A Study of Autologous Anti-Immunoglobulins in Mice

A Thesis

Presented To

The Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment

Of The Requirements For The Degree

Master of Science

---

by

Murray Drulak, B.Sc.

November, 1973

ACKNOWLEDGEMENTS

I would like to thank Miss Joan Cook for teaching and guiding me to proficiency in all experimental techniques used in this thesis and for her helpful comments on it.

I would also like to thank Mrs. S. Krzywdzinski for her invaluable work in preparing and separating hemolysin, preparing Dulbecco Buffered Saline and looking after experimental equipment I used.

I am extremely grateful to Dr. J.A. Romeyn not only for his continual help on my project but also for giving me a better insight into the scientific mode of thought.

Dr. N.L. Stephens of the Department of Physiology helped me plan the statistical analysis of data in this manuscript for which I thank him.

In closing, I would like to express my appreciation to the Department of Medical Microbiology and its head, Dr. J.C. Wilt, for providing me with the opportunity to pursue post-graduate studies in the field of my choice.

ABSTRACT

A Study of Autologous Anti-Immunoglobulins In Mice

Mouse plasma was diffused in agar plates containing sheep red cells sensitized by mouse hemolysin. Plates were incubated with complement (developed). Subsequent to complement incubation a ring of inhibited lysis (P ring) directly adjacent to the hole and an outer ring of facilitated lysis (L ring) as compared to background lysis on the plate appeared (see Figure 1). This was attributed to two functional types of naturally occurring (autologous) anti-immunoglobulin that diffused out to characteristic distances in the plate and reacted with antibody type immunoglobulin in the antigen-antibody complex. One type of anti-immunoglobulin inhibited lysis and the other type facilitated lysis.

The study was divided into three parts:

1) The plate test was analyzed by individually varying single factors. This not only contributed to the definition of limitations of the plate test but also revealed that the behaviour exhibited in the plates was similar to behaviour exhibited by artificially produced (heterologous) anti-immunoglobulin supporting the conclusion that the ring patterns were due to autologous anti-immunoglobulins.

2) Mean P and L diameter comparisons and mean $^{L}/_{P}$ ratio

comparisons were made for seven strains of mice, some predisposed to disease. Similar comparisons were made for the sexes of strains. This was done to gain an insight into whether there were any differences between strains, whether there was an increase in differences with age, and whether there were any differences between the sexes. 3) The mean P and L ring diameters and mean $^{L}/_{P}$ ratios were studied at various times during the primary and secondary responses. It was found that the two immunized groups of mice only differ from control groups in the first six hours of the immune response with a rise in mean $^{L}/_{P}$ value.

Murray W. Drulak
November, 1973

TABLE OF CONTENTS

CHAPTER		PAGE
	INTRODUCTION	1
I.	REVIEW OF THE LITERATURE	5
	Heterologous Anti-Immunoglobulins: Production and Behaviour	5
	Autologous Anti-Immunoglobulins: Incidence in Disease	16
	Autologous Anti-Immunoglobulins: Incidence in Normal Individuals	21
	Physical Properties of Anti-Immunoglobulins	26
	Possible Mechanisms for Stimulation of Anti-Immunoglobulin Production	29
II.	THEORY	32
	Theoretical Postulates Underlying the Study of Autologous Anti-Immunoglobulins	32
III.	MATERIALS AND METHODS	37
	Dulbecco Phosphate Buffered Saline	37
	Erythrocytes	37
	Plasma	38
	Mice	38
	Noble Agar	40
	Hemolysin	40

CHAPTER PAGE

Hemolysin Storage..... 40
Titration of Hemolysin..... 41
Glass Plates..... 42
Diethyl Amino Ethyl Dextran..... 42
Film..... 43
Camera..... 43
Complement..... 43
Agar Diffusion Plate Method..... 44
IV. PROCEDURES AND EXPERIMENTAL RESULTS..... 50
Correlation of Plasma Concentration With Ring Diameter..... 50
Procedure..... 50
Results..... 50
Effect of Complement Concentration on Ring Diameter..... 60
Procedure..... 60
Results..... 60
Effect of Hemolysin Dosage on Ring Diameter..... 64
Procedure..... 64
Results..... 64
Overnight Plasma Storage..... 70
Procedure..... 70
Results..... 71

CHAPTER PAGE

Variability in Plates Prepared on Different Days..... 73
Procedure..... 73
Results..... 73
Variability in Plates Prepared on the Same Day..... 76
Procedure..... 76
Results..... 76
The Effect of Warming Mice..... 79
Procedure..... 79
Results..... 79
Ring Diameter Comparison Between Strains. 82
Introduction..... 82
Analysis of Data..... 82
Data..... 88
Mean Ring Diameters ± S.E. and Mean $^{L}/_{P}$ ± S.E..... 88
Analysis of Data Results..... 94
Mean Ring Diameter Comparisons Between Strains (ANOVA)..... 94
Mean Ring Diameter Comparisons Between Strains (Tukeys Test).. 96
Mean Ring Diameter Comparisons Between The Sexes of Strains (Factorial Analysis)..... 101

CHAPTER PAGE

Mean Ring Diameter Comparisons Between the Sexes of Strains (Tukeys Test)..... 103
Results..... 105
Secondary Response Study..... 107
Changes in Ring Diameter During the Secondary Response Procedure..... 107
Results..... 109
V. DISCUSSION OF RESULTS..... 116
VI. SUMMARY..... 134
VII. BIBLIOGRAPHY..... 136
VIII. APPENDIX..... 141

LIST OF TABLES

TABLE		PAGE
I	Strains of Mice Used in the Autologous Anti-Immunoglobulin Study.....	39
II	Relationship Between Plasma Concentration and Mean Ring Diameter.....	51
III	Mean Ring Diameters and Standard Error at Different Plasma Concentrations (I) Whole Sensitized Plates.....	146
IV	Mean Ring Diameters and Standard Error at Different Plasma Concentrations (II) 7 S Sensitized Plates.....	147
V	Mean Ring Diameters at Different Complement Dilutions.....	148
VI	Mean Ring Diameters at Different HD_{50}'s of Hemolysin (I) Whole Sensitized Plates.....	149
VII	Mean Ring Diameters at Different HD_{50}'s of Hemolysin (II) 7 S Sensitized Plates.....	149
VIII	Comparison Between the Mean Ring Diameter of Fresh Plasma and Plasma Stored over-night.....	72
IX	Comparison of Ring Diameters of Plasmas Which Were Each Diffused on Plates Made on Different Days.....	75

TABLE		PAGE
X	Comparison of Ring Diameters of Plasmas Which Were Each Diffused on Plates made on Different Days.....	75
XI	Comparison of the Ring Diameters of Plasmas Diffused in Two Different Plates Prepared at the Same Time.....	78
XII	Comparison of the Ring Diameters of Plasmas Diffused in Two Different Plates Prepared at the Same Time.....	78
XIII	Comparison of Ring Diameters of Plasma Obtained From a Mouse With and Without Warming.....	81
XIV	Strains Whose Ring Diameters Were Compared.	83
XV-XXXII	Data for the Comparison of Different Strains of Mice	
	Mean Ring Diameters $\pm$ Standard Error	
	and Mean $^{L}/_{P} \pm$ Standard Error.....	88-93
	Trial I.....	88-89
	Trial II.....	90-91
	Trial III.....	92-93
XXXIII	Mean Ring Diameter Comparison Between Strains	
	One Way Analysis of Variance (ANOVA)	
	(95% Confidence Level)	
	(I) Whole Sensitized Plates.....	94

TABLE		PAGE
XXXIV	Mean Ring Diameter Comparison Between Strains One Way Analysis of Variance (ANOVA) (95% Confidence Level) (II) 7 S Sensitized Plates.....	95
XXXV	Mean Ring Diameter Comparison Between the Sexes of the Strains Factorial Analysis of Variance With Equal Replication in Each Cell (95% Confidence Level) (I) Whole Sensitized Plates.....	101
XXXVI	Mean Ring Diameter Comparison Between the Sexes of the Strains Factorial Analysis of Variance With Equal Replication in Each Cell (95% Confidence Level) (II) 7 S Sensitized Plates.....	102
XXXVII	Mean Ring Diameter Comparison Between the Sexes of Each of the Strains Tukeys Multiple Range Test (95% Confidence Level) (I) Whole Sensitized Plates.....	103
XXXVIII	Mean Ring Diameter Comparison Between the Sexes of Each of the Strains Tukeys Multiple Range Test (95% Confidence Level)	

TABLE		PAGE
	(II) 7 S Sensitized Plates.....	104
XXXIX-XLII	Mean Ring Diameters at Different Times After Injection.....	150-153
	Secondary Response.....	150
	Primary Response.....	151
	DBS Injected Controls.....	152
	Noninjected Controls.....	153
XLIII-XLVI	Mean $^{L}/_{P}$ at Different Times After Injection	154-157
	Secondary Response Mice.....	154
	Primary Response Mice.....	155
	DBS Injected Controls.....	156
	Noninjected Controls.....	157
XLVII	Summary of ANOVA Results.....	122
XLVIII	Percentage of Tukey Comparisons That Show Differences Between Strains.....	125
XLIX	Tukey Ring Diameter Comparisons From Age to Age.....	127
L	Mean Ring Diameters of the Three Groups of Strains on 7 S Plates.....	130

LIST OF FIGURES

FIGURE		PAGE
1.	An agar diffusion plate containing mouse sensitized sheep red cells.....	49
2.	Whole sensitized plates. Trial I: P mean ring diameter ± S.E. vs plasma concentration.....	53
3.	Whole sensitized plates. Trial II: P mean ring diameter ± S.E. vs plasma concentration.....	54
4.	7 S sensitized plates. Trial I: P mean ring diameter ± S.E. vs plasma concentration....	55
5.	Whole sensitized plates. Trial I: L mean ring diameter ± S.E. vs plasma concentration.....	56
6.	Whole sensitized plates. Trial II: L mean ring diameter ± S.E. vs plasma concentration.....	57
7.	7 S sensitized plates. Trial I: L mean ring diameter ± S.E. vs plasma concentration....	58
8.	7 S sensitized plates. Trial II: L mean ring diameter ± S.E. va plasma concentration.....	59
9.	Mean P ring diameter vs complement dilution..	62
10.	Mean L ring diameter vs complement dilution..	63

FIGURE PAGE

11. Mean P ring diameter vs HD_{50} of whole hemolysin..... 66

12. Mean L ring diameter vs HD_{50} of whole hemolysin..... 67

13. Mean P ring diameter vs HD_{50} of 7 S hemolysin. 68

14. Mean L ring diameter vs HD_{50} of 7 S hemolysin. 69

15. Tukeys test at a 95% confidence level.
I. Whole sensitized plates..... 97

16. Tukeys test at a 95% confidence level.
II. Whole sensitized plates..... 98

17. Tukeys test at a 95% confidence level.
I. 7 S sensitized plates..... 99

18. Tukeys test at a 95% confidence level.
II. 7 S sensitized plates..... 100

19. Trial I: Mean $^{L}/P$ vs time after antigen injection..... 110

20. Trial II: Mean $^{L}/P$ vs time after antigen injection..... 111

21. Trial I: Mean P diameter vs time after antigen injection..... 112

22. Trial II: Mean P diameter vs time after antigen injection..... 113

23. Trial I: Mean L diameter vs time after antigen injection..... 114

24. Trial II: Mean L diameter vs time after antigen injection..... 115

INTRODUCTION

INTRODUCTION

An anti-immunoglobulin is an antibody formed in response to immune system stimulation by an immunoglobulin. Immunoglobulins as antigens are species specific, although this specificity is not absolute.

Heterologous anti-immunoglobulins can be produced by injecting serum from one species into another. It has been observed *in vitro* that heterologous anti-immunoglobulin reacts with antibody type immunoglobulin on the surface of sensitized red cells. There are two functional types of anti-immunoglobulin which are defined by the way they react with antibody coated red cells. Anti-immunoglobulin of one type reacts in such a way as to facilitate complement fixation and therefore facilitate lysis. Anti-immunoglobulin of the other functional type reacts in such a way as to inhibit complement fixation and therefore inhibit lysis.

Consider now the case of autologous anti-immunoglobulins. In animal serum, when a native protein becomes changed in some manner, which sometimes happens, that animal makes antibodies against its own changed component, which it now recognizes as foreign. This is true when a native immunoglobulin becomes changed. Such a change in immunoglobulin structure could take place continually *in vivo* due to catabolism of immunoglobulins. In a more specific case when immunoglobulins such as antibodies become changed due to reaction with antigen, antibodies can be formed

against these changed immunoglobulins. Najjar (1959) stated that qualities that made immunoglobulin a familiar and normal protein to the immune system of the host were chiefly its surface configuration, shape and size. If either or all of these are altered by strong association with either another molecule or by autolytic digestion, new groupings arise on the surface structure.

Such antibodies against these changed native immunoglobulins are called autologous anti-immunoglobulins. Autologous anti-immunoglobulin was found in normal rabbit and human sera by Watson and Collins in 1963. Watson in 1963 reported the presence of anti-immunoglobulin in normal sera from cows, dogs, guinea pigs, horses, rabbits and sheep which he demonstrated by the latex fixation test.

There may be two functional types of autologous anti-immunoglobulin, analogous to the two functional types of heterologous anti-immunoglobulin, although this is not mentioned in the literature. The experimental method used throughout this thesis has consistently yielded results that could be interpreted in this way.

An agar diffusion plate method was used to study the two functional types of autologous anti-immunoglobulins in mice. (See Materials and Methods.) The two functional types of anti-immunoglobulin diffused out to different distances in the plates, reacted with sensitized cells, and inhibited or facilitated lysis whichever the case may

be upon complement addition. Such a phemomenon resulted in a ring of unlysed cells around the hole where mouse plasma was diffused indicating protection from lysis due to anti-immunoglobulin that inhibited lysis taking precedence over the action of the facilitating anti-immunoglobulin which was also present in the area directly adjacent to the hole. Such a ring of inhibited lysis was termed a P ring. An outer ring where there was facilitated lysis as compared to the background indicated facilitating anti-immunoglobulin was acting there. Such a ring was called the L ring.

The P and L rings were measured and the $^{L}/P$ ratio was used as some indication of the relative amounts of the two types of anti-immunoglobulin in mouse plasma.

Much theorizing could be done on the functional significance of these apparent inhibiting and facilitating anti-immunoglobulins. Such theorizing can link their presence to the magnitude of the immune response to a foreign antigen, chiefly through considerations concerning the surface immunoglobulin on certain cells of the lymphocyte series, and to malignant disease through effects on immune surveillance, and to auto-immune disease. Such theorizing seems inappropriate at this stage and I am deliberately omitting it in favour of indicating at once what are the three main purposes of this work:

1) To investigate the experimental factors which influence the size of the above mentioned rings (that is, to investigate

technical matters).

2) To determine whether strains of mice which are particularly susceptible to leukemia and other diseases have plasma which produces rings of significantly different size from those produced by plasma from "normal" strains.

3) To determine whether there is a change in ring size during the primary and secondary response of mice to a foreign antigen.

CHAPTER I

REVIEW OF THE LITERATURE

I. HETEROLOGOUS ANTI-IMMUNOGLOBULINS: PRODUCTION AND BEHAVIOUR

Bordet (1904) was one of the pioneers in the field of heterologous anti-immunoglobulins. He prepared them by immunizing guinea pigs with either normal or immune rabbit sera. The resulting guinea pig serum was added to rabbit sensitized [I] bovine, avian or human red blood cells, which were incubated with complement. They were protected from lysis. The protective ability of the guinea pig serum could be removed by mixture with normal rabbit serum or by incubation with rabbit sensitized cells. He also provided evidence that the protection from lysis was not due to complement destruction.

Ehrlich (1906) confirmed Bordet's observation that anti-rabbit serum produced in guinea pigs would inhibit complementary lysis of sensitized cells. Ehrlich prepared his anti-immunoglobulin-containing serum in two different ways. One way was to inject into goats immune serum, more specifically the serum of rabbits previously immunized with ox cells. The other way was by injecting normal rabbit serum into goats. Ehrlich serially diluted each of the two types of anti-immunoglobulin containing serum and tested their ability to inhibit lysis at different dilutions.

[I] The term "rabbit sensitized cells" means cells coated with hemolysin produced in rabbits.

When serum from goats injected with normal rabbit serum was serially diluted and its ability to inhibit lysis tested, it was found that the inhibition of lysis increased as the dilutions of anti-immunoglobulin serum decreased. When sera from goats injected with immune rabbit serum was serially diluted and its inhibition of lysis tested, it was found that at middle dilutions maximum inhibition of lysis was obtained. At low dilutions of these goat sera, there was a reversal of the inhibition of lysis.

Ehrlich explained this phenomenon by suggesting that two substances were present in the antiserum, one of which caused inhibition of lysis of sensitized cells and one of which prevented inhibition. It is possible that these two substances Ehrlich described were the inhibiting anti-immunoglobulin and facilitating anti-immunoglobulin, respectively.

Bordet considered that specificity of action of an anti-immunoglobulin was a function of the species of animal which produced the immunoglobulin against which it was formed, but Ehrlich (1906, page 88) showed that an anti-immunoglobulin may cross react to lesser degrees with antibodies of species other than the one against which it was formed.

Ehrlich (1906, page 577) suggested that the antibodies of different species with which the anti-immunoglobulin reacted had a common complementophil group with which

the anti-immunoglobulin reacted and prevented complement from being fixed by the sensitized cells.

Friedberger and Moreschi (1907) injected rabbits with serum of goats previously immunized with rabbit cells. They also injected rabbits with normal goat serum. Subsequently, samples of serum were obtained from the rabbits and were incubated with five per cent rabbit cells sensitized with anti-rabbit goat serum. After these mixtures were centrifuged, the supernatant was removed and the sensitized cells were resuspended in saline before complement was added. It was found that sera from rabbits injected in both ways facilitated the lysis of sensitized cells in the presence of complement.

Altmann (1912) injected rabbit red cells that were sensitized by goat hemolysin into three rabbits. The sensitized cells were washed. The serum of all three rabbits potentiated lysis of sensitized cells indicating a facilitating anti-immunoglobulin in the serum. He also injected the washings from rabbit cells treated with (1) anti-rabbit cell goat serum, (2) normal goat serum, into different rabbits. The resulting serum from rabbits injected in both ways did not potentiate lysis of sensitized cells, which indicated that production of facilitating anti-immunoglobulin was stimulated by goat antibody type immunoglobulin and not by the normal goat serum constituents in the washings. Such facilitating anti-immunoglobulin containing serum lost its activity upon heating at 56°C

for thirty minutes and thus differed from the facilitating anti-immunoglobulin serum prepared by Friedberger and Moreschi (1907).

In early work, the fact that anti-immunoglobulin containing serum produced by injecting either normal or immune serum of one species into another produced inhibition of lysis of sensitized cells in the presence of complement, suggested that a species specific anti-immunoglobulin had been produced rather than an anti-immunoglobulin formed specifically against a certain antibody present in the injected serum. However, several workers presented evidence that contradicted this.

La Porte et al. (1950) injected the serum of a horse previously immunized with sheep cells into a sheep. The anti-horse sheep serum thus produced inhibited lysis of sheep cells in the presence of horse hemolysin and guinea pig complement. The serum from sheep previously injected with normal horse serum did not inhibit lysis. He added diluted hemolysin, diluted anti-immunoglobulin containing serum, sheep cells and complement at the same time and incubated this mixture at 37°C for thirty minutes.

La Porte also noticed that there was decreased inhibition of lysis with increased hemolysin and increased inhibition of lysis with increased concentrations of anti-immunoglobulin containing serum.

Van den Ende (1940) injected a group of guinea pigs

with a suspension of specific precipitate from rabbit anti-pneumococcal serum. The serum obtained from these guinea pigs in subsequent bleedings contained anti-immunoglobulin. Another group of guinea pigs was injected with alum precipitated rabbit globulin. The sera from this group contained anti-immunoglobulin formed against the injected rabbit immunoglobulin. Precipitation tests were carried out, where each type of anti-immunoglobulin serum was tested against each of the antigens injected into the guinea pigs. The tests showed that anti-immunoglobulin prepared by immunizing guinea pigs to specific precipitate from rabbit anti-pneumococcal antiserum contained precipitins capable of reacting as well with the purified antibody as with the total immunoglobulins from normal rabbit serum. Anti-immunoglobulin containing sera from guinea pigs immunized to the total normal globulins reacted only with the homologous antigen.

Adler (1956) showed that Van den Ende's observations were applicable to various immune systems. Strong immune sera against the immunoglobulins of mice and guinea pigs were readily produced by injection of immune precipitates containing mouse or guinea pig immunoglobulin into guinea pigs and rabbits, respectively. Foreign sera, their immunoglobulin fraction or washed immune aggregates containing foreign antibody were tested for their ability to evoke antibody formation. The antibody response against immuno-

globulin was measured by two agglutination methods. In one of these sub-agglutinating amounts of the foreign antibody type immunoglobulin were absorbed on red cells; in the other the foreign antibody type immunoglobulin against polysaccharide, obtained by dissociation of immune precipitate, was absorbed on tanned red cells. The experimental data showed the guinea pigs injected with normal rabbit serum or its immunoglobulin fraction failed to produce appreciable amounts of antibody against rabbit antibody immunoglobulin. In contrast, injection into guinea pigs of washed immune aggregates formed by the reaction of rabbit immune sera with their correcponding antigens elicited production of potent antibody against antibody rabbit immunoglobulin.

Bordet's work was repeated in a more quantitative fashion by Onysko (1962), Romeyn and Onysko (1964). Onysko drew the conclusion that a dynamic equilibrium existed between association and dissociation of sensitized cells and inhibiting anti-immunoglobulin, which resulted in competition between anti-immunoglobulin and complement for the same or closely related receptor sites on sensitized cells.

Onysko came to this conclusion from her titrating of heterologous anti-immunoglobulin produced by injection of guinea pigs with normal rabbit sera. This type of guinea pig sera obtained and subsequently titrated was referred

to as Type I sera. In such a titration the guinea pig serum was two fold serially diluted, with rabbit sensitized red blood cells being added to each tube. After an incubation period, complement was added to each tube followed by another period of incubation. The tubes were then read against controls using unsensitized cells in a spectrophotometer. The absorbance values were plotted against dilution of guinea pig serum.

Onysko varied factors in the test. It was found with an increase in complement concentration there was a decrease in protection of sensitized cells from lysis. An increase in the incubation time of the sensitized cells, complement and guinea pig serum mixture decreased protection from lysis as did an increase in the sensitization of sheep cells by rabbit hemolysin. The amount of protection from lysis the guinea pig sera provided depended upon the ratio of anti-immunoglobulin and complement added to sensitized cells. In such experiments complement was shown to be active in supernatants from tubes in which cells were protected from lysis indicating it was not destroyed. Onysko also found that anti-immunoglobulin could be absorbed from the serum using sensitized cells and afterwards eluted from these cells.

She also injected guinea pigs with rabbit sensitized guinea pig red blood cells. The serum from the injected guinea pigs, which was designated as Type II serum was

effective in protecting sensitized cells from lysis when undiluted and its effectiveness decreased with dilution to a point where further dilution increased its efficiency. Finally, further dilution decreased the ability of the guinea pig serum to protect sensitized cells.

In 1965, McIllmurray tried to characterize anti-immunoglobulin in Onysko's Type I and Type II sera. He fractionated both sera by sucrose density gradient centrifugation. The top and bottom fractions of each type were titrated for inhibition of lysis by Onysko's method.

In Type I serum it was found that the top fraction which contained immunoglobulin G was responsible for nearly all the anti-immunoglobulin inhibitory action. It was observed that inhibition of lysis increased as anti-immunoglobulin dilution decreased. In Type II serum it was observed that the top fraction was responsible for most of the inhibiting anti-immunoglobulin activity presumably due to immunoglobulin G and was also responsible for reversal of inhibition seen at lower dilutions. The bottom fraction which contained immunoglobulin M showed "inhibiting" anti-immunoglobulin activity at low dilutions.

The effect of Type II sera on lysis was also studied using an agar diffusion method. In this method serum was added to a hole punched on a coverslip coated with sensitized red blood cells in agar. After the serum components had been given time to diffuse into the agar and had reacted

with the sensitized cells, the coverslips were immersed in complement. This study was carried out parallel to the study in which Type II sera was titrated by Onysko's method. He compared the tube titration curves with the patterns of facilitated and inhibited rings of lysis on the agar diffusion plates for a number of sera. The results of the two tests corresponded in every case. The method of comparison of results obtained by the two methods is illustrated below.

For example, suppose in a tube titration of an anti-immunoglobulin containing serum that the following observations are made. At low dilutions of serum there is inhibition of lysis, and at middle serum dilutions there is facilitation of lysis. At high serum dilutions there is again inhibition of lysis. This titration curve would correspond to a ring pattern in a suitable sensitized slide whence directly adjacent to the hole there is a ring of inhibited lysis as compared to background lysis on the slide. Outside of this, there is a ring of facilitated lysis and beyond this there is another ring of inhibited lysis. The underlying principle behind the comparison of anti-immunoglobulins behaviour is that an increase in dilution of anti-immunoglobulin in the tube test corresponds to an increase in the distance of anti-immunoglobulin diffusion from the well in the slide.

Ross (1968) further analyzed anti-immunoglobulin

action on the hemolysin in red cell hemolysin. He fractionated both the hemolysin used to sensitize the red cells and the Type II sera into 7 S and 19 S fractions using gel filtration with Sephadex G-200. All possible combinations of titrating a certain fraction of anti-immunoglobulin serum using red cells sensitized with a fraction of hemolysin were done.

Results revealed that 7 S anti-immunoglobulin titrated using sheep cells sensitized with 7 S rabbit-produced hemolysin showed facilitation of lysis. Inhibition of lysis resulted when 7 S anti-immunoglobulin was titrated using sheep red blood cells sensitized with 19 S hemolysin. In both cases when 19 S anti-immunoglobulin was titrated using cells sensitized with 7 S or 19 S hemolysin, there was inhibition of lysis.

Heterologous anti-immunoglobulins have been used as immunological tools in various laboratory techniques. Riha (1964) discovered that antibodies bound at the erythrocyte surface were detected by a hemolytic reaction in a conventional Coombs Test when heterologous anti-immunoglobulin and complement were added. Such facilitating anti-immunoglobulin increased the titer of the anti-erythrocyte serum.

Sterzl and Riha (1965), Dresser (1965) and Sterzl (1966) devised a way to detect cells producing 7 S antibodies of low hemolytic ability in the Jerne Plaque technique (Jerne, 1963), using anti-immunoglobulin. They added serum containing

facilitating anti-immunoglobulin which helped 7 S antibodies that were emitted from the antibody producing cell to lyse red blood cells and form a plaque. This procedure, therefore, increased the sensitivity of detection of 7 S plaque-forming cells which was particularly useful in the secondary response (Riha, 1964).

II. AUTOLOGOUS ANTI-IMMUNOGLOBULINS: INCIDENCE IN DISEASE

Autologous anti-immunoglobulins associated with various diseases has been noted by several workers.

Waaler (1940) was one of the first to discover the presence of anti-immunoglobulin in sera of patients with rheumatoid arthritis. The sera of such patients agglutinated sheep red cells sensitized with subagglutinating doses of rabbit anti-sheep hemolysin. Such anti-immunoglobulin was called rheumatoid factor.

Franklin et al. (1957) isolated and characterized the rheumatoid factor from patients with rheumatoid arthritis using ultracentrifugation, electrophoresis and immunologic reactions (precipitation and agglutination). They found in the sera of a number of patients with rheumatoid arthritis an unusual, high molecular weight protein component. This material sedimented more rapidly than the normal 19 S component. Similar components were not observed in a limited control series. The high molecular weight material was present in the immunoglobulin fraction of serum and joint fluid. It had an $S_{20,w}$ of approximately 22 S and could be dissociated into two immunoglobulin containing fractions, one of which had a sedimentation coefficient of approximately 19 S which was rheumatoid factor and the other had a 7 S sedimentation coefficient.

Evidence for a direct relationship between the 22 S

component and the precipitation which occurred in mixing rheumatoid serum with aggregated immunoglobulin was obtained. Adsorption of serum with aggregated immunoglobulin removed the 22 S component. There also appeared to be a connection with the sheep cell agglutination reaction and the latex fixation test. The 22 S fraction was always observed in sera giving the most positive tests.

Heimer et al. (1962) tested human sera for their ability to inhibit complement fixation in a test system where a diluted sample of the test serum was added to a standard amount of sensitized human thyroglobulin and a standard amount of complement. The criterion used for inhibition of lysis was by comparing the extent of lysis in serum containing tubes with a tube that contained only sensitized thyroglobulin and complement. It was found that sera from active rheumatoid arthritis cases inhibited complement fixation by greater than 50% in all fourteen cases studied.

Romeyn and Bowman (1967) studied the inhibitory effect of rheumatoid sera. The test involved serially diluting the test serum, then adding a constant amount of ORh negative human cells sensitized by $3HD_{50}$ rabbit hemolysin and incubating for twenty minutes at 37°C. Guinea pig complement was then added to each tube and there was a further incubation at 37°C for twenty minutes. The extent of lysis was measured in a spectrophotometer

by reading each tube against equivalent unsensitized cell controls. Results of tests for sera of twenty seven patients with rheumatoid arthritis showed that as the serum dilution decreased there was marked inhibition of lysis, and that this inhibition was much greater than that produced by normal sera.

Torrigiani et al. (1970) stated that rheumatoid factor demonstrable by the agglutination of sheep red cells coated with rabbit antibody is a characteristic serological feature of approximately 80% of patients with "definite" or "probable" rheumatoid arthritis as defined by the American Rheumatism Association (1959). Such patients were termed seropositive. In the remaining 20%, the sheep cell agglutination test was persistently negative. Such patients were termed seronegative. It was found by a quantitative immunoadsorption isolation technique and subsequent reaction with immunoglobulin class specific rabbit antisera that both seropositive and seronegative patients sera had large amounts of immunoglobulin G class anti-immunoglobulin which did not give positive results in the classical tests for immunoglobulin M rheumatoid factor.

The immunological distinction between seronegative and seropositive rheumatoid arthritis lies in the immunoglobulin class of the anti-immunoglobulin factors present and in the methods used to detect them, rather than in some fundamentally different pathogenetic mechanism.

A substance similar to rheumatoid factor was detected by the latex fixation test but not with the sheep cell agglutination test in the sera of patients with sarcoidosis without concomitant rheumatoid arthritis. Analytic ultracentrifuge experiments on the sera of patients with sarcoidosis and extremely high latex fixation test titers showed a 22 S complex similar to that observed in patients with rheumatoid arthritis (Kunkel et al., 1958).

Peltier and Christian (1959) found that sera from patients having syphilis gave positive tests for rheumatoid factor in 11% of 147 cases studied when the latex fixation procedure was used. The sensitized sheep cell agglutination test was negative for all but one of the syphilitic sera studied indicating the syphilitic sera may react specifically with human immunoglobulin which was used in the latex fixation test.

