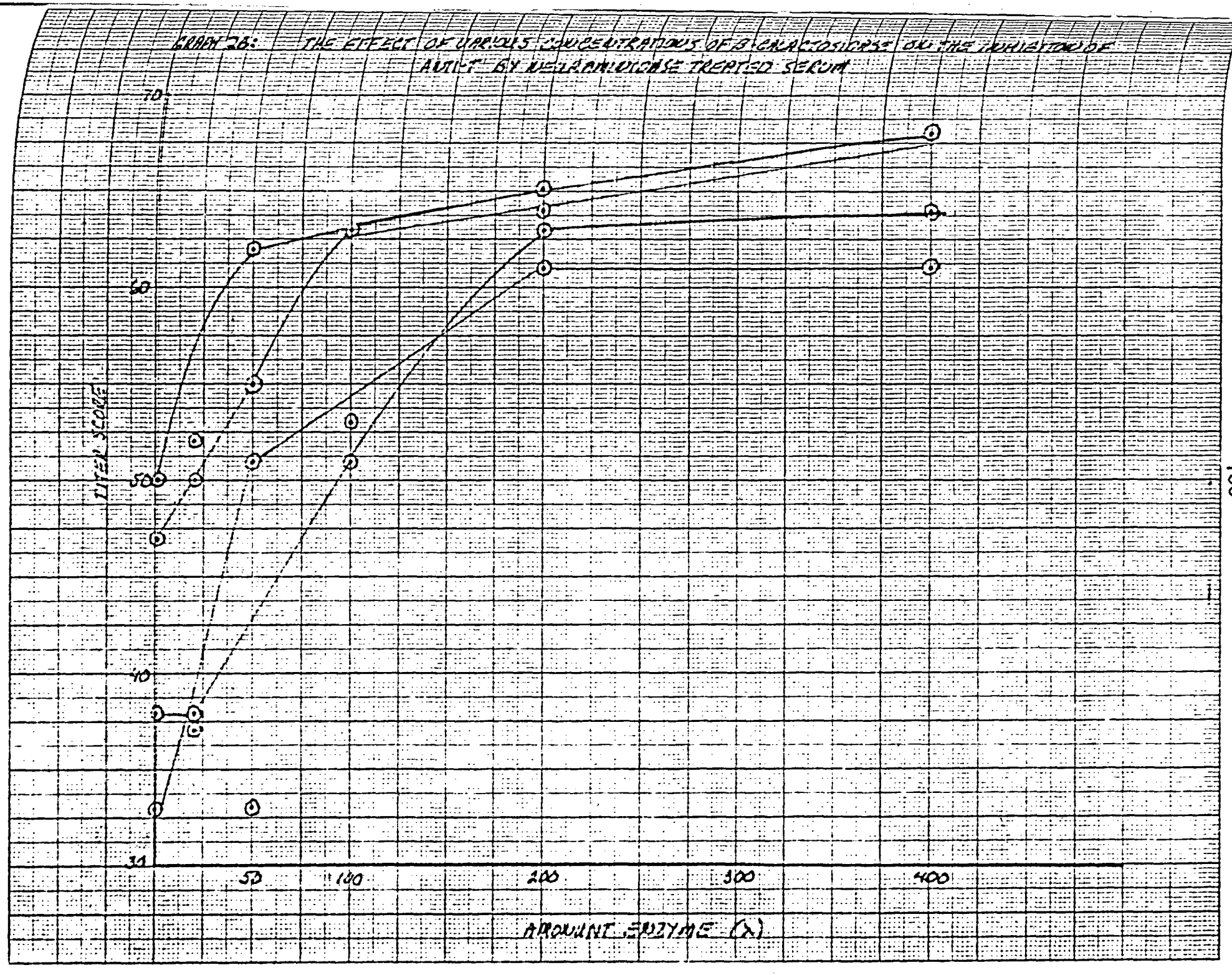


Table 34: The effect of various concentrations of Beta-galactosidase on the inhibition of anti-T by neuraminidase treated serum.

Amount of enzyme (λ)	Amount of saline (λ)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0	400	38	50	33	47	
20	380	38	52	37	50	
50	350	33	62	51	55	
100	300	51	63	53	63	
200	200	63	65	61	64	
400	0	64	68	61	68	

Table 35: The effect of neuraminidase treatment of saliva on the inhibition of A. hypogea and V. graminea

Sample no.	Percent inhibition (A. hypogea)	Percent inhibition (V. graminea)
1	40.3	36.7
2	28.9	8.8
3	52.9	19.6
4	34.2	16.7
5	73.3	61.2
6	22.4	42.0
7	26.3	5.6
8	35.8	27.5
9	62.8	16.3
10	30.4	26.8



samples. At first gradually increasing amounts of enzyme resulted in marked increases in the destruction of T substance as reflected by increases in titer scores. With further addition of enzyme the inrease in titer score levels off (at enzyme concentrations of 100 to 200 λ/cc serum) and remained at approximately the same level during further increases in enzyme concentration.

Results of experiments with Vicea graminea

The results of inhibition studies with neuraminidase treated saliva and V.graminea are given in Table 35. This data shows that treatment of saliva samples with neuraminidase always causes increases in the amount of inhibition of V. graminea and A. hypogea. The amount of inhibition of V. graminea caused by neuraminidase treatment of saliva rangedfrom 8.8% to 61.2% with a mean percent inhibition value of 40.7%. A striking difference between the inhibition of A. hypogea and V. graminea is that untreated saliva samples always inhibited the anti-T lectin to some degree, but never inhibited the V. graminea lectin.

