

THE IMMUNO-IDENTIFICATION OF THE BLOODMEALS
OF ERYTHROPHAGOUS ARTHROPODS

BY

MARTIN JUDE MICHAEL FALLON
B.Sc. (HONS) BRUNEL UNIVERSITY

Thesis submitted for the Degree of Doctor of Philosophy
University of London
and for the Diploma of Imperial College

Imperial College of Science & Technology
Department of Pure & Applied Biology
Silwood Park
Ascot
Berkshire
England

November 1986

The truth of it is, the first rudiments
of education are given very indiscreetly
by most parents

Richard Steele

1729

To whom this thesis is dedicated

Thank you

ABSTRACT

The identification of arthropod vector bloodmeals is of epidemiological importance, yielding information on host feeding preferences, insect behaviour and possible zoonoses. Over the last century or so various methods such as precipitin and passive haemagglutination inhibition have been employed. These methods were useful up to a degree, but fall short by today's standards, in their levels of sensitivity and specificity. In this research project efforts to improve these two criteria have been made.

A more versatile and sensitive assay has been developed, the enzyme linked immunosorbent assay (ELISA). This has been augmented by the preparation of some monoclonal antibodies to attempt to improve specificity.

Absorption studies on polyclonal rabbit antisera were performed with solid phase affinity columns and polymerized antigen. These experiments yielded specific immunoglobulins binding to blood antigens. These antigens were identified using immunoelectrophoresis and double gel diffusion, and were shown to be beta lipoproteins and complement component C_4 . Beta lipoprotein was isolated from human serum by ultracentrifugation and incorporated into a modified inhibition ELISA. In order to increase sensitivity and as a step towards a field method, this assay was converted to a 'dot blot' assay using nitro-cellulose acetate paper as the solid phase.

Due to the availability of an anti-human IgG light chain monoclonal antibody, a similar modified inhibition ELISA was prepared using human immunoglobulin as the antigen. The results were encouraging both for specificity and sensitivity, which led to a series of animal systems being set up, all incorporating immunoglobulin as the antigen.

To improve sensitivity further various non-soluble substrates have been tested in the 'dot-blot' assay with this series of animal systems.

A series of Anopheles stephensi mosquitoes were fed upon a human volunteer and kept at 25°C, being sequentially killed every 3 hours up to 70 hours post feeding. These were used to perform degradation studies on the bloodmeal components.

A number of approaches were used: 'dot blot' assay with both Ig and beta lipoprotein as the target antigens, Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) stained with coomassie blue and silver stain, and SDS-PAGE followed by Western transfer onto nitro-cellulose paper. The nitro-cellulose paper was then probed with a variety of antibodies and stained in a similar manner to that of the 'dot blot'.

Twenty six proteins were studied, Immunoglobulin, Beta lipoprotein and albumin remained intact the longest. Immunoglobulin and beta lipoprotein show the most promise for use as conjugated antigens in a inhibition ELISA.

ACKNOWLEDGEMENTS

I would like to offer my respect and thanks to my supervisor Dr.R.S.Bray, for his help, encouragement and friendship throughout the course of my studies, he has had to put up with me a lot more than I have had to put up with him.

I am also grateful for the interest and friendship shown by both my advisors, Dr.R.Killick-Kendrick and Dr.K.Hudson. I would also like to express my thanks to Professor E.U.Canning, Dr.R.E.Sinden, Dr.J. Alexander and Dr.D.J.Wright for their help and ideas.

I am deeply indebted to both Dr.D.Catty and Chandra Raykundalia from Birmingham University, who made my stay in Birmingham very enjoyable and profitable, and in whose laboratory much of the work contained in this thesis was performed. I also thank the staff of the Immunodiagnostic Research Laboratory (IDRL), situated at Birmingham University who supplied me with many antisera.

I thank Dr.Larry Winger and his wife Carrie who guided me successfully through the learning stages of tissue culture and extended much of their time and all of their facilities. I extend my gratitude to Dr. Ian Sinclair and David Wassel from the Central Veterinary Laboratories, Weybridge, who have been friends before and throughout these studies, and have always continued to assist and advise me whenever it was necessary, which was often.

For their technical assistance and superior experience I would like to thank Paul Nicholas, Carol Broadly and Veronica Gardiner.

Finally, I would like to show great appreciation to my friends, Gabriella Gibson, Muktari Amino Kano, Andrew Hamilton, Mohammed Jasim, Tony Wilkes, Golly Wrighton, Victoria Estaun-Morel, Jonathon Booth, Glenn Hall, David Noel, Nic. Prestwich, Janet Keen, Sally Thompson, Sally Spurrel, Karn Nielson and Karina Powell who have supported me in many different ways throughout my stay at Silwood Park.

CONTENTS

	Page
ABSTRACT	
ACKNOWLEDGEMENTS	
CONTENTS	
<u>INTRODUCTION</u>	1
Non-serological methods	1
i) Field observations	1
ii) Cytological Studies	2
iii) Other Studies	3
Serological methods	4
i) Precipitin test	4
ii) Passive haemagglutination Inhibition	8
iii) ELISA	9
iv) Dot blot	17
v) Other methods for Arthropod Bloodmeal	19
Identification	
Production of Antisera	22
Cross absorption procedures	29
Applications of blood meal identification	30
Aims	37

<u>CHAPTER 1</u>	38
PREPARATION OF BLOODMEALS IDENTIFICATION ASSAY	
1.1 INTRODUCTION	38
1.2 MATERIALS AND METHODS	41
1.2.1 Antisera production	41
1.2.2 Monoclonal antibody preparation	43
1.2.3 Specificity Test - ELISA	51
1.2.4 Isotyping of the monoclonal Antibodies	53
1.2.5 Gel diffusion with Specific antisera	54
1.2.6 Characterization of the monoclonal antibodies	57
1.2.7 Western transfer	59
1.2.8 Development of Western transfer with autoradiography	60
1.2.9 Counter current immunoelectrophoresis (CCIE) a modified precipitin test	61
1.2.10 Solid phase absorption	62
1.2.11 Insoluble gluteraldehyde polymerised antigen	65
1.3 RESULTS	67
1.3.1 Testing antisera used in bloodmeal identification	67
1.3.2 Solid phase absorption	75
1.3.3 Specificity testing murine monoclonal antibodies prepared to dog antigen	90
1.3.4 Sandfly host identification	92
1.4 DISCUSSION	99

<u>CHAPTER 2</u>	103
IDENTIFICATION OF THE BINDING CHARACTERISTICS OF THE REMAINING SPECIFIC ANTIBODIES POST SOLID PHASE ABSORPTION	
2.1 INTRODUCTION	103
2.2 MATERIALS AND METHODS	104
2.2.1 Immunelectrophoresis	104
2.2.2 Two dimensional electrophoresis (2-DIEP)	106
2.2.3 Gel diffusion	108
2.2.4 Beta lipoprotein extraction and purification by ultracentrifugation	110
2.3 RESULTS	111
2.4 DISCUSSION	121
 <u>CHAPTER 3</u>	 125
DEVELOPMENT OF INHIBITION ELISA FOR INSECT BLOODMEAL IDENTIFICATION	
3.1 INTRODUCTION	125
3.2 MATERIALS AND METHODS	129
3.2.1 Preparation of antigen and antibodies	129
3.2.2 Purification of IgG from antisera using DEAE column	130
3.2.3 Enzyme Linked Immunosorbent Assay (ELISA)	131
3.2.4 Development of Dot blot assay	132
3.2.5 Bloodmeal testing with dot blot assay	135
3.3 RESULTS	136
3.3.1 Inhibition ELISA	136
3.3.2 Dot blot ELISA	171
3.4 DISCUSSION	189

<u>CHAPTER 4</u>	192
DEGREDAATION STUDIES ON THE EFFECTS OF DIGESTION ON INSECT BLOODMEALS	
4.1 INTRODUCTION	192
4.2 MATERIALS AND METHODS	195
1. Preparation of mosquito bloodmeals	195
2. SDS-PAGE and Western transfer	195
3. Immunoprobng after Western transfer	195
4.3 RESULTS	198
4.4 DISCUSSION	204
 <u>CHAPTER 5</u>	 207
GENERAL DISCUSSION	
 REFERENCES	 210
 APPENDIX 1	 234

LIST OF FIGURES

Figure		Page
0.1	Haemagglutinin inhibition test	10
0.2	The competitive method of ELISA for assaying antigen	13
0.3	The double antibody sandwich ELISA for measuring antigen	15
0.4	Modification of the indirect ELISA for assay of antigen	16
0.5	The primary and secondary response of a rabbit when injected with antigen on two separate occasions	23
0.6	Synthesis of IgG and IgM antibody classes in the primary and secondary responses to antigen	25
1.1	Patterns of wells on a gel diffusion plate	56
1.2	Diagram showing the affinity column used for solid phase absorption	64
1.3	Activity of rabbit antisera measured on ELISA with Man antigen	68
1.4	Activity of rabbit antisera measured on ELISA with Dog antigen	69
1.5	Activity of rabbit antisera measured on ELISA with Ox antigen	70
1.6	Activity of rabbit antisera measured on ELISA with Badger antigen	71
1.7	Activity of rabbit antisera measured on ELISA with Buffalo antigen	72
1.8	Activity of rabbit antisera measured on ELISA with Avian antigen	73
1.9	Activity of rabbit antisera measured on ELISA with Reptile antigen	74
1.10	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Man antigen	77
1.11	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Dog antigen	78
1.12	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Ox antigen	79

Figure		Page
1.13	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Pig antigen	80
1.14	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Avian antigen	81
1.15	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Horse antigen	82
1.16	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Badger antigen	83
1.17	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Cat antigen	84
1.18	Activity of absorbed rabbit antisera, anti-Man, anti-Dog and anti-Ox measured on ELISA with Goat antigen	85
1.19	Activity of Rabbit anti-Man antisera prior and post absorption measured on the precipitin test	87
1.20	Activity of Rabbit anti-Dog antisera prior and post absorption measured on the precipitin test	88
1.21	Activity of Rabbit anti-Ox antisera prior and post absorption measured on the precipitin test	89
1.22	Comparison of the specificities of murine monoclonal antibodies, anti-Dog, tested with five antigens	91
2.1	Diagram of immunoelectrophoresis gel	105
2.2	Diagram of immunodiffusion gel	107
2.3	Diagram of two dimensional immunoelectrophoresis gel	109
2.4	Two dimensional electrophoresis, non-absorbed anti-human serum	112
2.5	Two dimensional electrophoresis, affinity column absorbed anti-human serum	113
2.6	Double gel diffusion. Absorbed antiserum with known anti-beta region proteins antisera	115
2.7	Two dimensional electrophoresis. Absorbed anti-human serum with human very low density lipoprotein	117
2.8	Two dimensional electrophoresis. Absorbed anti-human serum with human low density lipoprotein	118

Figure		Page
2.9	Two dimensional electrophoresis. Absorbed anti human serum with human intermediate density lipoprotein	119
2.10	Two dimensional electrophoresis. Absorbed anti human serum with human complement component C ₄	120
3.1	Standard curve of beta lipoprotein - HRP conjugate with sheep anti beta lipoprotein antibody	138
3.2	Competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti beta lipoprotein antibodies, testing lipoprotein fractions	142
3.3	Specificity test of competitive inhibition ELISA with beta lipoprotein - HRP conjugate and anti beta lipoprotein antibodies	146
3.4	Competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti beta lipoprotein antibodies, testing whole human blood and mosquito bloodmeal samples	150
3.5	Standard curve of IgG - HRP conjugate with the monoclonal antibody anti IgG	153
3.6	Competitive Inhibition ELISA, with IgG-HRP conjugate and the monoclonal antibody anti IgG, testing whole human blood samples	157
3.7	Inhibition ELISA, testing specificity of Dog antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Dog antibodies	159
3.8	Inhibition ELISA, testing specificity of Ox antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Ox antibodies	160
3.9	Inhibition ELISA, testing specificity of Hamster antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Hamster antibodies	161
3.10	Inhibition ELISA, testing specificity of Pig antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Pig antibodies	162
3.11	Inhibition ELISA, testing specificity of Rat antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Rat antibodies	163
3.12	Inhibition ELISA, testing specificity of Horse antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Horse antibodies	164

Figure		Page
3.13	Inhibition ELISA, testing specificity of Chicken antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Chicken antibodies	165
3.14	Inhibition ELISA, testing specificity of Guinea Pig antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Guinea Pig antibodies	166
3.15	Inhibition ELISA, testing specificity of Rabbit antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Rabbit antibodies	167
3.16	Inhibition ELISA, testing specificity of Sheep antigen conjugate, with all other species antigens, both prior and post absorption of the Rabbit anti-Sheep antibodies	168
3.17	Inhibition ELISA, testing specificity of Mouse antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Mouse antibodies	169
3.18	Dot blot inhibition ELISA (IgG-AP) with Napthol AS MX phosphate and fast blue BB base substrate.	173
3.19	Dot blot inhibition ELISA (IgG-AP) with Napthol AS MX phosphate and fast red BB base substrate.	175
3.20	Dot blot inhibition ELISA (IgG-AP) with Bromo chloro indolyl phosphate (BCIP) substrate.	177
3.21	Dot blot inhibition ELISA (IgG-AP) with with BCIP and Nitro blue tetrazoleum (NBT) substrate.	179
3.22	Dot blot inhibition ELISA (IgG-HRP) with amino ethyl carbazole substrate.	181
3.23	Dot blot inhibition ELISA (IgG-HRP) with alpha chloro napthol substrate.	183
3.24	Dot blot inhibition ELISA (IgG-HRP) with amino ethyl carbazole substrate kept for 15 days at 4°C.	185
3.25	Dot blot inhibition ELISA (IgG-HRP) with amino ethyl carbazole substrate kept for 15 days at 25°C.	186
3.26	Dot blot inhibition ELISA (IgG-HRP) with amino ethyl carbazole substrate kept for 30 days at 4°C.	187
3.27	Dot blot inhibition ELISA (IgG-HRP) with amino ethyl carbazole substrate kept for 30 days at 25°C.	188

LIST OF TABLES

Table		Page
1.1a	Identification of 230 sandfly bloodmeals using both the ELISA and CCIE, a comparison of the results.	93
1.1b	Summary table of the comparison of the results of ELISA and CCIE for sandfly bloodmeals.	98
3.1	Absorbance values for the standardization of the beta lipoprotein - HRP conjugate with different concentrations of sheep anti-beta lipoprotein antibody.	137
3.2a	Competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing lipoprotein fractions, absorbance values.	140
3.2b	Competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing lipoprotein fractions, percentage inhibition values.	141
3.3a	Specificity test of competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies, absorption values.	144
3.3b	Specificity test of competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies, percentage inhibition values.	145
3.4a	Competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing whole human blood and mosquito bloodmeal samples, absorbance values.	148
3.4b	Competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing lipoprotein fractions, percentage inhibition values.	149
3.5	Absorbance values of the standardization of the IgG - HRP conjugate and the monoclonal antibody anti-IgG.	152
3.6a	Competitive Inhibition ELISA, with IgG - HRP conjugate and the monoclonal antibody, anti-IgG, testing whole blood absorbance values.	155
3.6b	Competitive Inhibition ELISA, with IgG - HRP conjugate and the monoclonal antibody, anti-IgG, percentage inhibition values.	156
4.1	Summary table of Western transfer and immunoprobng results of <u>Anopheles stephensi</u> digested bloodmeals.	199

INTRODUCTION

The technique of serological identification of arthropod bloodmeals has been pursued for almost a century (Uhlenhuth, Weidanz and Angeloff, 1908) and has continued during that period to provide useful data in the various fields of its application.

The overall intention of the use of this technique is to contribute to the study of epidemiology of vector borne diseases, usually with a view to their control.

An essential feature of the study of arthropod borne diseases is the determination of the host preferences as sources of nutrient. These studies help to produce an accurate epidemiological picture, including the diagnosis of zoonotic reservoirs and the confirmation of vectoral capability including a man-biting rate.

Non-Serological Methods.

1) Field Observations.

Many Diptera are difficult to observe feeding in their natural state because of their small size and rapid feeding makes observation, at any reasonable distance, impossible. Observation at a closer range, in most cases introduces an unacceptable variable. If one is able to observe accurately, a wide range of observations is vital for any such data to be of use.

Despite these difficulties some workers have attempted this. Harrison, (1940), observed tsetse feeding on giraffe and warthog, but never on zebra; an observation later to be corroborated within the laboratory (Weitz 1952). Fiske (1920) and Swynnerton (1936) also

observed Glossina species feeding naturally, but it is doubtful if their conclusions are of any value.

Senior-White (1947) and Gillies (1956) monitored mosquitoes in an attempt to establish a correlation between host availability and the feeding habits of the insects. Their results were later not substantiated by Havelly (1958) with the result of laboratory identification tests.

Host preferences of the less dynamic arthropods, such as ticks can easily be studied by observation, in fact require no other techniques. (Hoogstraal, 1956).

ii) Cytological Studies.

Some determined efforts have been made to use cytological methods to identify the bloodmeals of tsetse. Lloyd, Johnson, Young and Morrison (1924) measured the diameter of the blood cells of numerous potential hosts of West African tsetse flies. Jackson and Vanderplank (1941) performed similar work in East Africa. The technique involved separation based upon cellular size and shape. At best such a method can only give an approximation. Not only is it necessary to appreciate the comparative morphology of the cellular components of the blood, but alterations which may follow digestion must also be taken into account. The digestion of cellular elements is often swift. Spriggs and Sladden (1958) pointed out that red blood cell size may vary in different pathological conditions and accordingly to the size of the animal. Age also can effect red blood cell size (Price Jones, 1933; Diamond, 1950).

Non-mammalian identification can be more easily performed with this method, the presence of nucleated cells allows of a reasonably accurate

result. Work by various authors confirmed that Glossina palpalis (Rob. Desv.) and G. tachinoides (Westwood) both feed on crocodiles and lizards. (Bruce, 1910; Fiske, 1920; Lloyd, Johnson, Young and Morrison, 1924; Johnson and Lloyd, 1923; Taylor, 1930).

iii) Other Studies

There have been a number of biochemical, but non-serological methods of differentiating serum species. They have either been impracticable or not used in practise. Munro - Fox (1945) with the aid of a Hartridge spectroscope distinguished the haemoglobin absorption bands of human and rabbit blood. Biocca (1950) and Grjebine and Fine (1954) suggested that the shape of the crystals of oxy-haemoglobin could be used, an idea first presented by Stirling and Brito (1882) studying the digestion of blood in the common leech. More recently Washino and Else (1972) considered the technique as a tool to supplement the precipitin test, when for various reasons it might fail. Boyd (1949) stated that plasma is rapidly excreted, causing the density of red blood cells in the mosquito gut to double, in view of this the authors felt the use of a test that did not depend on serum proteins might be more desirable.

Allison and Monton (1953) and Fine (1957) considered utilizing the Coombs' reaction but such methods are practicable only for the identification of human blood so far. Weitz (1960) after reviewing Cabannes and Serain (1955) wondered if chromatography and electrophoresis of the serum proteins might be useful. At the time he felt that the techniques were not sufficiently developed. Quite recently Chow, Yen and Bourgeois (1980) employed cellulose acetate and starch gel electrophoresis to identify mosquito bloodmeals as well as using precipitin and passive haemagglutination inhibition tests. Their conclusions were that starch

gel electrophoresis lacked any comparable sensitivity, only being able to identify bloodmeals up to 6 hours post ingestion. In contrast cellulose acetate electrophoresis performed consistently as well as the precipitin test. It remains to be seen whether there are any further developments in these areas.

Serological Methods.

i) Precipitin test.

The precipitin test has been the principle technique employed in arthropod bloodmeal identification and has been used extensively for over seventy years with only minor modifications.

Following the discovery by Richard Kraus (1897) that both filtrates of Bacillus pestis (= Yersinia pestis), the plague bacillus, and the cholera spirillum (= Vibrio), precipitate their respective antisera, the attention of workers turned to similar reactions with other proteins.

Tchistovitch's (1899) classical studies with eel serum led to the production of many antisera. Uhlenhuth (1901) found that human, horse and ox blood still gave reactions when extracted with physiologic saline. The work of mammouth proportions described in Nuttals 'Blood Immunity and Blood Relationships' (1904) established the importance and delicacy of the precipitin test in the identification of unknown bloods. His book, summarising over 16,000 precipitin reactions, using almost entirely the serum or egg proteins of vertebrates, demonstrated that the degree of reaction between a precipitating antisera for the proteins of one species and similar proteins of other species was correlated with the systematic positions of these species. The most intense reaction occurred when antisera were used with proteins of closely related

species. Little or no reaction was obtained when the proteins of distantly related species were tested. The degree of reaction was thus generally proportional to the closeness of the philogenetic relationships of the species tested. Bordet (1899) had reported a lack of species specificity with his rabbit antisera which was raised against chicken and yet reacted as well with serum from pigeons. Other workers, in similar studies have also used the precipitin test (Wolfe, 1939 and Boyden, 1942).

Uhlenhuth (1901) suggested a medico-legal application for the precipitin test, the identification of blood stains; Clarke (1914) analysed foodstuffs, while Goettsch and Kendall (1935) and Hewitt (1937) found the method a useful tool in protein analysis. Chow (1947) employed the test in pathological examinations.

Uhlenhuth, Weidanz and Angeloff (1908) first applied this method to the identification of the blood meals of arthropods, viz., bugs, fleas, lice and mosquitoes. They suggested its practical applications in the epidemiological studies of insect borne diseases, involving among others the still unknown virus of yellow fever!

Ascoli (1902), and Fernet & Muller (1910) made the test more delicate by the use of small glass tubes, looking for a ring of precipitate at the interface of the two reagents, as opposed to a general flocculation by just mixing the reagents; they called this the 'ring test' (Shicht-Oder ring probe).

Gozony, Hindle and Ross (1914) looked at the persistence of the 'precipitins' in antisera stored in vitro with preservatives for over 12 years, a heritage from Prof. Nuttal. The same authors did some of the first work on the digestion rates of bloodmeals and its effects upon the

reaction. They showed that bloodmeals from the ticks Ornithodoros moubata and Argas persicus still gave positive results after 23 months post feeding; whilst the louse Pediculus vestimenti gave positive results up to 3 days post feeding.

Hektoen (1918) published a work on the precipitin ring test for blood, confirming many of Nuttal's original observations. He suggested that specific absorption was not going to be of value as this reduced the sensitivity, however he also thought that diluting the antisera to reduce cross-reactions would increase the specificity of the test.

In the 1920's the method of precipitin testing for the determination of host selection by mosquitoes became of particular interest. Roubaud (1921) proposed a new theory of the competition among Anopheles for food, writing of A. maculipennis (Meigen), he postulated the existence of two physiological races with dissimilar feeding behaviours, one 'anthropophilic', the other 'zoophilic', more or less showing a preference for animals. Roubaud emphasized the epidemiological importance of 'animal deviation' in the spontaneous disappearance of malaria. Following these ideas Bull & King (1923) used the method of precipitin in the study of anopheline bloodmeals.

Further work was continued by Darling (1925). Within a decade or so a number of major studies had been published concerning the feeding habits of haematophagous arthropods. Glossinae (Prates, 1928, Buxton, 1948), mosquitoes (Rice and Barber, 1934, Davis and Philip, 1931, Rempel, Riddell and McNelly, 1946, Riddell, Rempel and McNelly, 1947 and Senior-White, 1947) and sandflies (Lloyd, Napier and Smith, 1924).

The next major review of the techniques concerned with bloodmeal identification was prepared by Weitz (1952) in his comprehensive 'The antigenicity of sera of man and animals in relation to the preparation of specific precipitating antisera'.

The author discussed all the methods involved in the procedure of identification, and included the results of some experiments performed. The paper concluded that animal sera contain two distinct groups of antigenic components; the homologous group (species specific) and the heterologous group (family specific). He demonstrated that absorption of the heterologous immunoglobulins from the antisera does not influence the titre of the homologous antibodies. He described the homologous antigenic component as specific for the species of the animal from which the serum used as antigen was derived and that this major specific component had associated with it minor components which were group specific for antigens in the sera of species of closely related mammals.

Conversely the heterologous antigen was said to be a more complex group of components and responsible for the production of immunoglobulins which reacted with the antigens present in most mammalian sera. Weitz concluded that this antigen must contain several components because it was possible to remove part only of its reacting immunoglobulins by absorption of an antisera with the serum of another animal species.

Cross immunization experiments between closely related species demonstrated the presence of specific antigenic material in the serum of individual species.

The precipitin test, though, had its short comings; it was not sufficiently specific for some of its applications, e.g. distinguishing

between the African bovid feeds of Glossina species. This problem led to the development of a more sensitive technique.

ii) Passive haemagglutination inhibition

Keogh, North and Warburton (1948) developed a method of sensitising sheep red blood cells with various chemicals which then allowed proteins to be absorbed onto the surface of the cells. If these cells were then exposed to an antiserum raised against the antigen that was absorbed onto the cell surface, agglutination of the cells occurred. This method amplified the antibody : antigen binding reaction to a greater degree than precipitin which required much larger amounts of material for a positive result to be visible.

Boyden (1951) experimenting with more sensitizing agents, like Tannic Acid showed that if the agglutinating antisera were first exposed to a sample of the absorbed antigen, agglutination did not occur and was in fact inhibited.

Weitz (1955 and 1956) exploited this for bloodmeals analysis, attempting to improve the schematic approach of his bloodmeal analysis system. In many cases, especially with tsetse bloodmeals, it had not been possible to identify beyond the family, but to go further was vital in the case of meals taken from bovid and/or suids. Passive haemagglutination inhibition offered a sufficiently higher degree of sensitivity and specificity to overcome the limited resolution power of the precipitin test and bring blood meal analysis beyond the family level.

Kirsh and Murray, (1969), described the process in more detail and showed it to be more sensitive than the capillary precipitin test (Murray, 1970) and the Ouchterlony gel diffusion method (Ouchterlony, 1948).

Other authors applied it to the identification of the bloodmeals of Culicoides (Murray, 1970) and mosquitoes, (Reid and Weitz, 1961; McClelland and Weitz, 1963; McCrae, et al., 1976 and Tempelis and Rodrick, 1972).

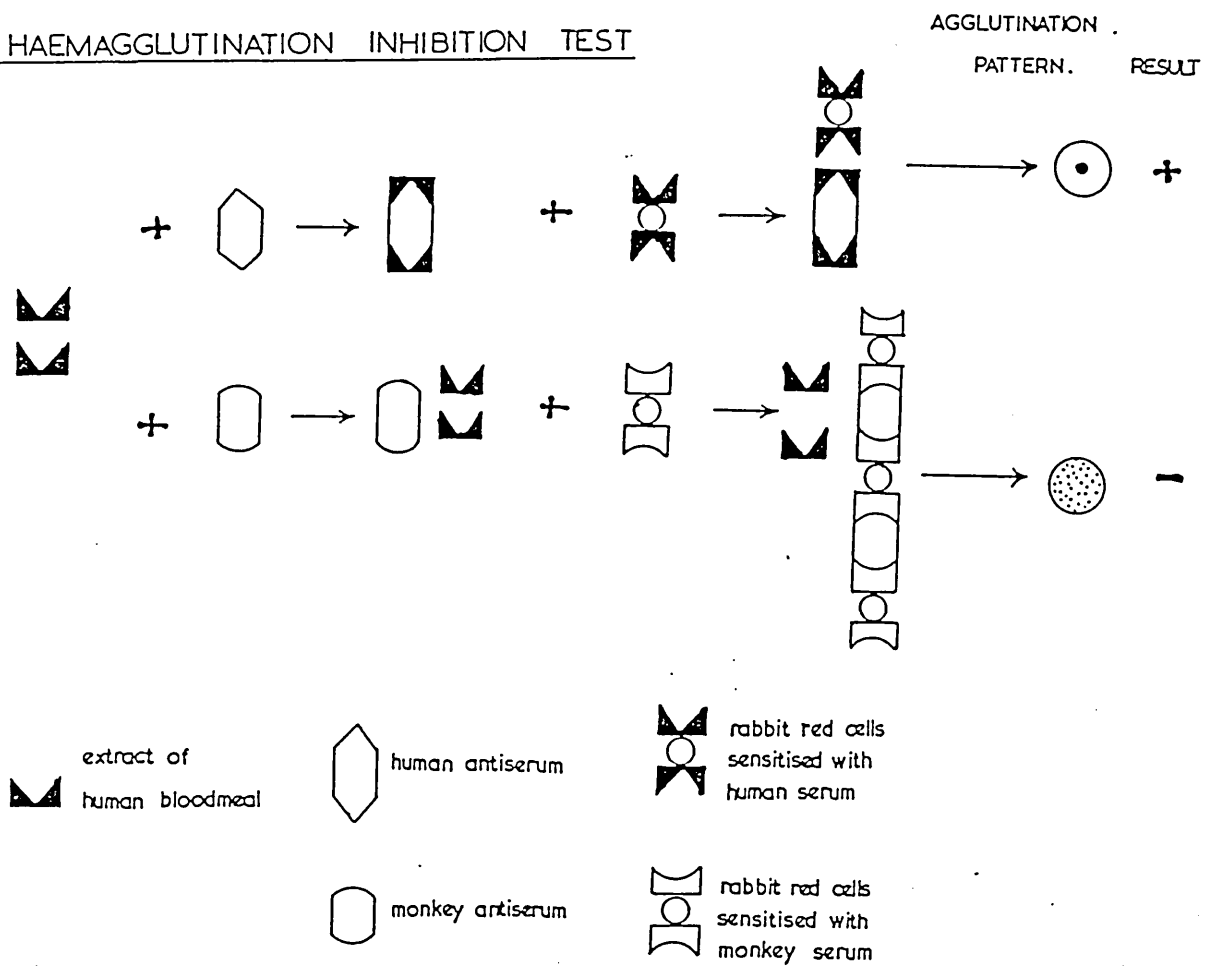
Boreham (1972) presented a schematic illustration of the haemagglutination inhibition test, which is used to identify bloodmeals from closely related species. (See Fig.0.1). The passive haemagglutination inhibition test is elaborate and not an easy method to perform. Experienced handling is necessary, for every batch of antisera being used, a calibration of dilutions needs to be performed accurately for the test to be of any use. Such practical problems restricted the use of the test to vital assessments only. Weitz (1956) prepared a procedure for the systematic analysis of arthropod bloodmeals. The scheme is a binary system of either/or and as soon as a result is achieved no further testing is performed. This is a weakness in the system because multiple feeds would not be recognised, the implication of which may be important epidemiologically. The scheme was a labour and material saving device, the tests with highest probability were performed first.

iii) ELISA

Carlsson et al., (1972), published a paper that described a new immunodiagnostic technique, the enzyme linked immunosorbent assay. This method is extremely versatile and adaptable and has been of tremendous value throughout the whole of the diagnostic sciences.