Howell et al. (1959) concluded that an agent similar in several important respects to rheumatoid factor was elaborated in the presence of chronic hepatic and other visceral diseases. Beck et al. (1961) noticed a substance similar to rheumatoid factor in patients with pulmonary tuberculosis and bronchial infection.

Singer et al. (1961) performed the latex fixation test on sera from 245 patients with active pulmonary tuberculosis and obtained positive tests for "rheumatoid factor" in 13% of the cases. The reactive sera were studied by a

number of methods, including analytical and density gradient ultracentrifugation, DEAE cellulose column chromatography, quantitative precipitation with human immunoglobulin and mercaptoethanol treatment. These procedures demonstrated that the fraction responsible for positive serological activity was a component of the macroglobulin fraction of the patients sera. They also stated that macroglobulins similar to rheumatoid factor were found in the sera of patients with systemic lupus erythematosis, sarcoidosis, syphilis and cirrhosis of the liver.

III. AUTOLOGOUS ANTI-IMMUNOGLOBULINS: INCIDENCE IN NORMAL INDIVIDUALS

It was previously mentioned that anti-immunoglobulin could be artificially produced by injection of foreign immunoglobulin into an animal. High titers of anti-immunoglobulin accompany various diseases. Such an incidence of anti-immunoglobulin gave investigators insight into when anti-immunoglobulin was formed but also created the impression that anti-immunoglobulin was only formed when the body was exposed to unusual conditions, such as severe disease or foreign immunoglobulin injection. Many workers though, uncovered evidence of anti-immunoglobulin in normal individuals.

Ehrlich (1906) found low titers of anti-immunoglobulin in normal nonimmunized goats. He suggested that anti-immunoglobulin was normally produced and artificially induced anti-immunoglobulin production was only an increase of normal output. Pike et al. (1951) found that samples of serum from normal animals including rabbits, goats, sheep, dogs, cows, guinea pigs, and horses increased agglutination of sheep red blood cells sensitized by rabbit hemolysin indicating the presence of a rheumatoid factor-like substance. Sheep, goat, guinea pig and horse sera gave mean titers above the range found with normal human sera. No samples of animal serum were found which agglutinated sensitized sheep cells in dilutions as high as those observed for the

sera of rheumatoid arthritis patients. Normal human sera in low dilutions increased the sheep cell agglutination titer of rabbit anti-sheep cell serum.

Unger et al. (1958) found in a series of 3,487 sera from healthy adult blood donors that 21 contained an agglutinating substance for human adult RhO positive red blood cells that were sensitized with particular anti-RhO sera.

Beck (1961) found that certain human sera which do not contain irregular haemagglutinins were capable of agglutinating group O Rh positive erythrocytes which had been sensitized by strong incomplete anti-D serum. Beck found anti-immunoglobulin present in 0.8% of blood donors surveyed as well as in 3.7% of the hospital patients surveyed. This 0.8% figure for normal persons is comparable with 0.5% found in Poland (Milgrom et al., 1956), 0.33% found in France (Ropartz et al., 1958a) and 0.6% in the U.S.A (Unger et al., 1958). Beck stated that the somewhat lower incidences reported by other investigators was due to use of older erythrocytes in the agglutination test, which causes the test to be less sensitive.

Watson and Collins (1963) partially characterized two functional types of anti-immunoglobulin found in normal rabbit sera. Samples of normal rabbit sera from 96 rabbits were used in the study. In these samples it was found that one type of anti-immunoglobulin was responsible for

agglutination of sensitized sheep cells. The other type of anti-immunoglobulin was demonstrable by the latex fixation test. The factor responsible for agglutination of sensitized sheep red cells (Waaler Rose Factor) was found in all normal rabbit sera tested in titers of 1/64 to 1/2048. They discovered this factor to be primarily localized in the water soluble fraction of rabbit serum, that it was heat stable at 56°C and could be absorbed from rabbit serum with sheep erythrocyte stroma highly sensitized with rabbit hemolysin. Waaler-Rose factor could not be removed from the sera by absorption with latex particles coated with human Cohn Fraction II. The factor demonstrable by the latex fixation test had titers of 1/20 to 1/512. Unlike the Waaler-Rose factor, the latex fixation factor was found to be primarily confined to the water insoluble fraction of sera and was completely inactivated when the sera were heated at 56°C for fourteen minutes. The latex fixation factor was completely absorbed from rabbit sera by latex particles coated with human Cohn Fraction II. The adsorption of rabbit sera with sheep erythrocyte stroma sensitized with rabbit hemolysin removed 75% of the latex fixation factor. The Waaler-Rose factor and latex fixation factor in rabbit sera were both differentiated from the Forssman heterophile antibody and were not associated with conglutinin activity, and were different from one another.

Watson (1963) tested sera from sixty normal adult

humans and found a heat labile (56°C, 8 minutes) latex fixation factor was present in the water insoluble euglobulin fractions. This factor was not usually detected in samples of fresh normal whole sera by the latex fixation test because of a heat labile (56°C, 4 minutes) inhibitor that prevented the reaction of the anti-immunoglobulin with the Cohn Fraction II coated on latex particles. This anti-immunoglobulin shared many characteristics with the heat labile anti-immunoglobulin described previously in Watson's work on normal rabbit sera.

Heimer et al. (1962) tested human sera for their ability to inhibit complement fixation in a test system where a diluted sample of the test serum was added to a standard amount of sensitized human thyroglobulin and a standard amount of complement was then added. The criteria used for inhibition of lysis was comparison of the serum containing tubes with a tube that only contained sensitized thyroglobulin and complement. It was found that normal human sera inhibited complement fixation above 50% in three out of twenty-nine cases studied. The other twenty-six normal sera inhibited complement fixation to extents less than 50%. Fractionation of a number of sera of healthy donors by DEAE cellulose chromatography revealed two inhibitors to complement fixation. In addition to these substances a third compound occurred in sera of individuals with rheumatoid arthritis. The compound was purified four

hundred fold and was shown to be a macroglobulin and was distinct from rheumatoid factor. This macroglobulin was found to be only present in minute amounts of normal serum suggesting increased amounts were due to disease.

Mackenzie et al. (1967) screened more than two thousand normal human sera for antibodies to immunoglobulin M. They used an agglutination test where samples of sera to be tested were mixed with type O Rh positive red cells coated with purified immunoglobulin M proteins from patients with Waldenstroms macroglobulinaemia. They found forty-two sera that had agglutinin activity. They concluded though that these anti-immunoglobulin M agglutinins did not seem to be antibodies to immunoglobulin M since attempts at adsorption of human sera with Rh positive cells heavily coated with anti-Rh saline agglutinin did not remove the agglutinating activity.

Romeyn and Bowman (1967) detected inhibition of complementary lysis of rabbit sensitized O Rh negative human cells in the presence of dilutions of twenty sera from normal humans although the inhibition of lysis was produced only at high concentration (1:2 and 1:4).

IV. PHYSICAL PROPERTIES OF ANTI-IMMUNOGLOBULINS

Treffers et al. (1942) injected rabbits with a washed specific precipitate composed of pneumococcus specific polysaccharide and the corresponding anticarbohydrate from anti-pneumococcus horse serum. Serum from rabbits contained heterologous anti-immunoglobulin formed against the anticarbohydrate antibody. It was determined by electrophoresis such anti-immunoglobulin had a mobility of an immunoglobulin. The anti-immunoglobulin was a macroglobulin with a sedimentation rate of 19 S.

The activity of heterologous anti-immunoglobulin was destroyed by heating at 100°C for thirty minutes (Bordet, 1904; Ehrlich, 1906) but such activity was not lost at incubations of 70°C and 56°C.

Beck (1960) studied autologous anti-immunoglobulin in human sera by its agglutination of human group O Rh positive cells sensitized with human anti-D serum. He found that titrations incubated at 4°C, 16°C and 37°C produced identical results. He also determined that agglutination was optimal between pH 7.5 and pH 8 and just as for heterologous anti-immunoglobulin, the autologous anti-immunoglobulin migrated with immunoglobulin on electrophoresis. He found that the activity of the autologous anti-immunoglobulin was stable after incubation at 56°C for 30 minutes and that human sera stored at -16°C for eight months did not show a decrease in autologous anti-

immunoglobulin titer. The activity of such human autologous anti-immunoglobulin was found to be enhanced by a non-dialysable cofactor which was present in normal human serum but which was destroyed upon prolonged dialysis.

Fudenberg (1964) determined anti-immunoglobulin activity in rabbit serum was confined to the 19 S containing fractions obtained by DEAE column chromatography. The 7 S and 19 S containing fractions similarly obtained from normal rabbit sera were devoid of such activity, indicating that the preparation process was not responsible for the activity in the 19 S containing peak of anti-immunoglobulin serum. The 19 S nature of the anti-immunoglobulin was demonstrated by the abolition of its agglutinating activity upon treatment of whole serum or of the 19 S containing fraction with 0.1 M mercaptoethanol. Such 19 S anti-immunoglobulin reacted specifically with 7 S rabbit immunoglobulin altered either by combination with antigen or by certain nonspecific methods. The anti-immunoglobulin combined with antigenic determinants on Fragments I and II, but not III of the papain digested 7 S immunoglobulin molecule.

Milgrom and Witebsky (1960) prepared anti-immunoglobulin containing serum by autoimmunizing rabbits with their own immunoglobulin. Such immunoglobulin caused anti-immunoglobulin to be formed against it because the ammonium sulphate precipitation technique used for its isolation from rabbit serum made a small amount of the immunoglobulin

antigenically foreign to the rabbit. Such anti-immunoglobulin retained its ability to agglutinate sheep cells sensitized with rabbit anti-sheep cell hemolysin after being incubated at 56°C for thirty minutes. It was found to be associated with the immunoglobulin fraction of the rabbit serum by immunoelectrophoretic analysis.

V. POSSIBLE MECHANISMS FOR STIMULATION OF ANTI-IMMUNOGLOBULIN PRODUCTION

When an animal is injected with foreign immunoglobulin it produces anti-immunoglobulin. The same is true when a native immunoglobulin becomes changed in some manner. Such a change in immunoglobulin structure could take place continually in "vivo" due to catabolism of immunoglobulins. In a more specific case, when immunoglobulins such as antibodies become changed due to reaction with antigen, antibodies are formed against these changed immunoglobulins. Several workers suggested mechanisms for anti-immunoglobulin production.

Eagle (1930) stated that in an immune reaction that an originally hydrophilic antibody type immunoglobulin became water insoluble and therefore denatured, upon combination with antigen. He suggested in the case of immune reactions that denaturation of antibody immunoglobulin was due to the fact that its specificity was determined by hydrophilic groups. When these combined with the antigen, hydrophobic groups face outwards causing the antibody to be regarded as a foreign immunoglobulin. The result was that anti-immunoglobulin production was stimulated.

Marrack (1938) suggested that the new antigenic determinants appearing in particulate antigen-antibody complexes were produced by changes in the antibody immunoglobulin. The properties exhibited by the antibody immuno-

globulin in the complexes were very similar to those of proteins denatured by heat. As with heat denatured proteins, complexed antibodies were flocculated when their surface potential had fallen below a critical level. Such flocculation was inhibited by high salt concentration.

Milgrom et al. (1956) showed that 0.5% of human sera possessed a thermostable anti-immunoglobulin that agglutinated O Rh positive red cells sensitized with anti-D antibodies. They stated this anti-immunoglobulin reacted only with immune antibodies after their denaturation in serological reactions and therefore was not neutralized by normal serum immunoglobulin.

Najjar and Robinson (1959) stated the qualities that made immunoglobulin a familiar and normal protein to the immune system of the host were chiefly its surface configuration, shape and size. If either or all of these were altered by strong association with another molecule or by autolytic digestion, new groupings might appear on the surface. An antibody molecule, normally considered familiar and non-antigenic, may as a consequence of altered configuration be rendered unfamiliar, "foreign" and therefore antigenic following its union with antigen. This was strongly indicated by studies on antigen-antibody interaction using yeast alcohol dehydrogenase and egg albumin as antigens. It was subsequently supported by experiments showing that when antibody-antigen complexes were used for immunizing rabbits, some of

the antibody produced in rabbits would react only with the complex but not with the antigen.

CHAPTER II

THEORY

THEORETICAL POSTULATES UNDERLYING THE STUDY OF AUTOLOGOUS ANTI-IMMUNOGLOBULINS

The hypothesis upon which my work is based is that the two types of autologous anti-immunoglobulin act as an *in vivo* control mechanism for the immune response. It is postulated that such a control mechanism works at two levels. It works at the level of the antibody-antigen complex, where one type of anti-immunoglobulin facilitates the ability of the complex to fix complement and the other type inhibits the ability of the complex to fix complement. The extent to which complexes can fix complement depends on the relative amounts of the two types of anti-immunoglobulin.

Such a mechanism is also postulated to work at the lymphocyte level of the immune response. It is postulated that the immune response is used as a degradative process with respect to body components. More specifically as B lymphocytes, plasma cells and possibly T lymphocytes' age, the antigenic determinants on their surface gradually change to a certain extent, causing these cells to be recognized as foreign by the body. The result is that antibody is formed against them and this antibody reacts with the surface of these cells causing an antibody-lymphocyte complex to form.

Mond (1973) indicated the presence of immunoglobulin on the surface of plasma cells. Greaves (1970) demonstrated

the presence of immunoglobulin on the surface of B cells. He also demonstrated a small amount of immunoglobulin on the surface of T cells. It may be that immunoglobulin on T cells is actually antibody against T cells and it is only natural that such an antibody would be present only in minute amounts in the body. It also may be that some of the immunoglobulin on the surface of B cells and plasma cells is antibody directed against these cells. It is postulated that the two types of anti-immunoglobulin control the degradative rate of B cells, plasma cells and T cells. The relative amounts of the two types of anti-immunoglobulin determine the rate at which complement may be fixed by the antibody-lymphocyte complex and therefore determine the B cell, T cell and plasma cell levels in the body.

Mond (1973) examined rabbit spleen cells which were sensitized to sheep erythrocytes to determine their ability to evoke a secondary response to sheep erythrocytes in the presence or absence of various anti-immunoglobulin sera in the cultures. After four days in culture enumeration of plaque forming cells revealed that all the anti-immunoglobulin containing sera used caused 85% inhibition or more as compared to control plaque forming cells values. This indicated that anti-immunoglobulin reacted with the immunoglobulin receptors on the surface of the cells and blocked their reacting with antigen for which they are specific.

No complement was added to the cultures in this experiment. Since anti-immunoglobulin reacts with the immunoglobulin receptors of these cells it is conceivable that anti-immunoglobulin would also react with the hypothetical antibody directed against the spleen cell.

Mond also found that after preincubation of immune mouse spleen cells in vitro with heterologous anti-immunoglobulin directed against mouse immunoglobulin and complement, the PFC response was much less than 25% of the control values.

Mond stated that the concentration of anti-immunoglobulin required to interfere with B cell function in experiments where no complement was added to mixtures of anti-immunoglobulin and B cells, did not significantly decrease the T cell proliferative response to antigen. Actually addition of anti-immunoglobulin to T cells stimulated the proliferative response. One of the conclusions Mond drew from these experiments was that none of the immunoglobulin determinants were readily accessible on T cells for interaction with anti-immunoglobulin if they were at all responsible for the specific T cell response to antigen.

From the results of Mond's work, I postulate that anti-immunoglobulin inhibits B cell function in two ways. It reacts with the immunoglobulin receptors on the B cell

surface and prevents antigen from reacting. There is also the possibility that anti-immunoglobulin reacts with the hypothetical antibody against the lymphocyte and causes lysis. These two phenomena of inhibition of the immune response could be caused by inhibiting and facilitating anti-immunoglobulin, respectively.

In the case of T cells, it may be that the immunoglobulin determinants Mond refers to are antibody against T cells. A significant drop in the T cell proliferative response due to lysis of T cells in the presence of complement and anti-immunoglobulin was not detected, however, probably for two reasons. One reason is that the antibody against T cells in the T cell population studied in vitro was present only in minute amounts. Coupled with the resulting slow rate of immune degradation is the stimulating action of anti-immunoglobulins on the proliferative response of T cells. These two factors combine to prevent the worker from observing the immune degradation of lymphocytes.

Takahashi (1971) found that incubation of spleen cells from mice immunized against sheep erythrocytes, with anti-immunoglobulin directed against mouse immunoglobulin, and complement, caused a reduction of their plaque forming activity, which was concluded to be due to lysis of lymphocytes.

It should be noticed that the total postulated

action of the two functional types of anti-immunoglobulin at the two different levels of the immune response is contradictory. For example, if there is a greater amount of facilitating anti-immunoglobulin in the body relative to inhibiting anti-immunoglobulin, then the total effect of this at the antigen-antibody complex level is greater complement fixation than if the amounts of the two functional types were reversed. On the other hand, at the B cell level a greater relative amount of facilitating anti-immunoglobulin means increased lysis of B cells and plasma cells and a lesser humoral immune response. The opposing action of anti-immunoglobulin at the two levels control the level of intensity in the immune response.

It is postulated that there is a definite need for such a control of the immune response in the body, for the immune response is not only used to destroy "foreign invaders" but also is used as a degradative process for the body's own components. So it is essential that the immune response be strong enough to deal with foreign substances or organisms in the body but also be weak enough to insure that in a reaction where antibody destroys an old or malignant body component that the antibody is not present in large enough quantities so that it is able to cross-react with normal tissue to a significant extent and damage organs and other components of the body.

CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

Dulbecco Phosphate Buffered Saline (DBS)

The diluent used throughout was DBS modified by addition of 0.00015 molar Ca^{++} and 0.0005 molar Mg^{++}. The pH is 7.3 to 7.4 (See Appendix 1).

Erythrocytes:

i) Sheep Red Blood Cells

Sheep blood from National Biological Laboratory (P.O. Box 1325, Winnipeg, Manitoba) was used. It was at least three days old and less than three weeks old.

ii) 2.25 OD Sheep Red Blood Cell Suspension

Sheep cells were washed three times in cold DBS in a fifty ml plastic centrifuge tube, at 700 g for ten minutes. A suspension of sheep red cells was prepared from the washed red cells, with a concentration such that 1/10 dilution in distilled water gave a lysate after centrifugation with an absorbance of 0.225 on a Unicam SP 500 Series 2 spectrophotometer at λ 545 nanometers (approximately 0.9% of volume, approximately 22.5 million cells per ml). Such a suspension was referred to as a 2.25 OD sheep red blood cell suspension.

iii) Guinea Pig Red Blood Cells

Guinea pig erythrocytes were obtained by cardiac puncture of normal laboratory guinea pigs, mixed with an equal volume of sterile Alsevers Solution (See Appendix 2) and refrigerated.

iv) 50% Guinea Pig Red Blood Cell Suspension

50% guinea pig red blood cell suspensions were prepared by centrifuging the guinea pig blood in Alsevers Solution at 700 g for ten minutes. The cells were washed three times in cold DBS in a 50 ml plastic conical centrifuge tube at 700 g for ten minutes each time. The red blood cells were then resuspended in an equal volume of DBS.

Mice:

The CFI female mice used in the experiments in this thesis were bred in the Department of Medical Microbiology. The strain was originally obtained from Carworth Farms, P.O. Box 176, Portage, Michigan 49081.

Mice of all other strains used in this thesis were obtained from Jackson Laboratory, Bar Harbor, Maine.

Table I briefly describes these strains.

Plasma:

i) Bleeding

Mice were heated for up to five minutes in an 18.5 cm by 24.5 cm metal box with a light (150 watt General Electric bulb) shining into a cage positioned a few inches away from it. Blood was obtained from the tail in Stevens heparinized capillary tubes. The tubes were plugged at one end with plasticene.

ii) Plasma Preparation

The plasticene plugged capillary tubes were placed in test tubes with a swab of cotton in the bottom and centrifuged

TABLE I

STRAINS OF MICE USED IN THE AUTOLOGOUS ANTI-IMMUNOGLOBULIN STUDY

Strain	Condition Predisposed to	Age of Onset of Condition	Reference
AKR/J	80% to 90% develop lymphoid leukemia	6 to 8 months for both sexes	Handbook on Jax Mice, Jackson Laboratory, Bar Harbor, Main, U.S.A.
RF/J	43 to 49% develop leukemia	12 months for both sexes	"
C58/J	73% develop leukemia	12 months for both sexes	"
NZB/BINJ	Hemolytic anemia	6 to 9 months males, 12 months females	Personal correspondence with Jackson Laboratory
SM/J	Amyloidosis	After 8 months for both sexes	"
CFI	NONE	-	Personal correspondence with Carworth Farms
C57BL/6J	NONE	-	Handbook on Jax Mice

at 325 g for twenty minutes in a refrigerated centrifuge. Drummond (calibrated) Ziptrol Tubes, Drummond Scientific Company, Bromall, Pennsylvania, were used.

Noble Agar:

The agar consisted of 0.75 grams of Difco Special Noble Agar powder in 80 mls of DBS or multiples thereof. After preparation, the agar was dispensed in 8 ml quantities into screw cap Pyrex tubes. A batch of prepared agar was kept at 4°C for up to one month after the date of preparation. All agar used in experiments reported in this thesis was prepared from one bottle of Difco Agar Powder (Control 533307).

(For other details of preparation see Appendix 3.)

Hemolysin:

Mouse anti-sheep red blood cell hemolysin was produced by injecting CFI female mice with washed sheep red cells. The 7 S fraction of whole hemolysin was prepared in the following way. Frozen whole hemolysin is thawed and dialyzed in Tris HCl (pH = 8.0) for forty-eight hours. The dialyzed preparation was then passed through a Sephadex G-200 column with Tris HCl (pH = 8.0) buffer used as the eluant. The 7 S fraction obtained from the column was concentrated to the original volume using the Amicon concentrator. The 7 S fraction was then dialyzed in DBS for twenty-four to forty-eight hours.

Hemolysin Storage:

The whole mouse anti-sheep red blood cell hemolysin was diluted 1/100. Both the whole and 7 S fraction were

titrated, then dispensed in ten ml quantities and stored at -20°C. When hemolysin was to be used in an experiment, the number of bottles of each type to be used was estimated on the basis of the previous hemolysin titration results. Then the contents of these bottles were thawed, pooled and titrated.

Titration of Hemolysin:

The titration procedure was divided into two parts, the rough titration and fine titration.

In the rough titration, one ml doubling serial dilutions of hemolysin were made in DBS. The starting dilution of hemolysin used for the serial dilution was 1/100 for whole hemolysin and 1/15 for 7 S dilution.

One ml quantities of 2.25 OD sheep red blood cell suspension was added to each tube and the dilution series was incubated at 37°C for twenty minutes. Then one ml of DBS and one ml of 1/25 guinea pig complement was added with a subsequent thirty minute incubation at 37°C. The tubes were cooled rapidly in an ice bath and centrifuged at 550 g for ten minutes. The tubes were read in a spectrophotometer against a control tube containing unsensitized sheep cells, DBS and complement. The thickness of the spectrophotometer tube used was one centimeter. Appropriate control tubes were set up.

The purpose of the rough titration was to determine approximately the dilution of hemolysin that caused 50% lysis of the volume of red blood cell suspension used in the test.

The results of the rough titration enabled the experimenter to pick a dilution of hemolysin slightly less than the

dilution that caused 50% lysis in the test system and by means of a fine titration to determine the exact dilution that caused 50% lysis in the test system

In the fine titration the same procedure was used as in the rough titration but here one ml harmonic dilutions of hemolysin were made. In such a titration, .1 ml to .9 ml volumes of the hemolysin dilution picked by the experimenter on the basis of rough titration results were made up to one ml with the appropriate amount of DBS.

The strength of hemolysin was determined by plotting per cent lysis vs volume of hemolysin used in the fine hemolysin titration on probability paper. Per cent lysis for each tube was calculated by dividing the absorbance value by 0.56 which was determined to be the absorbance value for 100 per cent lysis of the red blood cell suspension.

The strength of hemolysin could be obtained from the graph and was expressed in terms of HD_{50}'s.

HD_{50} (50% hemolytic dose) is defined as the amount of hemolysin in 1 ml of a dilution of hemolysin that will lyse 50% of a 1 ml volume of 2.25 OD sheep red cell suspension in the presence of 1 ml of DBS and 1 ml of 1/25 complement.

Glass Plates:

The plates were made from 70 mm squares of Kodak glass, edged by 9 mm wide and 1.3-1.7 mm thick strips of plastic to retain the agar.

Diethyl Amino Ethyl Dextran:

DEAE Dextran was prepared by mixing .8 grams of

Pharmacia DEAE Dextran (Uppsala, Sweden) with 40 mls of DBS. The same bottle of DEAE Dextran powder was used in preparation of the DEAE Dextran used in all the experiments in this thesis.

Film:

Kodak Verichrome Pan film (VP120) was used. (For film development, see Appendix 4.)

Camera:

The camera used was a Graflex General Precision camera.

Complement:

Two pools of guinea pig complement were used in the experiments reported in this thesis. Only for those experiments in which the same pool of complement was used were the results compared.

The guinea pig complement was obtained from North American Laboratory Supply Co., Gunton, Manitoba, and stored at -87°C.

Pool #4 complement titrated at 1 $C'H_{50}$ = 1 ml of 1/75 to 1/97 complement.

Pool #5 complement titrated at 1 $C'H_{50}$ = 1 ml of 1/101 to 1/129 complement.

1 $C'H_{50}$ is defined as the amount of complement in 1 ml of a certain dilution of complement that lyses 50% of a 1 ml volume of 2.25 OD sheep red blood cell suspension

sensitized by 4 HD_{50} rabbit anti-sheep hemolysin and in the presence of two mls of DBS.

Method:

Agar Diffusion Plate Method of Detecting Autologous Anti-Immunoglobulins

The following method was used to examine mouse plasma. Twenty-four mls of a 2.25 OD suspension of washed sheep red blood cells in DBS was mixed well with twenty-four mls of mouse produced hemolysin containing a certain HD_{50} (50% hemolytic dose). These were mixed in a 125 ml Erlenmeyer flask. The 2.25 OD red blood cell suspension was prepared fresh on the day of its use.

After incubation at 37°C in a water bath for twenty minutes (it was mixed after ten minutes), the contents of each flask were transferred to a 50 ml conical plastic centrifuge tube. After centrifugation, the supernatant was removed and the sensitized cells were resuspended by adding 2.2 mls of DBS. Noble agar (.75 gr/80 mls in DBS), 8 mls in a test tube, was melted and held at 47°C in a Temp-Blok nodule heater. To each agar tube 0.5 mls of DEAE Dextran (20 mg/ml in DBS) and 2 mls of the sensitized cell suspension were added, after warming both to 45°C in the nodule heater for up to five minutes. It was essential that DEAE Dextran be added only just before the sensitized cells were added to the tube of agar.

In order to ensure homogeneity, the mixture was

inverted several times following the addition of DEAE Dextran and then again after the sensitized cells were added.

With a warmed pipette 2 ml amounts of the agar mixture were transferred to each glass plate and spread over the plate's surface with the pipette tip. The plates were on a levelling board at the time of addition to ensure uniform distribution of the blood agar mixture. It was essential to transfer the blood agar to plates immediately after it was mixed together to obtain best results.

Each poured plate was placed in a petri dish and stored at 4°C for at least thirty minutes to ensure proper hardening of the blood agar. Four wells were then made in the agar using a metal cylinder 4.5 mm in outside diameter, sucking the agar out through a piece of rubber tubing attached to the cylinder. The holes were filled with 5 ml of plasma each, using Ziptrol capillary tubes as the method of plasma transfer from the heparinized capillary tube to the hole.

The plates were then stored in their petri dishes at 4°C for 44 hours to allow plasma diffusion to take place. Subsequently each plate is flooded with 2 mls of 1/30 guinea pig complement. At the end of a one hour incubation at 37°C, the complement was washed off using DBS. After these plates were stored in their petri dishes a further

25 hours at 4°C, they were photographed using dark field illumination.

Plates that contained unsensitized cells were also prepared by the above method.

The principle behind the method was as follows. There are two functional types of autologous anti-immunoglobulin. One type reacts in such a way with the mouse antibody in the mouse antibody sheep red cell complex as to facilitate fixation of complement and the other type reacts with the antibody in the complex in such a way as to inhibit fixation of complement.

These two functional types of anti-immunoglobulin present in the plasma diffused out to different characteristic distances from the hole and reacted with the antigen-antibody complexes. Upon complement addition there was a certain uniform level of background lysis on all parts of the plate except around the hole where there was a ring of inhibited lysis immediately adjacent to the hole, presumably due to the fact that when both types of anti-immunoglobulin are present, inhibition takes precedence over facilitation. There is a ring of facilitated lysis outside the inhibited lysis ring, presumably due to the presence of facilitating anti-immunoglobulin only.

Because dark field illumination was used in photography the more transparent areas on the negatives were the areas showing greater lysis. Therefore the ring of facilitated

lysis was lighter than the background lysis on the plate and the ring of inhibited lysis was darker than the background (see Figure 1).

The ring of inhibited lysis was termed the P ring or protective ring and the ring of facilitated lysis was termed the L ring or lytic ring.

A millimeter scale was included when each plate was photographed to determine the degree of magnification of ring diameter due to the photographic process. A Vernier caliper was used to measure the diameters of the rings on the negatives, in terms of millimeters on the plates.

Unless otherwise stated, all the experiments reported in this manuscript were carried out using the previously described standard method.

The term "sensitized" plate used throughout the thesis refers to the sheep red blood cells in the blood agar plates being complexed with a specific dose of mouse anti-sheep red blood cell hemolysin, whether it was 7 S or whole hemolysin. "Whole plates" mean that whole hemolysin was used; "7 S plates" that 7 S hemolysin was used.

The unsensitized plates were included in each experiment reported in this thesis as controls to discount the possibility of a toxic substance in the mouse plasma which lysed sheep red blood cells or of antibody against sheep red blood cells in mouse plasma causing a ring of lysis.

In every instance no rings appeared on the unsensitized

control plates after development which is a very strong proof for the validity of the plate method as an assay system for autologous anti-immunoglobulins.

