CONCENTRATION AND ACID-ALKALINE EFFECTS ON RIBONUCLEIC ACID INHIBITION OF ANTI-Rh ANTIBODIES

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Lois Leona Conrad

1962

This is to certify that the

thesis entitled

CONCENTRATION AND ACID-ALKALINE EFFECTS
ON RIBONUCLEIC ACID INHIBITION OF ANTI-Rh ANTIBODIES

presented by

LOIS LEONA CONRAD

has been accepted towards fulfillment
of the requirements for

PH.D. degree in BIOLOGICAL SCIENCE

Major professor

Date May 3, 1962

O-169

CONCENTRATION AND ACID-ALKALINE EFFECTS ON RIBONUCLEIC ACID INHIBITION OF ANTI-Rh ANTIBODIES

By

Lois Leona Conrad

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Division of Biological Science

1962

ABSTRACT

CONCENTRATION AND ACID-ALKALINE EFFECTS
ON RIBONUCLEIC ACID INHIBITION OF ANTI-Rh ANTIBODIES

by Lois Leona Conrad

It was the purpose of this study to conduct a further investigation of three inhibitor substances discovered by Hackel et al., namely, adenosine-2'-3'-monophosphate (AMP), cytidine-2'-3'-monophosphate (CMP) and uridine-2'-3'-monophosphate (UMP) and their effects at various concentrations and pH's on antibodies in the Rh system. The original work involved the inhibitory effects of these substances on various antisera at a 2% concentration and a pH 6.8. This investigation includes adenylic, cytidylic and uridylic acids at concentrations of 2%, 4%, 6% and 8% at the following pH's: 6.6, 6.8, 7.0, 7.2, 7.4 and 8.0. The Rh antisera involved were anti-D, anti-C and anti-E. The inhibitory effects were tested by use of the hemagglutination inhibition.

Increased inhibition of all three ribonucleic acid derivatives with anti-Rh sera was demonstrated with increase in concentration. It was postulated that the more inhibitor molecules in solution, the more antibody-inhibitor combinations would occur. This would leave less antibody to combine with the specific antigen on the erythrocytes.

Also, the results indicated that a monomolecular reaction was taking place between antibody and inhibitor molecule because of the steady rise in inhibition as the concentration of the inhibitor substance was increased from 2% up to 8%.

This investigation further demonstrated that alkaline pH's of all three inhibitors resulted in an increase of their inhibitory effectiveness. It was speculated that a certain amount of hydroxyl ions must be of more importance than hydrogen ions in effecting this inhibition reaction. It was further postulated that the ability of the hydroxyl ion to remove hydrogen atoms from the inhibitor molecule or antibody molecule could effect a chemical combination. Suggested bonds were hydrogen bonds through amino and/or carboxyl groups, nitrogen-nitrogen bonds, and oxygen bonds through phosphate groups.

The specificity of an antibody for an antigen may depend not only upon the determinant groups within each molecule but also upon the spatial arrangement of these groups. Thus, the fact that one inhibitor was more effective than another could be explained further by the spatial arrangements of the determinant groups within the molecules. For example, the weaker inhibition of one inhibitor compared to another could be due to the failure of oppositely charged groups to correspond perfectly in position because of differences in the spatial arrangements of the determinant groups within each individual inhibitor molecule.

Taking into consideration both increased concentration and alkaline pH, adenylic acid was found to be the most effective inhibitor with anti-D, anti-C and anti-E sera. Cytidylic and uridylic acid were almost equal in inhibitory effects. All of the inhibitors were most effective with anti-E and anti-D sera, respectively, and were least effective with anti-C.

CONCENTRATION AND ACID-ALKALINE EFFECTS ON RIBONUCLEIC ACID INHIBITION OF ANTI-Rh ANTIBODIES

By

Lois Leona Conrad

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Division of Biological Science

1962

ACKNOWLEDGMENTS

Most deeply and most directly, I am indebted to Dr. Emanuel Hackel, my advisor, for his many suggestions, patience and guidance during this thesis work. Also, I wish to express my appreciation to the other members of my committee, Dr. Rollin H. Baker, Dr. Richard U. Byerrum, Dr. Herman L. King, Dr. Chester A. Lawson and Dr. John M. Mason, for their helpful suggestions and criticism. I am further grateful to Dr. Amos Cahan of Knickerbocker Biologicals and Dr. Philip Levine and Robert T. McGee of Ortho Pharmaceutical Corporation who donated the serum used in this experimentation, as well as to all others who assisted me in any way during the writing of this thesis.

TABLE OF CONTENTS

Page

LIST OF TABLES. iv

LIST OF GRAPHS. v

LIST OF FIGURES vi

CHAPTER

I. INTRODUCTION. 1

II. REVIEW OF THE LITERATURE. 5

History of the Rh Factor. 5
Nature of Rh Antigens 12
Nature of Rh Antibodies 17
Hemagglutination Inhibition Studies 24

III. THE EXPERIMENTATION 28

Materials and Methods Used. 28
Procedure 29
Results 33
Discussion. 39

IV. SUMMARY 74

BIBLIOGRAPHY. 77

LIST OF TABLES

Table — Page

I. AMP versus Anti-D; % Inhibition 47

II. AMP versus Anti-C; % Inhibition 48

III. AMP versus Anti-E; % Inhibition 49

IV. UMP versus Anti-D; % Inhibition 50

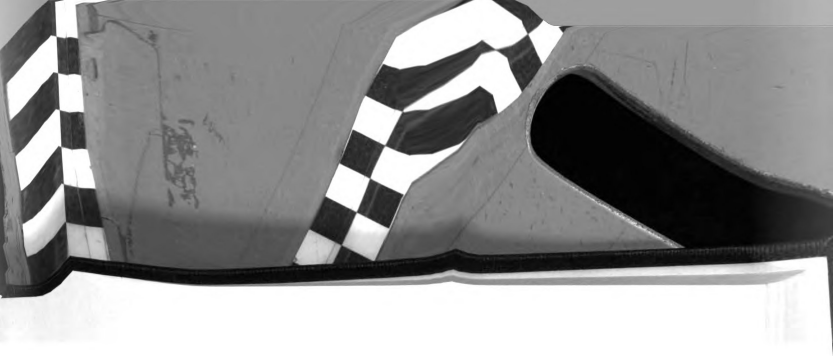
V. UMP versus Anti-C; % Inhibition 51

VI. UMP versus Anti-E; % Inhibition 52

VII. CMP versus Anti-D; % Inhibition 53

VIII. CMP versus Anti-C; % Inhibition 54

IX. CMP versus Anti-E; % Inhibition 55



LIST OF GRAPHS

Graph		Page
Ia.	AMP versus Anti-D; % Concentration.	56
IIa.	AMP versus Anti-C; % Concentration.	57
IIIa.	AMP versus Anti-E; % Concentration.	58
IVa.	UMP versus Anti-D; % Concentration.	59
Va.	UMP versus Anti-C; % Concentration.	60
VIa.	UMP versus Anti-E; % Concentration.	61
VIIa.	CMP versus Anti-D; % Concentration.	62
VIIIa.	CMP versus Anti-C; % Concentration.	63
IXa.	CMP versus Anti-E; % Concentration.	64
Ib.	AMP versus Anti-D; pH of Inhibitor.	65
IIb.	AMP versus Anti-C; pH of Inhibitor.	66
IIIb.	AMP versus Anti-E; pH of Inhibitor.	67
IVb.	UMP versus Anti-D; pH of Inhibitor.	68
Vb.	UMP versus Anti-C; pH of Inhibitor.	69
VIb.	UMP versus Anti-E; pH of Inhibitor.	70
VIIb.	CMP versus Anti-D; pH of Inhibitor.	71
VIIIb.	CMP versus Anti-C; pH of Inhibitor.	72
IXb.	CMP versus Anti-E; pH of Inhibitor.	73

LIST OF FIGURES

Figure Page

1. The structure of 3'-monophosphates is illustrated. The 2'-monophosphates differ in that the phosphate group is attached to the 2'-carbon of the sugar rather than the 3'-carbon. 3

2. Sample illustration of the test set-up and scoring procedure. 32

CHAPTER I

INTRODUCTION

A number of investigators, Kabat (56) and Morgan et al. (2, 3, 4, 39, 40), have found the technique of specific serological inhibition valuable in characterization of A, B, H and Lewis substances from various sources. Through such a technique it was possible for these investigators, during purification procedures of blood group substances, to determine in which body fluids and/or tissues these substances were distributed. In addition, this technique was of value, during the assays, in determining when degradation of the blood group substances was occurring. It was possible by this knowledge to avoid procedures that would cause degradation. These investigators demonstrated that isolated blood group substances can prevent their specific antibodies from causing agglutination.

Hackel et al. (46, 48) have discovered chemical substances other than isolated blood group substances which would specifically produce inhibition with anti-Rh and Lutheran sera. By adding one of these chemical substances to a serum with a specific antibody, it was possible for them to demonstrate that the resulting hemagglutination reaction was decreased. In other words, erythrocytes

added to an antibody by which they would be agglutinated, were not as strongly agglutinated in the presence of these substances as would have been the case without the addition of the chemical substances.

Their investigations included testing sixty different reagents consisting of sugars, amino acids, short chain polypeptides, purines, pyrimidines, desoxyribonucleic acid (DNA) derivatives and ribonucleic acid (RNA) derivatives. They found one nucleoside (cytidine) and three nucleotides (adenylic, cytidylic and uridylic acids), all ribonucleic acid derivatives, effective in the inhibition of anti-Rh and anti-Lutheran sera. As pointed out by Hackel et al. (48, p. 407), "With respect to the conceptual scheme which places ribonucleic acid in the key position between desoxyribonucleic acid as genetic material and protein as phaenogenetic material, there have been few demonstrations of specificity attributable to ribonucleic acid."

It was the purpose of this study to conduct a further investigation of three of these inhibitors discovered by Hackel et al. (48), namely, adenosine-2'-3'-monophosphate (AMP), cytidine-2'-3'-monophosphate (CMP) and uridine-2'-3'-monophosphate (UMP) and their effects at various concentrations and pH's on antibodies in the Rh system.

These substances are all derivatives of ribonucleic acids which occur, primarily, in the cytoplasm of living cells and are also the main component in some viruses.

Ribonucleic acids are thought to play an active role in protein synthesis of the cell. They consist of a nitrogenous base (either a purine or pyrimidine), a sugar (d-ribose) and a phosphoric acid group. The bases used in this particular study were either adenine, cytosine or uracil. Figure 1 illustrates the chemical structures of the inhibitors used in this investigation. Mixtures of 2'-3' derivatives were employed by Hackel in his early investigations and were used in this study.

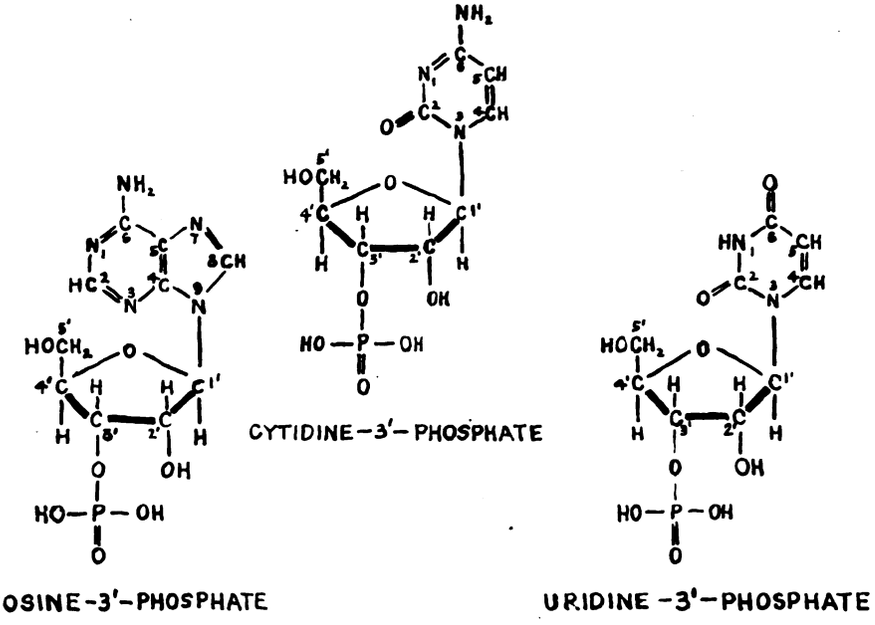


Fig. 1. The structure of 3'-monophosphates is illustrated. The 2'-monophosphates differ in that the phosphate group is attached to the 2'-carbon of the sugar rather than the 3'-carbon.

It was thought that through a further investigation of the effects of these inhibitors by varying their concentration and pH, it might be possible to gain a better understanding of the chemical nature of the antigens involved, as well as the immunological specificity. It is realized, however, that the problem chosen by this author is but one of many facets of the total situation involving antigen-antibody reactions.

CHAPTER II

REVIEW OF THE LITERATURE

I. History of the Rh Factor

Darrow (29), in 1938, made a review of the literature and an analysis of published data concerning hemolytic disease of the newborn. He was one of the first to conclude that an appropriate etiological explanation for this disease could be attributed to an antigen-antibody reaction. The kind of reaction involved was determined the following year in 1939.

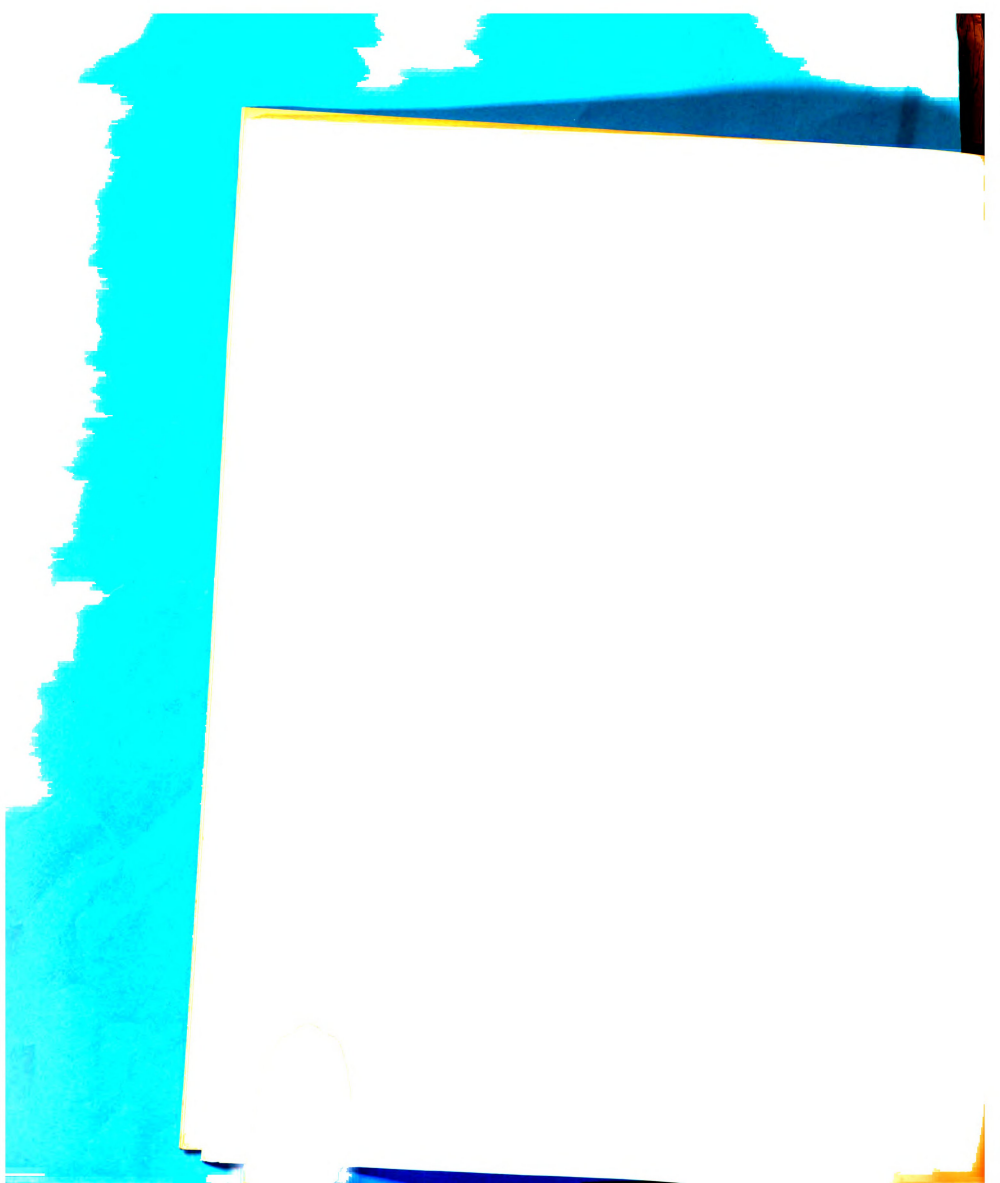
The beginning of an unierstanding of the Rh factor was the work of Levine and Stetson (66) in 1939. They studied the blood of a woman who had given birth to a fetus which had been dead for six weeks prior to delivery. An unusual antibody was found in this patient's serum which agglutinated the red blood cells of 80% of the other individuals tested having the same A-B-O group as that of the patient. It was thought, by them, that the retention of the dead fetus was responsible for the unusual maternal antibodies. They concluded that the fetus had inherited an antigen from the father which caused this maternal immunization.

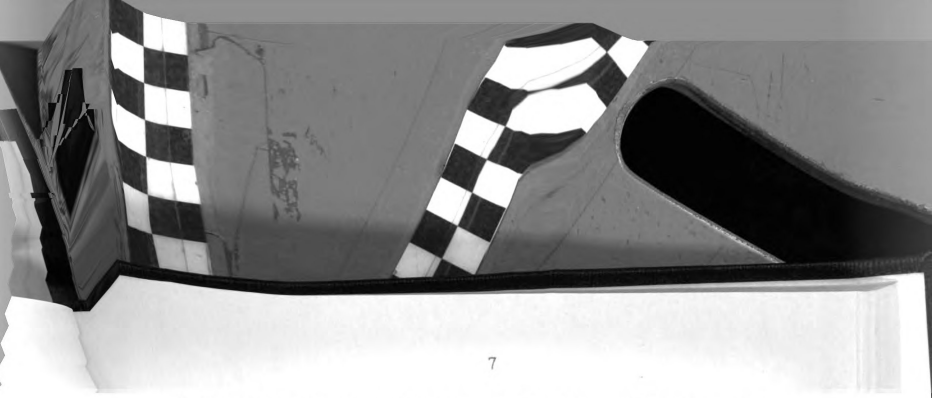
However, the true implication of their discovery and interpretations were not clear at the time. As mentioned by Race and Sanger (76, p. 116), "had Levine and Stetson given a name to the blood group system which they had discovered, it, and not Rh, would have been the title to [their] chapter and of a thousand other publications."

In 1940, Landsteiner and Wiener (58) injected rabbits and guinea pigs with blood cells from the rhesus monkey (Macaca mullata). They found after absorption of the rabbit serum to remove antibodies characteristic of the species that there was left an agglutinable factor which agglutinated not only the monkey red blood cells but also 85% of the blood of white people tested in New York City. The authors named this new agglutinin, anti-Rh, after the rhesus monkey whose blood cells had produced the original antibody. Thus, those people whose blood cells were agglutinated by this antibody were called Rh positive, whereas those, whose cells were not agglutinated by this antibody, were known as Rh negative individuals.

Wiener and Peters (88) studied the blood of three patients who suffered severe transfusion reactions in spite of receiving compatible A-B-O group blood. They demonstrated the presence of antibodies whose action resembled that of antibodies in the original anti-Rh serum.

Davidsohn and Toharsky (30) and Murray (73) demonstrated through absorption experiments, antigenic differences





between the Rh factors in the blood of man and that of the rhesus monkey. Essentially, they found that the animal antisera, after absorption with human Rh positive cells, also contained agglutinins for rhesus erythrocytes. Fisk and Ford (37) also confirmed the earlier fact that there were differences in their demonstrations with cord blood of 312 infants. All of them gave positive reactions with serum derived from guinea pigs, whereas 8.8% gave negative reactions to one human serum, and 13.6% to another.

Thus, it was obvious that the anti-Rh serum derived from animals was related to the human anti-Rh variety, but was not identical to it. In fact, for this very reason, anti-Rh serum derived from animals cannot be used today in accurate typing of human bloods for the Rh factor.

In 1941, Levine, Katzin and Burnham (64) examined bloods of sixteen women who had given birth to fetuses thought to have died of erythroblastosis fetalis (hemolytic disease of the newborn attributable to the Rh factor). They found that fourteen of these women were Rh negative, six had demonstrable agglutinins and that all of the fathers and fetuses tested were Rh positive.

Later the same year, Levine, Katzin and Vogel (65) summarized their observations and presented blood studies on 153 women who had given birth to infants with hemolytic disease. Ninety-three per cent of these patients were Rh negative and seven per cent were Rh positive. Of 141 women

tested, 42 showed the presence of anti-Rh agglutinins. Many, who had no agglutinins, had not been pregnant for over a year; furthermore, all of the 80 fathers and 76 affected infants were Rh positive.

From the observations cited above and similar ones by other investigators (8, 50, 60, 78), the role of the Rh factor in blood transfusions and hemolytic disease of the newborn became increasingly apparent.

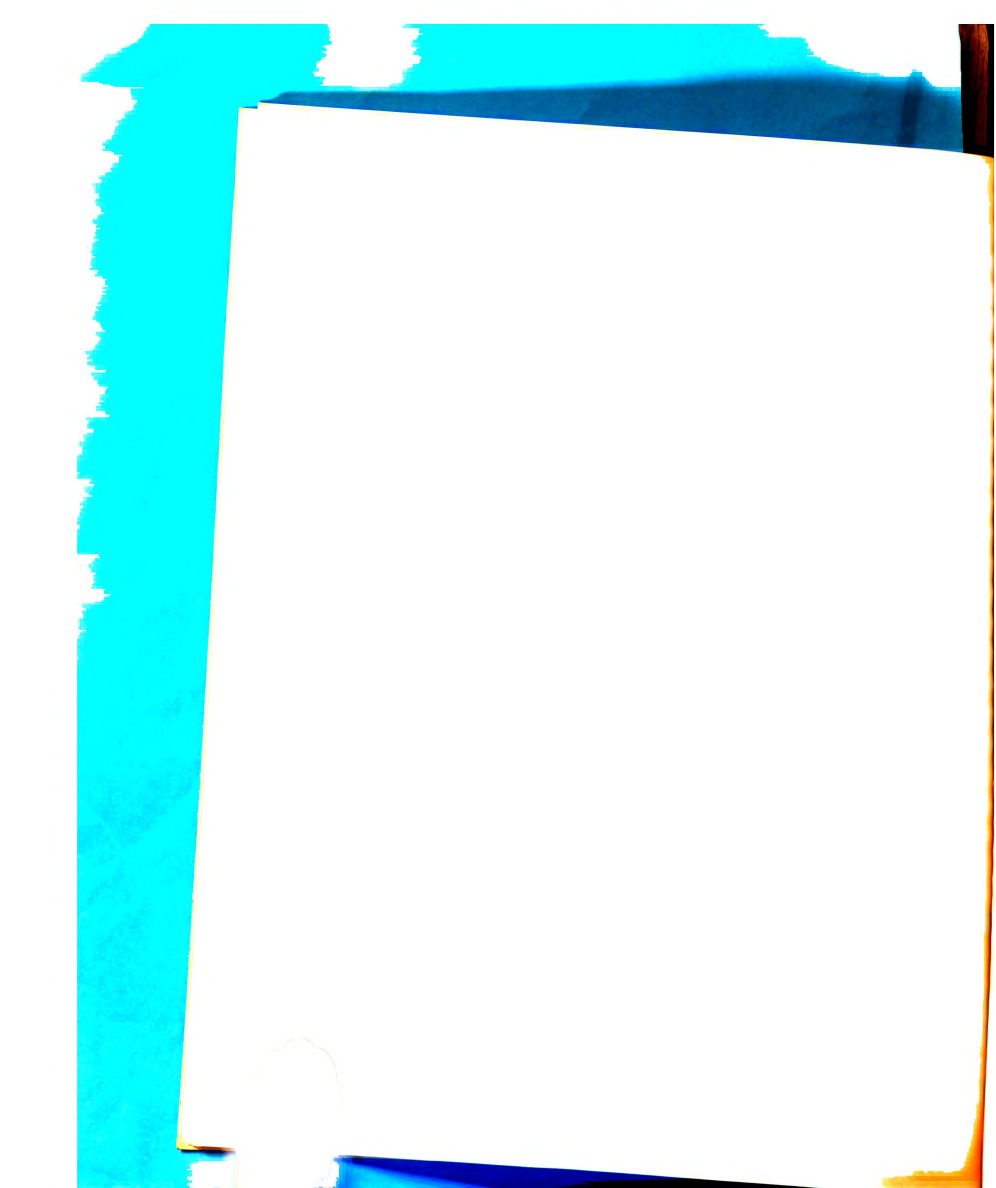
It was evident that the Rh agglutinin was not a naturally occurring antibody but was produced by Rh negative individuals upon introduction of the antigen into their systems. This was brought out by the fact that Rh negative individuals who had received one or more blood transfusions displayed Rh positive antibodies.