The ELISA offers many advantages over other possible assay systems based on immunodiffusion, immunoelectrophoresis, agglutination and immunofluorescence. These assay systems are less sensitive than ELISA,

Figure 0.1 THE HAEMAGGLUTINATION INHIBITION TEST



SPECIFIC IDENTIFICATION OF THE HOSTS OF TSETSE FLIES.

or radioimmunoassay (RIA) and require subjective interpretations of the antigen:antibody reaction. RIA has widespread usage due to its sensitivity and the fact that antigen:antibody reaction can be measured objectively by use of generally available laboratory instrumentation. However, although RIA is extremely valuable in many situations, a number of disadvantages are associated with the use of radioactive reagents. These include the relatively short half life of available gamma-emitting isotopes, the need for gamma - counting equipment, and the potential radiation hazard of the radioactive reagents.

The ELISA system offers the potential of great sensitivity but utilizes an objective non-radioactive indicator system. ELISA utilizes enzyme linked immuno reactants bound to a solid phase by a series of antigen:antibody reactions and measured by an enzyme - substrate reaction. Since a single molecule of enzyme can catalyse the conversion of a large number of molecules of substrate, the use of an enzyme in a reaction system provides the potential for extensive magnification of the antigen:antibody reaction. The enzyme conjugates used in ELISA systems are stable under a variety of storage conditions, and so can be stored until required (Averameas and Ternyck, 1971 and Pesce et al., 1976). The product of the enzyme reaction is visibly coloured, this has the advantage over other indicators such as agglutination, fluorescence or radioactivity in that the antigen:antibody reaction can be monitored by eye and measured quantitatively with colorimeters (Bullock and Walls, 1977; Yolken et al., 1977). Microtitre plate colorimeters are available that can measure a large number of reactions in a short period, making ELISA adaptable to large scale testing. Due to these advantages ELISA has been used not only for bloodmeal identification

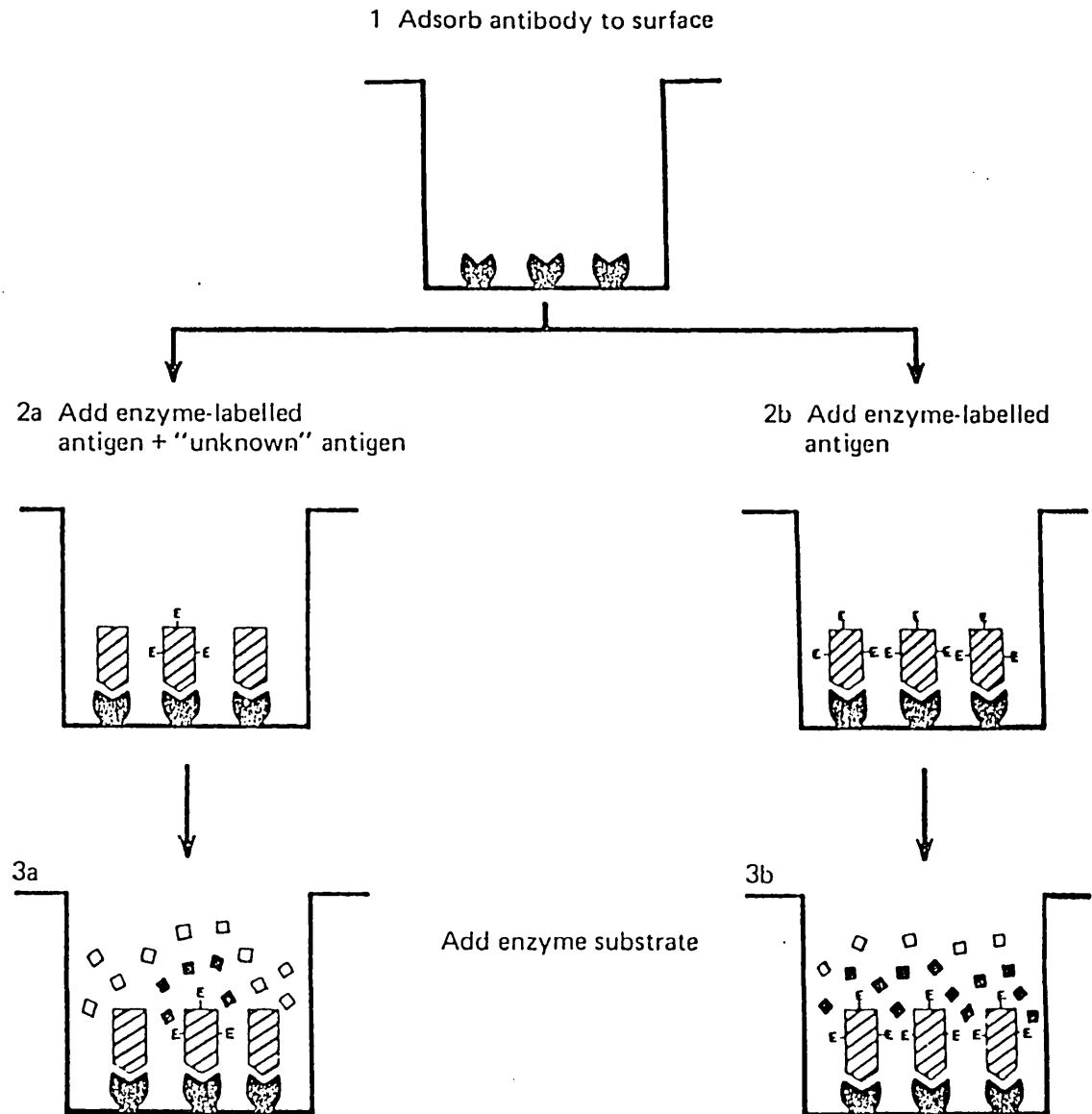
(Burkot et al., 1981) but a wide range of other diagnostic tests, bacterial (Pepple et al., 1980), viral (Yolken et al., 1977) parasitic (Voller et al., 1976) and fungal antigens (Warren et al., 1977) as well as immunoglobulin (Engvall and Perlmann, 1971), hormones (Pal, 1978), drugs (Gushaw et al., 1977), serum proteins (Belanger et al., 1976) and tumor antigens (Masseyeff, 1978).

Formulations of ELISA systems

There are a number of ways in which ELISA systems can be formulated to provide for the detection and measurement of antigen. The labelled-antigen competitive assay (Figure 0.2) has been widely used for the measurement of drugs, hormones and lipids in body fluids (Pal, 1978; Kominami et al., 1980 and Holmquist, 1980). This form of ELISA requires sufficient quantities of purified antigen, which are not always available. However, large quantities of antibody can be obtained by immunization of animals. Thus in many cases it is usually more practical to devise ELISA systems that make use of enzyme labelled antibody rather than enzyme-labelled antigen.

The ELISA utilizing labelled antibody can be performed in either a competitive or a non-competitive manner. In both cases, it is necessary to separate labelled antibody which has reacted with antigen from that which has remained unbound. This separation is generally accomplished by the immobilization of one of the reactants onto a solid phase (Scharpe et al., 1976). The use of a solid phase avoids the need for more complicated separation procedures such as precipitation and centrifugation, thus reducing performance time and simplifying the task of testing a large number of specimens (Herrman and Collins, 1976).

Figure 0.2 The competitive method of ELISA for assaying antigen



Substrate hydrolysis = labelled (antigen)

Difference between 3a & 3b = "unknown" (antigen)

THE PRINCIPLE OF THE ENZYME-LABELLED
ANTIGEN COMPETITIVE ASSAY FOR
MEASURING ANTIGEN.

The simplest form of a non-competitive assay is the direct assay, the double antibody sandwich ELISA, Figure 0.3, an unlabelled antibody is bound to a solid phase such as the well of a microtitre plate. This binding can be accomplished either by simple absorption of immunoglobulin to the solid phase or by covalent linkage (Yolken, 1980; Catt and Tregear, 1967; Herrmann and Collins, 1976).

In addition to coating the solid phase with antibody, antigen can be used, with the sandwich layer being one stage shorter and the performance time increased. This type of assay can either be a non-competitive or competitive assay (Figure 0.4).

The ELISA has been applied to bloodmeal identification. Burkot, Goodman and De Foliart (1981) and Edrissian and Hafizi (1982) independently described ELISA systems for mosquito bloodmeal identification. Both systems were sensitive and specific, yet there was a problem, after 24 hours of digestion of the bloodmeal, the test failed. The cruder precipitin technique was less sensitive to digestion as shown by West and Eligh, (1952).

Burkot and De Foliart (1982) successfully used the ELISA to study the bloodmeal sources of Aedes triseriatus (Say) and Aedes vexans (Meigen) in Southern Wisconsin, showing that the ELISA required far less material allowing many more tests to be performed.

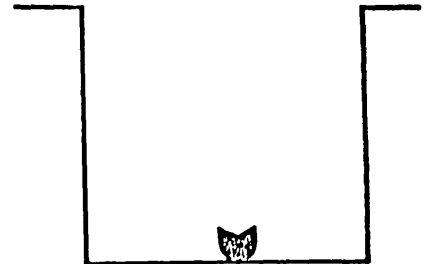
For the moment the future of successful bloodmeal identification seems to rest with the ELISA method. The system is highly sensitive, and as specific as the antisera used.

Kohler and Milstein, (1975) described the production of monoclonal antibody which may allow the ELISA to become as specific as necessary. In many cases monoclonal antibodies could be too specific, but the use

Figure 0.3 The Double Antibody Sandwich ELISA for measuring antigen

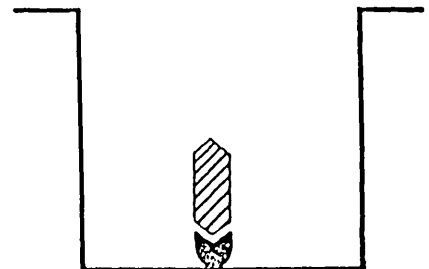
1 Antibody adsorbed to plate

wash



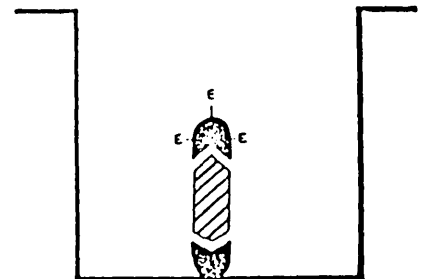
2 Test solution containing antigen added

wash



3 Add enzyme labelled specific antibody

wash



4 Add enzyme substrate

Amount hydrolysis $\equiv$ amount antigen present

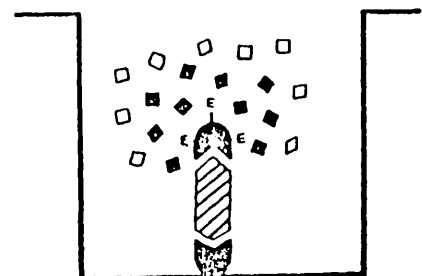
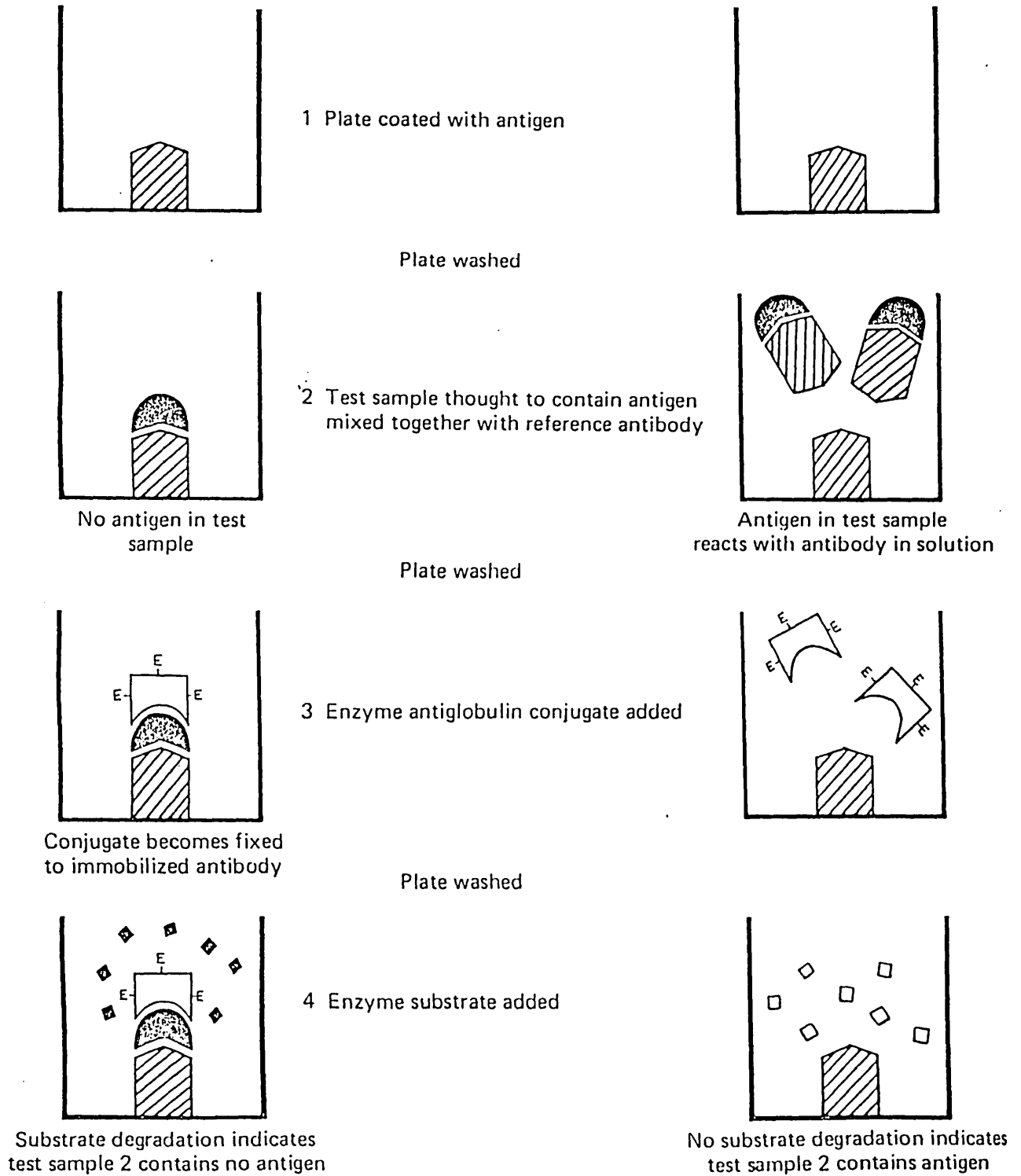


Figure 0.4 Modification of the Indirect ELISA for assay of antigen



of a cocktail of monoclonal antibodies, covering a range of key antigens should produce the appropriate assay necessary for species identification.

Burkot et al., (1984) used monoclonal antibodies to identify parasite material within mosquitoes, demonstrating the resolving power of the assay.

A development of the ELISA system is the Dot Blot assay, this technique is performed in exactly the same way except the whole process takes place upon cellulose acetate paper. It gives promise of a field assay system.

iv) DOT BLOT

In the past decade the enzyme linked immunosorbent assay (ELISA) has gained widespread application for rapid diagnosis of both antigens and antibodies (Sack et al., 1980; Voller et al., 1980; Iacona et al., 1980; Lunde et al., 1979; Hommel et al., 1978 and Pappas et al., 1983).

In ELISA, antigen attachment to a solid phase by passive adsorption is closely correlated to the binding characteristics of the selected carrier. The most commonly employed solid phase supports are microtitre plates made of polystyrene (Voller et al., 1977). The actual concentration of antigen bound per well is not known and certain antigens may bind weakly or not at all to the well surface. Sagonen and Vakein (1979) reported that only 10% of the amount of IgG initially applied bound to the polystyrene well. Towbin et al., (1979) demonstrated the excellent binding characteristics of nitrocellulose for protein binding in quantitative blotting procedures. Recently, Hawkes et al., (1982) described a modification of the 'dot hybridization' method of Kafatos et

al., (1979), where minute volumes of monoclonal antibodies were directly applied to nitrocellulose.

Nitrocellulose is produced by allowing thin films of nitric acid esterified cellulose, solubilized in a mixture of organic solvents, to solidify by controlled evaporation of the solvents. (Handman and Jarvis, 1985). Nitrocellulose was designed as a 'surface filter' for micro filtration of bacteria, which are retained at the surface of the matrix. In its use for blotting, nitrocellulose serves as a 'depth filter' in which macromolecules are absorbed throughout the matrix, predominantly by chemical interaction rather than sieving (Gershoni and Palade, 1983). It is not clearly understood how macromolecules bind to the nitrocellulose matrix, but hydrophobic effects are believed to play a role. (Gershoni and Palade, 1983; Schneider, 1980 and Farrah et al., 1981.)

Nitrocellulose has thus proved to be a suitable solid phase upon which to perform modified ELISA tests (Pappas et al., 1983; Bode et al., 1984; Kumar, et al., 1985; O'Connor and Ashman, 1982; Berger et al., 1985; and Lombardi and Esposito, 1986). These authors have described an increase in sensitivity compared to that of the conventional ELISA. An 8 to 10 fold increase in sensitivity is seen and attributed to the better binding properties of nitrocellulose compared with polystyrene. (Bode, et al., 1984). Hawkes (1982) describes a visible dot at concentrations of 0.1 ng. of antigen. Using nitrocellulose, the antigen is completely immobilised, which is better for producing dots of known concentration and thus overall reproducibility, unlike soluble antigen coating in the conventional ELISA (Berger, Thornburg and Pirone, 1985).

Nitrocellulose ELISA sensitivity compares favourably with some of the recently developed highly sensitive ELISA carriers, e.g. high affinity carriers for Ig, Poly vinyl chloride (Lems Van Kan et al., (1983) or covalent linkage (Sack, et al., 1980).

The use of various insoluble substrates to the enzyme commonly employed in histochemical studies can improve sensitivity. Better results being achieved with a greater contrast between the background and the colour of the dot. (Pappas et al., 1984). The application of a number of possible substrates have been tested in these studies.

Kumar, et al., (1985) showed that antigen remained stable for at least 4 months both at room temperature and at 37°C. This aspect has also been investigated with a view to applications in the field.

Lombardi and Esposito (1986) described a method of identification of mosquito bloodmeals by the immunobinding of peroxidase - antiperoxidase complexes on nitrocellulose. The authors described a comparable sensitivity with that of conventional ELISA, and 25 fold increase in sensitivity with bloodmeals that had been digested for longer than 20 hours. These results are an improvement on the ELISA described by Burkot et al., (1981) where only 75% of mosquito bloodmeals were successfully identified within 12 hours after feeding. This technique has been developed to identify only human bloodmeal.

The present studies include work for a dot blot ELISA system to identify a comprehensive range of sera of animals to the species level.

v) Other Methods for Arthropod Bloodmeal Identification

Many of these methods represent adaptations and modifications of the commonly used precipitin test, designed to improve the technique.

Ouchterlony (1948) described an agar gel diffusion of antigen and antibody through agar gel so that precipitate reactions leave a distinct band in the gel. Chamberlain and Sudia (1967) first applied this method to bloodmeal identification. A number of authors have since used and assessed this technique, Crans (1969) who tested over 10,000 field collected engorged mosquitoes, reported that it does not allow for large numbers of tests at any one time because of the agar slide preparation. It does allow the retention of a permanent record after the slides have been dried and stained. Chamberlain and Sudia (1967), commented on the advantage of this method over the precipitin test when using cloudy antisera which can obscure the results, but remarked that its sensitivity is less than that of the capillary precipitin method. Eliason (1971) suggested that relatively large amounts of reagents are required. However, Carpenter (1975) reported that gel diffusion can be 3 to 100 times more sensitive than the capillary precipitin test, now that agar production has been improved.

Boreham and Gill (1973) used another gel precipitin method, known as counter current immunoelectrophoresis (CCIE). The method is almost identical to gel diffusion except the antibodies and bloodmeal are electrophoresed towards each other. This method is said to be useful when analysing small bloodmeals such as from sandflies.

Rao (1984) developed a simple assay that could be used 'on the spot' in a field situation, despite these reports and those of others (Herndon and Ringle, 1967; and Collins et al., 1983) this technique has not been used generally.

Eliason (1971) described a similar method, gel surface precipitation. In this method a slide coated with agar is 'charged' with a small

amount of antisera. The eluted bloodmeals are added dropwise to the coated slide. Those bloodmeals that react with the antisera form a precipitate. Its sensitivity is claimed to be comparable to that of other methods, yet is really only practical for testing human feeds on single species.

Another modification of the precipitin test has been described by Tesh et al. (1971), the microtitre flocculation test. The author was working with phlebotomine sandflies and experienced difficulties with the capillary precipitin test. Small amounts of bloodmeals are placed in the wells of microtitre plates which contain antibody, allowed to stand for 3 hours. The formed precipitate is then centrifuged and the resultant reaction is viewed from below with a mirror. Its major drawback is that a lot of material is required.

As well as ELISA other antibody labelled techniques have been described, Gentry, Moore and Hayes (1967) developed a fluorescent antibody technique. The procedure used a direct immunofluorescence, small discs prepared from nitrocellulose filter papers were dipped into a buffer containing the bloodmeal antigen, dried, then a small drop of conjugated antibody added to the paper disc, dried again and washed in fresh buffer containing no antigen. The results were viewed under a U.V. lamp. The method failed to identify bloodmeals taken from mosquitoes after 24 hours of digestion. This technique represents a very early attempt at the technique of dot blot assays, which will undoubtedly begin to use chemiluminescent substrates.

McKinney, Spillane and Holden (1972) pursued similar studies, but not involving nitrocellulose paper, instead smears of the mosquito bloodmeals on microscope slides were prepared. The authors encountered

problems with cross reactions, yet had more success with digested bloodmeals, getting positive results up to 44 hours post ingestion by the mosquito. This improvement would be due to their use of a U.V. microscope to record the results, far more sensitive than a U.V. lamp.

Production of Antisera

When a foreign protein or antigen is inoculated into a vertebrate animal it stimulates the production of antibodies. These antibodies have various properties in vivo, they can also be harvested by bleeding and utilised in vitro. In vitro a major function is to bind to the antigen that stimulated their production. This binding often results in precipitation of the antigen from a soluble state to an insoluble antigen:antibody complex. In this way the occurrence of the antigen:antibody union is visible. This binding function is the basis of all the immuno assays developed.

When an antigen is first inoculated into a rabbit, the primary response occurs. This induces the activation of the small lymphocyte population, which leads to the production of antibody and the proliferation of an expanded population of antigen sensitive memory cells, which are capable of a faster response to the antigen. This combination of increase in cell number and more rapid maturation after a further antigen triggering, is responsible for the characteristically heightened course of the secondary antibody response. See Figure 0.5.

To improve antibody responses adjuvants are used (Freund et al., 1940). An adjuvant is a substance incorporated into or injected simultaneously with antigen which potentiates the immune responses (L.adjuvate = to help).

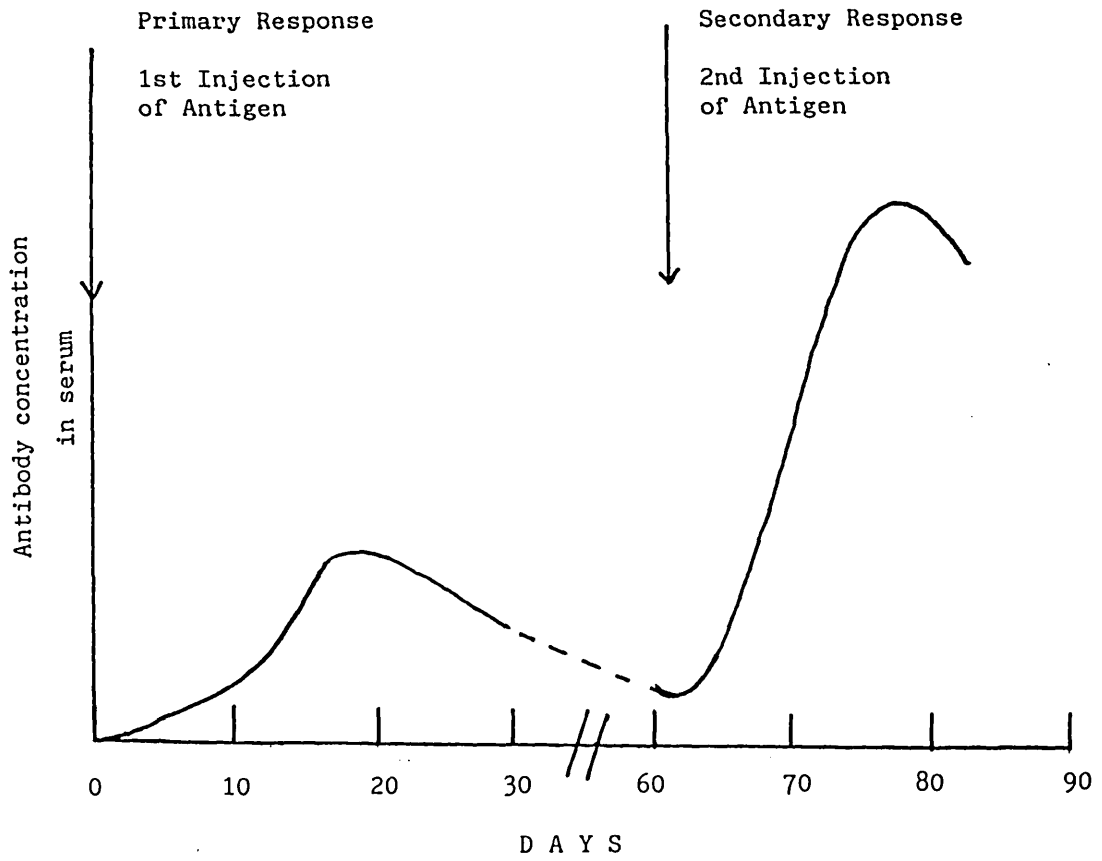


Figure 0.5 A rabbit is injected with antigen on two separate occasions. The antibody response on the second contact with antigen is more rapid and more intense. (After Roitt)

Adjuvants have a number of modes of action.

- 1) Depot effects: Free antigen very quickly disperses from local tissues, repository adjuvants counteract this by providing a long lived reservoir of antigen, at an extra-cellular location or within macrophages. Examples of these are aluminium compounds and Freund's incomplete adjuvant, a water in paraffin oil emulsion. Both types increase the antibody response but the emulsions tend to increase the antibody response to include a broader spectrum of the epitopes.
- 2) Macrophage activation: Under the influence of repository adjuvants, macrophages form granulomata which provide sites for interaction with antibody forming cells as well as further enclosing the antigen providing a further depot effect. The maintenance by the depot of persistent antigen concentrations, particularly on the macrophage surface, ensures that as antigen sensitive cells proliferate in the granuloma their progeny will be likely to be sensitised and stimulated by the antigen. This amplifies the effect.

Freund's complete adjuvant (FCA) (i.e. incomplete with added killed mycobacteria) acts on macrophages through T cells. Macrophages improve immunogenicity, with increased surface antigen and efficiency of its presentation to lymphocytes. They provide accessory signals and release interleukin I which assists B cell proliferation and thus antibody production.

The mycobacteria component of FCA have striking direct effects upon lymphocytes, enhancing T-helper cell activity and therefore B cell proliferation and antibody production.

IgM is a pentameric, intra vascular molecule, preceeding IgG in the phylogeny of the immune response. Due to its high valency it is a good precipitin and agglutinin. See Figure 0.6 (Roitt, 1984).

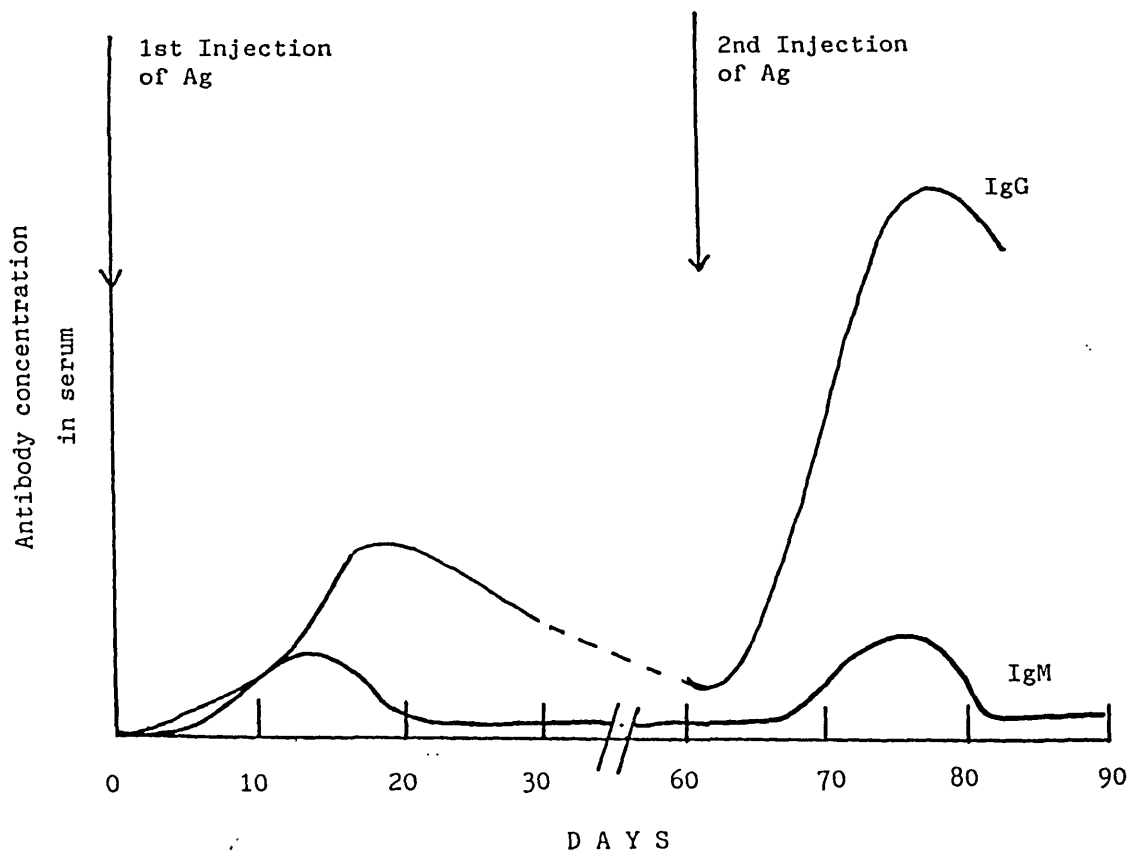


Figure 0.6 Synthesis of IgG and IgM antibody classes in the primary and secondary responses to antigen. (After Roitt)

In order to produce an antiserum of high specificity and precipitating ability one should evoke the primary response, then the secondary response, harvesting the antisera at the optimum for IgM and IgG.

The immunization protocols of different workers for preparing antisera are very varied. Bull and King (1923) gave a series of four intravenous injections on alternative days. Prates (1928) stated that all investigators are agreed that the intravenous injection produces prompter and more complete immunization.

