STUDIES ON THE HEAT LABILE HYALURONIDASE INHIBITOR OF MONKEY SERUM

I. Serum Inhibitor Levels in Monkeys infected with the Virus of Poliomyelitis

II. Kinetics of the Action of Hyaluronidase and its Inhibition by Normal Monkey Serum

by

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STUDIES ON THE HEAT LABILE HYALURONIDASE INHIBITOR OF MONKEY SERUM

I. INTRODUCTION

The spreading factors of biological origin (group A of Duran-Reynals) are believed to owe their activity to the enzyme hyaluronidase which they contain.

It was through the effect of tissue extracts upon the course of infectious processes that these factors were first discovered. The enzymatic activity displayed by these factors is directed primarily against hyaluronic acid which is an integral component of mammalian tissue. The ability of these substances to increase the permeability of animal tissue is of great interest to the microbiologist and has led more or less directly to the concept of invasive as distinguished from virulent infection. Further research was stimulated when the capacity of the organism to react to an invasive type infection with the production of highly specific anti-spreading antibody became known. More recently, studies have been reported which indicate that the blood of a number of mammalian species brings about the inactivation of hyaluronidase. This capacity must be distinguished from that due to the presence of a specific antibody produced in response to a given hyaluronidase. There is no evidence to indicate that this non-specific or physiological inhibitor is the result

of specific immunization. Relatively little information is as yet available regarding the chemistry of this substance. It is fortunate, however, that non-specific inhibitor can easily be distinguished from specific anti-hyaluronidase by virtue of its lack of stability at 56°C. Anti-hyaluronidase, like most antibody, is comparatively heat stable.

In recent years the variation of serum non-specific inhibitor levels under a variety of conditions has been studied. Despite conflicting reports, mainly due to differences in assay techniques, it is well established that these inhibitor levels rise in a variety of infectious diseases as well as in various malignancies.

Attempts to identify and purify this inhibitor have not been completely successful. It is generally agreed that this factor is to be found entirely within the serum, however, electrophoresis and "salting out" techniques have so far presented conflicting data as to the location of this factor within the serum. The mechanism of the inhibition is equally obscure despite the various theories in existance.

The purpose of this investigation was twofold: (1) to study the variation of physiological inhibitor levels in the sera of monkeys infected with the virus of poliomyelitis, and (2) to obtain some basic physico-chemical data as to the nature of the inhibition of hyaluronidase by normal monkey sera.

The significance of the changes in serum inhibitor

levels in various infectious disease processes must necessarily await the characterization of the inhibiting substance. Despite widespread attempts to determine the chemical nature of this substance definitive kinetic studies as to the nature of the inhibition are completely lacking. Such studies should aid in the ultimate characterization of this factor.

II. REVIEW OF THE LITERATURE

A. General Considerations

The observation, made by Duran-Reynals in 1928 (1). that extracts of rodent testis greatly increased the infectivity of vaccinia virus in rabbits aroused great interest in the spreading factors of biological origin. Subsequently it was found that not only infection but the spreading of various dyes (2), hemoglobin (3), toxins (4) bacteria (5), and viruses (6), among other things, was promoted by this factor. It was not until 1939, however, that Chain and Duthie (7) isolated this substance and identified it as the mucolytic enzyme hyaluronidase. Further study (8), coupled with the earlier work on hyaluronic acid by Meyer and his associates (9) (10), led to the tempting hypothesis that the phenomenon of "spreading" was due to the action of hyaluronidase. Although this hypothesis is widely accepted it can not yet be regarded as conclusive (11).

The discovery that certain pathogenic bacteria such as the hemolytic streptococci (groups A & C) (12), some staphylococci (13), pneumococci (14, 15, 16), and clostidia (welchi and perfringens) (17), produced this enzyme gave impetus to an era of vigorous research culminating in the concept of invasive infection, as distinguished from virulent. First suggested by Duran-Reynals (5, 18) this hypothesis rests upon the observation later confirmed by

Sprunt (6), that testicular extract always enhanced but never supressed the lesions caused by vaccinia, Shope fibroma, and Virus III in the experimental animal. This data plus the observation, also by Duran-Reynals (18), that testicular extract enhanced the infectivity of bacteria of high virulence but suppressed that of bacteria of low virulence led to the following conclusions; that the spread of an infectious agent may favor at times the host, and at other times the infecting agent, and further that viruses do not behave as ordinary bacteria but rather as an extremely virulent bacteria.

Reaction to these stimuli produced a great deal of research into the subject of bacterial invasiveness as evidenced by the tremendous of literature produced during this period. This material is exhaustively reviewed by Duran-Reynals (19) in a paper which may be regarded as the most comprehensive piece of literature on the subject.

Recent work by McCrea and Duran-Reynals (20) reports the inactivation of vaccinia virus by a diffusible component of hydrolyzed hyaluronic acid. It is suggested that glucuronic acid interacts with vaccinia virus in a way similar to its conjugating action in the detoxification mechanism (21), thus playing an integral part in the defense mechanisms when the ground substance is attacked during infection. Apparently Duran-Reynals has revised his earlier concepts of the role of hyaluronidase in viral infections.

The complexities of the relationship between hyaluronidase and hyaluronic acid are poorly understood. Hyaluronic acid is a polysaccharide often common both to bacteria and their hosts. Hyaluronidase is also often present in both. It is believed by many that no one bacterial species contains both the polysaccharide and the enzyme although this is known to occur in mammals (1,22,4,23). The further interrelationships in the host with the estrogenic and gonadotropic hormones serves only to make the overall picture more confusing. These hormones, as reported by Lurie (24), and Sprunt (6), act in decreasing and increasing respectively, the permeability of the ground substance. The mechanism of the action is not understood.

B. Chemical Studies

It is difficult to get a uniform picture of the properties of hyaluronidase from the existing literature. There are appreciable discrepancies between the data given by various investigators. The enzyme is filterable and non-dialyzable with a molecular weight in the vicinity of 10,000 (25), (26). The enzymic activity is closely associated with a rather large particle that is not necessarily of pure protein nature (23, 26). During the various purification procedures this carrier is partially or completely removed, depending upon the nature of the procedure. This progressive removal may account for the decrease in the sedimentation constant

as the degree of purification increases (26). The most highly purified preparations thus far subjected to electrophoresis have proven not to be homogenous (27). This has since been confirmed by several investigators using widely different criteria; chief among these is Meyer (28,29, 30). The variation existing between enzyme preparations (especially the isoelectric point and sedimentation constant) might well be reflected in the kinetics of the hydrolysis or hyaluronic acid by hyaluronidase thus accounting for the conflicting data found in the existing literature.

Duran-Reynals (19) states that the action of hyaluronidase is wholly specific. This viewpoint is widely challenged (46,34,28). The final answer awaits the study of a completely isolated and homogenous enzyme.

In composition, hyaluronic acid is an equimolar combination of glucuronic acid and N-acetylglucosamine of unknown structure. This is polymerized to very large and elongated molecules exhibiting molecular weights ranging from 200,000 to 500,000 (30). During hydrolysis, as by hyaluronidase, the polysaccharide is first depolymerized, and then hydrolyzed. The chemistry of hyaluronic acid is well described by Meyer (31) the leading authority on the subject.

The biological function of hyaluronic acid appears to be mainly architectural. Some of the most common sources of the substance are vitreous human(32), umbilical cord (9), and synovial fluid (31).

C. Physical Methods

The direct result of the increased emphasis on basic research has resulted in improved physical methods. The application of these in the form of the invitro assay of hyaluronidase have been the subject of numerous publications. The most commonly used method in the past was the viscosity reduction assay (33,34,35). This method, due to its intrinsic nature, is of doubtful value as a quantitative assay technique and is completely unreliable for kinetic studies. The mucin clot prevention test of McClean (36) is extremely limited in its application. The streptococcal decapsulation test of Fulton, Marcus, and Robinson (37) is extremely sensitive but not well suited for strict quantitative work. The turbidity reducing methods of assay are the most studied, the most popular and the most applicable . Originally described in 1944 by Kass and Seastone (38) this technique has subsequently been modified many times (39.40,41,42). More recently this assay technique has been improved by Bachtold and Gebhardt (42) by the utilization of well established physico-chemical methods.

Due to the increased efficiency of these methods, coupled with an improved understanding of the physical characteristics of the hyaluronidase-hyaluronic acid system, it is suggested that much of what has been done in the past might well be re-examined.

D. Immunological Properties

The immunological properties of hyaluronidase appear to be those of any proteinacious material. Antibodies are demonstrable both as precipitians and by complement-fixation (19). There appears to be present a species but not a type specificity (43).

Hyaluronic acid is not antigenic. Efforts to produce sensitivity or immunization have been uniformly unsuccessful (19,44,45).

E. The Non-specific or Physiological Inhibitor

It is believed by some, that the presence of hyaluronidase inhibitor in the blood of infected and convalescent animals represents a means of defense to invasive infection (47,5). The discovery by Hobby et al. (11) in 1941 that normal human and rabbit sera contained an inhibiting substance, revealed what is perhaps one of the most interesting and least understood aspects of the in vivo role of hyaluronidase. Hobby (11) mistakenly ascribed this effect to salt formation between serum albumin and hyaluronic acid and postulated, therefore, that the inhibition was primarily due to the effect upon the substrate rather than upon the enzyme. Haas (35,48,49) in 1946 observed that the substance responsible for the behavior is heat labile as contrasted to the heat stable specific antibody. This observation affords the best means of separation of these two inhibiter types. The heat labile fraction is generally known as non-specific or physiological (50) inhibitor. On the basis of

time and temperature Haas (35) concludes that this inhibiting substance in the blood is an enzyme. To support this scheme Haas has constructed an elaborate scheme of opposing enzymes in the blood designated as pro and anti-invasins and theorizes a gradient series of these, each exhibiting a checking effect upon the other. It is reported (50) that Adner, on the basis of similar experiments, concludes very cautiously that Haas may be correct. On the other hand, Dorfman, Ott, and Whitney (51) claim to have evidence which rules out any possibility of inhibition due to an enzyme. Similar conclusions have been arrived at independently by Hechter et al. (52) and by Hadidian and Pirie (53). More recent work (54) indicates that hyaluronidase of testicular origin posesses some proteolytic activity which appears to be responsible for the ultimate release of the inhibition of hyaluronidase by non-specific inhibitor. This activity, probably identical with Haas' "proinvasin I" is very likely due to the fact that everthing but the most highly purified enzyme preparations represent a mixture of at least two distinct and separate enzyme.

The interaction of hyaluronidase with the clotting mechanism has been suspected for some time. As early as 1946 Haas (35) postulated, then disproved, the identification of non-specific inhibitor as heparin. In 1948 an attempt was made to identify the inhibitor with complement and prothrombin (55). Fiala et al.(56, 57) have shown that dialyzed bovine hyaluronidase acts as an anticoagulant in

vitro by virtue of its capacity to counteract the thromboplastic effect of platelets. These investigators believe that the interaction between hyaluronidase and the clotting mechanism represents a competitive inhibition between platelets and enzyme. An inhibition of the enzymatic degradation of hyaluronic acid due to tissue thromboplastin has also been noted by Fiala (57). More recently Roth (58) suggests that hyaluronidase inhibition is intimately associated with proconvertin activity despite evidence which suggests that inhibitor and proconvertin are not chemically identical. With both complement and proconvertin preparations hyaluronidase inhibiting activity is lost rapidly upon freezing. The activity can promptly be restored by addition of inactivated serum. Both Fulton et al. (59) and Roth (58) suggest that this indicates that full inhibition depends upon the presence of a heat stable co-factor which is present in inactivated serum but is destroyed upon freezing.

Several investigators have studied the variation of hyaluronidase inhibitor under a variety of conditions. Among these are Glick and Gollan (60) who have reported studies on the inhibitor levels of human and monkey blood after infection with the virus of poliomyelitis; Grais and Glick (61) who studied inhibitor levels in a variety of skin diseases and Fulton et al. (59) who have studied the level of hyaluronidase inhibitor in various malignancies.

The explanation of these findings is as yet obscure. The observation by Winzler et al. (62), and Seibert et al. (63), that elevated levels of plasma mucoproteins occur in patients with a variety of metabolic and infectious diseases offered a tempting solution to the origin of the non-specific inhibitor in view of the observations of Glick and Gollan (60). Subsequently work by Glick et al. (64) indicated, however, that hyaluronidase inhibition is not associated with these plasma mucoproteins.

III. MATERIALS AND METHODS

A. Preparation of Enzyme

Hyaluronidase was extracted from fresh bull testes by essentially the same method as that described by Hahn (27). The resulting liquid concentrates were stored at 4^{0}C. for considerable periods of time. No appreciable loss of activity was observed over these periods. Fresh enzyme preparations were made for each experiment from the liquid concentrate.

B. Preparation of Substrate

Potassium hyaluronate was prepared from human umbilical cord collected in 2 per cent aqueous phenol.

The purification of hyaluronate depends primarily upon the removal of various proteins from the umbilical cords. Different methods of purification vary in the manner in which this is accomplished. The method described by Tolksdorf (41) proved highly satisfactory and was therefore routinely used to effect the preparation of the substrate in this laboratory.

The powdered substrate thus obtained was stored at room temperature. From this stock sufficient hyaluronate was made up in pH 6.0 buffer to run each experiment. Once in solution, the storage of substrate is not practical since turbidity producing properties are lost rapidly. This necessitates the preparation of a fresh substrate solution for each experiment thus involving certain weighing errors

which, in turn, are reflected in the range of turbidities obtained.

C. Buffer Solutions

A 0.5 M acetic acid-sodium acetate mixture of pH 6.0 containing 0.73 per cent sodium chloride, and a 0.5 M acetate buffer of pH 4.2 were used throughout the experiments. These buffers are known to allow the development of maximum turbidity in a given substrate preparation under the proper conditions. The buffer solutions were stored at 4^{0}C. to prevent microbial growth.