Table 36 shows the results of testing untreated and neuraminidase treated saliva samples by HIA against the V.graminea lectin using both normal N cells and neuraminidase treated N cells. These data confirm the previous data seen in Table 34 that demonstrates that all saliva samples will inhibit V. graminea lectin after treatment with neuraminidase. This data also shows that performing the HIA using neuramin-

idase treated cells or normal cells has no effect on the results of the inhibition studies. The mean percent inhibition using neuraminidase treated cells is 30.8% while the same samples tested with normal cells is 34.1%. The inhibition of V. graminea using untreated saliva showed a mean titer score 48.8 using normal cells and showed a mean titer score of 49.2 with neuraminidase treated cells. The saline control with both neuraminidase treated cells and with normal cells was 52.

Table 37 and Graph 27 presents data from experiments using various concentrations of neuraminidase and measuring the resulting inhibition of V. graminea by HIA. These data show that initially small increases in the amount of neuraminidase cause large decreases in the titer score up to doses of neuraminidase between 100 and 200 Å/cc. As the dose of neuraminidase is further increased there is a slight increase in the amount of inhibition.

Table 38 demonstrates the results of testing 20 serum samples with neuraminidase and measuring the amount of inhibition of V. graminea Ten of these samples were tested with A. hypogea as a comparison. As seen in the results in Table 38 neuraminidase treated serum samples always inhibit V. graminea to some degree. The series tested had a mean percent inhibition of 40.4%. The quantity of inhibition measured by V. graminea is not related to the amount of inhibition of A. hypogea. The untreated serum samples never showed any inhibition of V. graminea.

Table 36: A comparison of percent inhibition values for neuraminidase treated saliva samples using the HIA and Vicea graminea lectin and neuraminidase or normal red cells.

Sample no.	Percent inhibition (Normal cells)	Percent inhibition (Neuraminidase treated cells)
1	47.9	48.9
2	3.6	5.6
3	10.6	8.2
4	54.2	38.0
5	54.3	53.2

Table 37: The effect of various concentrations of neuraminidase on the amount of inhibition of Vicea graminea produced in saliva.

Amount of Enzyme(λ)	Amount of Saline(λ)	Sample 1	Sample 2	Sample 3
20	380	21	47	44
50	350	17	38	36
100	300	18	35	27
200	200	14	42	28
400	0	14	37	29

Table 38: The effect of neuraminidase treatment on the inhibition of Vicea graminea by serum.

Sample no.	Percent inhibition (Vicea graminea)	Percent inhibition (Arachis hypogea)
1	65.1	19.2
2	50.6	57.7
3	43.4	32.0
4	66.3	46.2
5	59.0	38.5
6	53.0	59.3

Table 38:(Continued)

Sample no.	Percent inhibition (Vicea graminea)	Percent inhibition (Arachis hypogea)
7	60.2	38.5
8	48.2	50.0
9	39.8	12.0
10	53.0	50.0
11	48.6	NT
12	47.1	NT
13	26.5	NT
14	25.7	NT
15	51.4	NT
16	23.5	NT
17	54.3	NT
18	51.4	NT
19	47.1	NT
20	28.6	NT

NT = not tested

Table 39: The effect of Beta-galactosidase on the inhibition of Vicea graminea by neuraminidase treated serum and saliva.

Sample no.	Percent inhibition (Neuraminidase treated)	Percent inhibition (Beta-galactosidase treated)
Serum 1	48.6	20.0
2	47.1	11.8
3	26.5	17.6
4	25.7	0.0
5	51.4	11.4
6	23.5	11.8

Table 39: (Continued)

Sample no.	Percent inhibition (Neuraminidase treated)	Percent inhibition (Beta-galactosidase treated)
7	54.3	11.4
8	51.4	25.7
9	47.1	8.8
10	28.6	20.0
Saliva 1	37.1	17.1
2	25.7	14.3
3	48.5	6.1
4	31.4	5.7
5	26.5	2.9
6	25.7	0.0

GRAPH 7: THE EFFECT OF CONCENTRATION OF MERCURIC CHLORIDE ON THE INHIBITION OF UREA (2%) HYDROLYSED BY SWAZI BEAN SAMPLES

The effect of Beta-galactosidase on neuraminidase induced inhibition of V. graminea in serum and saliva

Table 39 shows the results of treating serum and saliva samples with neuraminidase and testin them by HIA using V. graminea lectin as the antiserum, and then treating the samples with Beta-galactosidase and assaying the amount of inhibition of V. graminea again. These data show that the inhibition of V. graminea produced by neuraminidase treatment of serum and saliva is partially destroyed by Beta-galactosidase. The mean inhibition of the neuraminidase treated serum samples is 40.4% but when treated with Beta-galactosidase the mean percent inhibition is decreased to 12.9%. The series of saliva samples tested showed similar results. The mean percent inhibition of the saliva samples is 38.9% and after treatment with Beta-galactosidase it is reduced to 9.2%.

These results demonstrate that Beta-galactosidase has the ability to destroy the antigen that is produced in saliva and serum by neuraminidase treatment and is recognized by V. graminea.

DISCUSSION

The results of the experiments that measure the inhibition of anti-T by various body fluids after treatment with neuraminidase show that there is a molecule or population of molecules present in all serum and saliva samples that have the ability to inhibit anti-T reagents afterthey are treated with neuraminidase. Saliva has molecules present in a form that can inhibit anti-T to varying degrees before neuraminidase treatment , but none of the serum samples tested inhibited anti-T before neuraminidase treatment. This is expected, because all normal adult sera contain anti-T and if the molecules present in serum could inhibit anti-T then the antibodies would be neutralized.