Davis and Philip (1931), Laurel (1931) and Wolfe (1935), who used a shorter period of immunization all used the intravenous route, as did Romana (1939). Symes and McMahon (1937), Roy and Ganguli (1943), Arnold, Simmons and Fawcett (1946), Rempel, Riddell and McNelly (1946) and Schubert and Holdeman (1956) immunized over a longer period. Proom (1943) recommended the use of a single injection of alum-precipitated serum intra-muscularly, as species specificity was obtained more regularly. When he immunized the animals again, he found the sensitivity of the antisera increased but with a complete loss of specificity. This was because he used an adjuvant that exposed the antigen for a much longer period, thus allowing a response to the less immunogenic but more broadly based determinates. Leone (1952) obtained similar results. Weitz (1952) repeated Proom's method but was unable to obtain 'a specific antiserum'; in order to achieve the required specificity Weitz absorbed the antisera with other animal sera to remove the immunoglobulin component that bound to the 'heterologous' antigen. Weitz (1952) also avoided the use of preservatives by freeze drying the antisera after removing the lipid fraction with ether (McFarlane, 1942).

Studies carried out during the Second World War showed that birds were playing an important role in the epidemiology of mosquito-borne arbovirus diseases (Reeves and Hammon, 1962). It became apparent that a good method was needed to produce antisera that would differentiate bloodmeals from within the avian group. West (1950) had raised antibird sera in rabbits, unfortunately their sera showed no specificity, attempts were made to absorb the sera, this only rendered them completely inactive. Tempelis and Reeves (1962) and Tempelis and Lofy (1963), introduced the widespread use of the chicken as the antibody producer. Prior to this, the chicken had been shown to be a good precipitin producer, (Johnson and Rawson, 1927; Wolfe and Dilks, 1946). The antisera produced in the chicken was order-specific for all the avian species, and species specific for the gallinaceous birds. Tempelis and Reeves (1962) produced antichickens serum in the pheasant. Tempelis and Lofy (1963) reported improved antibody specificity to mammalian serum proteins. Other workers used these recommended procedures, Pennington and Phelps (1968); Edman (1971); Cupp and Stokes (1976). However it seems that the greater success reported by these authors is not a reflection of the animal used, but the immunization protocol that was followed.

Boreham and Gill (1973) published a protocol for producing high titrated, specific antisera with small amounts of antigen. They simply followed the procedure outlined at the beginning of this section, i.e. inducing a primary response, a secondary, with the harvest occurring at the optimum time after the second injection. They also used Freund's complete adjuvant and inoculated the antigen into the popliteal and axillary lymph nodes, i.e. lymphocyte centres. The authors quoted a

titre of 1 in 100,000, this sounds tremendously high but an anomaly exists. Most immunologists express an antiserum titre as the relative measure of the amount of antibody in the fluid, usually serum that is the greatest dilution of the fluid capable of producing a particular detectable antibody-antigen reaction. It is expressed as the proportion to which the fluid has been diluted. The relative amounts of antibody present can thus be compared in different specimens of the fluid (Abercrombie, Hickman and Johnson, 1975). The figure quoted by Boreham and Gill (1973) represents the antigen dilution against whole antiserum. Throughout the whole of the literature up until the 1980's this representation exists. It also suggests that no serious attempts were made to dilute the antisera, a device that often reduces cross reactivity because the predominant population of immunoglobulins tend to be raised against the most immunogenic component. It was found that reactions said to have a titre of 1 in 100,000 in fact employed sera of a titre of only 1 in 16 - 128 (Bray, pers. comm.). In this light much of the system could have been improved. It makes a nonsense to compare two antisera with two different titres when the comparison that is being made is between the two antigens. Throughout this project any titre quoted refers to the dilution of the antisera and not the antigen.

Despite confusion, immunizing techniques have finally evolved to an efficient and productive degree. The exact protocols vary in antigen concentrations, the number of inoculations and time intervals between inoculations, but the principles are the same. The procedures for immunising mice in preparation of monoclonal antibodies are the same, the difference is that spleen cells are harvested for the fusion and not serum.

Cross Absorption Procedures

Proom (1943) and later other workers observed that specificity of antisera decreases progressively as the course of immunization is repeated. Weitz (1952) performed some experiments to improve the specificity of the antisera. Weitz performed a preliminary titration of the 'heterologous' antigen with each antiserum. A constant amount of antiserum was introduced into each tube and varying amounts of the serum antigen were added as a crude absorption method. The tubes were incubated then centrifuged to remove the precipitate. Each sample was then tested with precipitin test to determine the presence or absence of heterologous antigen separately. The proportion of antigen to antiserum required for absorption of each heterologous antibody was indicated by the tube in which the antiserum had been absorbed with the highest dilution of antigen and whose supernatant fluid gave no positive reaction with the corresponding antigen.

The bulk of the antiserum was then absorbed by adding the correct amounts of neat antigen, previously determined by the preliminary titration. These antisera were then tested with both the homologous antigen and heterologous antigen. It was shown that considerable specificity had been achieved, it was from these experiments that the term homologous and heterologous antigen appeared.

Weitz stated that the homologous reaction takes place to a varying degree with the sera of zoologically related species whilst the heterologous reaction occurs with all mammalian sera from zoologically related or unrelated species.

Weitz 's expression of titre is a measure of dilution of the antigen not the antisera, the implications of which have been discussed

previously, He might well have achieved improved results by diluting his antisera. Liquid phase absorption is not entirely satisfactory at removing all the cross reactivity antibodies. With both antigen and antiserum in the liquid phase it is very difficult to remove all the complexed antibody. No amount of centrifugation will entirely remove the complexes, leave some remaining which can deviate. The free antibodies then interfere in assays subsequently performed with the antisera. This is more critical with sensitive assay like ELISA, but also cross reactions have been seen with the precipitin test after liquid phase absorption (Bray pers. comm.). For these reasons solid phase absorption is more desirable.

Applications of bloodmeal identification

The blood-feeding contact of insects and man is a key factor in determining the role of insects as vectors of human disease. Very few species of haematophagous insects feed exclusively on man as the sole host. The presence of other potential mammalian hosts, such as domestic animals, causes them to deviate from man to a varying degree.

The successful transmission of some insect-borne diseases demands a higher and more frequent degree of biting contact with man than others. With malaria, urban filariasis, urban yellow fever and dengue, for example effectively the mosquito can only become infected by initially feeding on an infective human subject. These cited pathogens all undergo developmental changes or multiplication in the mosquito host before the mosquito becomes infective, the time varies with each pathogen. After this time the mosquito is capable of transmitting the infection to a new host whilst feeding. If the mosquito feeds indis-

criminally on other mammalian hosts as well as man, it might never become infected in its lifetime. If it only feeds infrequently on man, the host may not be in an infective stage. The mosquito may acquire an infection by feeding on an infective host, yet then feed on another mammal, insusceptible to the human pathogen or the mosquito may not live long enough to become infective. Considering these possibilities, the successful transmission of such human pathogens relies on the mosquito taking a minimum of two human bloodmeals on different hosts in the course of its life. These feeds also need to be separated by an interval long enough for the infection acquired in the first feed to develop to the infective stage in the mosquito.

Those human diseases in which animals (mammal or bird) other than man act as reservoirs of infection are called zoonoses, and the minimum two blood feeds on man is not essential to secure transmission. The insect vectors normally acquire their infections by feeding on an animal which will tend to be the major host. Once the infection has developed to an infective stage, a single feed on man is sufficient to transmit the disease pathogen.

Examples of zoonoses are varied, including sandflies and leishmaniasis, tsetse flies and trypanosomiasis, triatomid bugs and Chagas' disease. The majority of mosquito borne zoonoses are viral diseases, including jungle yellow fever and various encephalitides such as Western Equine encephalitis, Eastern Equine encephalitis and St. Louis encephalitis.

In studying the feeding patterns and host selection of insects, the emphasis and the approach to the problem differs according to which of the two categories above is involved. With the non-zoonotic diseases

the main interest is to establish the relative attraction of mosquito to man versus his domestic animals and the extent to which these animals provide alternative sources of bloodmeal. In the case of the zoonoses group, alternative hosts to be considered are frequently wild or feral animals, as well as domestic animals, which are the natural reservoirs of the diseases, and the animals on which the insects must feed in the first instance in order to acquire infection.

Studies on arthropod host preferences have concentrated on one side on man versus domestic animals in the domestic environment, and on the other on man versus wild animals as alternative hosts in the wild or feral environment. The different approaches can be grouped under five headings:

- 1) Captures of insects feeding on man and on tethered domestic animals in the village environment.
- 2) The use of immunoassays to identify the source of bloodmeals from captured engorged insects. The capture preferably not involving a source of a bloodmeal.
- 3) Trapped animal comparison experiments, offering a choice to an insect.
- 4) Comparison of insect captures in traps baited with different feral mammals and birds.
- 5) Combination of two or more of the above.

A knowledge of insect-host relationships is important to understand the epidemiology of insect borne diseases of man and animals. Host preference studies that have been described yield a lot of information which in most cases is presented in a crude form, however, much work has been done to utilize these data in a more useful way.

Garrett-Jones (1964), expanding the original work of Ross (1911) and MacDonald (1957) in pioneering methods to model and measure the rate of disease (malaria) transmission by haematophagous insects devised the term 'vectorial capacity'. The ability of a disease to persist in a community is defined as the 'basic reproductive rate, R_0 ', which is directly proportional to the entomological component of the disease, the vectorial capacity, C . So, $R_0 = C/r$, where $1/r$ is the duration of the infection in man. Vectorial capacity can be obtained from the product of the 'man biting rate', i.e. the number of bites per person per day and the 'man biting habit' which is the mean frequency with which an individual female mosquito bites man, together with 'longevity factor', the expectation of the mosquito's infective life.

Garrett-Jones (loc. cit) defined the term the human blood index (HBI), which had earlier synonyms such as 'anthropophilic index' and 'human blood ratio', as the most important element, it represents the proportion of fed Anopheles found to contain human blood. This being determined by mosquito bloodmeal analysis. The HBI is stressed as being important because it forms a component of both the man biting rate and the man biting habit, thus vectorial capacity is proportional to the square of HBI.

The HBI of a population can only be assessed by sampling, and it is never possible to know whether the samples that are taken are fully representative (Garrett-Jones et al., 1980). All the methods of taking samples using baited traps, introduce bias (Service, 1976), for example there may be, in any similar situation, an unrecorded disparity in the prevalence (or availability) of the alternative resting places. A second variable to be considered is the density of blood fed insects in different capture stations. Thus the relative prevalence of two or more biotopes used by resting fed flies and the relative densities of blood fed females in each biotope, are essential data for interpreting the HBI of any vector population.

Another epidemiologically important factor to consider when studying the feeding preferences of insects, is relative availability of alternative hosts. Some mosquitoes are described as 'fixed feeders' preferentially feeding on one host, this feeding pattern obviously deviates if the preferred host is unavailable. Hess et al. (1968) made use of the forage ratio technique to study these questions. The technique was originally used to study the food habits of fishes (Hess and Rainwater, 1959) and water fowl (Bellrose and Anderson, 1943). The procedure simply compares the percentage use with the percentage abundance. Applied to mosquitoes, this is the percentage of engorged flies fed on a given vertebrate host divided by the percentage which it comprises of the total population of hosts available in the mosquitoes biotope.

A forage ratio of one (or near one) indicates neither preference nor avoidance of the indicated host animal; forage ratios significantly greater than one indicate selective preference; and values less than one indicate avoidance in favour of other hosts.

This technique though has major difficulties associated with it, obtaining even reasonable estimates of the population of possible vertebrate host in a given biotope proves very difficult. However, Hess et al., (loc. cit.) stated that with rough estimates of the animal populations, the results were much more meaningful than they would have been without any information on host availability. Hayes et al., (1973) have used this method to investigate the possible seasonal shifts in feeding patterns by mosquitoes.

Edman (1971) criticising the forage ratio stated that the technique neglected ecological and behavioural differences among host and mosquitoes. He went on to conclude that each situation where the forage ratio was used must be evaluated separately and on the basis of the prevailing circumstances. Edman and Kale, 1979 considering the limitation of the forage ratio, defined a further term concerning host preferences, the 'feeding index' which is defined as "the proportion of feeds on one host with respect to another divided by the expected comparative proportions of feeds on those 2 host based on factors affecting feeding". These factors include abundance and size of hosts, their temporal and spatial concurrence with the mosquito and its feeding success.

This can be expressed mathematically as:

$$FI = \frac{Ne/Ne^1}{Ef/Ef^1} \quad \text{Where FI equals the feeding index,}$$

Ne , the number of feeds on host I,

Ne^1 , the number of feeds on host II,

Ef , the expected population of feeds on host I,

Ef^1 , the expected population of feeds on host II.

An index of 1.0 indicates equal feeding on the two hosts being compared while figures less than one or greater than one indicate a decrease or increase in feeding on the first host relative to second. Similar problems were faced with this method, calculating abundance of hosts proved difficult and when the authors considered host biomass as a factor all their results reversed contrary to previous observations and expectations. The authors stated that the main advantage of the technique is that it departs from the inference that feeding patterns are attributable to innate host preference, although they may well be, but also draws attention to other multiple influences that operate, eg. host size, abundance, zooprophyllaxis, rate of exhalation of host, and spatial and temporal relativities between the hosts and mosquitoes.

AIMS

Bloodmeal identification still has a very important role to play in helping to understand the epidemiology of insect borne diseases. In the 1950's much insecticidal work was performed in an attempt to reduce malaria transmission and disease incidence. Limited success only was achieved. Various factors, such as resistance to insecticides and anti-malarial drugs have compounded the problem causing the situation to deteriorate to an even more critical level.

The pressure on epidemiologists to understand fully the transmission dynamics of the disease, both parasitologically and entomologically, is even greater. This is where the availability of a simple, accurate and reliable identification assay is required.

The aim of this study has been to investigate the areas where improvements in blood meal analysis can be made. There are four major areas of concern: sensitivity, specificity, simplicity and economy. A further aim has been to adapt the improved assay for use in the field.

CHAPTER 1

PREPARATION OF BLOODMEALS IDENTIFICATION ASSAY

1.1 INTRODUCTION

The key step in any bloodmeal immunoassay is the reaction of the specific antibody with the unknown antigen. The kinetics of these interactions can be described by the law of mass action (Ekins, 1980 and Pesce et al., 1978). The concentration of antigen:antibody complex is proportional to the concentration of antigen, the concentration of antibody utilized, and the affinity constant of the antibody. In an immunoassay the antigen can be detected and measured only when it is complexed with the specific antibody. Thus the sensitivity of an immunoassay depends largely on the concentration of antibody and the avidity of the antibody utilized. Because the non-competitive systems involve multiple washing steps, the affinity constant is particularly important since disassociation of antigen:antibody complexes will lead to elution of bound antigen from the system (Pesce et al., 1978). Therefore it is important that only high-avidity antibodies be utilized in solid-phase immunoassay systems if maximal sensitivity is to be attained.

The second important determinant of sensitivity is the concentration of specific antibody. This is determined not only by the concentration of immunoglobulin in the antisera but also by the proportion of immunoglobulin specifically directed at the antigen molecule. Many polyclonal antisera have been shown to only contain 3 to 10% specific antibodies of the total immunoglobulin content (Kalica

et al., 1944). Since the use of antibody at a concentration lower than the affinity constant of that antibody results in the binding of a small fraction of antigen to antibody, the concentration of antibody is the principal limiting factor in the sensitivity of solid phase immunoassay systems.

To achieve accurate host species identification of arthropod bloodmeals, the assay needs to be sensitive and specific. The most important component, the antiserum, should be both monospecific and of high titre, unfortunately this is not always the case.

Normally the supply of antibody is from an immunised animal such as a rabbit or sheep. Such antisera contain a variety of antibody populations, some specific others less so. A good antiserum is one that shows a high titre for the specific antibody.

Immunization protocols are designed to harvest antisera at an optimum for the specific antibody population, nonetheless many cross reactions commonly occur. The reasons for this are because the antigens used to immunise the animals contain many antigenic determinants or epitopes that either occur simultaneously between different species or are similar between the different species.

Weitz (1956) described these similar occurring antigenic epitopes as the 'heterologous' antigen. He stated that antibodies that reacted with these antigens in any antisera could be removed by absorption with exposure to that antigen. In this way the specificity of the antisera could be enhanced but at a large expense of sensitivity. Such absorption techniques massively reduced the titres of the antisera.

In this chapter, an ELISA assay has been developed for bloodmeal identification. Various antisera were prepared and tested with the ELISA, cross reaction were removed by solid phase absorption. The absorbed sera were then tested by the precipitin test to compare it with the ELISA test.

A number of sandfly bloodmeals were identified both with the ELISA using the absorbed antisera and with counter current immunoelectrophoresis, the traditional method used in the laboratory, the results are compared and discussed.

Some murine monoclonal antibodies were prepared against dog serum as antigen and these have been tested for specificity.

1.2 MATERIALS AND METHODS

1.2.1 Antisera production.

Antigen preparation.

The antigens used for immunization of the antibody producing animals were either whole animal serum or ammonium sulphate precipitated albumin fractions, which were dissolved in water.

Animal whole serum preparation.

The animal of choice was bled and the blood placed in a universal tube and allowed to coagulate overnight at 4°C. The serum was pipetted off after centrifugation at 2000x g for 10 mins.

This serum was then stored in the freezer at -20°C.

Ammonium sulphate precipitation.

A saturated $(\text{NH}_4)_2\text{SO}_4$ solution (i.e. 550 gms in 600 ml distilled H_2O) was prepared, adjusted to pH 6.5 with NH_4OH (1N). 1 volume of this solution was added to 2 volumes of serum dropwise. This was allowed to equilibrate at 4°C for at least one hour and then centrifuged at 10,000xg for 10 mins. The supernatant (albumin) was collected and placed in dialysing tubing. This was dialysed in 1 litre of 0.9% (w/v) $(\text{NH}_4)\text{HCO}_3$ at 4°C for 48 hours, with the $(\text{NH}_4)\text{HCO}_3$ change after 24 hours. The antigen solution was then removed from the tubing and the solution was then freeze dried to collect the albumin.

Emulsification with Freund's complete adjuvant.

Antigens at various concentrations that were prepared for immunization were always emulsified with Freund's complete adjuvant. Two volumes of the adjuvant were mixed with one volume of the antigen. The two were thoroughly mixed until an emulsion was formed. This was achieved by putting the mixture into a small glass syringe and then sucking it in and out of the nozzle of the syringe with the plunger. The mixing was shown to be complete when a droplet would not disperse when put onto water.

Immunization protocol.

For Rabbits.

One ml of the emulsified antigen preparation was inoculated subcutaneously (s.c.) into the nape of the neck of a New Zealand White Rabbit, 21 days later a further 1 ml volume of the antigen:adjuvant preparation was inoculated s.c. into the flank of the rabbit. This was repeated with the other flank 11 days later. The rabbit was then test bled from the ear. If test bleeds were positive as determined below, the rabbit was exsanguinated from the heart.

For Mice.

This procedure is the same as that used for the immunization of mice for monoclonal antibody preparation, in the latter case the spleen is harvested for hybridoma production instead of the serum. 0.5 ml of the antigen:adjuvant preparation containing 300 µg of whole animal serum was inoculated into Balb/c female mice via the interperitoneal (i.p.) route and repeated again 21 days later. 10 days later 0.1 ml volume

containing 100 µg of antigen alone was inoculated intravenously (i.v.) into the tail. The tail was first placed under an infra-red lamp for 3 mins, to dilate the veins and make the injection more simple. 4 days later a test bleed was taken from the tail, if the test was positive the mouse was bled from the heart for serum or the spleen was dissected out for monoclonal antibody preparation.

The blood was allowed to coagulate overnight at 4°C, then centrifuged at 10,000xg for 10 mins. The serum was pipetted off and stored in the freezer at -20°C.

1.2.2 Monoclonal antibody preparation.

Materials.

a) Equipment

37°C incubator, with a controlled atmosphere of CO₂ and humidity.

Laminar flow station for sterile work.

8 channel micropipette (Flow)

Liquid nitrogen storage.

Water bath.

Inverted and ordinary microscope.

b) Reagents, media and supplements, and supplies.

10 x RPMI stock (Flow)

Gentamycin sulphate (Flow) 50 mg/ml

Hypoxanthine (Sigma) $5 \times 10^{-3} \text{M}$

Thymidine (Sigma) $8 \times 10^{-4} \text{M}$

L. Glutamine (Gibco) 200 mM

Aminopterin (Sigma) $2 \times 10^{-5} \text{M}$

Pyruvate (Gibco) 100 mM

Pristane (Sigma)

DMSO (Dimethyl sulphoxide, Sigma)

Trypan Blue (Sigma)

Polyethene glycol M.W.4000 (Sigma)

Foetal calf serum (FCS, Gibco)

24- and 96- well plates, sterile, flat bottomed with covers. (Nunc, Costar)

Plastic petri dishes (Falcon)

Plastic culture flasks, 50 and 120 ml, sterile (Falcon)

Plastic sterile centrifuge tubes, 15 and 50 ml.(Falcon)

1.8 ml liquid nitrogen storage vials (Nunc)

200 ml graduate media flasks (Scholt.)

Dog serum

c) Animals and cell lines.

Balb/c mice bred for immunization (Olac)

Myeloma cell line: X 63/Ag 8.653 (Flow)

Procedures.

Media preparation.

a) Basic medium

This was prepared in 5 litre volumes, with distilled milli Q water, (highly purified and filtered water that is prepared with a filter system designed by Millipore), and 10x stock RPMI.

The pH was adjusted to 7.0 by adding 2 g of $\text{Na}(\text{CO}_3)_2$ (Sigma). The medium was sterilized by filtration through a Millipore 0.22 μm membrane and stored at room temperature in the dark.

b) Complete medium

This was prepared just before use in 200 ml graduated media flask.

200 ml basic medium

20ml FCS

1 ml 200 mM L.Glutamine solution

200 ul Gentamycin solution

1 ml Pyruvate solution

c) HAT Medium

Prepared a day prior to use and stored at 4°C.

200 ml Complete Medium

1 ml Hypoxanthine solution

1 ml Thymidine solution

1 ml Aminopterin solution

d) HT Medium

Prepared a day prior to use and stored at 4°C.

200 ml Complete Medium

1 ml Hypoxanthine solution

1 ml Thymidine solution

e) 20% FCS Medium

40 ml Complete Medium

10 ml FCS

f) Incomplete Medium

As complete medium but without FCS.

Preparation of polyethylene glycol (PEG) Solution (30% PEG).

Aliquots of previously molten PEG were prepared in 300 μ l volumes and stored at room temperature. For fusion, an aliquot was heated in a water bath at 45°C, 600 μ l of incomplete medium and 100 μ l of DMSO was added when the PEG was molten. This solution was then transferred to another water bath at 37°C.

Cell Maintenance.

The myeloma line was grown in the complete medium described above. The doubling time was about 20 hours.

The cells were routinely counted to determine their concentration and viability. The cells were passaged every 4 or 5 days, transferring about 10^5 cells to 4 ml of new medium. When high viability was needed for a fusion, the cells were maintained in the logarithmic phase.

The cells were counted and tested for viability using the method of Trypan blue exclusion. 0.4% Trypan blue in saline (w/v) was mixed with an aliquot of cells volume for volume and viewed under the microscope on a Neubauer haemocytometer.

The Neubauer haemocytometer has different regions where cells can be counted. The region where the cells were counted, were the four large (so-called W) squares, each with 16 smaller squares, flanking the central cross pattern of lines. The cells occurring in the 16 small squares were counted. The cell count was converted to cells per ml by multiplying the figure counted by 1×10^4 (the volume correction factor). Total number of cells was calculated by multiplying the cell counter per ml by the total volume of cell suspension.

The incubator was kept at 37°C, well humidified and with a CO₂ concentration of 6%.

Storage and Recovery.

Both the myeloma and hybridoma cell lines were stored in liquid nitrogen. The cryo-preserved agent used was 90% FCS with 10% DMSO. The cells were stored in 1 ml aliquots placed in storage vials. For freezing the cells were left overnight at -80°C then transferred directly to liquid nitrogen.

Recovery was made by rapidly thawing the cells in a water bath at 37°C , followed by cooling in an ice bath for 10 mins. The cells were transferred to centrifuge tubes and washed in 10 ml of complete medium. The cells were then centrifuged (1000 rpm for 10 mins.) and added to 1 ml of complete medium. The material was then transferred to a culture plate and incubated.

Preparation of macrophage feeder layers.

A Balb/c mouse was sacrificed by dislocation of the vertebrae, the whole animal dipped into 70% ethanol and fixed to a dissecting board. Under sterile conditions, the belly skin was opened, exposing the peritoneum whilst avoiding any perforations. Then with a sterile disposable syringe with a 21g needle, 5 ml of cold incomplete medium was injected into the peritoneum. The media was drawn back into the syringe and transferred to a centrifuge tube standing in an ice bath. The media was kept cold to minimise macrophage adherence. This procedure was repeated with as many mice as necessary to coat the amount of plates needed.

The cells were counted, then centrifuged (1500 rpm for 10 mins.) and adjusted to 2×10^6 cells/ml in HAT medium. This was then aliquoted into tissue culture plates : 0.1 ml per each well of a 96-well plate or 1 ml per each well of a 24-well plate and placed into the incubator.

Cell fusion and selection of hybrids.

Different protocols for cell fusion have been described, with similar results (Chaflin and Williams, 1978; Goding, 1980; Kohler, 1981; Pearson and Anderson, 1980). They vary mainly in relation to the step of PEG addition or the time for the addition of selective medium. The precise ratio of myeloma to spleen cells is not crucial and good results have been achieved with ratios of spleen : myeloma cells from 1:1 to 10:1 (Goding, 1980). The method outlined here is a modification of Kennett et al. (1978).

a) Preparation of myeloma cells.

The myeloma cells were harvested by gently pipetting the media in a tissue culture flask with a sterile pipette so as to loosen the adhered cells. The cells were centrifuged (1500 rpm for 5 mins.) and resuspended in 5 ml of complete medium, counted and checked for viability. Only the cells with viability greater than 98% were used. The necessary amount of cell preparations were then transferred to 50 ml centrifuged tube and kept at room temperature until needed.

b) Preparation of Spleen Cells.

A mouse immunized against dog serum was sacrificed by dislocation of the vertebrae, dipped into 70% ethanol and fixed to a dissecting board. The spleen was then carefully dissected out and placed in a petri dish with complete medium. Using a 1 ml sterile syringe the spleen was gently teased and perfused with medium so as to release the cells within. This medium containing the cells was then collected, placed in a 15 ml centrifuge tube and left to stand for 10 minutes to

allow cell clumps to sediment. The suspended cells were aspirated off and placed in another 15 ml centrifuge tube. A small aliquot of cells was taken and diluted 1/20 in basic medium. This sample was then used for counting the cells with Trypan blue exclusion.

c) Cell Fusion.

The necessary number of myeloma cells were collected, centrifuged (1000 rpm for 5 mins.), washed twice in 10 ml of incomplete medium, and finally resuspended in 5 ml of incomplete medium.

The spleen cells were then treated to remove the red blood cells. The cells were centrifuged (1000 rpm for 5 mins.) and the pellet resuspended in 5 ml of cold 0.17 M NH_4Cl . This was placed on ice. After 10 mins. 10 ml of cold 20% complete medium was mixed with the cells, which were then centrifuged (1000 rpm for 5 mins.) and washed twice in 10 ml of incomplete medium. The cells were then resuspended in an appropriate volume of incomplete medium and mixed with the myeloma cells (ratio of spleen : myeloma 10:1). The mixture was transferred to a round bottomed tube to increase the area of the pellet after centrifugation (1000 rpm for 5 mins.). The supernatant was discarded and the pellet loosened by gently tapping the tube.

A stop watch was set for 8 mins. during which time 200 μl of 30% PEG in incomplete medium was added directly to the pellet. The cells were then immediately placed in the centrifuge and spun for 6 mins. at 200 x g. The cells were then diluted dropwise with 5 ml of incomplete medium, then 5 ml of 20% complete medium was added. The cells were spun down again for 6 mins. at 200 x g and resuspended in 100 ml of HAT medium. This was transferred to the previously prepared macrophage

feeder layer culture plates, 0.1 ml in 96-well plate and 1 ml in 24-well plates, and then incubated.

After 7 days the cells were fed, replacing half the medium of each well with fresh HAT medium. This was then repeated every other day.

The supernatants were tested when the colonies were macroscopically visible. The expansion of positive hybrids was done gradually, always with a feeder layer until eventually each clone was transferred to a 50 ml flask.

Positive hybrids (screened as described below) were frozen before and after cloning as a positive hybrid can often lose the ability to produce antibodies.

d) Cloning by limiting dilutions.

Hybridoma cells must be cloned as soon as possible after fusion as cells from the same culture well producing irrelevant or no antibody may otherwise overgrow the specific antibody producing cells. Cloning by limiting dilution was always done in the presence of a macrophage feeder layer because hybridoma depend on appropriate cell density for growth.

Feeder layer plates were prepared as previously described in complete medium. The hybrid cells were then counted and checked for viability. Dilutions of the cells were made (1, 5, 10 cells/ml) in complete medium. Then an aliquot of 100 μ l of the diluted cells was transferred in tissue culture plates with feeder layers and placed in the incubator. After 10 days, growth of single clones was checked. Further specificity tests (see below) were performed with the supernatant. After expansion of the clones, remaining positives were cloned yet again so as to insure monoclonality.

e) Preparation of Ascites.

A number of syngeneic BALB/c mice were injected with 1 ml of pristane intra peritoneally 12 days prior to use.

The positive clone to be expanded was centrifuged (1000 rpm for 5 mins.) resuspended and counted. 3×10^6 cells were then inoculated into the peritoneal cavity of each of the pristane treated mice. These mice were then left for about 10 days, then the peritoneal fluid removed with a pipette after sacrifice. This fluid was centrifuged and the supernatant was taken off and stored at -20°C . The cells were either reinoculated into more pristane treated mice or stored in liquid nitrogen as previously described.

1.2.3 Specificity Test - Enzyme Linked Immunosorbent Assay (ELISA)

a) Materials.

Automatic ELISA reader (Dynatech)

Micropipettes with disposable tips in 5 - 500 μl range (FLOW)

96-well microtitre plates with lids (Dynatech,

Immulon II)

8 Channel pipette (Flow)

Storage boxes

Refrigerator at 4°C

Incubator at 37°C

Plastic wash bottles

Casein (Sigma)

Enzyme conjugated rabbit anti mouse immunoglobulin (Nordic)

b) Buffers

Coating buffer 0.05M Carbonate, pH. 9.6

Incubation buffer - PBS pH. 7.4

PBS - Tween:

0.5 ml Tween-20 added to 1 litre of PBS

Substrate buffer - Citrate, pH 5.0 for Orthophenylene diamine OPD

Stopping solution 2.5 M HCl

Enzyme substrate for peroxidase conjugate OPD 40 mg/100 ml

Substrate buffer 40 μ l 30% H_2O_2 /100 ml added immediately before use. This was placed in a light protected container.

c) Procedure.

Coating the plates with antigen.

The antigen being used to screen the monoclonal supernatant was the one used to immunise the mice, i.e. whole animal serum, (DOG) prepared as previously described. This was dissolved in carbonate coating buffer to a concentration of 100 μ g/ml. Then 200 μ l of this antigen solution was introduced into the wells of the microtitre plate and incubated at 4°C overnight in a damp storage box. The plates were washed 3 times with PBS-Tween by immersing the plate into a tank, allowing at least 3 mins. between each wash. After emptying the plate for the last time it was shaken and tapped onto a pile of bench tissue to remove all excess liquid.