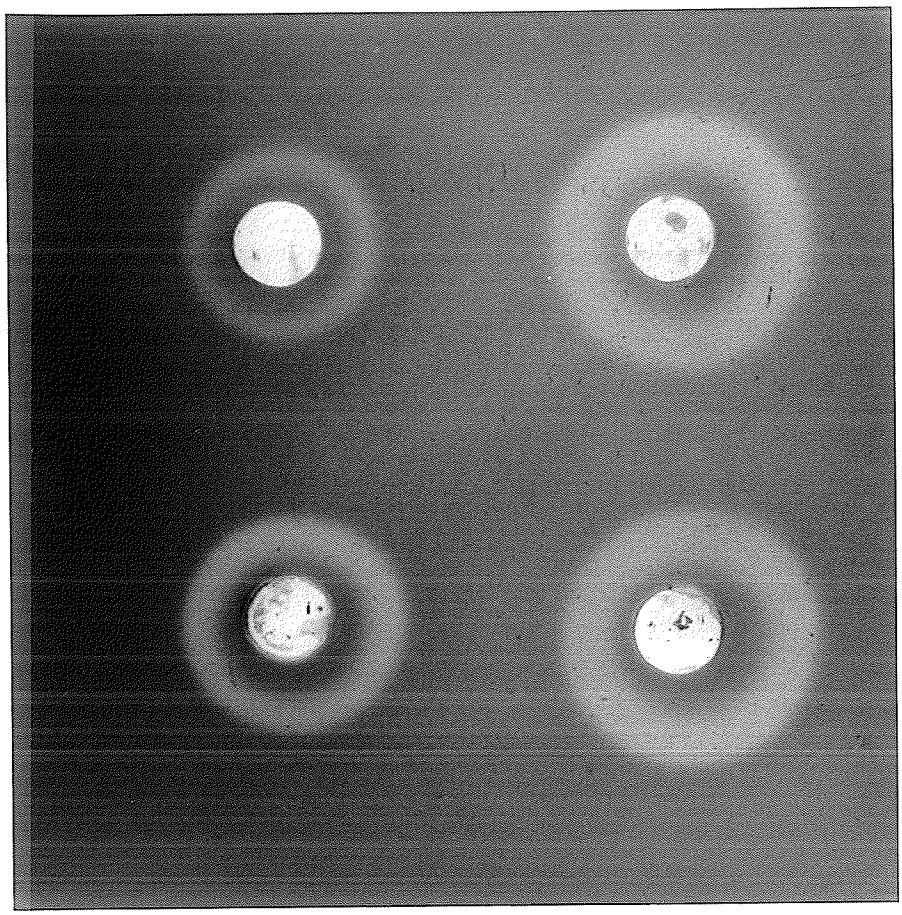


Figure 1. An agar diffusion plate containing mouse sensitized sheep red cells. A mouse plasma was diffused in each of four wells. The dark rings of unlysed cells immediately adjacent to the well and the light outer rings of lysed cells (as compared to background lysis) were caused by inhibiting and facilitating anti-immunoglobulin, respectively (subsequent to complement addition) which diffused out to characteristic distances.

CHAPTER IV

PROCEDURES AND EXPERIMENTAL RESULTS

CORRELATION OF PLASMA CONCENTRATION WITH RING DIAMETER

Procedure:

Samples of plasma were obtained from eight CFI female mice. Amounts of plasma ranging from 0.5 µls to 4.5 µls were mixed with the appropriate amount of DBS to make the mixture up to 5 µls. Each of these 5 µl plasma dilutions was diffused in one, 1 HD_{50} whole sensitized plate and one, 1 HD_{50} 7 S sensitized plate. A 5 µl quantity of undiluted plasma from each mouse was diffused in one, 1 HD_{50} whole sensitized plate, one 1 HD_{50} 7 S sensitized plate, and one unsensitized control plate. DBS controls were also set up, consisting of 5 µl quantities of DBS alone being diffused in one 1 HD_{50} whole sensitized plate and in one 1 HD_{50} 7 S sensitized plate.

This experiment was done twice. The first trial utilized 4.5 µl to 1 µl amounts of plasma diluted appropriately. The mice were 36 ± 2 weeks old. The second trial consisted of 4 µl to 0.5 µl amounts of plasma diluted as above. The mice were 37 ± 2 weeks old.

Results:

Both the mean P and mean L ring diameter values were calculated from eight individual ring diameters produced upon diffusion of plasma samples from the eight mice used in the experiment.

It was statistically determined in each of the eight cases in Table II whether there was a linear regression

TABLE II

RELATIONSHIP BETWEEN PLASMA CONCENTRATION AND MEAN RING DIAMETER

TYPE OF SENSITIZED PLATE	TRIAL	RING TYPE	LINEAR REGRESSION AT THE 95% CONFIDENCE LEVEL
WHOLE	I	P	YES
	II		YES
	I	L	YES
	II		YES
7 S	I	P	YES
	II		NO
	I	L	YES
	II		YES

relationship between the plasma concentration and mean ring diameter.

In seven out of the eight cases in Table II, there is a linear regression relationship at the 95% confidence level. The appropriate plots for each of the seven linear regressions can be seen in Figures 2 to 8.

It is probable that there really is a linear regression relationship in the 7 S sensitized plate, trial 2, P ring case but since this ring "faded out" on the plate at lower plasma concentrations, it was not visible on the negatives of the plates and therefore could not be measured. The result was that only three mean P ring diameters at high plasma concentrations were obtained and plotted.

The control plates in which DBS alone was diffused yielded no rings.

(See Appendix 5 for data in Tables III and IV.)

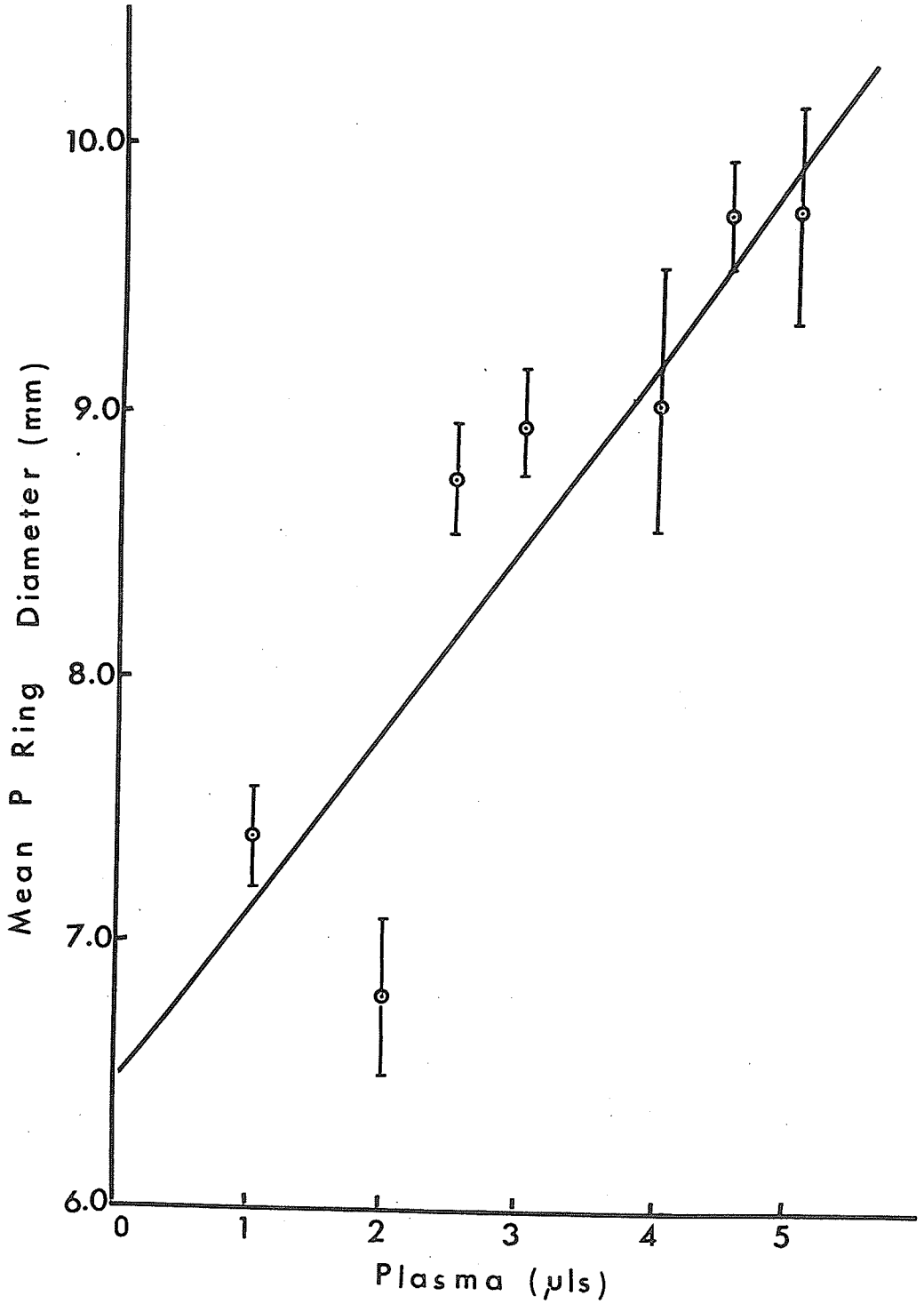


Figure 2. Whole sensitized plates.

Trial I: P mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of µls of plasma diluted with DBS to a 5 µl total. Each mean P diameter was calculated from eight individual P diameters.

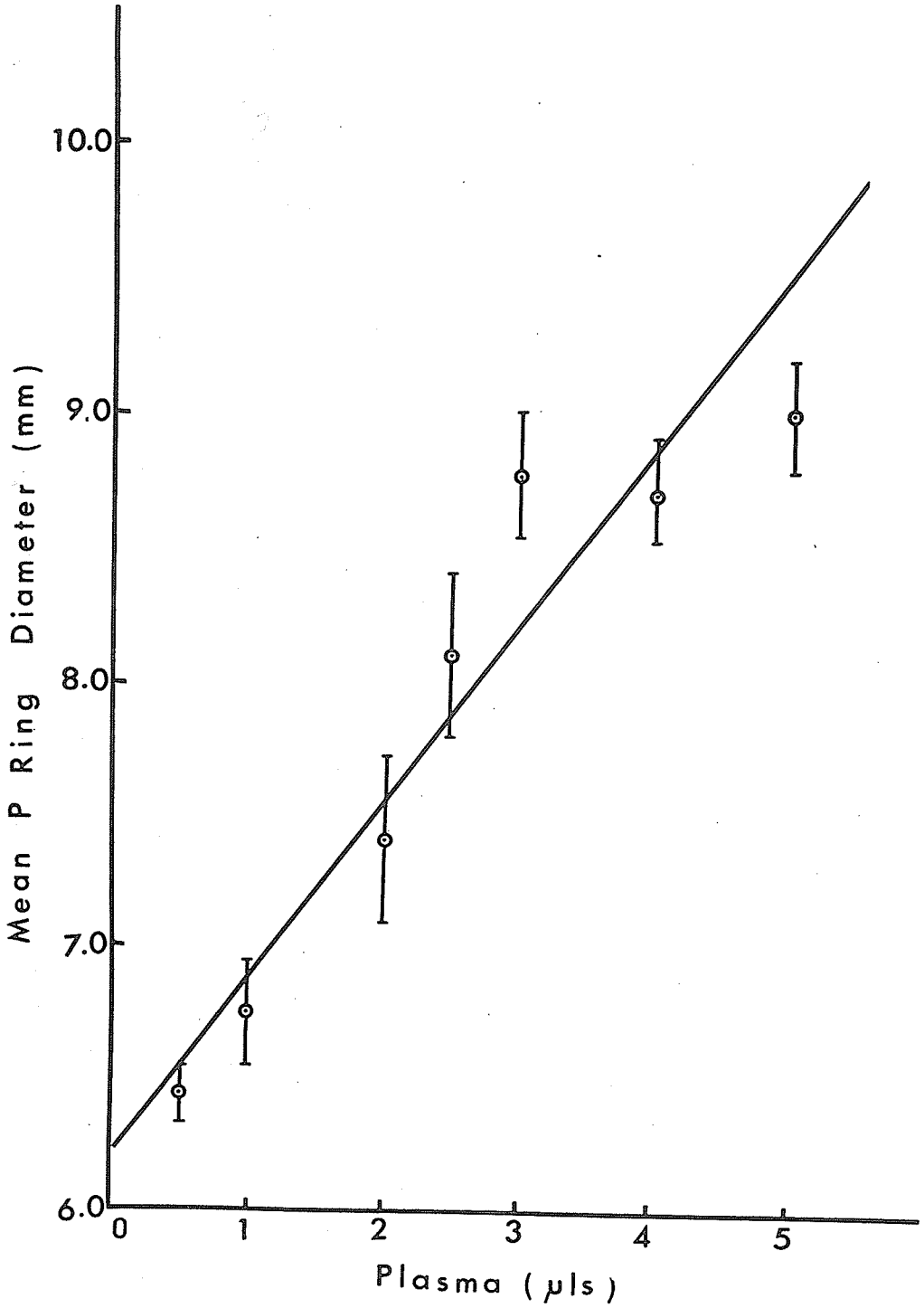


Figure 3. Whole sensitized plates.

Trial II: P mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of µls of plasma diluted with DBS to a 5 µl total. Each mean P diameter was calculated from eight individual P diameters.

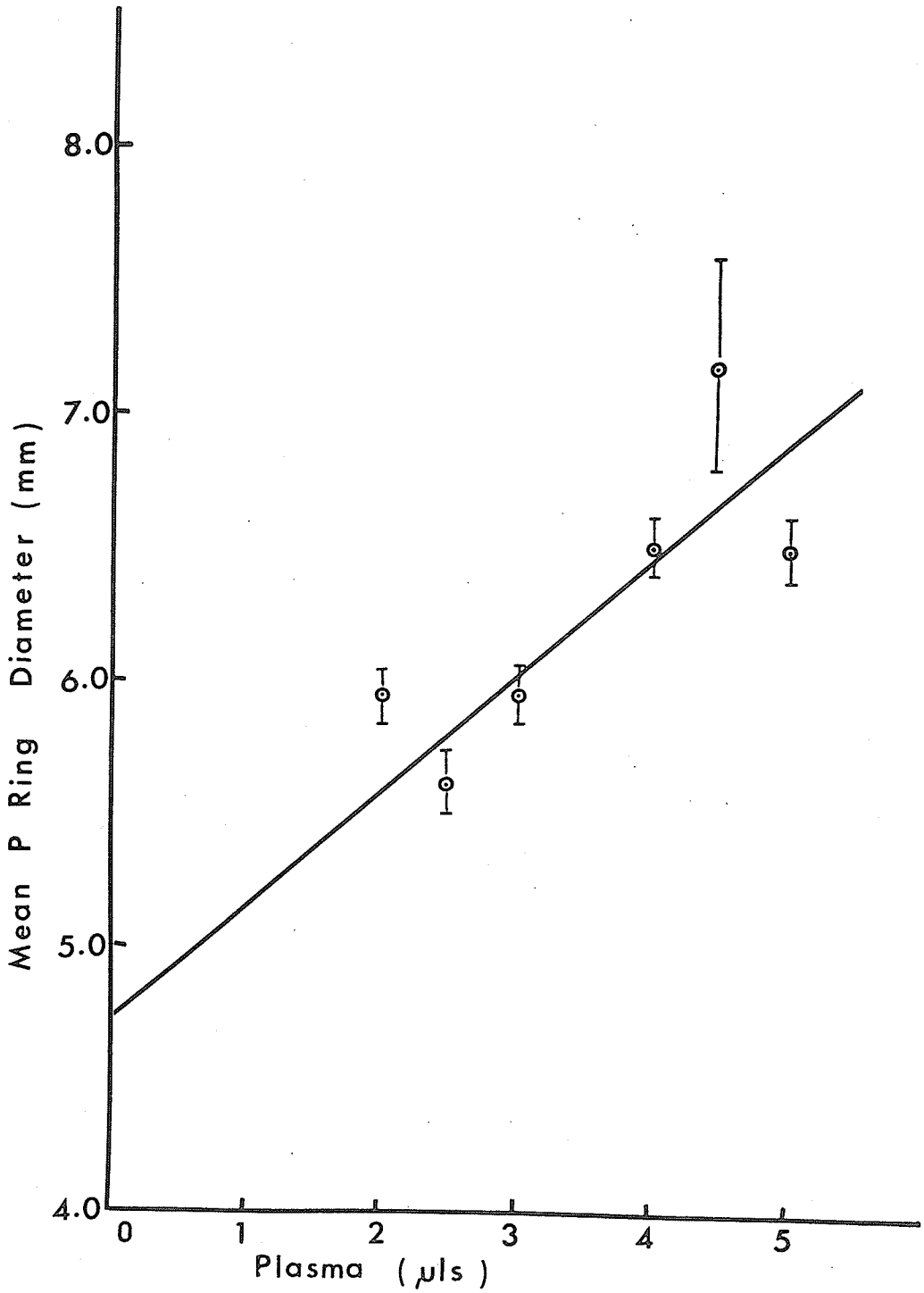


Figure 4. 7 S sensitized plates.

Trial I: P mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of µls of plasma diluted with DBS to a 5 µl total. Each mean P diameter was calculated from eight individual P diameters.

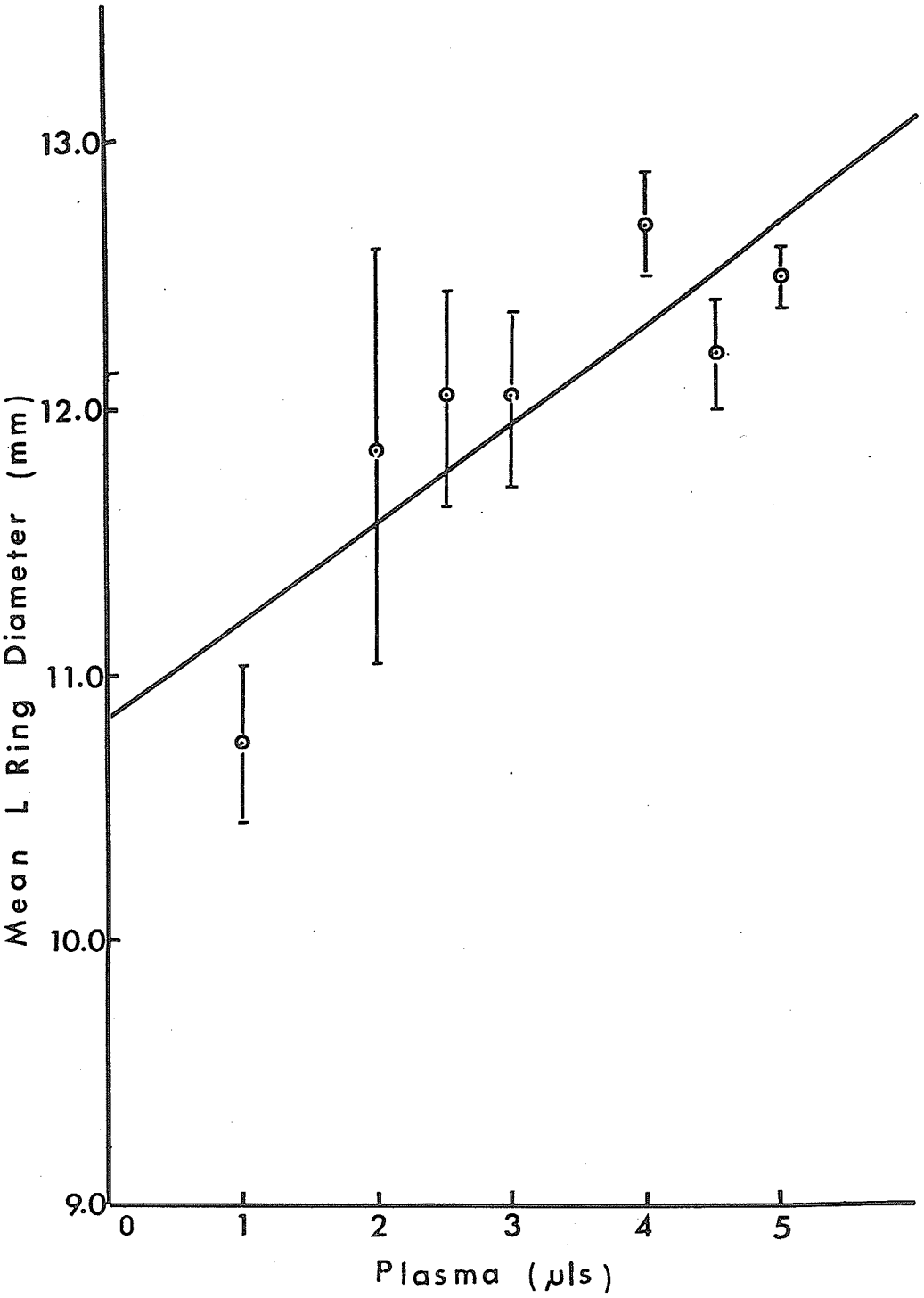


Figure 5. Whole sensitized plates.

Trial I: L mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of µls of plasma diluted with DBS to a 5 µl total. Each mean L diameter was calculated from eight individual L diameters.

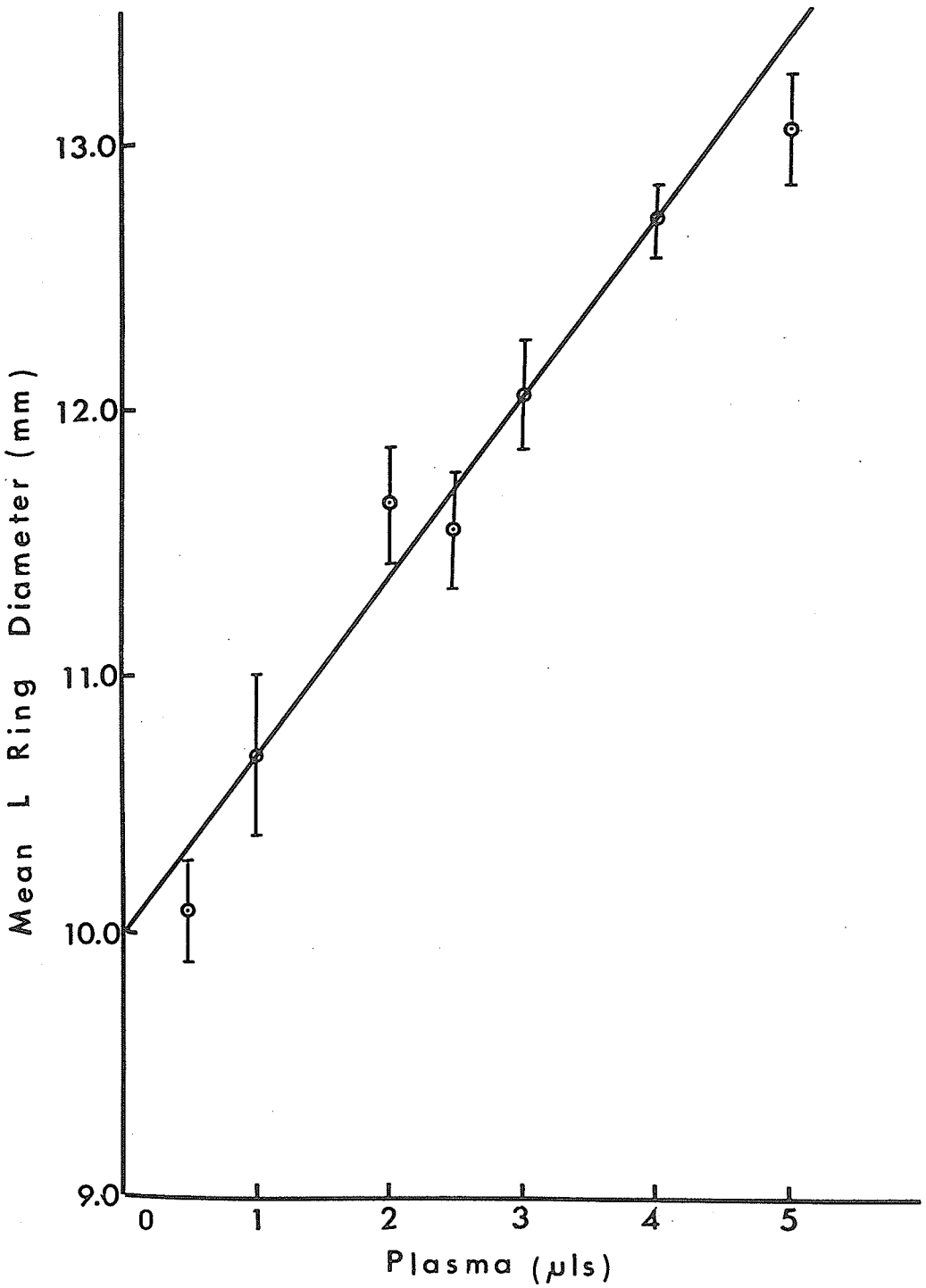


Figure 6. Whole sensitized plates.

Trial II: L mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of µls of plasma diluted with DBS to a 5 µl total. Each mean L diameter was calculated from eight individual L diameters.

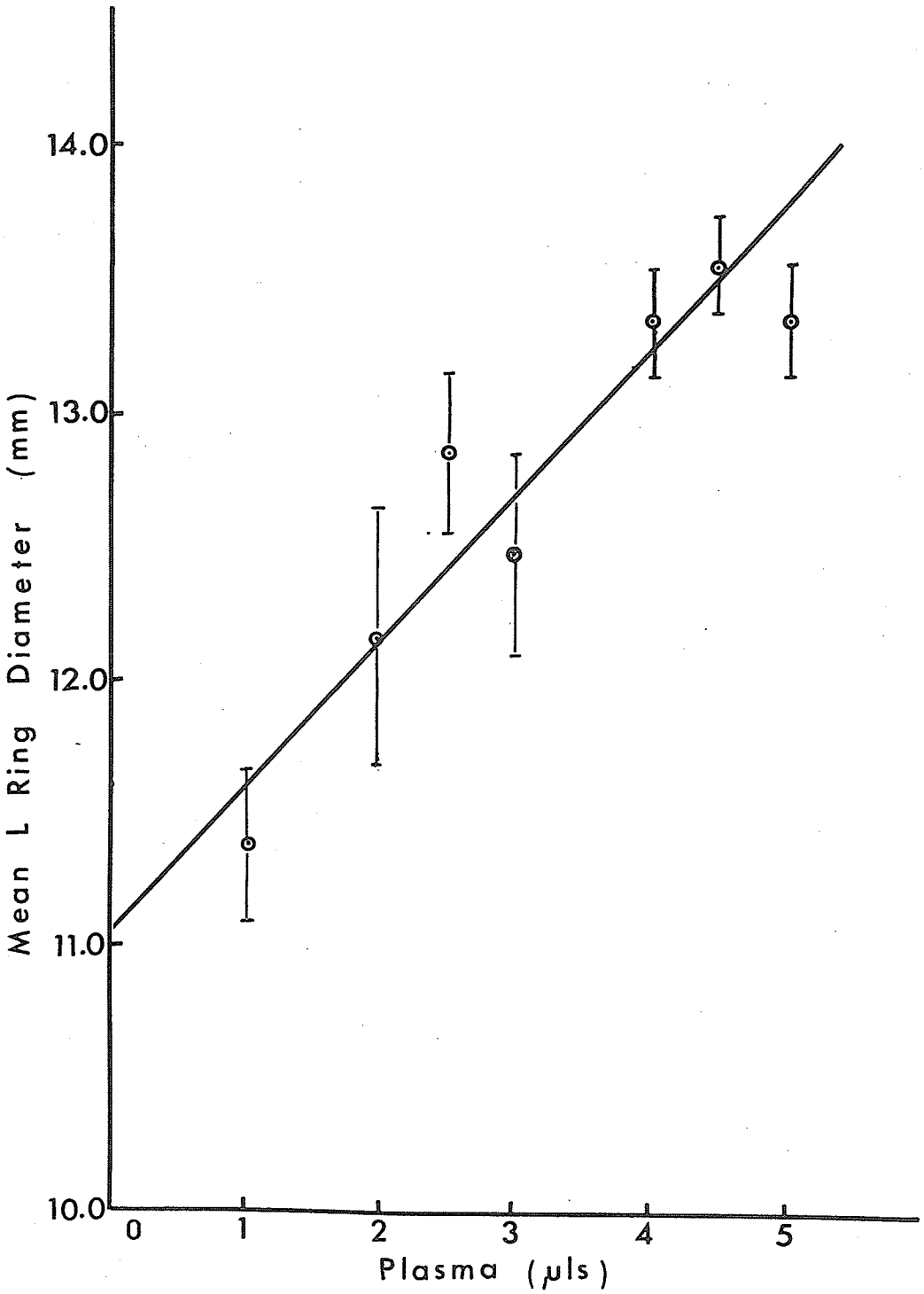


Figure 7. 7 S sensitized plates.

Trial I: L mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of µls of plasma diluted with DBS to a 5 µl total. Each mean L diameter was calculated from eight individual L diameters.

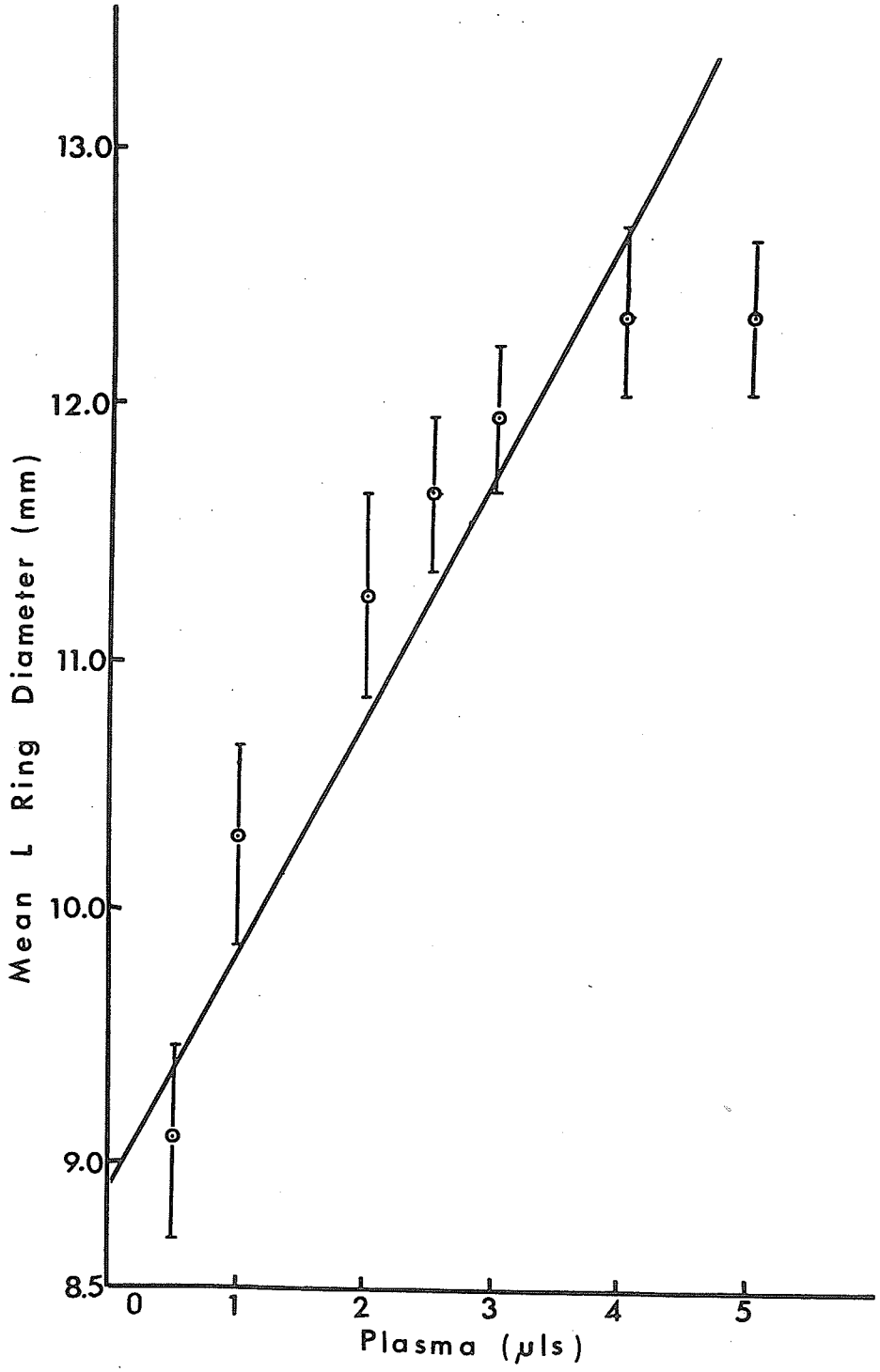


Figure 8. 7 S sensitized plates.