D. Acidified Plasma

Human plasma was obtained from local blood banks, acidified, then aged according to the method described by Tolksdorf, et al. (65).

E. Monkey Serum

Monkey sera was collected aseptically by cardiac puncture from anesthetized experimental animals. After centrifugation the serum was removed and stored in ampules at -60^{0}C. until needed.

F. Normal Serum Pools

Serum samples were collected from 20 normal monkeys and pooled. This material was then placed im ampules, sealed, and stored at -60^{0}C. until used. Serum from this pool was

used throughout the entire study in order to eliminate variations in inhbitor levels which would interfere with the accuracy of the kinetic studies.

G. The Hyaluronidase Inhibition Test

1. Collection of serum.

At least two serum samples were obtained from each animal previous to infection with poliomyelitis virus. From these samples, a mean normal resting level of inhibition was ascertained. Blood was drawn at two day intervals following infection until the first symptoms of poliomyelitis were noted. It was found necessary, due to the rapid progress of the infection, to draw blood upon the day of first symptoms and each day thereafter until the height of paralysis and the consequent inability of the animal to feed itself.

The sera thus collected were sealed in ampules and stored at -60^{0}C until the series of serum samples for the animal under observation was complete. The entire group of sera were then run in one experiment with a single substrate and enzyme preparation to determine the extent of the rise in inhibitor.

2. Test for nonspecific (physiological) inhibitor.

The technique used to assay the degree of hyaluronidase inhibition was essentially that described by Bachtold and Gebhardt for the assay of enzyme preparation (42).

For each serum sample a series of tubes was set up, each containing 0.5 ml. of substrate solution and 0.1 ml. of a 1.3 dilution of the serum sample. Bothe the substrate and the

serum were prepared in pH 6.0 buffer. To each tube was added 0.4 ml. of enzyme containing 2.0 turbidity reducing units (T.R.U.). These tubes were then incubated for varying periods of time in a 37°C water bath. A suitable series of control tubes was also run, each tube containing 0.5ml. of substrate, 0.1 ml. of pH 6.0 buffer and 0.4 ml. of enzyme. Following the incubation interval the reaction was stopped by immersing the tubes in a 60°C water bath for 10 minutes. The tubes were chilled in an ice bath then 3 ml. of pH 4.2 buffer and 1.0 ml. of acidified protein were added. After a period of 30 minutes to allow turbidity to develop, the absorbancy was determined with a Coleman Junior spectrophotometer set at a wave length of 580 ml.

As has been shown by other investigators (42,66) the concentration of hyaluronate in each tube can be determined from the absorbancy observed. The concentration of hyaluronate present after the period of hydrolysis can then be substituted into the familiar first order equation and used to calculate the specific reaction rate constant k as shown below:

$$k = \frac{2.303}{t} \log \frac{C_o}{C_t}$$

C_o = initial concentration of substrate

C_t = concentration of substrate after the reaction interval (t)

From the mean k value so obtained for each serum sample (k_s), the following relationship can be established with the k value obtained from the enzyme control (k_e):

$$\frac{k_c - k_s}{k_c} \cdot 100 \quad \% I$$

The symbol, %I, represents the per cent inhibition of enzyme activity.

3. The test for specific antihyaluronidase.

Specific antihyaluronidase refers to the specific antibody to hyaluronidase which may be readily distinguished from the physiological inhibitor of hyaluronidase in any given serum sample by means of it's stability at 56°C. At this temperature the physiological or nonspecific inhibitor is completely inactivated at the end of 10 minutes. Any remaining inhibitory properties of the heated serum sample may then be attributed solely to the presence of specific antihyaluronidase.

The assay procedure for specific antihyaluronidase is the same as that described for physiological inhibitor with but one exception; the serum sample to be assayed is heated at 56°C for 10 minutes previous to dilution of 1.3 with pH 6.0 buffer. The heated,diluted, serum is then added to the test system as described previously.

H. Procedures For Kinetic Studies

1. Determination of optimal enzyme concentration.

The optimal enzyme concentration varies depending upon the nature of the experiment. If, in any given experiment, the enzyme concentration chosen is too great, hydrolysis of the substrate proceeds to such a degree during the reaction interval that it is impossible to accurately measure the

amount of substrate remaining unhydrolyzed. The reverse situation occurs when the enzyme concentration is too small.

Bearing in mind the range of hydrolysis that is desirable in the proposed experiment, a series of tubes containing the proper concentration of substrate is prepared. To each tube is added a successively higher dilution of enzyme. The reaction is allowed to proceed over the allotted time interval then stopped as previously described. The amount of substrate remaining in each tube is determined and from this data the degree of hydrolysis in each tube is calculated. The enzyme dilution corresponding to the desired range of hydrolysis is thus chosen directly or by interpolation.

2. Changes in the reaction system.

In order to get the precise results required for any kinetic study it was found necessary to revise the procedure used for the inhibition studies. The factors responsible for the inaccuracies present in the original assay system were primarily due to temperature variations, timing and pipetting errors.

In order to eliminate, as far as possible, the error due to pipetting the ratio of the various reactants was altered from that originally used in the inhibitor studies. The concentrations of substrate and enzyme were adjusted so that for each a volume of 1 ml. was used per tube. This made possible the use of volumetric pipettes. The various substrate concentrations necessary for each test were obtained

by careful dilution. One ml. aliquots of the dilutions were pipetted into the reaction tubes. The various dilutions of sera, when used, were added in a 0.2 ml. volume. Such a small volume was considered to be permissible since the amount of serum was found to be less critical than that of enzyme of substrate. The final volume of the reaction system was then 2.2 cc as compared to 1.0 cc used in the original inhibition studies.

The utilization of a highly accurate constant temperature water bath made it possible to keep temperature fluctuation within $\pm 0.2^{0}$C.

3. The low temperature apparatus.

The majority of the kinetic studies were carried out at temperatures varying from 4^{0}C to 15^{0}C. It was found necessary to use low temperatures for the reaction for two reasons; at low temperature, the reaction velocity could be retarded to the degree where the reaction time interval could be prolonged sufficiently to minimize the timing error resulting from a short reaction interval; secondly, a sufficiently high enzyme concentration could be utilized to insure that the reaction would proceed as a monomolecular or first-order reaction.

The constant temperature bath was equipped with a Fenwal thermoregulator, a high speed stirring motor, and one or two (as was needed) hollow steel cylinders into which small pieces of dry ice were placed. By varying the number of these cylinders, the depth at which they were immersed in

the bath, and the adjustment of the thermoregulator, any temperature from 4^0 to 37^0C could be maintained to within $\pm 0.2^0$C.

4. The calculation of the reaction velocity

In order to calculate reaction velocity one must know the time interval of reaction (t), the initial substrate concentration (Co), and the amount of substrate remaining at the end of the interval of reaction (C). Both the time interval and the initial substrate concentration are chosen by the investigator, hence one need only calculate the amount of substrate remaining unhydrolyzed at the end of the reaction interval. This is done by comparing the turbidity developed in the reaction tube with that of a series of graded substrate concentrations. The relationship between absorbancy and substrate concentration is established diagrammatically. One may, from the resultant curve, determine the amount of substrate from any given absorbancy value. Having established the amount of substrate remaining after a reaction interval of (t) one may then calculate velocity from the following relationship:

$$\frac{C_o - C_x}{t} = v$$

The symbol v represents velocity and is expressed in units of milligrams hydrolyzed per minute.

The value Co - C_x represents the amount of substrate hydrolyzed within the reaction interval (t).

5. Determination of the reaction order

Both the original and the revised assay procedures were utilized, depending upon the nature of the experiment, for the determination of the reaction order of the hydrolysis of hyaluronate. With either method of assay the rate of the reaction under the conditions imposed was established by determining how this rate was influenced by the concentration of the reacting substance or substances. Values for the concentration of unhydrolyzed substrate were obtained at various time intervals during the reaction; the temperature and enzyme concentration being held constant throughout any one run. The order of the reaction was then determined solely by the best fit of the data so obtained to one of the rate equations. Because the data invariably fitted so closely either the zero or first order equations, depending upon the experimental conditions, it proved unnecessary to calculate a fractional order for any of the reactions studied.

6. The effect of substrate concentration upon reaction velocity.

In order to calculate various physical constants it is necessary to establish the effect exerted upon the reaction velocity by the initial substrate concentration. The range of substrate concentrations which can be utilized in such a study is limited by the nature of the turbidimiteric method of assay. Because this range is extremely narrow, all substrate concentrations were prepared by the dilution of a standard substrate solution. In any one experiment the the enzyme concentration, temperature, and time interval of

reaction were held constant, the only variable being the substrate concentration. The velocity of the reaction for a given initial substrate concentration was determined by dividing the amount of substrate hydrolyzed in each group by the reaction time interval. The values thus obtained were plotted in various ways against the corresponding initial substrate concentrations as described under experimental results.

7. The effect of temperature.

In order to gain a further insight into the nature of the hydrolysis of hyaluronate by hyaluronidase, an attempt was made to determine the effect of temperature upon the reaction. Substrate and enzyme concentrations were adjusted so that the reaction proceeded as near as possible to the first order over as large a temperature range as possible. The reaction was allowed to proceed to the same degree of completion (within experimental limits of error) at each temperature level. The variables were therefore temperature and time, enzyme and substrate concentrations being held constant. These conditions were imposed in order to minimize the variation from the mean "k" values that occured (at certain temperature levels) as the degree of hydrolysis progressed. This occured because the reaction did not proceed strictly in accordance with the first order equation at these temperatures but was, nevertheless, close enough to justify the use of this equation (provided the degree of hydrolysis

was rigidly controlled). The "k" value for the reaction at each temperature level was calculated by means of the first order rate equation and plotted against the corresponding temperature as shown under experimental results. The revised assay technique was utilized throughout these studies in order to obtain more precise data.

The effect of temperature upon the inhibition of the reaction by normal monkey serum was determined by essentially the same method as described above. To each tube, with the exception of the controls, was added 0.2 cc of a 1:3 dilution of normal monkey serum. The reaction was allowed to proceed to the same degree of completion at each temperature as was previously described and the degree of inhibition was calculated for each temperature level. Per cent inhibition was plotted as the ordinate versus the corresponding temperatures.

IV. EXPERIMENTAL RESULTS I

A. Nature of the Hyaluronidase Inhibitor

The inhibition of hyaluronidase by unheated monkey serum (I_x) may be attributed to the sum of two factors;

$$I_x = I_{n\text{-}s} + I_s$$

the heat labile, non-specific inhibitor ($I_{n\text{-}s}$) plus the heat stabile antibody, antihyaluronidase (I_s).

Antihyaluronidase (I_s) was observed in approximately 60 per cent of the animals tested, but in such low levels that it was difficult to detect. In addition, there has been no consistant correlation between the progress of the poliomyelitis infection and the serum level of this antibody. The few cases in which a rise in this antibody was observed (Figure 1) might well be due to the secondary invasion frequently observed in terminal paralysis.

The contribution of antihyaluronidase to the observed total inhibition (I_x) may therefore be considered sufficiently small as to be disregarded thus leaving I_x solely as a function of the non-specific inhibitor ($I_{n\text{-}s}$);

$$I_x \cong I_{n\text{-}s}$$

B. Significance of Hyaluronidase Inhibitory Levels

In order to quantitate changes in serum hyaluronidase inhibitory levels, one must first establish a normal resting range for this capacity. The establishment of such a value

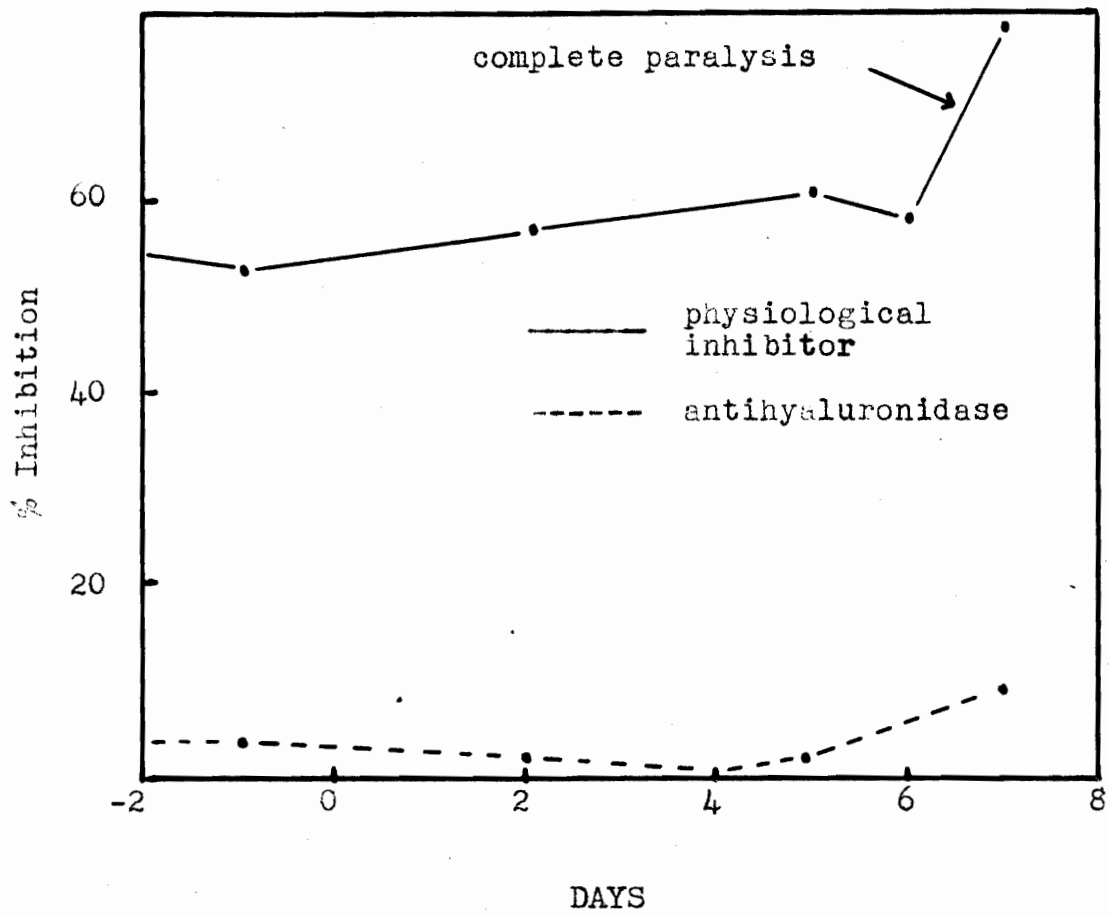

Figure 1. Comparitive levels of physiological inhibitor and antihyaluronidase in a poliomyelitis infected monkey.

depends upon two major factors: the day-to-day variation in normal resting inhibitory levels and secondly, those errors inherent in any analytical test of this nature.