All saliva samples tested except one and all serum samples showed increased inhibitory activity towards anti-T after neuraminidase treatment. This supports the hypothesis that there is a correlation between the red cell cryptantigen, T, and the T antigen demonstrated by HIA in serum and saliva. The T antigen is present on all red cells, regardless of expression of other blood group antigens, and the T antigen in secretions is apparently found in the saliva and serum of all people, regardless of other genetic differences. The one saliva sample tested that showed no further inhibition after neuraminidase treatment showed a large degree of inhibition before any treatment (82.9%). Because the sample was retest-

ed and gave the same results technical error is unlikely. The possibility exists that the donor's saliva had already been T-activated *in vivo* by bacterially produced neuraminidase. This explanation fits with the data from dosage studies of neuraminidase on saliva that showed that after a certain point increasing the amount of neuraminidase causes no further increase in the amount of inhibition of anti-T. Diplococcus and some diptheroid bacilli are fairly common organisms in the normal flora of the mouth, and both of these organisms produce neuraminidase (8). This donor may have already had a large amount of neuraminidase in his mouth which caused his saliva to be neuraminidase treated to a point where further addition of neuraminidase caused no further increase in the amount of T antigen. The red cells from this donor were easily T-activated to the same extent as controls. Saliva samples from this donor were subsequently tested many times and never again failed to produce inhibition after neuraminidase treatment, which also supports the hypothesis that the original sample was maximally treated with neuraminidase *in vivo*. This concept could also explains why untreated saliva samples always inhibit anti-T to some extent and why they do so to varying degrees while serum never does. It is possible that normal flora in the mouth produce neuraminidase which removes sialic acid from the molecules that carry the T antigen and expose it.

Other data from the experiments point towards the fact

that there are molecules present in saliva and serum that carry T antigenic determinants that are exposed by neuraminidase and that the results are not the result of some experimental anomaly. Both of the populations studied for the amount of inhibition of anti-T after neuraminidase treatment of serum and saliva and the population studied for the amount of inhibition of anti-T in untreated saliva samples showed normal distribution patterns. The mean and nedian of each series tested were very close, and when data was plotted on graphs as the intervals of percent inhibition against the number of individuals in each interval a normal curve results.

The molecules in serum and saliva samples that have T specificity may bear no genetic relationship to the T antigen on red cells, indeed the genetic control of the T antigen is not known. The fact that the T antigen of red cells is a simple carbohydrate, galactose (47), makes it possible that there are oligosaccharide molecules in all sera and saliva that have a terminal NANA molecule and a subterminal galactose molecule but that they are produced by totally different glycosyl transferases which are specified by different genes. The genetics of the production of these molecules would be very difficult to study.

Studies done on the amount of inhibition of anti-T produced by neuraminidase treatment of samples from the same donors, collected at the same time of day, and at the same time after food ingestion at intervals of one two and three

weeks showed a high degree of variability in the amount of inhibition present in untreated saliva samples and in the amount of inhibition produced by neuraminidase treatment. This variation could also be the result of the amount of neuraminidase present in the mouth of the donor. The amount of in vivo pretreatment with neuraminidase would effect the amount of inhibition seen in the baseline sample, the amount of inhibition produced by neuraminidase, and the calculated percent inhibition. This would account for the variability of the per cent inhibition values seen for one donor over a period of time. The variability could also be due to a fluctuation in the amount of T antigen present in the sample. Many constituents of body fluids show a marked variation in the concentration that they are present when measured over a period of time. The variability of the amount of T substance in saliva and in neuraminidase treated serum or saliva could be due to either of these causes or to a combination of both.

In the experiments testing the effect of neuraminidase treatment on serum and saliva in relation to the degree of inhibition of anti-T there were many variables that had to be examined individually in some detail, to insure that the observed increase in the amount of inhibition of anti-T after neuraminidase treatment actually was due to the enzymatic activity of neuraminidase and then the specific inhibition of anti-T and not the result of one of the experimental con-

ditions.

The factors that played a role in the experimental procedure that were examined were:

1. The effect of heat on neuraminidase activity.
2. The effect of heating serum and saliva on the inhibition of A. hypogea.
3. The effect of neuraminidase on A. hypogea

Three other factors that were unrelated to the experimental variables, but which were important to determining whether or not the increased inhibition observed after neuraminidase treatment was actually due to the specific enzymatic activity of neuraminidase were:

1. The effect of concentration of neuraminidase and incubation time on the amount of inhibition of anti-T.
2. The effect of neuraminidase treatment of serum and saliva on the inhibition of other anti-T reagents.
3. The effect of treating saliva with various proteases on the ihhibition of anti-T.

In all of the experiments with neuraminidase treatment of serum or saliva, the neuraminidase was inactivated before the HIA was performed or before other enzymes were used. The neuraminidase in saliva was inactivated by placing the sample in a boiling water bath for ten minutes, and the neuraminidase in serum samples was inactivated by placing them in a 56C water bath for 30 minutes to avoid excessive denaturation of serum proteins. It was critical that the neuraminidase be inactivated, because if it was still active when the

the sample was tested by HIA it could possibly inactivate the lectin used in the assay, or it could act on molecules in the lectin preparation and neutralize the serological activity, or it could alter the degree of T-activation of the red cells used as indicator cells. Any of these effects would be highly undesirable, so it was necessary to be certain that the inactivation process actually did destroy the activity of neuraminidase.

To assay neuraminidase activity, its ability to T-activate red cells was used as a criterion. The red cells were tested with a serial dilution of A. hypogea to measure the degree of T-activation produced by the neuraminidase. As shown in the results neuraminidase activity is relatively stable for long periods at 37C (a minimum of eight hours). When incubated at 56C the enzymatic activity falls sharply and after 25 minutes its ability to T-activate red cells is lost. The effect of temperatures of 100C is more dramatic and after only two minutes the neuraminidase activity is completely destroyed.

These experiments show that the activity of neuraminidase is rapidly heat labile at temperatures of 56C or higher. These experiments demonstrate that the methods used to inactivate neuraminidase in these experiments were adequate to destroy its activity and therefore the variables that could be caused by the enzymatic activity of neuraminidase in HIA or studies with other enzymes were eliminated.