Trial II: L mean ring diameter ± S.E. vs plasma concentration. Plasma concentration was expressed as the number of uls of plasma diluted with DBS to a 5 ul total. Each mean L diameter was calculated from eight individual L diameters.

EFFECT OF COMPLEMENT CONCENTRATION ON RING DIAMETER

The object of this experiment was to determine how the complement dilution used to "develop" 1 HD_{50} whole sensitized plates affected the mean diameter of the P and L rings.

Procedure:

Plasma samples from four AKR/J female mice were obtained. Twenty plates sensitized with 1 HD_{50} whole hemolysin were prepared and each plate contained a sample of plasma from each of the four mice. Also, ten unsensitized plates were similarly prepared.

These plates were divided into ten, three plate sets each consisting of two sensitized plates and one unsensitized plate. Each three plate set was "developed" using a different complement dilution.

This experiment was done both when the mice were fourteen weeks old (trial 1) and when they were twenty-one weeks old (trial 2). Only in trial 2 were ten plate sets prepared to test ten complement dilutions. In trial 1, six plate sets were prepared to test six complement dilutions.

Results:

In Figure 9 and Figure 10, the mean P and mean L ring diameter are respectively plotted against complement dilution. Only trial 2 data were plotted. Figure 9 indicates that as complement dilution used to "develop" plates decreases so

does the mean P ring diameter. This is apparent only for plates "developed" with dilutions of complement less than 1/20. In the case of the L ring (Figure 10) data from both trials indicates there may or may not be a decrease in the L ring diameter as complement dilution decreases. Results are therefore inconclusive.

(See the Appendix 6 for trial 1 and 2 data, in Table V.)

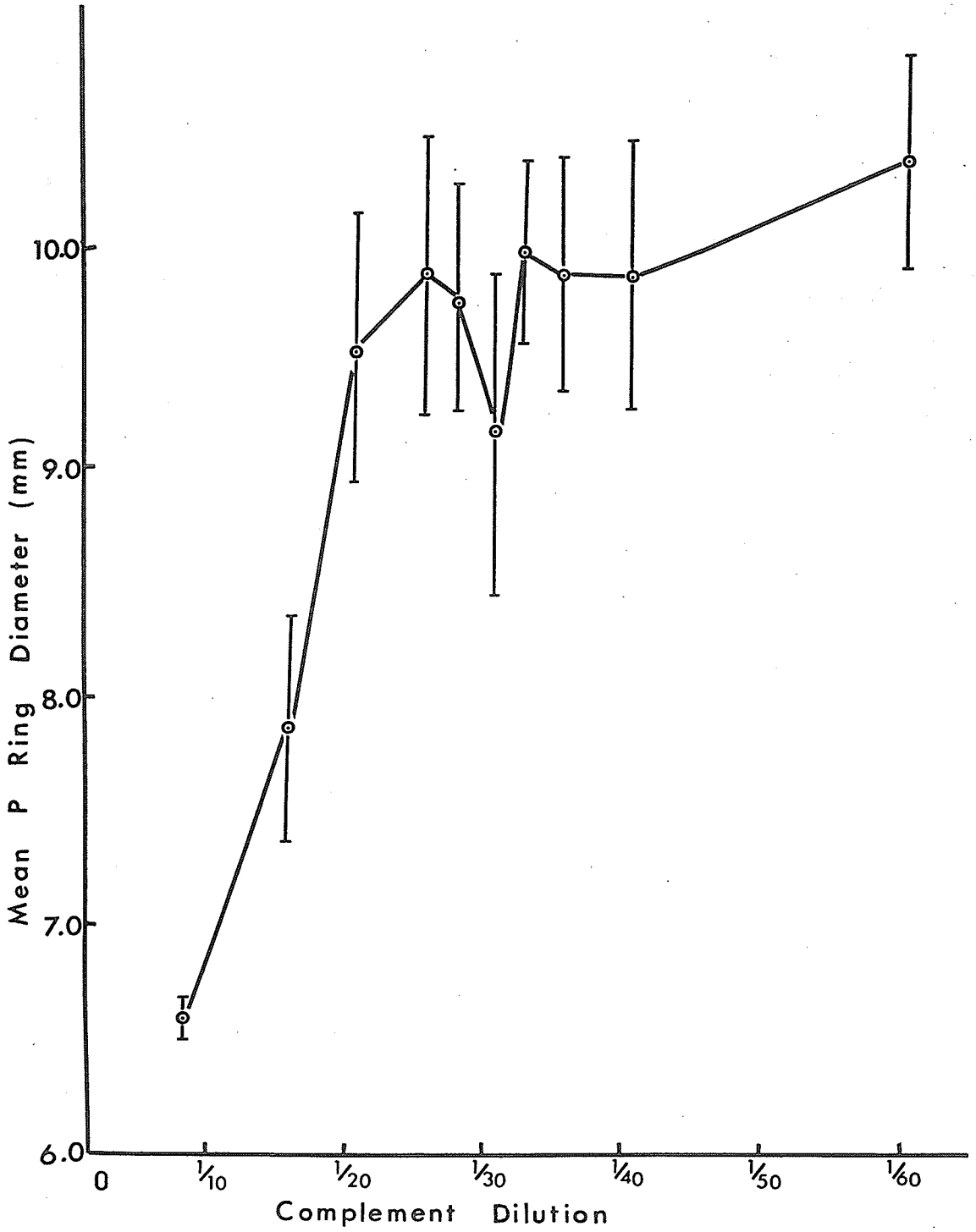


Figure 9. Mean P ring diameter vs complement dilution. Each mean P diameter was computed from six to eight observations.

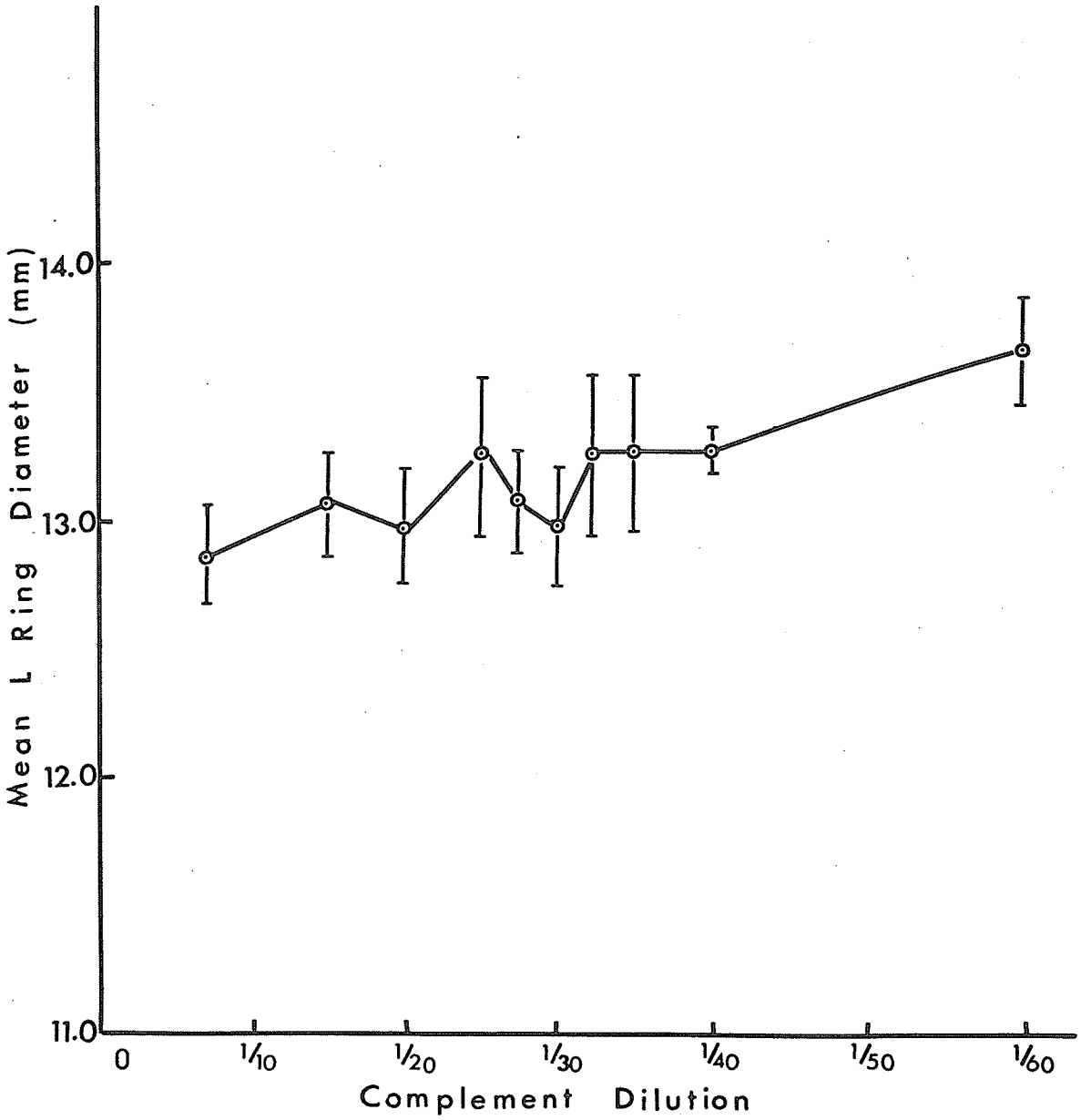


Figure 10. Mean L ring diameter vs complement dilution. Each mean L diameter was computed from six to eight observations.

EFFECT OF HEMOLYSIN DOSAGE ON RING DIAMETER

The object of this experiment was to determine how the dose of 7 S and whole hemolysin used to sensitize plates affected the mean diameter of the P and L rings.

Procedure:

A sample of plasma was obtained from each of four CFI females used in this study. Plasma from each mouse was diffused in two plates sensitized with whole hemolysin ranging in dosage from 1 to 4 HD_{50} and in two plates sensitized with 7 S hemolysin ranging in dosage from 0.5 to 2 HD_{50}.

Plasma from each mouse was also diffused in an unsensitized control plate.

This experiment was done twice. The CFI females were 41 ± 2 weeks old when trial 1 was done and were 68 ± 2 weeks old when trial 2 was done.

A certain optimum level of background lysis was necessary on the plate for the rings to show up on the film. Only a certain range of HD_{50}'s unique for each type of hemolysin gave the level of background lysis that enabled rings to show up on film. The range of HD_{50}'s tested for their effect on mean ring diameter was chosen with this in mind.

Results:

In Figures 11 to 14, the mean ring diameter of each ring type on each type of sensitized plate was plotted vs HD_{50}. In each case the mean ring diameter was calculated

on 8 individual ring diameters, since the four mice used each had samples of their plasma diffused in duplicate plates.

Referring to the trial 2 data plotted in Figures 11 to 14, the following conclusions were reached.

In the case of the P ring on whole sensitized plates (Figure 11), there is over a 1 millimeter decrease in the mean P ring diameter as the sensitization on the plate increases from 1 to 4 HD_{50}. The mean L ring diameter on both whole (Figure 12) and 7 S (Figure 14) sensitized plates exhibits no noticeable trend as hemolysin dosage is varied. The mean P ring diameter on 7 S plates (Figure 13) decreases with increase in hemolysin dosage.

All trends in date discussed above are reproducible in trial 1 and 2 of the experiment. The actual mean ring diameters of whole and 7 S sensitized plates in trial 1 and 2 are found in Tables VI and VII in the Appendix 7.

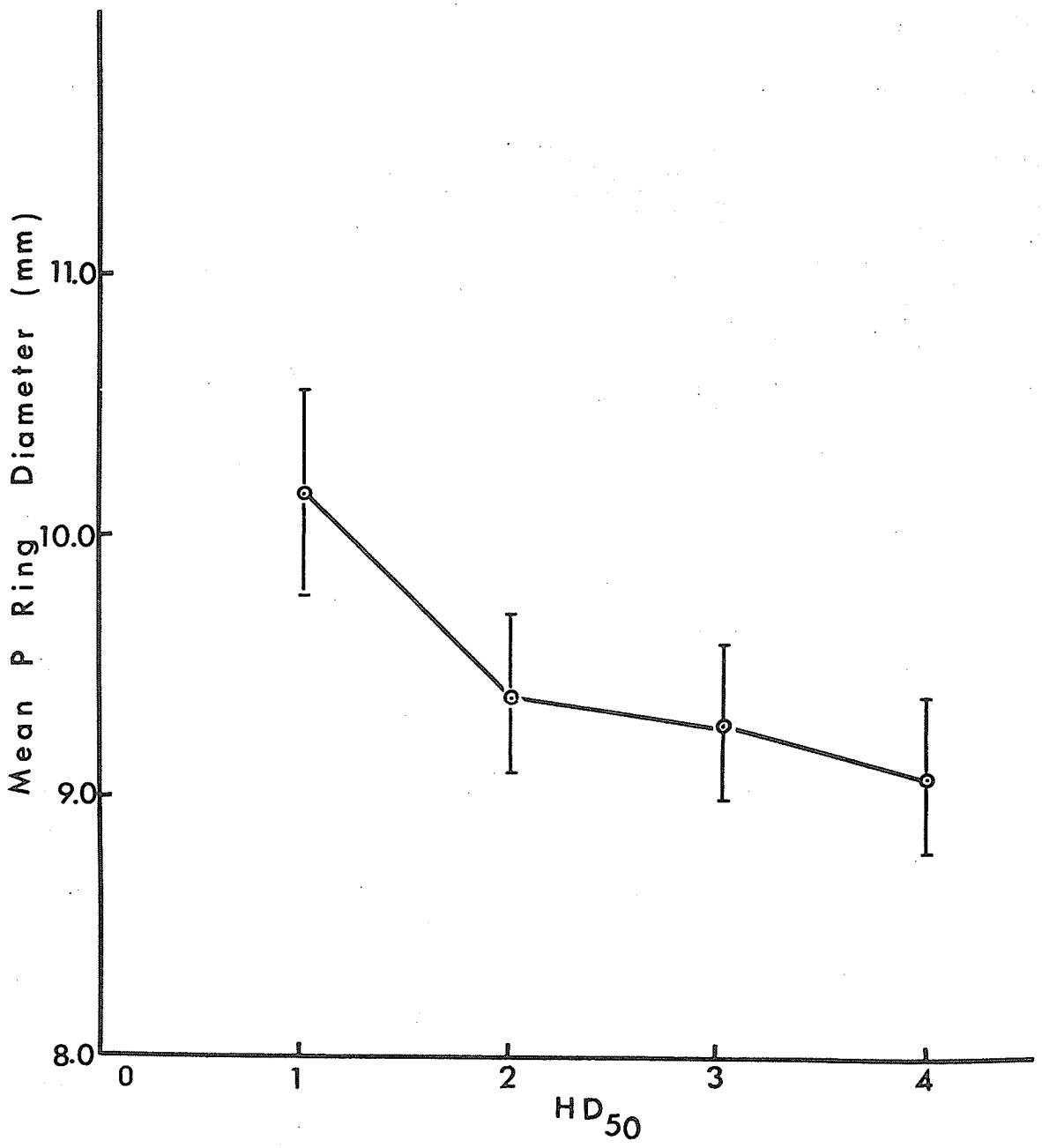


Figure 11. Mean P ring diameter vs HD_{50} of whole hemolysin. Each mean P diameter was calculated from eight individual P diameters.

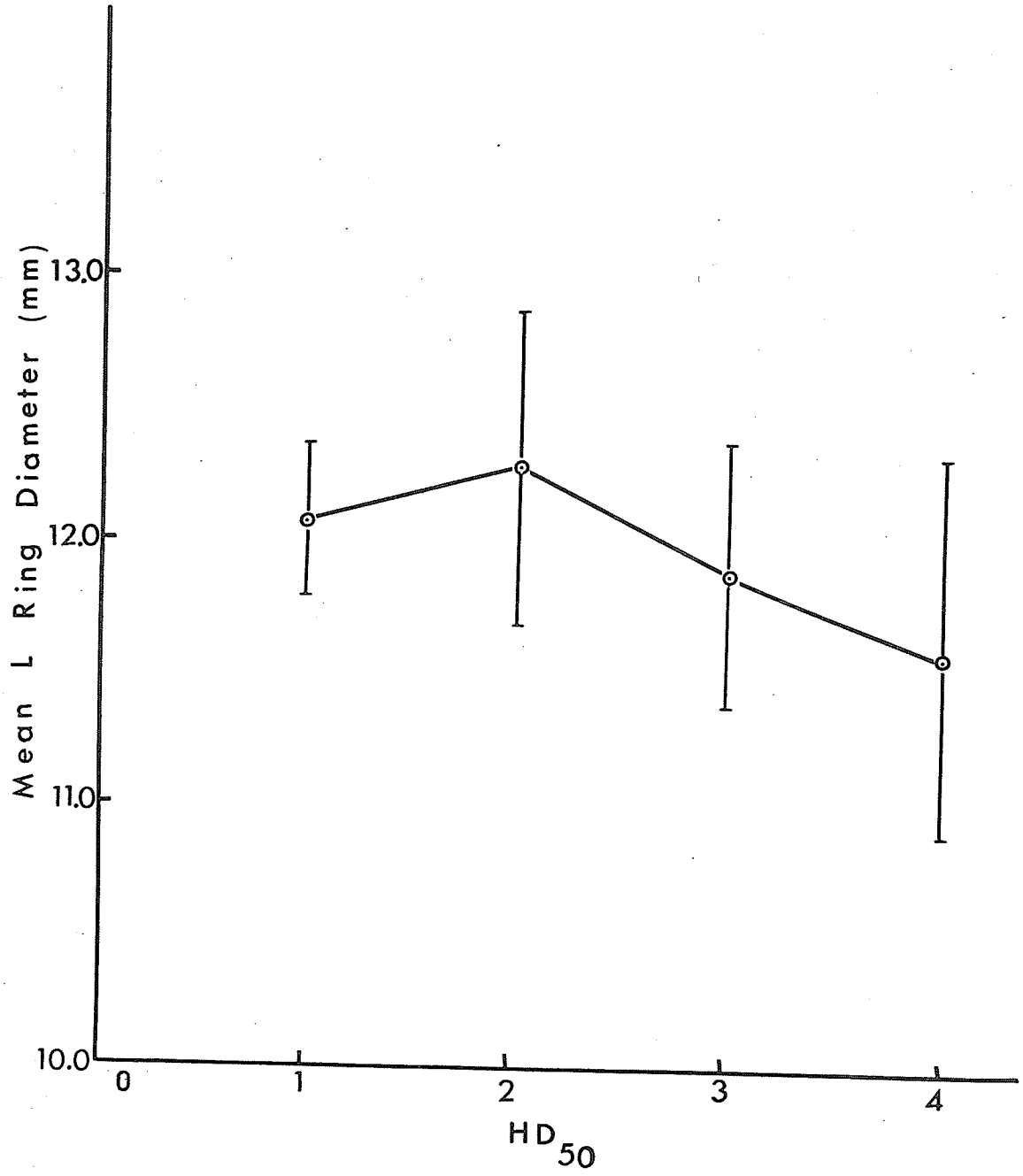


Figure 12. Mean L ring diameter vs HD_{50} of whole hemolysin. Each mean L diameter was calculated from eight individual L diameters.

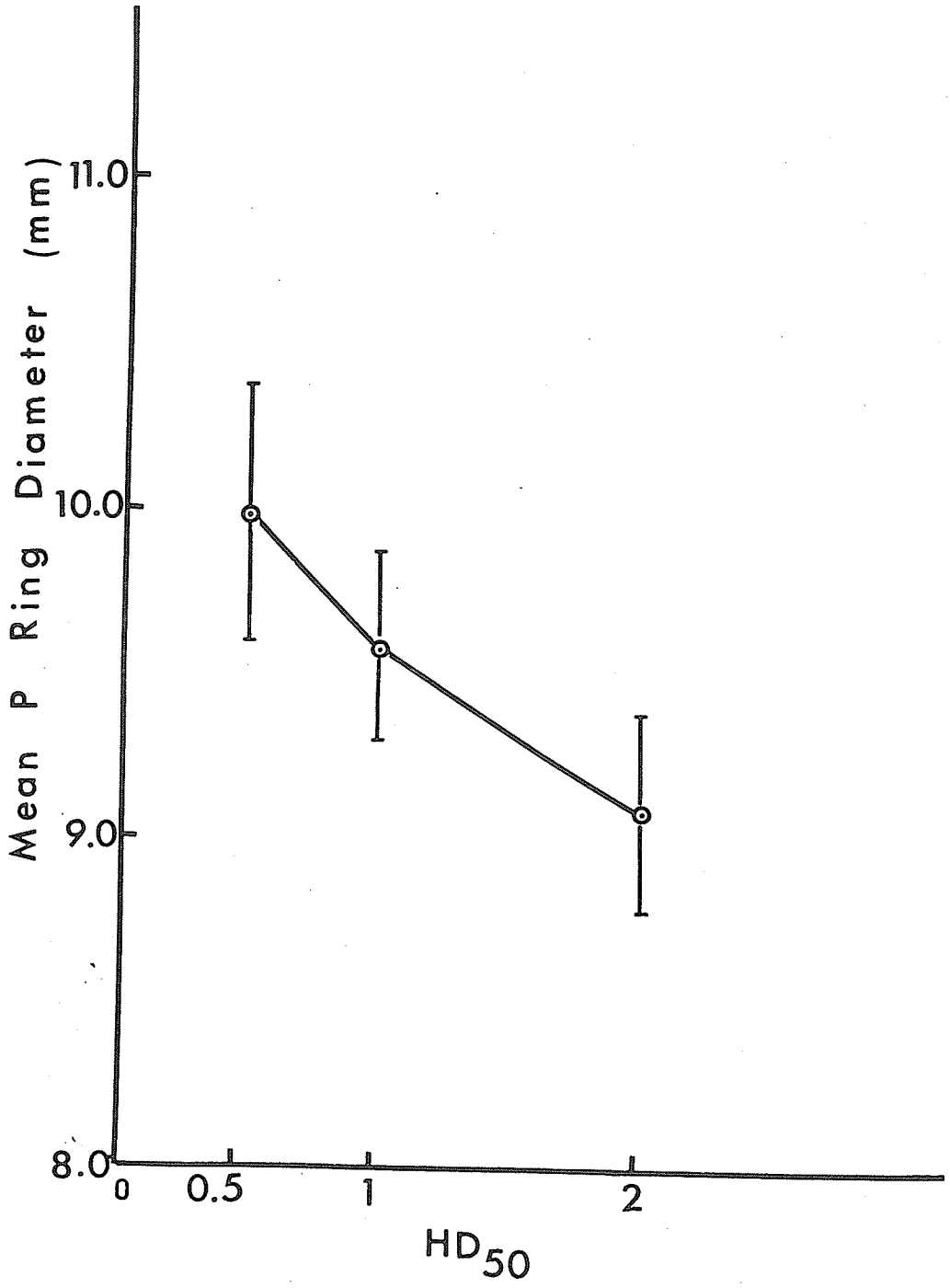


Figure 13. Mean P ring diameter vs HD_{50} of 7 S hemolysin. Each mean P diameter was calculated from eight individual P diameters.

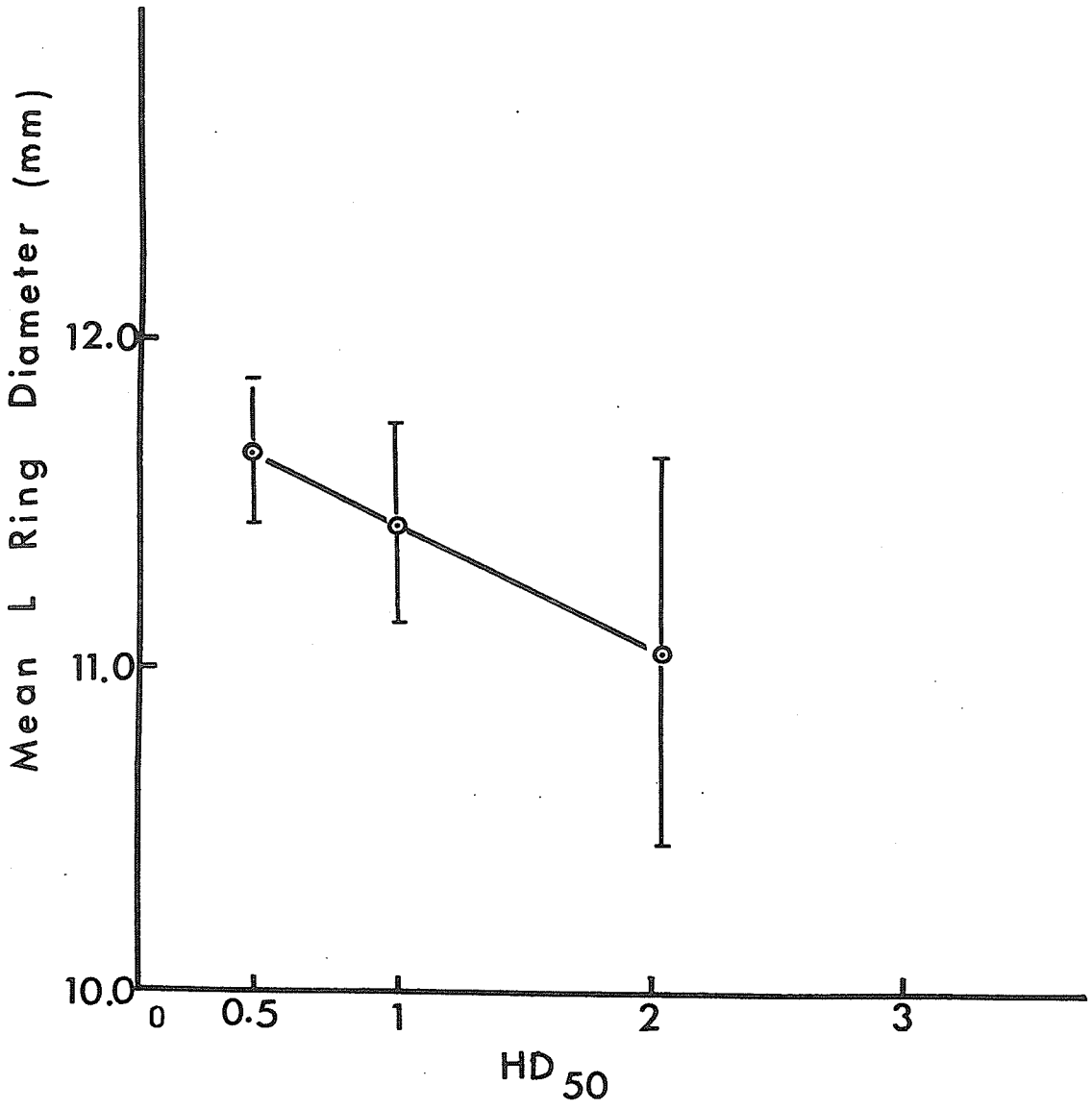


Figure 14. Mean L ring diameter vs HD_{50} of 7 S hemolysin. Each mean L diameter was calculated from eight individual L diameters.

OVERNIGHT PLASMA STORAGE

The object of this experiment was to see if overnight storage of plasma would significantly alter results. This was done by comparing the mean ring diameter of plasmas diffused immediately after being obtained from mice to the mean ring diameter of plasmas obtained from these same mice and kept twenty-four hours at 4°C before being diffused.

Procedure:

This experiment was divided into two days work.

On day one, seven male and seven female RF/J strain mice (9-11 weeks of age) were bled. Their plasmas were stored 24 hours at 4°C in spun down form*. On day two the same mice were bled.

Also on day two the plasma from each mouse obtained on day one and stored overnight was diffused on one, 1 HD_{50} whole sensitized plate and the plasma from the same mice obtained on day two was diffused on two 1 HD_{50} whole sensitized plates.

Both sets of plasmas were diffused on unsensitized control plates as well.

This experiment was repeated using seven C57BL/6J males and seven C47BL/6J females (10-12 weeks of age).

*spun down form refers to plasma separated from red blood cells in the heparinized capillary tube by centrifugation. The plasma is the supernatant on the red blood cell precipitate.

Results:

Comparisons were made between the mean ring diameter of day one plasmas kept overnight and fresh day two plasmas for each ring type. The ring types of each sex and strain were compared separately. Unpaired two sided t tests at a 95% confidence level were used for comparing the mean diameters. Whenever variances of the two groups to be compared were nonhomogeneous an unequal variance t test was used. The results in Table VIII show that in all t test comparisons there were no significant differences between the mean ring diameters of plasmas from the same mice diffused when fresh and after storage.

TABLE VIII

COMPARISON BETWEEN THE MEAN RING DIAMETER OF FRESH PLASMA AND PLASMA STORED OVERNIGHT

RING TYPE	STRAIN	SEX	MEAN RING DIAMETER OF OVERNIGHT* PLASMA (MM)	S.E.	MEAN RING DIAMETER OF DAY 2** PLASMA (MM)	S.E.	T TEST RESULT AT THE 95% CONFIDENCE LEVEL
P	RF/J	male	10.1	.3	9.9	.1	NO DIFFERENCE
		female	8.6	.4	8.5	.2	NO DIFFERENCE
	C57	male	9.9	.1	10.1	.1	NO DIFFERENCE
		female	8.7	.4	8.5	.1	NO DIFFERENCE
L	RF/J	male	13.1	.2	13.0	.6	NO DIFFERENCE
		female	13.1	.3	13.1	.1	NO DIFFERENCE
	C57	male	13.1	.1	13.4	.1	NO DIFFERENCE
		female	12.9	.1	13.0	.1	NO DIFFERENCE

* Each mean diameter for overnight plasma was calculated from 7 individual ring diameters.

** Each mean diameter for day two plasma was calculated from 14 individual ring diameters.

VARIABILITY IN PLATES PREPARED ON DIFFERENT DAYS

The object of this experiment was to determine whether plates prepared on different days caused fresh samples of a certain plasma to produce rings of significantly different diameter.