The following experiments were designed to ascertain the magnitude and relative importance of these factors, and hence to establish the normal resting levels within carefully defined limits.

Blood was drawn from three normal monkeys every second day for 10 days. The total inhibitory level (I_x) was then calculated for each sample (Table I). These values represent a combination of day-to-day variation and any analytical error.

TABLE 1

Total Inhibitory Levels and Accompanying Std. Deviations in Three Animals

Monkey Number	Number of Samples*	Mean I_x ($\bar{I}_x$)	Std. Deviation from the Mean (σ_x)
1	5	26.2%	3.1%
2	5	37.6%	2.6%
3	5	34.4%	2.8%
Average std. deviation (σ) = $\pm$ 2.83%			

* Samples drawn at 24 hour intervals

It is apparent from these data shown in Table 1 that the variation in the resting level of individual animals is large. The standard deviation involved is almost twice

the calculated value for the standard deviation within each group.

In order to determine the contribution of the analytical error to the observed standard deviation, four samples of sera were tested repeatedly for hyaluronidase inhibitory activity (Table II).

TABLE II

Estimation of Analytical Error
(Repeated Observations on Single Serum Samples)

Sample Serum	% (I_x)	Mean I_x ($\bar{I}_x$)	Std. Deviation σ
1	37,35,35,32,37	34.2%	2.2%
2	29,37,37,37,37	35.4%	3.6%
3	21,28,26,27,28	26.4%	3.2%
4	21,26,26,27,30	26.0%	3.2%

Average std. deviation (σ) = ± 3.1%

Upon comparison of the average standard deviations from Tables I and II it is apparent that the contribution of the day-to-day inhibitory variations to the observed standard deviation is so slight that it is of questionable significance. The variation about the normal resting level must, therefore, be due almost completely to analytical error.

If the experimental data could be assumed to follow closely the normal or "Gaussian" distribution, any level of hyaluronidase inhibition greater than or equal to two

standard deviation (2) above the mean resting value could be considered to fall within the 5 per cent (0.05) level of confidence (Figure 2). Since, however, the experimental population is routinely rather small (4 to 6 samples) it was deemed necessary to replace this value with "t" standard deviations (t.σ). This value plus the normal resting mean represents the lower level of significance. The "t" for an experimental population of 6 samples (5 degrees of freedom, n-1) is found to be 2.6 at the 5 percent level of confidence; hence, "t" standard deviations is 8.1 per cent (t = 2.6 x 3.1%). Any increase of serum inhibitor values in excess of the sum of the mean resting value plus "t" standard deviations was therefore considered to indicate a significant rise in the inhibitory level of the sera. This is demonstrated in Figure 3.

It is important to note that as the number of samples approaches 20, the distribution of the significance ratio (t) approaches the shape of the normal curve (67).

C. The Rise of Hyaluronidase Inhibitory Levels

An identical pattern in the rise of serum hyaluronidase inhibitor (I) was noted in animals infected via the intranasal and intracerebral routes (Figure 4). This similarity is not surprising since in both routes, the virus enters the brain rapidly and more or less directly without a humoral phase. With either method the period preceeding paralysis is essentially the sam.

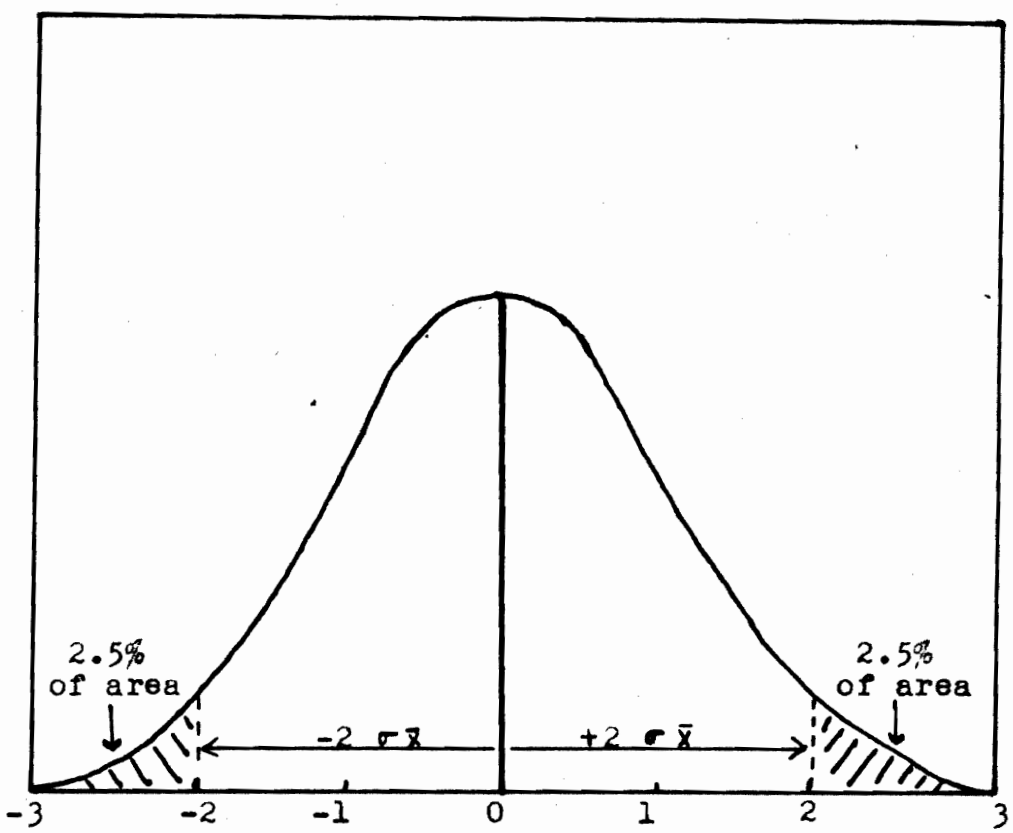

Figure 2. The normal distribution curve.

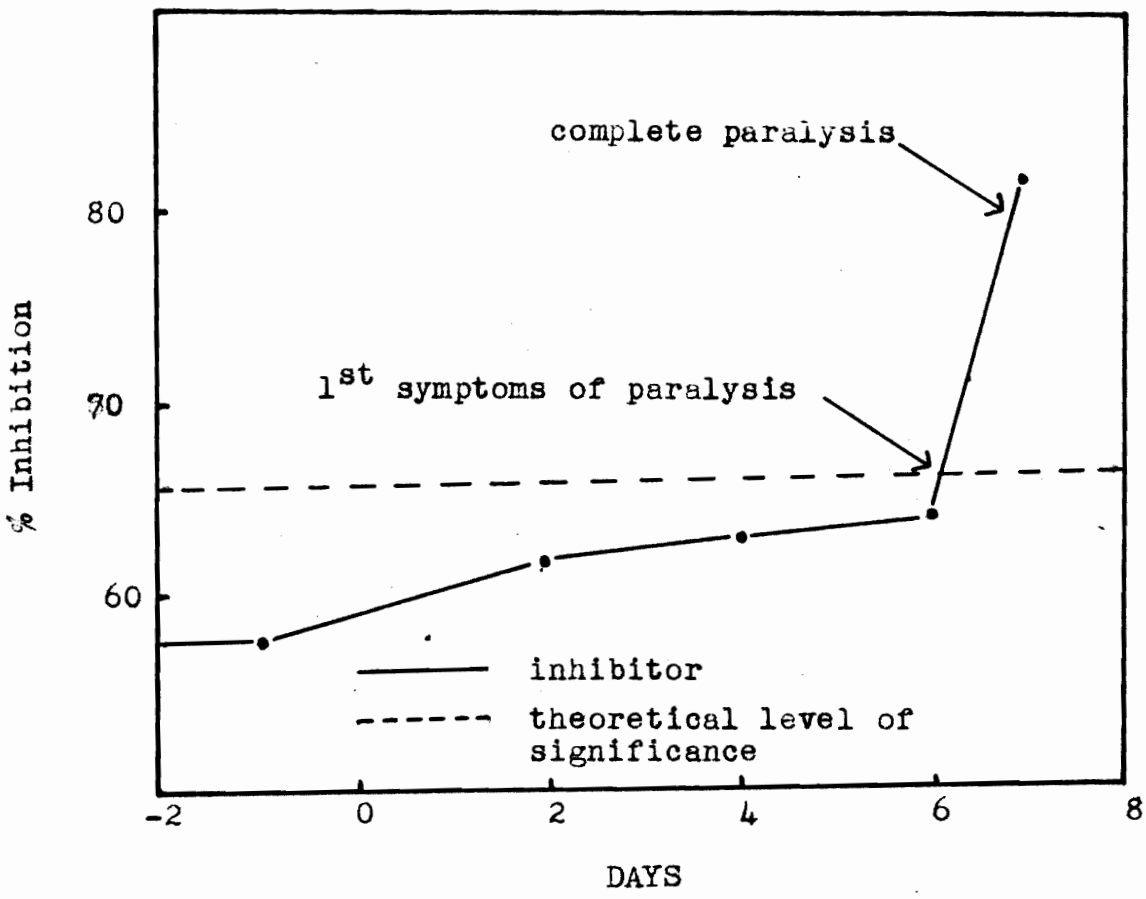

Figure 3. The rise of physiological inhibitor to levels of significance.

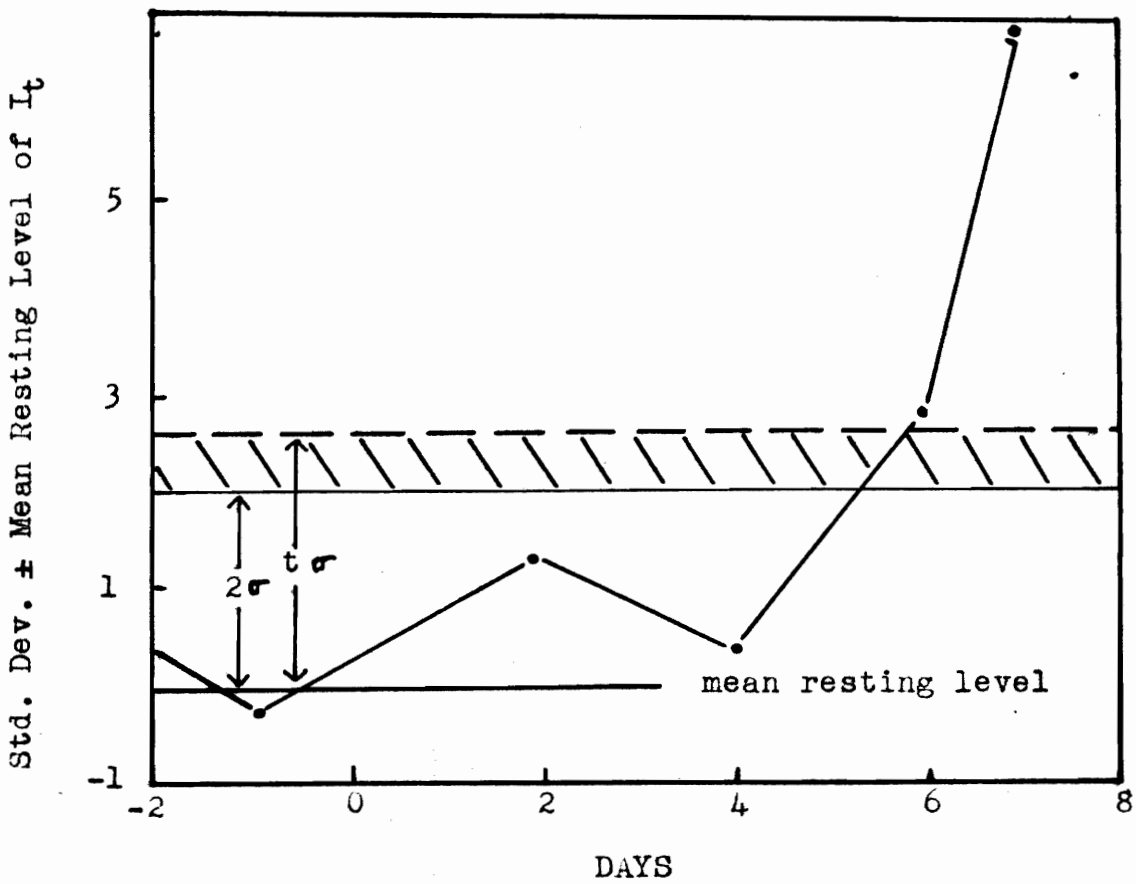

Figure 3a. The rise in serum inhibitor expressed as the number of std. dev. in excess of the mean resting level. The dotted line represents "t" or 2.6 std. deviations.