The second control experiment tested the effect of heating saliva and serum samples in the absence of neuraminidase to determine if hetaing alone caused any alteration in any of their constituents into a form in which they could inhibit anti-T. This was important to investigate, because all serum and saliva samples were heated in order to inactivate the enzymes used in the experiments. If heating caused any of the component molecules to be altered so that they would inhibit anti-T then the results of HIA performed after heating would show inhibition which would be incorrectly attributed to neuraminidase. It is possible, though unlikely that heating at 56C for 30 minutes or at 100C for ten minutes could cause the oligosaccharide components of the sample to be partially hydrolyzed so that some of the terminal NANA molecules would be removed and inhibition of anti-T would result. Conditions that cause hydrolysis are usually more severe, such as heating at 100C for eight hours at low pH values (25).

The results of heating experiments demonstrated that heating saliva samples at 100C for periods of up to 15 minutes caused no signifigant inhibition of anti-T. The percent inhibition values for the samples remained essentially the same, which also shows that whatever causes the inhibition of anti-T in untreated saliva samples is relatively heat stable and is not effected by the temperatures used in these

experiments.

The results of heating serum samples at 56C for periods up to 30 minutes showed essentially no change in the percent inhibition of anti-T. This demonstrates that the amount of inhibition caused by the serum and saliva samples after treatment with neuraminidase could not be caused by the amount of heating used to inactivate neuraminidase.

The third experimental variable that had to be investigated was whether the neuraminidase added to the test system had any inhibitory effect on A. hypogea lectin used in HIA. This seems to be a remote possibility, but the immunodominant group of the T antigen is galactose and many enzymes contain carbohydrates as part of their chemical constituents. There is therfore the possibility that the molecules of neuraminidase, independently of enzymatic activity could inhibit anti-T because of exposed galactose molecules.

Three separate preparations of A. hypogea lectin were tested in these experiments. Despite increasing the dosage of neuraminidase to 400 λ/cc of lectin, there was never any inhibition of the lectin when it was tested against T-activated red cells. This indicates that the neuraminidase used in the experiments had no inhibitory capacity toward anti-T.

This experiment also points out that neuraminidase has no ability to destroy the anti-T activity of A. hypogea. It was previously discussed that neuraminidase if not inactivated could possibly inactivate the serologically active mole-

cules of the lectin, or remove sialic acid from molecules in the lectin and inhibit the anti-T activity. These experiments suggest that neuraminidase, even in high concentrations, does not inactivate or inhibit the serological activity of anti-T.

Any inhibition of A. hypogea lectin observed by HIA after treatment with neuraminidase co ld not result from either simple neutralization of the lectin or by inactivation of the lectin by the enzymatic activity of neuraminidase.

All enzyme mediated chemical reactions are dependent upon the concentration of enzyme in the system and on the time that the reaction is exposed to conditions favorable for its activity (25). A valuable indication of whether the inhibition of anti-T observed after saliva and serum were treated with neuraminidase was whether the amount of inhibition produced b neuraminidase was related to the concentration of enzyme used and to the incubation time.

The data from experiments that measured the amount of inhibition produced by various concentrations of neuraminidase when plotted as the concentration of enzyme versus the amount of inhibition produced showed the type of curve that is characteristic of enzymatic reactions. At first, small increases in the amount of enzyme added to the system caused large increases in the amount of inhibition, but as more enzyme was added the increase in the amount of inhibition produced became progressively smaller. The curve from the data from these

experiments is highly suggestive of this type of curve. The minor discrepancies that are apparent are probably the result of the methods used to measure the activity of neuraminidase. Increasing amounts of neuraminidase were added to saliva samples and incubated. The neuraminidase was heat inactivated and the amount of inhibition of anti-T was measured by HIA. So, the amount of neuraminidase activity was measured indirectly. Enzyme studies are most accurate when performed with chemical assays that directly measure the amount of product formed or the amount of decrease in the substrate. The slight discrepancies seen for some of the data points in these experiments can be attributed to the indirect method of measuring the product of the enzymatic activity, rather than an actual deviation from the classical enzymatic reaction curve.

The other parameter measured in these experiments was the effect of incubation time on the amount of inhibition produced by neuraminidase. Normally, enzymatic reactions vary with time in the same manner that they vary with enzyme concentration. In the begining of the curve, samples taken after small time intervals show large increases in the amount of inhibition produced, while samples taken at longer time intervals show little increase in the amount of inhibition produced because the reaction is approaching the maximum amount of product that can be formed. This is exactly

the pattern of the data from these experiments. When the data is plotted on a graph the points do not fit perfectly. This is a function of two factors. First, the measurement of the amount of product formed is an indirect measurement Second, the values for percent inhibition are calculated from baseline values that are obtained by testing the untreated saliva samples by HIA. These values differ slightly due to variables in HIA and these slight shanges in the baseline values cause the percent inhibition values to vary. In order to partially overcome this effect, the graph was also plotted as the reciprocal of the titer score from HIA. This is valid because the changes in the baseline values are small and this method disregarded these changes and still allows the curve to have a positive slope.

These experiments show that the amount of inhibition of anti-T produced as the result of neuraminidase treatment varies with the length of incubation time and with the concentration of enzyme used exactly as enzyme mediated reactions classically should. This data strongly indicates that the inhibition of anti-T in serum and saliva samples is a result of enzymatic activity of neuraminidase.

If the inhibition of anti-T by neuraminidase treated saliva and serum is actually the result of production of T antigen, then this inhibition should also be demonstrable when measured by different types and different preparations of anti-T reagents. To determine if this actually occurs, sal-

iva and serum were treated with neuraminidase and tested by HIA using one example of A. hypogea, two examples of human anti-T serum, two examoles of human anti-T eluates, two examples of rabbit anti-T serum, and two examples of rabbit anti-T eluates. The percent inhibition was calculated from the results of HIA using each different reagent.