Procedure:

The experiment was divided into day one and day two parts.

On day one a plasma sample was obtained from seven RF/J male and seven RF/J female mice (9-11 weeks of age) and each plasma was diffused in two plates sensitized with 1 HD_{50} whole mouse anti-sheep red blood cell hemolysin and one unsensitized plate.

On day two plasma samples were obtained from the same mice and diffused as on day one.

For the plasmas each ring type on day one was compared to the equivalent ring type on day two by factorial analysis of variance at the 95% confidence level only in those cases with homogeneous variance. In the cases with nonhomogeneous variances an unequal variance t test at the 95% confidence level was used.

This experiment was repeated using seven C57BL/6J males and seven C57BL/6J females (age 10-12 weeks).

Results:

Results in Tables IX and X show that in five out of

eight cases there are significant differences in the ring diameters of samples of plasma from the same mice, diffused in plates prepared on different days. This strongly indicates that plates prepared on different days may or may not cause significant variability in the ring diameters of plasma samples from the same mice. It is concluded then that it is not a valid procedure to compare ring diameter results obtained on plates prepared on different days.

TABLE IX

COMPARISON OF RING DIAMETERS OF PLASMAS WHICH WERE EACH DIFFUSED ON PLATES MADE ON DIFFERENT DAYS

RING TYPE	SEX	STRAIN	FACTORIAL ANALYSIS RESULT AT THE 95% CONFIDENCE LEVEL
P	MALE	RF/J	SIGNIFICANT DIFFERENCE
		C57	NO SIGNIFICANT DIFFERENCE
	FEMALE	RF/J	SIGNIFICANT DIFFERENCE
L	MALE	C57	SIGNIFICANT DIFFERENCE
	FEMALE	RF/J	SIGNIFICANT DIFFERENCE
		C57	SIGNIFICANT DIFFERENCE

TABLE X

RING TYPE	SEX	STRAIN	UNEQUAL VARIANCE T TEST RESULT AT THE 95% CONFIDENCE LEVEL
P	FEMALE	C57	NO SIGNIFICANT DIFFERENCE
L	MALE	RF/J	NO SIGNIFICANT DIFFERENCE

VARIABILITY IN PLATES PREPARED ON THE SAME DAY

The object of this experiment was to determine whether different plates prepared at the same time caused a certain plasma to have significantly different ring diameters.

Procedure:

Seven RF/J males and seven RF/J females (9-11 weeks of age) each had a sample of plasma diffused in two different plates sensitized with 1 HD_{50} whole mouse anti-sheep red blood cell hemolysin. Each plasma was also diffused in one unsensitized plate.

For each plasma the diameters of each ring type (P and L) on the two plates were compared by factorial analysis of variance at the 95% confidence level only in those cases with homogeneous variance. In the cases with nonhomogeneous variance an unequal t test at the 95% confidence level was used.

This experiment was repeated using seven C57BL/6J males and seven C57BL/6J females (10-12 weeks of age).

In plate preparation a common lot of agar, 2.25 OD cell suspension and mouse hemolysin were used.

Results:

Results in Tables XI and XII show that in seven out of eight cases there are no significant differences in the ring diameters of plasmas diffused in two plates. This

strongly indicates that different plates produced at the same time do not cause significant variability in the ring diameter of a common plasma diffused in them. The one case in eight that showed a significant difference in the ring diameter of plasmas diffused in two different plates could mean that there is a statistical difference in terms of the statistical procedure employed rather than a true difference for although the confidence level for each one of the eight individual comparisons was 95%, the level at which we are confident that all eight comparisons are non-significant when considered as a unit is very much less than 95% (63%), so it could be expected that in studying eight comparisons at least one will deviate in results from the other seven.

TABLE XI

COMPARISON OF THE RING DIAMETERS OF PLASMAS DIFFUSED IN TWO DIFFERENT PLATES PREPARED AT THE SAME TIME

RING TYPE	SEX	STRAIN	FACTORIAL ANALYSIS RESULT AT THE 95% CONFIDENCE LEVEL
P	MALE	RF/J	NO SIGNIFICANT DIFFERENCE
		C57	NO SIGNIFICANT DIFFERENCE
	FEMALE	RF/J	NO SIGNIFICANT DIFFERENCE
L	MALE	C57	NO SIGNIFICANT DIFFERENCE
	FEMALE	RF/J	NO SIGNIFICANT DIFFERENCE
		C57	SIGNIFICANT DIFFERENCE

TABLE XII

RING TYPE	SEX	STRAIN	UNEQUAL VARIANCE T TEST RESULT AT THE 95% CONFIDENCE LEVEL
P	FEMALE	C57	NO SIGNIFICANT DIFFERENCE
L	MALE	RF/J	NO SIGNIFICANT DIFFERENCE

THE EFFECT OF WARMING ON MICE

The mice used in all experiments described in this thesis were warmed for up to five minutes to facilitate collection of blood (see Materials and Methods).

Warming the mouse may have been a stress factor which could have changed the plasma in some way resulting in a significantly different ring diameter than the one obtained from a plasma sample taken from the same mouse without warming. This experiment was done to determine whether or not this was so.

Procedure:

Plasma was obtained from six CFI female mice (seven months old) that were warmed for five minutes. Four hours later plasma was obtained from the same six CFI without prior warming. Each plasma, in both sets of plasmas (warmed and unwarmed), was diffused in one 1 HD_{50} whole sensitized plate, one 1 HD_{50} 7 S sensitized plate and one unsensitized plate.

Results:

The ring diameter of plasma obtained with warming, from a mouse, was compared with the ring diameter of plasma obtained without warming from the same mouse. The following separate comparisons were made using factorial analysis of variance at a 95% confidence level.

(i) P ring on whole sensitized plates.

(ii) L ring on whole sensitized plates.

(iii) P ring on 7 S sensitized plates.

(iv) L ring on 7 S sensitized plates.

This experiment was done twice with the same mice and the data from each of the experiments was combined appropriately for each of the above comparisons.

The results in Table XIII show that in all comparisons of the factorial analysis that there was no significant difference in the ring diameter of plasma obtained from each mouse with and without warming at the 95% confidence level.

TABLE XIII

COMPARISON OF RING DIAMETER OF PLASMA OBTAINED FROM A MOUSE WITH AND WITHOUT WARMING

TYPE OF SENSITIZED PLATE	RING TYPE	FACTORIAL ANALYSIS RESULT AT THE 95% CONFIDENCE LEVEL
WHOLE	P	NO DIFFERENCE
	L	NO DIFFERENCE
7 S	P	NO DIFFERENCE
	L	NO DIFFERENCE

RING DIAMETER COMPARISON BETWEEN STRAINS

The strains listed in Table I had their ring diameters and $^{L}/_{P}$ ratios compared. Table XIV lists the sex and condition to which the strains used in the comparisons were predisposed to.

The number of mice for each sex of each strain listed in Table XIV was seven.

Each mouse in the Table had a sample of plasma diffused in:

(i) Two plates sensitized with 1 HD_{50} whole hemolysin.

(ii) Two plates sensitized with 0.5 HD_{50} 7S hemolysin.

(iii) One plate unsensitized.

The mice were at the following mean ages during the three trials of the experiment.

Trial I - 15 $\pm$ 2 weeks

Trial II - 22 $\pm$ 2 weeks

Trial III - 30 $\pm$ 2 weeks

Analysis of Data:

I. Comparison of the Mean Ring Diameters of the Different Strains

Ring diameter comparisons between strains were done separately for each ring type on each type of sensitized plate. Only different strains of the same sex were compared.

TABLE XIV

STRAINS WHOSE RING DIAMETERS WERE COMPARED

STRAIN	SEX	CONDITION PREDISPOSED TO
AKR/J	Male Female	leukemia
RF/J	Male Female	leukemia
C58/J	Male	leukemia
NZB/BINJ	Male	hemolytic anemia
SM/J	Male Female	amyloidosis
CFI	Male Female	NONE
C57BL/6J	Male Female	NONE

Comparison Between Strains Of:

(i) The P ring on whole sensitized plates for males of the different strains.

(ii) The L ring on whole sensitized plates for males of the different strains.

(iii) The P ring on 7 S sensitized plates for males of the different strains.

(iv) The L ring on 7 S sensitized plates for males of the different strains.

(v) The P ring on whole sensitized plates for females of the different strains.

(vi) The L ring on whole sensitized plates for females of the different strains.

(vii) The P ring on 7 S sensitized plates for females of the different strains.

(viii) The L ring on 7 S sensitized plates for females of the different strains.

Each of the above statistical comparisons consisted of three parts:

(i) Bartletts Test for homogeneity of variance.

This test was used to insure that the variances of the ring diameters of the strains that were compared were homogeneous at a confidence level of 95%. (Homogeneity of variance is a prerequisite for one way analysis of variance.)

(ii) One way analysis of variance (ANOVA).

This analysis compared the mean ring diameters of the different strains and determined whether any of the strains were significantly different from each other.

(iii) Tukeys Multiple Range Test.

This test determined specifically which strains had significantly different mean ring diameters.

II. Comparison of the Different Strains $^{L}/_{P}$ Ratios

The mean $^{L}/_{P}$ ring diameter ratio of each of the strains was computed and a comparison of these ratios between strains was made.

Comparison Between Strains Of:

(i) The $^{L}/_{P}$ ratios on whole sensitized plates for the males of the different strains.

(ii) The $^{L}/_{P}$ ratios on whole sensitized plates for the females of the different strains.

(iii) The $^{L}/_{P}$ ratios on 7 S sensitized plates for the males of the different strains.

(iv) The $^{L}/_{P}$ ratios on 7 S sensitized plates for the females of the different strains.

Each of the above statistical comparisons consisted of the same three procedures used in part I to compare mean ring diameters between strains.

III. Comparison of the Mean Ring Diameters Between the Sexes

Comparisons of the mean ring diameters between the

sexes of each strain were carried out, with separate comparisons for each ring type on each type of sensitized plate.

Comparisons Between the Sexes of:

(i) The P ring on whole sensitized plates for the different strains.

(ii) The L ring on whole sensitized plates for the different strains.

(iii) The P ring on 7 S sensitized plates for the different strains.

(iv) The L ring on 7 S sensitized plates for the different strains.

Each of the above statistical comparisons consisted of three parts.

(i) Bartletts Test for homogeneity of variance.

(ii) Factorial Analysis.

(iii) Tukeys Multiple Range Test.

IV. Comparison Between the Sexes of the $^{L}/_{P}$ Ratio

The mean $^{L}/_{P}$ ring diameter ratio for each sex of each strain was computed and a comparison between the sexes of each strain was made.

Comparisons Between the Sexes of:

(i) The $^{L}/_{P}$ ratio on whole sensitized plates for the different strains.

(ii) The $^{L}/_{P}$ ratio on 7 S sensitized plates for the different strains.

Each of the above statistical comparisons consisted of the same three procedures used in part III to compare mean ring diameters between strains.

DATA: MEAN RING DIAMETERS

Mean ring diameter and standard error in millimeters is listed for each strain on whole sensitized plates and for each strain on 7 S sensitized plates. There were three trials of this experiment, each with a separate set of data.

TRIAL I: MEAN RING DIAMETERS AND S.E. (mm)

I. WHOLE SENSITIZED PLATES

TABLE XV

MEAN P RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	9.8±.2	10.0±.1	10.9±.3	10.6±.2	10.9±.1	10.4±.2	N.D.
FEMALES	7.6±.2	7.3±.2	7.1±.1	N.D.	N.D.	N.D.	7.3±.1

TABLE XVI

MEAN L RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	13.5±.2	13.2±.1	13.8±.1	13.0±.4	14.0±.1	13.5±.1	N.D.
FEMALES	14.0±.1	13.5±.1	13.7±.1	N.D.	N.D.	N.D.	13.6±.1

TABLE XVII

MEAN $^{L}/_{P}$ RING DIAMETER RATIOS AND STANDARD ERROR

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	1.39±.04	1.32±.02	1.27±.02	1.23±.02	1.28±.02	1.33±.04	N.D.
FEMALES	1.85±.05	1.85±.05	1.94±.04	N.D.	N.D.	N.D.	1.90±.04

N.D. - Strain not done.
Each mean is based on 14 individual ring measurements.

TRIAL I:

II. 7 S SENSITIZED PLATES

TABLE XVIII

MEAN P RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	8.8±.2	9.6±.2	9.5±.3	10.1±.3	10.2±.3	9.6±.4	N.D.
FEMALES	6.5±.1	6.7±.3	6.4±.1	N.D.	N.D.	N.D.	6.3±.1

TABLE XIX

MEAN L RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	13.0±.2	12.9±.1	13.6±.2	13.1±.1	13.6±.1	13.3±.1	N.D.
FEMALES	13.4±.1	13.0±.2	13.6±.1	N.D.	N.D.	N.D.	13.2±.1

TABLE XX

MEAN $^{L}/_{P}$ RING DIAMETER RATIOS AND STANDARD ERROR

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	1.48±.04	1.36±.03	1.45±.06	1.31±.03	1.35±.04	1.43±.06	N.D.
FEMALES	2.06±.03	1.97±.07	2.12±.03	N.D.	N.D.	N.D.	2.12±.03

N.D. - Strain not done.
Each mean is based on 14 individual ring measurements.

TRIAL II: MEAN RING DIAMETERS AND S.E. (mm)

I. WHOLE SENSITIZED PLATES

TABLE XXI

MEAN P RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	10.2±.3	10.1±.2	10.2±.6	11.1±.1	11.3±.2	10.5±.1	10.8±.3
FEMALES	7.4±.2	8.1±.4	8.0±.4	N.D.	N.D.	7.8±.2	7.9±.4

TABLE XXII

MEAN L RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	12.8±.2	12.5±.2	13.5±.1	12.8±.1	13.3±.1	13.3±.1	12.7±.2
FEMALES	13.2±.2	12.6±.1	12.8±.2	N.D.	N.D.	13.5±.1	12.7±.1

TABLE XXIII

MEAN $^{L}/_{P}$ RING DIAMETER RATIOS AND STANDARD ERROR

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	1.26±.04	1.28±.05	1.38±.09	1.15±.01	1.19±.02	1.26±.02	1.17±.02
FEMALES	1.79±.06	1.59±.06	1.65±.08	N.D.	N.D.	1.75±.05	1.67±.09

N.D. - Strain not done.
Each mean is based on 14 individual ring measurements.

TRIAL II:

II. 7 S SENSITIZED PLATES

TABLE XXIV

MEAN P RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	8.8±.3	9.4±.2	10.7±.3	10.5±.2	10.4±.3	10.5±.2	10.3±.2
FEMALES	6.6±.1	6.7±.1	6.6±.1	N.D.	N.D.	6.8±.1	7.3±.4

TABLE XXV

MEAN L RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	12.5±.2	11.7±.1	13.0±.1	12.4±.2	12.7±.1	12.7±.1	12.2±.1
FEMALES	12.4±.3	12.1±.1	12.4±.2	N.D.	N.D.	13.1±.1	11.9±.1

TABLE XXVI

MEAN $^{L}/_{P}$ RING DIAMETER RATIOS AND STANDARD ERROR

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	1.44±.06	1.25±.03	1.21±.03	1.18±.02	1.24±.06	1.21±.02	1.18±.03
FEMALES	1.90±.04	1.82±.03	1.87±.03	N.D.	N.D.	1.93±.04	1.68±.08

N.D. - Strain not done.
Each mean is based on 14 individual ring measurements.

TRIAL III: MEAN RING DIAMETERS AND S.E. (mm)

I. WHOLE SENSITIZED PLATES

TABLE XXVII

MEAN P RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	10.3±.2	10.3±.1	11.5±.2	11.2±.1	11.7±.1	11.3±.2	10.5±.1
FEMALES	9.4±.2	9.1±.2	9.8±.2	N.D.	N.D.	9.5±.2	9.4±.3

TABLE XXVIII

MEAN L RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	12.9±.1	12.5±.1	13.6±.2	13.3±.2	14.0±.2	14.0±.1	12.7±.2
FEMALES	13.1±.2	12.1±.1	12.1±.2	N.D.	N.D.	13.8±.2	12.5±.3

TABLE XXIX

MEAN $^{L}/_{P}$ RING DIAMETER RATIOS AND STANDARD ERROR

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	1.25±.02	1.22±.02	1.19±.02	1.21±.02	1.19±.01	1.24±.02	1.25±.04
FEMALES	1.41±.03	1.34±.04	1.25±.02	N.D.	N.D.	1.46±.04	1.34±.05

N.D. - Strain not done.

Each mean is based on 14 individual ring measurements.

TRIAL III:

II. 7 S SENSITIZED PLATES

TABLE XXX

MEAN P RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	10.2±.2	10.1±.1	11.4±.1	11.1±.1	11.4±.2	10.8±.1	10.2±.2
FEMALES	9.2±.3	9.3±.1	9.8±.2	N.D.	N.D.	9.2±.2	8.4±.2

TABLE XXXI

MEAN L RING DIAMETERS AND STANDARD ERROR (mm)

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	12.8±.2	12.4±.1	13.4±.1	13.1±.1	13.6±.1	13.5±.1	12.5±.2
FEMALES	13.1±.1	11.8±.1	11.9±.2	N.D.	N.D.	13.5±.2	12.1±.2

TABLE XXXII

MEAN $^{L}/_{P}$ RING DIAMETER RATIOS AND STANDARD ERROR

STRAINS	CFI	C57BL/6J	RF/J	NZB/BINJ	C58/J	AKR/J	SM/J
MALES	1.26±.02	1.22±.01	1.18±.01	1.18±.01	1.19±.01	1.24±.02	1.23±.02
FEMALES	1.44±.04	1.27±.02	1.22±.02	N.D.	N.D.	1.47±.04	1.46±.04

N.D. - Strain not done.
Each mean is based on 14 individual ring measurements.

TABLE XXXIII

MEAN RING DIAMETER COMPARISON BETWEEN STRAINS
ONE WAY ANALYSIS OF VARIANCE (ANOVA) (95% CONFIDENCE LEVEL)

I. WHOLE SENSITIZED PLATES

		TRIAL I (15 wks of age)		TRIAL II (22 wks of age)		TRIAL III (30 wks of age)	
RING	SEX	STRAINS COMPARED	ANOVA RESULTS	STRAINS COMPARED	ANOVA RESULTS	STRAINS COMPARED	ANOVA RESULTS
P	MALE	CFI RF/J NZB/BINJ C58/J AKR/J	STRAINS ARE DIFFERENT	C57BL/6J NZB/BINJ C58/J AKR/J SM/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J NZB/BINJ C58/J AKR/J SM/J	STRAINS ARE DIFFERENT
L	MALE	CFI RF/J C58/J AKR/J	STRAINS ARE THE SAME	C57BL/6J RF/J NZB/BINJ C58/J AKR/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J SM/J NZB/BINJ C58/J	STRAINS ARE DIFFERENT
L/P	MALE	C57BL/6J NZB/BINJ C58/J AKR/J	STRAINS ARE DIFFERENT	C58/J AKR/J SM/J	STRAINS ARE DIFFERENT	CFI C57BL/6J NZB/BINJ RF/J C58/J AKR/J	STRAINS ARE THE SAME
P	FEMALE	CFI C57BL/6J RF/J SM/J	STRAINS ARE THE SAME	CFI C57BL/6J RF/J SM/J	STRAINS ARE THE SAME	CFI C57BL/6J RF/J AKR/J SM/J	STRAINS ARE THE SAME
L	FEMALE	CFI C57BL/6J RF/J SM/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J SM/J AKR/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J AKR/J SM/J	STRAINS ARE DIFFERENT
L/P	FEMALE	CFI C57BL/6J RF/J SM/J	STRAINS ARE THE SAME	CFI C57BL/6J RF/J SM/J AKR/J	STRAINS ARE THE SAME	CFI C57BL/6J RF/J AKR/J SM/J	STRAINS ARE DIFFERENT

TABLE XXXIV

MEAN RING DIAMETER COMPARISON BETWEEN STRAINS
ONE WAY ANALYSIS OF VARIANCE (ANOVA) (95% CONFIDENCE LEVEL)

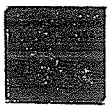
II. 7 S SENSITIZED PLATES

		TRIAL I (15 wks of age)		TRIAL II (22 wks of age)		TRIAL III (30 wks of age)	
RING	SEX	STRAINS COMPARED	ANOVA RESULTS	STRAINS COMPARED	ANOVA RESULTS	STRAINS COMPARED	ANOVA RESULTS
P	MALE	C57BL/6J RF/J NZB/BINJ C58/J AKR/J	STRAINS ARE THE SAME	CFI C57BL/6J RF/J NZB/BINJ C58/J AKR/J SM/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J NZB/BINJ C58/J AKR/J SM/J	STRAINS ARE DIFFERENT
L	MALE	CFI C57BL/6J RF/J NZB/BINJ C58/J AKR/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J NZB/BINJ C57/J SM/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J NZB/BINJ C58/J AKR/J SM/J	STRAINS ARE DIFFERENT
L/P	MALE	CFI C57BL/6J RF/J NZB/BINJ C58/J AKR/J	STRAINS ARE THE SAME	C57BL/6J RF/J NZB/BINJ AKR/J SM/J	STRAINS ARE THE SAME	CFI C57BL/6J C58/J AKR/J SM/J	STRAINS ARE THE SAME
P	FEMALE	CFI RF/J SM/J	STRAINS ARE THE SAME	CFI C57BL/6J AKR/J RF/J	STRAINS ARE THE SAME	CFI RF/J AKR/J SM/J	STRAINS ARE DIFFERENT
L	FEMALE	CFI C57BL/6J RF/J SM/J	STRAINS ARE DIFFERENT	C57BL/6J SM/J AKR/J	STRAINS ARE DIFFERENT	CFI C57BL/6J RF/J AKR/J SM/J	STRAINS ARE DIFFERENT
L/P	FEMALE	SM/J CFI RF/J	STRAINS ARE THE SAME	CFI C57BL/6J RF/J AKR/J	STRAINS ARE THE SAME	CFI RF/J AKR/J SM/J	STRAINS ARE DIFFERENT

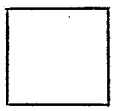
TUKEYS MULTIPLE RANGE TEST

Tukeys multiple range test was carried out in each of the cases after ANOVA at the 95% confidence level to determine which strains had significantly different mean ring diameters and mean $^{L}/_{P}$ ratios.

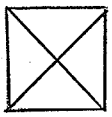
CODING FOR SUBSEQUENT RESULT TABLES



Tukeys test at the 95% confidence level indicated a significant difference in mean ring diameter or mean $^{L}/_{P}$ ratio of the two strains compared.



Tukeys test at the 95% confidence level indicated NO significant difference in mean ring diameter or mean $^{L}/_{P}$ ratio of the two strains compared.



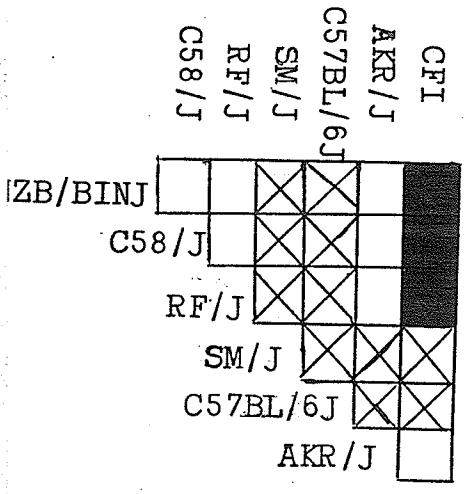
Not analysed due to non-homogeneity of variance as determined by Bartletts test at the 95% confidence level.

FIGURE 15: TUKEYS TEST AT A 95% CONFIDENCE LEVEL

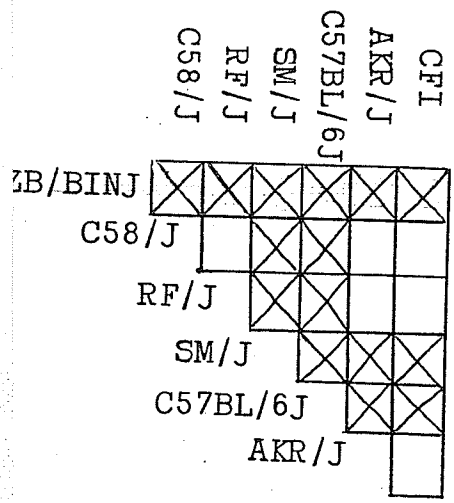
I. WHOLE SENSITIZED PLATES

TRIAL I

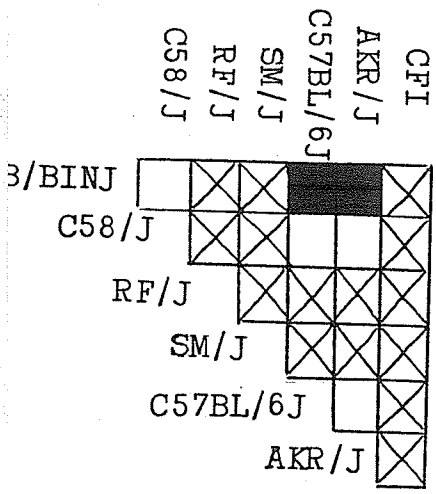
MALE P RING



MALE L RING

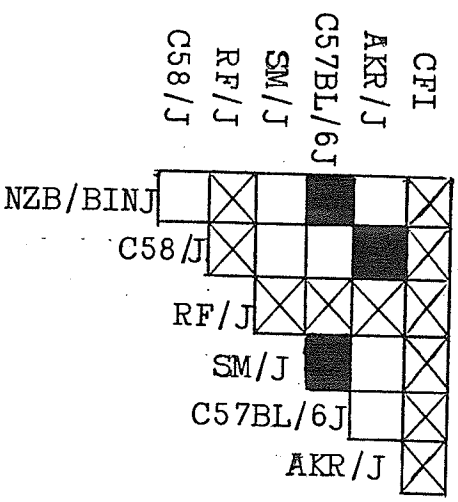


MALE L/P

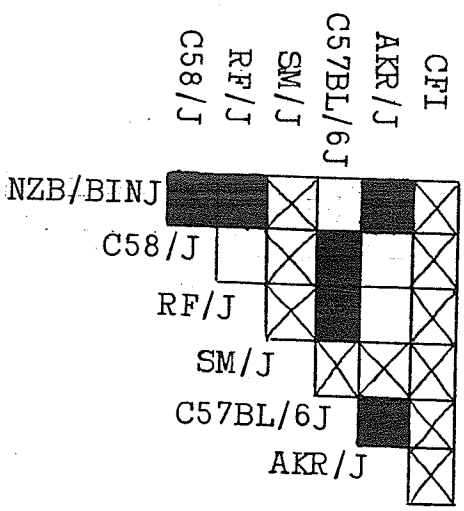


TRIAL II

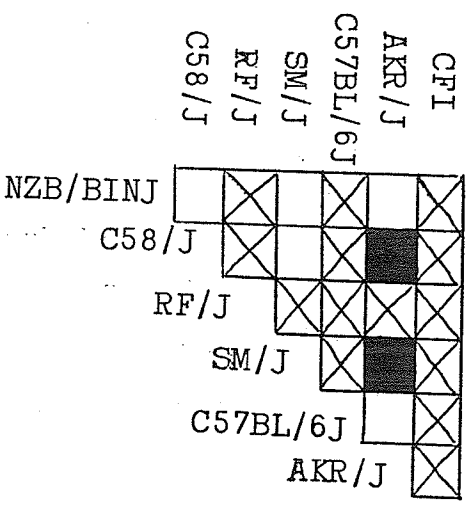
MALE P RING



MALE L RING

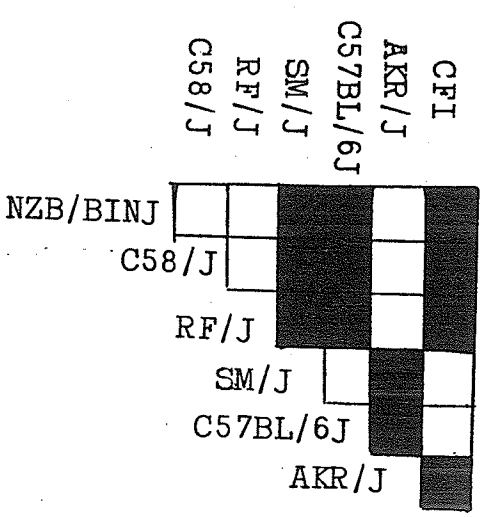


MALE L/P

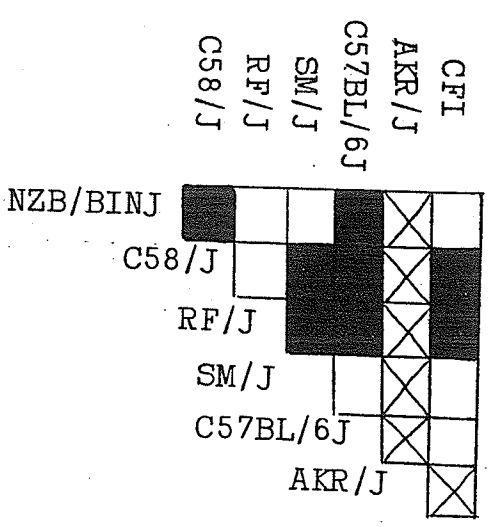


TRIAL III

MALE P RING



MALE L RING



MALE L/P

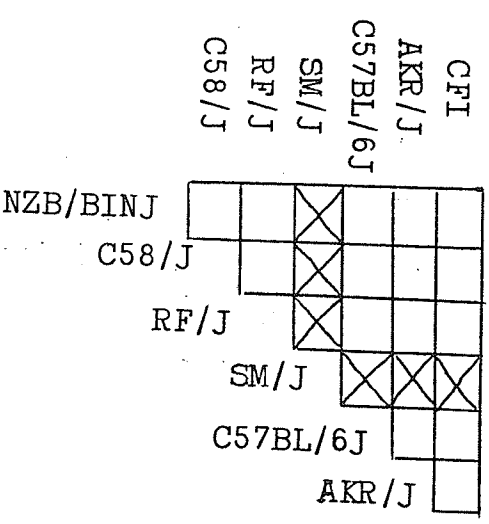
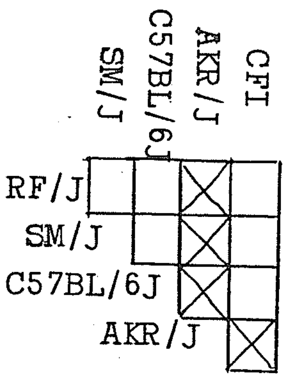