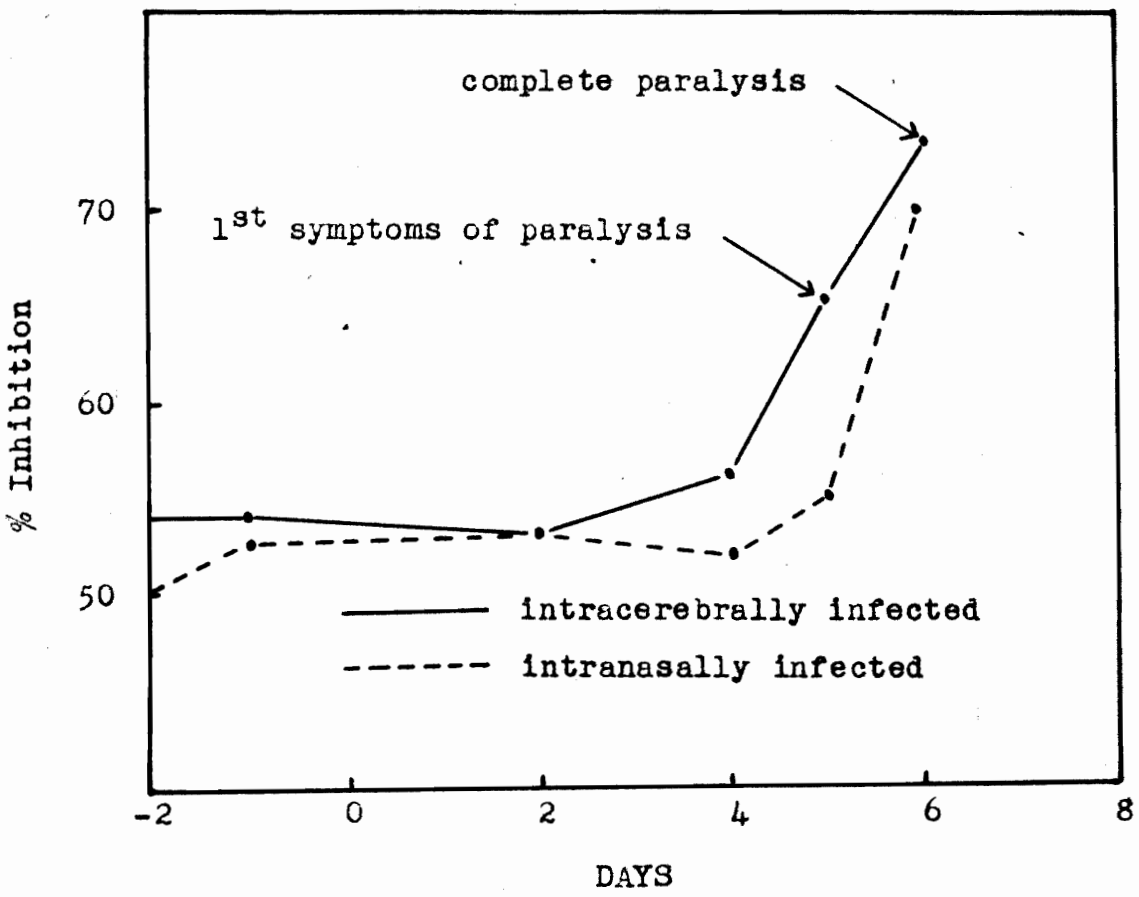

Figure 4. The rise of serum inhibitor in an intranasally and an intracerebrally infected animal.

The rise in the hyaluronidase inhibitory level of the infected animals' sera is not gradual but, to the contrary, rose rather abruptly. A significant increase in the serum inhibitory level just preceeds or is concomittant with the onset of paralysis (Figures 1,3, and 4).

When a vertical line is drawn to the abcissa from the point of intercept of the serum inhibitor curve with the line representing the theoretical level of significance, the time necessary for the serum to rise from resting to significant inhibitor levels is indicated (Figures 3 and 5). The mean value for this period in a series of 10 animals was found to be 5.1 days in contrast to mean 6.2 days for the onset of first paralytic symptoms and 6.9 days until paralysis was complete. (Table III).

The magnitude of the rise in serum hyaluronidase inhibitory levels of individual animals is relatively constant. Conversely, the normal resting inhibitor levels in individual animals varies widely as illustrated in Table IV.

The mean value for the magnitude of serum hyaluronidase inhibitor (I_t) rise is 20.5 per cent possessing a standard deviation of 5.3 per cent. If the error due to the test procedure (3.1%) was substracted from this value (5.3% - 3.1%) the resulting deviation is 2.2 per cent. This indicates that the magnitude of the serum inhibitor (I_t) rise in individual animals is amazingly constant. On the other hand, the resting mean value of 34.8 per cent for serum inhibitor, possessed the extremely large standard deviation

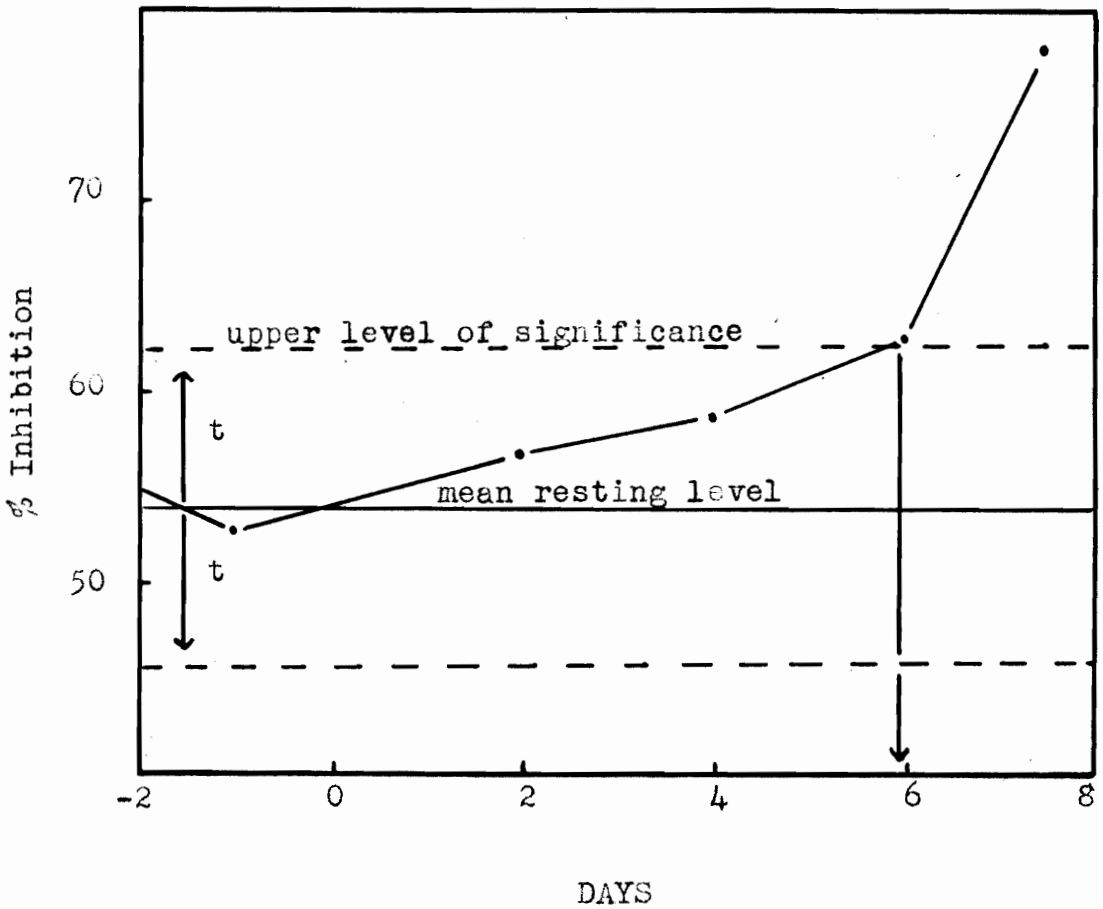

Figure 5. Method by which the time interval of rise of serum inhibitor was calculated. The interval necessary for the inhibitor levels to rise to significant heights was, as can be seen, 5.8 days.

TABLE III

Comparison of Significant Inhibitor Levels to the Time Intervals of the Infection

Monkey Number	Interval Preceeding Significant Inhib. Level	1st Paralytic Symptoms	Total Paralysis
1	5.3 days	5 days	5 days
2	6.2 days	7 days	8 days
3	5.0 days	5 days	6 days
4	5.6 days	6 days	6 days
5	5.3 days	6 days	7 days
6	2.5 days	5 days	6 days
7	6.3 days	7 days	7 days
8	5.2 days	8 days	8 days
9	4.0 days	6 days	7 days
10	5.8 days	7 days	9 days
	Mean = 5.1 days	Mean = 6.2 days	Mean = 6.9 days

of 10.9 per cent. Correction for errors of measurement fail to reduce this value significantly. This indicates that a marked variation in the resting level of serum inhibitor (I_t) exists among individual animals.

D. Failure to Observe a Rise in the Hyaluronidase Inhibitory Level of Sera

The probability of an infected animal exhibiting paralysis or clinical symptoms is much less in the gastro-

intestinally infected animal than in an animal infected by either the intracerebral or intranasal route. Ordinarily 95 per cent to 100 per cent of the animals innoculated by either of the latter two routes exhibit some evidence of poliomyelitis within 5 to 11 days after introduction of the virus. When the virus is introduced into the stomach by means of a catheter only 20 per cent to 60 per cent of the animals exhibit clinical symptoms or paralysis and the incubation period varies from 7 to 18 days.

TABLE IV

A Comparison of the Magnitude of the Rise in Serum Inhibitor Level in Several Animals

Animal Number	Resting (I_r)	Maximum (I_x)	Relative I_x Rise
1	24 %	41 %	17 %
2	59 %	74 %	15 %
3	37 %	60 %	23 %
4	24 %	50 %	26 %
5	32 %	46 %	14 %
6	48 %	69 %	21 %
7	32 %	60 %	28 %
8	32 %	58 %	26 %
9	31 %	52 %	21 %
10	29 %	43 %	14 %

Resting mean = 34.8 % ± 10.9 %

Mean relative rise = 20.5 % ± 5.3 %

Twenty animals were infected by the gastro-intestinal route. Eight animals or 40 per cent exhibited paralysis following incubation periods of 8 to 16 days. None of the 8 paralysed animals exhibited a rise in serum hyaluronidase inhibitory level either preceeding or during paralysis (Table V).

TABLE V

Total Inhibitory Levels in Monkey Infected with Poliomyelitis via the Gastro-Intestinal Route

Animal Number	Resting I_x	I_x values Preceeding Paralysis (%)	I_x at the time of & subsequent to Paralysis	Incubation Period
1	22%	26,26,25,23,28,23	24,23	16 days
2	32%	35,33,31,31,33	32,33	10 days
3	43%	40,38,45,43,43	45,44	10 days
4	38%	39,39,40,42,36,40	40,39	11 days
5	26%	22,26,23,25	25	8 days
6	31%	28,31,29,35,33	31,35	10 days
7	28%	31,31,28,31,33,28	30,31	12 days
8	49%	52,53,50,46,49,49	50	11 days

E. The Relationship of Poliomyelitis Antibody Level to the Serum Hyaluronidase Inhibitor.

The rise in the hyaluronidase inhibitory levels of monkey sera during the course of poliomyelitis was markedly

similar in both intranasally and intracerebrally infected animals. However, there is no corresponding rise in the inhibitory levels of monkey infected via the oral or gastro-intestinal route.

It has frequently been observed in this laboratory as well as elsewhere (68,69), that monkeys infected either intracerebrally or intranasally fail to exhibit detectable of humoral antibody to poliomyelitis virus at the time of paralysis or up to two months subsequent to paralysis. On the other hand, orally infected animals rarely fail to show detectable virus antibody levels at the time of , or shortly after, paralysis (70).

The failure to exhibit a virus antibody titer associated with a tise in hyaluronidase inhibitory levels and, conversely high virus antibody titer associated with the failure to observe a rise in hyaluronidase inhibitor levels suggests a reciprocal relationship between enzyme inhibition and virus antibody. Whether or not this relationship is a coincidence is not clear. The analogy is, however, too tempting to discard without mention.

F. Inhibition of Hyaluronidase by the Hydrolytic Products of Hyaluronic Acid

The rise of serum hyaluronidase inhibitory levels has been observed in a variety of infectious processes (71,61). The possibility that this rise might be due to the accumulation in the blood of the breakdown products of ground

substance or, in essence, the hydrolytic products of hyaluronic acid is worth investigation. It is theorized that under certain conditions this enzymatic reaction may be self limiting due to the accumulation of reaction by-products which may manifest an inhibitory effect upon the reaction.

The effects exerted upon hyaluronidase by the hydrolytic products of hysluronic acid were determined as shown in Figure 6.

It was noted that following overnight dialysis (step 2). the concentration of hyaluronic acid in the dialysate, (measured turbidimetrically) remained constant (step 2a). To this dialysate a small amount of an extremely dilute solution of hyaluronidase was added (step 3). This was enough to assure complete breakdown of the substrate yet not enough to interfere with the subsequent analysis of the dialysate for enzyme inhibitory qualities. Since a small residual amount of active enzyme was in every case present in the dialysate after hydrolysis, it proved necessary to devise a method to estimate the magnitude of this residue. Because of this a parallel control was run in which an identical concentration of enzyme, prepared in buffer pH 6.0, was submitted to the same procedures as was the original enzyme-substrate preparation. It was assumed that after hydrolysis the same amount of active enzyme would be present in the hydrolysate as was found to be present in the control preparation.

Hydrolysis of the substrate was found to be complete at the end of 10 hours (step 3), at which time the hydrolysate was divided into two aliquots. Various fraction of these aliquots were tested with suitable controls for hyaluronidase inhibitory activity. The results of these tests are shown in Table VI.