Also, it became clear that the phenomenon of erythroblastosis fetalis occurred in a situation where the Rh negative mother was carrying an Rh positive fetus, with the Rh positive factor necessarily inherited from the father. It was thought that the Rh antigens of the red blood cells of the fetus, in some way, crossed the placenta, escaping into the maternal circulation. Since the mother lacked these antigens, antibodies would be produced against these foreign intruders. These antibodies, then, could pass back through the placenta into the circulation of the fetus where they combined with and coated the red blood cells of the fetus, destroying them by hemolysis.

It was noted that one or two incompatible pregnancies were usually required to immunize the mother which meant that the first and second Rh positive infants were quite frequently unaffected. It is now thought that this phenomenon concerning the effect of the antibody on the fetus depends upon the titer (quantity) and avidity (strength) of the antibody present in the maternal circulation.

Besides this, it was discovered that many of these Rh negative women exposed to this kind of antigenic stimuli never became immunized. It would seem that it was due to the fact that the maternal circulation was not exposed to the antigen on the erythrocytes of the fetus; but why and how this occurs in some cases and not in others is not known as yet.

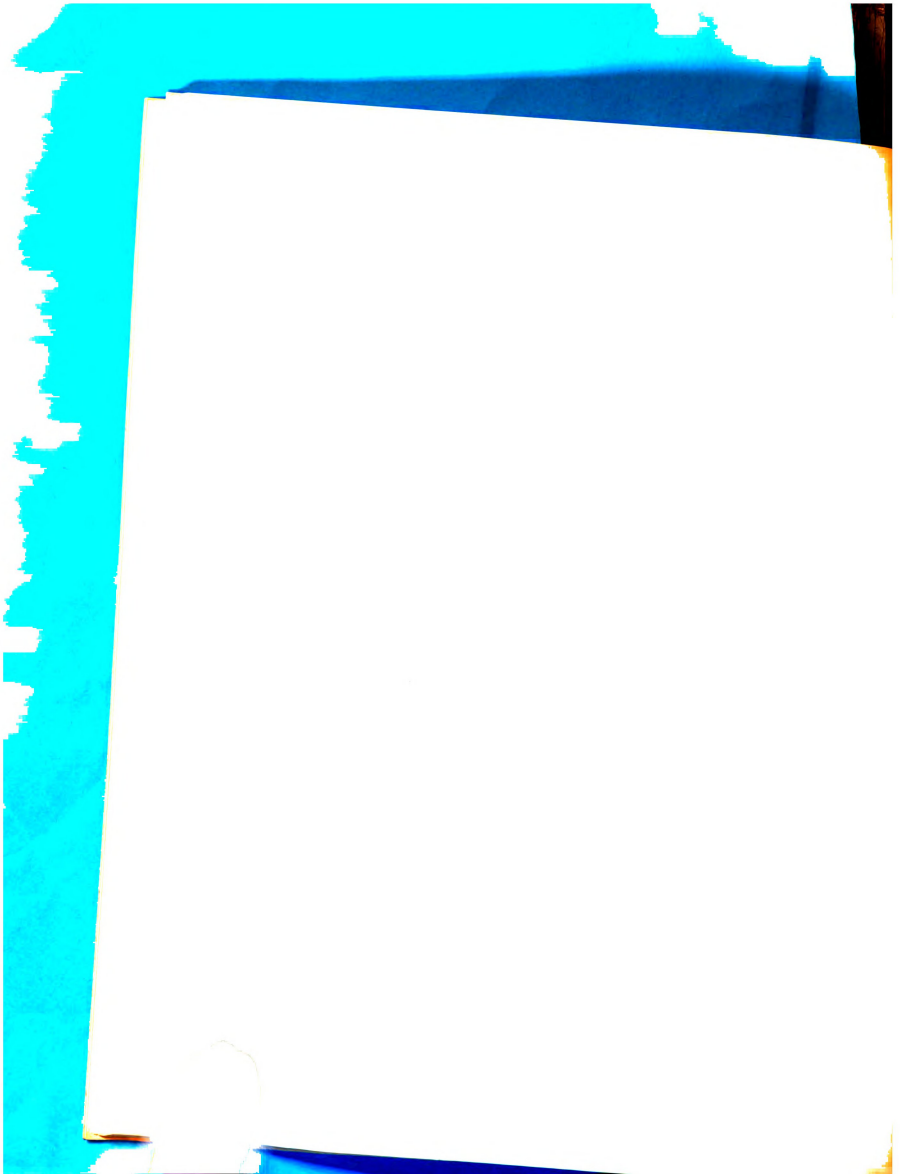
Researchers began to realize that there was apparently more than one variety of Rh factor. In 1941, Levine et al. (65) made mention that not all Rh antibodies found in all human serums were identical. They found blood that was Rh positive to anti-Rh serum and also contained Rh agglutinins of a different specificity. Landsteiner and Wiener (59) described and verified this and commented on the fact that human serums varied to some extent in their ability to cause agglutination. Still another variety was discovered by Race et al. (79) in 1943.



In 1943 Fisher, cited in Race and Sanger (76), postulated allelic genes which would produce antigens and in appropriate individuals would cause the production of antibodies. He further postulated that these antibodies, produced as a result of antigens that originated from allelic genes, would give an antithetical reaction. Thus, he recognized the fact that two of the anti-Rh sera described by Levine et al. (65) and also by Landsteiner and Wiener (59) were antithetical and gave them the names, anti-C and anti-c. Since the two remaining sera, the original anti-Rh described by Landsteiner and Wiener in 1940 (58) and that discovered by Race, did not react in an antithetical manner, he further postulated that they, too, had antithetical forms. He called the original Rh antibody, anti-D, and the latter one, anti-E. The postulated antithetical forms he labelled anti-d and anti-e.

Anti-e was discovered by Mourant in 1945 (72) as predicted. Diamond, cited in Race and Sanger (76), in 1946, first reported anti-d followed by two other examples reported by Haberman et al. (43) in 1948. However, later, considerable doubt was raised as to the specificity of the anti-d sera.

Fisher theorized that there were three genes with contrasting alleles responsible for the antigens C, c, D, d, E and e found on the red blood cells. He felt that these genes were closely linked. Wiener disagreed with this,



pointing out that instead of three genes, only one gene with multiple alleles could be responsible for the expression of the Rh antigens on the red blood cells. However, as Race and Sanger (76, p. 103) later stated in 1958, "The existence of three sites where Mendelian substitution can go on seems to us unassailable, and to argue whether the three sites are to be placed within or without the boundary of one gene appears particularly unprofitable at the present time when no one seems to know what the boundaries of a gene are."

In addition to the three main antibodies and the antithetical forms already mentioned, subgroups of these were found, most of them being fairly rare. This author shall limit the discussion to those already mentioned since these are the only ones considered in the experimentation of this paper. A very thorough discussion of the other variant forms of anti-Rh can be found in Race and Sanger (76).

Nomenclature in early investigations presented no problem, but, with the addition of more and more information, complications began to arise which necessitated a more standardized nomenclature. The following is an example of the terminology systems which are now widely used:

Gene Combinations	
Fisher	Wiener
DCe	R_1
DcE	R_2
Dce	R_o
DCE	R_z
dce	r
dCe	R'
dcE	R"
dCE	R_y

Rh Antigens	
Fisher	Wiener
D	Rh_o
C	rh'
E	rh"
d	Hr_o
c	hr'
e	hr"

II. Nature of Rh Antigens

As has been pointed out previously, Rh antigens are present in erythrocytes of Rh positive and negative individuals. Investigators such as Bornstein and Israel (10), Diamond (32), Potter (74), and Stratton (82) have demonstrated the presence of Rh antigens in fetuses at various stages of development, this being indicative of the fact that Rh antigens appear early in life.

Fisk and Ford (37) found that the Rh antigens in infants differ somewhat from those found in adults. Animal anti-Rh serum, derived from guinea pigs, was shown to agglutinate the red blood cells of infants up to one month of age whether the infant was Rh positive or negative. This was not the case with adults.

More recently in 1961, Levine and co-workers' findings (67) suggested that there is a "D-like" antigen in rhesus monkey, human Rh-positive and human Rh-negative red blood cells. They indicated that guinea pig reagents produced by injection of the above cells or their extracts defined an agglutinable property which differs distinctly from the human D antigenic determinant. The human anti-D will not agglutinate Rh negative cells, whereas in this experimentation they were able to recover eluates from Rh positive, Rh negative and rhesus monkey cells that gave D-like specificity. They concluded that the term "rhesus factor," as applied to the human D (Rh_o) antigen, appears to be a misnomer.

There have been investigations for the Rh antigen on body fluids and tissues other than erythrocytes. Levine and Katzin (62) were unable to find Rh antigens in saliva, sperm cells and seminal fluid. Wiener and Forer (89) confirmed this absence and concluded that the antigens were only present in erythrocytes. Boorman and Dodd (7) reported the antigen's presence in the liver, spleen and salivary glands of Rh positive individuals and also in the saliva of 27 of the 51 Rh positive individuals they tested. However, this has never been confirmed. Witebsky and Mohn (92) reported finding the Rh antigen in amniotic fluid in four-fifths of all pregnancies in which the fetus was Rh positive and none in the Rh negative fetuses.

Several investigators have undertaken the task of separating the Rh antigen from the red cell stromata through hemolysis. Belkin and Wiener (6) subjected erythrocytes to hemolysis and claimed that they obtained Rh antigen along with A and B substances. They mentioned that the inhibition and absorption titers obtained for the various Rh properties were consistently lower than the titers obtained for A-B-O substances. These findings suggested to them that the number of Rh "haptens" per cell may be less than the number of A, B or O "haptens." Calvin et al. (17) separated erythrocyte stromata into a protein fraction which they called stromatin and a lipoprotein known as elinin. From elinin they separated an ether-soluble fraction containing a still greater concentration of Rh antigen and proportionately lower concentration of A and B antigens.

In 1947 Carter (23) reported an ether-soluble fraction separated from group O Rh positive cells. This fraction was non-antigenic in experimental animals but was antigenic when injected simultaneously with a protein carrier. She claimed that this substance specifically inhibited the agglutinins present in anti-Rh serum and it resisted inactivation by heat. The substance was thought to be probably the Rh "hapten" in impure form.

Price et al. (75) attempted to purify Carter's substance and described the pure "hapten" as an acid, optically inactive, soluble in alkali with a melting point of 156.9° -

157.2°. It exhibited activity in dilutions of 1:5000 as measured by the complement fixation test with anti-Rh_o serum.

According to Landsteiner (57), a hapten is a specific protein-free substance which, although active in vitro, induces no, or only slight, antibody response. Thus, a hapten performs as an antigen in that it combines with an antibody in vivo and in vitro, but unlike an antigen it, of itself, will not elicit the production of antibodies. It was with this in mind that several investigators (16, 41, 42, 49, 53, 68) treated patients, with demonstrable Rh antibodies during pregnancy, with an Rh "hapten" using Carter's and/or Price's methods. It was thought that if this was truly Rh hapten, then an Rh negative pregnant woman actively sensitized at the time of treatment would be desensitized, the maternal titer falling in response to treatment with Rh hapten.

The results of this treatment were quite variable. In some cases the "Rh hapten" apparently seemed to help, in other cases, there was no response whatsoever. It became more and more apparent it could not be concluded that this "Rh hapten therapy" was the determining factor as to whether the mother gave birth to a normal child or the child died of erythroblastosis.

Besides this, the previously mentioned experiments of Belkin, Carter and Calvin were not found to be reproducible by other investigators. It also became apparent that

what they labelled "Rh hapten" was, in reality, some other substance, non-specific in nature.

In 1950, Stratton and Renton (83) followed Carter's original and modified methods for extracting Rh hapten. In all cases, control Rh negative cells were extracted at the same time as the Rh positive cells. They found that the Rh positive cell extracts had a very slight inhibitory effect on Rh antisera, but also, that extracts from Rh negative cells proved equally inhibitory. It was concluded that the phenomenon was of a non-specific character.

Evans and co-workers (36) made a study of the Rh factor in elinin. They could not obtain an active fraction of crude preparations of elinin by chemical or enzymatic means, nor could they repeat the work of Carter in extracting an active substance from red blood cells by alcohol and ether.

These later studies helped confirm the belief that an Rh hapten or antigen actually had not been isolated as first claimed by the earlier investigators. Thus, a purified form of Rh antigen has yet to be found.

There have been other studies such as that of Lubinski and Portnuff (69) who did an investigation of heat and formalin upon the Rh agglutinogen. They discovered that the addition of formalin to red blood cell suspensions reduced their agglutin-ability for anti-Rh serum much more than for A or B sera. It was also found that heating red blood

cells to 55° C for five to twenty minutes caused them to lose their reactivity with agglutinating as well as with blocking anti-Rh sera, whereas the anti-A, anti-B anti-M and anti-N sera were not affected. They speculated that these results might be due to the fact that the A, B, M and N agglutinogens are on the surface or the Rh agglutinogens are less numerous and/or there is a difference in chemical structure.

III. Nature of Rh Antibodies

Rh antibodies do not occur naturally but are produced as a result of the introduction of Rh antigens into the circulatory system of a susceptible individual, namely, one who is negative for the Rh antigen introduced.

Two types of Rh antibodies are recognized in vitro. They are (1) anti-Rh agglutinins which unite with Rh positive erythrocytes suspended in saline and (2) blocking or incomplete antibodies which unite with Rh erythrocytes but do not cause agglutination unless the cells are suspended in a protein-like material such as albumin or plasma. It is thought that, in vivo, both types of antibody cause the same kind of reaction, namely, hemolysis of the erythrocytes following the union with their specific antigens on the erythrocytes.

For the most part, these Rh antibodies occur in the serum of Rh negative individuals who have had an

incompatible blood transfusion or in Rh negative pregnant women carrying Rh positive fetuses. In addition to this, Witebsky and Heide (90, 91) have demonstrated Rh antibodies in the breast milk of recently delivered women who had the Rh antibody in their blood.

Potter (74) stated that it seemed probable that agglutinating antibodies were the earliest varieties formed in response to stimulation by Rh antigen. Blocking antibodies appeared later and were thought to be evidence of a greater degree of immunization and they frequently persisted much longer in the blood. However, either type of antibody produced hemolytic disease or could be responsible for transfusion reactions.

It has been suggested by Wiener (86) that differences in action between Rh agglutinating and blocking antibodies might be due to the number of combining groups (sites on the antibody where a chemical union could be affected with the corresponding antigen) which make up each antibody. The agglutinating antibody was thought to have two combining sites (bivalent) causing agglutination when each site was attached to a red blood cell. On the other hand, blocking antibodies were thought to have one combining site (univalent) which can attach to one erythrocyte but because of the lack of a second site it cannot attach to a second erythrocyte in order to hold two cells together.

The demonstration that incomplete antibody in a

suitable medium could cause agglutination called into question Wiener's theory on the univalence of the incomplete antibody. To overcome this, Wiener et al. (87) proposed that plasma or serum media caused the erythrocytes to "stick" together because of a substance called "conglutinin" within the media. In other words, it did not bring about proper agglutination.

A different approach for the detection of incomplete antibody was the antiglobulin or Coombs test described by Coombs et al. (27) in 1945. It was found that erythrocytes coated with incomplete Rh antibodies would agglutinate upon exposure to anti-human globulin. These anti-human globulins were produced by immunizing rabbits with globulins of human serum. This is a test widely used today for detection of incomplete antibodies.

Diamond and Abelson (33) have shown that agglutinating and blocking antibodies are similar in that they may unite with erythrocytes at room or icebox temperature, but the reaction is more rapid at 37° C. However, blocking antibodies are more thermostable than agglutinating antibodies, according to both Diamond (33) and Coombs (28).

Coombs and Race (28) state that blocking antibodies will not go through a collodion filter known to be permeable to proteins with a molecular weight of 30,000. They also found that the electrophoretic migration of Rh positive cells exposed to either kind of antibody was the same.

Boyd (11) demonstrated that Rh agglutinins were destroyed by exposure to pressures of 3000-4000 atmospheres for twenty-four hours, whereas blocking antibodies required pressures in excess of this for their activity to be inhibited.

In 1947, Coombs and Mourant (26) suggested from serological evidence that blocking antibodies were present in the gamma globulin fraction of human serum with the possibility of there being small amounts in the alpha and beta fractions.

In the late 1940's, Witebsky and Mohn (93), through dialysis of certain sera containing Rh antibodies, not only found blocking and saline agglutinating antibodies but a supposedly third order of antibody (reactive only in the antiglobulin test) in their various globulin fractions and supernatants.

Hill et al. (51) used the Reid-Jones fractionation method (80) utilizing ion-exchange resin materials also resulting in a third order of antibody which they termed "cryptagglutinoids." However, whether these antibodies represent a weaker reacting antibody detected only by means of the more sensitive anti-globulin test or whether they are a true "third order antibody" has not been convincingly demonstrated.

Cann and co-workers (21), in 1952, employed electrophoresis convection in the fractionation of Rh antibody.

A series of top fractions of the sera were removed by successive runs at progressively lowered pH, ranging from 8.1 to 5.3. Using four sera with Rh antibodies, they indicated that the saline and blocking antibodies were found not only in gamma globulin fractions of human serum but may also be associated with proteins possessing mobilities of alpha and beta globulins.

The same year Sturgeon and Brown (84) concluded that the electrophoresis convection technique did not serve to separate the agglutinating antibodies from the blocking antibodies. The electrophoresis convection data tended to show that both saline agglutinating and blocking antibodies are distributed in two fractions of different mobility, one of which is gamma globulin and the other, beta globulin. They felt that from an immunological standpoint the total antibody in the serum represented a spectrum with the saline agglutinating antibody at one end, and the blocking antibody at the other end. However, they felt that the heterogeneity in electrophoretic properties was unrelated to the heterogeneity in antibody properties.

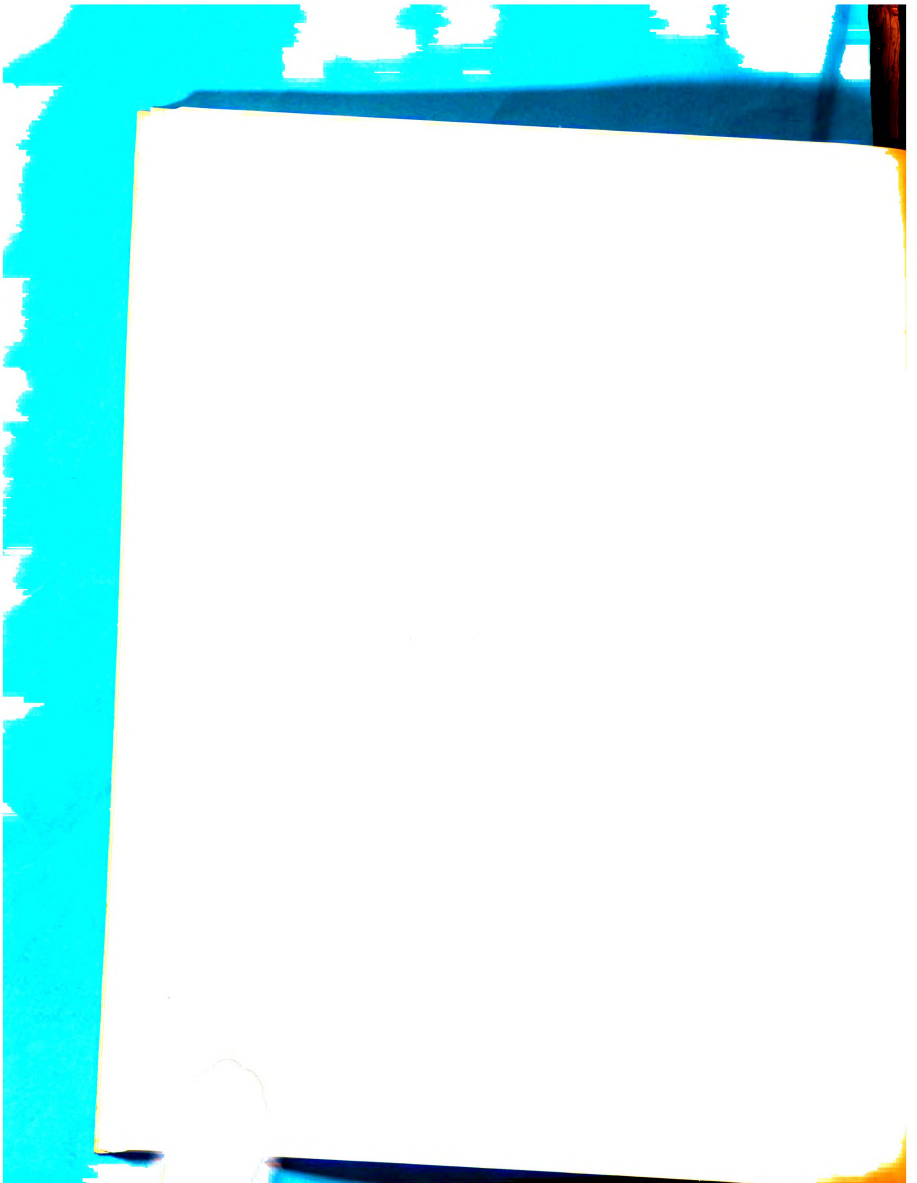
In 1953, Jankovic and Kuijnen (55), using three anti-D sera containing both agglutinating and blocking antibody, separated them through the use of paper electrophoresis. They found agglutinating and blocking antibody only in the gamma globulin fraction. They could not confirm the observations of Cann et al. (21) that other

globulin fractions also contain Rh antibodies. The authors felt that this discrepancy was due to the fact that a better separation of globulin fractions was possible by means of paper electrophoresis than by the electrophoresis convection method.

In 1959, Abelson and Rawson (1) employed yet another method known as exchange chromotography. They found that the incomplete antibodies were removed in a broad band, whereas the saline agglutinins were found in fewer aliquots of the eluting solutions. This, they felt, was in accordance with the theory that incomplete agglutinins represent a spectrum of molecules with slight variations while the saline-active antibodies may be more nearly homogeneous.

Campbell et al. (19) claimed that they isolated the agglutinating antibodies from the blocking antibodies by ultracentrifugation. They stated that the Rh saline agglutinins sedimented at a faster rate than the blocking type of antibodies. They came to the conclusion that the Rh saline agglutinins consist of molecules of a greater molecular weight than the blocking type. More recent studies have confirmed this plus the fact that saline antibodies were associated with protein of a molecular weight near 1,000,000, whereas incomplete antibodies possessed a molecular weight of 160,000 which is that of normal gamma globulin.

Chan and Deutsch (25), in 1960, did a study of the chemical and biological activities of Rh antibodies which



had been reported in the past. Additional information gained from this study was that saline agglutinins were destroyed by treatment with 2-mercaptoethanol, whereas the incomplete antibody was not affected. This caused a loss in direct agglutinating activity but reactivity was demonstrated with the Coombs test. The incomplete antibody, destroyed by papain digestion also resulted in a loss of the usual agglutinin reactions but maintained Coombs reactivity. They speculated that the loss of direct agglutinin activity could be attributed to the fact that the larger molecules, having been broken down into smaller ones, had fewer sites to offer for complete combination with antigen, but enough to affect Coombs reactivity.