As seen in Table 28, the results show that the T antigen in neuraminidase treated serum and saliva previuosly detected by HIA using A. hypogea is also easily demonstrable by using any of the other anti-T reagents tested.

These experiments demonstrate two important facts. First, the inhibition of anti-T from A. hypogea by neuraminidase treated serum and saliva that was previously demonstrated is a valid measurement of what is actually occuring, and not the result of an experimental artifact that is involved with the nature of the A. hypogea lectin. All five of the anti-T reagents clearly showed inhibition when incubated with neuraminidase treated serum or saliva. These experiments also confirm the earlier observation that untreated serum does not inhibit anti-T while untreated saliva samples do. The percent inhibition values calculate for the different reagents vary widely when compared to the values for the sample calculated with a different reagent. This is expected because the percent inhibition value is dependent on the baseline value obtained when the untreated sample is

is tested by HIA, and the baseline values differ widely because of the different strengths of the reagents. Even if the reagents had identical titer scores when tested with T-activated red cells, the percent inhibition values would probably differ because of differences in the affinity of the antibody molecules. The important fact demonstrated here is that the inhibition is seen with all of the reagents tested against untreated saliva samples and neuraminidase treated serum and saliva samples.

The second important fact demonstrated here is that the anti-T reagents prepared from A. hypogea, human serum and eluates, and rabbit serum and eluates all detect the T antigen produced as a result of neuraminidase treatment of serum and saliva. This indicates that the reagents from lectin, human, and rabbit sources all have a similar specificity. This differs from the situation seen in the MN blood group system which is also involved with carbohydrate molecules of NANA and galactose. In this system the specificity of antisera differ markedly. If the anti-T reagents from different sources had markedly different specificites then they would have shown greater variation in the demonstration of soluble T antigen. The fact that eluates prepared from human T-activated red cells should contain only antibodies that react with the T antigen on red cells, and that the eluates still detect the soluble T antigen of serum

and saliva, suggests that the T antigen in fluids and the T antigen on red cells must be identical or very close in structure.

The final factor that was investigated was the specificity of the reaction of saliva and serum with neuraminidase to produce a substance that could inhibit anti-T. A characteristic of enzymatic activity is the specificity of the reactions that they can mediate. Proteases, for example, will usually attack only the peptide bond between two specific amino acids (25). If the neuraminidase is responsible for producing the T antigen in serum or saliva, then other enzymes with other specificities should not produce any inhibition of anti-T.

The four enzymes tested were all proteases, ficin, bromelin, papain, and trypsin. The results of the study showed that none of the enzymes resulted in the inhibition of anti-T by saliva. The samples treated with proteases gave essentially the same score as the untreated control sample when tested by HIA. Each of the samples tested with proteases was also tested with neuraminidase to insure that the saliva samples were capable of inhibiting anti-T, and that the lack of inhibition was not due to the inability of the sample to form T antigen under appropriate conditions. In all of the samples tested only neuraminidase ever caused the inhibition of anti-T.

To insure that the proteases were used in sufficent

quantities samples of saliva were tested with various concentrations of the proteases. These studies demonstrated that even with concentrations as high as 400 λ of enzyme/cc of saliva there was no inhibition produced. These studies showed that the reason saliva treated with proteases does not cause inhibition of anti-T is that the proteases have no enzymatic effect and not that they were not tested in high enough concentrations.

To show that the reason there was no inhibition of anti-T produced by proteases was not due to insufficent incubation time, two different concentrations of proteases were tested at dfferent incubation times up to four hours. In no case, regardless of the protease, the concentration, or the length of incubation time was anti-T inhibited.

These studies indicate that neuraminidase induced inhibition of anti-T is a specific enzymatic reaction that requires neuraminidase. The possibility that the inhibition of anti-T demonstrated in these experiments is produced by some nonspecific experimental variable is virtually eliminated.

When a persons red cells become T-activated it is due to the production at the site of infection by bacteria or viruses of neuraminidase that cleaves NANA from the red cells and exposes the cryptantigen T. The process is reversible and disappears when the infection is controlled (44). One of

the hallmarks of any type of polyagglutination is the absence from the sera of the antibody that detects the particular antigen that is causing the polyagglutination. Several theories have been proposed to account for the the absence of these antibodies that are found in the sera of virtually all normal adults. The theories include absorption (11), immune tolerance (1), and immune paralysis (2).

In view of the finding that neuraminidase treatment of serum results in the production of soluble T antigen, neutralization of the anti-T is another possible explanation for the absence of anti-T from the serum of people whose cell's are T-activated. The neuraminidase produced at the site of infection could act on serum to produce a soluble T antigen that could neutralize the naturally occuring anti-T. To test this hypothesis, the sera of 25 normal adults were tested for the presence of anti-T, treated with neuraminidase and retested for anti-T activity. All of the sera tested either lost all of their anti-T or the anti-T was signifigantly weakened. This indicated that the neuraminidase did cause neutralization of the anti-T.

To insure that neuraminidase did not simply inactivate the IgM anti-T antibodies three examples of anti-D, shown to be all IgG and three examples of anti-M, shown to be all IgM, were titrated and then treated with neuraminidase and retested. The neuraminidase produced no inhibition of any of the

the blood group antibodies, which shows that neuraminidase has no inhibitory activity on antibody molecules, and that this explanation cannot be used to explain what happens to cause the disappearance of anti-T when sera are treated with neuraminidase.