TABLE VI

Enzyme Inhibitory Levels of Various Fractions of the Hydrolytic Products of Hyaluronic Acid

Fraction Tested	% Inhibition
Intact Hydrolysate Step 5b, (Figure 6)	3 %
Heat Inactivated Hydrolysate Step 5a, (Figure 6)	0 %
Dialysate of the Intact Hydrolysate Step 4a,(Figure 6)	0 %
Concentrated Diffusate of the Hydrolysate Step 4b, (Figure 6)	5 %

The inhibitory values noted above are expressed as the percentage of the theoretically hydrolysable substrate remaining unhydrolysed in the presence of a given amount of enzyme. These values are calculated as follows:

$$\frac{X_K - X_I}{X_K} \cdot 100 \quad \% I$$

X_K = mg. of substrated hydrolysed in the control.

X_I = mg. of substrate hydrolysed in the test.

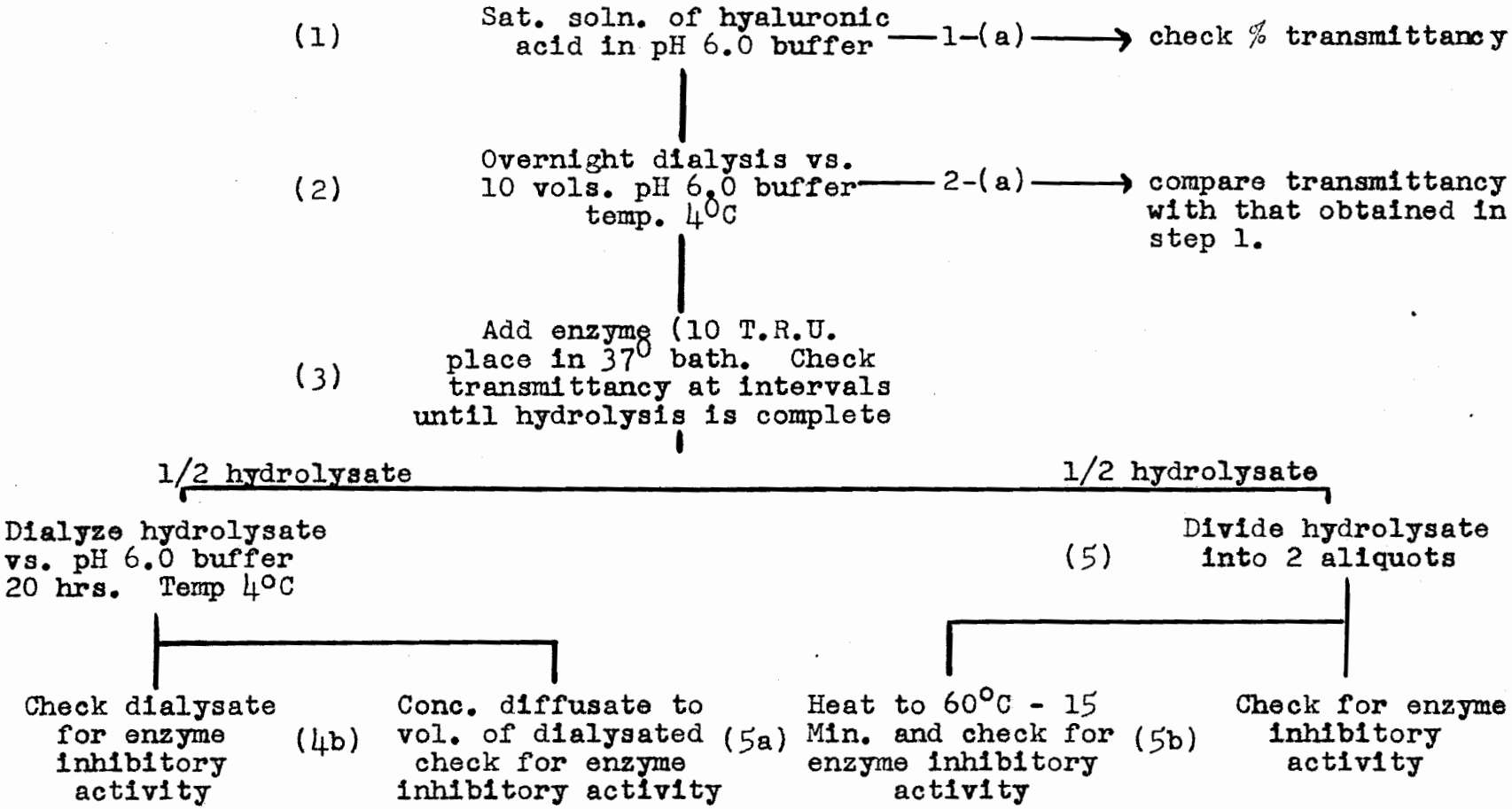

Figure 6. Flow sheet. This figure illustrates the steps in the procedure used to determine the effect of break-down products upon enzyme activity substrate.

Time, temperature, substrate concentration, and enzyme concentration are necessarily the same in both the control and test.

It is obvious that with the concentration of hydrolytic products obtained in the preceeding experiment the inhibition of hyaluronidase by these products in minimal; therefore, the rise in serum hyaluronidase inhibitory levels cannot be attributed to the presence of such by-products in the blood of infected animals.

V. EXPERIMENTAL RESULTS II (THE KINETICS OF THE INHIBITION HYALURONIDASE BY NORMAL MONKEY SERUM)

A. The Reaction Order

The order of the reaction was found to be influenced by the nature of the substrate preparation used. The reaction was observed to be of a fractional order with the best substrate preparation available in this laboratory. The data, however, fits so closely the conditions imposed by the first order equation

$$\frac{-dCa}{dt} = kCa$$

in both the enzyme-substrate and enzyme-substrate plus inhibitor systems, that it was felt justifiable to calculate inhibition on the basis of the "k" values obtained from the above equation (Figure 7, and Table VII).

It proved to be possible, with less highly refined substrate preparations, to push the reaction into the realm of the zero order as defined below.

$$\frac{-dCa}{dt} = K$$

This may be due to the fact that it is possible, with this type of substrate preparation, to get a high concentration of substrate without the flocculation which occurs in these same concentration ranges when using more highly refined substrate preparations.

TABLE VII

The Calculation of Per Cent Inhibition from the "k" Values Obtained from the First Order Data

System	Time	Mean "k"	% Inhibition
Enzyme	5 min.	0.1180	14 %
Enzyme n.m.s.	5 min.	0.1020	14 %
Enzyme	10 min.	0.0948	
Enzyme n.m.s.	10 min.	0.0720	24 %
Enzyme	15 min.	0.0805	
Enzyme n.m.s.	15 min.	0.0610	24 %
Enzyme	20 min.	0.0682	
Enzyme n.m.s.	20 min.	0.0555	19 %

n.m.s. = normal monkey serum

B. The Effect of Substrate Concentration

The effect of the concentration of substrate (hyaluronic acid) upon the velocity of the hyaluronidase catalyzed reaction is shown in Figure 8. The basis for the mathematical treatment of this type of data was first formulated by Michaelis and Menten (74), however the estimation of the enzyme-substrate complex dissociation constant (K_s) and the maximum velocity (V) is readily accomplished by the less cumbersome method of Lineweaver and Burk (72). The use of this method for the calculation of Ks and V for a typical hyaluronidase catalyzed reaction is shown in Figure 9. The slope of the line in Figure 9 is Ks/V and the ordinate intercept is 1/V. From this data Ks and V are readily obtainable

Several such determinations were run on different hyaluronidase hyaluronic acid systems. The results with their

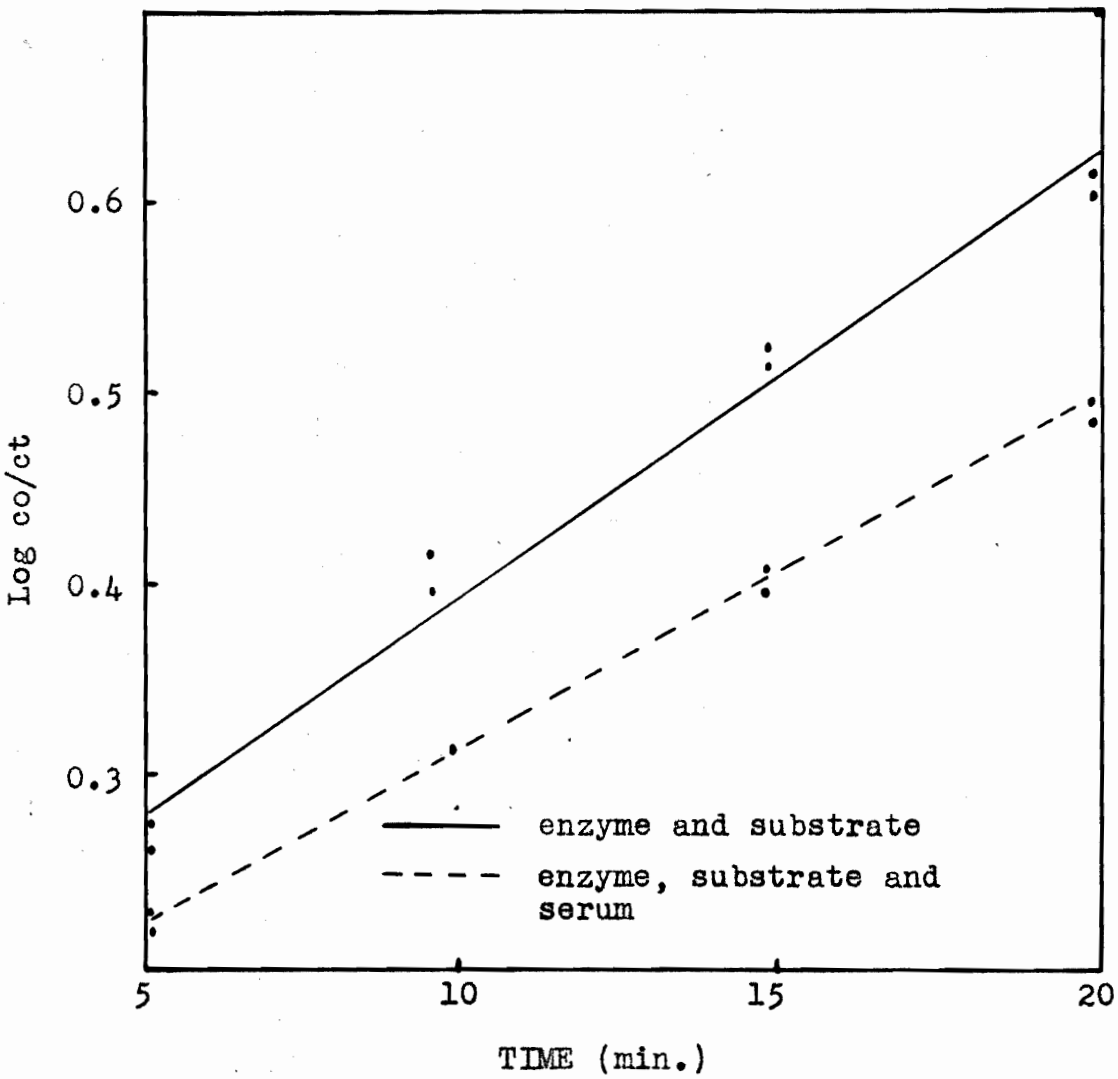

Figure 7. The enzymatic hydrolysis of hyaluronic acid plotted as a first order reaction. The lines were drawn by the method of least squares. The slope is equal to 2.303/k in each case.

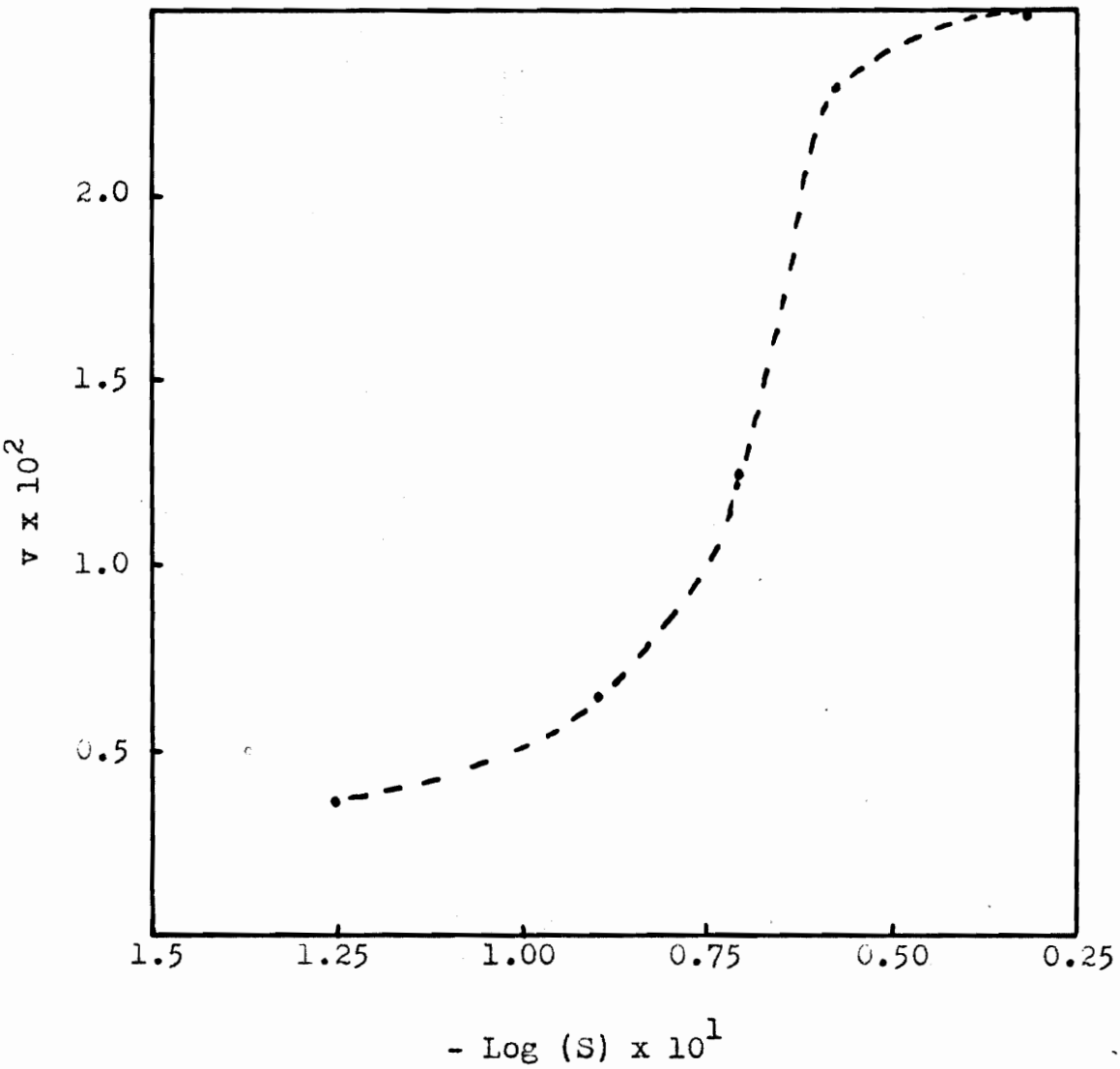

Figure 8. Michaelis - Menten log substrate - velocity curve for typical hyaluronidase catalyzed reaction.

accompanying standard deviations are shown in Table VIII. The large variances demonstrated in Table VIII indicates that the system is not especially well suited for such precise studies. However, the values obtained from these experiments are adequate to indicate the magnitude of the physical constants involved in the hyaluronidase catalyzed reaction.