Blocking the plates with Casein.

To minimise non-specific binding the plates were blocked with a non-reactive protein that would prevent the immunoglobulin binding. 1% casein in carbonate buffer was used, 100 μ l/well. This was left at 37°C for 1 hour then washed in PBS - Tween as before.

Removing the supernatants.

Using a micropipette with sterile tips a 100 μ l portion of the culture supernatant was removed from each well and transferred to the ELISA plate. This was then incubated for 1 hour at 37°C and then washed in the same manner as before.

Addition of conjugated immunoglobulin.

A 1/2000 dilution of rabbit anti-mouse Ig/peroxidase (Nordic) conjugate was prepared in PBS - Tween buffer. 100 μ l/well was added and incubated for 1 hour at 37°C, then washed in the same way.

Addition of substrate.

100 μ l of substrate solution, which had been prepared just prior to use was added to each well and the plate was incubated at 37°C for 20 - 30 mins. When enough colour had formed the reaction was stopped with 25 μ l of 2.5 M HCl and the plate read on the automatic ELISA reader.

1.2.4 Isotyping of the monoclonal antibodies.

Concentration of immunoglobulin in supernatants by negative pressure dialysis.

a) Materials.

500 ml Buchner Flask

Pressure tubing

Suction pump

Bored bung

Filter funnel

Gate clips

Visking tubing 8/32

b) Procedure.

The visking tubing containing the supernatant samples were placed into the Buchner funnel and sealed. The apparatus was then attached to a suction pump. After a few minutes the suction was closed off, a partial vacuum was formed, and the apparatus was left for ultra filtration to occur.

When the process was complete, the concentrated supernatants were stored at -20°C .

1.2.5 Gel diffusion with specific antiserum.

a) Materials.

Water bath

Horizontal table and spirit level

Glass plates 8 cm x 8 cm

Gel punches

Hamilton micropipettes 10 μl and 100 μl

Whatman No.1 filter papers

b) Chemicals.

Low endosmosis agarose (Oxoid)

Barbitone (BDH)

Sodium barbitone (BDH)

Polyethylene glycol (MW 1500 BDH)

Methanol (BDH)

Acetic acid (BDH)

Coomassie brilliant blue R (Sigma)

c) Immunologic reagents.

Specific antisera to IgM
 IgG - all subclasses
 IgA

d) Barbitone Buffer.

0.92 g Barbitone
5.15 g Sodium barbitone dissolved in 500 ml of distilled water

e) Agarose gel

5 g Agarose
15 g Polyethylene glycol (M.W.1500) dissolved in
 500 ml of hot barbitone buffer

f) Protein stains

Stain solution : mixture of water 5
 acetic acid 1
 methanol 5

2.5 g Coomassie brilliant blue dissolved in 500 ml stain solution

g) Preparation of plates.

9.6 ml molten agarose gel was poured on to the glass plates 8 cm x 8 cm on table after levelling and allowed to set. Wells were cut in the pattern shown in Figure 1.1.

The wells were then filled with the concentrated supernatants in the centre well and the specific class and subclass antisera in the surrounding wells. Where a positive result occurred, a line of precipitate was seen between the centre well and one of the outside wells.

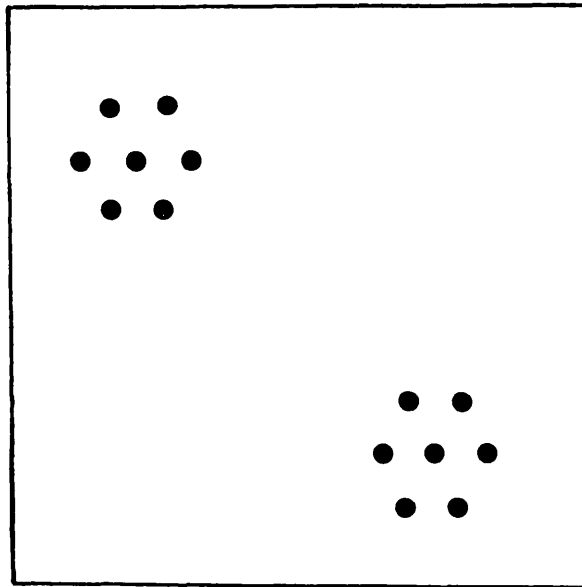


Figure 1,1 Patterns of wells on a gel diffusion plate

1.2.6 Characterization of the monoclonal antibodies with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer.

SDS-PAGE

The SDS polyacrylamide gels were prepared from a technique adapted from Laemmli (1970).

a) Separating gel. (11%)

14.90 ml H₂O

14.65 ml Acrylamide (28.4% Acrylamide and 1.6% BIS-acrylamide)

1.5 M Tris HCl (pH 8.8)

1.4 ml 10% sodium dodecyl sulphate (SDS)

0.1 ml 10% Ammonium persulphate

25 µl TEMED (N, N, N' N' - Tetra methyl ethylene - diamine, Sigma)

b) Stacking Gel. (4%)

6.1 ml H₂O

2.5 ml Acrylamide

0.1 ml 10% SDS

50 µl 10% Ammonium persulphate

10 µl TEMED

c) Electrode buffer.

6 gm Tris

28.8 gm Glycine

10 ml 20% SDS

made up to 2 litres with distilled water.

d) Sample buffer.

2 ml 20% SDS
 1 ml 1 M Tris HCl pH 7.0
 1 ml 60% Sucrose
 15 ml H₂O
 4 drops Bromophenol blue

e) Coomassie brilliant blue stain.

0.12 gm Coomassie blue in 400 ml of solvent

Staining solvent:

260 ml H₂O
 100 ml Ethanol
 40 ml Acetic acid

f) Destain solvent:

425 ml H₂O
 50 ml Ethanol
 25 ml Acetic acid

g) Procedure.

The gels were prepared in LKB gel assembly kits by pouring in the separating gel first. This gel was then overloaded with distilled water and allowed to set. The water was then aspirated and the stacking gel poured onto the separation gel; this gel was also overloaded with water. Immersed into the stacking gel was a plastic comb which when removed provided the sample wells. After the gel had set and the comb removed the upper buffer chamber was attached and the whole apparatus immersed in the running tank.

The proteins for gel electrophoresis were boiled for 5 mins. in sample buffer to ensure that all the proteins present were in solution

and bound with SDS. Between 50 - 70 μ l of sample was loaded into each sample well. The gels were focused at 20 mA until the protein front had almost reached the end of the stacking gel. The separation gels were run at 40 mA.

The gels were then taken out and the apparatus dismantled in preparation for Western transfer. If gels were stained, they were placed in Coomassie brilliant blue for 30 mins. at room temperature and then destained overnight.

1.2.7 Western transfer

An adaption of the technique of Towbin et al., (1979).

a) Equipment

Bio-Rad Western transfer tank

Bio-Rad power pack

Whatman No.1 filter paper

Nitro cellulose paper (Bio-Rad)

b) Transfer buffer:

15 g Tris

72 g Glycine

1 dm³ Methanol

4 dm³ H₂O

c) Procedure

After the gel frames were dismantled, the gels were carefully laid down on to filter paper soaked in transfer buffer, avoiding the formation of air bubbles. On top of the gel was placed the soaked nitro-cellulose paper again ensuring that there were no air bubbles.

More filter paper was then laid down to make a thick sandwich. This was then placed in the plastic electrode clamp which was immersed into the running chamber.

The side of the sandwich with nitro-cellulose paper was always connected to the positive electrode. The transfer process took 2 hours at 0.4 A. Afterwards the nitro-cellulose paper was carefully removed and placed in between two sheets of polythene plastic for storage.

1.2.8 Development of the Western transfer with autoradiography

a) Materials

1% Casein - PBS

PBS Tween

I¹²⁵ Goat anti mouse Ig. 100 µCi/ml. (Amersham International)

Photographic equipment

b) Procedure

After the western transfer, the nitro-cellulose paper was placed in a container with 300 ml of 1% casein - PBS and gently rocked for an hour at 37°C, then left at 4°C for 15 hours. The paper was then washed three times in PBS - Tween. Each sample lane was cut out and placed in between polythene sheets which were later heat sealed to form liquid tight bags. The monoclonal antibody ascitic fluids were introduced into the polythene bags before the final seal. These were left at 37°C for 2 hours.

The nitro-cellulose strips were then washed three times in PBS - Tween and again placed in between polythene sheets. The I¹²⁵ Goat anti-mouse Ig antibody was introduced into each bag, sealed and left shaking at 37°C for 1 hour. The strips were again washed in PBS - Tween

1.2.9 Counter Current Immunelectrophoresis (CCIE), a modified precipitin test.

This technique is very similar to the agar gel diffusion method described by Crans (1969), with the addition of assisted diffusion by electrophoresis. The immunoglobulin migrate in the opposite direction to the albumin of the antigen, thus allowing a more concentrated reaction to occur, increasing the sensitivity. This method is used for sandfly bloodmeal identification due to the low volume of bloodmeal.

a) Materials

As described for double gel diffusion.

Electrophoresis tank Power Pack (Volkan)

b) Procedure

The gels were poured and the wells cut in pairs side by side 5 mm apart. The sandfly bloodmeals were dissected out of the abdomen and placed in a glass test tube containing 0.2 ml of saline. Each bloodmeal was crushed and washed with a glass rod and left to stand overnight at 4°C. 5 µl of the bloodmeal eluate were added to the right-hand well and 5 µl of the polyclonal antibody to the left-hand well. The plates were then left until the solutions were diffused into the gel. Then the plates were laid inverted onto the two lint wicks of the electrophoresis tank. The electrode buffer used was barbitone buffer pH 8.8. The power pack was set to 0.2 mA per pair of wells and ran for 2 hours. The plates were dried under Whatman No.1 filter paper overnight.

After drying the results were developed by staining in Coomassie blue for 1 hour and destained overnight. These results were recorded by visual inspection.

1.2.10 Solid phase absorption

Activated Affinity Supports 'Affigel 10' (N-Hydroxy-succinimide esters of cross linked agarose gel).

Use of the Affigel 10 for solid phase absorption enables the immobilization of the cross absorbing antigen and subsequent removal of the cross reactive antibody from the absorbed antiserum.

Three columns were prepared to adsorb three antisera.

Rabbit anti-Man

Rabbit anti-Dog

Rabbit anti-Bovine

a) Materials

Affigel 10 (Bio-Rad)

Cold distilled water

PBS - Azide (0.001% Sodium Azide)

3 - 20 ml syringes

glass wool

3 way peristaltic pump

b) Procedures

Three rabbit antisera were prepared as previously described. They were tested with the ELISA against 10 cross reacting animal sera : Man, Pig, Dog, Cat, Horse, Mouse, Avian, Reptile, Ox and Goat. From the results antigen cocktails were prepared for loading onto each column for the absorption of the three antisera.

16 ml of the Affigel 10 suspension was placed into a Nalgene suction filter and washed three times with an equal volume of 4°C distilled water. This was completed within 20 mins.

3.5 ml of the washed affigel was then placed in 3 10 ml tubes which contained an equal volume of the pooled antigen sera, a different cocktail being prepared for each antisera.

Pooled Sera :

For:	Anti-Man	Anti-Dog	Anti-Bovid
A Cocktail of:-			
	Ox serum	Ox serum	Man serum
	Goat serum	Goat serum	Pig serum
	Dog serum	Man serum	Dog serum
	Pig serum	Pig serum	Mouse serum
	Avian serum	Avian serum	Horse serum
	Mouse serum	Mouse serum	Avian serum
	Horse serum	Horse serum	Reptile serum
	Reptile serum	Reptile serum	Cat serum
	Cat serum	Cat serum	

This mixture was rotated for 1 hour at room temperature, then placed in the fridge at 4°C for 24 hours. The antigen agarose mixture was placed into 10 ml syringes plugged with glass wool and connected to the peristaltic pump (Figure 1.2). 50 ml of PBS - Azide was pumped through the column to wash away any excess unbound antigen, the last 1 ml being read spectroscopically at 0.D.280 N.M. to detect protein. The column was then loaded with antisera for adsorption. The antisera was pumped and re-pumped through the column continuously for 4 hours at room temperature, this ensured a total of 5 circuits through the column for

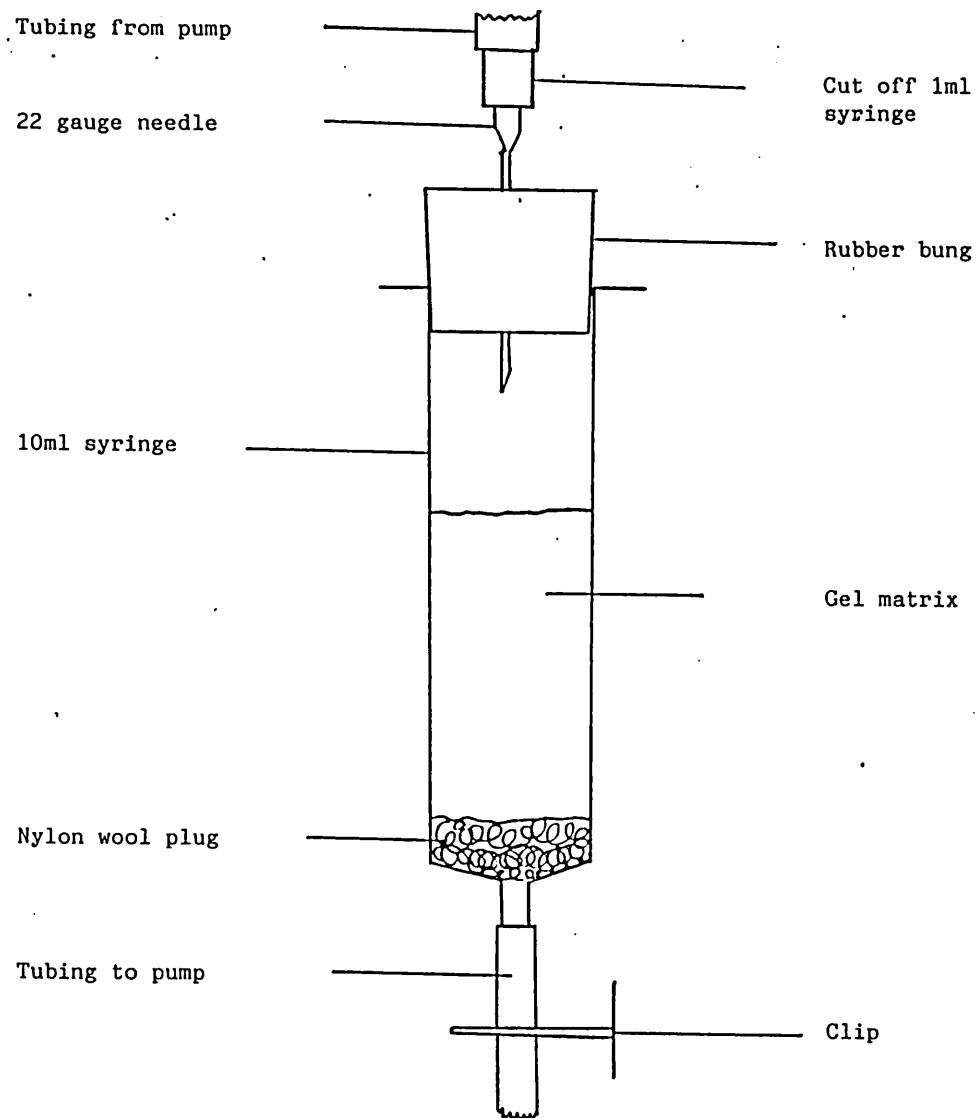


Figure 1.2 Diagram showing the affinity column used for solid phase absorption

maximum adsorption. When adsorption was completed the antisera was collected, aliquoted and stored at -20°C . The columns were then rewashed with a further 50 ml of PBS - Azide, sealed and stored at 4°C for further use.

1.2.11 Insoluble gluteraldehyde polymerised antigen

Soluble protein antigens can be rendered insoluble by cross-linking with gluteraldehyde.

a) Materials

Soluble antigens

0.1 M PO_4 buffer pH 7.0

2.5% Gluteraldehyde

0.2 M PO_4 buffer pH 7.4

0.1 M glycine buffer, pH 3.2

PBS, pH 7.4

b) Procedure

The animal antisera used in the previous section were also used for this technique. 100 μl of each animal serum were pooled together. 500 μl of this was then dissolved in 10 ml of 0.1 M PO_4 buffer, pH 7.0. Whilst stirring, 2 ml of 2.5% aqueous solution of gluteraldehyde was added dropwise to the antigen solution. The gel that formed was kept for 3 hours at room temperature and then mashed into fine particles.

This was washed copiously with distilled water and then with 0.2 M PO_4 buffer, pH 7.4, 0.1 M glycine - HCl buffer pH 3.2 and then repeatedly with PBS, pH 7.4 to remove any remaining gluteraldehyde. The gel was then stored in PBS, pH 7.4 at 4°C . When being used to absorb antisera, it was mixed in an equal volume with the antisera and stirred at room temperature for several hours.

The absorbed antisera was recovered after centrifugation and if necessary, the adsorbent reconstituted by washing the gel with 0.1 M glycine - HCl buffer or acetic acid at pH 3.2. All the absorbed antisera were stored at -20°C .

1.3 RESULTS

1.3.1 Testing the antisera used in bloodmeal identification by ELISA for specificity.

Seven antisera were prepared in rabbits and tested against each of the seven antigens used to prepare the antisera. Figure 1.3 to 1.9 shows the activity of the antisera tested against all the antigens. The table below shows a summary of the results. All the antisera prepared to mammalian serum(^) antigens cross react extensively. The antisera prepared to avian and reptilian serum antigens(*) show no cross reactivity. All the anti-mammalian antisera cross react, only the antisera prepared to reptilian antigen is order specific, this is because the reptiles are the most distantly related species.

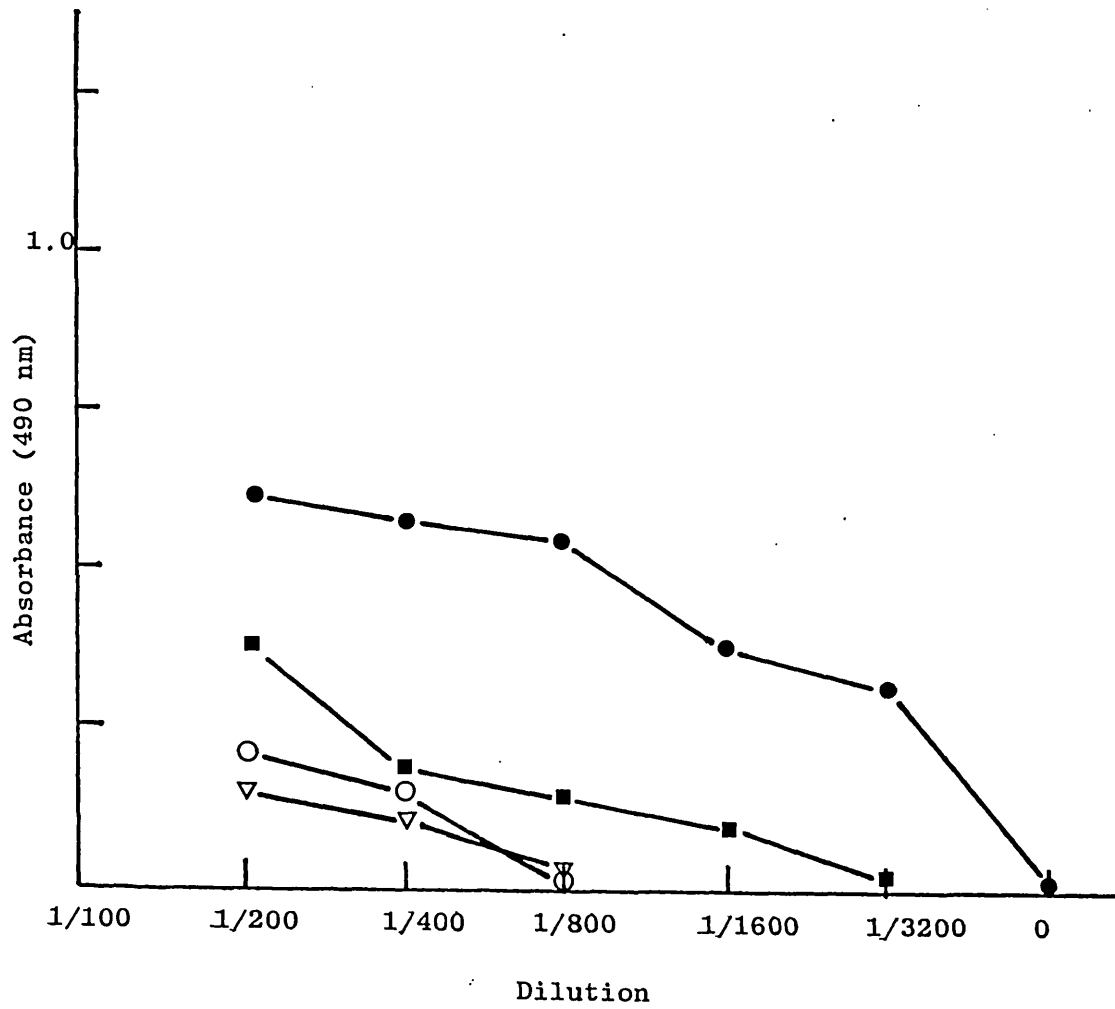
	Antigen						
	Man^	Dog^	Ox^	Badger^	Buffalo^	Avian	Reptilian
Anti-Man	+++	++	+	+	++	-	-
Anti-Dog	+	+++	+	++	+	+	-
Anti-Ox	-	+	+++	-	+++	-	-
Anti-Badger	++	++	+++	++	+++	++	-
Anti-Buffalo	+	+	+++	+	+++	-	-
Anti-Avian*	-	-	-	-	-	++	-
Anti-Reptilian*	-	-	-	-	-	-	+++

^ = mammalian sera

* = antisera to Avian and Reptile

The values shown in this table are a subjective approximation taken from the data in Figs. 1.3 to 1.9.