FIGURE 16: TUKEYS TEST AT A 95% CONFIDENCE LEVEL

II. WHOLE SENSITIZED PLATES

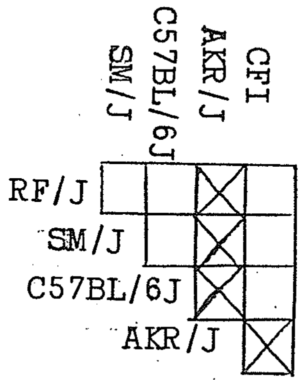
TRIAL I

FEMALE P RING



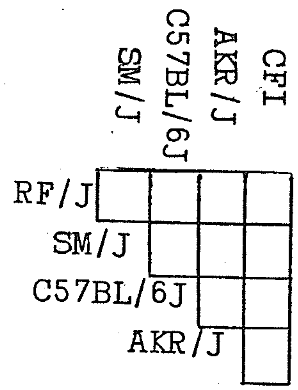
TRIAL II

FEMALE P RING

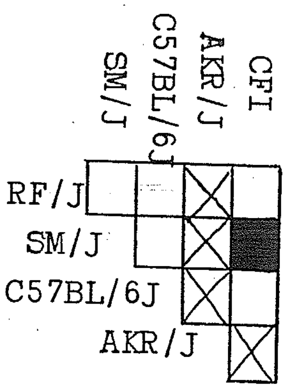


TRIAL III

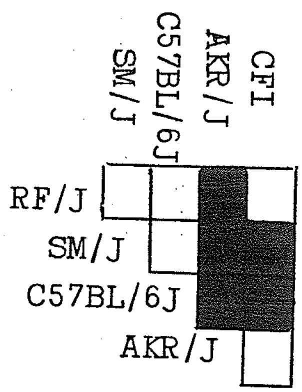
FEMALE P RING



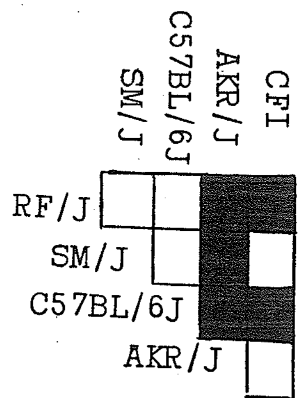
FEMALE L RING



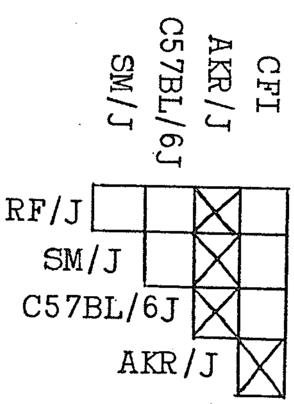
FEMALE L RING



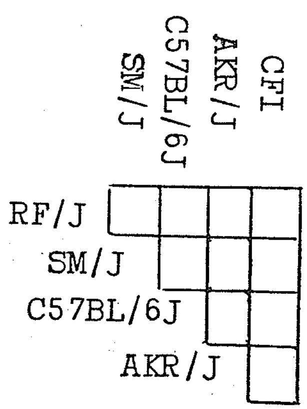
FEMALE L RING



FEMALE L/P



FEMALE L/P



FEMALE L/P

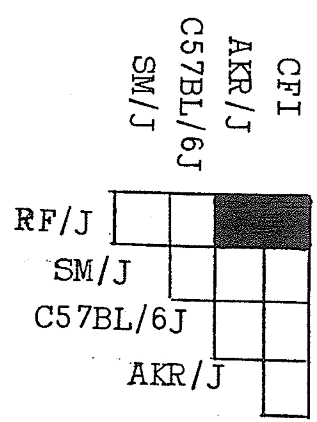
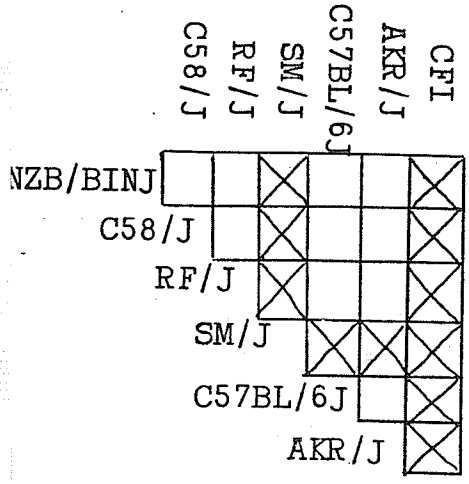
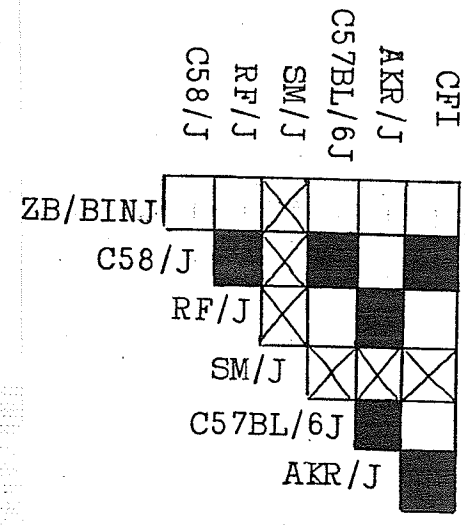


FIGURE 17: TUKEYS TEST AT A 95% CONFIDENCE LEVEL
I. 7 S SENSITIZED PLATES

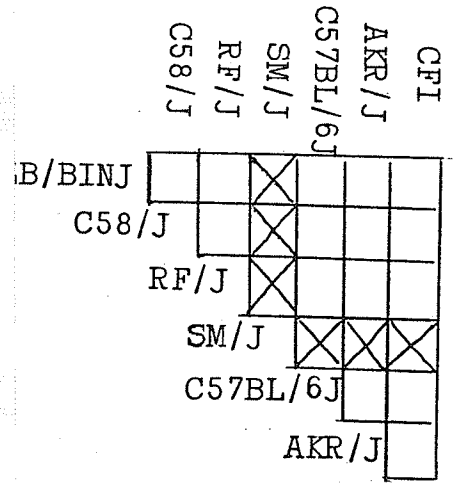
TRIAL I
MALE P RING



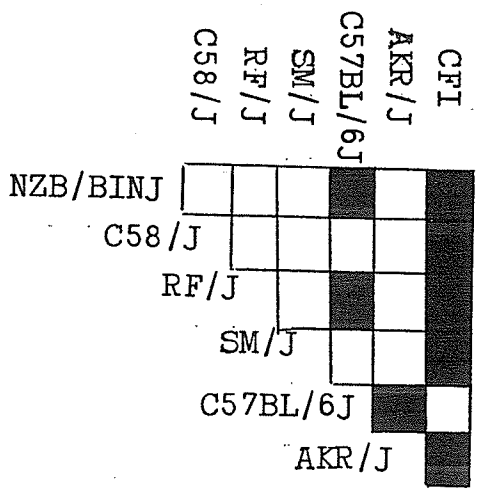
MALE L RING



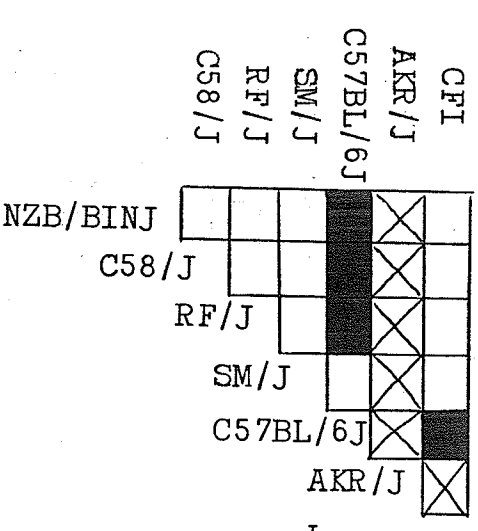
MALE L/P



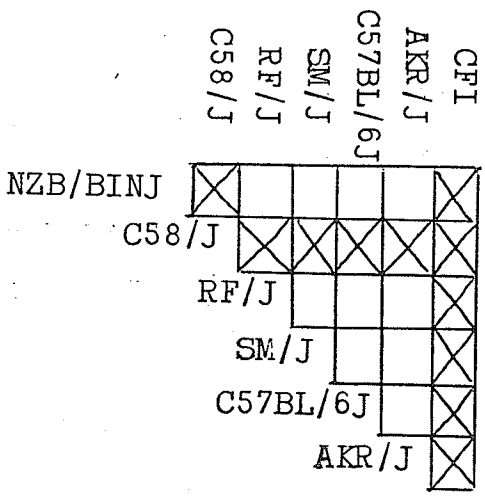
TRIAL II
MALE P RING



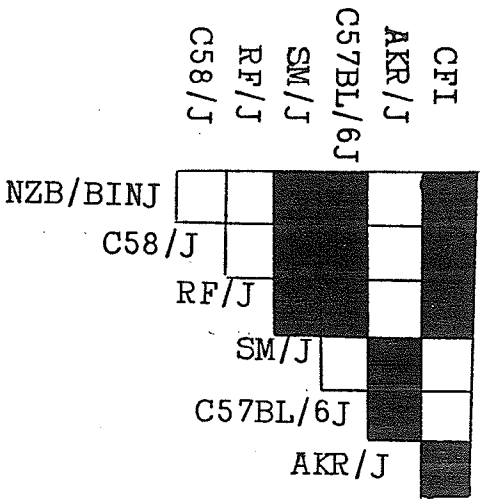
MALE L RING



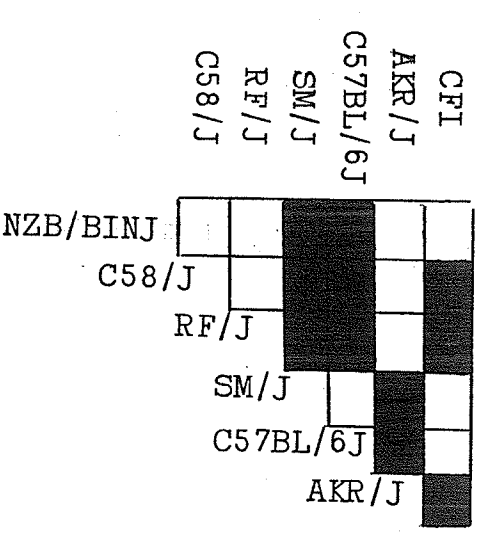
MALE L/P



TRIAL III
MALE P RING



MALE L RING



MALE L/P

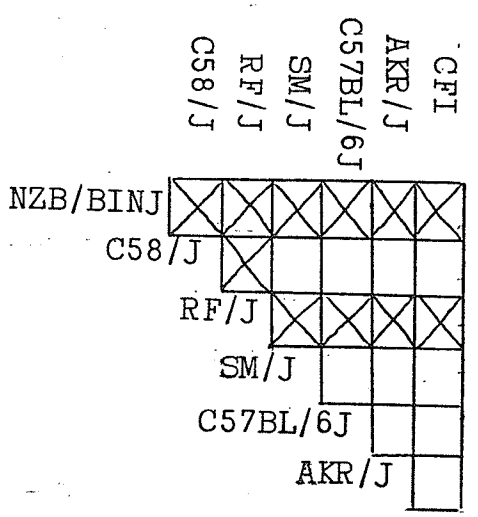


FIGURE 18: TUKEYS TEST AT THE 95% CONFIDENCE LEVEL

II. 7 S SENSITIZED PLATES

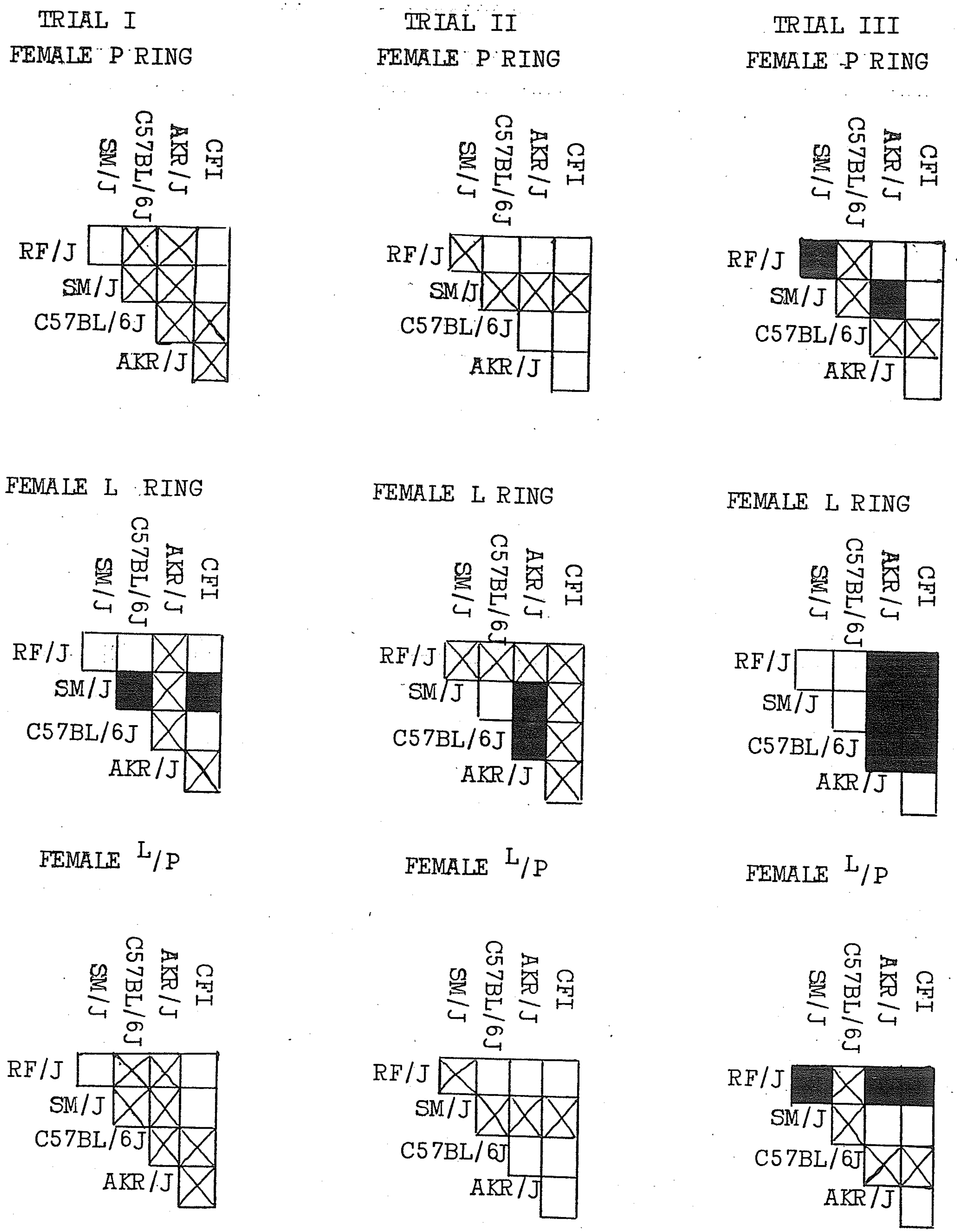


TABLE XXXV

MEAN RING DIAMETER COMPARISON BETWEEN THE SEXES OF THE STRAINS FACTORIAL ANALYSIS OF VARIANCE WITH EQUAL REPLICATION IN EACH CELL (95% CONFIDENCE LEVEL)

I. WHOLE SENSITIZED PLATES

	TRIAL I		TRIAL II		TRIAL III	
RING	STRAINS WHOSE SEXES WERE COMPARED	FACTORIAL RESULTS	STRAINS WHOSE SEXES WERE COMPARED	FACTORIAL RESULTS	STRAINS WHOSE SEXES WERE COMPARED	FACTORIAL RESULTS
P	CFI RF/J	THE SEXES ARE DIFFERENT	CFI AKR/J	THE SEXES ARE DIFFERENT	CFI C57BL/6J AKR/J SM/J RF/J	THE SEXES ARE DIFFERENT
L	CFI RF/J	THE SEXES ARE DIFFERENT	C57BL/6J RF/J AKR/J	THE SEXES ARE DIFFERENT	CFI AKR/J SM/J RF/J	THE SEXES ARE DIFFERENT
L/P	CFI RF/J	THE SEXES ARE DIFFERENT	CFI C57BL/6J RF/J	THE SEXES ARE DIFFERENT	CFI RF/J	THE SEXES ARE DIFFERENT

TABLE XXXVI

MEAN RING DIAMETER COMPARISON BETWEEN THE SEXES OF THE STRAINS FACTORIAL ANALYSIS OF VARIANCE WITH EQUAL REPLICATION IN EACH CELL (95% CONFIDENCE LEVEL)

II. 7 S SENSITIZED PLATES

	TRIAL I		TRIAL II		TRIAL III	
ING	STRAINS WHOSE SEXES WERE COMPARED	FACTORIAL RESULTS	STRAINS WHOSE SEXES WERE COMPARED	FACTORIAL RESULTS	STRAINS WHOSE SEXES WERE COMPARED	FACTORIAL RESULTS
P	NO COMPARISONS DUE TO NONHOMO-GENEITY OF VARIANCES	X	C57BL/6J AKR/J	THE SEXES ARE DIFFERENT	CFI AKR/J SM/J RF/J	THE SEXES ARE DIFFERENT
L	CFI C57BL/6J RF/J	THE SEXES ARE DIFFERENT	C57BL/6J RF/J SM/J	THE SEXES ARE DIFFERENT	CFI C57BL/6J AKR/J SM/J RF/J	THE SEXES ARE DIFFERENT
/P	CFI	THE SEXES ARE DIFFERENT	C57BL/6J RF/J AKR/J	THE SEXES ARE DIFFERENT	C57BL/6J	THE SEXES ARE DIFFERENT

TABLE XXXVII

MEAN RING DIAMETER COMPARISON BETWEEN THE SEXES OF EACH OF THE STRAINS
TUKEYS MULTIPLE RANGE TEST (95% CONFIDENCE LEVEL)

I. WHOLE SENSITIZED PLATES

	TRIAL I		TRIAL II		TRIAL III	
RING	EACH STRAIN HAD SEXES COMPARED	TUKEY RESULTS	EACH STRAIN HAD SEXES COMPARED	TUKEY RESULTS	EACH STRAIN HAD SEXES COMPARED	TUKEY RESULTS
P	CFI RF/J	✓ ✓	CFI AKR/J	✓ ✓	CFI C57BL/6J AKR/J SM/J RF/J	✓ ✓ ✓ ✓ ✓
L	CFI RF/J	✓ X	C57BL/6J RF/J AKR/J	X X	CFI AKR/J SM/J RF/J	X ✓ X X
L/P	CFI RF/J	✓ ✓	CFI C57BL/6J RF/J	✓ ✓ ✓	CFI RF/J	✓ ✓

✓ - Indicates the sexes of the certain strain have significantly different mean ring diameters as determined by Tukeys test at 95% confidence level.

X - Indicates the sexes of the certain strain have NO significant difference in mean ring diameter as determined by Tukeys test at 95% confidence level.

TABLE XXXVIII

MEAN RING DIAMETER COMPARISON BETWEEN THE SEXES OF EACH OF THE STRAINS
RUKEYS MULTIPLE RANGE TEST (95% CONFIDENCE LEVEL)

II. 7 S SENSITIZED PLATES

	TRIAL I		TRIAL II		TRIAL III	
RING	EACH STRAIN HAD SEXES COMPARED	TUKEY RESULTS	EACH STRAIN HAD SEXES COMPARED	TUKEY RESULTS	EACH STRAIN HAD SEXES COMPARED	TUKEY RESULTS
P	NO COMPARISON DUE TO NONHOMOGE-NEITY OF VARIANCE		C57BL/6J AKR/J	✓ ✓	CFI AKR/J SM/J RF/J	✓ ✓ ✓ ✓
L	CFI C57BL/6J RF/J	✓ X X	C57BL/6J RF/J SM/J	✓ ✓ ✓	CFI C57BL/6J AKR/J SM/J RF/J	X ✓ X ✓ ✓
L/P	CFI	✓	C57BL/6J RF/J AKR/J	✓ ✓ ✓	C57BL/6J	✓

✓ - Indicates the sexes of the certain strain have significantly different mean ring diameters as determined by Tukeys test at a 95% confidence level.

X - Indicates the sexes of the certain strain have NO significant difference in mean ring diameter as determined by Tukeys test at a 95% confidence level.

Results:

In Tables XXXIII and XXXIV, ANOVA results indicate that strains of male mice compared have differences in their mean P ring diameters at all three ages tested on whole sensitized plates and the last two ages (22 and 30 weeks) on 7 S plates. Strains of females compared show no mean P ring diameter differences at any age on whole and only at the last age tested on 7 S sensitized plates.

Both in the case of strains of males and strains of females compared, ANOVA indicates at all ages tested on both whole and 7 S plates there are significant differences between the mean L ring diameters of the strains with only one exception (male strains L ring on whole plates at age 15 weeks).

The ANOVA results show that there is no significant difference between mean $^{L}/_{P}$ for strains of males on 7 S plates. On whole plates mean $^{L}/_{P}$ values for strains of males are shown to be different at 15 and 22 weeks.

Mean $^{L}/_{P}$ values for strains of females compared show that on both whole and 7 S plates only at 30 weeks do differences show up.

Tukeys Test results (Figures 15 to 18) show visually that as age increases there are more differences between strains. (Note the increase in the number of dark boxes.)

It is also observed that the number of strains not compared due to nonhomogeneous variance decreased each time

the experiment was done, possibly indicating increased efficiency by the experimenter in using the plate technique.

In the cases of comparison of $^{L}/_{P}$ values there were very few differences.

Since the tables are standard in arrangement of strain comparisons, it is possible to see a pattern of differences with increased age. But results show that there doesn't seem to be a common pattern of differences from age to age.

In comparisons of the mean ring diameters of the sexes of strains, it was found by factorial analysis (Tables XXXV and XXXVI) that in all cases the sexes compared indicated differences.

In Tukey comparisons between individual pairs (sexes of a strain) it was found: (Tables XXXVII and XXXVIII)

1) On both whole and 7 S sensitized plates the mean P ring diameters are always different.

2) On both whole and 7 S sensitized plates the mean L ring diameters may or may not be significantly different.

3) On both whole and 7 S sensitized plates the mean $^{L}/_{P}$ values are always different.

SECONDARY RESPONSE STUDY

CHANGES IN RING DIAMETER DURING THE SECONDARY RESPONSE

Ring diameter was studied in the four groups of mice below by bleeding the mice at various times during an immune response. The size of each ring type was compared between the four groups as the immune response progressed. All injections in this experiment are intraperitoneal.

I. Secondary Response Mice

Four CFI females were previously injected with 0.1 mls of a 50% guinea pig red blood cell suspension every three days over a period of 15 days when the mice were approximately five months old. On the day of commencement of this experiment each of these mice was injected with 0.1 mls of 50% guinea pig red blood cell suspension.

II. Primary Response Mice

Four CFI females not previously injected with guinea pig red blood cells were injected with 0.1 mls of 50% guinea pig red blood cell suspension on the day of commencement of the experiment.

III. DBS Control Mice

Four CFI females not previously injected with guinea pig red blood cells were injected with 0.1 mls of DBS on the day of commencement of this experiment.

IV. Control Mice

This group consisted of four CFI females that were not previously injected with guinea pig red blood cells and on the day of commencement of this experiment were not injected.

Experimental Schedule:

A sample of plasma was obtained from each mouse in the four groups of mice at the below specified bleeding times and each was diffused in two plates sensitized with 1 HD_{50} whole hemolysin and one plate that was unsensitized.

Preinjection bleeding of the four groups.

Injection of groups I, II, III.

2 hours post injection bleeding of the four groups.

6 hours post injection bleeding of the four groups.

12 hours post injection bleeding of the four groups.

24 hours post injection bleeding of the four groups.

48 hours post injection bleeding of the four groups.

72 hours post injection bleeding of the four groups.

1 week post injection bleeding of the four groups.

2 weeks post injection bleeding of the four groups.

This experiment was done twice. The CFI females' ages were:

	Age of Mice
Trial I	13 months $\pm$ 2 weeks
Trial II	16 months $\pm$ 2 weeks

Results:

Secondary and primary response groups of mice are different from the two control groups in the plot of their $^{L}/_{P}$ ratios vs time.

In both trials of the experiment the two immune response groups of mice have an increase in mean $^{L}/_{P}$ values between the time of injection and six hours after (Figures 19 and 20). This could be predominantly due to decrease in the P ring diameter (Figures 21 and 22), and a constant L ring diameter (Figures 23 and 24) during this time period. The two control groups mean $^{L}/_{P}$ values either stayed constant or decreased during this time. Whether or not the increase in the two immune response groups is significant or not cannot be determined at this time.

After six hours the two immune response groups and two control groups do not deviate to any noticeable extent in trends of $^{L}/_{P}$ ratios. After the six hour mark, it may be that other factors blur naturally occurring $^{L}/_{P}$ changes during the immune response. An example of one of these factors is stress caused by repeated bleeding of the mice (five times in a 24 hour period).

See Appendix 8 for data, Tables XXXIX to XLVI.

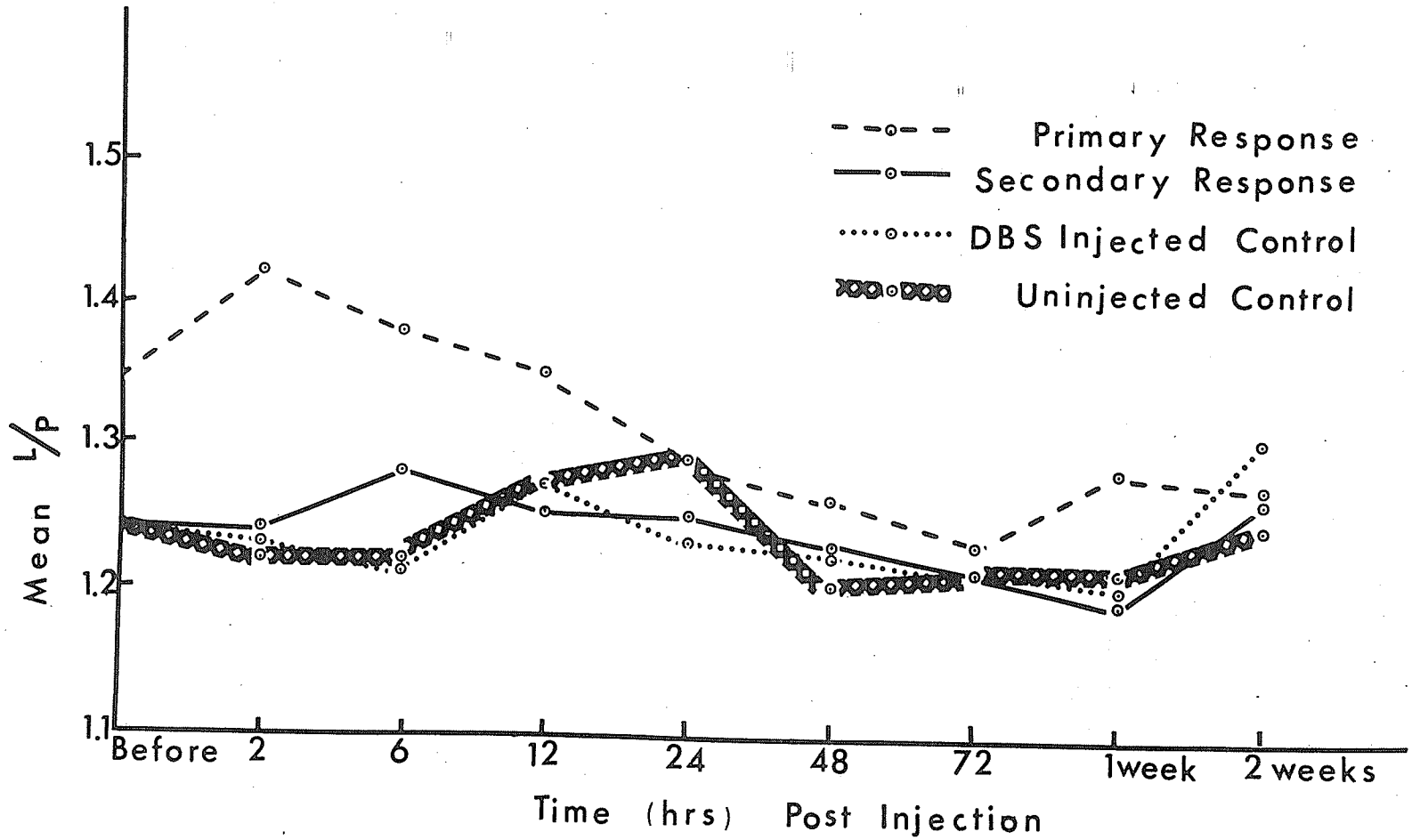


Figure 19. Trial I: Mean $^{L}/_{P}$ vs time after antigen injection. Each mean $^{L}/_{P}$ was calculated from eight individual $^{L}/_{P}$ values.

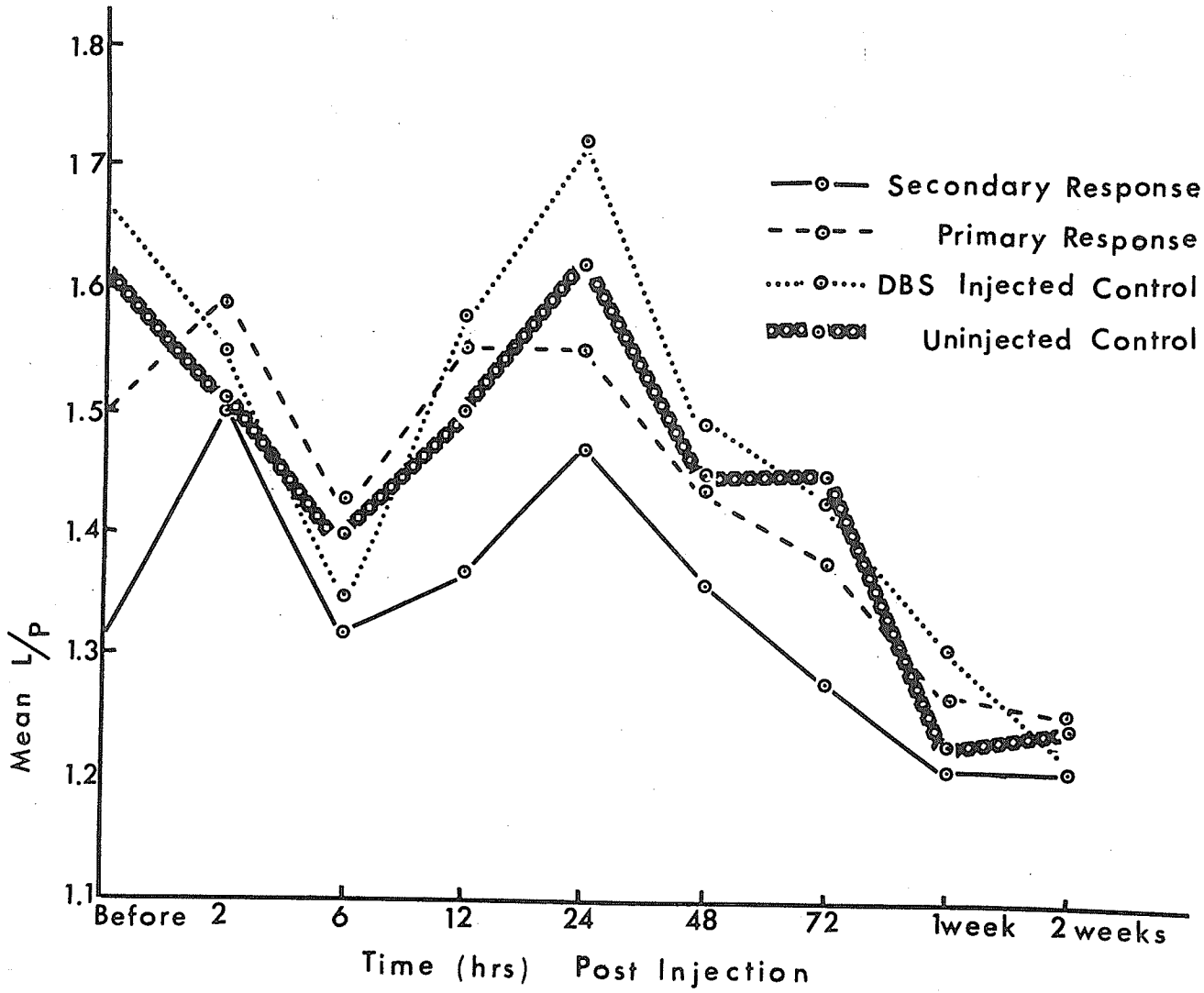


Figure 20. Trial II: Mean L/P vs time after antigen injection. Each mean L/P was calculated from eight individual L/P values.