These experiments demonstrate that the probable explanation of the disappearance of anti-T from the serum of people whose red cells are T-activated is at least partially due to simple inhibition of the anti-T due to the production of T antigen in a soluble form in neuraminidase treated serum. The actual concentration of neuraminidase in the serum of people whose red cells are T-activated is unknown, but even very low levels circulating in the serum would have the opportunity to produce a substantial amount of T antigen because of the good stability of the enzyme at 37C.

This experiment also points out the necessity for carefully wahing red cells when testing them for polyagglutination. If red cells are weakly T-activated and are being tested with anti-T in a suspension of autologus serum, the soluble T antigen may neutralize the anti-T reagent, and a false negative reaction could result.

It is well known that typing sera used in blood grouping work is often prepared from human or rabbit serum and may contain anti-T. This can result in false positive reactions due to the T-anti-T reaction rather than to the reaction of the antibody that the reagent is supposed to con-

tain. It has been noted that in sera that have been stored for a long period of time the anti-T antibodies disappaer (5, 15, 44). This could be due to the labile nature of the IgM immunoglobulin, but in view of the effect of neuraminidase on serum producing a soluble T antigen capable of neutralizing anti-T the effect of anti-T disappearing in stored sera could be due to bacterially produced neuraminidase producing a soluble T antigen and then neutralizing the anti-T. This effect would be expected to be especially pronounced in reagents that have been stored for extended periods in nonsterile conditions and unfrozen.

To test this theory ten serum samples that had been stored at 4C for periods of four to nine years were tested against T-activated cells to determine if there was nay anti-T activity. None of the samples contained any anti-T. The aged sera were then tested for their ability to neutralize anti-T by HIA. None of the sera showed any capacity to inhibit anti-T. This argues against the theory that of neutralization of anti-T in aged sera, but it is still possible that the T antigen was produced, neutralized the anti-T, and then disappeared due to effects of aging. There is no data available concerning the long term stability of T antigen in serum. If the soluble T antigen can be shown to be labile over a long period of time then the neutralization hypothesis would still be possible.

The problem with anti-T contaminating blood grouping reagents has to be overcome by absorbing the reagent with T-

activated cells that are negative for the antigen that the antiserum detects, and then testing the same T-activated cells to be sure that all of the anti-T is removed. Another method for removing anti-T from typing reagents may be to treat them with neuraminidase and neutralize the anti-T. If the reagents have been diluted to the point where the levels of serum are very low, neuraminidase treated group AB serum could be added to neutralize the anti-T without effecting the serological activity of the antibodies that are in the reagent.

The fact that neuraminidase treatment of serum or saliva produces a substance that is recognized as T antigen by anti-T reagents is now well documented. All of the experiments performed show that this process does occur and that it is a direct result of the activity of neuraminidase.

Neuraminidase removes a carbohydrate molecule, NANA, from the terminal position of an oligosaccharide chain where it is bound in alpha linkage to the subterminal carbohydrate. When red cells are treated with neuraminidase the NANA molecules are removed and the sugar that has T specificity is exposed to the activity of anti-T antibodies. The same process probably occurs in serum and saliva. There are molecules present with oligosaccharide components some of which have NANA as their terminal molecule. When neuraminidase is allowed to react with these molecules the terminal NANA is removed and the T antigenic determinant is then exposed. Some

of the molecules present in untreated saliva already have their terminal NANA removed from the chains by the neuraminidase produced by normal flora in the mouth. These molecules account for the inhibition present in untreated saliva samples.

The following methods were used to try to determine the type and characteristics of molecules that are responsible for the T activity of serum and saliva:

1. Protein free filtrates.
2. Immunodiffusion
3. Dialysis studies
4. Column chromatography with Sephadex
5. Enzymatic studies

The T antigen itself is undoubtedly a carbohydrate. This has been studied extensively and well documented (3, 28). Many carbohydrate molecules are attached to a carrier molecule, especially proteins or lipids. If the carbohydrate moieties with T specificity were attached to protein or lipid or any other large molecule, then making protein free filtrates from serum or saliva should prevent neuraminidase from causing the production of T substance. Conversely, if neuraminidase treatment of protein free filtrates of saliva or serum causes the inhibition of anti-T there would be strong evidence that the carbohydrate molecule with T specificity is not attached to a carrier molecule, but is rather

a small independent molecule.

When protein free filtrates of serum and saliva were tested there were two effects observed. When the untreated samples of saliva were tested they always showed some degree of inhibition of anti-T. When protein free filtrates of the same samples were tested the inhibitory activity of the untreated samples almost disappeared, dropping from 25.7% in the raw samples to 1.4% in the protein free filtrates. This was signifigant because it was possible that the inhibition of anti-T by untreated samples was caused by free galactose or other small carbohydrate molecules, but these molecules would have readily passed through the ultrafiltration membrane and would have caused an equal amount of inhibition in the protein free filtrates. If small carbohydrates caused some of the inhibition in untreated saliva samples but not all of it, then the values for percent inhibition of the untreated samples protein free filtrates would have fallen but not disappeared. The protein free filtrates of the untreated saliva samples have a very small amount of inhibition (1.4%) but this is attributable to the assay used to quantitate inhibition rather than to a small amount of carbohydrate. This is because if small amounts of free galactose were remaining in the protein free filtrates there should have been a small amount of inhibition in all of the samples. As seen in Table 25 some of the samples showed negative inhibition values

values and this indicates that this is due to experimental procedure rather than to small amounts of inhibition due to free carbohydrates.

The other major effect of making a protein free filtrate of both serum and saliva samples was that when the samples were treated with neuraminidase they could no longer produce any inhibition of anti-T. This was shown for all samples tested. The raw saliva samples when treated with neuraminidase showed a mean percent inhibition of 57.5% and the neuraminidase treated protein free filtrates showed a mean percent inhibition of 0.04%. The raw serum samples had a mean percent inhibition of 53.0% after neuraminidase treatment while the protein free filtrates of serum had a mean percent inhibition of 0.04% after neuraminidase treatment.