TABLE VIII

Typical Values of Ks* and V** Obtained with Hyaluronidase Catalyzed Systems

Test #	Ks	V
1	3.30×10^{-2}	5.00
2	3.04×10^{-2}	4.08
3	2.27×10^{-2}	2.20
4	5.51×10^{-2}	6.00
5	5.90×10^{-2}	6.40
6	1.42×10^{-2}	1.56
7	5.30×10^{-2}	7.81

$Ks = 3.82 \times 10^{-2} \pm 1.75 \times 10^{-2}$

$V = 4.72 \pm 2.29$

*Ks = The dissociation constant of the enzyme substrate complex

**V = The maximum reaction velocity.

All tests were run either in duplicate or triplicate. It was found necessary to maintain the reactions at low temperature (4^0 to 6^0C). At higher temperatures, hence necessarily lower enzyme concentrations, the reaction proceeded in a

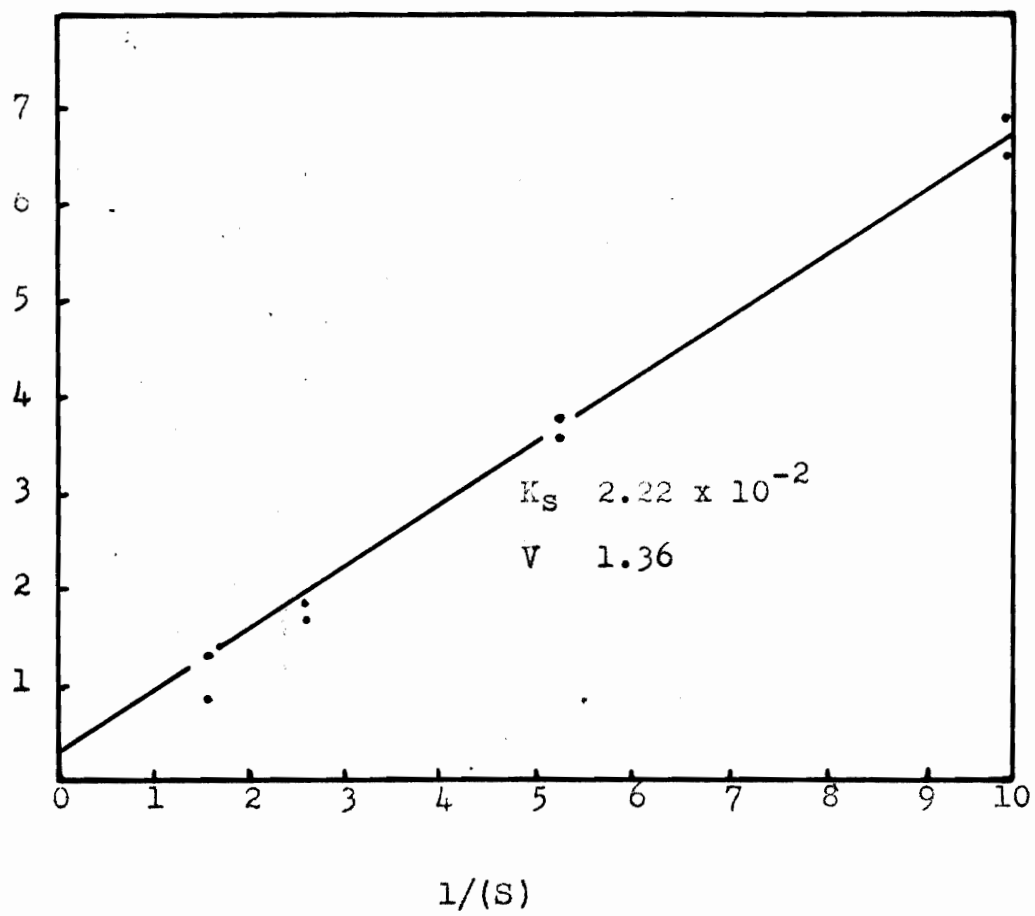

Figure 9. Calculation of max. velocity (V) and enzyme - substrate dissociation constant (K_S) by the method of Lineweaver and Burk. The line was drawn by the "least square" method.

manner analogous to a zero order reaction. It was only when the reaction velocity could be restricted (lower temperatures) that the enzyme concentration could be raised to the level where the reaction velocity would respond to changes in the substrate concentration.

C. The Energy of Activation

In 1887, Arrhenius pointed out that a reasonable equation for the variation of rate constant (k) with temperature might be

$$\frac{d \ln k}{dT} = \frac{Ea}{RT^2}$$

The quantity Ea is known as the energy of activation of the reaction.

If Ea can be assumed to be temperature independent the above equation can be integrated to the form

$$\log k = \frac{-Ea}{2.303R} \frac{1}{T} + A$$

where A is the constant of integration. This is the classical Arrhenius equation.

It is obvious from this that a plot of the logarithm of the rate constant (k) against the reciprocal of the absolute temperature should be a straight line with a slope equal to

$$\frac{-Ea}{2.303\ R}$$

From this data the activation energy for the reaction is readily calculated.

Figure 10 illustrates the Arrhenius diagram for the hyaluronidase catalyzed system. The slope of the straight line is 3.0×10^3 and from this the energy of activation (Ea) is calculated to be 13.7 K-calories.

D. The Effect of Temperature Upon the Degree of Inhibition

Ideally in temperature studies of this type the only variable in a system of enzyme, substrate, inhibitor, and time would be the temperature itself. It was reasoned, however, that if the time interval was kept constant, regardless of the various temperatures at which the tests were run, the end of each test would find the reaction in a different stage of completion. An enzymic reaction may be studied at different stages by varying the time interval of reaction. Table IX indicates the variation in percent inhibition found at various stages of substrate hydrolysis in a typical hyaluronidase catalyzed reaction.

The large standard deviation observed within the 5 minute group is to be expected. In this short interval those errors involved in the timing of the test are magnified. The overall standard deviation (5.06%) of the inhibitory values found at various stages of the reaction is rather large. For this reason the reaction intervals, at the various temperatures, were chosen so as to allow the hydrolysis of the substrate to proceed to the same degree of completion in every case.

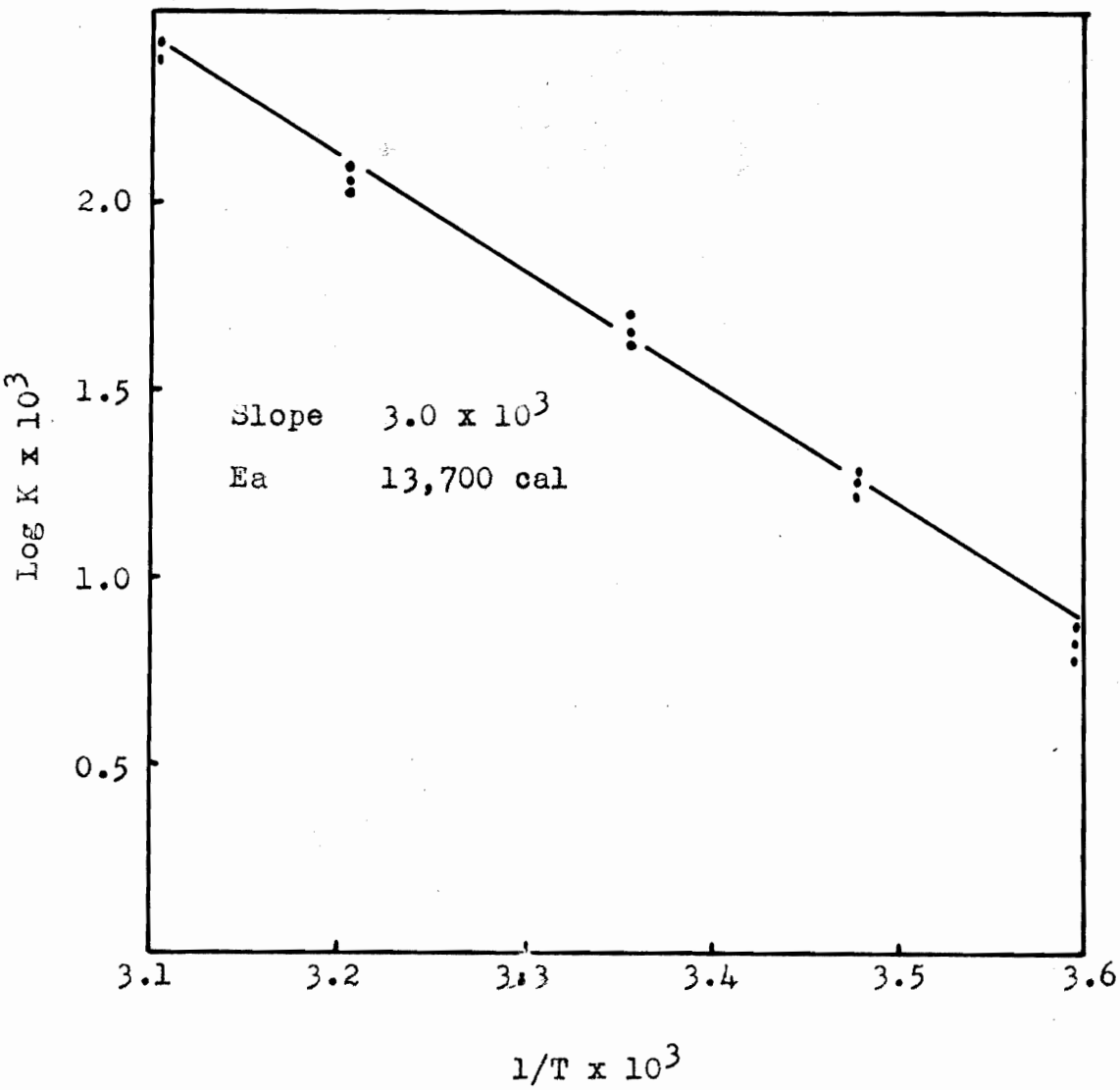

Figure 10. Arrhenius diagram for hyaluronidase - hyaluronic acid system.

The variables in these studies were, therefore, temperature and time. The results of the temperature experiments are shown in Figure 11 and 12.

TABLE IX

Per Cent Inhibition of Hyaluronidase at Various Stages of the Hydrolysis

Tube	Time	% Inhibition	Mean % Inhibition	Std. Dev. σ
1	5 min.	15		
2	5 min.	22	15.2	5.75 %
3	5 min.	8		
4	5 min.	16		
5	10 min.	21		
6	10 min.	21	23.5	2.90 %
7	10 min.	26		
8	10 min.	26		
9	15 min.	23		
10	15 min.	23	24.5	1.73 %
11	15 min.	26		
12	15 min.	26		
13	20 min.	19		
14	20 min.	19	19.0	3.25 %
15	20 min.	23		
16	20 min.	15		

Mean % Inhibition = 20.5 %

Overall Std. Dev. = ± 5.06 %

E. The Determination of Hyaluronidase Inhibitor in Monkey Serum

A linear relationship between hyaluronidase inhibited and the amount of inhibitor would seem to indicate that the blood plasma inhibitor forms a stoichiometric, non-dissociable

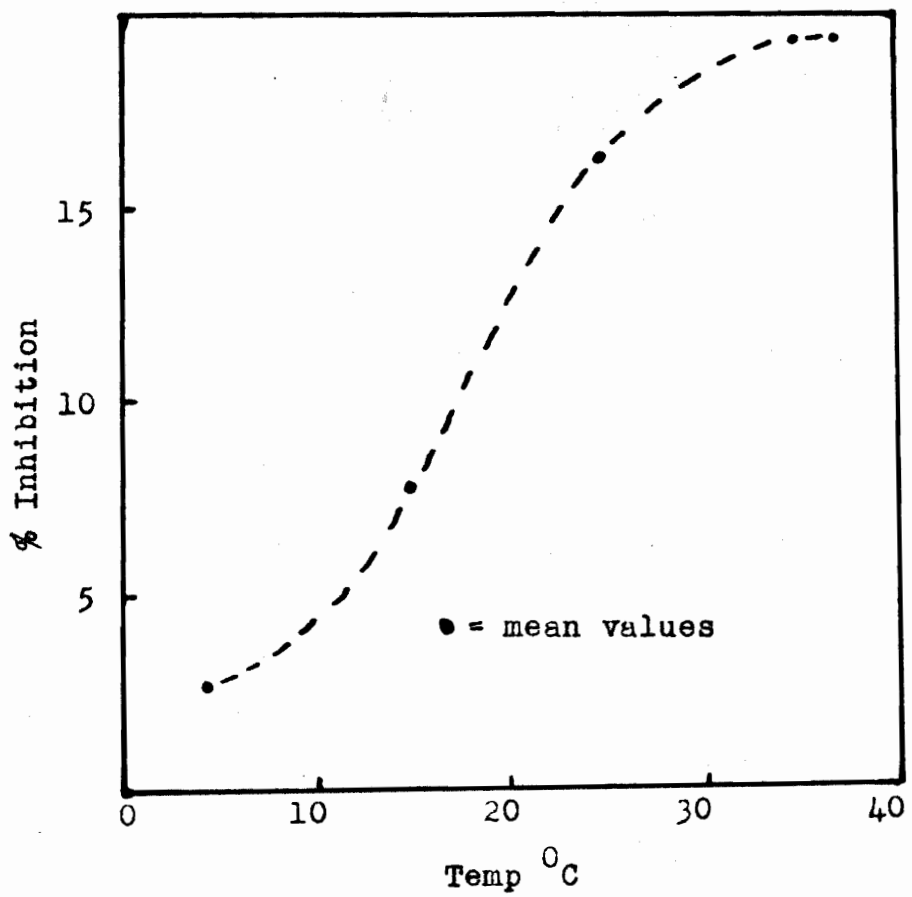

Figure 11. Effect of temperature upon inhibition. Per cent inhibition values calculated by "k" ratios.