Figure 1.3: Activity of Rabbit antisera measured on ELISA



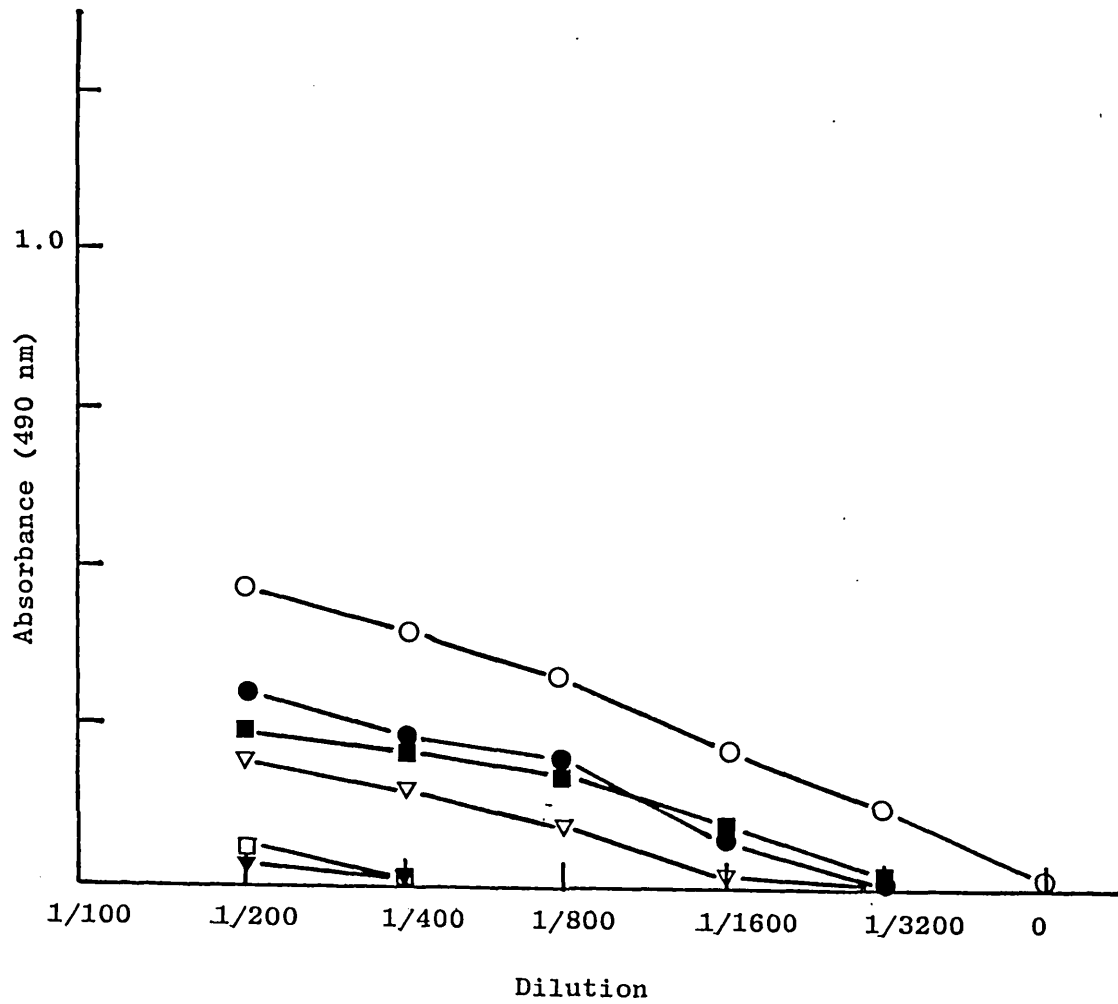
Antigen: MAN

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

Figure 1.4: Activity of Rabbit antisera measured on ELISA



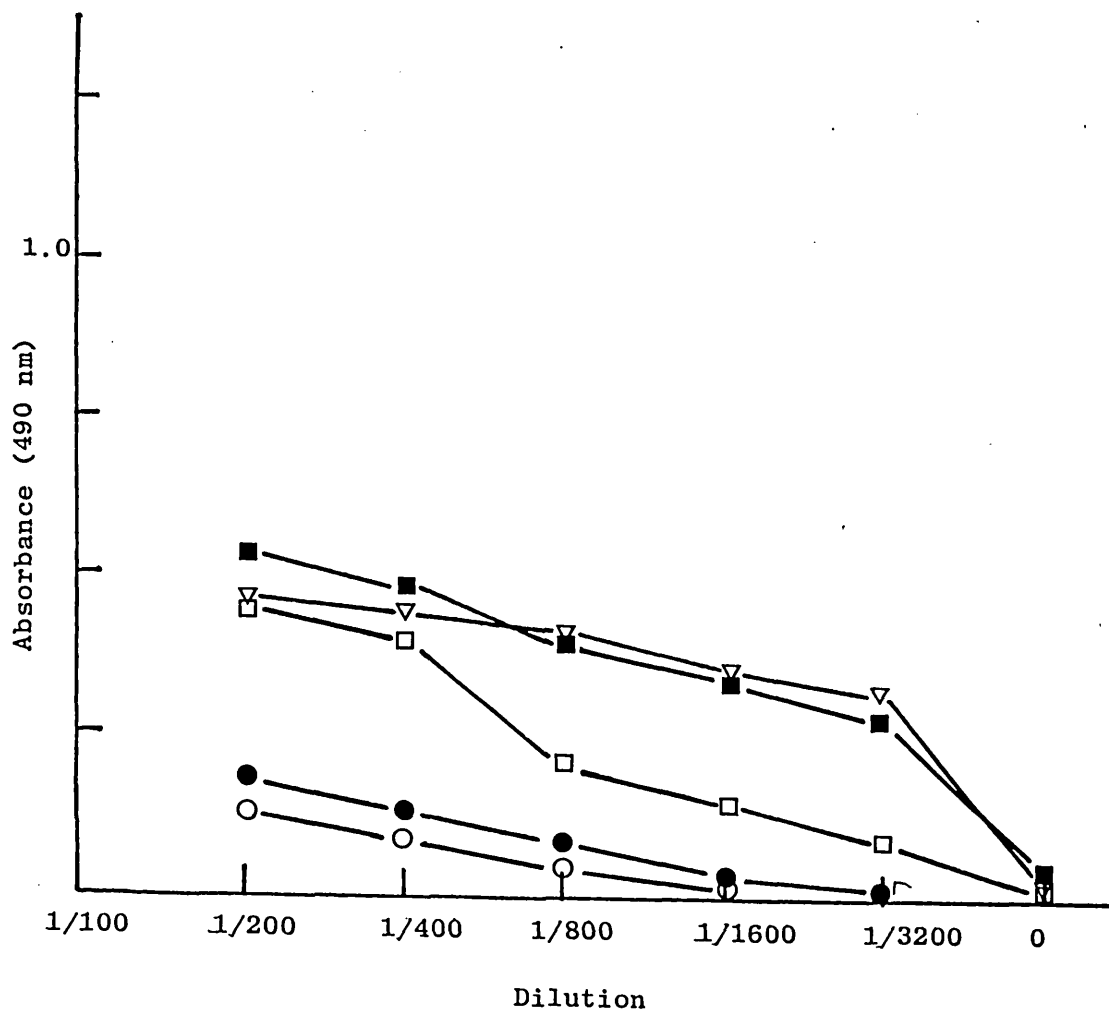
Antigen; DOG

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

Figure 1.5: Activity of Rabbit antisera measured on ELISA



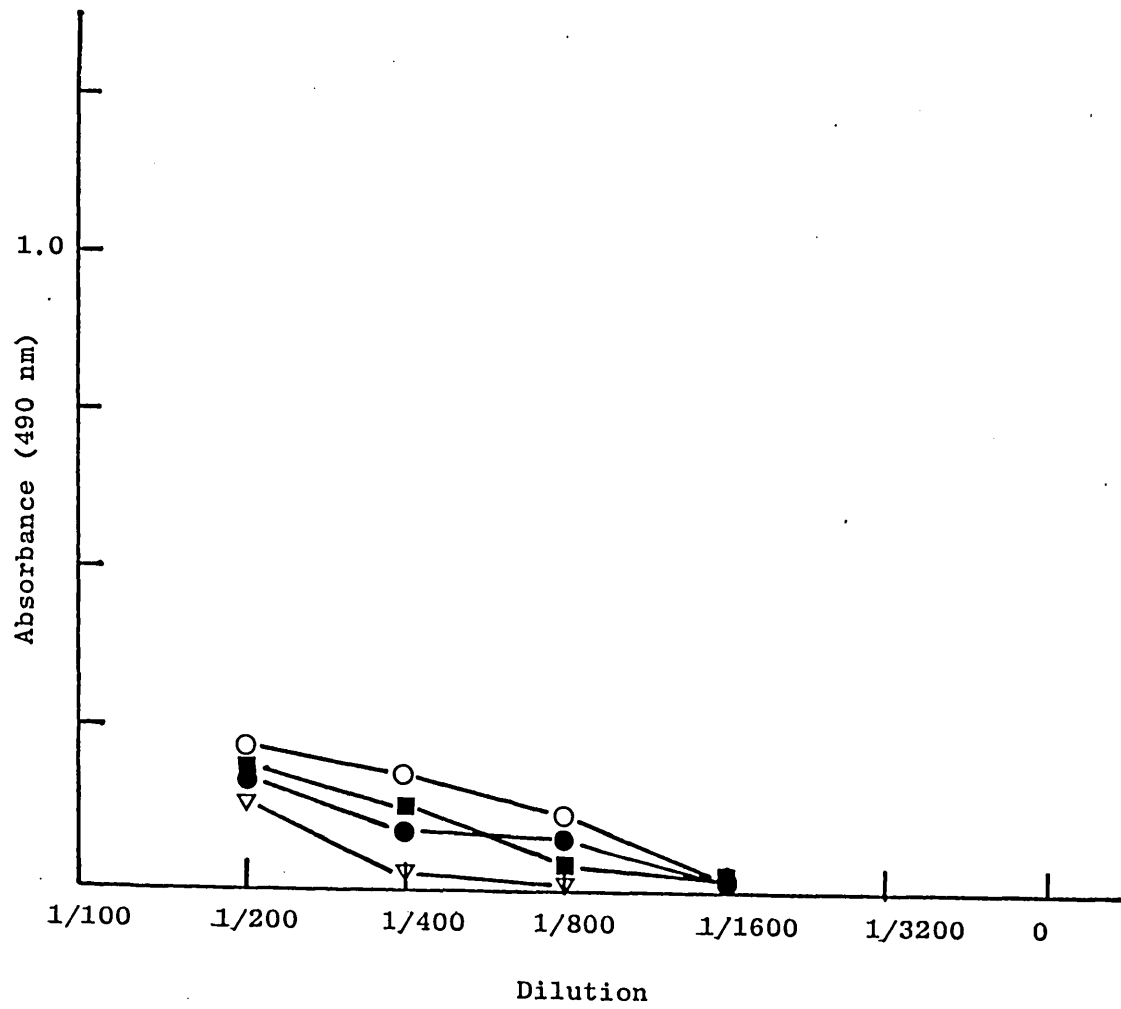
Antigen: OX

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

Figure 1.6: Activity of Rabbit antisera measured on ELISA



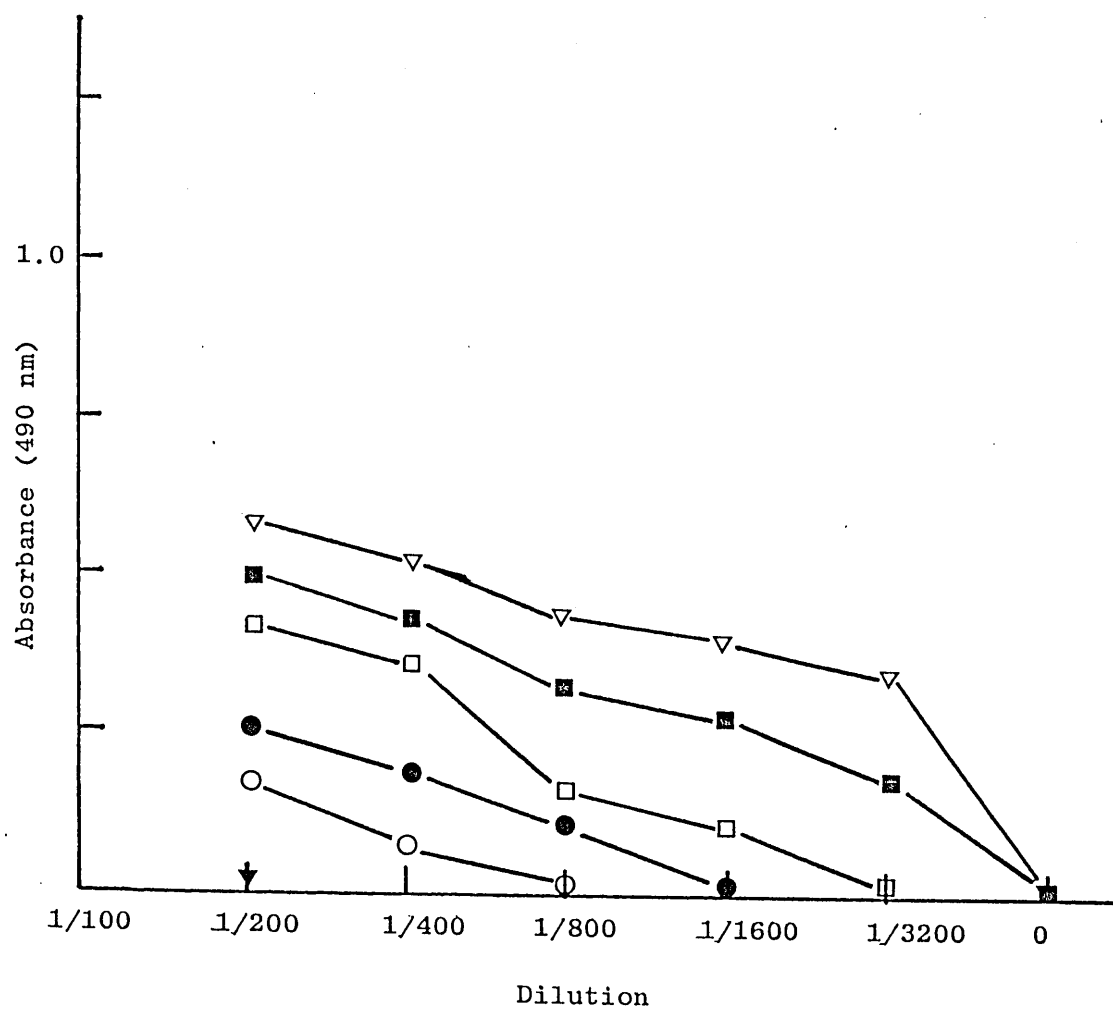
Antigen: BADGER

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

Figure 1.7: Activity of Rabbit antisera measured on ELISA



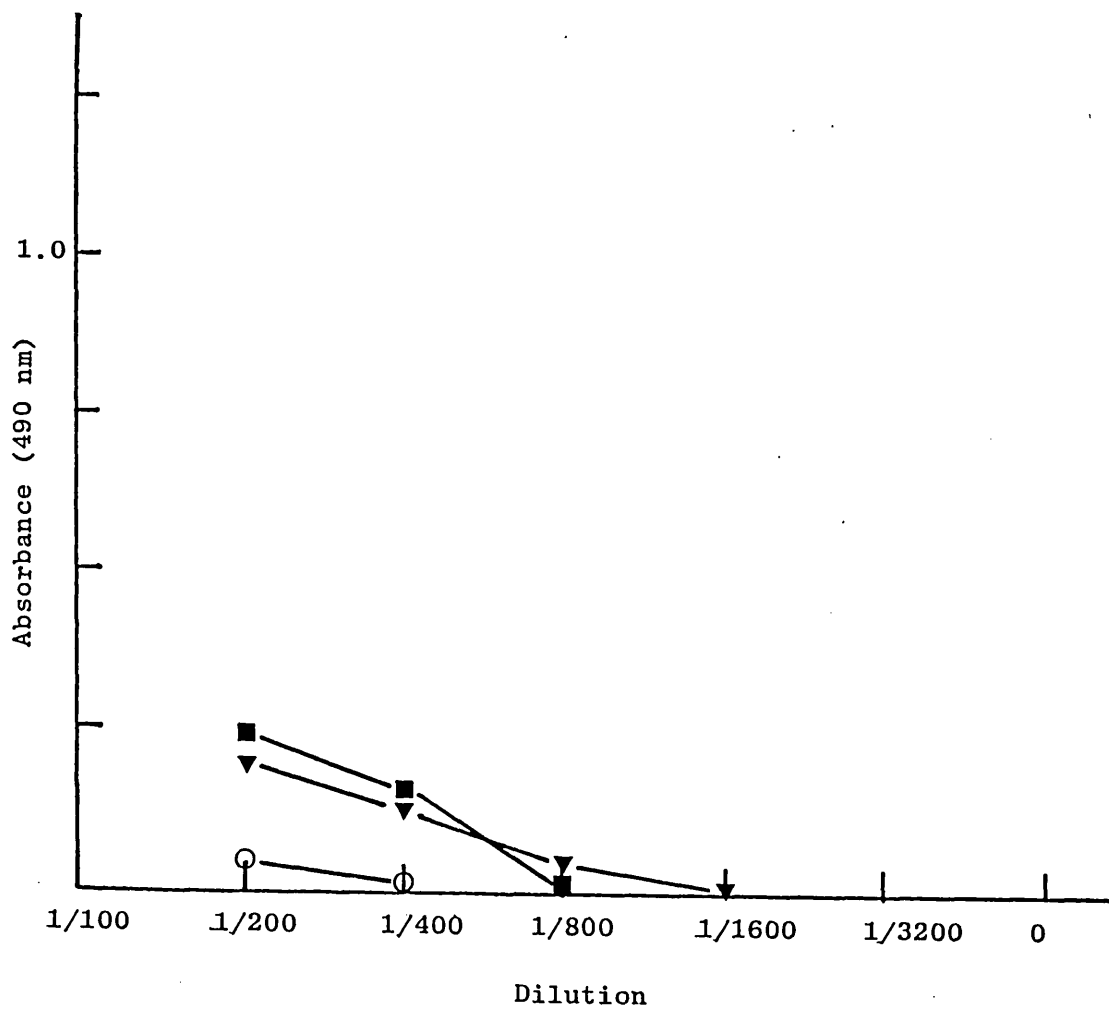
Antigen: BUFFALO

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

Figure 1.8: Activity of Rabbit antisera measured on ELISA



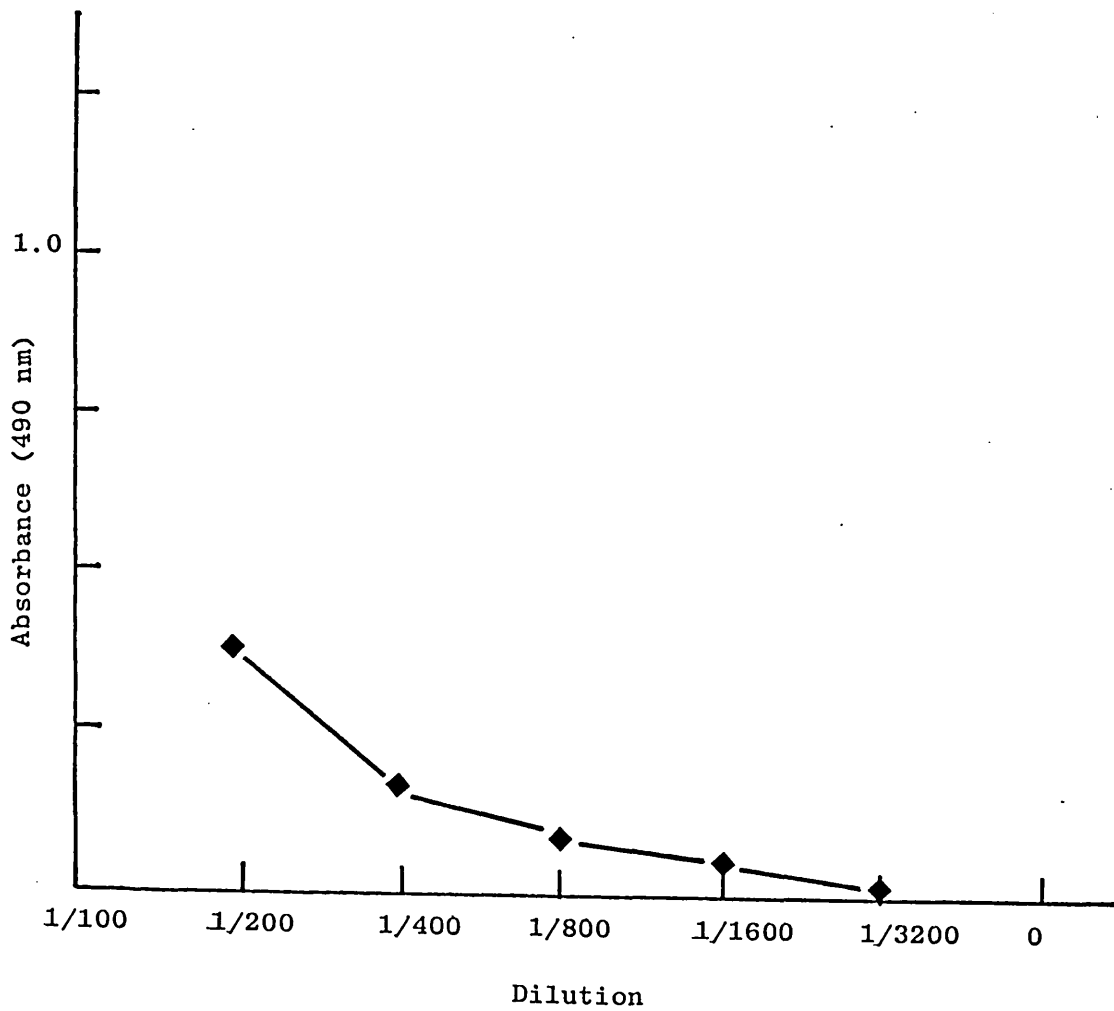
Antigen: AVIAN

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

Figure 1.9: Activity of Rabbit antisera measured on ELISA



Antigen: REPTILE

KEY

Antisera to

Man	●
Dog	○
Ox	□
Badger	■
Buffalo	▽
Avian	▼
Reptile	◆

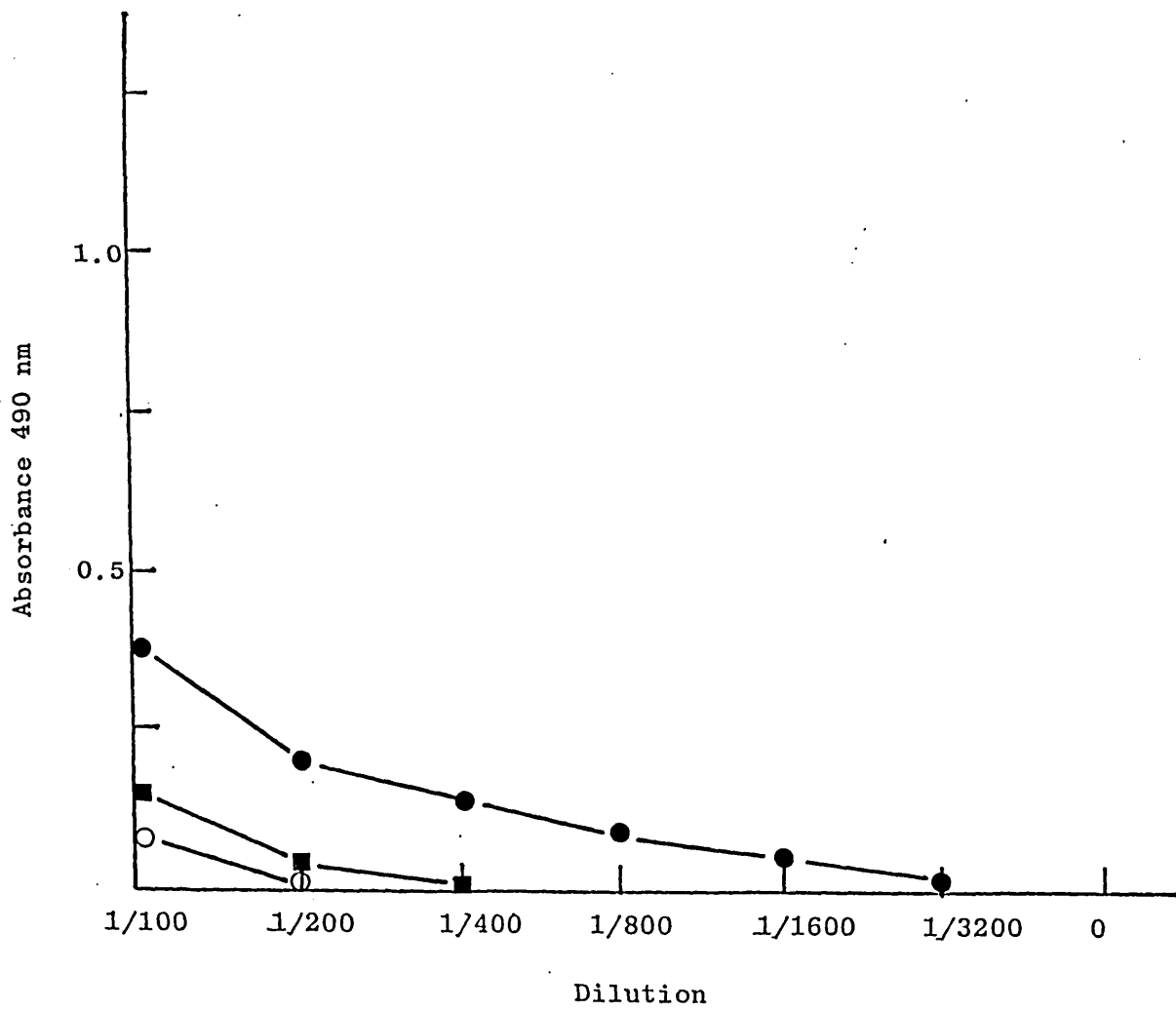
1.3.2 Solid phase absorption

Three antisera, anti-Dog, -Man and -Ox were chosen for solid phase absorption. Three affigel columns were prepared each containing a cocktail of cross reacting antigens. The antisera were absorbed then tested with the ELISA for cross reactivity. The animal antigens used to prepare the cocktails for the column absorption were not identical to those originally tested. The scarce antigens were not included, these were Badger and Buffalo (which is very closely related to Ox). Other antigens were incorporated in addition, these were Horse, Mouse, Cat and Pig. These were used to cover a broader spectrum of species.

Figures 1.10 to 1.18 show the results of the antigens tested with the ELISA using the three absorbed antisera. The antigens tested included all the antigens used in the cocktails with the exception of the Reptile and Mouse antigen. Badger antigen was in enough supply. Reptile antigen was not tested as it had already been shown not to cross react. Mouse antigen was not used as this cross reacted with the anti-Rabbit enzyme conjugate antibody which had not been adsorbed because it was obtained commercially. The results show a reduction in sensitivity but specificity of the antisera has been achieved, see Figures, 1.10, 1.11 and 1.12 compared with figures 1.3, 1.4 and 1.5. Figures 1.13 to 1.18, i.e. the tests which were performed with non-target antigens, show slight cross reactions at the lowest dilution of the absorbed antisera but these are minor when compared to the activity of the antisera with the target antigens. The only exception to this is seen in Figure 1.18, where the antigen being tested is goat antigen, which still shows activity with the anti-Ox antisera. The bovids are very difficult to separate as they are very closely related.

The activity with goat antigen is not equal to that seen with ox antigen (Figure 1.12) it is approximately 25% less. A successful separation of these two animals would have to rely on knowledge of the animal populations in the area of the catch.