We find, then, in a review of the properties of Rh antibodies, that the investigators have been able to effect a separation of the antibodies through various physical and chemical methods. There was agreement that these antibodies are found in the globulin fraction of serum as opposed to albumin fractions, and, at first, there was disagreement as to which globulin fractions contain the antibodies. However, it is now agreed upon by most investigators that antibodies are found primarily in the gamma globulin fraction of serum.

There has been some attempt on the part of several to differentiate the molecular size of the two types of antibody which has resulted in apparent agreement that the

saline agglutinin has a molecular weight of approximately 1,000,000, whereas the incomplete antibody has a molecular weight of about 160,000.

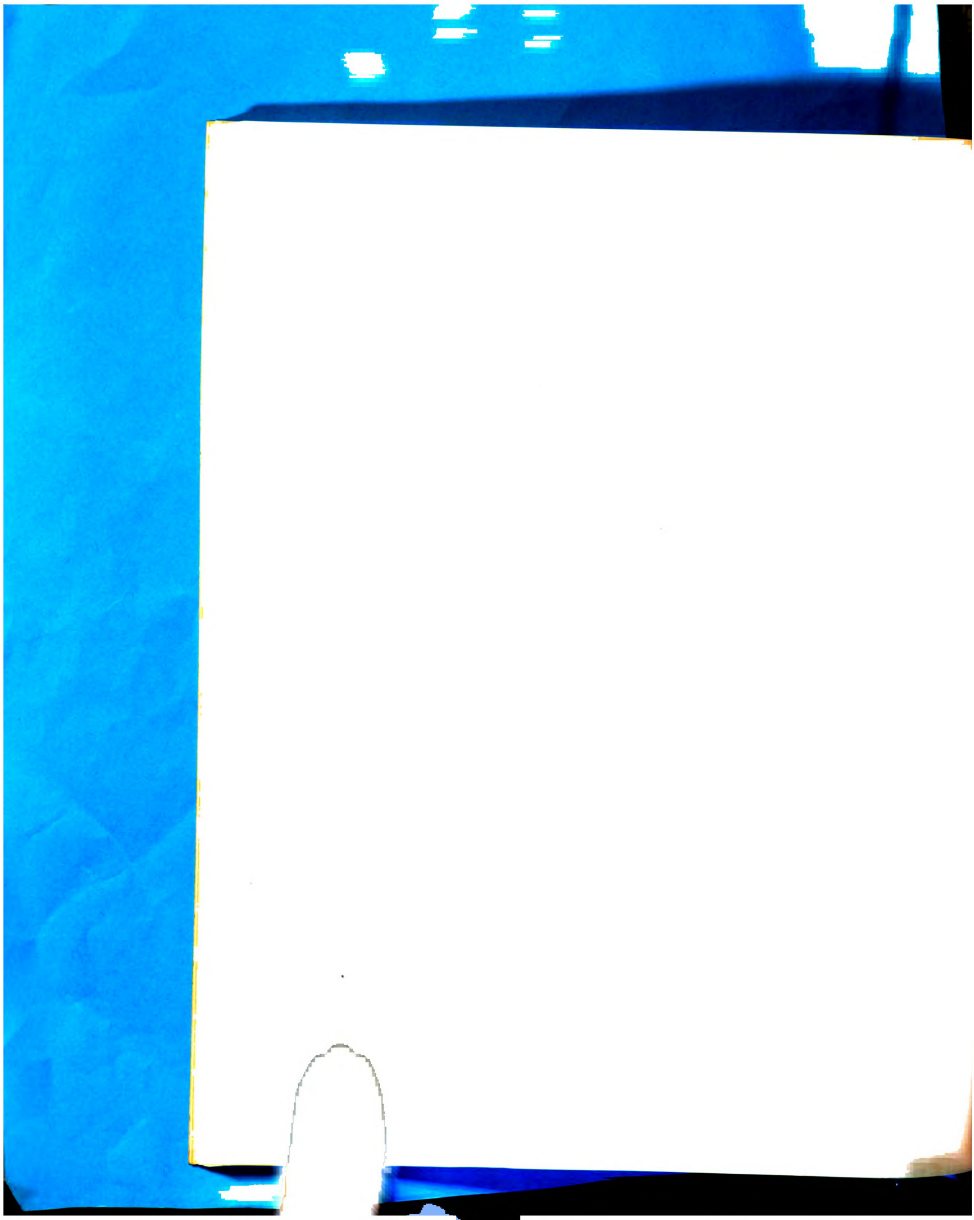
From the aforementioned information, it may be observed that although there have been many reports concerning the physical and immunological properties of both the Rh antigens and antibodies, very little is known about the chemical properties of either.

IV. Hemagglutination Inhibition Studies

Landsteiner (57) stated that immune antibodies all have the property of specificity in common, i.e., they react, as a rule, only with the antigens that were used for immunization.

Here was a clue to a method for elucidating the chemical nature and structure of antigens of unknown composition, namely, the hemagglutination inhibition test. This test has been used effectively in the isolation and study of some blood group substances. It was employed by Morgan et al. in the purification of A, B, H and Lewis substances. Also, Kabat (56) and Boyd (12) have summarized inhibition studies of other investigators and described the inhibition technique.

The inhibition reaction results from the union of an artificial antigen or haptenic substance with the antibody in question. This artificial antigen or haptenic



substance is thought to combine with the antibody by virtue of its similarity to the original antigen. In the case of hemagglutination the reaction takes place as follows: A chemical or haptenic substance is added to a serum with a known antibody. To this are added red blood cells with the antigen specific for the known antibody. If agglutination of the red blood cells does not take place as expected, it could then be assumed that the chemical substance has inhibited agglutination of the red blood cells. The rationale is that the chemical substance or napten must exhibit some of the properties of the original antigen in order to combine with the antibody. Thus, in turn, it leaves less, or no, antibody free for combination with antigens on the red blood cells.

The work of Morgan and his associates (2, 3, 39, 40) has demonstrated that blood group substances A, B, H and Lewis antigens consist of mucopolysaccharide-protein complexes. They found that acid hydrolysates of preparations of A, H and Lewis substances from ovarian cyst fluid contained hexosamine (35%), L-fucose (13%), galactose (17%) and a variety of α-amino acids (40%). Blood group substance B resulted in a higher proportion of L-fucose (19%) and less hexosamine (21%) than the other three. They also found that the hexosamine fraction of the acid hydrolysates contains both glucosamine and galactosamine; the glucosamine/galactosamine ratio is in the range 1.4 to 2.8.

Hackel et al. (46, 48) have reported that anti-D, anti-C, anti-E, anti-c, anti-e and Lutheran sera are specifically inhibited by four ribonucleic acid derivatives, suggesting that the Rh and Lutheran antigens are at least partly nucleotide in nature. This was further supported by Hackel and Smolker (47) in their treatment of erythrocytes containing Rh and Lutheran antigens with ribonuclease. The rationale was that if any antigenic specificity was due to ribonucleic acid derivatives, then treatment with enzyme, ribonuclease, should remove these from the cell, lowering the agglutinability. They found that the treated cells did lose part of their Rh and Lutheran specificities, whereas the other antigens tested for were unaffected.

Boyd et al. (15) reported finding weak inhibition of anti-D serum by three monosaccharides (L-glucose, L-mannose and d-gulose). Anti-C was weakly inhibited by L-glucose and anti-C and anti-E were both inhibited (less strongly than anti-D) by streptomycin (a natural glycoside of N-methyl-L-glucosamine) and rutinose.

In 1960, Dodd et al. (35) reported that they had discovered substances which specifically inhibited anti-D but not anti-C or anti-E. They observed specific anti-D inhibition with crude and crystalline N-acetyl neuraminic acid, less inhibition by its glycol derivatives and weaker inhibition by its degradation products, N-acetyl-mannosamine and D-mannose. A beef-brain ganglioside containing 17%

neuraminic acid and a Pseudomonas polysaccharide were almost as effective inhibitors as the crude and crystalline preparations of neuraminic acid.

In 1961, Boyd and Reeves (13) reported that they, too, found specific inhibition of anti-D antibody. The substance that caused this effect was colominic acid in a relatively low concentration (0.006 M). This substance was produced by certain strains of Escherichia coli and was thought to be a polymer of N-acetyl nueraminic acid.

CHAPTER III

THE EXPERIMENTATION

I. Materials and Methods Used

The inhibitors used in this study were adenylic acid (AMP), cytidylic acid (CMP) and uridylic acid (UMP), all of them consisting of 2'-3' mixtures.

Solutions of two, four, six and eight per cent concentrations were made of each inhibitor. This meant that the molarity of each inhibitor at each concentration was as follows:

	concentration	molarity
AMP	2%	.058
	4%	.116
	6%	.174
	8%	.232
CMP	2%	.062
	4%	.124
	6%	.186
	8%	.248
UMP	2%	.062
	4%	.124
	6%	.186
	8%	.248

These inhibitors were dissolved and adjusted to the proper pH by using 0.1 M of acid (pH 4.0) or alkaline (pH 9.0) phosphate buffer solutions in conjunction with minute amounts of 0.1 M potassium hydroxide. Further dilutions

to the appropriate concentration were made with a 0.1 M phosphate buffer having a pH of one of the following: pH 6.6, pH 6.8, pH 7.0, pH 7.2, pH 7.4 and pH 8.0.

The red blood cells used as the indicator in the hemagglutination inhibition test were prepared by washing the cells three times with physiological saline (0.9%) solution. This was accomplished by centrifuging the cells three times, adding fresh physiological saline after each centrifugation, and mixing. The third time, the supernatant saline was pipetted off, leaving packed red blood cells in the bottom of the tube. One drop of packed red blood cells from an appropriately calibrated pipette was added to a measured volume of physiological saline resulting in a 2% suspension of red blood cells. The kinds of erythrocytes used for anti-D and anti-C serums were of the genotype CDe/CDe (R_1 R_1) or CDe/cde (R_1r) and for anti-E serum, cDE/cde (R_2r) or cDE/cDE (R_2 R_2). The age of the erythrocytes varied from fresh cells up to those held in refrigeration for two weeks. Both, the kind and age of erythrocytes used, depended upon the availability of these at the time the tests were conducted.

II. Procedure

A series of dilutions, consisting of ten tubes, were made of each antiserum, using 0.9% saline as the diluent. These dilutions consisted of the following: full strength, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256,

1:512. Each serum dilution was transferred in 0.01 cc. volumes to a smaller tube and to each of these tubes was added a 0.01 cc. volume of the desired inhibitor, followed by a 2% red blood cell suspension, also in 0.01 cc. aliquots. This mixture was incubated at 37° C for one-half hour or one hour, depending upon the specifications of the serum used. It was then read microscopically, recording the varying degrees of agglutination and scoring the same according to the Race scale (76) as follows:

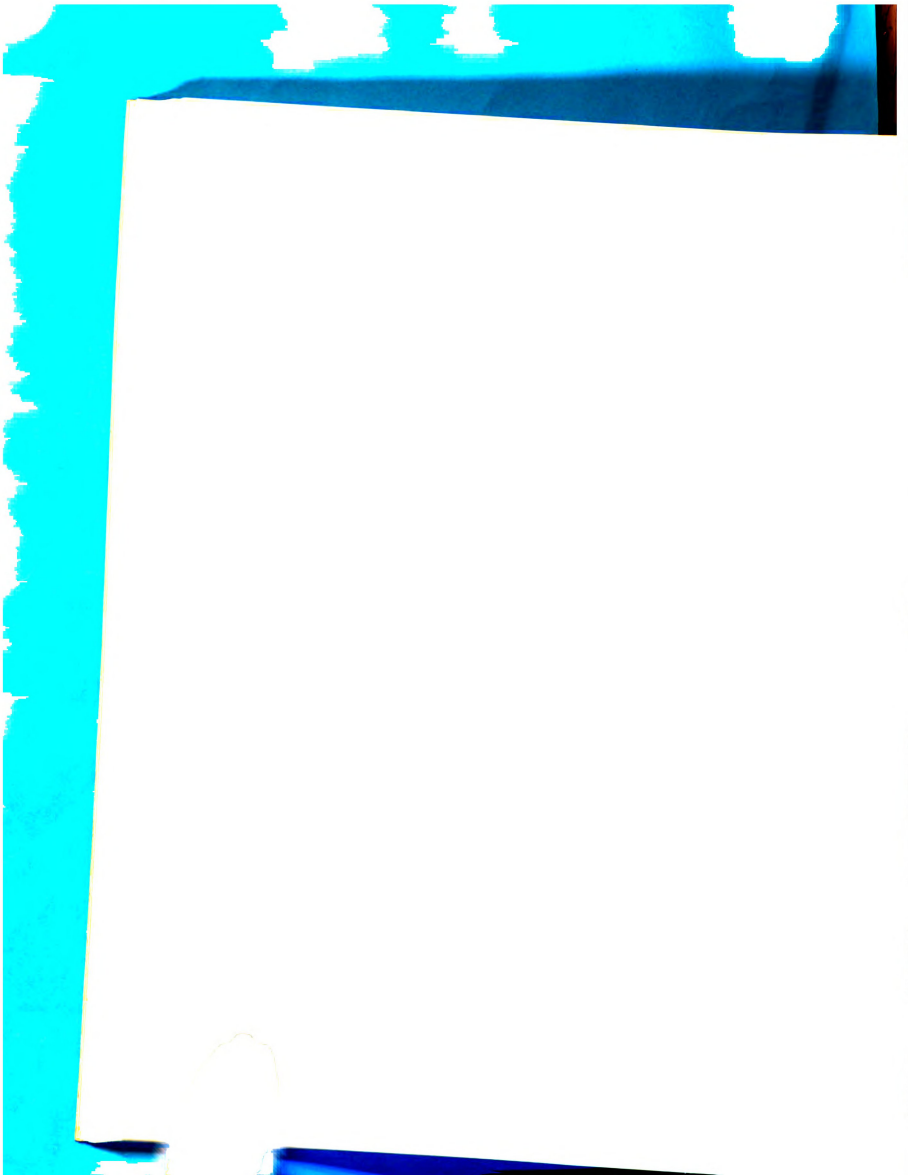
Degree of agglutination		Score
$++^{V}$	= agglutination clearly visible to the naked eye	10
++	= very large agglutinates seen microscopically	8
+	= large agglutinates seen microscopically	5
(+)	= smaller agglutinates seen microscopically	3
w	= the smallest definite agglutinates	2
-	= no agglutination and cells evenly distributed	0

These numerical values, of course, are arbitrary but, without them, direct comparisons could not be made between a control and test situation. This also makes it possible to go beyond a qualitative comparison. Through setting up a series of dilutions or standardized titration of serum, quantitative aspects of antibody present in the serum can be determined. This is done by adding the scores together, obtained at individual dilutions within the series

of the titrated serum, resulting in a total score. The total score, then, indicates the amount of antibody present.

The aforementioned was applied in the present study in the following manner: The total score of each titrated serum with inhibitor was subtracted from the total score of the titrated control serum containing saline in place of the inhibitor, giving a measure of the inhibitor's effect. Hackel (44) calls this the "Inhibition Score." If control scores of various serums were always the same, direct comparisons could be made by using the inhibition score alone. However, since this was not always the case, it was necessary to go one step further in obtaining an index of the inhibiting power on the serum being tested by dividing the total control titration score into the inhibition score, resulting in a "per cent inhibition" score.

In this experimentation, wooden blocks were used that could accommodate fifty small test tubes per block. Therefore, fifty tests were run at a time, consisting of five rows. Each row had ten tubes of the same titrated serum. The saline (0.9%) control was added to the first row. The 2%, 4%, 6% and 8% concentration of inhibitor, respectively, were added to the other four rows. The appropriate erythrocytes were added to all tubes, followed by incubation and reading of the fifty tests, microscopically. Figure 2 illustrates the aforementioned.



AMP (pH 6.6) vs. Anti-D

R_1 R_1 cells

37° C (1 hr.)

		Concentration of Inhibitor				
		Saline Control	2%	4%	6%	8%
Dilutions of antisera	Full-strength	++^{V}	++^{V}	++^{V}	++^{V}	++
	1:2	++^{V}	++^{V}	++	++	+
	1:4	++^{V}	++^{V}	++	++	(+)
	1:8	++	++	+	(+)	w
	1:16	++	+	(+)	w	-
	1:32	+	(+)	w	-	-
	1:64	(+)	w	-	-	-
	1:128	w	-	-	-	-
	1:256	-	-	-	-	-
	1:512	-	-	-	-	-
	Total score	56	48	36	28	18
	"Inhibition score"		8	20	28	38
	% inhibition		14	36	50	68

Fig. 2. Sample illustration of the test set-up and scoring procedure.

The above procedure was run with each inhibitor at pH 6.6, pH 6.8, pH 7.0, pH 7.2, pH 7.4 and pH 8.0, with three different serums of anti-D, anti-C and anti-E. Controls using phosphate buffer at each pH rather than inhibitor were run with each serum during the first of the three test repetitions

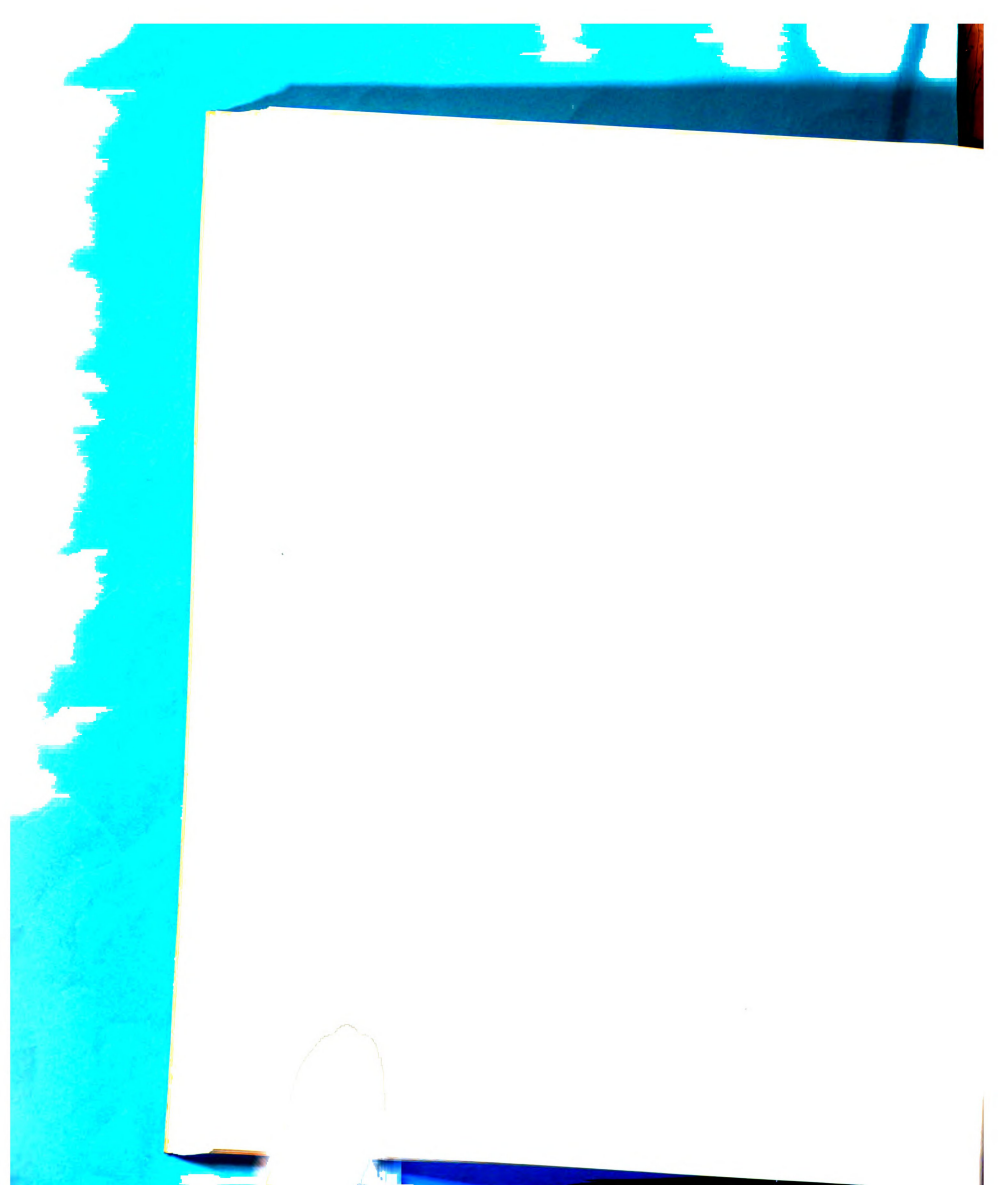
but were discontinued since the results did not differ from the saline controls.

III. Results

The derived scores of per cent inhibition for each inhibitor at different concentrations and pH with the different antisera are depicted in Tables I through IX. The average per cent inhibition scores for the three repetitive runs are also given.

There were differences between the three samples of the same antiserum used as well as concentration and pH differences of inhibitors. Examples illustrating these individual differences between different samples of the same kind of antiserum can be found in every one of the following tables. For example, in Table II, at 2% concentration and pH of 6.0, serum sample I gave 0% inhibition and serum sample II gave 8% inhibition, whereas serum sample III gave 18% innibition; or at the other end of the scale, as depicted in Table I, serum sample III at 8% concentration and a pH of 8.0 gave 100% inhibition, whereas sample II gave 79% inhibition and sample I, 86% inhibition.

To accomplish a more generalized impression of what is occurring with these results, the average "per cent inhibition" scores at different concentrations were plotted against pH of inhibitor in Graphs Ia through IXa. A



comparison of the anti-serum with the three different inhibitors, adenylic, uridylic and cytidylic acids, respectively, clearly demonstrated, in each case, that the greater the concentration, the greater was the percentage inhibition. This was indicated by each plotted line, representing concentration, being completely separated from every other plotted line with only a few individual exceptions. In Graph IIa (AMP vs. anti-C) and Graph VIIIa (CMP vs. anti-C), the 2% concentration exhibits slightly more inhibition than the 4% concentration at a pH 8.0, the differences being 1% and 5%, respectively. There was no difference in per cent inhibition between the 2% and 4% concentrations in Graph IVa (UMP vs. anti-D) at pH 8.0. AMP vs. anti-E exhibited only 1% difference, whereas UMP vs. anti-C showed only 3% difference between the 2% and 4% concentrations and no difference between the 4% and 6% concentrations at pH 8.0. With concentrations of 6% and 8%, in all cases, the separation of the curves were complete throughout the pH series, with the 8% concentration exhibiting a greater inhibitory effect than the 6% concentration.

With anti-D vs. the three different inhibitors (Graphs Ia, IVa and VIIa), it can readily be seen that the 8% concentration curve for adenylic acid is significantly higher than the same concentrations of uridylic and cytidylic acids. Also, with the exception of pH 7.2, the curve for 6% concentration of adenylic acid is significantly

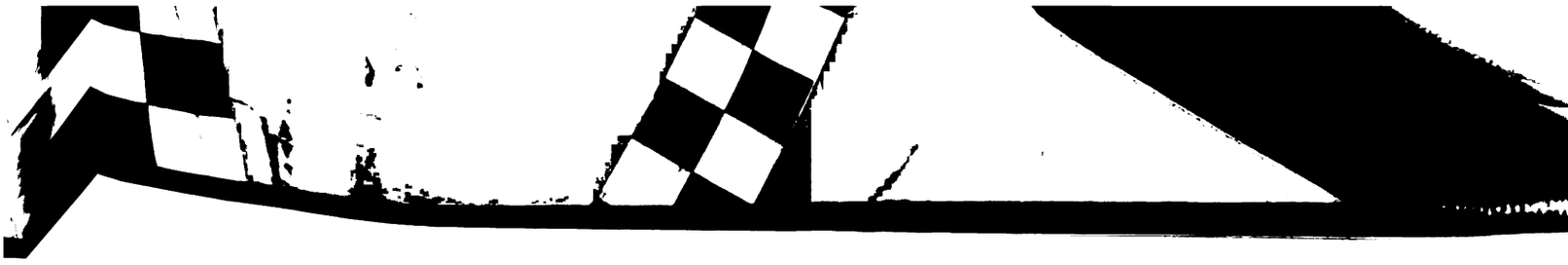
higher than that of uridylic and cytidylic acids. The 4% concentration curve of AMP is increased over uridylic acid but it is not as clear-cut with cytidylic acid except at the higher pH's from about 7.2 up to 8.0. The range of all the curves of per cent concentration for uridylic and cytidylic acid are about the same but fluctuate within this range depending upon pH. Cytidylic acid appears to be more erratic in its variations from one pH to the next, as compared to uridylic acid.