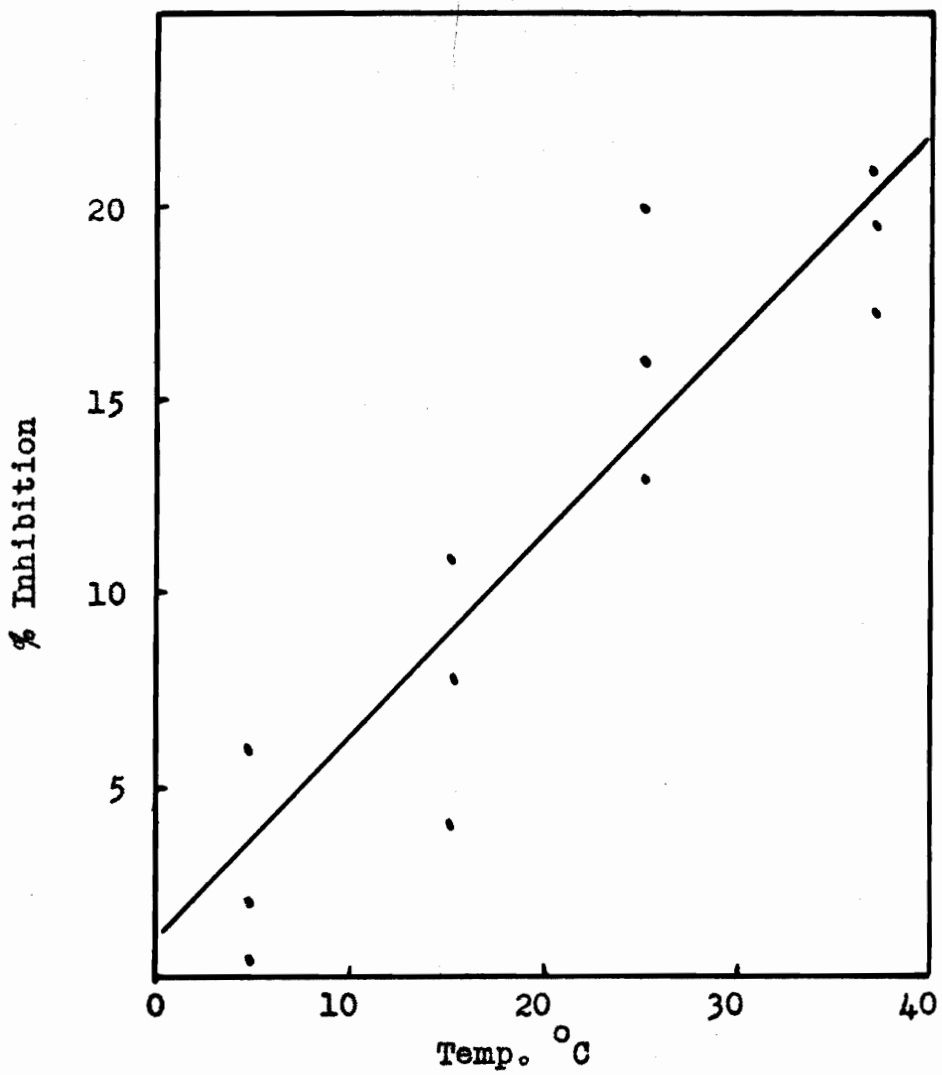

Figure 12. Same data as presented in Figure 11 calculated by the "least square" method.

compound with hyaluronidase. If this proved to be the case then a method similar to that of Kunitz (73), might be adapted to the determination of hyaluronidase inhibitor in animal sera. In order to determine if this linear relationship existed, various serum diluations were made with pH 6.0 buffer to obtain various inhibitor concentrations. The degree of hyaluronidase inhibition was expressed as per cent inhibition. This value can be determined either from the degree of substrate hydrolysis (Figure 13) or by means of the "k" values obtained from the first order relationship. The agreement between the values obtained by the two methods is fairly good as shown in Figure 14.

When one plots percentage inhibition versus decreasing inhibitor concentrations, the relationship between these values approaches a linear function (Figure 15). Unfortunately at the serum concentrations necessary to produce a completely linear relationship, the degree of inhibition can no longer be measured with precision.

F. The Type of Inhibition Involved in the Hyaluronidase Serum System

Inhibitors of enzymic reactions are frequently divided into two types: competitive and non-competitive. Competitive inhibition may be described as the competitive between the inhibitor and the substrate for specific groups on the enzyme; thus, the decrease in enzymic activity is dependent upon the relative concentration of both substrate

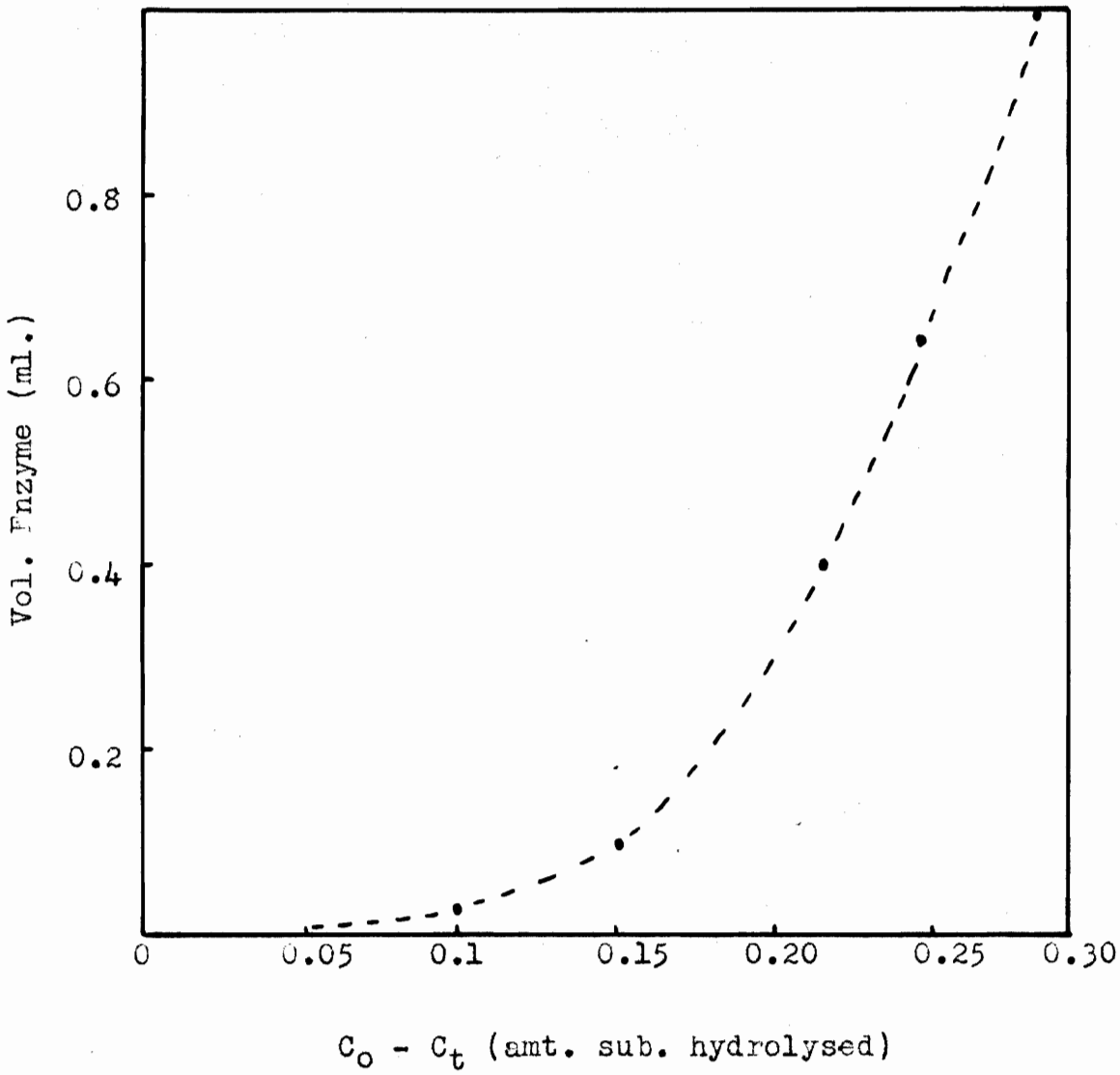

Figure 13. Relationship between amt. enzyme added and amt. sub. hydrolysed. Used to calculate percent inhibition (%I).

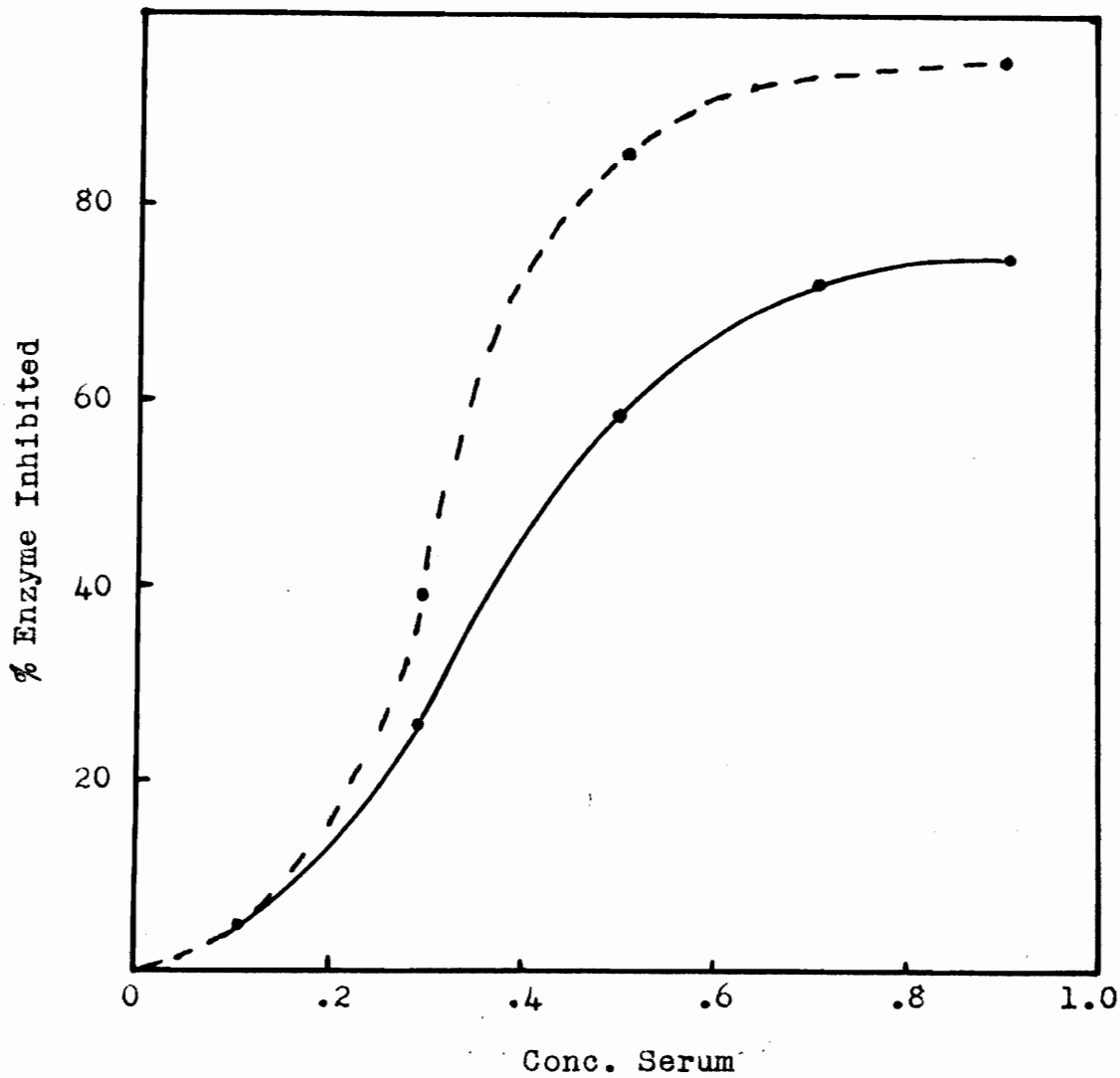

Figure 14. Demonstration of % inhibition values obtained using Figure 13 (dotted line) and "k" values (solid line) to determine enzyme inhibition.