Figure 1.10 Activity of Rabbit antisera measured on ELISA



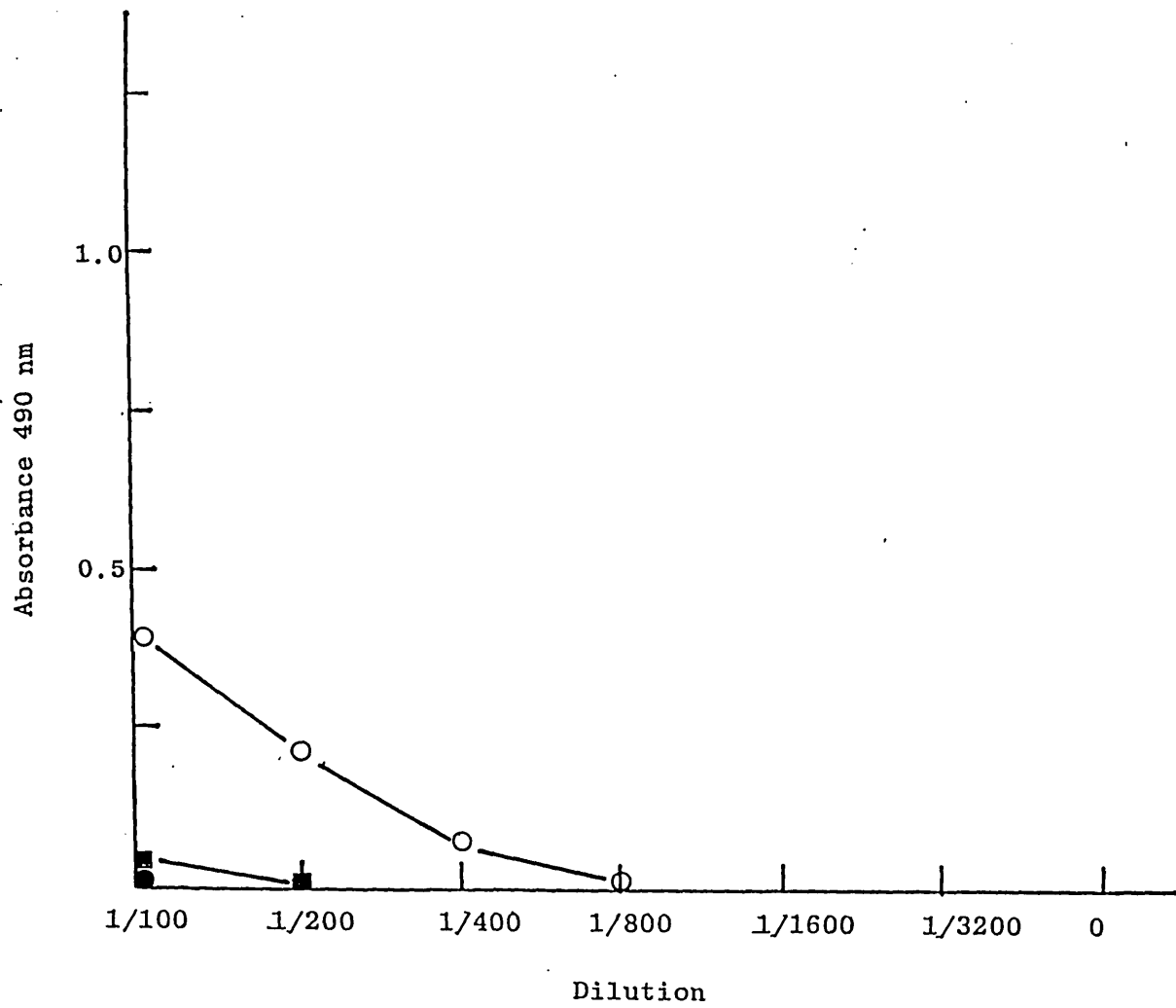
Antigen tested on ELISA: MAN

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

Figure 1.11 Activity of Rabbit antisera measured on ELISA



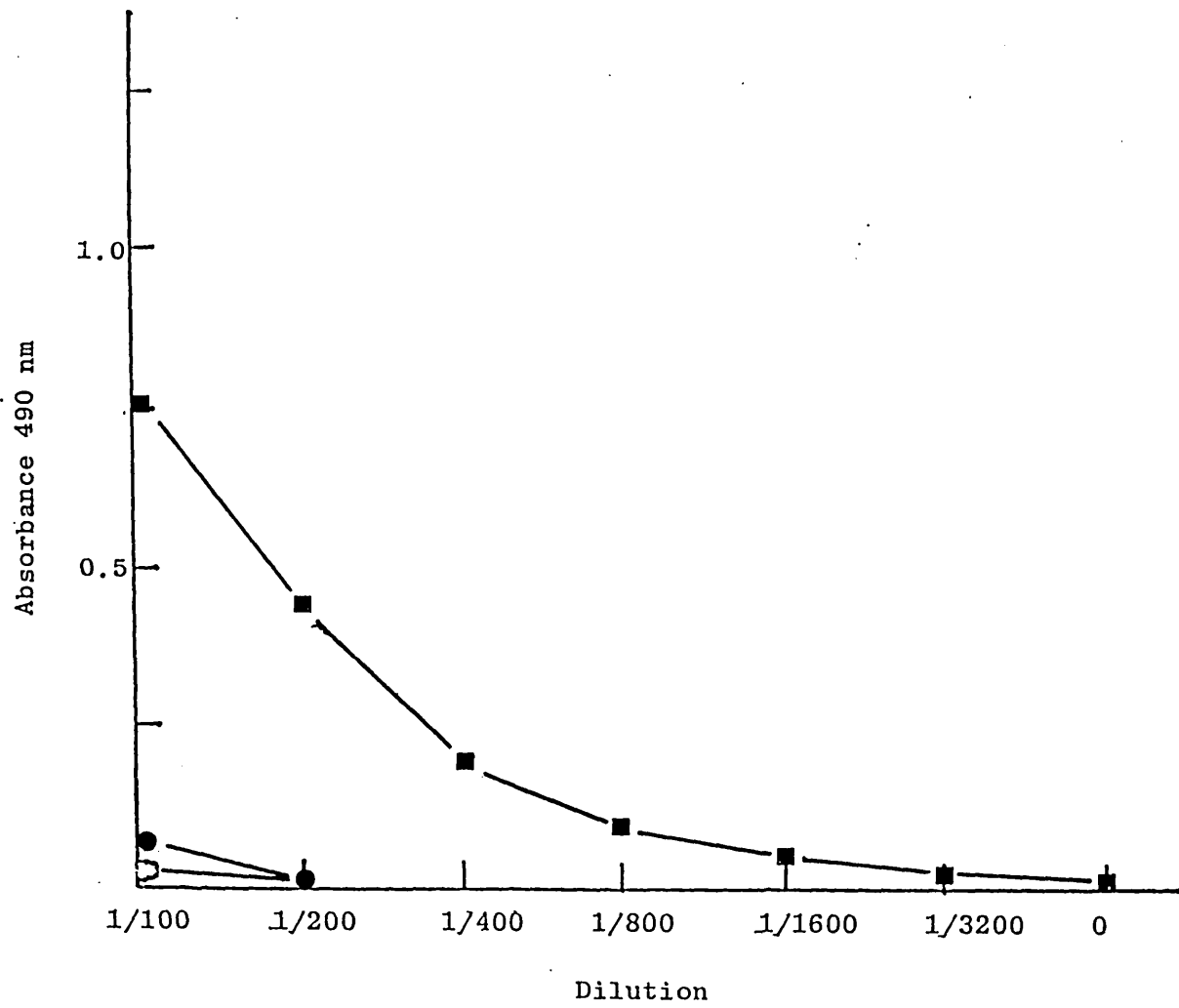
Antigen tested on ELISA: DOG

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

Figure 1.12 Activity of Rabbit antisera measured on ELISA



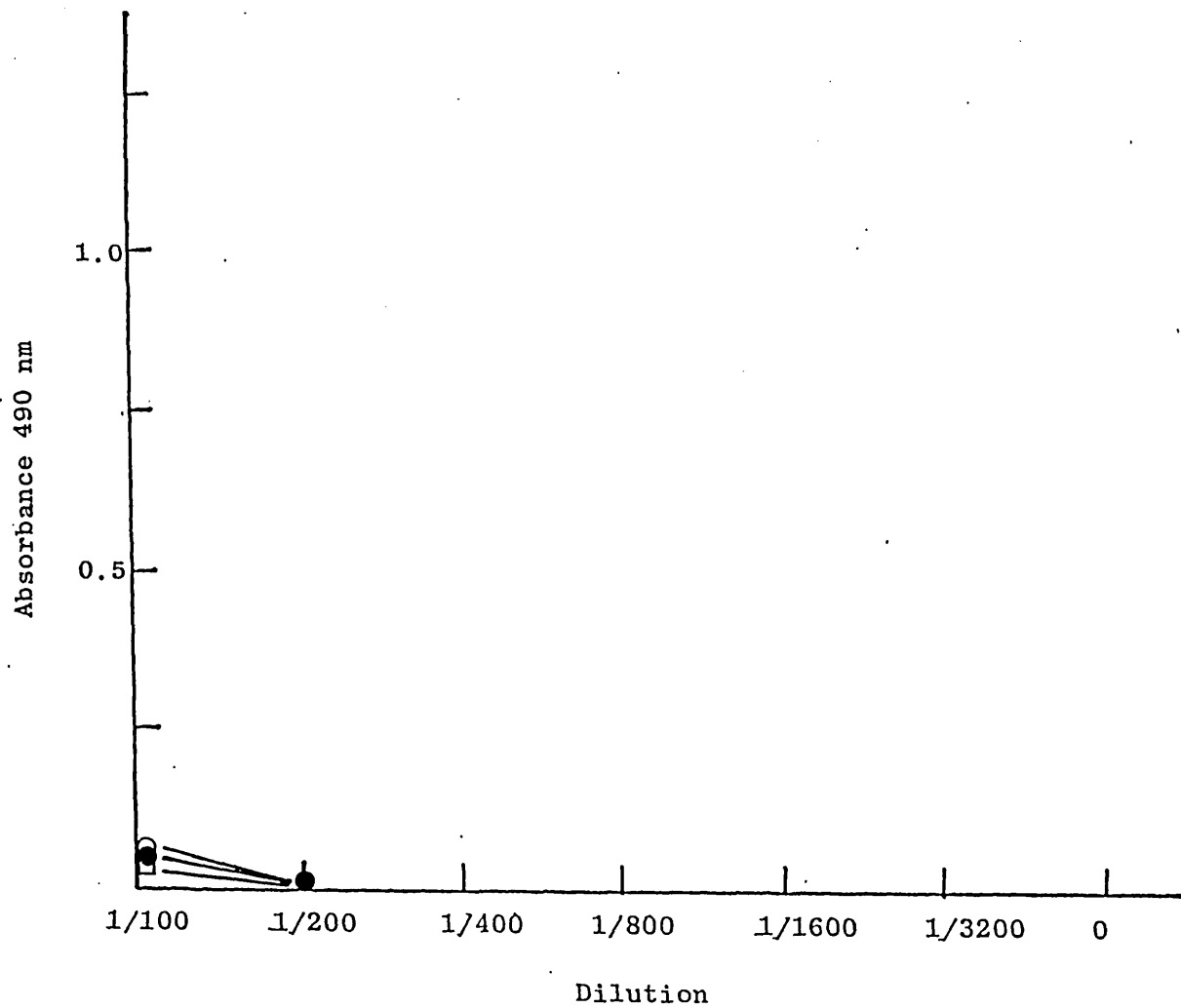
Antigen tested on ELISA: OX

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

Figure 1.13 Activity of Rabbit antisera measured on ELISA



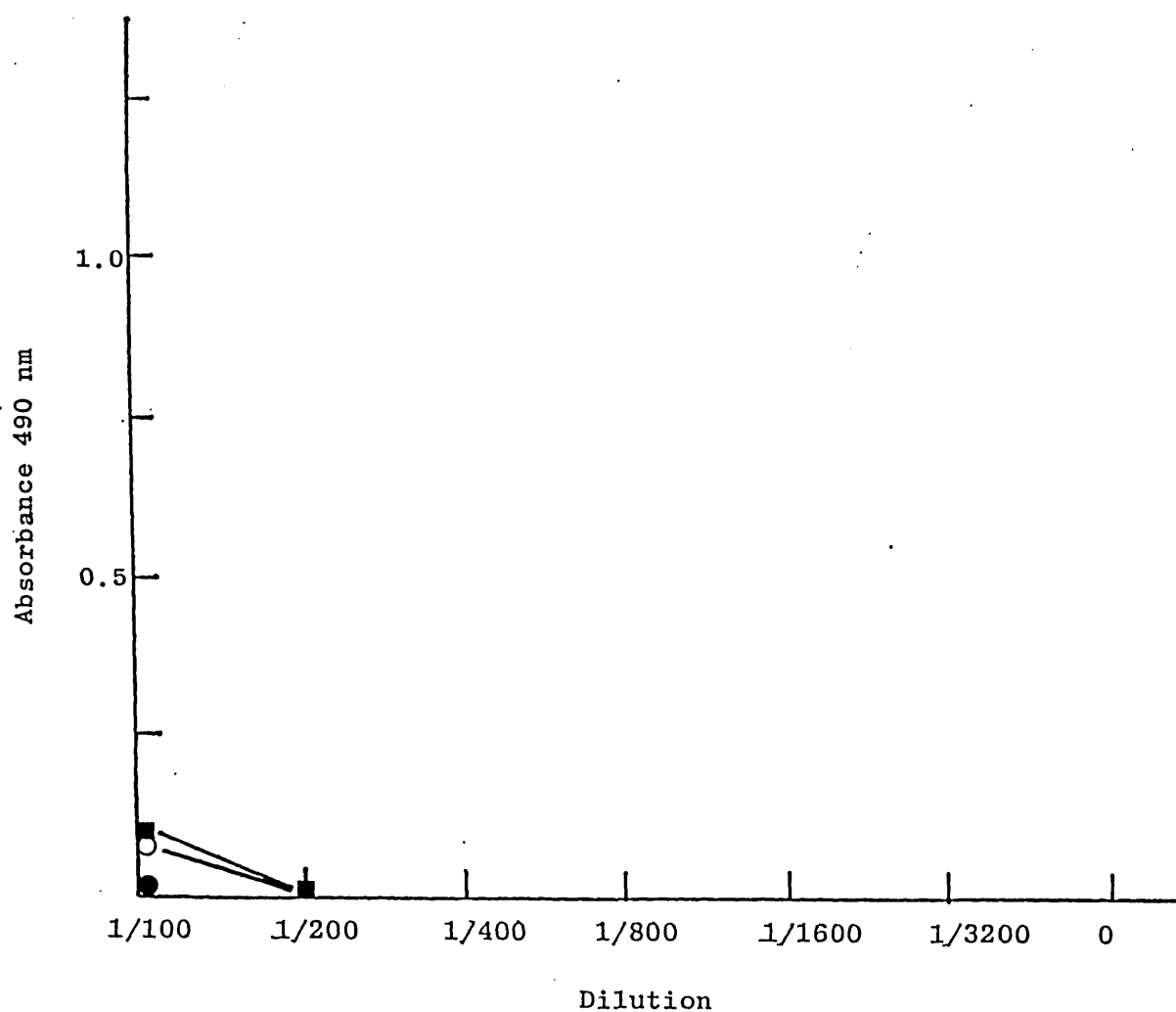
Antigen tested on ELISA: PIG

KEY

Antisera to

Man	●	
Dog	○	
Ox	□	Absorbed

Figure 1.14 Activity of Rabbit antisera measured on ELISA



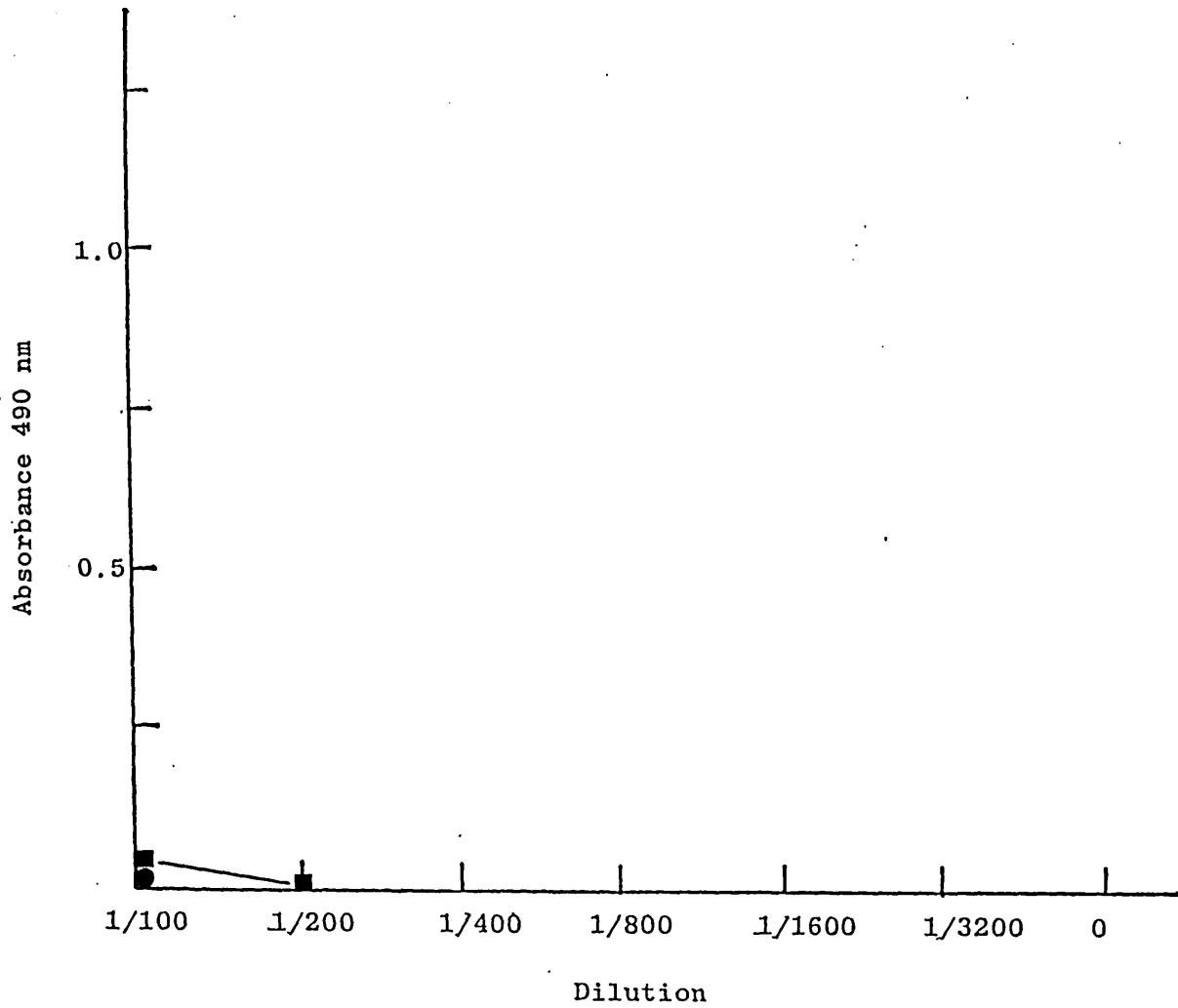
Antigen tested on ELISA: AVIAN

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

Figure 1.15 Activity of Rabbit antisera measured on ELISA



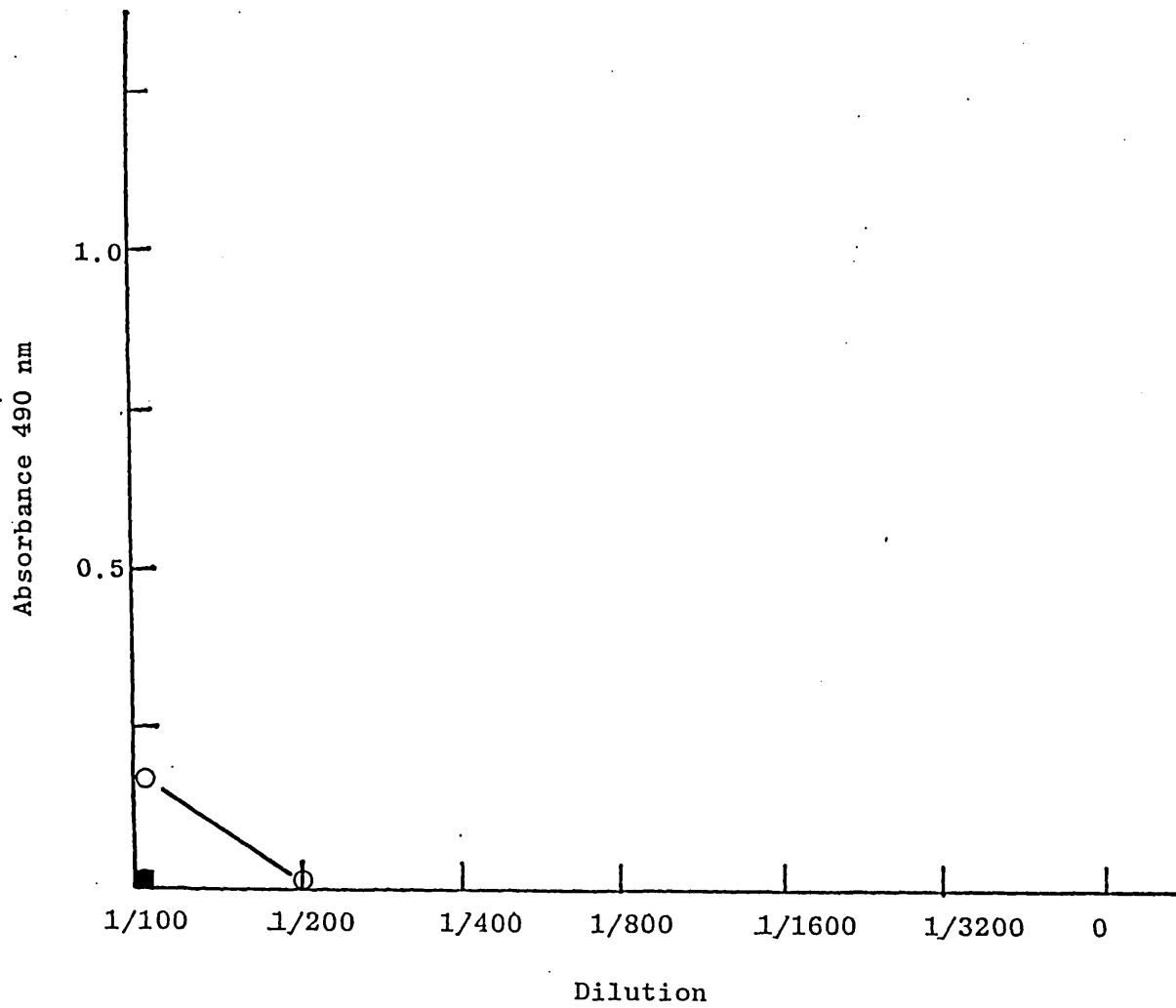
Antigen tested on ELISA: HORSE

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

Figure 1.16 Activity of Rabbit antisera measured on ELISA



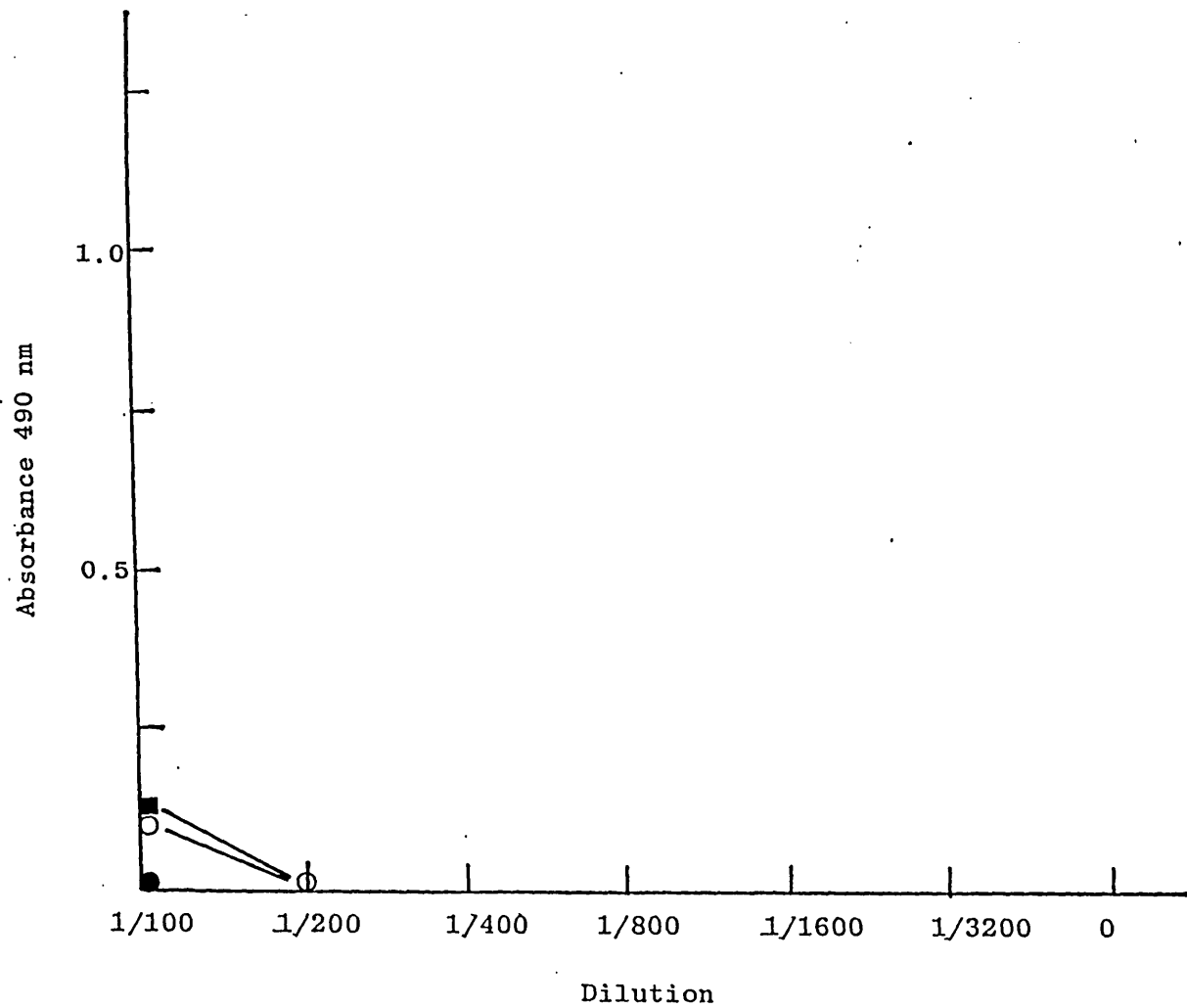
Antigen tested on ELISA: BADGER

KEY

Antisera to

Man	●	
Dog	○	
Ox	□	Absorbed

Figure 1.17 Activity of Rabbit antisera measured on ELISA



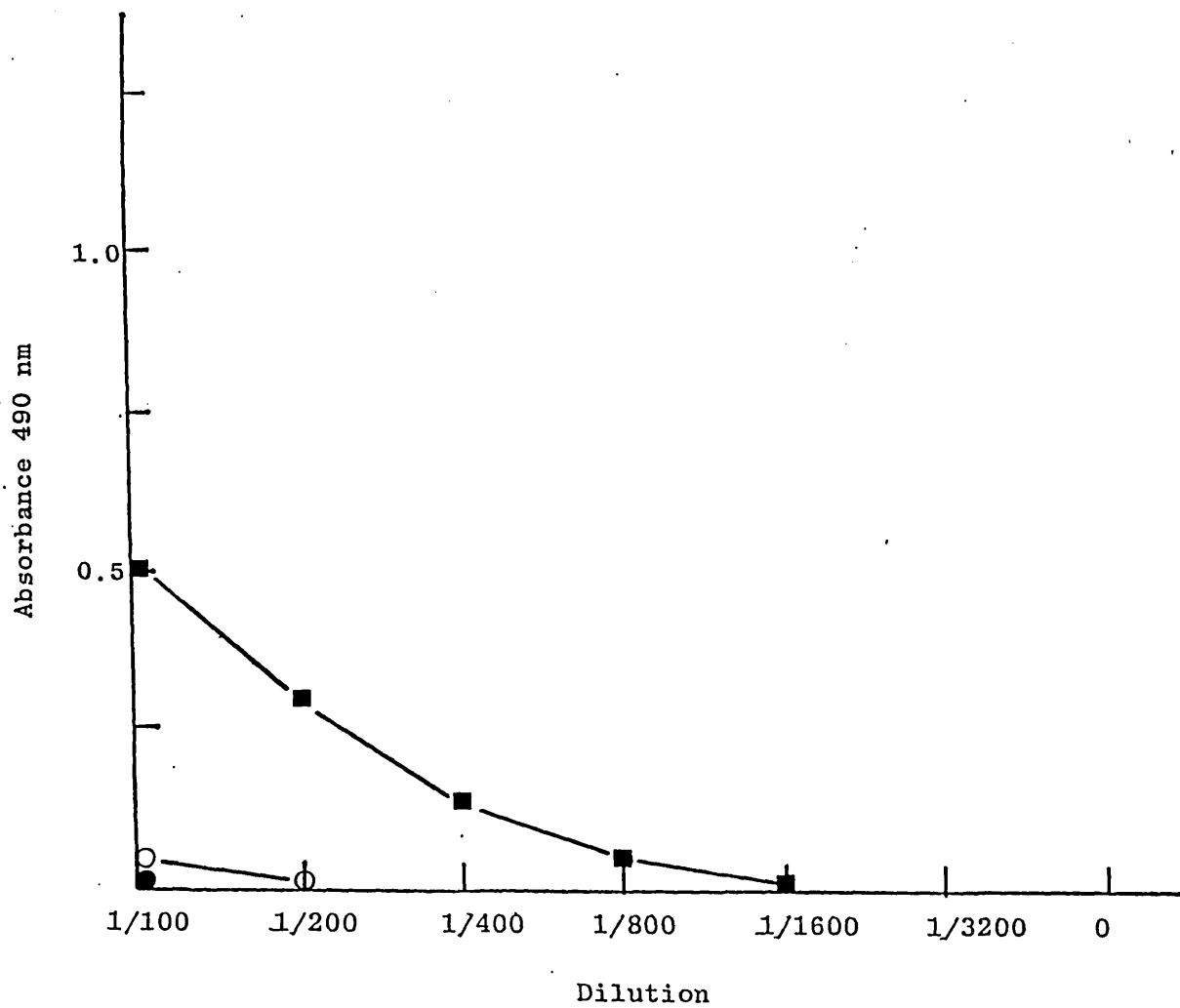
Antigen tested on ELISA: CAT

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

Figure 1.18 Activity of Rabbit antisera measured on ELISA



Antigen tested on ELISA: GOAT

KEY

Antisera to

Man	●	
Dog	○	
Ox	■	Absorbed

A parallel experiment was performed using the precipitin test. Figures 1.19 to 1.21 show the cross reactivity of the antisera prior to and after absorption with the heterologous antigens, after absorption the sensitivity of the test is reduced, but the cross reactivity is almost eliminated with the exception of anti-Ox antiserum and Goat antigen. A result similar to that obtained with ELISA is seen. In fact in this case there is no differentiation whatsoever.

Figure 1.19 Activity of Rabbit anti-Man antisera prior and post absorption measured on the precipitin test.

Dilution:		1/2	1/4	1/8	1/16	1/32	1/64
Antigen:							
Man		+++	+++	+++	++	+	
Ox							
Goat		+					
Pig		+					
Dog		++	+				
Cat		++	+				
Horse		++	++	+			
Rabbit							
Avian		++	++				
Reptile		+	+	+			

Dilution:		1/2	1/4	1/8	1/16	1/32	1/64
Antigen:							
Man		+++	+++	+			
Ox							
Goat		+					
Pig							
Dog							
Cat							
Horse							
Rabbit							
Avian		+					
Reptile		+					

Prior to absorption

Post absorption

Antigen: 1/50 dilution of animal sera

Figure 1.20 Activity of Rabbit anti-Dog antisera prior and post absorption measured on the precipitin test.

Dilution:		1/2	1/4	1/8	1/16	1/32	1/64
Antigen:							
Dog		+++	+++	++			
Cat		++					
Man							
Ox		+					
Goat		+					
Pig		+					
Horse		++					
Rabbit		+					
Avian							
Reptile							

Dilution:		1/2	1/4	1/8	1/16	1/32	1/64
Antigen:							
Dog		++					
Cat							
Man							
Ox							
Goat							
Pig							
Horse							
Rabbit							
Avian							
Reptile							

Prior to absorption

Post absorption

Antigen: 1/50 dilution of animal sera

Figure 1.21 Activity of Rabbit anti-Ox antisera prior and post absorption measured on the precipitin test.

Dilution:	1/2	1/4	1/8	1/16	1/32	1/64
Antigen:						
Ox	+++	+++	+++	+		
Goat	+++	+++	+++	+		
Man	++	++	+			
Pig	++	+				
Dog	+	+				
Cat	+	+				
Horse	+++	+++				
Rabbit						
Avian						
Reptile						

Dilution:	1/2	1/4	1/8	1/16	1/32	1/64
Antigen:						
Ox	+++	+				
Goat	+++	+				
Man						
Pig						
Dog						
Cat						
Horse						
Rabbit						
Avian						
Reptile						

Prior to absorption

Post absorption

Antigen: 1/50 dilution of animal sera

1.3.3 Specificity testing of murine monoclonal antibodies prepared against dog antigen.

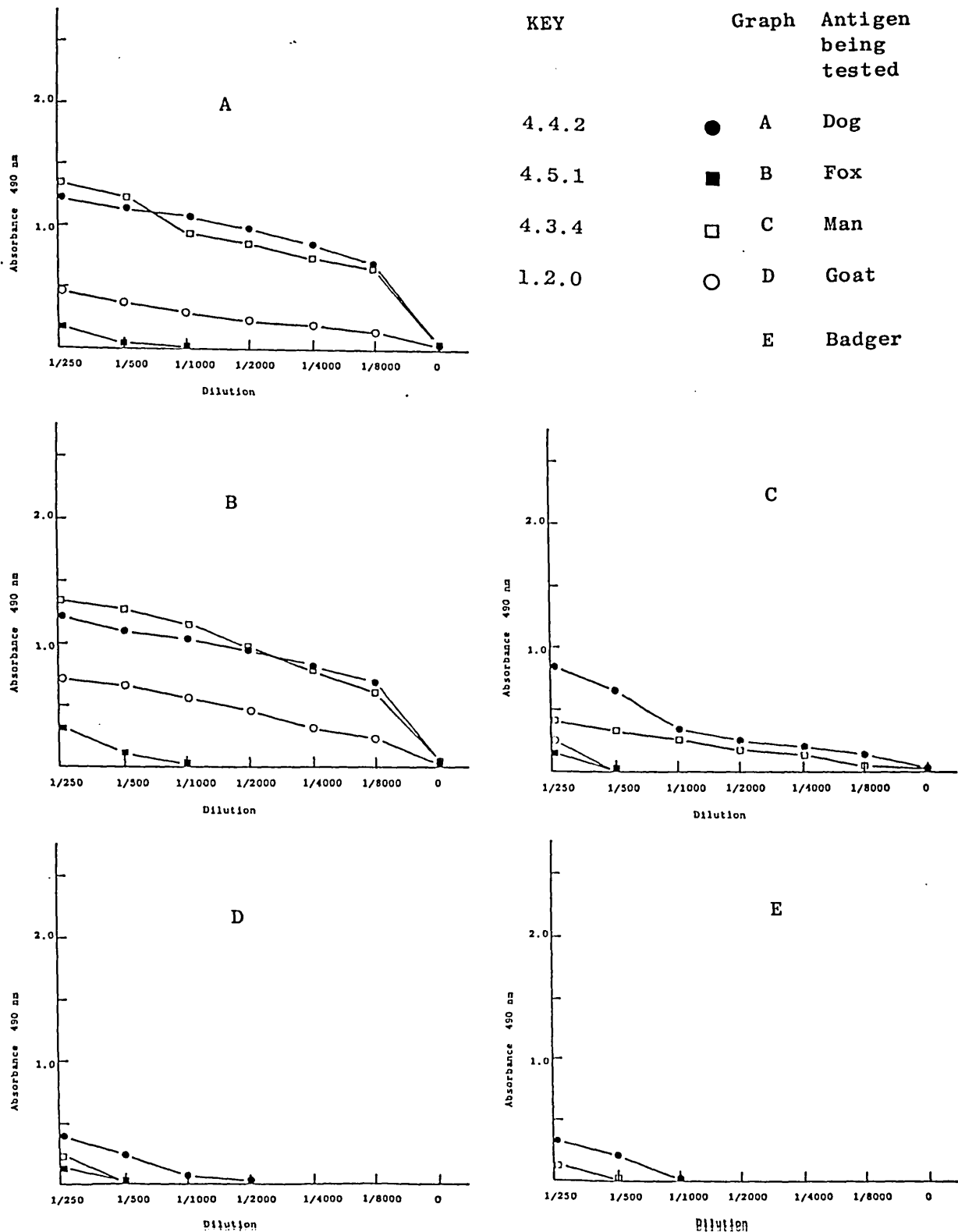
Four reactive monoclonal antibodies were prepared to Dog antigen (whole serum). Western transfer and radiolabelling of the antibodies showed that all four antibodies were binding to the immunoglobulin component of Dog serum. Isotyping of the antibodies with immunodiffusion showed the class of the monoclonal antibodies to be IgG.

These monoclonal antibodies were tested on the ELISA for species specificity. Figure 1.22.

All antibodies reacted with Dog antigen, albeit antibody 4.5.1 showed much less activity than the other three (graph A).

When tested with Fox antigen, all the antibodies reacted as they had with Dog antigen, showing no species specificity whatsoever. There was less activity with the other antigens tested, Man, Goat and Badger (graphs C, D, and E respectively), but cross reactions still occurred.

Figure 1,22: Comparison of specificities of murine anti-Dog monoclonal antibodies



1.3.4 Sandfly host identification, a comparison between the ELISA and the CCIE technique.

Table 1.1(a & b) shows the results of parallel analysis of 230 fed sandflies collected from the Cevennes, France. The ELISA was able to identify 88% of the flies, CCIE 85%. The ELISA detected 5 double feeds between Man and Dog, the CCIE none.

Only 39% of the results were common between the two tests.

Table 1.a: Identification of 230 sandfly bloodmeals using both the ELISA and CCIE, a comparison of results.

Sandfly	Host Identity		Similar result
	ELISA	CCIE	
1	DOG	DOG	x
2	DOG	DOG	x
3	DOG	MAN	
4	---	---	x
5	MAN	MAN	x
6	DOG	DOG	x
7	DOG	MAN	
8	DOG	MAN	
9	DOG	---	
10	DOG	MAN	
11	DOG	MAN	
12	DOG	MAN	
13	DOG	DOG	x
14	DOG	MAN	
15	DOG	MAN	
16	DOG	MAN	
17	DOG	DOG	x
18	DOG	DOG	x
19	DOG	MAN	
20	DOG	---	
21	DOG	---	
22	DOG	MAN	
23	DOG	MAN	
24	DOG	MAN	
25	DOG	DOG	x
26	DOG	MAN	
27	DOG	MAN	
28	DOG	---	
29	DOG	MAN	
30	GOAT	DOG	
31	DOG	MAN	
32	---	MAN	
33	---	DOG	
34	DOG	MAN	
35	DOG	MAN	
36	DOG	MAN	
37	DOG	DOG	x
38	DOG	MAN	
39	DOG	MAN	

Sandfly	Host Identity		Similar result
40	DOG	MAN	
41	AVIAN	DOG	
42	DOG	MAN	
42	DOG	MAN	
44	DOG	DOG	x
45	----	DOG	
46	----	----	x
47	DOG	MAN	
48	AVIAN	----	
49	DOG	DOG	x
50	DOG	MAN	
51	DOG	DOG	x
52	DOG	DOG	x
53	DOG	----	
54	DOG	DOG	x
55	DOG	MAN	
56	DOG	MAN	
57	DOG	----	
58	DOG	----	
59	DOG	DOG	x
60	DOG	----	
61	DOG	DOG	x
62	MAN/DOG	----	
63	DOG	DOG	x
64	MAN/DOG	DOG	X
65	DOG	MAN	
66	DOG	----	
67	DOG	MAN	
68	DOG	MAN	
69	DOG	MAN	
70	DOG	MAN	
71	DOG	MAN	
72	DOG	MAN	
73	DOG	DOG	x
74	DOG	DOG	x
75	MAN/DOG	MAN	x
76	DOG	DOG	x
77	DOG	DOG	x
78	DOG	DOG	x
79	----	DOG	
80	----	DOG	
81	----	DOG	
82	DOG	DOG	x
83	----	----	x
84	DOG	DOG	x
85	DOG	DOG	x
86	DOG	MAN	
87	DOG	MAN	
88	DOG	DOG	x
89	DOG	MAN	
90	DOG	MAN	
91	DOG	DOG	x

Sandfly	Host Identity		Similar result
92	DOG	DOG	x
93	DOG	MAN	
94	DOG	DOG	x
95	DOG	DOG	x
96	DOG	DOG	x
97	DOG	DOG	x
98	DOG	DOG	x
99	DOG	---	
100	DOG	MAN	
101	DOG	MAN	
102	DOG	MAN	
103	DOG	---	
104	DOG	MAN	
105	DOG	MAN	
106	DOG	MAN	
107	DOG	MAN	
108	DOG	MAN	
109	DOG	---	
110	DOG	DOG	x
111	DOG	DOG	x
112	DOG	DOG	x
113	DOG	DOG	x
114	DOG	DOG	x
115	DOG	DOG	x
116	DOG	---	
117	---	DOG	
118	DOG	MAN	
119	---	DOG	
120	DOG	RODENT	
121	DOG	DOG	x
122	DOG	DOG	x
123	DOG	DOG	x
124	DOG	DOG	x
125	DOG	MAN	
126	DOG	MAN	
127	DOG	DOG	x
128	---	DOG	
129	---	---	x
130	---	MAN	
131	---	DOG	
132	---	---	x
133	---	---	x
134	DOG	MAN	
135	DOG	DOG	x
136	DOG	MAN	
137	DOG	MAN	
138	DOG	---	
139	DOG	DOG	x
140	DOG	---	
141	DOG	MAN	
142	DOG	MAN	
143	---	---	x

Sandfly	Host Identity		Similar result
144	DOG	DOG	x
145	DOG	DOG	x
146	DOG	DOG	x
147	----	----	x
148	GOAT	DOG	
149	MAN	DOG	
150	MAN	DOG	
151	DOG	DOG	x
152	----	----	x
153	----	----	x
154	----	----	x
155	----	----	x
156	----	DOG	
157	DOG	DOG	x
158	----	DOG	
159	DOG	DOG	x
160	MAN	----	
161	MAN	----	
162	DOG	DOG	x
163	DOG	----	
164	DOG	----	
165	----	MAN	
166	DOG	DOG	x
167	DOG	DOG	x
168	DOG	MAN	
169	DOG	MAN	
170	MAN	DOG	
171	MAN	DOG	
172	MAN	----	
173	DOG	DOG	x
174	DOG	DOG	x
175	DOG	DOG	x
176	----	----	x
177	----	DOG	
178	DOG	----	
179	DOG	----	
180	----	DOG	
181	DOG	DOG	x
182	DOG	----	
183	DOG	MAN	
184	DOG	----	
185	DOG	----	
186	DOG	DOG	x
187	DOG	DOG	x
188	DOG	DOG	x
189	MAN	MAN	x
190	MAN	MAN	x
191	----	DOG	
192	DOG	----	
193	DOG	DOG	x
194	DOG	MAN	
195	DOG	MAN	

Sandfly	Host Identity		Similar result
196	DOG	DOG	x
197	DOG	DOG	x
198	DOG	DOG	x
199	DOG	DOG	x
200	DOG	MAN	
201	DOG	DOG	x
202	DOG	DOG	x
203	DOG	MAN	
204	DOG	DOG	x
205	DOG	MAN	
206	DOG	DOG	x
207	DOG	---	
208	DOG	MAN	
209	DOG	MAN	
210	DOG	DOG	x
211	---	DOG	
212	DOG	DOG	x
213	DOG	---	
214	---	DOG	
215	---	DOG	
216	DOG	DOG	x
217	MAN/DOG	MAN	x
218	DOG	DOG	x
219	DOG	DOG	x
220	DOG	DOG	x
221	DOG	---	
222	DOG	---	
223	DOG	MAN	
224	DOG	MAN	
225	DOG	DOG	x
226	---	DOG	
227	---	---	x
228	---	DOG	
229	DOG	DOG	x
230	BADGER	DOG	

Table 1.b: Summary table of the comparison of the results of ELISA and CCIE for sandfly bloodmeal identification

HOST IDENTITY	ELISA	CCIE
DOG	177	105
MAN	10	79
AVIAN	2	-
BADGER	1	-
RODENT	-	1
GOAT	2	-
DOUBLE FEED	5	-
MAN & DOG	-	
NEGATIVE	33	45
	<u>230</u>	<u>230</u>

NO. OF SIMILAR RESULTS WITH EACH TEST = 89

1.4 DISCUSSION

The crucial aspects of immuno-identification of arthropod blood-meals are the specificity and sensitivity of the antiserum being used. The immunization protocol used was designed to produce high titre antiserum, this being achieved in most cases. However the antibodies were not specific. Whole serum (the antigen) is a very complex array of proteins containing thousands of antigenic epitopes, all of which can stimulate an antibody population. Many of these epitopes occur simultaneously in all mammals only a very few epitopes may represent the species of the donor. Thus strong cross reactions were found between all the antisera prepared with mammalian antigen. The antisera prepared with non-mammalian antigens, i.e. reptilian and avian showed little cross reactions with the other mammalian antigens, in fact they were significantly specific enough for clear identification.