Interestingly, in a consideration of these three inhibitors vs. anti-C (Graphs VIa, Va and VIIIa), the concentration curves follow the same general pattern as was found with anti-D. One significant exception is that none of the inhibitors was as effective with anti-C as with anti-D serum. Another rather apparent trend is that there does not appear to be as much difference of all three inhibitors in inhibitory effects between the 2% and 4% concentrations as there was with anti-D. This can be observed by comparing the distances between the 2% and 4% plotted concentration curves. Also, the 6% and 8% concentration curves are much more separated than the 2% and 4% concentrations.

With anti-E (Graphs IIIa, VIa and IXa), adenylic acid at all four concentrations exhibits the greatest inhibitory effects of the three inhibitors. In a comparison between uridylic and cytidylic acid, the 6% and 4%

concentrations of cytidylic acid exhibit slightly stronger innibitory powers than uridylic acid from a pH of 7.0 up to 8.0. The 8% concentration of these two inhibitors are much more alike at higher pH's but cytidylic acid inhibits more effectively in lower ranges. The 2% concentrations are somewhat erratic in an attempt of comparisons, although cytidylic acid is obviously greater in inhibitory effects at a pH of 8.0 than uridylic acid.

In Graphs Ib through IXb, the average per cent inhibition scores at different pH's were plotted against per cent concentration of inhibitor. It will be noted, in a general consideration of all these graphs, that with adenylic acid vs. anti-D (Graph Ib) and anti-C (Graph IIb) and with uridylic acid vs. anti-C (Graph Vb), the pH 8.0 and pH 7.4 curves are clearly separated from the others. With adenylic acid vs. anti-E (Graph IIIb), uridylic acid vs. anti-E (Graph VIb) and cytidylic acid vs. anti-E (Graph IXb), the pH 7.2 curve as well as pH 7.4 and pH 8.0 curves are clearly separated from the others, exhibiting more inhibitory effect. In Graph IVb, with uridylic acid vs. anti-D, cytidylic acid vs. anti-D (Graph VIIb) and cytidylic acid vs. anti-C (Graph VIIIb), the separation between the higher pH's is not very clear. However, with these as well as all the other graphs in this series, it can be observed that the least effective inhibition occurs in pH's ranging from 6.6 through 7.2.



In a comparison of the three inhibitors with anti-D (Graph Ib, IVb and VIIb), adenylic acid exhibits the greatest per cent inhibition, at all pH's taken collectively, than the other two inhibitors. With cytidylic acid, there appears to be a wider range of fluctuation from the least inhibitory effect involving the pH 6.8 curve to the greatest effect consisting of the pH 6.6 and 7.2 curves. With uridylic acid, the range of fluctuation is smaller as well as a difference in the reflection of pH's exhibiting the least and greatest inhibitory effect, the least being the pH 6.6 curve and the greatest, the pH 8.0 curve.

With anti-C serum, adenylic acid at pH 8.0 has a curve that is widely separated with much greater inhibitory powers than at any other pH. Again, adenylic acid appears to be more effective at all pH's than uridylic or cytidylic acid. Uridylic acid has a wider range of inhibitory effects than cytidylic acid with a pH 8.0 and 7.4 being most effective and 6.6 being least effective. Cytidylic acid appears to be least effective at pH's of 7.2 and 6.6, whereas all other pH's appear to be about equal in effectiveness except at 8% concentration, where pH of 6.8 becomes most effective.

Adenylic acid is most effective with anti-E at a pH of 8.0. However, cytidylic acid at a pH of 8.0 becomes next most effective in inhibitory powers. The third most effective of the three inhibitors is adenylic acid at pH of 7.4 and 7.2, respectively. From that point on, cytidylic

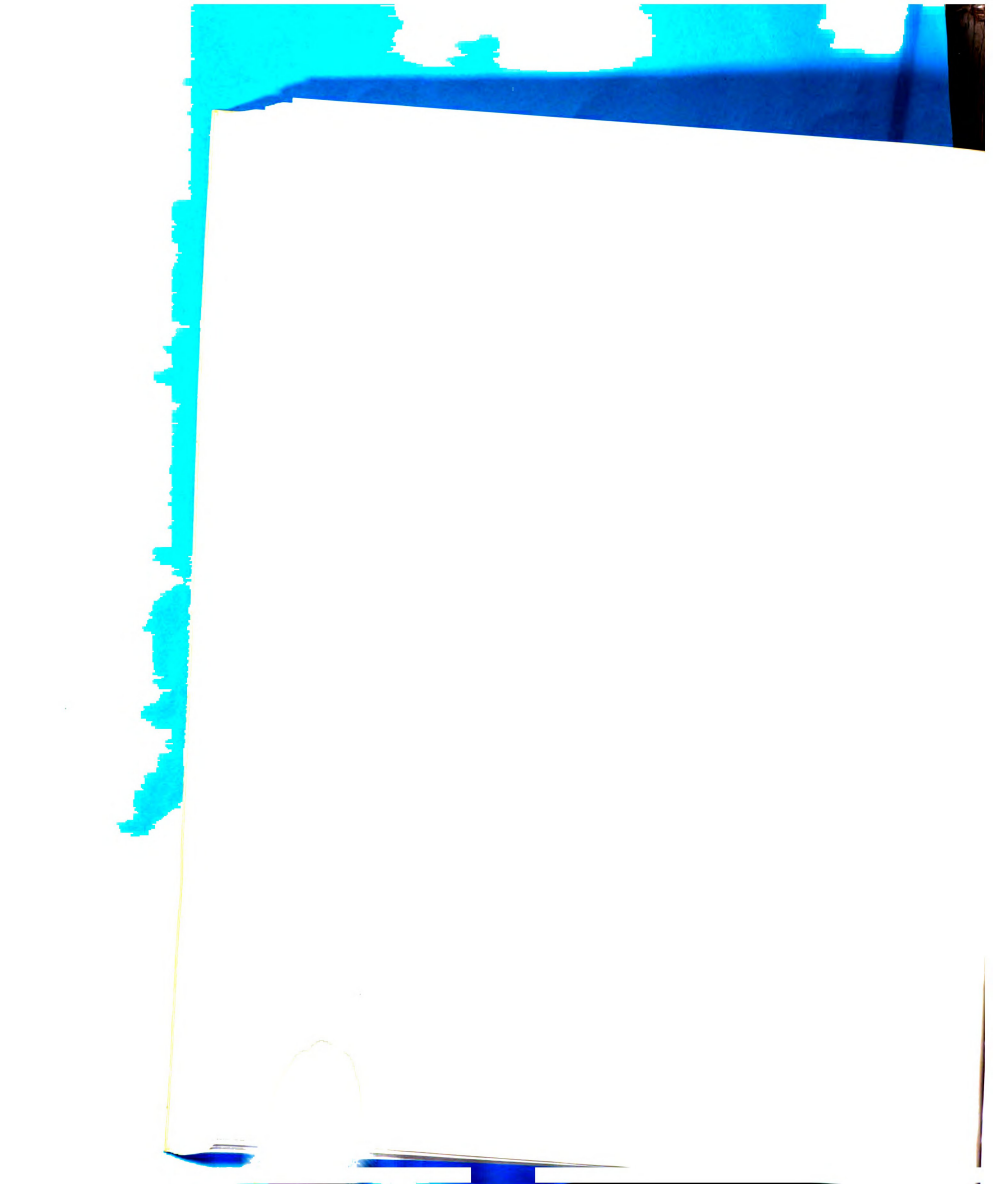
and uridylic acids have almost the same range of effect. The least effective occurs at pH 6.6 with cytidylic acid and pH 7.0 with uridylic acid.

From observations of a comparison of the general trends of these graphs concerning pH and its inhibitory effects, it appears, then, that adenylic, uridylic and cytidylic acids are all most effective with anti-D and anti-E sera, being less effective with anti-C. One general trend is that all the inhibitors appear to be most effective with higher alkaline pH's but that there is much fluctuation within that range. That is, which pH is most effective depends upon the inhibitor and antiserum involved.

The following is the most effective individual average "per cent inhibition" of each inhibitor for each antiserum as evaluated from data recorded in the tables:

	Inhibitor	pH	concentration	average % inhibition
Anti-D sera	AMP	7.4	8%	89
	UMP	8.0	8%	76
	CMP	6.6, 7.2	8%	74
Anti-C sera	AMP	8.0	8%	74
	UMP	7.4	8%	53
	CMP	7.4	8%	52
Anti-E sera	AMP	8.0	8%	95
	CMP	8.0	8%	88
	UMP	8.0	8%	86

As can be observed from above, without exception, the most effective individual inhibition takes place at 8% concentration. Concerning pH, the results are somewhat more



variable. However, with one exception, the most effective individual inhibition takes place with the inhibitor on the alkaline side. Also, adenylic acid has the most individual effectiveness with all antisera, being most effective with anti-E. The difference in individual effect between uridylic and cytidylic acids is very slight, with all three antisera.

IV. Discussion

Kabat (56) and Morgan et al. (2, 3, 4, 39, 40), through the use of the hemagglutination inhibition test, demonstrated the fact that isolated blood group substances caused inhibition of their specific antibodies. This was found to be true, not only of blood group substances but also of other substances. Thus, any substance which would inhibit a specific antibody could be interpreted as being similar to the antigen in question.

Hackel and co-workers (46, 48) demonstrated specific inhibition of anti-Rh and anti-Lutheran sera with chemical substances other than blood group substances. Of 60 reagents used in 2% concentrations, adjusted to pH 6.8, they found ribonucleic acid derivatives effective in this respect. These included three nucleotides, namely, 2'-3' mixtures of adenylic, cytidylic and uridylic acids and one nucleoside, cytidine sulfate.

The present study is an extension of Hackel's

original studies on the previously mentioned three nucleotides. In this study, 4%, 6% and 8% concentrations of each inhibitor were used as well as the 2% concentration and for each concentration, the pH used ranged from 6.6 to 8.0.

The results demonstrated differences between samples of the same kind of antisera. One explanation for this might be differences in strength of sera used. However, as explained previously, the derived "per cent inhibition" score should compensate for this. Another explanation might be the difference in the genotype of the cells used, i.e., whether cells from a heterozygous or homozygous individual were employed, although it has not been proven with Rh factor whether the genotype of the erythrocytes causes a difference (dosage effect) or not. It is known that the strength of agglutination of erythrocytes becomes weaker with age. Thus, this could have contributed to differences of reaction of individual sera, in part. Also, it cannot be ignored that human error could play a part in performing such a sensitive test as the hemagglutination inhibition test. The fact that new serum dilutions were made up each time the tests were performed would allow for slight variations in the titrated serum used; and since very small quantities (0.01 cc.) of material were used in the actual test, an error in pipetting would be exaggerated more than if greater quantities of substance were involved. Or it could be that these differences actually exist between different serum

samples. However, this problem could be solved only by further experimentation.

It was hoped that by taking an average "per cent inhibition" score of three sample sera in each situation the individual differences would be minimized to a degree. Because of the aforementioned, then, the emphasis in discussing the results was placed on the plotted trend curves of the average "% inhibition" scores. This emphasis was not so much in a consideration of the fluctuation within each curve but of a comparison of the spatial relationship differences existing between each curve within a graph, e.g., as is indicated by a comparison between the separations of 2%, 4%, 6% and 8% concentration curves.

Generally speaking, then, there was a marked increase of inhibition with all three inhibitors used with anti-C, anti-D and anti-E serum when the concentration of the inhibitor was increased and in the alkaline range.

It should be emphasized, once again, that each inhibitor in this study consisted of 2'-3' mixtures. Since this research was completed, there has been an investigation by Hackel (45) of 2' and 3' as well as 5' isomers of these mixtures. (The 5' isomer means that the phosphate group is attached to the 5' carbon of d-ribose within the molecule of the ribonucleic acid derivative.) He found that for anti-D and anti-E, the 3' uridylic and cytidylic acids had approximately twice the inhibitory effect as did

the 2'-3' mixture. With anti-C, the 3' isomers of each inhibited no more or no less than the 2'-3' mixtures. The 5' isomers of these two inhibitors had no inhibition effect on any of the antibodies with which they were tested. For adenylic acid, on the other hand, the 2' isomer was the most effective with anti-D and anti-E, even though the others had some inhibitory effect. For anti-C, the 5' was as much involved in inhibitory effect as the 2' isomer, the 2'-3' mixture not being as effective. These studies were carried out at the 2% concentration level and a pH 6.8.

It can be seen on the basis of Hackel's work that it is possible, if certain isomers of these inhibitors had been used in the present study, the inhibition effect, in some cases, may have been increased. This, of course, could be explained by the fact that one would be coming closer, chemically, to mimicry of the specific antigen involved.

The fact that the greater the concentration of the inhibitor the greater the inhibitory effect could be explained simply in that an increase in the amount of inhibitor combines with just that much more antibody in the antisera. Thus, it leaves less antibody present in the serum to combine with the antigens on the red blood cells, which, in turn, causes a reduction in the hemagglutination reaction. Also, the increased inhibition effect of increased concentration is to be expected if a monomolecular reaction is taking place. In other words, one molecule

of inhibitor substance is combining with one molecule of antibody, thus interfering with the antibody molecule's effective combination with the specific antigen. It might be suspected that increase in concentration was causing a non-specific reaction. However, Hackel (personal communication) pointed out that 8% to 12% concentrations of other substances reported on earlier had no inhibitory effect. Also, preliminary work done by this author with 8% concentrations of these inhibitors at pH 6.8 had no inhibitory effect upon anti-A and anti-B serum.

It is not quite so easy to explain the fact that inhibition, in general, is increased with the increase in alkalinity of the inhibitors but that there are exceptions in that it is not always most effective at a pH of 8.0; and that in the case of CMP with anti-D, it is as effective at a pH of 6.6 as well as at an alkaline pH. It could be postulated that obviously hydroxyl ions are more important than hydrogen ions (with the one exception) in effecting a chemical bond between the antibody and inhibitor involved. It can be ruled out that pH alone is responsible for this inhibition effect since the phosphate buffers used at the different pH's had no inhibitory effect.

It is possible that the hydroxyl ions could remove a hydrogen of the NH_2 group on the inhibitor molecule. This molecule could then effect a hydrogen bond with an appropriate site of the antibody; or the hydroxyl ion could

remove a hydrogen from the antibody (such as from an amino group), making it possible for a hydrogen bond between this site on the antibody and a C =O group of the pyrimidine part of the inhibitor molecule. A nitrogen-nitrogen bond could be effected through removal of hydrogen atoms from nitrogen atoms of both the inhibitor molecule and antibody; or through removal of a hydrogen from a phosphate group, an oxygen could combine the antibody molecule with the inhibitor. As can be seen, the speculations are numerous concerning the kinds of bonds possible.

However, another important feature in this connection is spatial relationship. In other words, the specificity of an antibody for an antigen may depend not only upon the determinant groups within each molecule but also upon the spatial arrangement of these groups. The weaker inhibition of one inhibitor as compared to another, then, could be due to the failure of oppositely charged groups to correspond perfectly in position.

It is evident, then, this investigation has demonstrated that increase in concentration and an increase of hydroxyl ions (alkaline pH) of all three inhibitors have caused them to be more effective with anti-Rh sera. In a comparison of the general trend curves, it was found that adenylic acid was most effective in inhibition with anti-D, anti-C and anti-E sera at all concentrations and most pH's than cytidylic or uridylic acid. All three inhibitors were

less effective with anti-C than with anti-D and anti-E serum. All three inhibitors were more effective, particularly at higher pH's, with anti-E than with anti-D.

In terms of the inhibition reaction theory, then, adenylic acid comes closest to mimicry of anti-D, anti-C and anti-E. All of the inhibitors come closest to imitating anti-E, with anti-D being a close second. Uridylic and cytidylic acids were about equal in their inhibition effects, with all three antisera even though they both have their own fluctuations depending upon the pH involved.

It is apparent that this study is but one small step in the process of elucidating the role of the ribonucleic acid derivatives in the inhibition of anti-Rh sera. Even though this investigation has proved the increased inhibition effectiveness of these inhibitors, it would be interesting to know what effects concentration and pH have on isomers of these inhibitors as compared to 2'-3' mixtures. It is known that these inhibitors are specific for anti-Rh sera and Lutheran sera at 2% concentration and pH 6.8. However, certainly further investigation of these inhibitors at other concentrations and pH's would have to be more thoroughly checked out than was done in this investigation. For example, these inhibitors at various concentrations and pH's other than 2% and pH 6.8 could be tested with other kinds of antisera to confirm their specificity or non-specificity. Thus, it would appear

that further study of these ribonucleic acid derivatives might be quite useful in further elucidating their exact roles in causing inhibition of anti-Rh sera.

TABLE I

AMP VERSUS ANTI-D; °/o INHIBITION

CONCEN-TRATION	SERUM SAMPLES	pH 6.6	pH 6 8	pH 7.0	pH 7 2	pH 7.4	pH 8.0
2 °/o	I	9.0	23.0	30.0	14.0	27.0	64.0
	II	36.0	17.0	50.0	39.0	57.0	63.0
	III	28.0	24.0	7.0	33.0	47.0	68.0
	AVERAGE	24.0	21.0	29.0	29.0	44.0	65.0
4 °/o	I	45.0	50.0	67.0	32.0	41.0	64.0
	II	50.0	33.0	36.0	57.0	63.0	63.0
	III	28.0	71.0	36.0	58.0	56.0	76.0
	AVERAGE	41.0	51.0	46.0	49.0	53.0	68.0
6 °/o	I	55.0	50.0	67.0	46.0	58.0	78.0
	II	68.0	61.0	54.0	67.0	89.0	73.0
	III	72.0	85.0	64.0	44.0	85.0	95.0
	AVERAGE	65.0	65.0	62.0	52.0	77.0	82.0
8 °/o	I	65.0	64.0	82.0	68.0	73.0	86.0
	II	77.0	72.0	82.0	96.0	100.0	79.0
	III	86.0	88.0	82.0	56.0	94.0	100.0
	AVERAGE	76.0	75.0	82.0	73.0	89.0	88.0
SERUM + PHOSPHATE BUFFER = 0°/o INHIBITION AT ABOVE pH's							

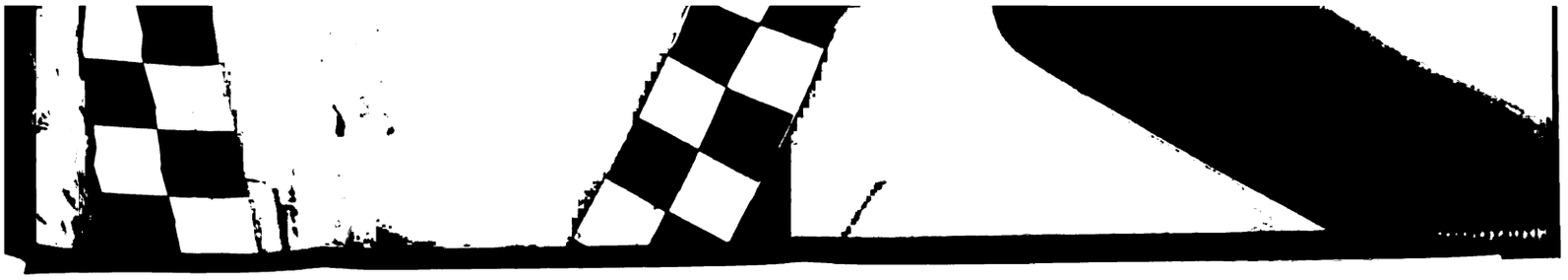


TABLE II

AMP VERSUS ANTI-C; % INHIBITION

CONCENTRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 %	I	0.0	0.0	16.0	17.0	18.0	57.0
	II	8.0	0.0	0.0	12.0	0.0	32.0
	III	18.0	26.0	26.0	21.0	0.0	67.0
	AVERAGE	9.0	9.0	14.0	17.0	6.0	52.0
4 %	I	21.0	32.0	26.0	21.0	32.0	53.0
	II	16.0	6.0	20.0	27.0	32.0	36.0
	III	23.0	26.0	26.0	21.0	33.0	63.0
	AVERAGE	20.0	21.0	24.0	23.0	32.0	51.0
6 %	I	21.0	36.0	35.0	35.0	36.0	53.0
	II	25.0	26.0	35.0	55.0	50.0	68.0
	III	36.0	37.0	26.0	10.0	39.0	89.0
	AVERAGE	27.0	33.0	32.0	33.0	42.0	70.0
8 %	I	42.0	59.0	65.0	52.0	50.0	66.0
	II	41.0	45.0	58.0	58.0	68.0	68.0
	III	50.0	37.0	45.0	21.0	59.0	89.0
	AVERAGE	44.0	47.0	56.0	44.0	59.0	74.0
SERUM + PHOSPHATE BUFFER = 0% INHIBITION AT ABOVE pH's							

TABLE III

AMP VERSUS ANTI-E °/o INHIBITION

CONCENTRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 °/o	I	15.0	29.0	35.0	19.0	35.0	92.0
	II	13.0	30.0	20.0	41.0	32.0	73.0
	III	26.0	17.0	32.0	47.0	71.0	62.0
	AVERAGE	18.0	25.0	29.0	36.0	46.0	76.0
4 °/o	I	50.0	32.0	52.0	32.0	52.0	85.0
	II	41.0	42.0	38.0	56.0	50.0	85.0
	III	50.0	50.0	56.0	71.0	79.0	62.0
	AVERAGE	47.0	41.0	49.0	53.0	60.0	77.0
6 °/o	I	58.0	50.0	69.0	50.0	69.0	92.0
	II	44.0	58.0	50.0	84.0	100.0	96.0
	III	61.0	90.0	56.0	77.0	85.0	83.0
	AVERAGE	54.0	66.0	58.0	70.0	85.0	90.0
8 °/o	I	58.0	68.0	83.0	82.0	83.0	92.0
	II	59.0	73.0	85.0	100.0	100.0	100.0
	III	61.0	90.0	63.0	85.0	88.0	92.0
	AVERAGE	59.0	77.0	77.0	89.0	90.0	95.0

SERUM+ PHOSPHATE BUFFER = 0% INHIBITION AT ABOVE pH's

TABLE IV

UMP VERSUS ANTI - D; % INHIBITION

CONCEN-TRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 %	I	18.0	18.0	18.0	21.0	21.0	29.0
	II	15.0	18.0	15.0	21.0	17.0	52.0
	III	22.0	32.0	22.0	18.0	28.0	28.0
	AVERAGE	18.0	23.0	18.0	20.0	22.0	36.0
4 %	I	18.0	32.0	36.0	35.0	42.0	32.0
	II	24.0	36.0	39.0	21.0	28.0	48.0
	III	36.0	45.0	50.0	36.0	36.0	28.0
	AVERAGE	26.0	38.0	42.0	31.0	35.0	36.0
6 %	I	32.0	50.0	50.0	38.0	42.0	32.0
	II	39.0	41.0	55.0	42.0	39.0	58.0
	III	50.0	61.0	50.0	59.0	72.0	72.0
	AVERAGE	40.0	51.0	52.0	46.0	51.0	54.0
8 %	I	50.0	59.0	50.0	52.0	63.0	68.0
	II	48.0	66.0	63.0	42.0	57.0	75.0
	III	60.0	74.0	72.0	77.0	86.0	86.0
	AVERAGE	53.0	66.0	62.0	57.0	69.0	76.0
SERUM + PHOSPHATE BUFFER = 0 % INHIBITION AT ABOVE pH's							

TABLE V

UMP VERSUS ANTI-C ; % INHIBITION

CONCEN-TRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 %	I	0.0	21.0	9.0	17.0	32.0	25.0
	II	0.0	0.0	0.0	0.0	10.0	46.0
	III	0.0	0.0	0.0	21.0	21.0	33.0
	AVERAGE	0.0	7.0	3.0	13.0	21.0	35.0
4 %	I	0.0	21.0	9.0	17.0	23.0	28.0
	II	0.0	3.0	9.0	17.0	26.0	41.0
	III	0.0	17.0	0.0	21.0	25.0	39.0
	AVERAGE	0.0	14.0	6.0	18.0	25.0	36.0
6 %	I	14.0	42.0	28.0	35.0	41.0	28.0
	II	15.0	17.0	18.0	35.0	29.0	41.0
	III	0.0	28.0	14.0	21.0	42.0	39.0
	AVERAGE	10.0	29.0	20.0	30.0	37.0	36.0
8 %	I	14.0	60.0	28.0	52.0	50.0	57.0
	II	27.0	35.0	32.0	39.0	45.0	54.0
	III	0.0	28.0	32.0	21.0	63.0	44.0
	AVERAGE	14.0	41.0	31.0	37.0	53.0	52.0
SERUM + PHOSPHATE BUFFER = 0% INHIBITION AT ABOVE pH's							