If the inhbition of anti-T in the untreated samples was due to a small carbohydrate molecule it would have caused inhibition in the protein free filtrates, and if the molecule that caused inhibition of anti-T after neuraminidase treatment is a small oligosaccharide then neuraminidase treatment of protein free filtrates should have caused the inhibition of anti-T. Neither of these conditions was what was actually observed. These studies suggest that the T antigen of saliva and serum is a carbohydrate molecule that is attached to a large carrier molecule.

Clues to structures of molecules can be obtained through immunodiffusion studies. These methods are well documented

and very sensitive (6). The demonstration of T antigen in serum and saliva samples would be excellent evidence that the T antigen does exist and is not due to experimental procedures. A great deal of experimental manipulation was done on the immunodiffusion system to try to demonstrate T antigen in saliva or serum.

Anti-T reagents tried in the immunodiffusion studies included A. hypogea lectin, as well as ammonium sulfate concentrates of A. hypogea prepared by the method of Grundbacher (10), human anti-T serum, human anti-T eluates, rabbit anti-T serum, and rabbit anti-T eluates. Lectins of U. europaeus and D. biflorus were tested both as saline extracts and as ammonium sulfate concentrates as controls to determine whether lectins could be made to form precipitin lines. Various other manipulations including dilution and concentration of samples and antisera, pH changes, buffer changes, various agar concentrations, various incubation temperatures and times were all tried without success.

The failure to demonstrate T substance in saliva or serum by means of immunodiffusion probably reflects a failure to use the proper condiditons for precipitation to occur. The most likely cause of the failure was the antisera. In order to demonstrate substances by immunodiffusion an antiserum with a large population of precipitating antibodies is necessary. U. europaes and D. biflorus lectins prepared by the same methods used to prepare the A. hypogea lectin also

failed to demonstrate H or A substance in saliva from known secretors.

The dialysis studies were undertaken to prove that the inhibition of anti-T seen in untreated saliva and in neuraminidase treated serum and saliva samples was not due to a small molecule such as galactose or a galactose-containing-oligosaccharide which should be readily dialyzed. If this T activity cannot be dialyzed then this would be further support for the data showing that the T antigen is attached to a large carrier molecule.

As shown in Table 27 and Graphs 14 and 15 none of the inhibitory activity of any sample could be dialyzed. The titer score of the untreated and neuraminidase treated samples were not altered by dialysis even after dialyzing for periods up to 72 hours. If any of the inhibition was caused by small carbohydrate molecules they would have dialyzed and this would have been evident as an increase in the titer score. These experiments provide further evidence that there is a large carrier molecule that is bound to the carbohydrate portion of the molecule that is responsible for the T specificity.

The data from the dialysis and protein free filtrate experiments are compatible with the theory that that the molecules that cause the inhibition of anti-T in untreated samples are neuraminidase treated <u>in vivo</u> by bacterially or virally produced neuraminidase, and that the reason that

samples tested from the same donor on different days show wide variation in the amount of T substance present in their saliva. If the inhibition of anti-T in untreated saliva was due to free galactose or other free carbohydrates, which these experiments show that it is not, then this theory would be invalid.

The experiments performed on separated components of saliva and serum that were separated on Sephadex was attempted to try and further characterize the molecules that cause the inhibition of anti-T. If the molecules are glycoproteins or glycolipids as the experiments with protein free filtrates and dialysis suggest, then the molecules should be able to be separated from each other. Because the fractions collected from the column are read for the O.D. at a wavelength of 280 m at which wavelength proteins show a strong absorption where most other molecules do not, if all of the inhibitory activity of anti-T consistently falls in one of these peaks then this would be further evidence that the carrier molecules bearing the T active structures are proteins or lipids of a constant molecular weight that are eluted with the protein peaks.

Graphs 16 to19 and the data in Table 29 show that saliva is always eluted as a single protein peak. All of the inhibitory activity found in untreated saliva is found in fractions from this peak. Only the fractions from this peak have the ability to inhibit anti-T after treatment with

neuraminidase. Fractions eluted before and after this peak are totally devoid of this activity. When saliva is neuraminidase treated and then chromatographed only fractions from the peak show inhibition of anti-T. These observations confirm the results of the earlier experiments that suggested that the inhibition of anti-T is involved with a large molecule, presumbably a lipid or protein.

When serum is eluted from Sephadex G-200 there are always three major peaks eluted (Graphs 20 to 22). These peaks are broad which suggests that they contain many different types of proteins of roughly the same molecular weight and configuration.

When the individual fractions are tested individually for their ability to inhibit anti-T there is never any signifigant inhibition found in the untreated samples. Treatment of the various fractions with neuaminidase and measuring the inhibition of anti-T shows that only fraction 1 samples has the ability to inhibit anti-T after neuraminidase treatment. If serum is treated with neuraminidase before it is chromatographed all of the inhibitory activity of the sample is found in fraction 1. This data also fits the results from the earlier experiments that suggests the inhibition of anti-T is caused by a large molecule that presumbably is of one specific type, as shown by the elution patterns from Sephadex.

Loton et. al. (28) have published evidence that the T

antigen of red cells is a D-galactose molecule linked in beta 1-3 linkage to an N-acetylgalactosamine molecule. If t the T antigen in serum and saliva is the same as the T antigen on red cells then the inhibitory activity against anti-T should be destroyed by the enzyme beta-galactosidase which specifically cleaves terminal beta linked galactose molecules.

When beta-galactosidase is incubated with normal untreated saliva samples the inhibition that is normally present is partially destroyed. As shown by using increasing concentrations and measuring the amount of inhibition that results the more beta-galactosidase that is used to treat the saliva samples the less inhibition of anti-T that results. This indicates that beta-galactosidase does destroy the T activity and the graph of the concentration versus the amount of inhibition shows that the process is enzymatic.