These common epitopes between the mammalian species were described by Weitz (1952) as the heterologous antigens. He demonstrated that the antibodies to these antigens could be removed from the antisera by exposure to the antigens. Weitz mixed antisera with sera from other mammalian animals and then tested the activity of the antisera using the precipitin test. He found a much higher specificity had been achieved, although the sensitivity had been vastly reduced, in most cases virtually to a non-useable degree.

G.Gill (pers. comm. 1984) found that the specificity of liquid absorbed antisera declined over quite short periods, eventually rendering the antisera useless. The reason for this is that the cross reacting antigens may not bind the antibodies with the same degree of

avidity, thus the antigen antibody complex have lower affinity constants. With freeze thawing and manipulation of the antisera, these antibodies disassociate from the antigen antibody complex. Centrifugation was used to remove antigen:antibody complexes but it could never successfully remove all the complexed antibody, thus these problems arose.

For these reasons solid phase absorption (affinity column) was employed, the antigens being bound to the solid phase are insoluble and removable. Any cross reacting antibody binding to them, will then be successfully removed from the antiserum. An alternative method for removing antigen:antibody complexes after liquid phase absorption would be to incubate the antiserum with the complement component Clq bound to a solid phase. Clq exclusively binds complexed antibody, leaving free the unbound antibody (Segal et al., 1979). This method works well but would include an extra stage in an assay.

Due to the amplifying nature of the ELISA system, the reduction in sensitivity after solid phase absorption was not significant. A reduction of 50% in the activity was observed but the titre was still high, e.g. 1/460. This compares favourably to that of the titre used in the precipitin test which was between 1/4 and 1/8.

In 1975 Kohler and Milstein described an in vitro system for producing monoclonal antibodies. This system is based on the fact that antibody production in an intact animal is the sum of production of antibody-producing B cell clones arising from single B lymphocytes. Such clones can be prepared in vitro by fusion of effector cells from an immunized animal with a continuous cell line capable of producing antibody (Herzenberg et al., 1979). Since each molecule of antibody is directed at the antigen in question, the specific antibody is not

diluted by irrelevant antibodies, and high concentrations of specific antibody can be obtained. Such antibodies have provided useful specific tools that have been extensively used in molecular biology. The use of monoclonal antibodies for bloodmeal identification seems an obvious avenue to pursue. However, the theoretical advantages of using monoclonal antibodies are not realised in practise. Hybridoma clones were produced with murine spleen cells stimulated with dog serum as the antigen. This antigen is extremely complex offering many possible clone products. The reactive clones that were produced all showed a lack of species specificity, they all totally reacted with fox antigen and to a lesser extent with all the other mammalian antigens tested. This cross reactivity is due to antigenic epitopes occurring simultaneously in all mammalian species, although they might vary quantitatively. Western transfer and autoradiography showed that all the monoclonal antibodies were bound to the immunoglobulin component of serum. Solid phase absorption would not be of any use in attempts to improve the specificity of the antibodies, due to the monospecific nature of each hybridoma cell line. If a particular antigenic determinant could be isolated that produced a species specific clone, this problem could be solved. But when starting with whole serum as the antigen it is comparable to the classic needle search in the haystack.

Jefferies et al. (1982) have experienced similar difficulties in preparing species specific monoclonal antibodies. These workers were attempting to raise a human specific anti IgG monoclonal antibody. They performed hundreds of fusions and produced thousands of clones and not once did they produce a species specific monoclonal antibody. Most

of the clones reacted with a whole range of vertebrate animals, the nearest they got was one clone that did react with human and gorilla IgG lambda light chains.

The production of a comprehensive library of species specific monoclonal antibodies, would be ideal, and is not beyond possibility, but would require a great deal of time and manpower. Such a project would need to be organised and financed by a well coordinated international organization.

The comparison of techniques of bloodmeal identification between counter current immunoelectrophoresis (CCIE) and ELISA shows enormous variation between the results. Only 39% of the results were agreed by both methods. This casts grave doubts on the validity of techniques such as CCIE and precipitin. They perform well enough at the family level but there is no specificity when attempting species identification.

The ELISA has been shown to be superior in both sensitivity and specificity after absorption of the antiserum by solid phase absorption.

CHAPTER 2IDENTIFICATION OF THE BINDING CHARACTERISTICS OF THE
REMAINING SPECIFIC ANTIBODIES POST SOLID PHASE ABSORPTION

2.1 INTRODUCTION

The results of the previous chapter demonstrate that solid phase absorption can enhance the specificity of an antiserum. The absorption procedure removes most antibodies that bind to common antigenic determinants that occur in vertebrate species.

The remaining specific antibodies bind to unknown specific antigenic epitopes. The identity of these antigens would be valuable information as it would allow the production of more specific antisera and the possible production of specific monoclonal antibodies by immunising the donor animals with a purified antigen extract that would reduce the possibilities of cross reaction.

This chapter describes the methods used to identify the binding characteristics of the specific antibodies prepared after solid phase absorption

2.2 MATERIALS AND METHODS

Column affinity absorption and gluteraldehyde polymerised antigen absorption were described in the previous chapter.

2.2.1 Immuno-electrophoresis

a) Materials

As for immunodiffusion described in the previous chapter.

b) Procedure

The gel was poured onto 8 x 8 cm glass plates and allowed to set. The antigen well was punched and a trough for the antisera cut in the gel as shown in Figure 2.1.

5 μ l of human serum was placed in each antigen well.

The plate was placed on to the electrophoresis tank and run for 5 hours at 2 mA per antigen well per plate, until separation was complete. This was indicated by the inclusion of a well containing bromophenol blue which can be visually followed.

After separation the plate was removed and approximately 150 μ l of antisera placed in the troughs so that they were nearly full. The plate was then placed in the refrigerator at 4°C overnight for diffusion and precipitation development.

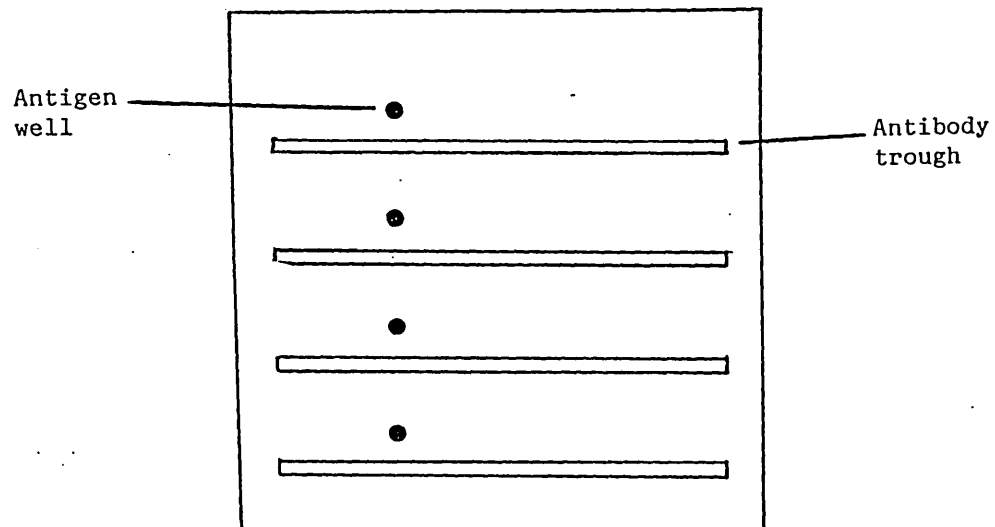


Figure 2,1 Immunoelectrophoresis gel.

2.2.2 Two-dimensional immunoelectrophoresis (2-DIEP)

This technique is similar to immunoelectrophoresis. The antigens are first separated by electrophoresis as described above.

Then an antibody containing gel is placed next to it and the antigens are electrophoresed into the antibody. Precipitin arcs occur and all the other non-precipitated proteins are removed by the electrophoresis.

The procedure is the same as described in the previous section until the plates are removed from the electrophoresis tank. After the electrophoresis the gel was sectioned with a secatome blade and transferred to another glass plate (Figure 2.2).

Meanwhile 7.5 ml of molten agarose at 56°C was mixed with 500 µl of antisera. The antisera/gel mixtures were poured next to the gel strips, care was taken to ensure that the gel abutted evenly against the first strip. After the gel had set the plate was replaced on the electrophoresis tank with the antigens moving towards the anode. The voltage was set to 20 volts (2.5 volts per cm of plate) and ran overnight (approx. 16 hours), the plates were press dried and stained as described for immunodiffusion.

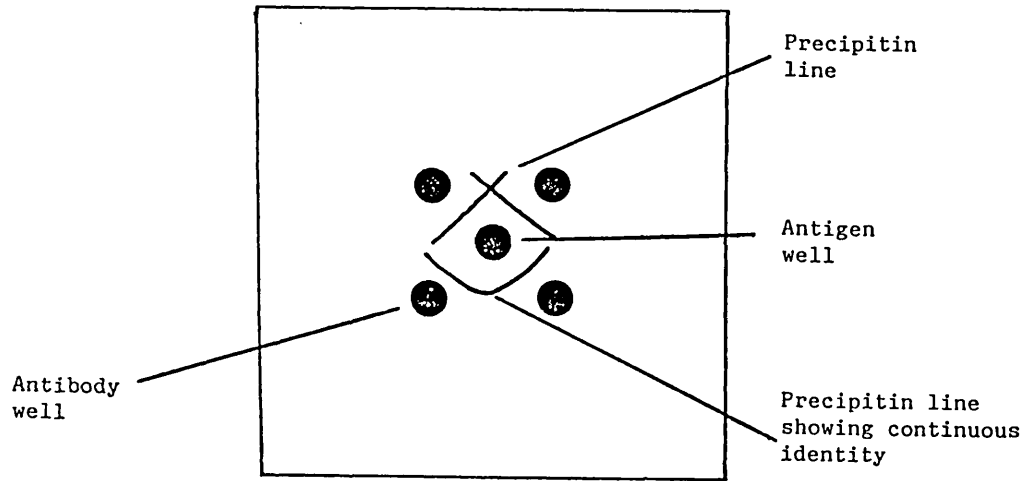


Figure 2.2 Diagram of immunodiffusion gel

2.2.3 Gel diffusion

Plate preparation is identical to that described for immunodiffusion. In this experiment the outer wells contained the absorbed antisera and a series of known antisera raised against the beta region proteins of human serum made available by the kindness of the Immunodiagnostic Research Laboratories, Birmingham, (IDRL). The centre well contained human sera, the antigen.

The absorbed antiserum was paired with each one of the known antisera, the results were read by looking for a line of continuous identity (Figure 2.3).

The plates were press dried and stained as described for immunodiffusion.

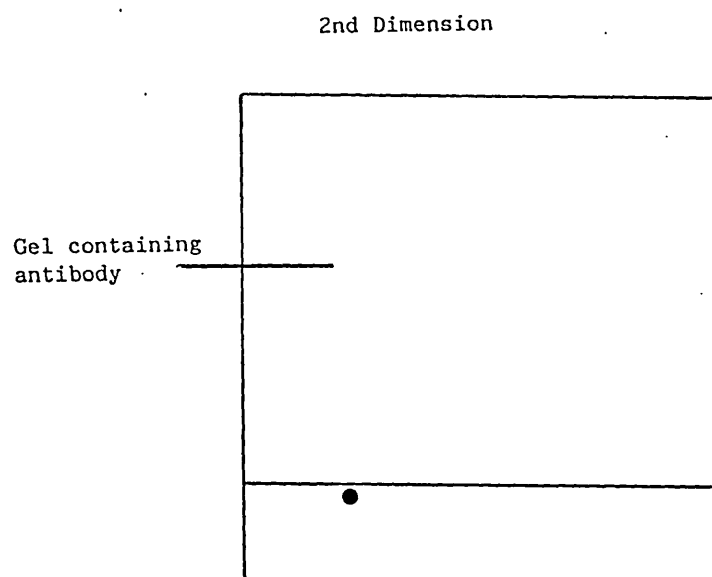
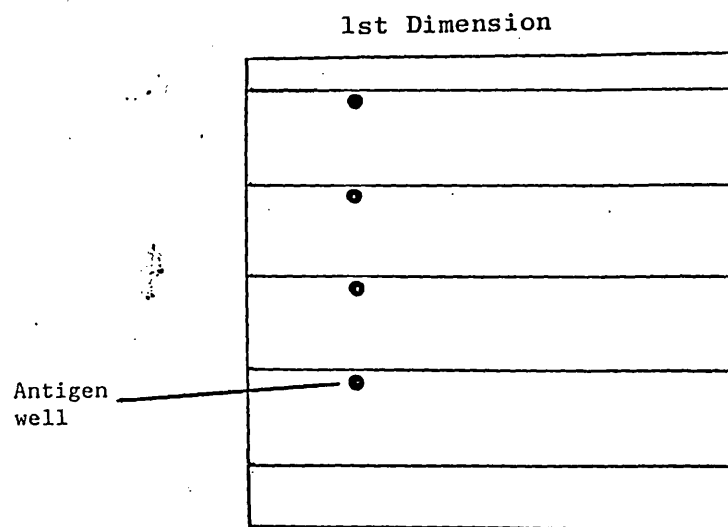


Figure 2,3 Two Dimensional Electrophoresis gel

2.2.4 Beta lipoprotein extraction and purification by ultracentrifugation

a) Materials

20 ml human serum

0.15 M saline containing 10 mg/100 M EDTA

2.0 M saline containing 10 mg/100 M EDTA

4 plastic (13.5 ml capacity) ultracentrifuge tubes

b) Procedure

5 ml of 2.0 M saline was placed in each of the 4 plastic ultracentrifuge tubes.

5 ml of serum was carefully layered onto the surface of the 2.0 M saline. The tubes were filled with 0.15 M saline, capped and placed into the No.40 rotor of a Spinco Model L ultracentrifuge. The tubes were then centrifuged with refrigeration, at 105,000 x g for 18 hours. The separated layers were carefully pipetted off and stored at -20°C.

2.3 RESULTS

The results of ELISA from the previous chapter demonstrated the presence of specific immunoglobulins, although the binding characteristics were completely unknown.

Two dimensional electrophoresis is a technique that can be used to visualize the different antigen and antibody complexes that are formed when an antiserum is mixed with the corresponding antigen. Figure 2.4 shows the results of running human serum into an antibody gel containing anti human antiserum. Many peaks are seen, including albumin, the alpha proteins; Immunoglobulin, the gamma globulin and the numerous beta region proteins. Figure 2.5 shows the effect of absorption of the same antiserum on the affigel column. Many of the peaks have gone, the remaining peaks occur in the beta region with a very slight trace in the gamma region.

Figure 2.4 Two dimensional electrophoresis
Non absorbed serum, Anti human

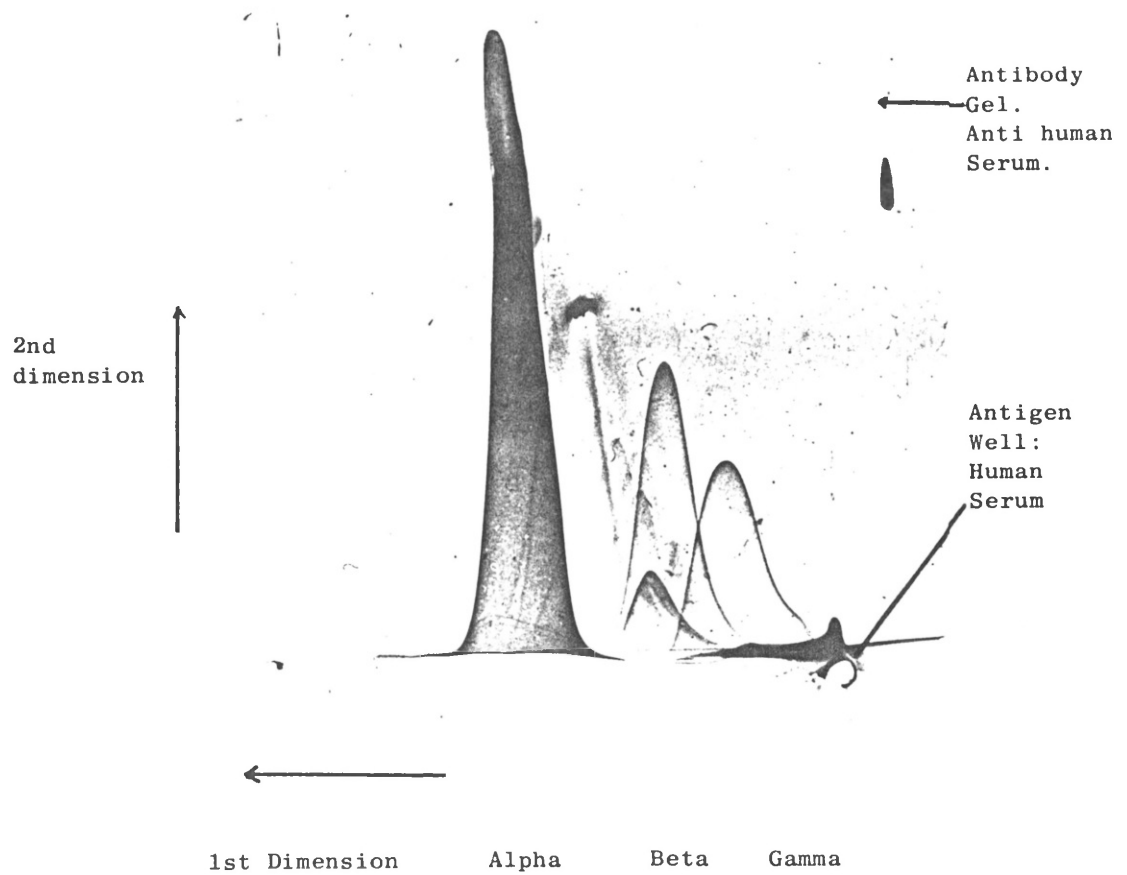


Figure 2.5 Two dimensional electrophoresis
Affinity column absorbed serum, anti human

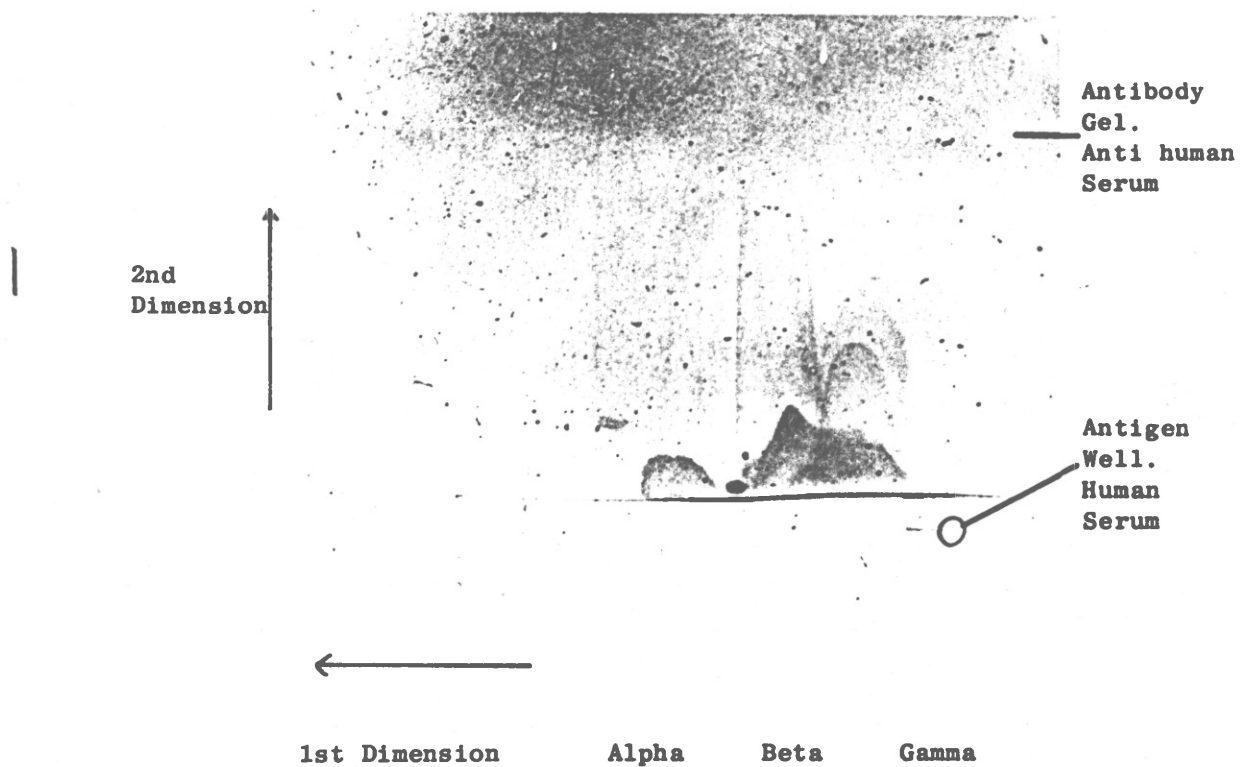


Figure 2.6 shows gel diffusion plates where the absorbed antisera was paired with a whole series of known antisera to human beta region plasma proteins. Continuous lines of identity occur between the absorbed sera and the known antisera to beta lipoprotein, complement component C4, Transferrin and Haptoglobin.

Figure 2.6 Double gel diffusion. Absorbed antiserum with known anti β -region proteins antisera.

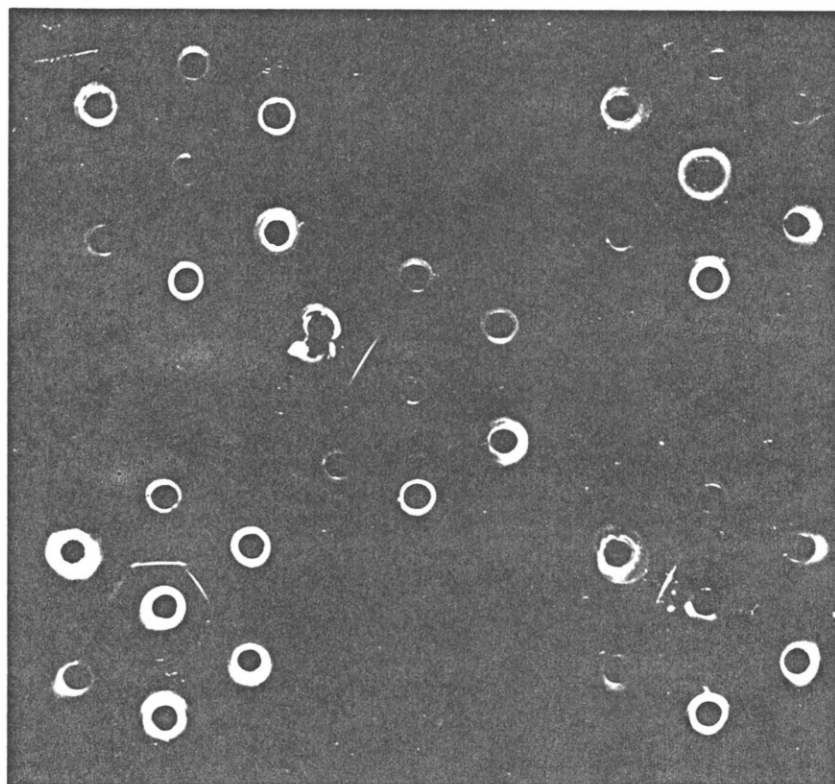
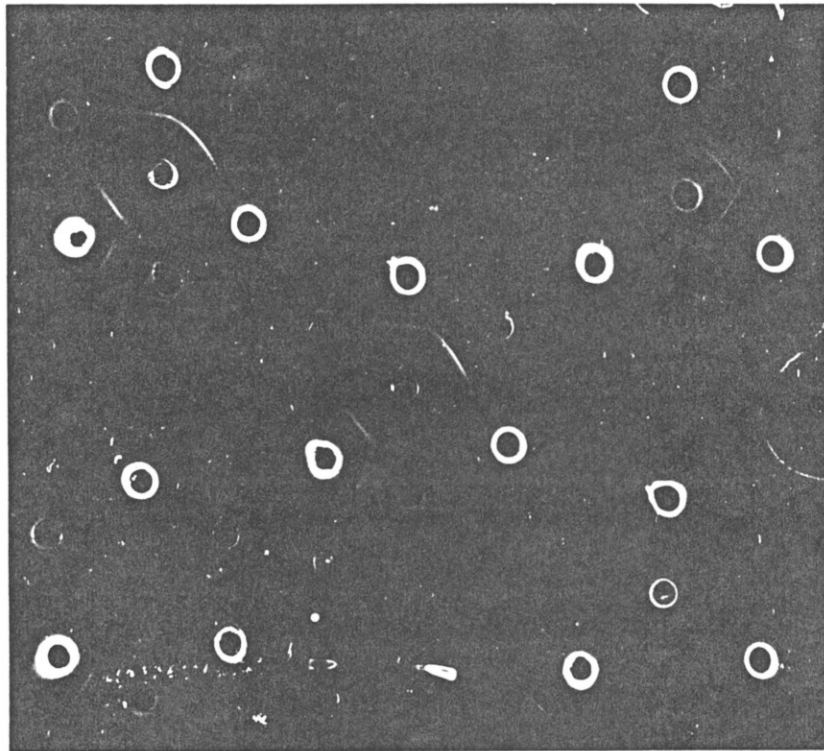


Figure 2.7 to 2.10 are further two-dimension electrophoresis gels with the absorbed serum and the available antigens identified with the gel diffusion. These figures confirm the results obtained in the gel diffusion shown in Figure 2.6.

It was not possible to obtain the two antigens haptoglobin and transferrin in high enough concentration to perform these analyses. It is known that many sera from malarious regions are ahaptoglobinaemic so the inability to use haptoglobins may not be any great loss. High density lipoprotein (HDL) was also excluded from this analysis because the separation technique separates the other fractions from the remaining plasma proteins but not HDL so that the two-dimensional analysis with HDL looks almost identical to that of using whole human serum as the antigen.

Figure 2.7 Two dimensional electrophoresis.

Antibody : absorbed anti human antiserum.

Antigen : Very low density lipoprotein

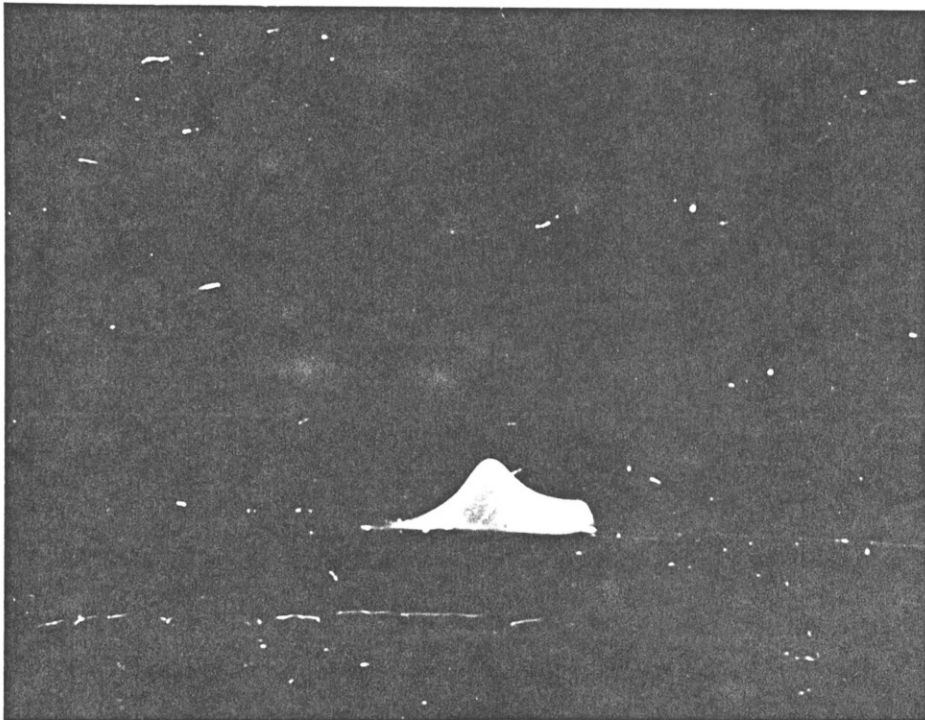


Figure 2.8 Two dimensional electrophoresis.
Antibody : absorbed antiserum.
Antigen : Low density lipoprotein.