TABLE VI

UMP VERSUS ANTI-E; % INHIBITION

CONCENTRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 %	I	12.0	21.0	3.0	13.0	21.0	23.0
	II	0.0	14.0	15.0	17.0	30.0	64.0
	III	14.0	50.0	40.0	44.0	33.0	33.0
	AVERAGE	9.0	28.0	19.0	25.0	28.0	40.0
4 %	I	42.0	26.0	21.0	28.0	41.0	32.0
	II	23.0	18.0	15.0	21.0	36.0	50.0
	III	52.0	50.0	50.0	72.0	47.0	67.0
	AVERAGE	39.0	31.0	29.0	40.0	41.0	50.0
6 %	I	42.0	38.0	40.0	48.0	54.0	55.0
	II	36.0	18.0	15.0	43.0	55.0	79.0
	III	52.0	75.0	65.0	78.0	67.0	87.0
	AVERAGE	43.0	44.0	40.0	56.0	59.0	74.0
8 %	I	58.0	52.0	52.0	56.0	71.0	73.0
	II	45.0	32.0	33.0	60.0	68.0	84.0
	III	76.0	90.0	80.0	89.0	87.0	100.0
	AVERAGE	60.0	58.0	55.0	68.0	75.0	86.0
SERUM + PHOSPHATE BUFFER = 0 % INHIBITION AT ABOVE pH's							

TABLE VII

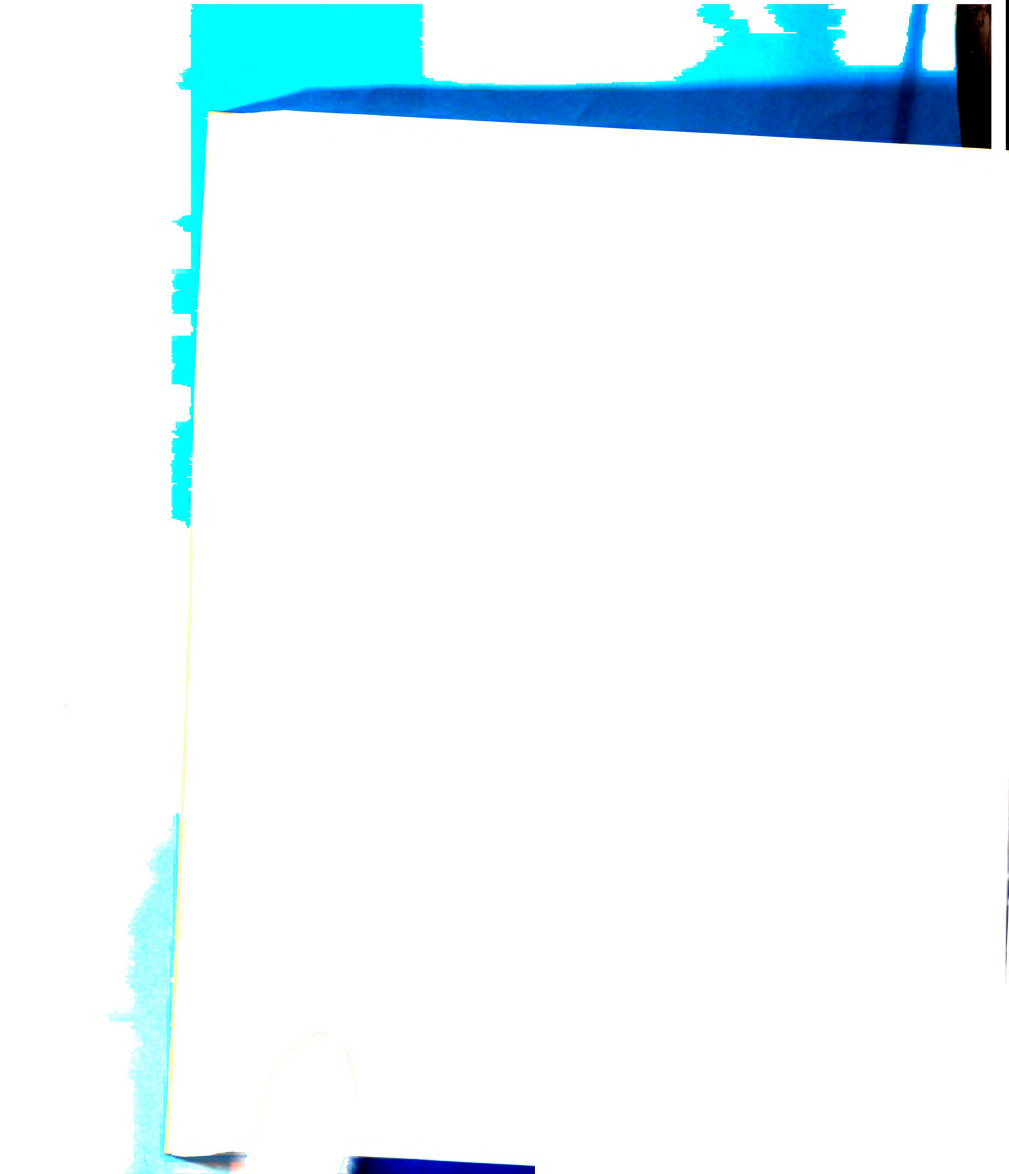
CMP VERSUS ANTI-D; % INHIBITION

CONCENTRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 %	I	0.0	24.0	30.0	18.0	9.0	35.0
	II	14.0	21.0	14.0	14.0	4.0	64.0
	III	63.0	0.0	22.0	31.0	10.0	12.0
	AVERAGE	26.0	15.0	22.0	21.0	8.0	37.0
4 %	I	17.0	32.0	50.0	48.0	35.0	43.0
	II	36.0	21.0	32.0	36.0	21.0	41.0
	III	80.0	22.0	50.0	50.0	35.0	32.0
	AVERAGE	44.0	25.0	44.0	45.0	30.0	39.0
6 %	I	50.0	31.0	36.0	50.0	58.0	64.0
	II	39.0	39.0	50.0	66.0	48.0	48.0
	III	90.0	44.0	50.0	71.0	65.0	32.0
	AVERAGE	60.0	38.0	45.0	62.0	57.0	48.0
8 %	I	57.0	56.0	50.0	77.0	52.0	57.0
	II	68.0	52.0	50.0	64.0	69.0	73.0
	III	96.0	58.0	64.0	81.0	90.0	51.0
	AVERAGE	74.0	55.0	55.0	74.0	70.0	60.0
SERUM + PHOSPHATE BUFFER = 0 % INHIBITION AT ABOVE pH's							

TABLE VIII

CMP VERSUS ANTI - C; °/o INHIBITION

CONCEN-TRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 °/o	I	0.0	13.0	9.0	0.0	15.0	26.0
	II	0.0	14.0	15.0	0.0	8.0	38.0
	III	5.0	11.0	17.0	0.0	0.0	22.0
	AVERAGE	2.0	13.0	14.0	0.0	8.0	29.0
4 °/o	I	6.0	18.0	9.0	0.0	30.0	26.0
	II	19.0	30.0	48.0	21.0	16.0	25.0
	III	16.0	22.0	22.0	5.0	10.0	22.0
	AVERAGE	10.0	23.0	26.0	9.0	19.0	24.0
6 °/o	I	15.0	26.0	19.0	26.0	48.0	31.0
	II	18.0	35.0	48.0	21.0	25.0	42.0
	III	35.0	40.0	39.0	16.0	26.0	36.0
	AVERAGE	23.0	34.0	35.0	21.0	33.0	36.0
8 °/o	I	33.0	34.0	28.0	53.0	57.0	41.0
	II	36.0	45.0	63.0	63.0	54.0	42.0
	III	47.0	50.0	39.0	35.0	45.0	50.0
	AVERAGE	39.0	43.0	43.0	50.0	52.0	44.0
SERUM + PHOSPHATE BUFFER = 0 °/o INHIBITION AT ABOVE pH's							



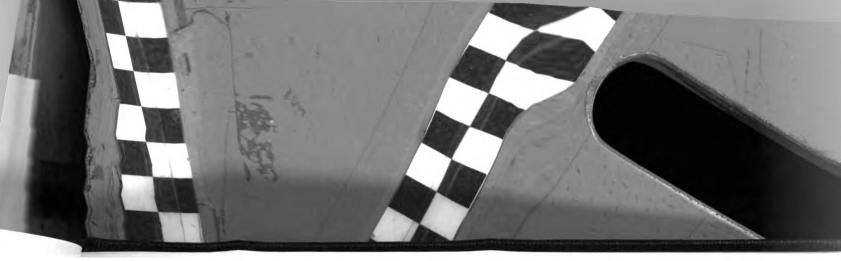


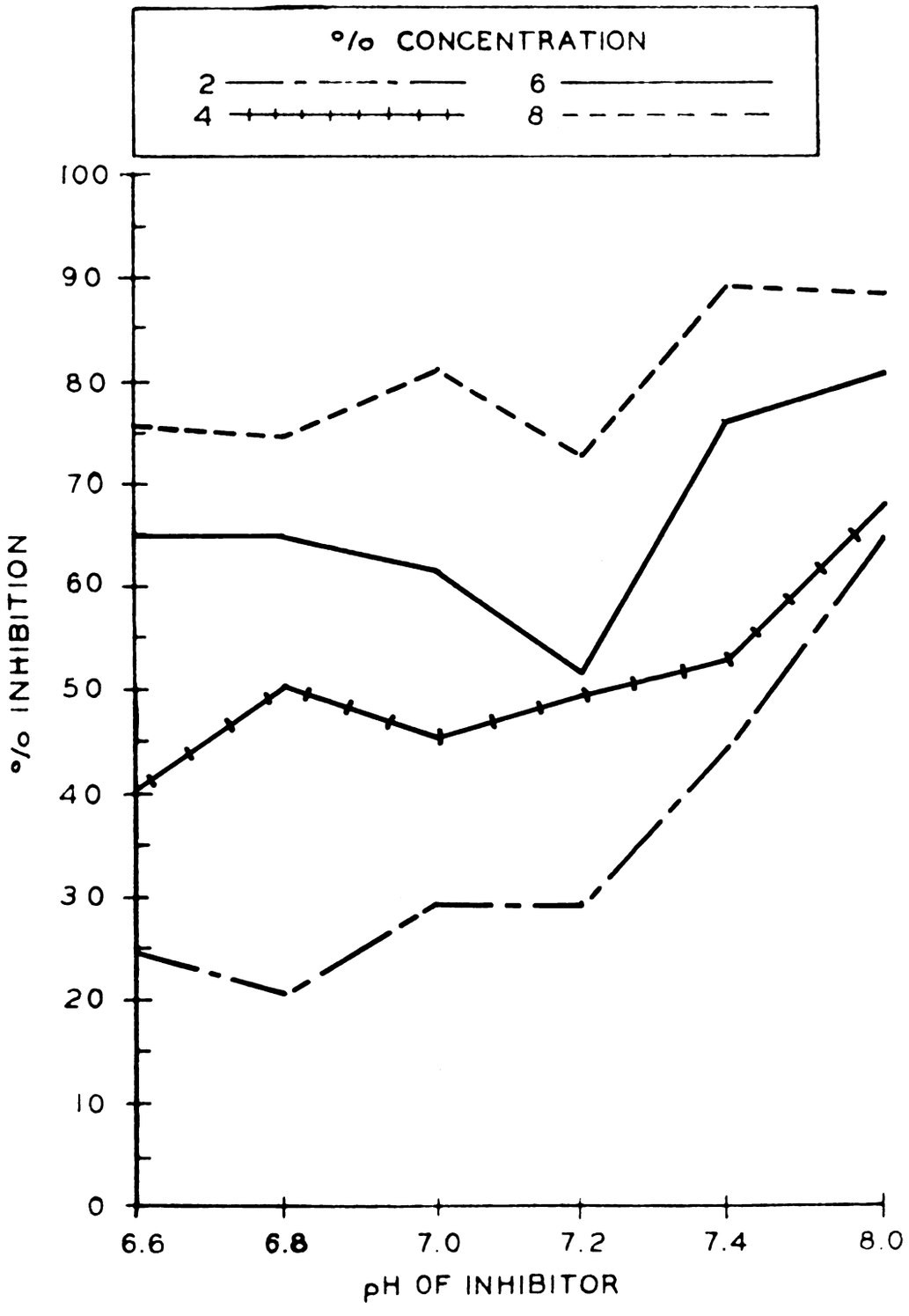
TABLE IX

CMP VERSUS ANTI-E; % INHIBITION

CONCENTRATION	SERUM SAMPLES	pH 6.6	pH 6.8	pH 7.0	pH 7.2	pH 7.4	pH 8.0
2 %	I	0.0	0.0	22.0	0.0	15.0	55.0
	II	19.0	21.0	32.0	17.0	17.0	82.0
	III	24.0	22.0	31.0	31.0	31.0	62.0
	AVERAGE	14.0	14.0	28.0	16.0	21.0	66.0
4 %	I	18.0	22.0	42.0	36.0	45.0	55.0
	II	28.0	35.0	32.0	33.0	38.0	82.0
	III	32.0	57.0	62.0	62.0	62.0	83.0
	AVERAGE	26.0	38.0	45.0	44.0	48.0	73.0
6 %	I	32.0	31.0	42.0	64.0	46.0	78.0
	II	28.0	35.0	68.0	39.0	69.0	95.0
	III	47.0	57.0	62.0	83.0	92.0	83.0
	AVERAGE	36.0	41.0	57.0	62.0	69.0	85.0
8 %	I	50.0	50.0	42.0	82.0	46.0	78.0
	II	47.0	52.0	77.0	67.0	91.0	95.0
	III	71.0	78.0	83.0	83.0	92.0	92.0
	AVERAGE	56.0	60.0	67.0	77.0	76.0	88.0
SERUM + PHOSPHATE BUFFER = 0 % INHIBITION AT ABOVE pH's							