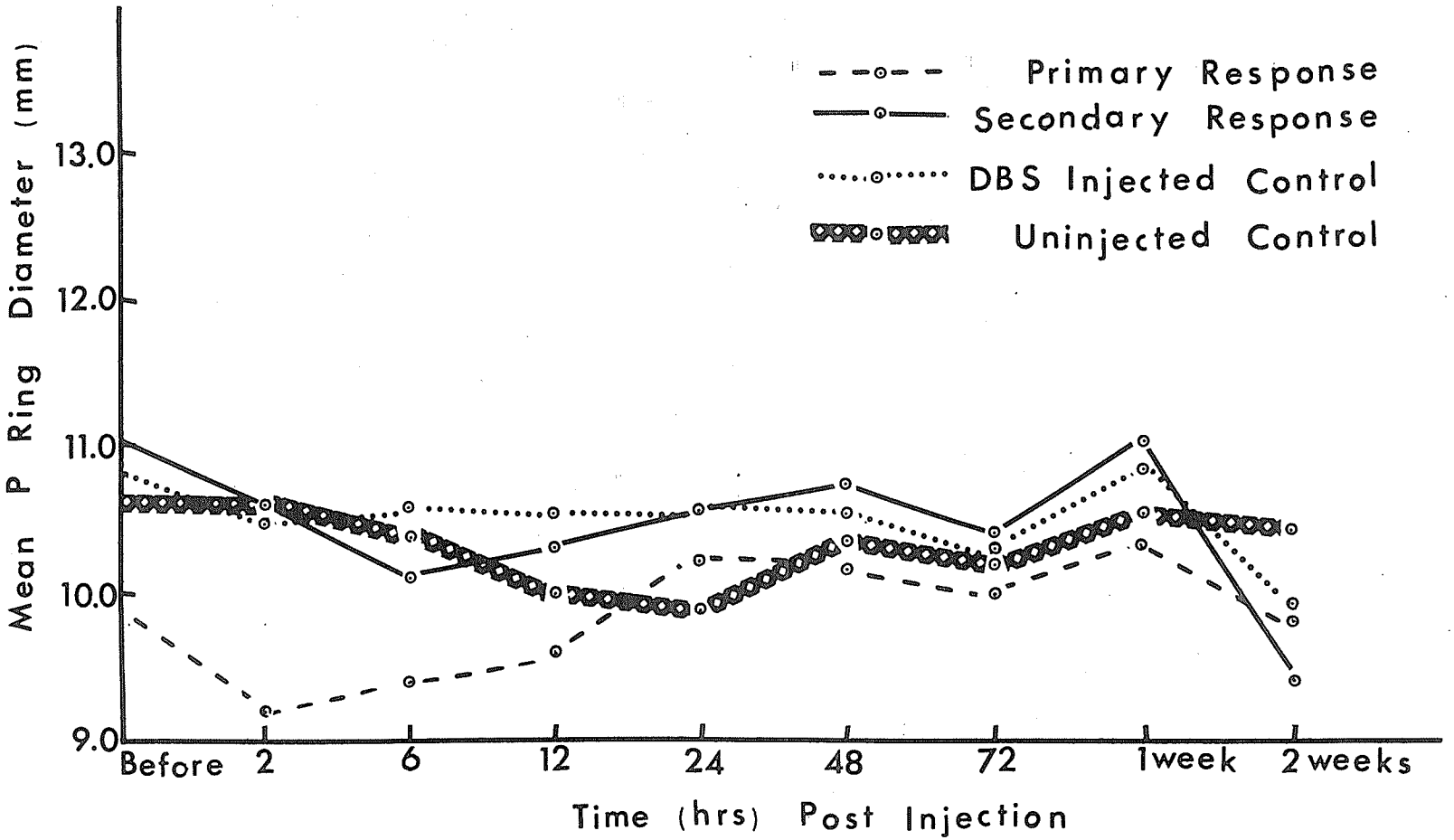


Figure 21. Trial I: Mean P diameter vs time after antigen injection. Each mean P diameter was calculated from eight individual P diameters.

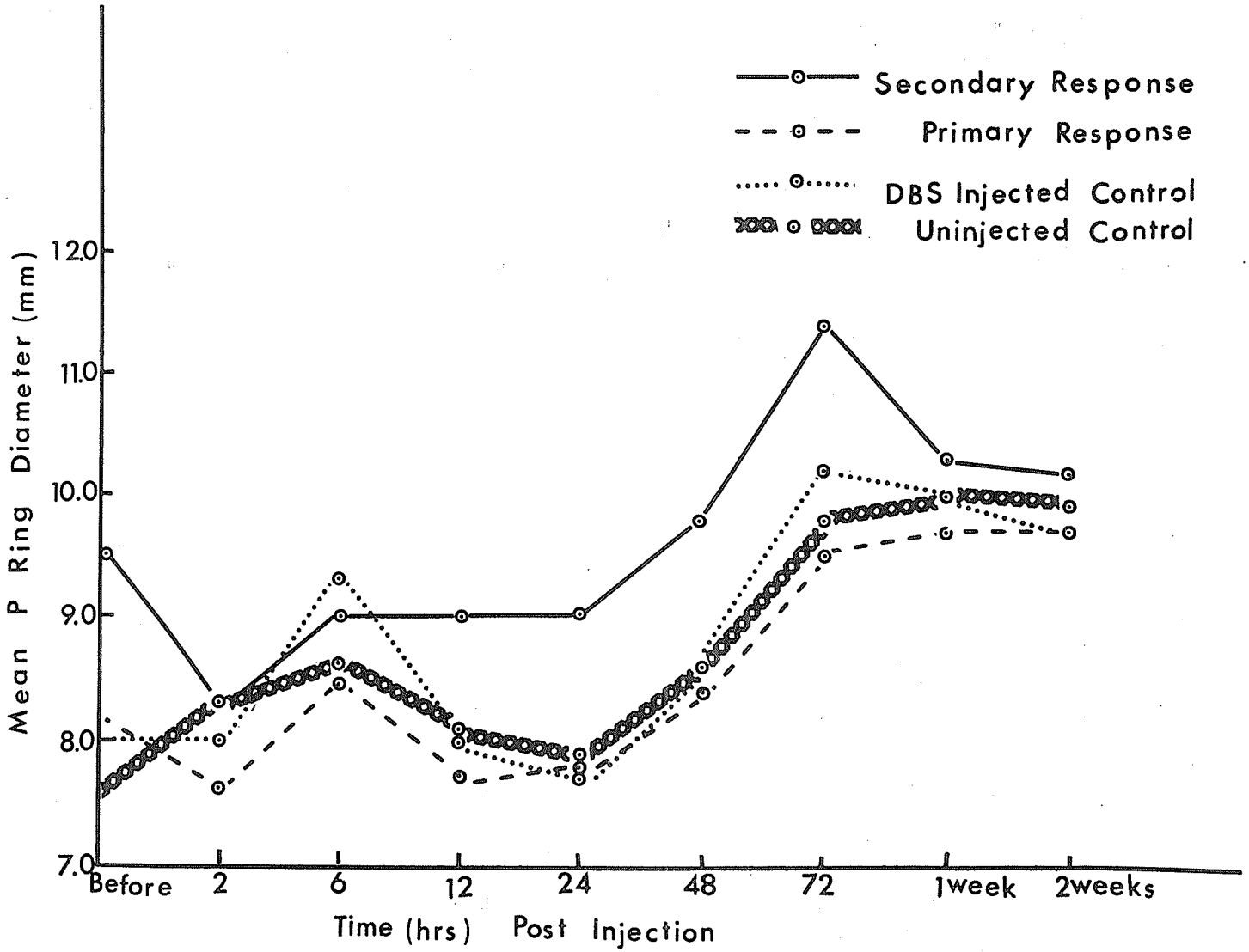


Figure 22. Trial II: Mean P diameter vs time after antigen injection. Each mean P diameter was calculated from eight individual P diameters.

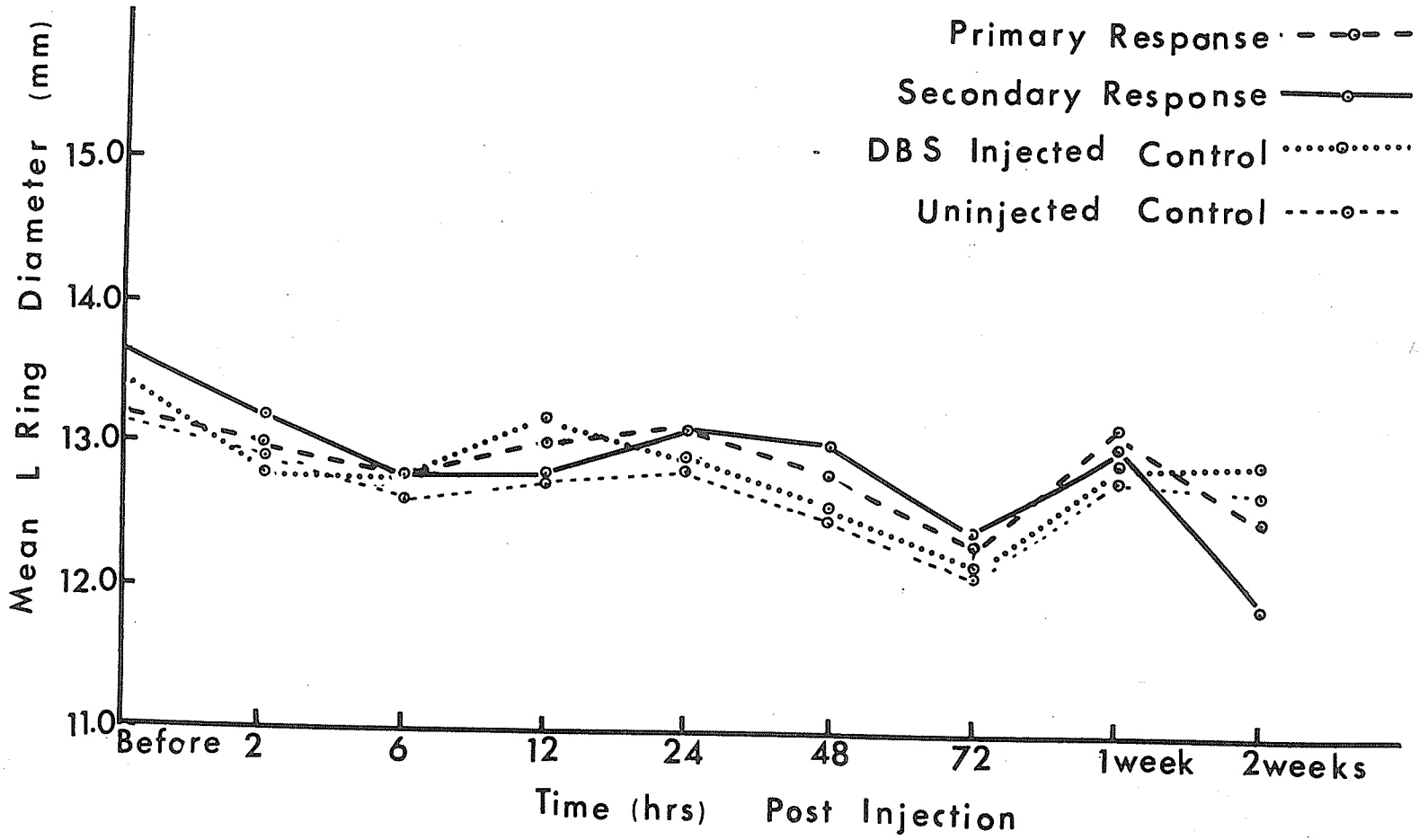


Figure 23. Trial I: Mean L diameter vs time after antigen injection. Each mean L diameter was calculated from eight individual L diameters.

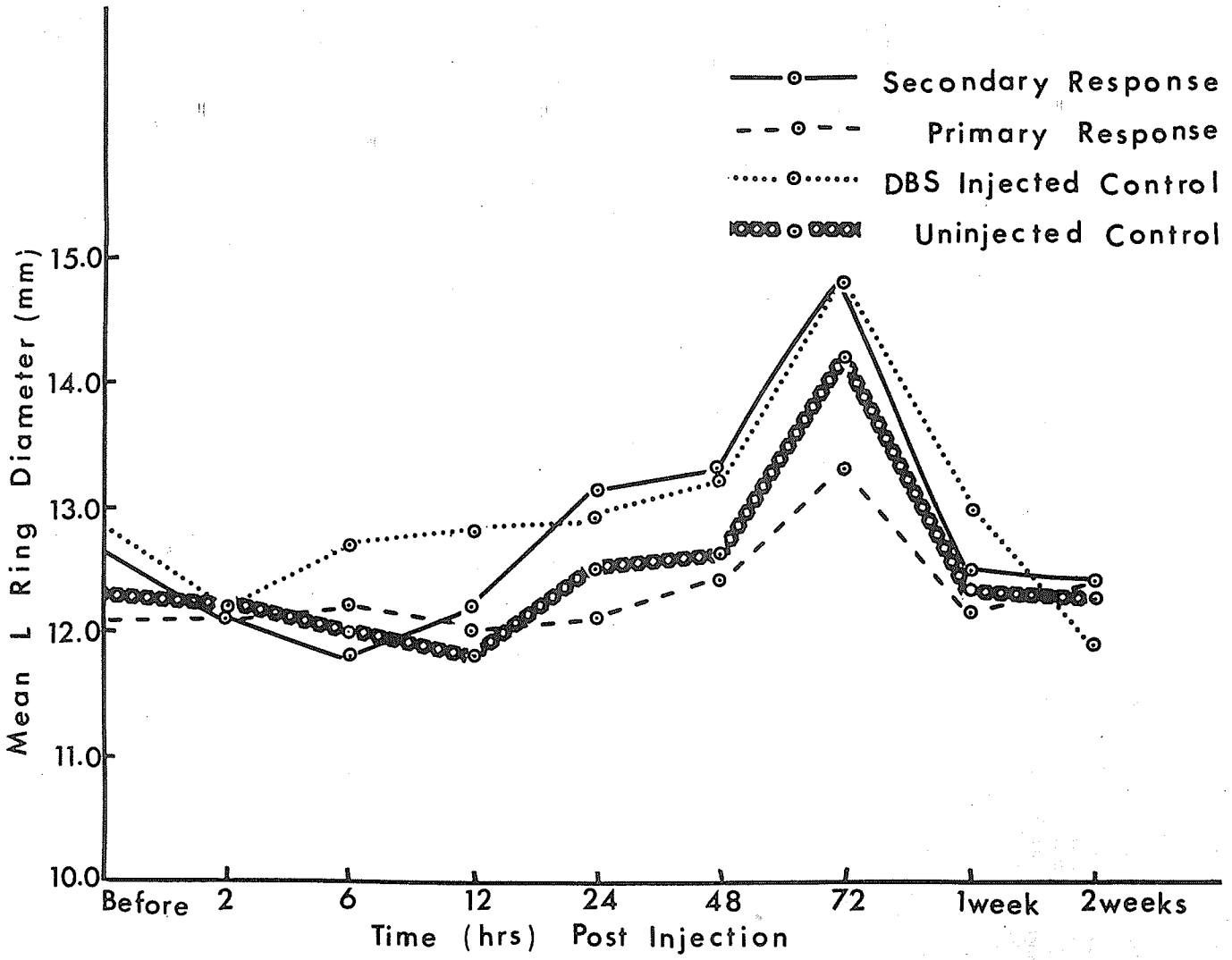


Figure 24. Trial II: Mean L diameter vs time after antigen injection. Each mean L diameter was calculated from eight individual L diameters.

CHAPTER V

DISCUSSION

The experimental work in this thesis was divided into three sections, as indicated in the Introduction (page 3):

i) Analysis of the Plate Test.

ii) A Study of Ring Diameter Difference Between Strains.

iii) Secondary Response Study.

which will be discussed in this order. The analysis of the plate test is devoted to defining the limitations of the plate test and also presenting evidence that autologous anti-immunoglobulins are responsible for the rings on the plates.

In the experiment where the ring diameters produced using different plasma concentrations were compared, it was found that there was a linear relationship between mean ring diameter (for both P and L rings) and plasma concentration. This was true for whole and 7 S sensitized plates and suggests that the plate method is a valid assay (Figures 2 to 8).

The effect of complement concentration was studied and it was found that as the complement concentration increases the diameter of the inhibited ring of lysis (P ring) decreases (Figure 9).

In the plate test it is likely that both inhibiting and facilitating anti-immunoglobulins are present directly adjacent to the hole, since they both diffuse out from the hole. It is noted that where both are present the inhibiting

anti-immunoglobulin predominates over the facilitating anti-immunoglobulin in action causing the P ring of inhibited lysis to be present directly adjacent to the hole (see Figure 1). Onysko postulated that anti-immunoglobulin blocks the complement fixing site on the antibody in the antibody-antigen complex. Complement competes with the anti-immunoglobulin for the site and therefore a higher concentration of complement used to develop plates decreases the diameter of the P ring. The fact that this result corresponds to the decreased inhibitory titre of known heterologous anti-immunoglobulin found by Onysko (1962) in her tube test when she used higher complement concentrations, suggests that the interpretation of the P ring being due to inhibitory anti-immunoglobulin is justified. The fact that the mean P ring diameter doesn't decrease when complement dilutions greater than 1/20 are used indicates only complement dilutions less than 1/20 contain enough complement to noticeably compete with the inhibiting anti-immunoglobulin for sensitized red cells.

An experiment to study the effect which hemolysin dosage had on ring diameters showed that as the hemolysin dosage increases in both whole and 7 S sensitized plates the P mean ring diameter decreases (Figures 11 and 13). The L mean ring diameter on both 7 S and whole plates doesn't change as hemolysin dosage changes.

The behaviour of the autologous inhibiting anti-immunoglobulin (P ring) is related to Onysko's work, for in her tube titrations of heterologous anti-immunoglobulin

inhibition of lysis decreased as the dose of hemolysin used to sensitize the red blood cells increased.

McIllmurray (1965) disproved the idea that heterologous anti-immunoglobulin inhibited lysis by destroying complement.

Najjar (1959) stated that qualities that made immunoglobulin a familiar and normal protein to the immune system of the host were chiefly its surface configuration, shape and size. If either or all of these are altered by strong association with another molecule such as an antigen, new groupings arise on the surface structure. Such new groupings cause the antibody to be regarded as foreign by the immune system and anti-immunoglobulin is formed against this antibody type immunoglobulin. We consider that this is the way that anti-immunoglobulin which acts in the plate test is produced.

In conclusion, the results for the hemolysin dosage experiments as well as the complement concentration experiments indicate that the rings produced on plates are due to autologous anti-immunoglobulin.

The effect that storing plasma overnight has on the P and L ring diameters was studied by diffusing samples of plasma stored overnight at 4°C and fresh samples of plasmas from the same mice. It was found that there was no significant difference between the mean ring diameters (P and L) of the two types of plasma on whole sensitized plates.

This result enables us to use overnight plasma storage without fear of distortion of results. In experiments where the plate method is being used to assay autologous anti-immunoglobulin it often entails more than one work day so overnight plasma storage is essential. There is a remote possibility, however, that stored plasma does change in ring diameters. The anti-immunoglobulin properties in mice may change randomly from day to day and therefore the ring diameters seen for fresh plasma would be different from that of fresh plasma obtained on the previous day from the same mice, but the fact you stored it overnight changed its anti-immunoglobulin properties in such a way that it ends up with ring diameters the same as the fresh plasma. This is very unlikely for the experiment was repeated twice and each time many different types of ring diameter comparisons were made (see Table VIII).

When plasmas are diffused in more than one plate there is the possibility that the ring diameters will be different on different plates due to variability in plate preparation. To test the possibility that different plates prepared at the same time may cause significant variability in ring diameters, each of a set of mouse plasmas was diffused in two such whole plates and the ring diameters obtained on the plates were compared. Referring to Tables XI and XII, it is seen in seven out of eight cases there is no significant difference in ring

diameter on different plates indicating that it is valid to compare ring diameters on different plates prepared at the same time.

A separate comparison was carried out to test for variability in ring diameter caused by preparing plates at different times.

In such a comparison sets of plasmas were obtained from a common group of mice on different days and in each case were diffused in plates prepared on these days. Referring to Tables IX and X, the ring diameters for the plasmas diffused in plates made on different days were compared and it was found in five out of eight cases that ring diameters were significantly different, indicating ring diameters may or may not be different due to variability in plates prepared on different days. Therefore, it is not valid to compare ring diameters on plates prepared on different days.

In an experiment to test whether heating mice to be bled had an effect on ring diameters, the ring diameters caused by plasmas obtained from heated mice were compared by factorial analysis to ring diameters of plasmas obtained from these same mice when they were not heated. Results in Table XIII show heating had no significant effect on ring diameter.

The ring diameters of several strains of mice were compared in the following ways.

Analysis of Variance:

The ring diameters of different strains were compared (Tables XXXIII and XXXIV, Figures 15 to 18). General comparisons of whether there were differences among the strains were carried out using a one way analysis of variance (ANOVA) at the 95% confidence level (Tables XXXIII and XXXIV). Due to nonhomogeneity of variance it was not possible to compare all possible strains although we do not believe this factor severely affected analysis.

Table XLVII is a summary of the ANOVA results showing the following:

(A) P Ring

i) In whole plates significant differences among the strains are noted at each age, for males but not for females.

ii) On 7 S plates differences are not observable at the youngest age but have appeared by the age of 30 weeks for both sexes.

(B) L Ring

i) On whole plates significant differences are not noted among the strains of males compared at 15 weeks, but do appear at 22 and 30 weeks. Among strains of females compared, differences are present at all ages.

ii) On 7 S plates differences are observed at all ages for both sexes.

TABLE XLVII

SUMMARY OF THE ANOVA RESULTS

SEX	AGE COMPARED (WEEKS)	WHOLE			7 S		
		P	L	$L_{/P}$	P	L	$L_{/P}$
MALE	15	X	O	X	O	X	O
	22	X	X	X	X	X	O
	30	X	X	O	X	X	O
FEMALE	15	O	X	O	O	X	O
	22	O	X	O	O	X	O
	30	O	X	X	X	X	X

X - ANOVA indicates differences in mean ring diameters of the strains compared.

O - ANOVA indicates no such differences.

(C) $\underline{L/P}$

i) On whole plates significant differences are noted among males at 15 and 22 weeks but not at 30 weeks. The reverse is true for females where no differences between strains are observed at 15 and 22 weeks but are seen at 30 weeks.

ii) On 7 S plates differences are not observed among males at any age and among females are not observed at 15 and 22 weeks but appear at 30 weeks.

These ANOVA results may be summarized more briefly as follows:

(a) Differences between strains with respect to the sizes of P and L rings are either present at 15 weeks or appear by 30 weeks, except for the P ring for strains of females on whole plates.

(b) Differences among strains in L/P values are much less apparent than differences among strains in P and L values.

Tukeys Test:

In contrast to ANOVA, Tukeys Test allows us to compare individual strains with one another (Figures 15 to 18). It should be emphasized that in the tables the blank squares mean that no difference was demonstrable between the members of the pair of strains in question; a dark square means that there was a difference demonstrated and ☒ means that a comparison could not be made because of

nonhomogeneity of variance.

Table XLVIII was calculated from Figures 15 to 18. It shows the percentage of comparisons by Tukeys Test which showed a difference between the strains compared. This figure will be referred to here as "percent differences".

The following conclusions were drawn.

1) The percentage of differences for all P ring comparisons tends to increase in number with age up to 30 weeks. This in general is not true for the L ring comparisons.

2) $^{L}/_{P}$ differences in all cases are much less numerous than P and L ring diameter differences. Indeed, the males' $^{L}/_{P}$ differences do not increase with age.

The following is another way of studying the trend of Tukeys comparisons as age increases. Referring to Figures 15 to 18 there are four possible ways that results of a Tukeys Test comparison between two strains at two ages can turn out.

Age 1 ⟶ Age 2

(15 weeks ⟶ 22 weeks
or
22 weeks ⟶ 30 weeks)

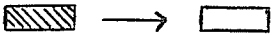
1) ☐ ⟶ ☐ Here, at each age the comparison between strains fails to indicate a difference between their mean ring diameters.

TABLE XLVIII

PERCENTAGE OF TUKEY COMPARISONS THAT SHOW DIFFERENCES BETWEEN STRAINS

RING TYPE	15 WKS	22 WKS	30 WKS
MALE P RING, WHOLE	30%	30%	57%
MALE L RING, WHOLE	0%	60%	50%
MALE L/P, WHOLE	33%	33%	0%
FEMALE P RING, WHOLE	0%	0%	0%
FEMALE L RING, WHOLE	17%	50%	50%
FEMALE L/P, WHOLE	0%	0%	20%
MALE P RING, 7 S	0%	38%	57%
MALE L RING, 7 S	40%	27%	53%
MALE L/P, 7 S	0%	0%	0%
FEMALE P RING, 7 S	0%	0%	33%
FEMALE L RING, 7 S	33%	67%	60%
FEMALE L/P, 7 S	0%	0%	50%

2)  Here, at each age the comparison between strains indicates there is a difference between their mean ring diameters.

3)  At age 1 the strains do not show different diameters. At age 2 the reverse is true.

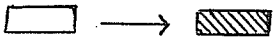
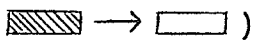
4)  The reverse of case 3).

Table XLIX indicates the percentage of the previous four cases present for the ring types and $^{L}/_{P}$ ratios with the total number of comparisons listed for males and females on whole and 7 S.

Since (2), (3) and (4) indicate that males have percentages roughly 2 to 2 1/2 times that of females, it may be that females do not exhibit as many differences as males or that differences in males show up better statistically because they are larger.

In both males and females, there is a definite trend of increasing differences with age. That is, as age increases the number of differences between mean diameters that decrease (by reverting ) is only 1/3 as large as the number of differences that come into being ().

Using the results of Tukeys Test for the mice at the age of 30 weeks, an attempt was made to divide the strains

TABLE XLIX

TUKEY RING DIAMETER COMPARISONS FROM AGE TO AGE

SEX OF THE STRAINS COMPARED	(1) □ → □	(2) ▨ → ▨	(3) ▨ → □	(4) □ → ▨	NUMBER OF TUKEY COMPARISONS
MALE	46%	16%	10%	28%	95
FEMALE	75%	9%	4%	11%	54

into groups such that within one group all pair comparisons failed to show a statistical difference between the members of the group; and pairs of strains that showed differences were placed in separate groups. This proved possible only for strains of male mice using P or L rings. When Tukeys results for L or P ring diameters were studied on whole and 7 S plates, two distinct groups of strains were observed.

Group 1 - CFI, C57BL/6J and SM/J.

Group 2 - AKR/J, RF/J, C58/J and NZB/BINJ.

Using the term "mistake" to mean instances when the rules for group formation were transgressed, the mistakes were as follows:

P ring on whole plates - no "mistakes".

P ring on 7 S plates - no "mistakes".

L ring on whole plates - NZB vs C58 - difference.
NZB vs SM/J - no difference.
NZB vs CFI - no difference.

L ring on 7 S plates - NZB vs CFI - no difference.

On the basis of these "mistakes" which suggest that NZB falls between group 1 and 2 with respect to the L ring only, we recognize three groups, as follows:

Group 1 - CFI, C57BL/6J and SM/J.

Group 2a - NZB/BINJ.

Group 2b - AKR/J, RF/J and C58/J.

These groups could not be detected using $^{L}/_{P}$ ratios. The mean P and L diameters and $^{L}/_{P}$ ratios were calculated for each of these groups and are shown in Table L. In this table the results on 7 S plates are listed. (The results on whole plates are almost the same, 0.1 to 0.4 mm more in all cases.)

The interesting point about these groups is that Group 1 included two strains of mice which are not characteristically subject to any disease (CFI and C57BL/6J) and one strain characterized by amyloidosis in later life.

Group 2a had only one strain, characterized by the development of auto-immune disease (high antibody production). The three strains in Group 2b are all subject to leukemia in later life. The possibility arises, therefore, that a characteristic level of inhibiting and facilitating autologous anti-immunoglobulin is related to the development of auto-immune disease and leukemia.

The fact that the $^{L}/_{P}$ ratio does not suffice to recognize these groups suggests that in health at least (all these mice were healthy and had not yet developed any of the diseases characteristic of the strains), there is some "balance" between the two types of anti-immunoglobulins. Thus, when the P ring is large, the L ring is also larger and vice versa.

Furthermore, the fact that the $^{L}/_{P}$ ratio is lowest in NZB/BINJ mice (Group 2a) which are the mice that are

TABLE L

MEAN RING DIAMETERS OF THE THREE GROUPS OF STRAINS ON 7 S PLATES

RING TYPE	GROUP I (mm)	GROUP IIa (mm)	GROUP IIb (mm)
P	10.2	11.1	11.2
L	12.6	13.1	13.5
L/P	1.24	1.19	1.20

difficult to distinguish from groups 1 and 2b on the basis of the L ring diameters, provides a suggestion that in this strain it is the ratio of L and P ring diameters which is associated with NZB strains auto-immune disease.

In NZB there is less facilitating anti-immunoglobulin relative to inhibiting anti-immunoglobulin than in the other strains. Therefore, it could be expected that the high antibody production by this strain may be due to its high lymphocyte level caused by inhibiting anti-immunoglobulins predominant action.