When saliva samples are initially treated with neuraminidase to produce a large amount of T substance and then treated with increasing amounts of beta-galactosidase then the amount of inhibitoin is gradually decreased as the amount of enzyme is increased up to a point where further increases in the amount of enzyme used causes no further decrease in the inhibition of anti-T. This is hown in Graph 23. These curves indicate that the process is enzymatic in nature and that the enzyme can destroy the T antigen in untreated and neuraminidase treated saliva.

If saliva samples are treated with neuraminidase and

and then treated with beta-galactosidase for various time periods and the amount of inhibition at each time interval is measured the results are consistent with those seen in enzymatic reactions. Initially there is a sharp increase in the amount of inhibition destroyed, but later the amount levels off at a maximum level and there are no further increases in the amount of inhibition destroyed with further increases in incubation time.

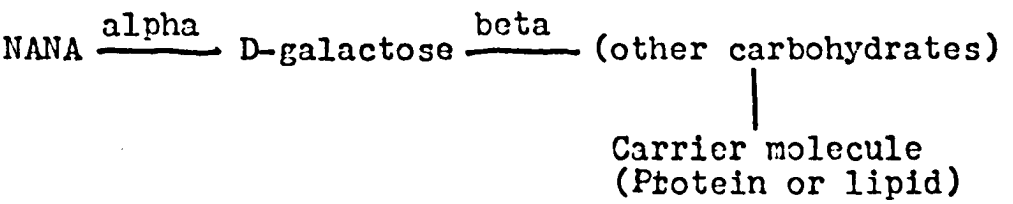
When serum is treated with neuraminidase and then treated with beta-galactosidase the amount of inhibition of anti-T is decreased as the amount of enzyme used is increased. This shows that beta-galactosidase also destroys the T antigen present in serum and does so by an enzymatic process. This is also evidence that the T antigen of serum is the same as the T antigen found in saliva.

In summary, the studies on the nature of the T antigen that is found in untreated saliva and in neuraminidase treated serum and saliva samples is an oligosaccharide molecule that is attached to a large carrier molecule, probably a protein or lipid. The antigenic determinant is a carbohydrate because it is revealed only after treatment with neuraminidase which specifically cleaves the alpha linked NANA molecules of a carbohydrate chain. If the molecule with NANA removed is then treated with beta-galactosidase which specifically removes terminal beta-linked galactose molecules, then the T antigen is destroyed. The dialysis and protein free

filtrate experiments show that the carbohydrate group is linked to a large carrier molecule. The experiments on Sephadex separated components show that molecules responsible for inhibition of anti-T always elute in a specific protein peak.

In view of these data the structure shown in Figure 4 is proposed for the structure of T antigen in body fluids.

Figure 4: Proposed structure of the T antigen in serum and saliva.



Because another lectin commonly used in blood group investigations, V. graminea, also has galactose as its immunodominant sugar and because the experiments discussed previously showed that the T antigen in saliva and serum has galactose as its immunodominant sugar, samples of untreated and neuraminidase treated serum and saliva samples were tested by HIA to determine if they could inhibit V. graminea.

Neuraminidase does induce a change in serum and saliva that allows them to inhibit V. graminea. When these results are compared to results of inhibition studies of A. hypogea with the same samples, it is seen that ther is no good correlation. A difference between the inhibition of A. hypogea and V. graminea by saliva samples is that untreated saliva

always inhibits A. hypogea to some degree but never inhibits V. graminea. This indicates that although both lectins are inhibited by neuraminidase treated saliva they do not have the same identical specificities.

When saliva samples are treated with increasing concentrations of neuraminidase and the resulting inhibition of V. graminea measured, the results shown in Table 37 and Graph 27 are obtained. This data gives the characteristic pattern of an enzymatic reaction. This indicates that the neuraminidase induced inhibition of V. graminea is due to an enzymatic process.

When saliva samples were treated with neuraminidase and tested by the HIA using normal N red cells and neuraminidase treated N red cells the results were essentially the same. The mean inhibition of the saliva samples tested with normal N red cells was 34.1% and using neuraminidase treated N red cells the mean percent inhibition was 30.8%. The difference between the means is not signifigant.

The results of neuraminidase treatment of serum samples and testing them for inhibition of V. graminea are shown in Table 38. These results show that treating serum with neuraminidase consistently results in inhibition of V. graminea. The percent inhibition produced by neuraminidase treated samples against A. hypogea when compared to the percent inhibition when tested against V. graminea show no good correlation. These results indicate that the molecules inhibiting A.

hypogea and the molecules inhibiting V. graminea do not have identical structures.

The V. graminea receptor and the A. hypogea receptor also differ on red cells. The A. hypogea lectin never reacts with red cells unless they have been neuraminidase treated. The V. graminea lectin reacts with all normal cells that carry the N antigen.

When neuraminidase treated serum or saliva samples were treated with beta-galactosidase the inhibition of V.graminea is partially destroyed. Because the immunodominant sugar of the V. graminea receptor is probably beta linked galactose this is as expected. Neuraminidase treatment removes sialic acid and exposes the galactose molecules that can inhibit V. graminea. When this galactose is selectively cleaved by beta-galactosidase then the molecules can no longer react with V. graminea, and this is seen in these experiments as a decrease in the inhibition. Because the receptors for A. hypogea and V. graminea are probably different, yet both are involved with galactose that is at least partially covered with NANA, the differences in the molecules must be very minor. The differences could be possibly due to the configuration of the molecules or to carbohydrate protein interactions such as hydrogen bonding or other non-covalent forces that alter the configuration of the molecules.

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