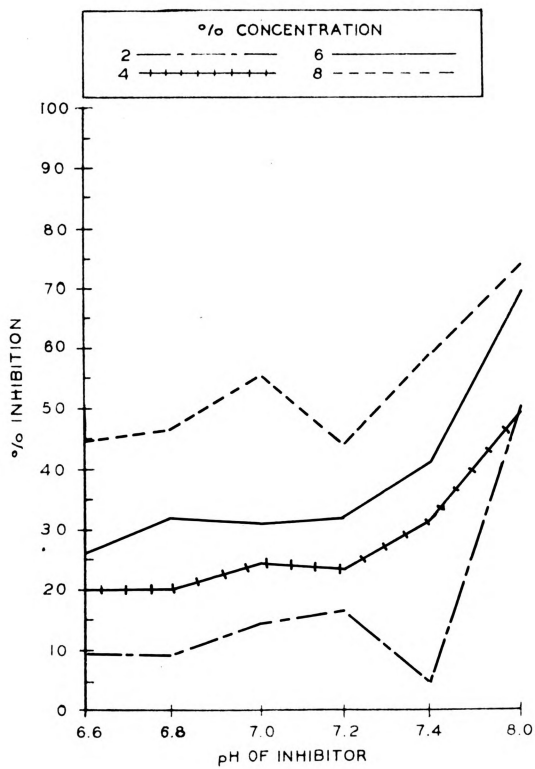
GRAPH Ia

AMP VERSUS ANTI-D



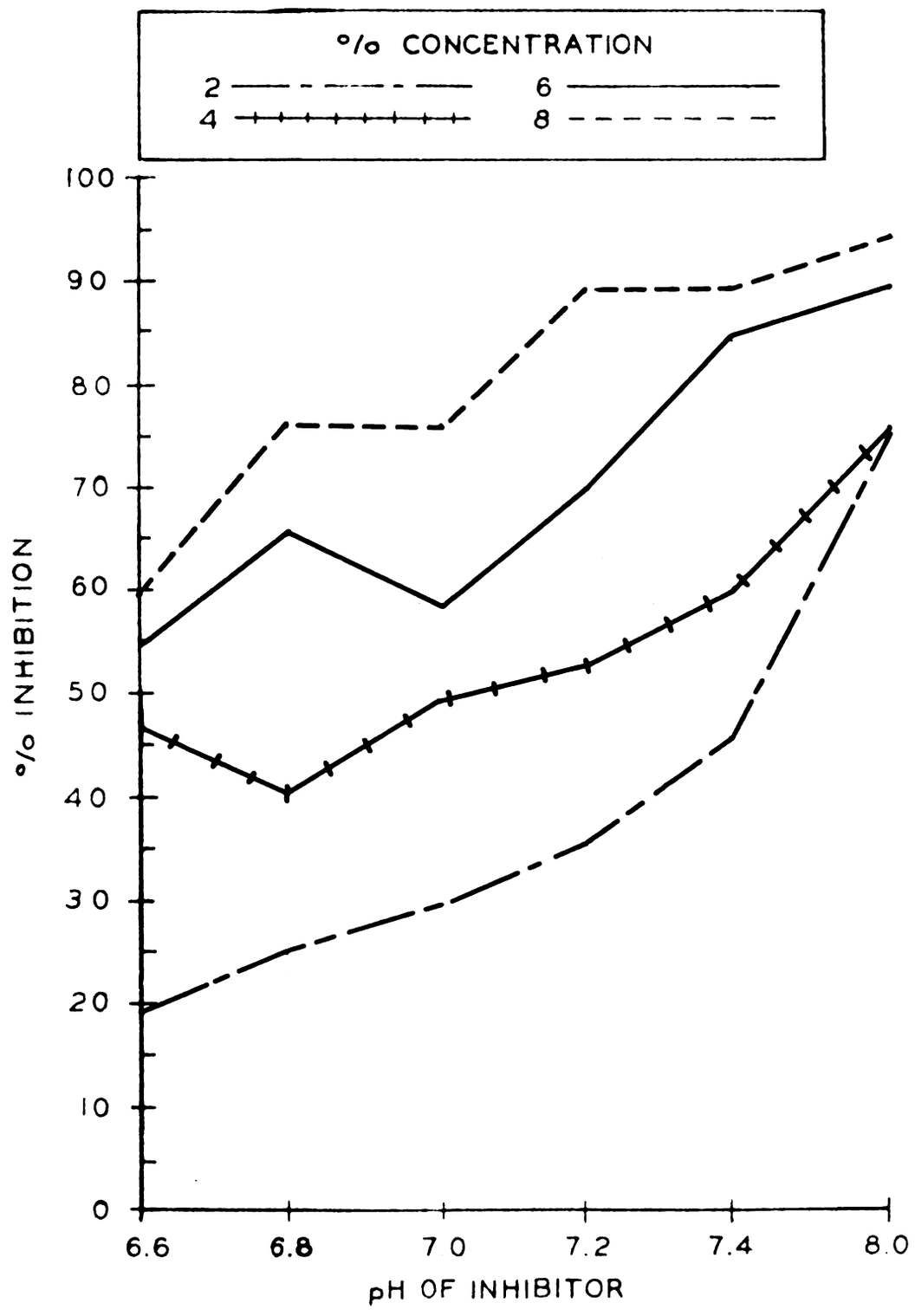
GRAPH IIa

AMP VERSUS ANTI-C



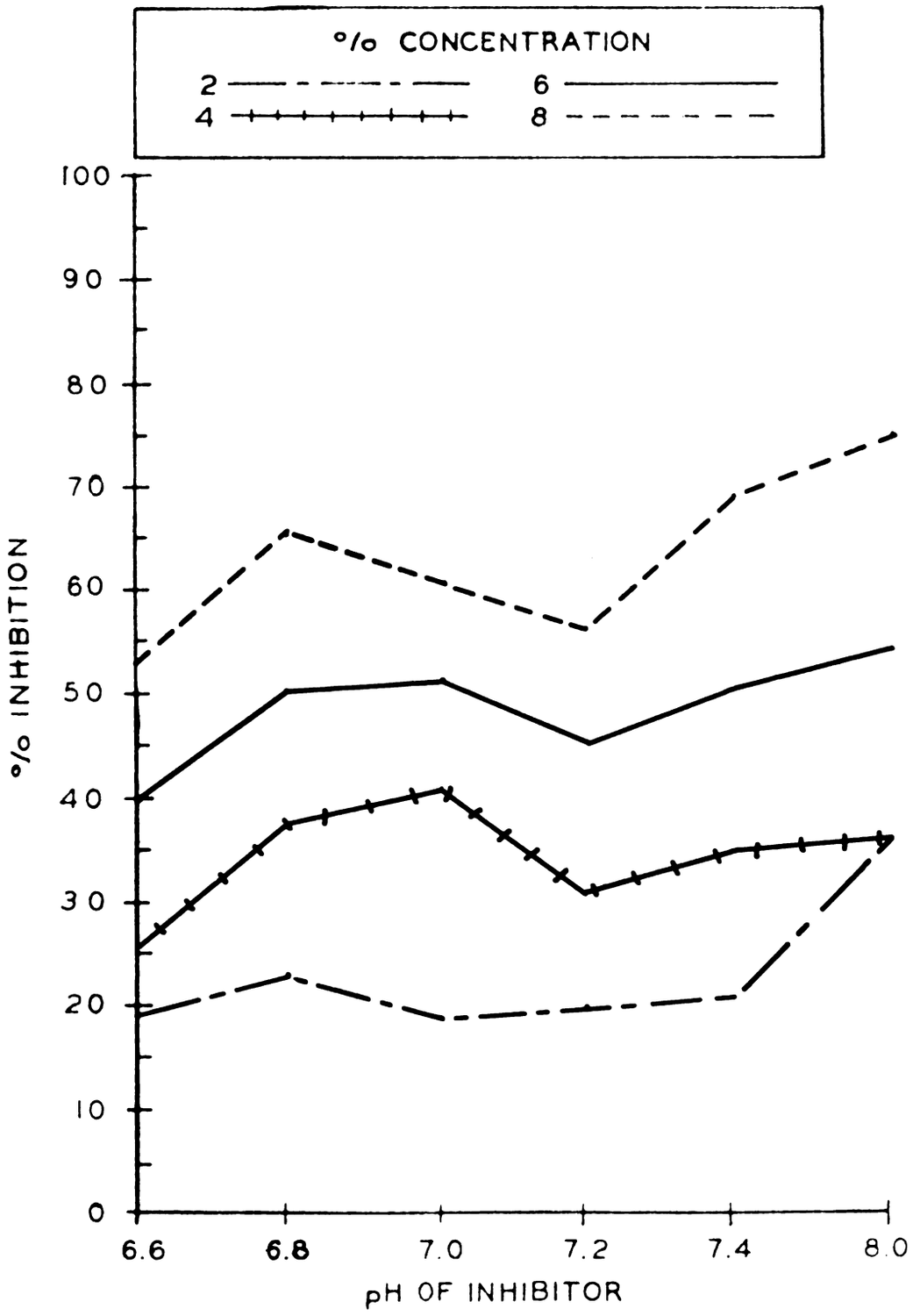
GRAPH IIIa

AMP VERSUS ANTI-E



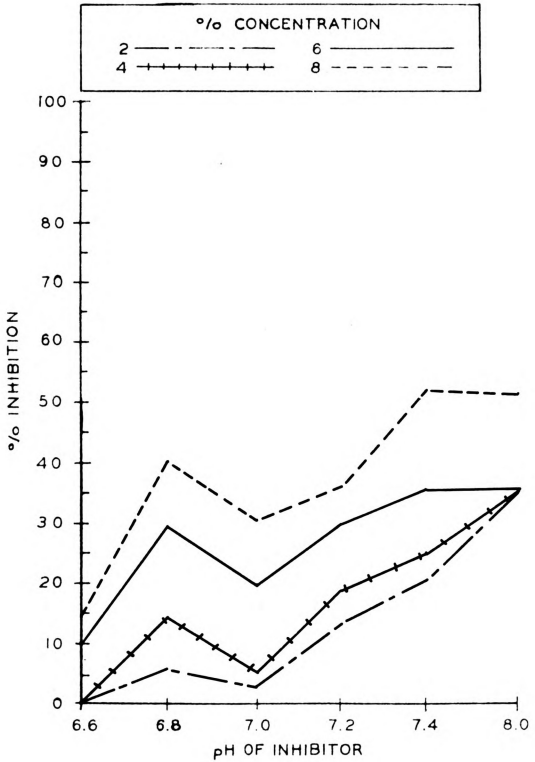
GRAPH IVa

UMP VERSUS ANTI-D



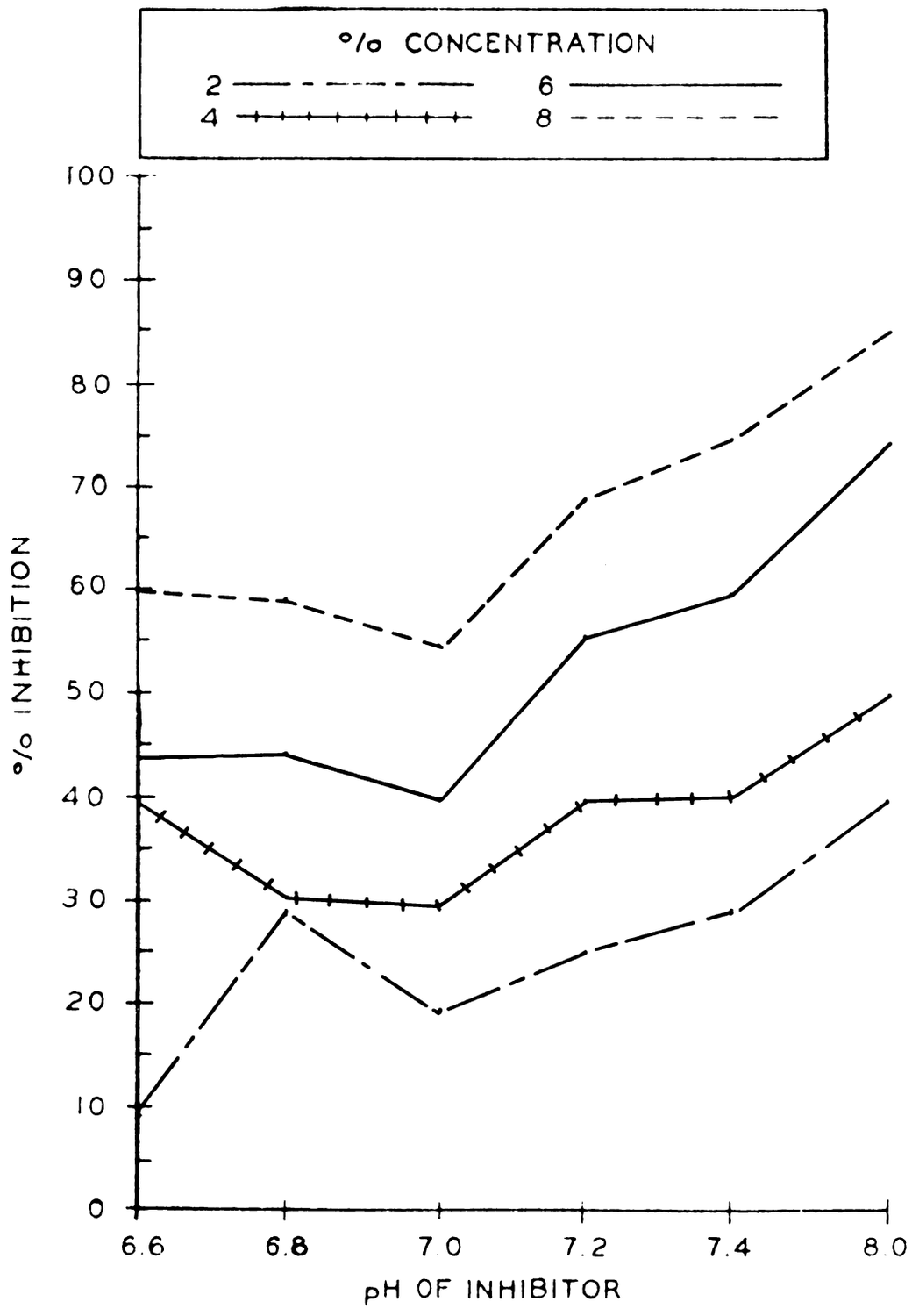
GRAPH Va

UMP VERSUS ANTI-C



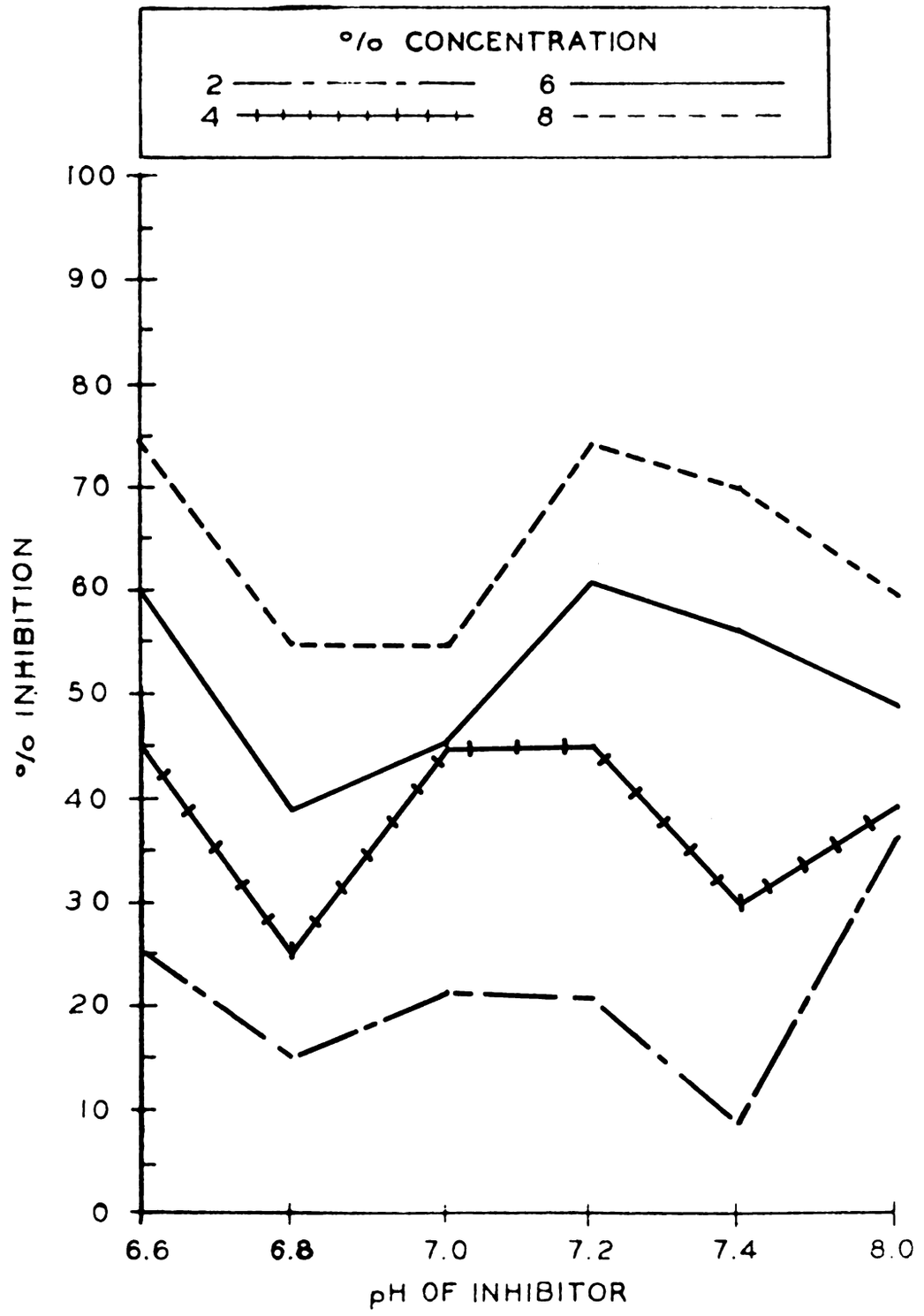
GRAPH VIa

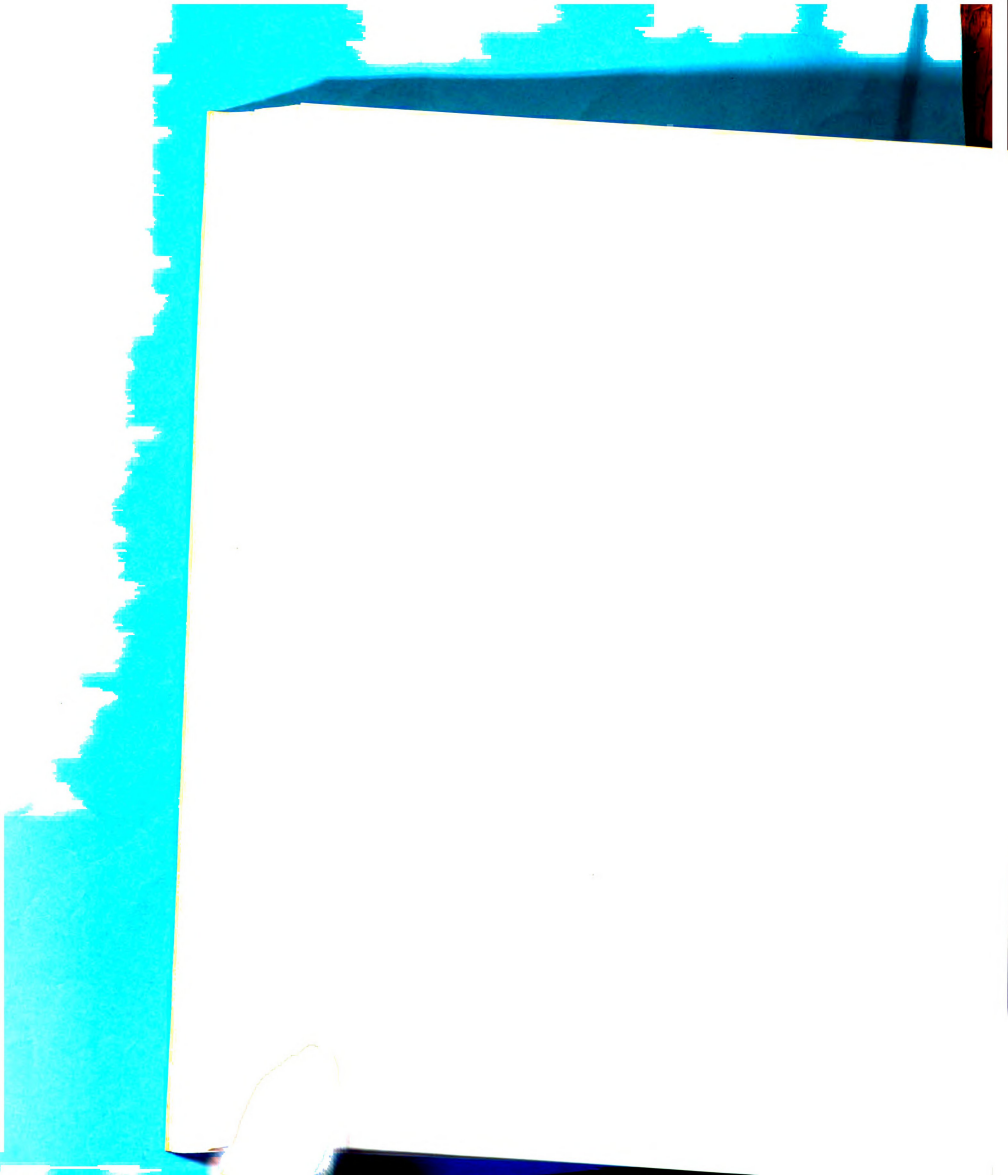
UMP VERSUS ANTI-E



GRAPH VIIa

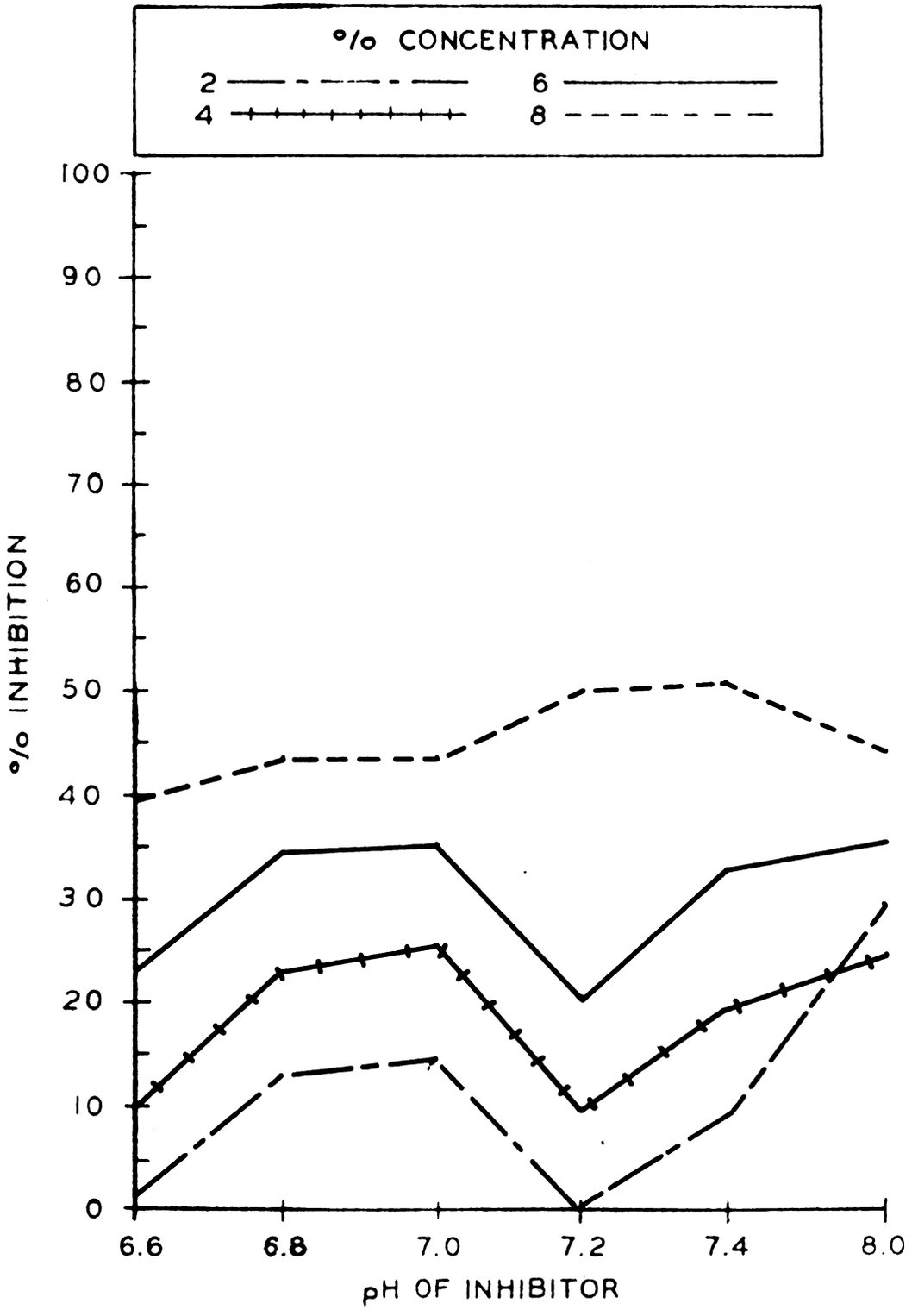
CMP VERSUS ANTI-D





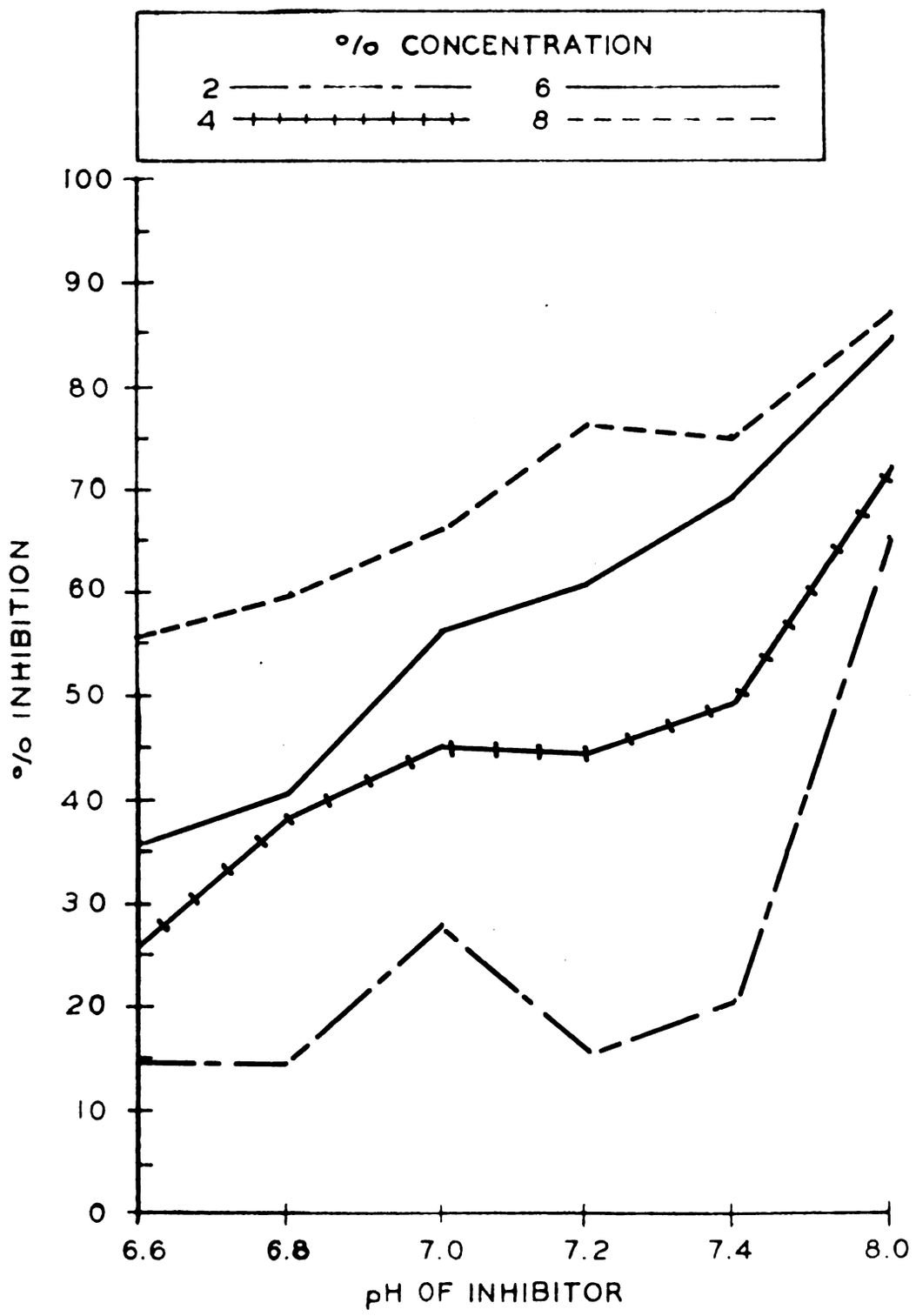
GRAPH VIIIa

CMP VERSUS ANTI-C



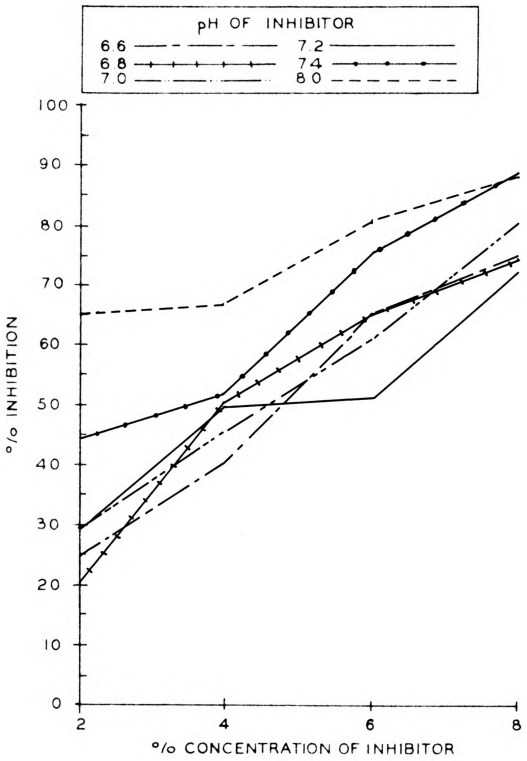
GRAPH IXa

CMP VERSUS ANTI-E



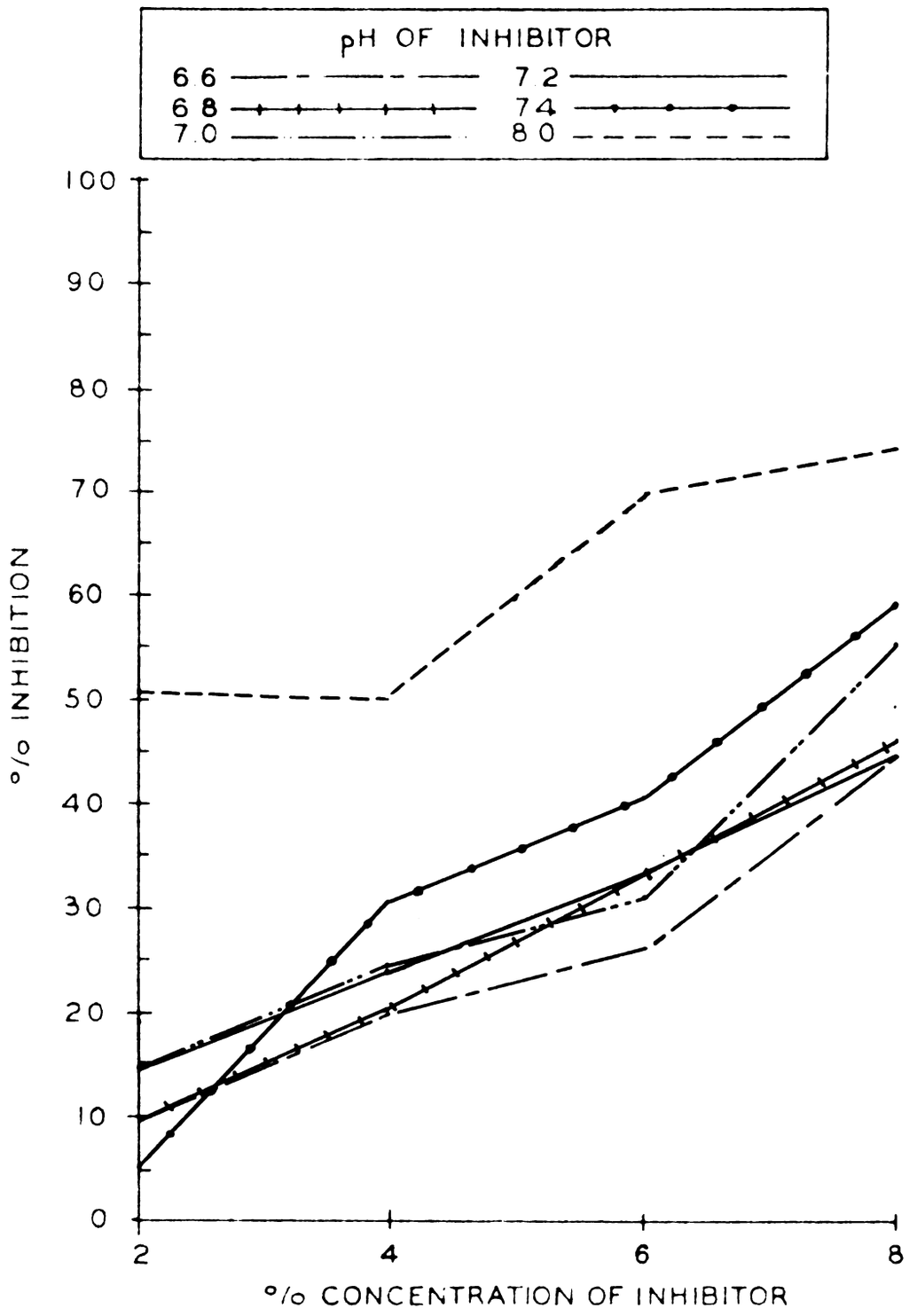
GRAPH Ib

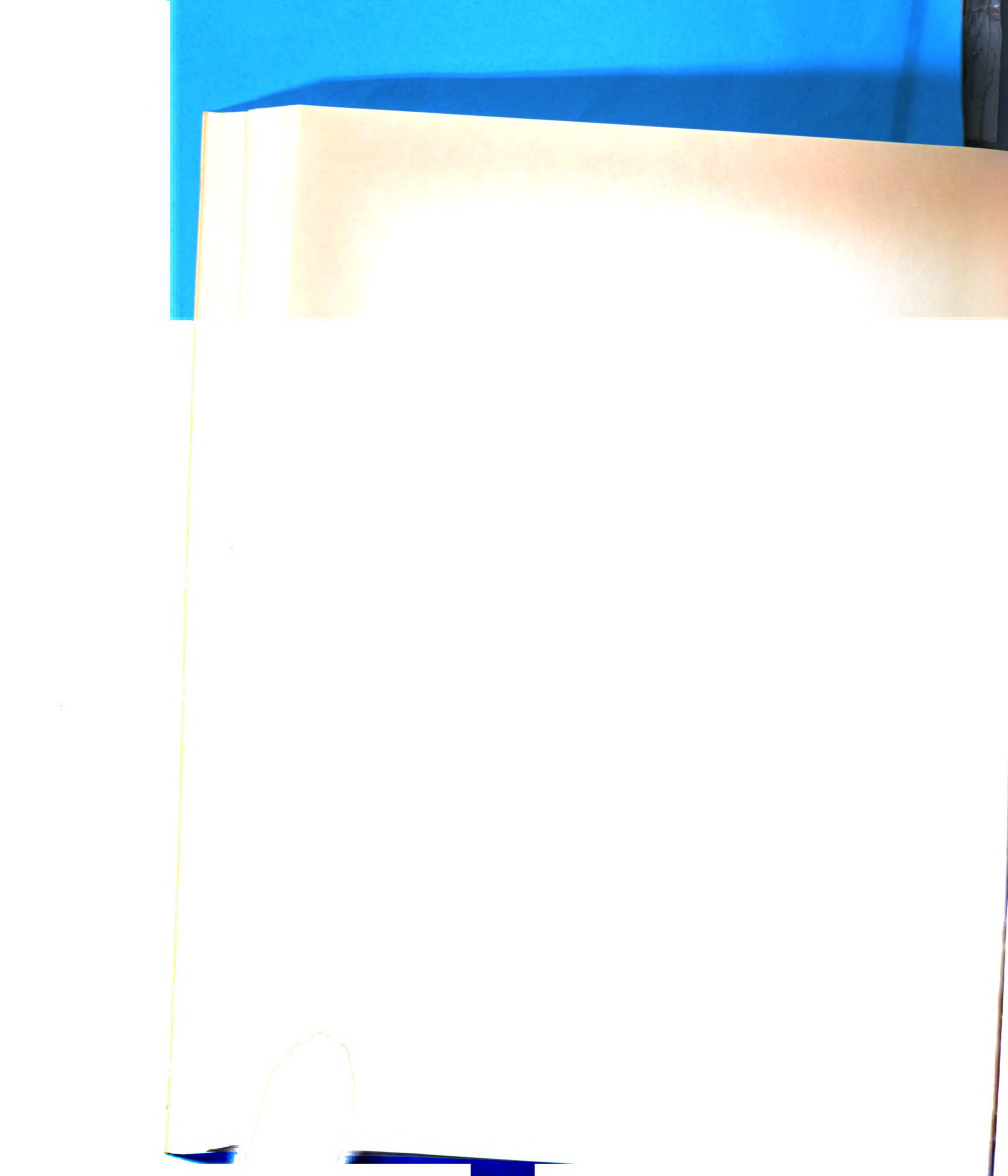
AMP VERSUS ANTI-D



GRAPH IIb

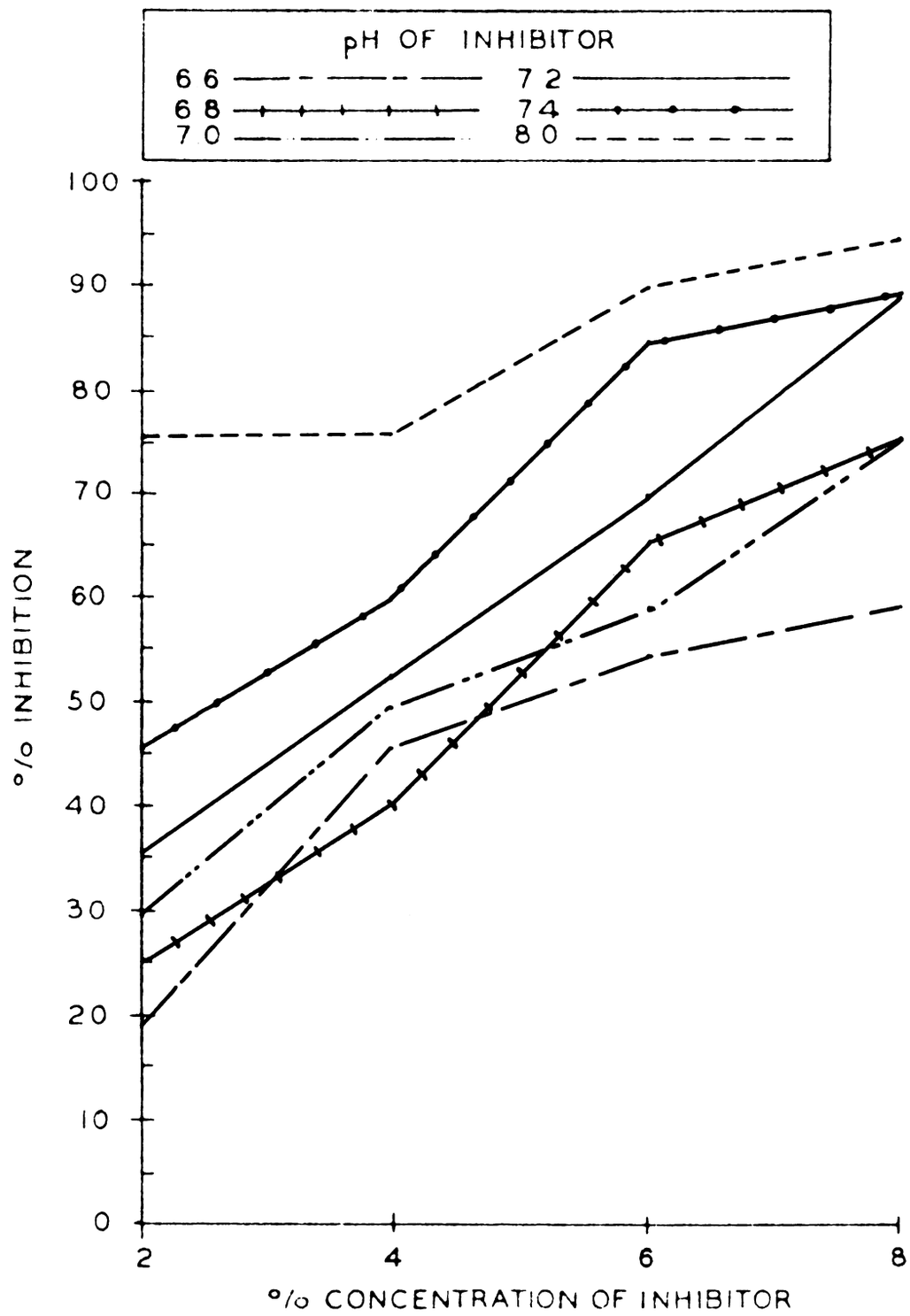
AMP VERSUS ANTI-C





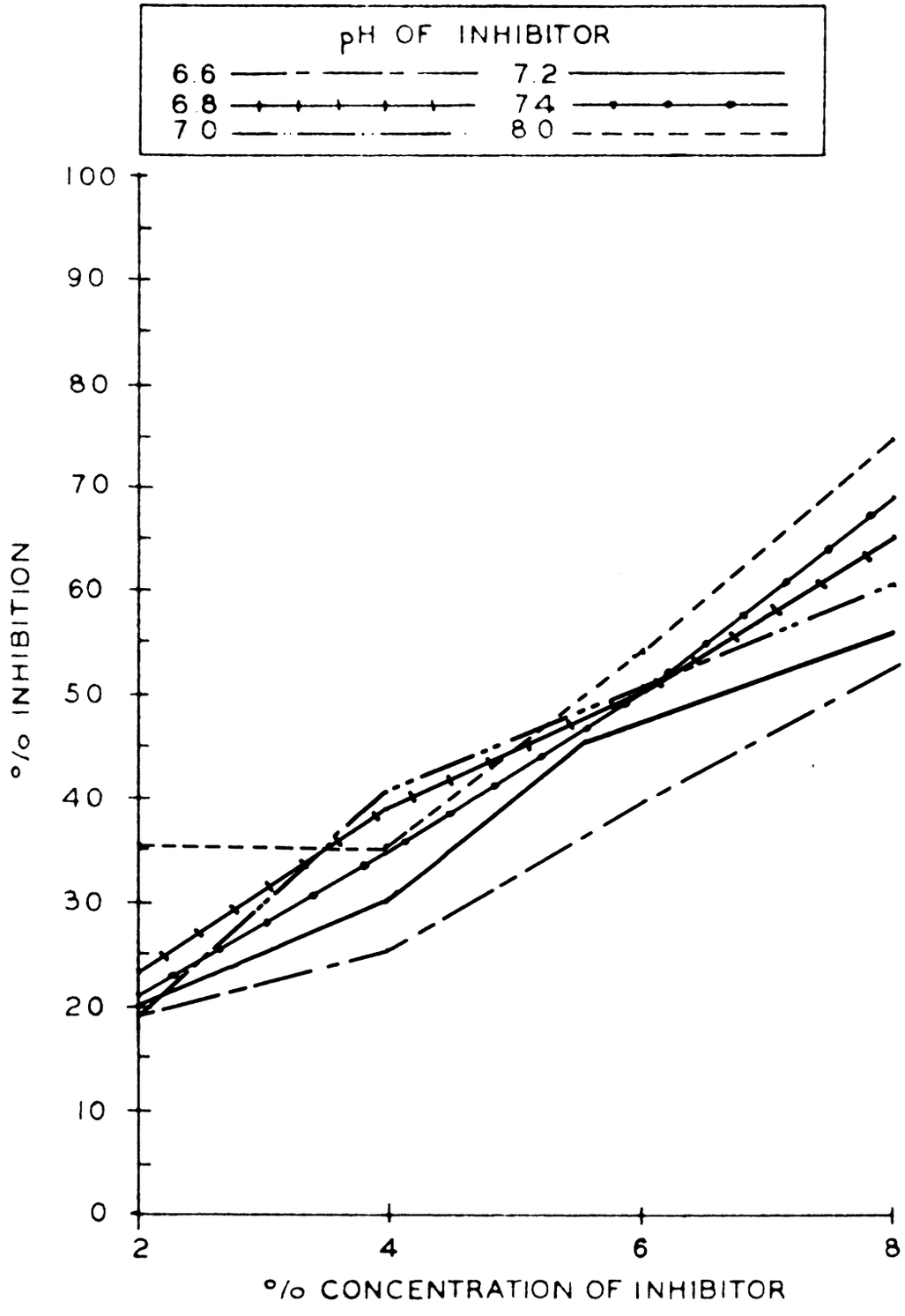
GRAPH IIIb

AMP VERSUS ANTI-E



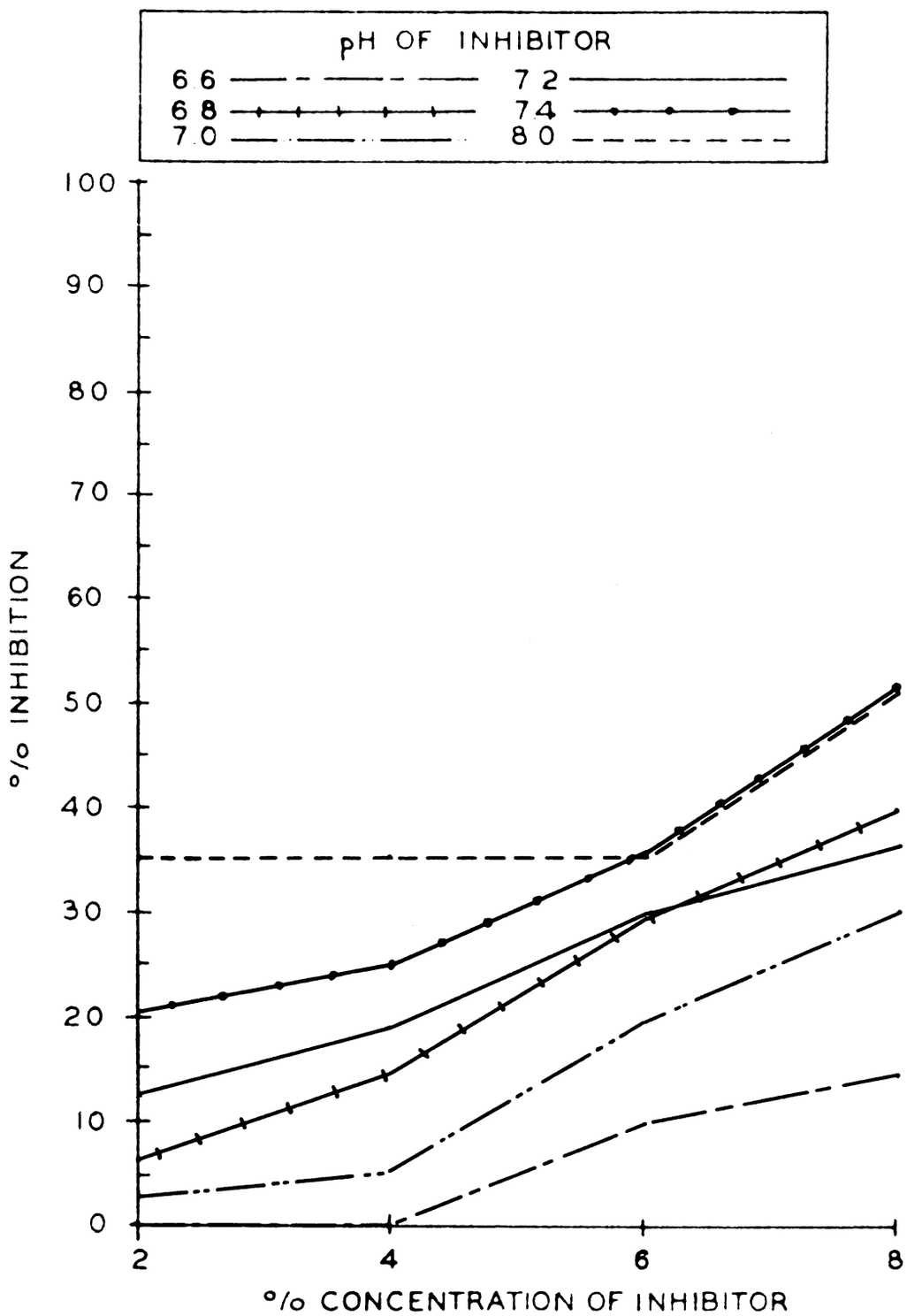
GRAPH IVb

UMP VERSUS ANTI-D



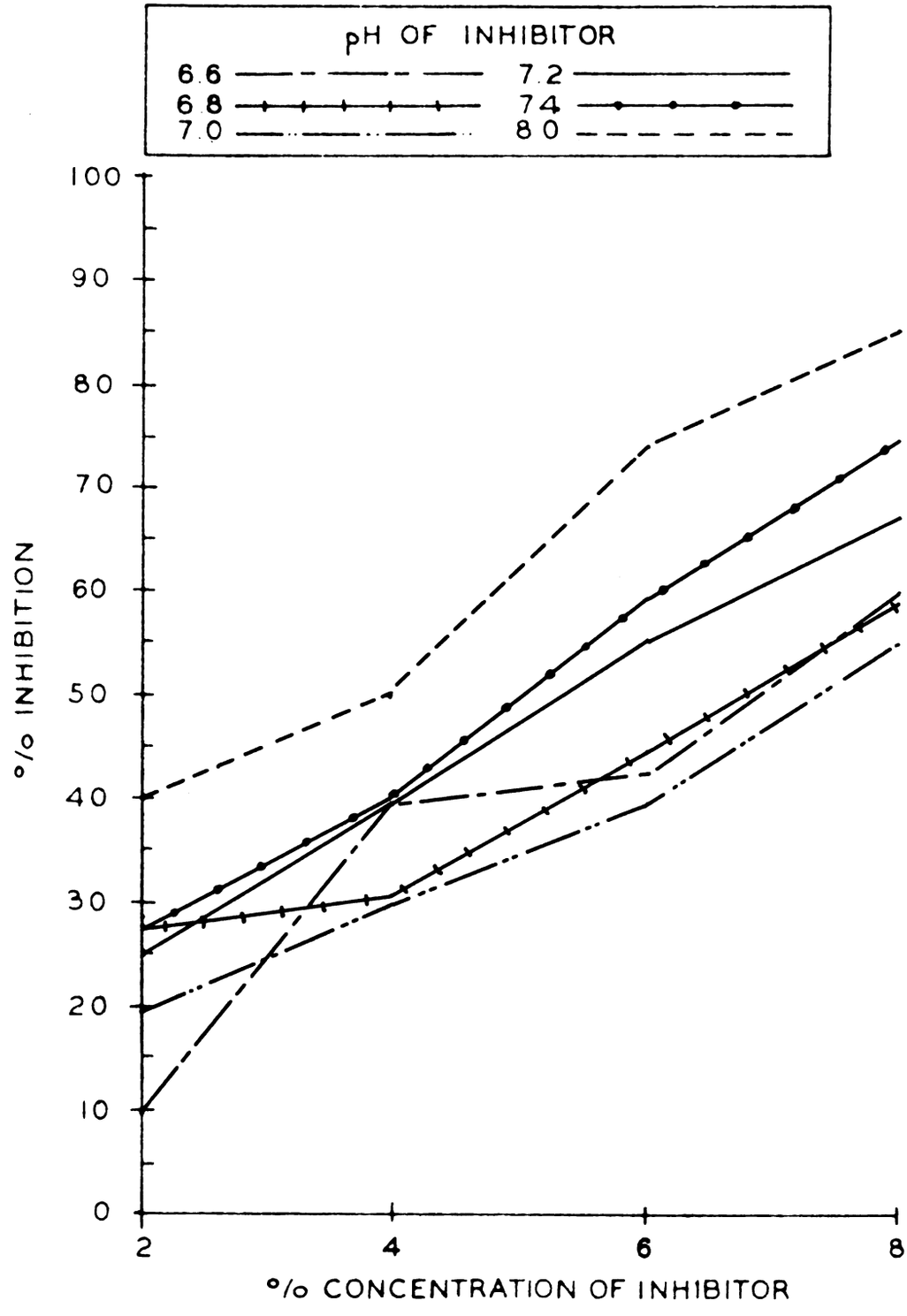
GRAPH Vb

UMP VERSUS ANTI-C



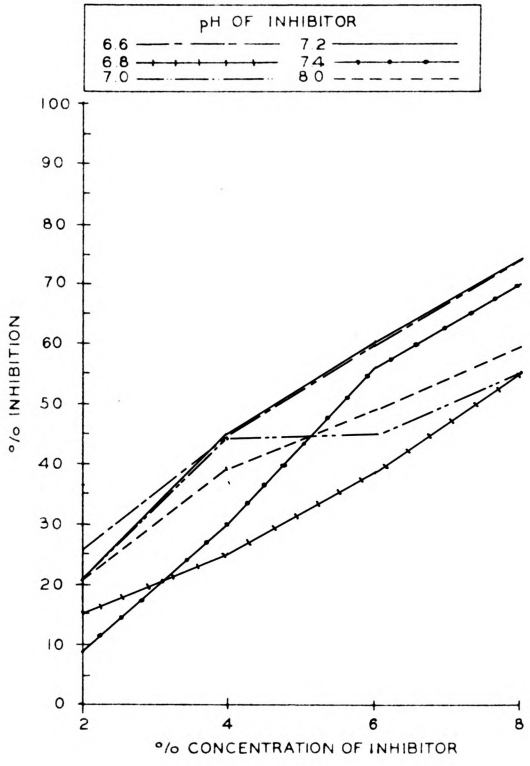
GRAPH VIb

UMP VERSUS ANTI-E



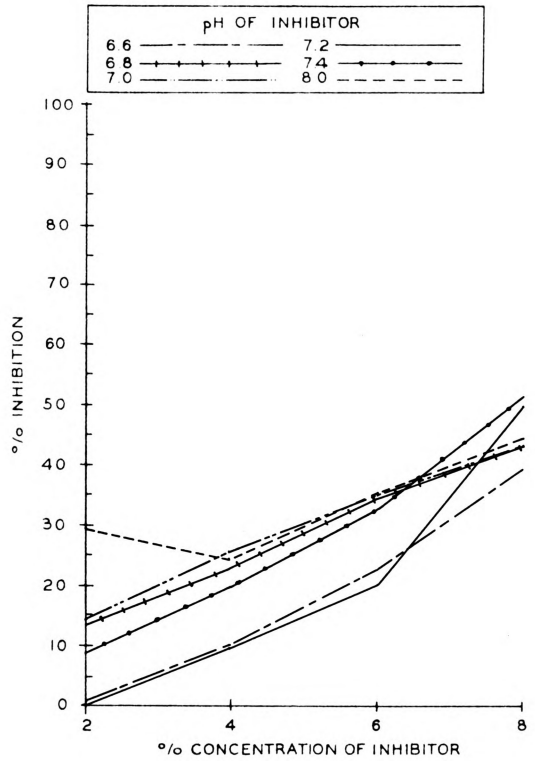
GRAPH VIIb

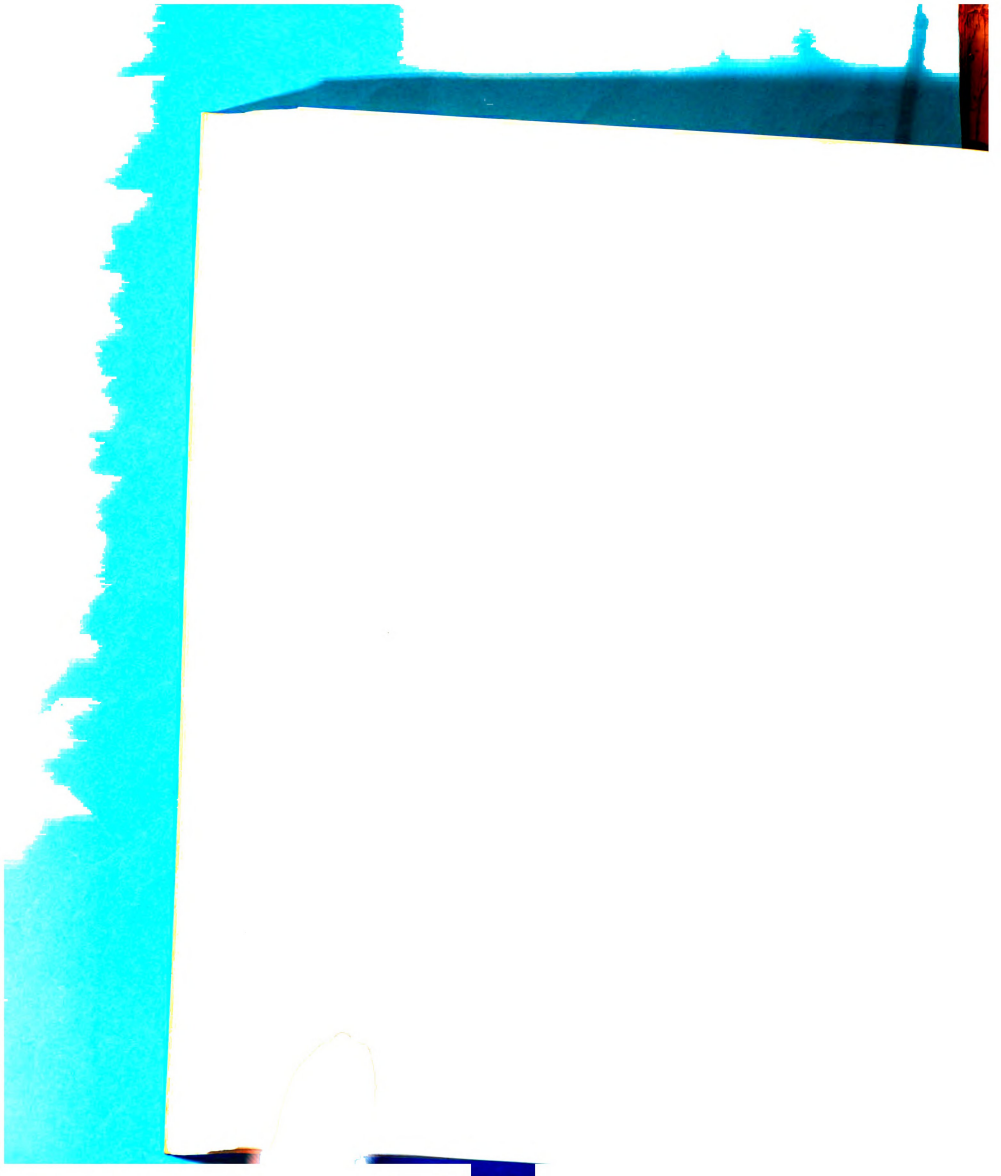
CMP VERSUS ANTI-D



GRAPH VIIIb

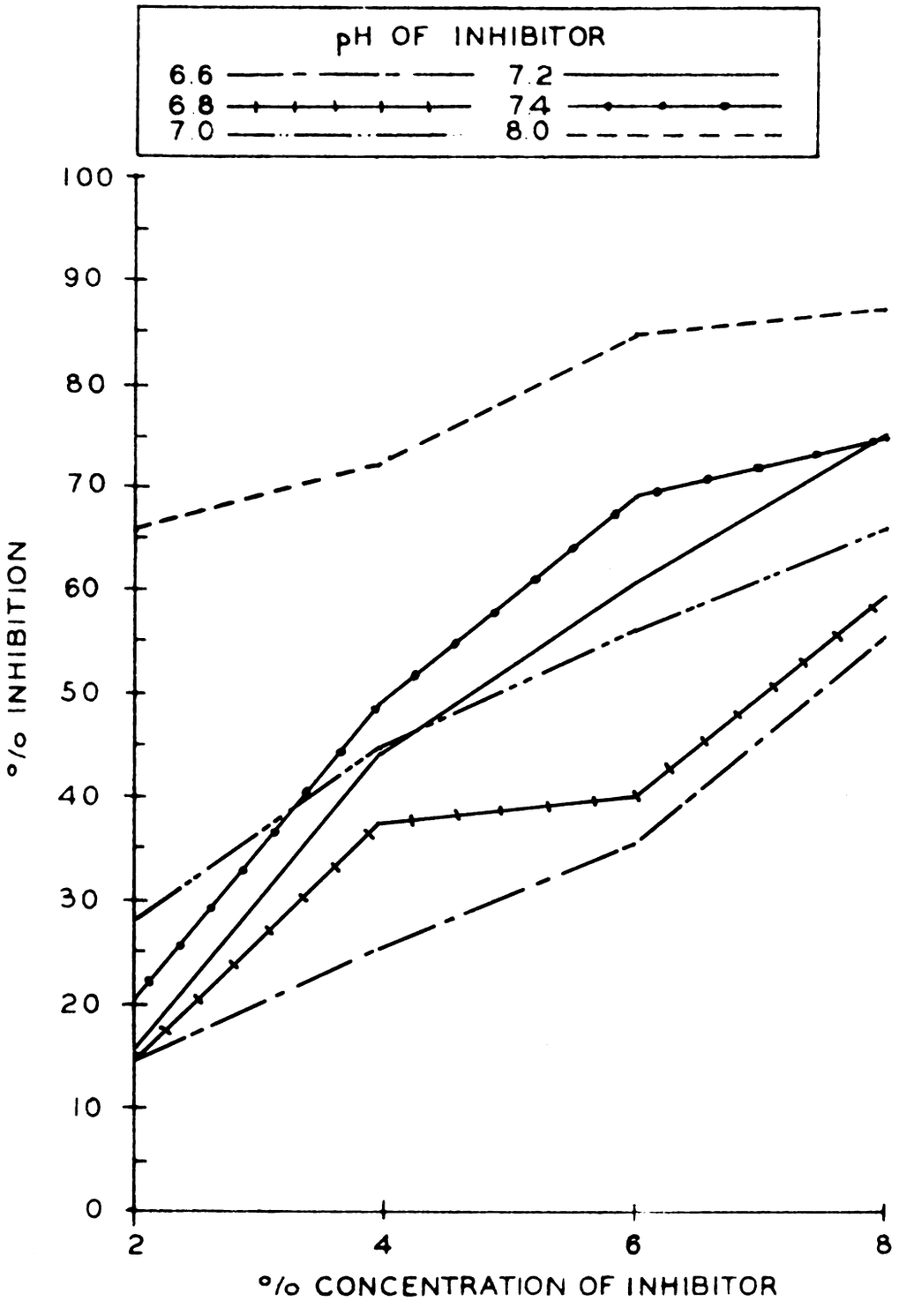
CMP VERSUS ANTI-C





GRAPH IXb

CMP VERSUS ANTI-E



CHAPTER IV

SUMMARY

The present study is an extension of Hackel and co-workers' (46, 48) investigations of three ribonucleic acids, namely, adenylic, cytidylic and uridylic acids, all 2'-3' mixtures. The original work involved the inhibitory effects of these substances on various antisera at a 2% concentration and a pH 6.8. This investigation included adenylic, cytidylic and uridylic acids at concentrations of 2%, 4%, 6% and 8% at the following pH's: 6.6, 6.8, 7.0, 7.2, 7.4 and 8.0. The Rh antisera involved were anti-D, anti-C and anti-E. The inhibitory effects were tested by use of the hemagglutination inhibition.

Increased inhibition of all three ribonucleic acid derivatives with anti-Rh sera was demonstrated with increase in concentration. It was postulated that the more inhibitor molecules in solution, the more antibody-inhibitor combinations would occur. This would leave less antibody to combine with the specific antigen on the erythrocytes. Also, the results indicated that a monomolecular reaction was taking place between antibody and inhibitor molecule because of the steady rise in inhibition as the concentration of the inhibitor substance was increased

from 2% up to 8%.

This investigation further demonstrated that alkaline pH's of all three inhibitors resulted in an increase of their inhibitory effectiveness. It was speculated that a certain amount of hydroxyl ions must be of more importance than hydrogen ions in effecting this inhibition reaction. It was further postulated that the ability of the hydroxyl ion to remove hydrogen atoms from the inhibitor molecule or antibody molecule could effect a chemical combination. Suggested bonds were hydrogen bonds through amino and/or carboxyl groups, nitrogen-nitrogen bonds, and oxygen bonds through phosphate groups.

Also, the specificity of an antibody for an antigen may depend not only upon the determinant groups within each molecule but also upon the spatial arrangement of these groups. Thus, the fact that one inhibitor was more effective than another could be explained further by the spatial arrangements of the determinant groups within the molecules. For example, the weaker inhibition of one inhibitor compared to another could be due to the failure of oppositely charged groups to correspond perfectly in position because of differences in the spatial arrangements of the determinant groups within each individual inhibitor molecule.

Taking into consideration both increased concentration and alkaline pH, adenylic acid was found to be the

most effective inhibitor with anti-D, anti-C and anti-E sera. Cytidylic and uridylic acid were almost equal in inhibitory effects. All of the inhibitors were most effective with anti-E and anti-D sera, respectively, and were least effective with anti-C.

Even though this investigation has demonstrated further increase of antigen mimicry of these ribonucleic acid derivatives in terms of the inhibition theory, there is much to be learned. For example, to be assured of the specificity of these reactions, further investigation is needed with these inhibitors at various concentrations and pH's other than anti-Rh sera. It would be interesting to know the effects of concentration and pH's on isomers of the 2'-3' mixtures used in this study. Also, further investigation of the individual differences between samples of the same kind of serum is needed--is it a true effect or not? Thus, it would appear that further experimentation would be quite helpful in further elucidating the exact role that ribonucleic acid derivatives play in inhibition of anti-Rh sera.

BIBLIOGRAPHY

1. Abelson, Neva M., and Rawson, Arnold J. 1959. Studies of blood group antibodies. II. Fractionation of Rh antibodies by anion-cation cellulose exchange chromotography. Journal of Immunology, 83:49-57.

2. Aminoff, D., Morgan, W. T., and Watkins, W. M. 1950. The isolation and properties of the human-blood-group A substance. Biochemistry Journal, 46:426-439.

3. Annison, E. F., and Morgan, W. T. 1952. The isolation and properties of Lewis (Le^a) human blood-group substance. Biochemistry Journal, 50:460-472.

4. _____ and _____. 1952. The isolation and properties of the human blood-group H substance. Biochemistry Journal, 52:247-260.

5. Baar, H. S. 1945. The Race-Wiener test in haemolytic disease of the newborn. Nature, 155:789.

6. Belkin, Ruth B., and Wiener, Alexander S. 1944. Demonstration of the properties A, B, M, N and Rh in red-cell stromata. Proceedings of the Society of Experimental Biology and Medicine, 56:214.

7. Boorman, K. E., and Dodd, B. E. 1943. The group-specific substances A, B, M, N, and Rh: Their occurrence in tissues and body fluids. Journal of Pathology and Bacteriology, 55:329.

8. _____, _____ and Mollison, P. L. 1944. The incidence of haemolytic disease of the foetus (erythroblastosis foetalis) in different families: The value of serological tests in diagnosis and prognosis. Journal of Obstetrics and Gynaecology, 51:1.