RING DIAMETER COMPARISONS BETWEEN SEXES

Factorial analysis, Tables XXXV and XXXVI, revealed that males and females had significantly different P, L and $^{L}/_{P}$ values on both whole and 7 S plates. Only the strains with homogeneous variances were included.

Tukeys Test results, Tables XXXVII and XXXVIII, indicated that for sexes of the strains compared on both whole and 7 S plates:

1) The mean P ring diameters of males and females are always different where comparison is possible.

2) The mean L ring diameters of males and females may or may not be significantly different.

3) The mean $^{L}/_{P}$ values of males and females are always different.

These results may indicate that the P ring diameter is the main determining factor as to whether $^{L}/_{P}$ values are significantly different or not for sexes of a strain.

Data tables for the experiment show that the mean P ring diameters for females of strains are consistently smaller than mean P ring diameters for males of the same strain.

In the experiment done to determine whether or not L ring diameters, P ring diameters and $^{L}/_{P}$ values changed with time during an immune response (Figures 19 to 24), there were not any noticeable differences in the trend of

L ring diameters of any of the four groups at any time.

The only definite differences between the antigen injected mice and the controls occur between the time of injection and six hours later.

In both trials, the $^{L}/_{P}$ ratios in the antigen-injected groups increase sometime during this time interval. From the P and L ring results over the same period of time, the increase is usually due largely to a decrease in the P ring rather than an increase in the L ring. The two control groups, on the contrary, do not show an increase in $^{L}/_{P}$ ratios during the first six hours. This indicates the P ring is the determining factor in the change in $^{L}/_{P}$ values which is similar to P ring being the factor in the sex comparisons of the "Strain Experiment" that most likely causes the male and female $^{L}/_{P}$ values to be statistically different.

This change in $^{L}/_{P}$ values indicates a change in the relative amounts of the facilitating (L ring) and inhibiting (P ring) anti-immunoglobulins between the time of injection and six hours after. Paraskevas (1972) discovered a factor in mouse plasma during the primary response that increases the number of immunoglobulin coated cells during this time period. This indicates a relation between the $^{L}/_{P}$ changes and the increase in immunoglobulin coated cells. Similarly the number of immunoglobulin coated cells decreases by 24 hours with the $^{L}/_{P}$ ratio decreasing due to an increase in the P ring diameter.

CHAPTER VI

SUMMARY

SUMMARY

From the experimental results obtained upon diffusion of mouse plasma in agar plates containing sensitized red cells, the following conclusions were drawn:

1) There is a linear relationship between plasma concentration and mean ring diameter for both P and L rings on both whole and 7 S plates.

2) As the complement concentration increases, the mean P ring diameter decreases.

Results are inconclusive at this time as to whether the mean L ring diameter changes as complement concentration changes.

3) As the hemolysin dosage increases, the mean P diameter decreases and the mean L diameter doesn't appear to change significantly.

4) It is found that the effect of warming mice prior to bleeding has a nonsignificant effect on mean ring diameters.

5) The effect of storing plasma overnight is not significant.

6) It is valid to compare ring diameter results obtained on plates prepared at the same time.

7) It is not valid to compare ring diameter results obtained on plates prepared at different times.

8) When strains of mice had their mean ring diameters compared at each of three ages, it was found that with increased age there is an increased number of differences

between strains. Considering Tukeys Test results for mean P diameters at age 30 weeks, the strains could be placed in two groups on the basis of P diameter.

	C58/J	
Predisposed to leukemia	AKR/J	Group 1
	RF/J	
Predisposed to hemolytic anemia	NZB/BINJ	
Not predisposed to disease	C57BL/6J	
	CFI	Group 2
Predisposed to amyloidosis	SM/J	

Strains could be classified into similar groups using Tukey results for mean L diameters at 30 weeks of age but here there are three groups, for NZB/BINJ is in a separate group by itself.

Comparison of sexes shows that the P ring diameter is always significantly larger for males than females. The mean L diameter may or may not be different between sexes and the mean $^{L}/P$ values are always different between sexes.

9) It is found that in the first six hours of the primary and secondary responses there is an increase in mean $^{L}/P$ values. There is a decrease in P diameters during this time. The significance of these results is not clear at this time.

CHAPTER VII

BIBLIOGRAPHY

BIBLIOGRAPHY

ADLER, F.L. Antibody formation after injection of heterologous immune globulin. Journal of Immunology, 76:217, 1956.

ALTMANN, K. Veber immunizierung mit ambozeptorbeladenen blutkörperchen. Z. Immunitoetsforsch., 13:219, 1912.

AMERICAN RHEUMATISM ASSOCIATION. Ann. Rheum. Dis., 18:49, 1959.

BECK, J.S. Some properties of human anti-antibody. Brit. J. Exp. Path., 42:7, 1961.

BECK, J.S., and LEES, A.W. Anti-antibody in pulmonary tuberculosis and other respiratory diseases. Tubercle, 42:245, 1961.

BORDET, J. Les proprietes des antisensibilisatrices et les theories chemiques de l'immunite. Ann. Inst. Pasteur, 18:593, 1904.

COOMBS, R.R.A., FEINSTEIN, A., WILSON, A. Immunoglobulin determinants on the surface of human lymphocytes. Lancet II, 1157, 1969.

DRESSER, D.W., and WORTIS, H.H. Use of an antiglobulin serum to detect cells producing antibody with low hemolytic efficiency. Nature, 208:859, 1965.

EAGLE, H. Specific agglutination and precipitation. I. The mechanism of the reactions. Journal of Immunology, 18:393, 1930.

EHRLICH, P. Studies on Immunology. (Translated by Charles Bolduan.) New York:John Wiley and Sons, 1906.

FRANKLIN, E.C., HOLMAN, H.R., MÜLLER-EBERHARD, H.J., and KUNKEL, H.G. An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. J. Exp. Med., 105:425, 1957.

FRIEDBERGER, E., and MORESCHI, C. Veber himolyse beschleunigende immun-substanzen. Zbl. Bakt. (Orig.), 45: 346, 1907.

FUDENBERG, H.H., GOODMAN, J., and MILTROM, F. Are "anti-antibodies" rheumatoid factors? Transfusion, 3:422, 1963.

FUDENBERG, H.H., GOODMAN, J.W., and MILGROM, F. Immunochemical studies on rabbit anti-antibody. Journal of Immunology, 92:227, 1964.

GREAVES, M.F. Biological effects of anti-immunoglobulins: Evidence for immunoglobulin receptors on T and B lymphocytes. Transplant Rev., 5:45, 1970.

Handbook on Genetically Standardized Jax Mice (Second Edition). The Jackson Laboratory, Bar Harbor, Maine, U.S.A.

HEIMER, R., LEVIN, F.M., and KAHN, M.F. Inhibition of complement fixation by human serum. II. The activity of a γ-1M globulin and rheumatoid factor in complement fixation reactions. Journal of Immunology, 91:866, 1963.

HEIMER, R., LEVIN, F.M., PRIMACK, A., CORCOS, J.M., and NOSENZO, C. Inhibition of complement fixation by human sera. Journal of Immunology, 89:382, 1962.

HOWELL, D.S., PIKE, R., and MALCOHN, J.M. The nature of FII agglutinating factor in patients with non-rheumatic diseases. Arthritis Rheum., 2:63, 1959.

JERNE, N.K., and NORDIN, A.A. Plaque formation in agar by single antibody producing cells. Science, 140:405, 1963.

KLEIN, E., ESKELAND, T., INONE, M., STROM, R., and JOHANSSON, B. Surface immunoglobulin moieties on lymphoid cells. Exp. Cell Res., 62:133, 1970.

KUNKEL, H.G., SIMON, H.J., and FUDENBERG, H. Observations concerning positive serologic reactions for rheumatoid factor in certain patients with sarcoidosis and other hyperglobulinemic states. Arthritis Rheum., 1:289, 1958.

LAPORTE, R., HARDRE DE LOOZE, L., and ROULIER, P. Immunserums antihemolytiques. Ann. Inst. Pasteur, 79:381, 1950.

MACKENZIE, M.R., MACKEY, G., and FUDENBERG, H.H. Antibodies to IgM in normal human sera. Nature, 216:691, (November 18, 1967).

MARRACK, J.R. The chemistry of antigens and antibodies. Med. Res. Council Spec. Report Series, No. 230, page 149, London, 1938.

McILLMURRAY, M.B. Characterization of anti-antibody. M.Sc. Thesis, Department of Bacteriology and Immunology, University of Manitoba, August, 1965.

MERCHANT, D.J., KAHN, R.H., and MURPHY, W.H. Handbook of Cell and Organ Culture, Page 162, Burgess Publishing Company, Minneapolis, Minnesota, 1961.

MILGROM, F., and WITEBSKY, E. Studies on the rheumatoid and related serum factors. J.A.M.A., 174:56, 1960.

MILGROM, F., and DUBISKI, S. Antigeneity of antibodies of the same species. Nature, Lond., 179:1351, 1957.

MILGROM, F., DUBISKI, S., and WOZNICZKO, S. A simple method of Rh determination. Nature, 178:539, 1956(2).

MOND, J., KAPLAN, R.E., and THORBECKE, G.J. Effect in vitro of anti-immunoglobulin sera on proliferative responses of lymphoid cells to antigen. European Journal of Immunology, 3:156, 1973.

NAJJAR, V.A. "Immunity of Virus Infection". Symposium. John Wiley and Sons, Inc., New York, 1959.

ONYSKO, E. The effects of anti-antibody on the activities of antibodies. M.Sc. Thesis, Department of Bacteriology and Immunology, University of Manitoba, August, 1962.

OSLER, A.G., STRAUSS, J.H., and MAYER, M.M. Diagnostic complement fixation. I. A method. American J. Syphil., 36:140, 1952.

PARASKEVAS, F., and LEE, S.T. Cell surface associated gamma globulins in lymphocytes. Journal of Immunology, 109: 1262, 1972.

PETTIER, A., and CHRISTIAN, C.L. The presence of the rheumatoid factor in sera from patients with syphilis. Arthritis Rheum., 2:1, 1959.

PIKE, R.M., SULKIN, S.E., and COGGESHALL, H.C. Serological reactions in rheumatoid arthritis. III. Increased agglutination of sensitized sheep erythrocytes in the presence of normal animal sera. Journal of Immunology, 66:107, 1951.

RIHA, I. Increase in hemolytic activity of anti-erythrocyte antibodies by anti-gamma globulin serum. Folia Microbiol., 9:304, 1964.

ROMEYN, J.A., and BOWMAN, D. Inhibition of complementary lysis by rheumatoid sera. Nature, 216:180, 1967.

ROMEYN, J.A., and ONYSKO, E. The effects of "anti-antibody" on immune hemolysis. Immunology, 7:217, 1964.

ROPARTZ, C., LENOIR, J., HUREL, R., HEMET, Y., and BRETEAU, G. Rev. Hémat., 13:511, 1958a.

ROSS, S. The effects of anti-antibody on immune hemolysis. M.Sc. Thesis, Department of Medical Microbiology, University of Manitoba, March, 1968.

SINGER, J.M., PLOTZ, C.M., PERALTA, F.M., and LYONS, H.C. The presence of anti-gamma globulin factors in sera of patients with active pulmonary tuberculosis. Annals of Internal Medicine, 56:No. 4, 545, April, 1962.

STERZL, J. Immunological tolerance on the result of terminal differentiation of immunologically competent cells. Nature, 209:416, 1966.

STERZL, J., and RIHA, I. A localized hemolysis in gel method for the detection of cells producing 7 S antibody. Nature, 208:258, 1965.

TAKAHASKI, T., OLD, L.J., McINTIRE, K.R., and BOYSE, E.A. Immunoglobulin and other surface antigens of cells of the immune system. Journal of Experimental Medicine, 134^2:815, 1971.

TORRIGIANI, G., ROITT, I.M., LLOYLD, K.N., and CORBETT, M. Elevated IgG antiglobulins in patients with seronegative rheumatoid arthritis. The Lancet, January 3, 1970.

TEFFERS, H.P., MOORE, D.H., and HEIDELBERGER, M. Quantitative experiments with antibodies to a specific precipitate. III. Antigenic properties of horse serum fractions isolated by electrophoresis and ultracentrifugation. Journal of Experimental Medicine, 75:135, 1942.

UNGER, L.J., WIENER, A.S., and KATZ, L. Studies on antibody-like substance in certain human serums, causing agglutination of red cells coated with Rh antibody. American J. of Clin. Path., 29:113, 1958.

VAN DEN ENDE, M.J. Reversed passive anaphylaxis in the guinea pig. J. Hyg., 40:377, 1940.

WAALER, E. Acta path. microbiol. scandinav., 17:172, 1940.

WATSON, R.G. Antiglobulin in normal serum. II. Demonstration of a heat labile antiglobulin in normal human sera. Journal of Immunology, 91:243, 1963.

WATSON, R.G., and COLLINS, N.M. Antiglobulins in normal serum. I. Characterization of two antiglobulins in normal rabbit sera. Journal of Immunology, 90:238, 1963.

CHAPTER VIII

APPENDIX

APPENDIX 1

PREPARATION OF DULBECCO BUFFERED SALINE (DBS)
(Modified from Merchant et al., 1961, page 162)

#1 - In each of two litre flasks:	80.0 gm NaCl
	2.0 gm KCl
	11.5 gm Na_2HPO_4
	2.0 gm KH_2PO_4
	1400.0 ml distilled H_2O
#2 - In a 500 ml flask:	10.0 gm dextrose
	400.0 ml distilled H_2O
#3 - In a 500 ml flask:	0.34 gm $CaCl_2$
	400.0 ml distilled H_2O
#4 - In a 500 ml flask:	2.0 gm $MgCl_2 \cdot 6H_2O$
	400.0 ml distilled H_2O

Autoclave all units separately at 10 lbs for 30 minutes.

To each of the two litre flasks (#1) add: 200 ml #2, 200 ml #3, and 200 ml #4, with sterile precautions.

Bottle with sterile precautions in sterile screw capped bottles. Dilute 1:5 with distilled water before use.

APPENDIX 2

PREPARATION OF ALSEVER'S SOLUTION
(Modified from Osler et al., 1952)

Mix: 10.25 gm dextrose

4.00 gm sodium citrate

2.10 gm sodium chloride

0.275 gm citric acid

500.0 ml distilled water

Adjust to pH 6.1.

Filter with Seitz filter and store in sterile containers.

APPENDIX 3

PREPARATION OF NOBLE AGAR SOLIDS

Batches of forty, 8 ml tubes were made. Each batch was labelled with the date it was made and was discarded if not used within one month.

Three grams of Difco Noble Agar was weighed out on a Sartorius balance.

This quantity of agar powder was mixed with 320 ml of DBS in a 1000 ml flask. The DBS was warmed at room temperature before the agar was added to it.

This mixture was then heated in boiling water until the agar powder was completely dissolved. This was a crucial step and the mixture had to be heated long enough that the agar dissolved completely (at least 15 minutes in boiling water).

The agar was mixed frequently during heating.

The melted agar was dispensed with a 25 ml pipette (which was warmed in a flame) in 8 ml amounts into screw capped pyrex tubes.

The agar was mixed frequently during this procedure.

The tubes were then capped loosely and autoclaved at 15 lbs for 20 minutes.

The tubes were allowed to cool, the caps were then tightened and the tubes were then stored at 4°C.

APPENDIX 4

DEVELOPMENT OF KODAK VERICHROME PAN VP120 FILM

Materials:

Developer: Kodak Dektol Developer,
Eastman Kodak Company,
Rochester, N.Y. 14650.

Prepare as directed on the package.

Fixer: Kodak Fixer,
Eastman Kodak Company,
Rochester, N.Y. 14650.

Prepare as directed on the package.

Stop Bath: Mix 2000 mls of H_2O with 250 mls of 28% acetic acid. To make 28% acetic acid mix 224 mls of acetic acid with 800 mls of H_2O.

Photo Flo: Kodak Photo Flo Solution,
Eastman Kodak Company,
Rochester, N.Y. 14650.

Mix 20 oz of distilled H_2O with 1/2 capful of Photo Flo solution.

Spool Case: Nikor Products,
Springfield, Massachusetts.

Method: Film Development

Put 16 oz quantities of developer and fixer in the

refrigerator for 10 minutes to lower their temperature to 68°F.

In total darkness separate the film to be developed from the spool and wind the film on the developing spool. Put this into the light proof spool case. The rest of the development procedure may be carried out with light.

Add developer into the light proof box and let it incubate for 4 minutes exactly. Shake the spool box periodically during this time. After the 4 minutes pour the Dektol out of the spool case.

Add stop bath solution into the spool case for 10-15 seconds, shake and pour it off.

Add fixer to the spool case for 5 minutes.

Then open the light proof spool case and rinse the spool containing the film still in the spool case under running water for 30 minutes. (The temperature of the water is 68°F.

Dip the film into photo flo, skim the photo flo off the film and hang the film up to dry.

APPENDIX 5

TABLE III

MEAN RING DIAMETERS AND S.E. AT DIFFERENT PLASMA CONCENTRATIONS

I. WHOLE SENSITIZED PLATES

PLASMA CONCEN-TRATION	AMOUNT OF PLASMA (µls)	AMOUNT OF DBS (µls)	TRIAL I				TRIAL II			
			N	MEAN P ± S.E. (millimeters)	N	MEAN L ± S.E. (millimeters)	N	MEAN P ± S.E. (millimeters)	N	MEAN L ± S.E. (millimeters)
5/5	5.0	0	6	9.8±.4	6	12.6±.1	8	9.0±.2	8	13.1±.2
4.5/5	4.5	0.5	6	9.8±.2	6	12.3±.2		N.D.		N.D.
4/5	4.0	1.0	8	9.1±.5	8	12.8±.2	8	8.7±.2	8	12.8±.1
3/5	3.0	2.0	8	9.0±.2	8	12.1±.3	8	8.8±.2	8	12.1±.2
2.5/5	2.5	2.5	4	8.8±.2	4	12.1±.4	8	8.1±.3	8	11.6±.2
2/5	2.0	3.0	3	6.8±.3	4	11.9±.8	8	7.4±.3	8	11.7±.2
1/5	1.0	4.0	8	7.4±.2	8	10.8±.3	8	6.7±.2	8	10.7±.3
.5/5	.5	4.5		N.D.		N.D.	2	6.4±.1	8	10.1±.2

N - The number of individual ring diameters used to compute the mean.
N.D. - Not done.

TABLE IV

MEAN RING DIAMETERS AND S.E. AT DIFFERENT PLASMA CONCENTRATIONS

II. 7 S SENSITIZED PLATES

PLASMA CONCEN-TRATION	AMOUNT OF PLASMA (µls)	AMOUNT OF DBS (µls)	TRIAL I				TRIAL II			
			N	MEAN P ± S.E. (millimeters)	N	MEAN L ± S.E. (millimeters)	N	MEAN P ± S.E. (millimeters)	N	MEAN L ± S.E. (millimeters)
5/5	5.0	0	8	6.5±.1	8	13.4±.2	5	7.5±.1	8	12.4±.3
4.5/5	4.5	0.5	8	7.2±.4	8	13.6±.2		N.D.		N.D.
4/5	4.0	1.0	8	6.5±.1	8	13.4±.2	4	6.5±.1	8	12.4±.3
3/5	3.0	2.0	6	5.9±.1	6	12.5±.4	8	5.9±.1	8	12.0±.3
2.5/5	2.5	2.5	4	5.6±.1	8	12.9±.3		NONE	8	11.7±.3
2/5	2.0	3.0	4	5.9±.1	8	12.2±.5		NONE	8	11.3±.4
1/5	1.0	4.0		NONE	8	11.4±.3		NONE	8	10.3±.4
.5/5	.5	4.5		N.D.		N.D.		NONE	7	9.2±.4

N - The number of individual ring diameters used to compute the mean.

NONE - This indicates no ring was observed on the plate at this plasma concentration.

N.D. - Not done.

APPENDIX 6

TABLE V

MEAN RING DIAMETERS AT DIFFERENT COMPLEMENT DILUTIONS

COMPLEMENT DILUTION	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E. (mm)	MEAN L RING DIAMETER (mm)	S.E. (mm)	MEAN P RING DIAMETER (mm)	S.E. (mm)	MEAN L RING DIAMETER (mm)	S.E. (mm)
1/60	8.7	.2	11.7	.2	10.4	.5	13.7	.2
1/40	8.8	.2	11.8	.2	9.9	.6	13.3	.1
1/35	NOT DONE		NOT DONE		9.9	.5	13.3	.3
1/32.5	NOT DONE		NOT DONE		10.0	.4	13.3	.3
1/30	8.5	.2	12.4	.1	9.2	.7	13.0	.2
1/27.5	NOT DONE		NOT DONE		9.8	.5	13.1	.2
1/25	8.4	.2	12.1	.1	9.9	.6	13.3	.3
1/20	8.1	.2	11.9	.1	9.6	.6	13.0	.2
1/15	NOT DONE		NOT DONE		7.9	.5	13.1	.2
1/7.5	7.2	.4	11.9	.1	6.6	.1	12.9	.2

Each mean value was computed from 6-8 observations.

APPENDIX 7

TABLE VI

MEAN RING DIAMETERS AT DIFFERENT HD_{50}'s OF HEMOLYSIN

I. WHOLE SENSITIZED PLATES (millimeter values)

HD_{50}	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.
1	9.7	.5	12.4	.3	10.2	.4	12.1	.3
2	8.3	.4	13.0	.5	9.4	.3	12.3	.6
3	6.8	.5	12.6	.6	9.3	.3	11.9	.5
4	8.1	.8	12.1	.5	9.1	.3	11.6	.7

TABLE VII

II. 7 S SENSITIZED PLATES (millimeter values)

HD_{50}	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.
2	6.3	.1	11.4	.5	9.1	.3	11.1	.6
1	7.1	.3	12.5	.5	9.6	.3	11.5	.3
.5	9.4	.2	12.4	.4	10.0	.4	11.7	.2

APPENDIX 8

TABLE XXXIX

MEAN RING DIAMETERS AT DIFFERENT TIMES AFTER INJECTION

I. SECONDARY RESPONSE MICE

TIME PLASMA WAS OBTAINED	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.
PREINJECTION	11.0	.4	13.6	.1	9.6	.2	12.6	.2
2 HOURS POST INJECTION	10.6	.3	13.2	.1	8.3	.4	12.2	.2
6 HOURS POST INJECTION	10.1	.4	12.8	.1	9.0	.3	11.8	.3
12 HOURS POST INJECTION	10.3	.2	12.8	.1	9.0	.3	12.2	.3
24 HOURS POST INJECTION	10.5	.3	13.1	.1	9.0	.3	13.1	.3
48 HOURS POST INJECTION	10.7	.7	13.0	.1	9.7	.2	13.2	.2
72 HOURS POST INJECTION	10.4	.2	12.4	.1	11.1	.3	14.6	.3
1 WEEK POST INJECTION	11.0	.3	13.0	.4	10.5	.3	12.6	.2
2 WEEKS POST INJECTION	9.4	.3	11.9	.3	10.3	.2	12.5	.3

Each mean is computed from 8 individual ring diameters.

TABLE XL

MEAN RING DIAMETERS AT DIFFERENT TIMES AFTER INJECTION

II. PRIMARY RESPONSE MICE

TIME PLASMA WAS OBTAINED	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.
PREINJECTION	9.8	.3	13.2	.4	8.1	.2	12.1	.5
2 HOURS POST INJECTION	9.2	.3	13.0	.2	7.7	.2	12.1	.5
6 HOURS POST INJECTION	9.4	.3	12.9	.3	8.5	.1	12.2	.6
12 HOURS POST INJECTION	9.6	.2	13.0	.2	7.7	.2	12.0	.6
24 HOURS POST INJECTION	10.2	.3	13.1	.3	7.9	.2	12.1	.6
48 HOURS POST INJECTION	10.2	.2	12.8	.3	8.6	.2	12.4	.5
72 HOURS POST INJECTION	10.0	.1	12.3	.2	9.6	.2	13.3	.6
1 WEEK POST INJECTION	10.3	.2	13.1	.3	9.7	.3	12.2	.6
2 WEEKS POST INJECTION	9.8	.2	12.5	.4	9.9	.1	12.3	.3

Each mean is computed from 8 individual ring diameters.

TABLE XLI

MEAN RING DIAMETERS AT DIFFERENT TIMES AFTER INJECTION

III. DBS INJECTED CONTROL

TIME PLASMA WAS OBTAINED	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.
PREINJECTION	10.8	.3	13.4	.2	8.0	.4	12.9	.4
2 HOURS POST INJECTION	10.5	.2	12.8	.3	8.0	.4	12.2	.5
6 HOURS POST INJECTION	10.6	.2	12.8	.2	9.3	.3	12.6	.4
12 HOURS POST INJECTION	10.5	.2	13.1	.2	8.0	.2	12.7	.4
24 HOURS POST INJECTION	10.5	.2	12.9	.2	7.8	.4	12.8	.4
48 HOURS POST INJECTION	10.5	.1	12.6	.2	8.8	.4	13.1	.5
72 HOURS POST INJECTION	10.3	.1	12.2	.1	10.2	.5	14.6	.7
1 WEEK POST INJECTION	10.9	.2	12.9	.2	10.0	.3	13.1	.5
2 WEEKS POST INJECTION	9.9	.3	12.9	.3	9.8	.2	11.9	.3

Each mean is computed from 8 individual ring diameters.

TABLE XLII

MEAN RING DIAMETERS AT DIFFERENT TIMES AFTER INJECTION

IV. NON-INJECTED CONTROL

(The times referred to in the plasma obtained column are in reference to the injection time for Group I, II and III)

TIME PLASMA WAS OBTAINED	TRIAL I				TRIAL II			
	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.	MEAN P RING DIAMETER (mm)	S.E.	MEAN L RING DIAMETER (mm)	S.E.
PREINJECTION	10.6	.2	13.2	.3	7.7	.2	12.3	.4
2 HOURS POST INJECTION	10.6	.3	12.9	.2	8.3	.3	12.3	.4
6 HOURS POST INJECTION	10.4	.3	12.6	.2	8.7	.3	12.0	.4
12 HOURS POST INJECTION	10.0	.2	12.7	.2	8.1	.5	11.9	.4
24 HOURS POST INJECTION	9.9	.1	12.8	.2	7.9	.4	12.5	.4
48 HOURS POST INJECTION	10.4	.1	12.5	.2	8.8	.3	12.6	.4
72 HOURS POST INJECTION	10.2	.1	12.1	.2	9.9	.4	14.2	.6
1 WEEK POST INJECTION	10.5	.2	12.8	.2	10.0	.3	12.3	.4
2 WEEKS POST INJECTION	10.4	.2	12.7	.2	9.9	.2	12.3	.3

Each mean is computed from 8 individual ring diameters.

TABLE XLIII

MEAN $^{L}/_{P}$ AT DIFFERENT TIMES AFTER INJECTION

I. SECONDARY RESPONSE MICE

TIME PLASMA WAS OBTAINED	TRIAL I		TRIAL II	
	MEAN $^{L}/_{P}$ RING RATIO	S.E.	MEAN $^{L}/_{P}$ RING RATIO	S.E.
PREINJECTION	1.240	.04	1.320	.04
2 HOURS POST INJECTION	1.240	.03	1.500	.08
6 HOURS POST INJECTION	1.280	.05	1.320	.04
12 HOURS POST INJECTION	1.250	.02	1.370	.05
24 HOURS POST INJECTION	1.250	.02	1.470	.06
48 HOURS POST INJECTION	1.220	.01	1.360	.03
72 HOURS POST INJECTION	1.190	.02	1.280	.02
1 WEEK POST INJECTION	1.180	.03	1.210	.02
2 WEEKS POST INJECTION	1.260	.02	1.210	.02

Each $^{L}/_{P}$ is computed from 8 individual $^{L}/_{P}$ values.

TABLE XLIV

MEAN $^{L}/_{P}$ AT DIFFERENT TIMES AFTER INJECTION

II. PRIMARY RESPONSE MICE

TIME PLASMA WAS OBTAINED	TRIAL I		TRIAL II	
	MEAN $^{L}/_{P}$ RING RATIO	S.E.	MEAN $^{L}/_{P}$ RING RATIO	S.E.
PREINJECTION	1.350	.06	1.500	.08
2 HOURS POST INJECTION	1.420	.06	1.590	.08
6 HOURS POST INJECTION	1.380	.06	1.430	.06
12 HOURS POST INJECTION	1.350	.04	1.560	.10
24 HOURS POST INJECTION	1.290	.05	1.560	.09
48 HOURS POST INJECTION	1.260	.02	1.440	.07
72 HOURS POST INJECTION	1.230	.02	1.380	.06
1 WEEK POST INJECTION	1.280	.03	1.270	.03
2 WEEKS POST INJECTION	1.270	.04	1.250	.01

Each $^{L}/_{P}$ is computed from 8 individual $^{L}/_{P}$ values.

TABLE XLV

MEAN $^{L}/_{P}$ AT DIFFERENT TIMES AFTER INJECTION

III. DBS CONTROL MICE

TIME PLASMA WAS OBTAINED	TRIAL I		TRIAL II	
	MEAN $^{L}/_{P}$ RING RATIO	S.E.	MEAN $^{L}/_{P}$ RING RATIO	S.E.
PREINJECTION	1.240	.02	1.660	.10
2 HOURS POST INJECTION	1.230	.02	1.550	.09
6 HOURS POST INJECTION	1.210	.01	1.350	.04
12 HOURS POST INJECTION	1.270	.01	1.580	.08
24 HOURS POST INJECTION	1.230	.02	1.720	.10
48 HOURS POST INJECTION	1.210	.01	1.490	.06
72 HOURS POST INJECTION	1.190	.01	1.440	.08
1 WEEK POST INJECTION	1.190	.01	1.310	.05
2 WEEKS POST INJECTION	1.300	.03	1.210	.02

Each $^{L}/_{P}$ is computed from 8 individual $^{L}/_{P}$ values.

TABLE XLVI

MEAN $^{L}/_{P}$ AT DIFFERENT TIMES AFTER INJECTION

IV. CONTROL MICE

TIME PLASMA WAS OBTAINED	TRIAL I		TRIAL II	
	MEAN $^{L}/_{P}$ RING RATIO	S.E.	MEAN $^{L}/_{P}$ RING RATIO	S.E.
PREINJECTION	1.240	.04	1.610	.09
2 HOURS POST INJECTION	1.220	.02	1.510	.10
6 HOURS POST INJECTION	1.220	.02	1.400	.08
12 HOURS POST INJECTION	1.270	.02	1.500	.10
24 HOURS POST INJECTION	1.290	.03	1.620	.10
48 HOURS POST INJECTION	1.200	.02	1.450	.06
72 HOURS POST INJECTION	1.190	.01	1.450	.09
1 WEEK POST INJECTION	1.230	.04	1.230	.05
2 WEEKS POST INJECTION	1.240	.03	1.240	.03

Each $^{L}/_{P}$ is computed from 8 individual $^{L}/_{P}$ values.