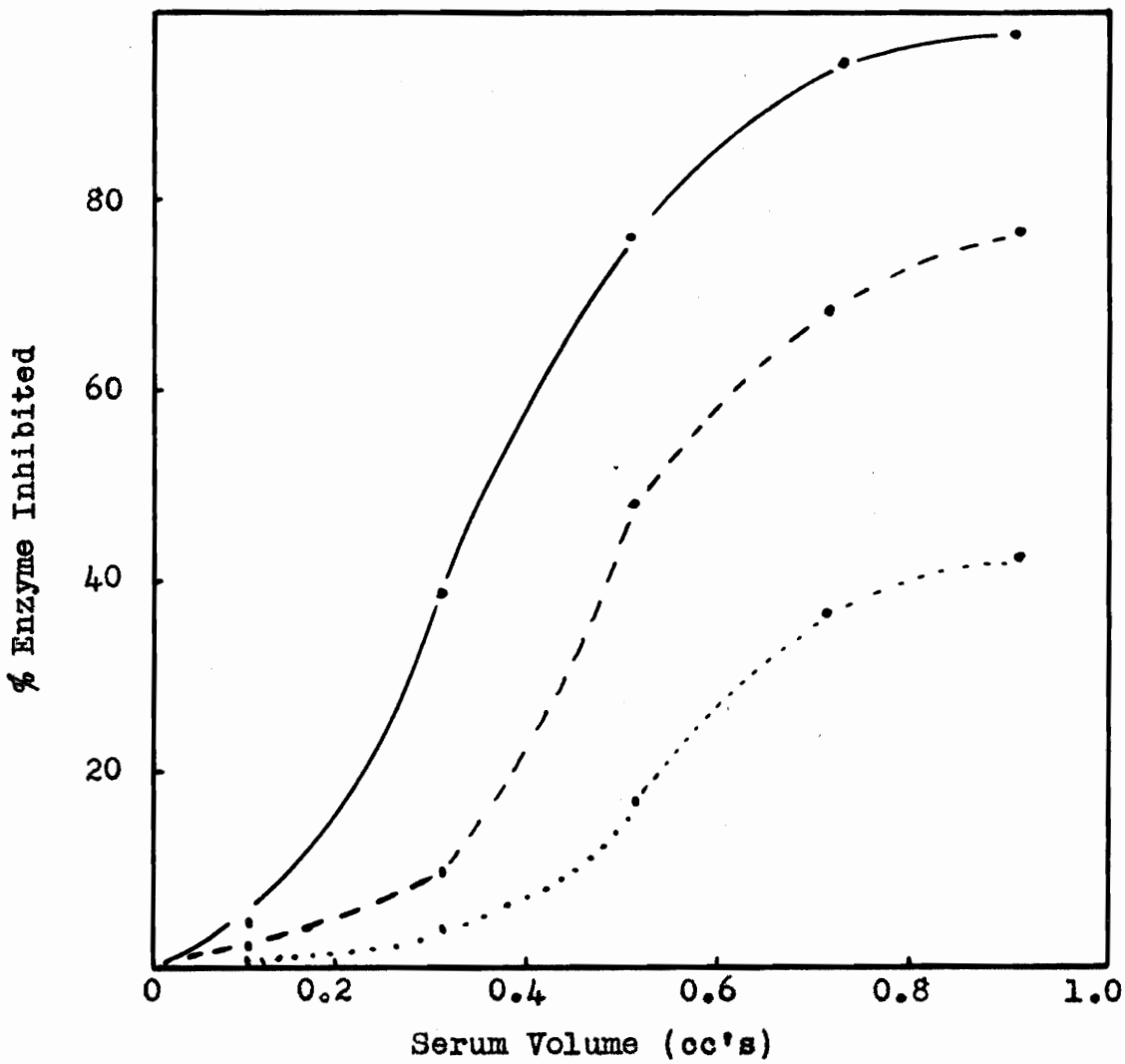

Figure 15. The effect of serum dilution and concentration upon the inhibition of hyaluronidase.

——— 1/5 dil. of serum
- - - - 1/10 dil. of serum
····· 1/15 dil. of serum

and inhibitor. In non-competitive inhibition the combination of inhibitor and enzyme does not interfere with the specific groups of the enzyme which bind the substrate and, hence, inhibition depends solely upon the concentration of the inhibitor.

The method used was based upon the mathematical formulation of Lineweaver and Burk (72), for competitive inhibition. Competitive inhibition is indicated by an increase in the slope of the reciprocal of the velocity versus the reciprocal of the substrate (1/v; 1/(S) plot accompanied by no significant change in intercept. Such is the case in the hyaluronidase-serum system as may be seen in Figure 16.

The effect of the competitive inhibitor is to increase the slope of the control line by the quantity $\frac{Ks\ (I)_1}{Ki}$

Ks = dissociation constant of (ES)

(I) = concentration of inhibitor

Ki = dissociation constant of (EI)

From this relationship it is possible to calculate Ki. In this case, however, the use of serum for inhibitor precluded an accurate evaluation of (I).

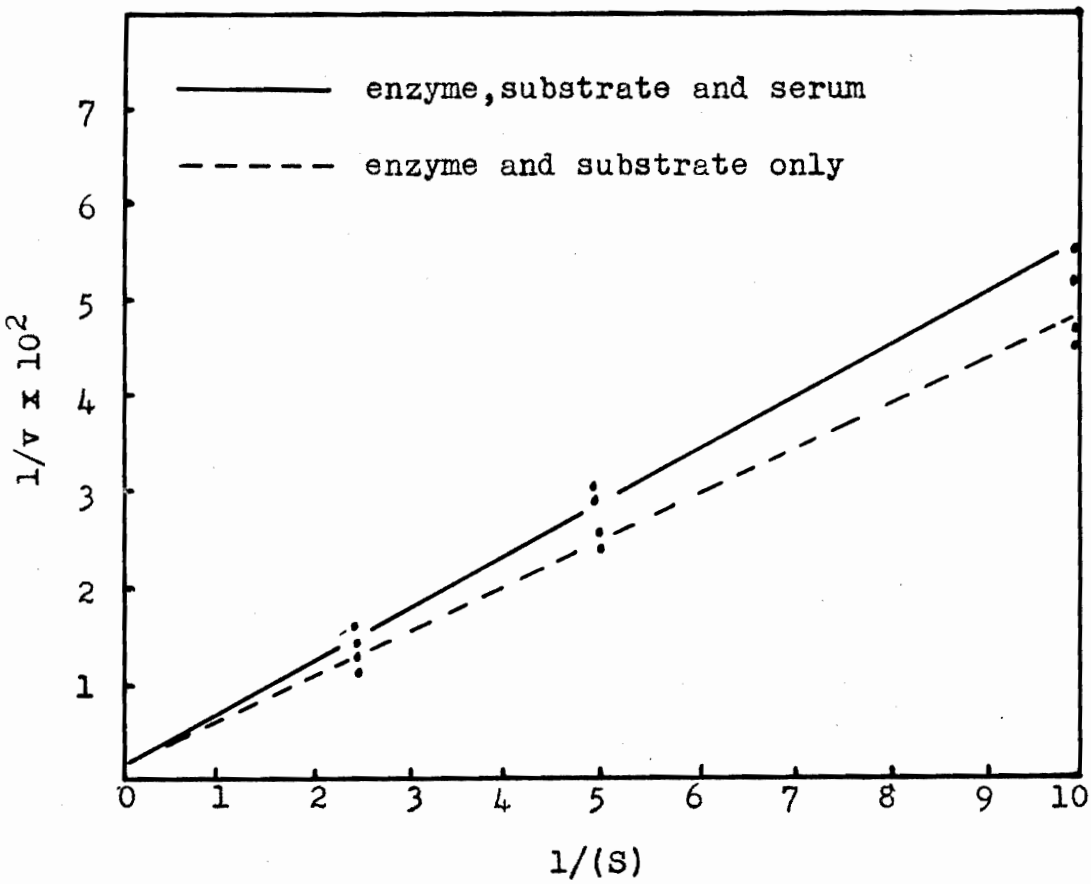

Figure 16. The competitive inhibition of hyaluronidase by normal monkey sera.

VI. DISCUSSION

The studies presented here were designed mainly to clarify two closely related occurences; the rise in the serum level of hyaluronidase inhibitor during the course of poliomyelitis and secondly, the physical nature of the inhibition of hyaluronidase by normal serum.

The fact that the blood of a number of mammalian species brings about the inhibition of hyaluronidase has been known for some. time. This occurence is due to the presence of a "physiological" or non-specific inhibitor as contrasted to the specific antibody produced in response to the antigenic stimulus of some specific hyaluronidase. A rise in the serum level of this non-specific inhibitor has been observed in monkeys injected with the virus of poliomyelitis by way of the intranasal and intracerebral routes. Those animals which failed to exhibit clinical poliomyelitis, as well as all the animals injected by means of the gastro-intestional route, demonstrated no rise in physiological inhibitor levels.

Animals injected via the intracerebral and intranasal routes rarely show a virus antibody titer in less than two months following injection. On the other hand, those animal injected via the gastro-intestional route routinely exhibit a titer shortly preceeding or concomittant with the first symptoms of paralysis. The possibility of a reciprocal relationship existing between virus antibody and enzyme inhibitor (mentioned in the experimental results) should not be ignored. If this relationship could be assumed to be correct

it would appear that the presence of humoral virus antibody may be related to the failure of physiological inhibitor levels to rise. The presence of a group common to both the virus particle and the physiological inhibitor molecule might then be speculated upon.

The rise in the serum inhibitor levels of injected animals was observed to be rapid. This rise either occured with or preceeded shortly the first signs of paralysis. This, in conjunction with the observation that no inhibitor rise occured in animals not exhibiting clinical symptoms, would appear to indicate that the rise in inhibitor is not a defense mechanism but, on the other hand, a consequence of cellular damage.

The presence of both hyaluronidase and hyaluronic acid in some tissues (75), suggests the consistancy of the ground substance may be controlled by a delicately balanced hyaluronic acid-hyaluronidase system. What role the physiological inhibitor plays in such a system is not understood as yet.

The role of hyaluronidase in viral infections is believed by some to be different than that in bacterial infections. The spreading effect of the hyaluronidase can only aid the virus in view of its intracellular habitat whereas the same effect serves to render most bacteria more readily accessible to the host defense mechanisms. Duran-Reynals (19), has stated that the critical concentration of virus per unit area is an infectious unit of virus. This

opinion is based upon the observation that testicular extract always enhanced and never suppressed the lesions of vaccinia, Shope fibroma and Virus III.

Relatively little information is as yet available regarding the chemistry of the hyaluronidase inhibitor of mammalian sera. The various purification procedures are of small value because of the apparent instability of this substance. The fact that this inhibitor is heat labile has, in fact, been used to distinguish this activity from the antibodies formed against specific hyaluronidase which are much more heat stabile. The possiblity that the inhibitory properties of serum were due to the presence of the breakdown-products of hyaluronic acid was investigated. The results obtained from these experiments indicate this is extremely unlikely.

The action of serum on hyaluronidase remains an intriguing but poorly understood phenomenon. Although frequent reference is made to non-specific or "physiological inhibitor" nothing is known as yet of the in vivo action of this substance.

Inhibition of hyaluronidase by normal human sera has been ascribed to salt formation between serum albumin and hyaluronic acid suggesting that inhibition was primarily due to an effect upon the substrate rather than on the enzyme (11). Haas (35), on the other hand, concludes (on the basis of time and temperature) that the inhibitory substance in the blood is an enzyme. The results of the kinetic studies reported here indicate that neither of the

above theories is correct.

The enzymatic hydrolysis of hyaluronic acid was found to be essentially first order in nature when a highly refined substrate preparation was utilized under optimal conditions. It was found, however, to be possible for the reaction to proceed as a zero order reaction in the presence of a large excess of substrate. It is not unlikely that this is the case in vivo. The effect of temperature upon the reaction in the presence and absence of inhibitor was observed. The data thus obtained proved adequate for the calculation of the heat of activation; an important physical constant. A refined turbidimetric method was devised which proved precise enough to measure the substrate enzyme dissociation constant by either the classical method of Michaelis and Menten (74), or the more refined method of Lineweaver and Burk (72). From this same type of experiment it was possible to establish the inhibition of hyaluronidase by serum as being competitive in nature.

The preciseness of the results obtained in these kinetic studies, especially those in which serum was used, would seem to indicate that the inhibitory substance is less complex in nature than previously thought. On the basis of these observations it is felt that more sophisticated methods might be successfully applied toward the elucidation of the nature of the physiological inhibitor of the serum.

VII. SUMMARY

1. The nature of the rise of non-specific hyaluronidase in poliomyelitis infected monkeys was established.

2. Animals infected with the virus of poliomyelitis by means of the gastro-intestional route failed to exhibit a rise in non-specific inhibition. Those animals infected by either the intranasal or intracerebral routes exhibited a rise in serum inhibitor levels at or near the onset of paralysis.

3. A possible relationship between virus antibody levels and the level of physiological inhibitor is suggested.

4. The kinetics of the enzymic hydrolysis of hyaluronic acid was studied in some detail by means of an improved turbidimetric method. The reaction order, enzyme-substrate dissociation constant, and the energy of activation were obtained by means of these studies.

5. The kinetics of the inhibition of hyaluronidase by normal monkey serum were studied. The type of inhibition involved in this reaction was found to be competitive in nature.

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