9. _____, _____ and Morgan, W. T. 1945. Enhancement of the action of immune haemagglutinins by human serum. Nature, 156:663.

10. Bornstein, S., and Israel, M. 1942. Agglutinogens in fetal erythrocytes. Proceedings of the Society of Experimental Biology and Medicine, 49:718.

11. Boyd, W. C. 1946. The effect of high pressures on hemagglutinating antibodies. Journal of Experimental Medicine, 83:401.

12. _____. 1947. Fundamentals of immunology, 503 pp. New York: Interscience Publishers, Inc.

13. _____, and Reeves, Egli. 1961. Specific inhibition of anti-D antibody by colominic acid. Nature, 191:511.

14. _____, Boyd, L. G., and Warshaver, E. R. 1945. Spontaneous loss of specificity of absorbed immune hemagglutinating serum. Journal of Immunology, 51:191.

15. _____, McMaster, M. H., and Waszczenko-Zacharczenko, E. 1959. Specific inhibition of anti-Rh serum by "unnatural" sugars. Nature, 184:989.

16. Brockland, Betty. 1949. The preparation and use of Rh hapten. American Journal of Medical Technology, 15:179-184.

17. Calvin, Melvin, Evans, Robert, et al. 1946. Rh antigen and hapten. Nature of antigen and its isolation from erythrocyte stroma. Proceedings of the Society of Biology and Medicine, 61:416.

18. Cameron, James W., and Diamond, Louis K. 1945. Chemical, clinical and immunological studies on the products of human plasma fractionation. XXIX. Serum albumin as a diluent for Rh typing reagents. Journal of Clinical Investigation, 24:793-801.

19. Campbell, Don H., Sturgeon, Phillip, and Vinograd, Jerome. 1955. Separation of complete and incomplete Rh antibodies by centrifugation. Science, 122:1091-1093.

20. Cann, J. R., Brown, R. A., Gajdusek, D. C., et al. 1951. Fractionation of Rh antiserum by electrophoresis-convection. Journal of Immunology, 66:137.

21. _____, _____, Kirkwood, J. G., et al. 1952. Studies of antibody distribution in the serum proteins of several Rh antisera. Journal of Immunology, 68:243-251.

22. Cappell, D. F., and McFarlane, M. N. 1946. Spontaneous loss of specificity of absorbed anti-Rh sera. Lancet, 1:558.

23. Carter, Bettina B. 1947. Preliminary report on a substance which inhibits anti-Rh serum. American Journal of Clinical Pathology, 17:646-649.

24. _____. 1949. Rh hapten: Its preparation, assay and nature. Journal of Immunology, 61:79-88.

25. Chan, P. C., and Deutsch, H. F. 1960. Immunochemical studies of human serum Rh agglutinins. Journal of Immunology, 85:37-46.

26. Coombs, R. R. A., and Mourant, A. E. 1947. On certain properties of antisera prepared against human serum and its various protein fractions: Their use in the detection of sensitization of human red cells with "incomplete"Rh antibody, and on the nature of this antibody. Journal of Pathology and Bacteriology, 59:105-111.

27. _____, _____, and Race, R. R. 1945. A new test for the detection of weak and "incomplete" Rh agglutinins. British Journal of Experimental Pathology, 26:255-266.

28. _____, and Race, R. R. 1945. Rh factor: Further observations on "incomplete" or "blocking" Rh antibody. Nature, 156:233.

29. Darrow, R. R. 1938. Icterus gravis (erythroblastosis) neonatorum. Archives of Pathology, 25:378.

30. Davidsohn, I., and Toharsky, B. 1942. The Rh blood factor: An antigenic analysis. American Journal of Clinical Pathology, 12:434-441.

31. _____, and Stern, Kurt. 1948. Interpretation of Rh antibodies. American Journal of Clinical Pathology, 18:690-699.

32. Diamond, L. K. 1945. The clinical importance of the Rh blood type. New England Journal of Medicine, 232: 447.

33. _____, and Abelson, Neva. 1945. The importance of Rh inhibitor substance in anti-Rh serums. Journal of Clinical Investigation, 24:122-126.

34. _____, and Denton, Ronald L. 1945. Rh agglutination in various media with particular reference to the value of albumin. Journal of Laboratory and Clinical Medicine, 30:821-830.

35. Dodd, Matthew C., Bigley, Nancy J., and Geyer, Virginia B. 1960. Specific inhibition of Rh_0 (D) antibody by sialic acids. Science, 132:1398-1399.

36. Evans, Robert S., Moskowitz, Merwin, and Calvin, Melvin. 1950. Studies on antigens of human red cells, II. Serological properties of Rh factor in elinin. Proceedings of the Society of Experimental Biology and Medicine, 73:637-642.

37. Fisk, R. T., and Ford, A. G. 1942. Observations on the Rh agglutinogen of human blood. American Journal of Clinical Pathology, 12:545.

38. Fruton, Joseph S., and Simmonds, Sofia. 1959. General biochemistry. Second edition, 1077 pp. New York: John Wiley & Sons, Inc.

39. Gibbons, R. A., and Morgan, W. T. 1952. Serological properties of human blood-group A and B substances. Nature, 170:77.

40. _____, _____, _____. 1954. The isolation and properties of substances of human origin possessing blood group B specificity. Biochemistry Journal, 57:283-295.

41. Goldsmith, J. W. 1950. Experiences with Rh hapten. American Journal of Obstetrics and Gynecology, 59:172-177.

42. Greenwalt, T. J. 1949. Preliminary report of experiences with Rh hapten. Journal of Laboratory and Clinical Medicine, 34:1603-1604.

43. Haberman, W. 1948. The demonstration and characterization of the anti-d agglutinin and antigen predicted by Fisher and Race. Blood, 3:682.

44. Hackel, Emanuel. 1960. Quantitative aspects of anti-Rh inhibition. Paper presented at American Society of Human Genetics. Unpublished.

45. _____. 1961. Inhibition studies on anti-Rh antibodies. Rome (in press).

46. _____, and Spolyar, K. S. 1960. Anti-c and anti-e inhibition by ribonucleic acid derivatives. Vox Sang, 5:517-522.

47. _____, and Smolker, R. E. 1960. Effect of ribonuclease on erythrocyte antigens. Nature, 187:1036-1037.

48. _____, _____, and Fenske, S. A. 1958. Inhibition of anti-Rh and anti-Lutheran sera by ribonucleic acid derivatives. Vox Sang, 3:402-408.

49. Hamilton, E. G., and Brockland, Mary E. 1950. The results of treatment with Rh hapten. American Journal of Obstetrics and Gynecology, 60:813-819.

50. Hill, J. M., Haberman, S., and Velez, O. A. 1945. The preparation of potent anti-Rh typing serum by injection of Rh positive blood into previously isoimmunized individuals. Journal of the American Medical Association, 128:944.

51. _____, _____, and Guy, R. 1949. Further evidence for antibodies of third order: Fractionation of agglutinins, blocking antibodies and cryptagglutinoids by physico-chemical methods. American Journal of Clinical Pathology, 19:134-140.

52. Howe, C. J. 1951. The influenza virus receptor and blood group antigens of human erythrocyte stroma. Journal of Immunology, 66:9.

53. _____, and Rustigan, R. 1950. Studies with the Rh_o (D) antigen. Journal of Immunology, 64:505-513.

54. Hubinont, P. O. 1948. Action of heating on Rh-positive human red cells. Nature, 161:642.

55. Jankovic, B. D., and Krijnen, H. W. 1953. Serological activity of globulin fractions of anti-D sera separated by paper electrophoresis. Nature, 171:982-983.

56. Kabat, E. A. 1956. Blood group substances, 330 pp. New York: Academic Press.

57. Landsteiner, Karl. 1947. The specificity of serological reactions, 310 pp. Cambridge: Harvard University Press.

58. _____, and Wiener, A. S. 1940. An agglutinable factor in human blood recognized by immune sera for rhesus blood. Proceedings of the Society of Experimental Biology and Medicine, 43:223.

59. _____ _____. 1941. Studies on an agglutinogen (Rh) in human blood reacting with anti-rhesus sera and with human isoantibodies. Journal of Experimental Medicine, 74:309-320.

60. Levine, P. 1943. The pathogenesis of erythroblastosis fetalis; a review. Journal of Pediatrics, 23:656.

61. _____. 1960. Blood group antigens and antibodies as applied to blood transfusion, 72 pp. New Jersey: Ortho Pharmaceutical Corporation.

62. _____, and Katzin, E. M. 1940. Isoimmunization in pregnancy and the varieties of isoagglutinins observed. Proceedings of the Society of Experimental Biology and Medicine, 45:343.

63. _____, _____, and Burnham, L. 1940. Atypical warm isoagglutinins. Proceedings of the Society of Experimental Biology and Medicine, 45:346.

64. _____, _____, _____. 1941. Isoimmunization in pregnancy, its possible bearing on etiology of erythroblastosis foetalis. Journal of the American Medical Association, 116:825.

65. _____, _____, _____, and Vogel, P. 1941. The role of isoimmunization in the parthogenesis of erythroblastosis fetalis. American Journal of Obstetrics and Gynecology, 42:925-937.

66. _____, and Stetson, R. E. 1939. An unusual case of intra-group agglutination. Journal of the American Medical Association, 113:126-127.

67. _____, Cellano, Marino, Fenichel, Richard, et al. 1961. A "D-like" antigen in rhesus monkey, human Rh positive and human Rh negative red blood cells. Journal of Immunology, 87:414.

68. Loughry, J., and Carter, B. B. 1948. Report of a case of erythroblastosis fetalis treated with Rh hapten. American Journal of Obstetrics and Gynecology, 55:1051-1052.

69. Lubinski, Hubert H., and Portnuff, Joseph C. 1947. The influence of heat and formalin upon the Rh agglutinogen. Journal of Laboratory and Clinical Medicine, 32:178-180.

70. Mohn, J. F., and Witebsky, Ernest. 1948. The demonstration of a third type of Rh antibody with blocking properties. Journal of Laboratory and Clinical Medicine, 33:1360-1368.

71. Moskowitz, M., Dandliker, W. B., Calvin, M., and Evans, R. S. 1950. The separation from human erythrocytes of a water soluble fraction containing the Rh, A, and B factors. Journal of Immunology, 65:383.

72. Mourant, A. E. 1945. A new rhesus antibody. Nature, 155:542.

73. Murray, J. 1952. Rh antigens of human and monkey blood. Journal of Immunology, 68:513.

74. Potter, E. L. 1948. Rh--Its relation to congenital hemolytic disease and to intragroup transfusion reactions, 344 pp. Chicago: The Yearbook Publishers, Inc.

75. Price, Charles C., Reed, David H., et al. 1948. The isolation of a substance with Rh hapten activity. Journal of the American Chemical Society, 70:3527-3528.

76. Race, R. R., and Sanger, Ruth. 1959. Blood groups in man, 377 pp. Third edition. Springfield, Illinois: Charles C. Thomas Publ.

77. _____, and Taylor, G. L. 1943. A serum that discloses the genotype of some Rh positive people. Nature, 152:300.

78. _____, _____, et al. 1943. The Rh factor and erythroblastosis. British Medical Journal, 2:289.

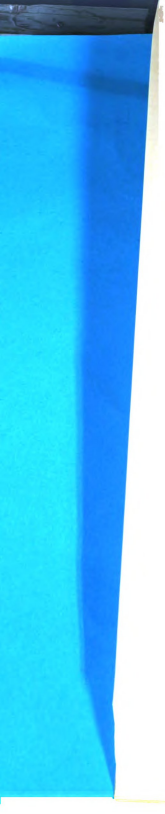
79. _____, _____, _____. 1943. Recognition of Rh genotypes in man. Nature, 152:563.

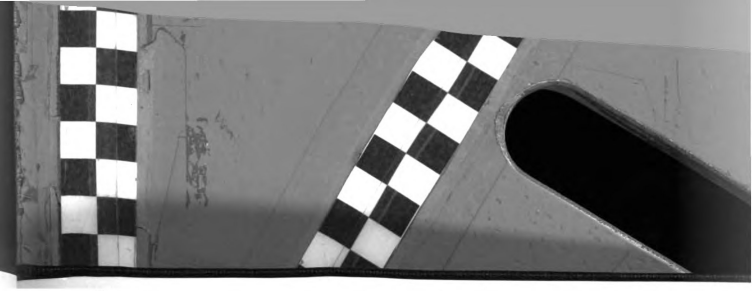
80. Reid, Allen F., and Jones, Frances. 1949. Production of therapeutic fractions of human blood serum. American Journal of Clinical Pathology, 19:10-15.

81. Renton, P. H., and Hancock, Jeanne A. 1958. The influence of antigen strength agglutinin and incomplete antibody on agglutination by anti-D sera. British Journal of Hematology, 4:89-96.

82. Stratton, F. 1943. Demonstration of the Rh factor in the blood of a 48 mm. embryo. Nature, 152:449.

83. _____, and Renton, P. H. 1950. The Rh hapten. Lancet, 258:326.





84. Sturgeon, P., and Brown, R. A. 1952. Studies of a "complete" and an "incomplete" Rh antiserum with cDe/cde and -D-/-D- cells. Journal of Immunology, 68:287.

85. Wiener, A. S. 1944. A new test (blocking test) for Rh sensitization. Proceedings of the Society of Experimental Biology and Medicine, 56:173.

86. _____. 1945. Conglutination test for Rh sensitization. Journal of Laboratory and Clinical Medicine, 30:662-667.

87. _____, Sonn, E. B., and Belkin, R. B. 1944. Nature of Rh agglutination reactions. Science, 100:98.

88. _____, and Peters, H. R. 1940. Hemolytic reactions following transfusions of blood of the homologous group with three cases, in which the same agglutinogen was responsible. Annals of Internal Medicine, 13:2306-2322.

89. _____, and Forer, S. 1941. A human serum containing four distinct isoagglutinins. Proceedings of the Society of Experimental Biology and Medicine, 47:215.

90. Witebsky, E., and Heide, Ann. 1943. Further investigations on presence of Rh antibodies in breast milk. Proceedings of the Society of Experimental Biology and Medicine, 52:280-281.

91. _____, Anderson, G. W., and Heide, Ann. 1942. Demonstration of Rh antibodies in breast milk. Proceedings of the Society of Experimental Biology and Medicine, 49:179-183.

92. _____, and Mohn, J. 1945. Investigations on the occurrence of Rh substances in amniotic fluid. Journal of Experimental Medicine, 82:143.

93. _____ _____. 1948. Studies on Rh antibodies. Journal of Laboratory and Clinical Medicine, 33:1353-1369.

94. _____, _____, et al. 1946. A simple method for the concentration of Rh agglutinins. Proceedings of the Society of Experimental Biology and Medicine, 61:1-5.