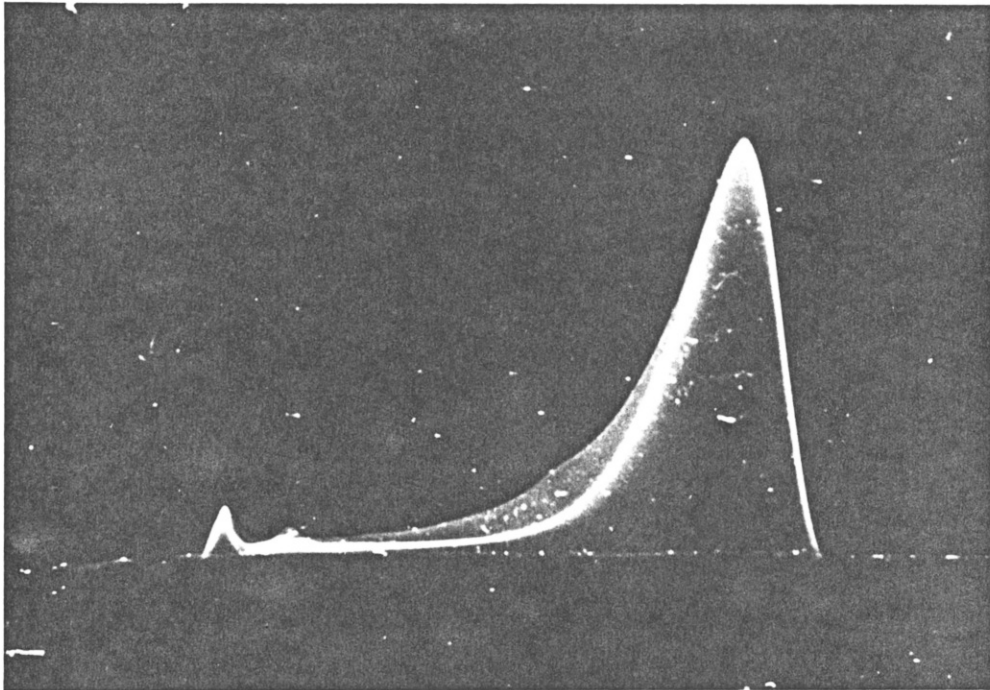


Figure 2.9 Two dimensional electrophoresis.

Antibody : absorbed anti human antiserum

Antigen : Intermediate density lipoprotein.

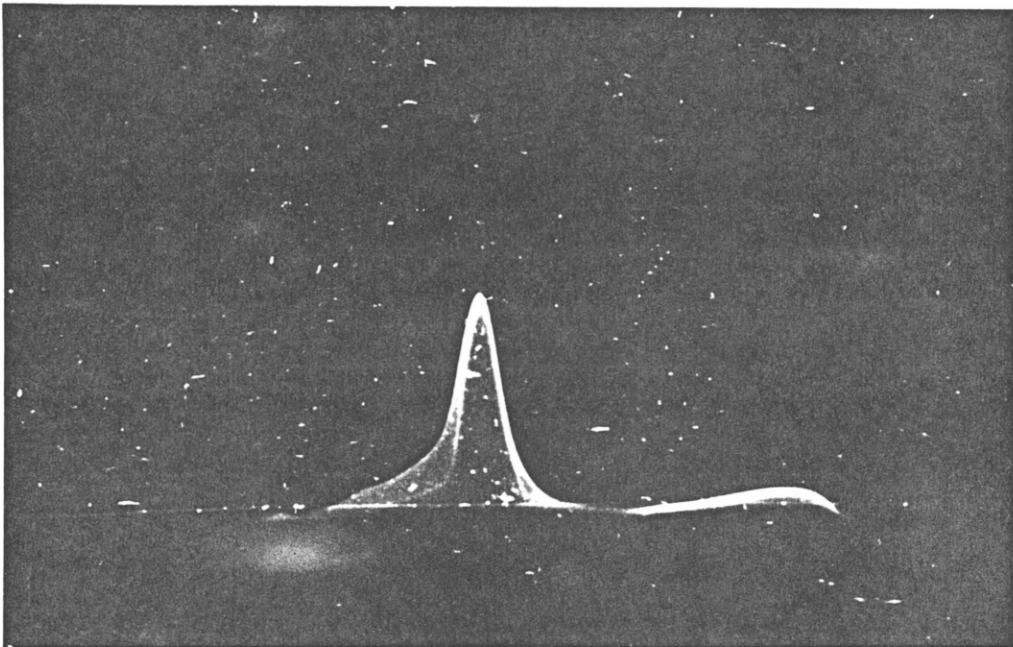
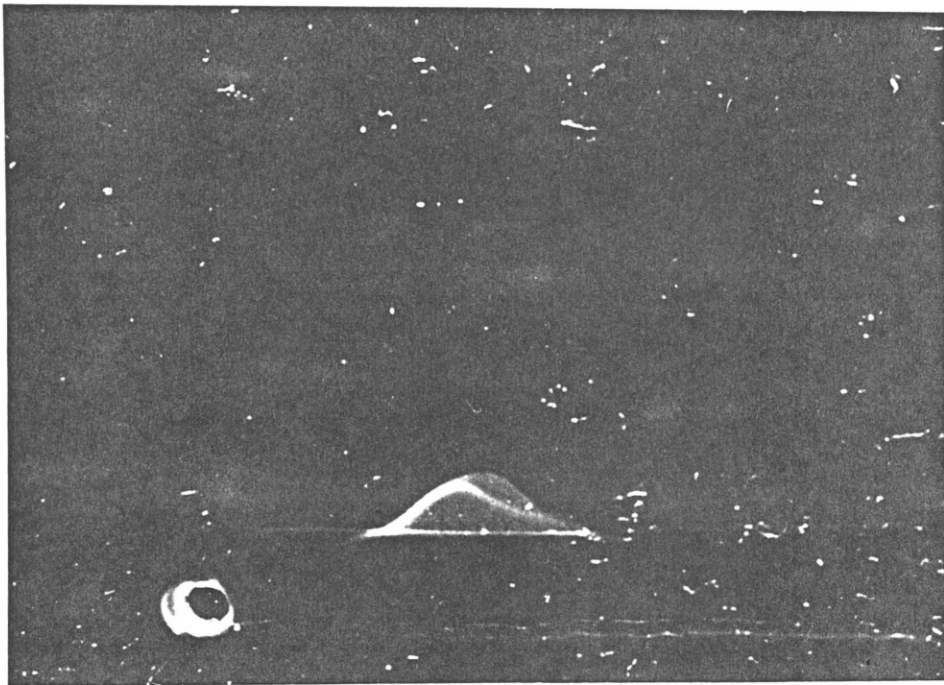


Figure 2.10 Two dimensional electrophoresis.

Antibody : absorbed anti human antiserum.

Antigen : Complement component C₄



2.4 DISCUSSION

Five molecules were identified as binding to the post-absorption specific antiserum, these were transferrin, haptoglobin, immunoglobulin, complement component C4 and beta lipoproteins. The reactions with the first two were very weak, this would be due to the lower serum concentrations at which the two molecules occur. Also the antibodies that bind to these molecules might be of a lower avidity and thus the precipitin reaction is not as strong.

Intra specific polymorphism occurs with transferrin and the haptoglobins, this intra specific diversity also then extends inter specifically, so much so that some of the antigenic epitopes are species specific.

The two dimensional electrophoresis with the absorbed antiserum showed a faint trace in the gamma globulin region. This is due to immunoglobulin, this result has been corroborated by the studies of Jefferies et al., (1982). This specific activity is very small as much of the anti-immunoglobulin antibody cross reacts with immunoglobulin of other species (Bauer, 1970; 1971a; 1971b; 1974; Esteves and Binaghi, 1972; Neoh et al., 1974; Vaerman et al., 1969; and Porter, 1979).

Complement component C₄ as well as C₂ and factor B are coded by genes of the major histocompatibility complex (Barger, 1982). These genes express much heterogeneity due to genetic polymorphism. Lachman (1979) demonstrated at least ten different patterns of electrophoretic mobility, again this intra specific diversity extends interspecifically.

Beta lipoproteins are a very heterogeneous group of macro molecules, comprising of a broad spectrum of size, hydrated density and in the content of protein, carbohydrate, phospholipid, triglycerides,

cholesteryl esters and free cholesterol. Butler, (1967) described the lipoproteins as one of the most complex groups of the serum proteins. Their chemical properties are determined by the protein moiety. It is mainly involved in the sequestration and transport of hydrophobic lipids.

Beta lipoproteins can be subdivided into three groups, determined by hydrated density. Very low density lipoprotein (VLDL), all substances of hydrated density less than 1.007 g/ml; low density lipoproteins (LDL) the class of compounds of density greater than 1.007 but less than 1.0653 g/ml; high density lipoproteins (HDL) those lipoproteins the density of which lies between the limits 1.063 and 1.21 g/ml.

Each major density class of lipoproteins contains six or more different polypeptide moieties, (Jonas, 1972). Further heterogeneity has been demonstrated due to genetic polymorphism, Albers and Dray (1968), Rapacz et al., (1969), Berg et al., (1971) and Butler (1967).

Immunological studies have been performed with the different density classes of lipoproteins and with the lipoproteins of different species. Albers and Hazzard (1973) demonstrated different protein groups and general heterogeneity between the different density fractions, and they showed that lipoprotein concentration was age independent, which contrasts with cholesterol levels.

Storiko and Fischer (1966) showed beta lipoprotein to be species specific immunologically. Kaminski et al., (1978) raised antisera to avian lipoproteins and found no cross reactions occurred with any mammalian species lipoprotein which is also true of other avian proteins.

Butler (1967) raised species specific precipitin to human LDL inoculated into rhesus monkeys. He showed that at birth, group specific antigens were not present, but that they develop quite quickly with infancy. Harvie and Schultz, (1970) showed that serum concentrations varied between species and within species.

Solyom et al., (1971) studying dog lipoproteins, found an immunologically distinct protein that was bound to the VLDL fraction. They found the protein to be a gamma globulin, suggesting that it was a specific auto antibody. This is interesting as a small peak in the gamma globulin region can be seen on the two dimensional electrophoresis of the LDL fraction, perhaps a similar phenomenon is occurring.

Mills and Taylaur (1971) compared the distribution and composition of serum lipoproteins between 18 animals. They found a difference between all species, but especially between the classes, birds, fish and mammals. Among the mammals no animal could duplicate the pattern and composition found in man. The nearest were the old world monkeys. All fraction occurred in all the species examined, except no LDL was found in the toad Bufo marinus. They demonstrated the wide variation in protein concentration in the different classes of lipoprotein between all the different species.

Using the method of ultra centrifugation described by De Lalla and Gofman (1954) the beta lipoproteins were separated from human serum and retested with the absorbed serum on the two dimensional electrophoresis. All fractions reacted with the antiserum, suggesting that each lipoprotein fraction had antigens that independently reacted with the antiserum as each lipoprotein fraction possess different protein moieties.

Some complement component C_4 antigen was kindly supplied by Professor Lachman, this was also tested on the two dimensional electrophoresis, the result was confirmed, albeit the reaction was not as strong as the reaction of the beta lipoprotein fractions. A source of transferrin and haptoglobin was not available for further electrophoretic work and none was carried out.

In summary, the specific antibodies remaining after solid phase absorption bind to at least five components of human serum, immunoglobulin, transferrin, haptoglobin, complement component C_4 and the beta lipoproteins. The beta lipoproteins are divided into three classes, all of which have binding activity with the antibodies. It must be stated that all of these serum components with the exception of complement component C_4 , occur at relatively high concentrations, compared with most other serum compounds. It is therefore quite probable that there are other specific components of serum, that have not been identified by the techniques used in this investigation. The sensitivity of all precipitation immunoassay is quite low and very much dependent upon high concentrations of antigens, which may not always be the case.

CHAPTER 3DEVELOPMENT OF INHIBITION ELISA FOR INSECT BLOODMEAL
IDENTIFICATION

3.1 INTRODUCTION

The most popular method of bloodmeal identification to date has been the precipitin test, or some variation such as counter current immuno electrophoresis. Passive haemagglutination has also been used, especially for separating closely related species, for example the bovids.

By current standards of immunological methods these tests are no longer suitable. Precipitin is very simple to perform but almost impossible to quantify, not at all sensitive and will be useless for monoclonal antibodies. Agglutination inhibition has proved to be complicated with the results so often irreproducible and inconsistent. More recently ELISA has been used, the results of which demonstrate better sensitivity but not always specificity.

Any serodiagnostic assay whether it be for bloodmeal identification or not needs to be specific for accurate results; sensitive because low concentrations of the target molecule only are available; simple, otherwise the cost of the assay can be prohibitive for its general use.

All enzyme immunoassays make use of the fact that a single molecule of enzyme can react with a large number of substrate molecules to produce a measurable product. Part of the sensitivity of ELISA systems is thus dependant on the turnover rate of enzyme linked to the immuno-reactant.

Enzymes that have been utilized in ELISA systems include alkaline phosphatase (Voller et al., 1980), peroxidase (Bullock and Walls, 1977), beta galactosidase (Guedson et al., 1979) and glucose oxidase (Guedson et al., 1977).

The reasons these enzymes are used in ELISA systems include the fact that all are available in purified forms from commercial sources and that they can be conjugated with immunoglobulin molecules with the retention of a significant proportion of their enzyme activity (Averameas et al., 1978).

The enzymes that have been most widely used in ELISA systems are peroxidase and alkaline phosphatase. Peroxidase is the least expensive, and since it is a plant product, it can be obtained easily in a large supply. Peroxidase molecules are glycoproteins with a carbohydrate content of about 10% - 15% (Averameas & Ternynck, 1971). The presence of carbohydrate offers the theoretical advantage of allowing for the linkage of enzyme to antibody via the carbohydrate portion of the enzyme molecule, thus leading to less interference with the enzyme function (Wilson and Nakane, 1981). The other advantage of peroxidase is that the visible substrate produces an intense dark colour that provides for more definitive visual determination (Waant et al., 1978).

There are a number of disadvantages inherent in the use of peroxidase for ELISA systems. For example, peroxidase conjugates can lose activity if they become contaminated with micro-organisms (Yolken, 1980). This problem cannot be overcome by the use of bacteriostatic agents since peroxidase activity is very sensitive to the action of antibacterial agents such as sodium azide and methanol (Schonbaum, 1973).

Another problem with the use of peroxidase is that some of the chromogens such as benzidine, O-toluidine and O-phenylene-diamine have been found to have carcinogenic or mutagenic effects in laboratory animals (Scharpe et al., 1976; Holland et al., 1974 and Venitt et al., 1976). Other chromogens, however, such as 5-amino salicylic acid and 3-methyl-2-benzothiazoline hydrazone, have not been shown to have any mutagenic effects.

Alkaline phosphatase is extremely stable under conditions of storage and is virtually resistant to the action of bacteriostatic agents (Belanger et al., 1976). The enzyme substrates are not known biohazards. The principal disadvantage of alkaline phosphatase is that enzyme of sufficient purity and specific activity for use in ELISA systems must be obtained from an animal source such as calf intestine. This makes the enzyme expensive and limits its supply.

Methods for increasing the sensitivity of enzyme immunoassays have been proposed by Self (1985). Such amplified enzyme immunoassays generally employ enzyme labels catalysing the formation of a trigger molecule which activates an 'amplifier' causing a detectable change, e.g. an increase in optical absorbance of a solution. There are many well known examples of biological amplifiers which may have applications in immunoassays, e.g. cascade mechanism of blood clotting, the mobilization of glycogen or a number of cyclic reaction sequences.

In this chapter an inhibition ELISA has been developed considering the criteria discussed above. Part of the specificity of any immunoassay relies upon the specificity of the antibodies used within the assay. The results from Chapter 2 have shown that solid phase absorption can successfully improve the specificity of an antiserum,

especially when the antibodies used are directed to a species specific antigen.

The antigens used in the inhibition ELISA are beta lipoproteins and fractions of the gamma immunoglobulins.

The conventional ELISA usually has the antigen immobilised to the solid phase i.e. the microtitre plate. The identifying antibody is then placed with the unknown antigen, if a positive reaction occurs it is then visualised by a second enzyme labelled antibody that binds to the first antibody. This combination is then exposed to the substrate and a colour change occurs. This method involves four stages, each with a wash.

The inhibition assay is more simple, the specific antibody is immobilised to the solid phase, the target antigen to be detected is then mixed with known enzyme labelled antigen. The target antigen will compete for the binding sites with the labelled known antisera causing an inhibition of reaction to occur. This degree of inhibition is a measure of the target antigen present.

The inhibition ELISA was further simplified by converting it into a dot blot inhibition ELISA, using nitro cellulose acetate paper as the solid phase. A variety of enzyme substrates were tested for both the conjugated enzymes, horseradish peroxidase (HRP) and alkaline phosphatase (AP). The results of these experiments are discussed in relation to bloodmeal identification.

DEAE cellulose columns were used to concentrate the immunoglobulin fraction of the antiserum so that enzyme conjugation could be performed for use in the inhibition assay. The standard inhibitor used as a control in each assay and the antigen used to prepare the antisera was also immunoglobulin, and was separated using DEAE columns for use in the assay.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of antigen and antibodies

Conjugation of antigens to horse raddish peroxidase (HRP) enzyme.

The peroxidase method introduced by Nakane and Kawaoi (1974) and modified by Wilson and Nakane (1978), gives a good recovery of the enzyme in the conjugate. This method involves the oxidation of peroxidase with metaperiodate and generates some proportion of conjugate in the polymeric form.

a) Buffers and solutions

0.1 M Sodium periodate

1.0mM Sodium acetate pH 4.4

0.2 M Sodium bicarbonate buffer, pH 9.5

0.01 M Sodium carbonate/bicarbonate buffer, pH 9.5

0.4% Sodium borohydride solution (4 mg/1 ml/ dH₂O)

0.1 M Borate buffer, pH 7.4

b) Procedure

4-8 mg horseradish peroxidase (P8375 Sigma) - activity : 285 pur-purogallin units (mg solid) was dissolved in 1 ml distilled water. 0.2 ml of freshly prepared 0.1 M sodium periodate solution was added to the HRP solution, which immediately turns green. This was then stirred gently for 20 mins. at room temperature. The reaction mixture was then dialysed overnight against a large excess of 1.0mM sodium acetate buffer, pH 4.4 at 4°C.

The pH was then raised to 9.0-9.5 by addition of 20 ul of 0.2 M sodium bicarbonate buffer, pH 9.5. Immediately afterwards 1 ml of the antigen solution (DEAE column separated Ig factions) to be conjugate was

added. The 1% antigen solution, was previously dialysed against 0.01 M sodium carbonate/bicarbonate buffer, pH 9.5. The mixture was stirred gently for 2 hours at room temperature in the rotary wheel mixer.

0.1 ml of freshly prepared sodium borohydride solution was added to reduce the free enzyme, this was left to stand for 2 hours at 4°C.

The conjugated mixture was dialysed against 0.1 M borate buffer, pH 7.4 overnight at 4°C.

The enzyme-antigen conjugate was separated from the uncoupled materials by passing the reaction mixture down a sephadex G200 column (1.6 x 35cm) in borate buffer as described below. The first peak was the enzyme active fraction. The conjugates were stored at -20°C.

3.2.2 Purification of IgG from antisera using DEAE column

a) Materials

DEAE (Diethylaminoethane cellulose) anion exchanger -
Whatman DE52.

0.01 M phosphate buffer, pH 7.2

Chromatography column 20cm x 1cm

5ml of rabbit antiserum and/or serum from each animal dialysed
against 0.01 M phosphate pH 7.2.

b) Procedure

DEAE cellulose needs to be washed and equilibrated before packing. This was done by making a suspension of the cellulose in 0.01 M phosphate, buffer pH 7.2, allowing it to settle by gravity for an appropriate time, then discarding the cloudy supernatant. This was repeated several times. The material was then further washed through a sintered glass filter under vacuum.

The cellulose was soaked in 0.3 M KH_2PO_4 (500ml per 50g dry DEAE) overnight, washed in the same buffer, washed copiously (5 times) with distilled water, then transferred to a beaker and washed (500 ml per 50g) in 0.5N NaOH through a filter funnel then suspended in ethanol (500 ml per 50g) for 1-2 hours. It was then washed with 800 ml of 0.05 N NaOH and 5-10 times with distilled water.

Finally the pH was adjusted to the appropriate level by careful titration with buffer, the cellulose could now be packed into a column.

The rubber tube at the base of the column was clamped, the column then filled with the initial buffer. Before adding, the cellulose was mixed with sufficient buffer to form a fine suspension. The column was filled up to a few cm from the top at room temperature to avoid air bubbles forming. The column was then washed with a further few 100 ml of buffer, until the eluate had the same pH of the washing buffer.

5 ml of the dialysed antisera was carefully layered onto the top and allowed to run into the column.

This was followed by buffer (0.01 M phosphate pH 7.2), the column was always topped up with the buffer.

The first 4 ml from the column were discarded, the next 12 ml collected. This fraction was equilibrated against PBS, pH 7.4 by dialysis. The samples were sometimes concentrated by vacuum dialysis as described in Chapter 1.

3.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

a) Preparation of ELISA

All the plates, buffers and diluents, the washing and substrate solution were the same as used in the ELISA to screen monoclonal antibodies as described in Chapter 1.

It is very important to ensure that components of the ELISA (e.g. antibody and antigen - conjugate) are titrated against each other to achieve optimal concentrations, as less than adequate coating of the solid phase will reduce sensitivity and afford opportunities for conjugate itself to bind to the plastic. This leads to high backgrounds in the quality control test obscuring the properties of the reference and test conjugates alike. Overcoating the solid phase may likewise lead to non-specific trapping of conjugate.

This was performed by setting up a quality control test as a chequer board and was done for every inhibition assay developed. A range of antibody concentrations used to coat the plates was tested against a serial dilution of the conjugate antigen.

Following this a range of concentrations of standard inhibitor were tested so that the results with unknown material could be calibrated.

These tests all completed, a range of cross reactivity assays were performed testing all the different antigen conjugates to react with against all the different IgG preparations that had been purified using DEAE columns previously described.

Any cross reactions recorded were then eliminated by solid phase absorption procedures as described in Chapter 1.

3.2.4 Development of 'Dot blot' assay

After developing the inhibition ELISA using plastic microtitre plates and soluble substrates, the assay was converted to the use of nitro-cellulose acetate paper as the solid phase.

The antisera used in the conventional ELISA were applied in the dot blot assay.

The antibody layer was prepared as a small dot (approx 4mm in diameter) which was prepared by slow addition (1 μ l at a time) of 5ul of antibody solution, 25 μ g/ml in carbonate coating buffer, pH 9.6.

The dots were left to dry at room temperature. The nitrocellulose paper was then quenched with 1% casein in carbonate coating buffer, pH 9.6 for 1 hour at 37°C. The dots were then washed three times in physiological saline - Tween 20 (0.05%). The nitrocellulose paper was placed into the antigen conjugate solution which varied in concentration as determined by the initial quality control tests for each antigen conjugate. Mixed with the antigen conjugate solution was either the standard inhibitor or the test material. This stage was incubated for 1 hour at 37°C. The nitrocellulose paper was again washed three times in physiological saline - Tween 20 (0.05%) then placed in the insoluble substrate solution. A number of substrates were tested to determine that which achieved the highest colour reaction with the lowest background.

Substrates used

I) For horseradish peroxidase/enzyme

a) O-Diaminobenzidine

The use of this substrate has been described by Horejri and Hilgert (1983). 5 mg of ortho-diamino benzidine (DAB) (Sigma) were dissolved in 20 ml 0.1 M tris HCl buffer, pH 7.6. containing 0.01% H_2O_2 . The substrate solution was filtered immediately before use. The samples were incubated for 20 mins then washed with tap water.

b) 3 amino-9-ethyl carbazole.

The use of this substrate has been described as above. 3 mg of aminoethyl carbazole dissolved in 1 ml of dimethyl formamide. 4 ml of 5 mM acetate buffer, pH 5 was then added and the solution complemented immediately with 20 ul of 3% H_2O_2 . The samples were incubated usually for 10 mins, as the reaction with the substrate occurred quickly. On the occasions of a strong reaction, the test was repeated using a 25% strength of the substrate solution to reduce the activity.

c) 4 chloro 1 naphthol

The use of this substrate was first described by Hawkes et al., (1982) and has also been described by Kumar et al., (1985).

A stock solution was initially prepared that keeps for about 8 weeks at $-20^{\circ}C$, This was prepared by dissolving 3 mg/ml of chloronaphthol (Sigma) in anhydrous methanol. When the substrate was required 2 ml of the stock solution was mixed with 10 ml of TBS (triethanolamine buffered saline, pH 7.5, see Appendix 1). This was then immediately complemented with 4 ml of 30% H_2O_2 . The samples were left to incubate for 1 hour at room temperature, then washed with tap water.

II) For alkaline phosphatase enzyme

a) 5 - bromo, 4 - chloro, 3 - indolyl phosphate (BCIP) and nitroblue tetrazoleum salt (NBT).

This substrate was described by Leary et al., (1983) and Bergher et al., (1985). 2.5 mg BCIP (Sigma) and 37.5 mg NBT (Sigma) were dissolved in a minimum volume of dimethylformamide and mixed with 0.1M

tris HCl buffer, pH 9.5, containing 0.1 M NaCl and 5 mM MgCl_2 to a final volume of 15 ml. The samples were incubated for 30 mins at room temperature, then washed with tap water.

b) Naphthol AS - MX phosphate with Fast Red salt

This is the method of Druguet and Pepys (1977) and has also been described by O'Connor and Ashman (1982). 0.4 mg/ml of Naphthol AS MX phosphate (Sigma) were dissolved in distilled H_2O , and an equal volume of this was then mixed with the Fast Red TR salt solution, 6 mg/ml of Fast Red TR (Sigma) in 0.2 M tris buffer, pH 8.2. The samples were incubated for 1 hour at room temperature, then washed in tap water.

c) Naphthol AS MX phosphate and Fast blue salt

This method is the same as the previous one except a different tetrazoleum salt is used. This method has been described by Mason and Sammons (1978).

0.2 mg/ml naphthol AS MX phosphate (Sigma) in H_2O (distilled) were mixed with an equal volume of 0.1 mg/ml Fast blue (Sigma) dissolved in 0.1 M tris buffer, pH 9.0. The samples were incubated for 1 hour at room temperature, then washed in tap water.

The nitrocellulose paper pieces with dots were then dried and stuck on paper with glue to show the results.

3.2.5 Bloodmeal testing with dot blot assay

The test having been developed was then used to identify the hosts of arthropod bloodmeals. The tests were performed in exactly the same way except the conjugate was mixed with a dilution of the eluate of a dried bloodmeal, prepared in 0.2 ml PBS - Tween 20 (0.05%).

3.3 RESULTS

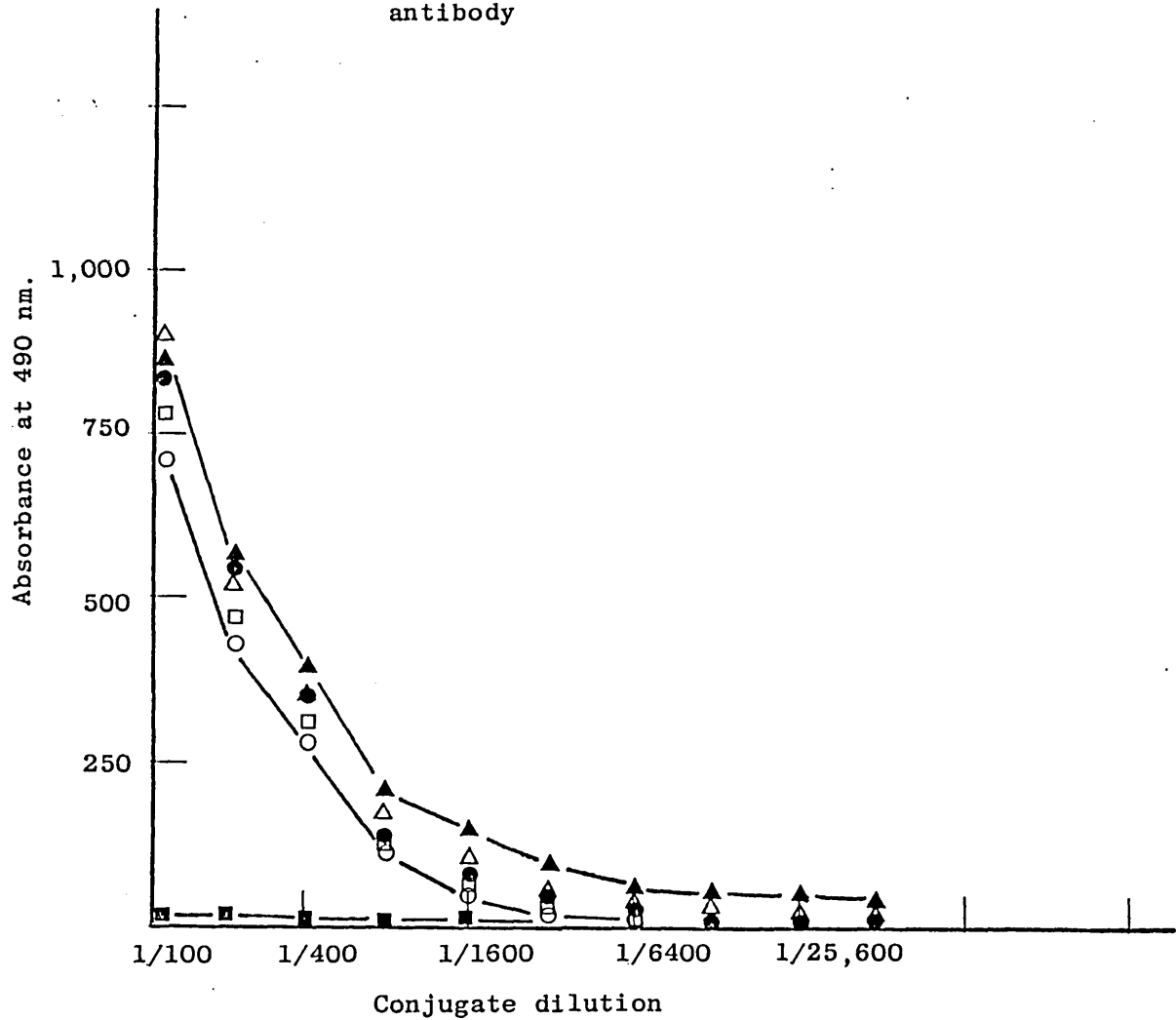
1. Inhibition ELISA

A horse radish peroxidase conjugate was prepared with human beta lipoproteins that had been isolated and purified as described in the previous chapter. The activity of this conjugate was measured using a conventional ELISA. Five concentrations of coating antibody were tested against ten dilutions of the conjugate, the absorbances values were read using an automatic ELISA reader (See Table 3.1 and Figure 3.1). An optimum antibody concentration, 2.5 µg/ml and conjugate dilution, 1/400 was chosen for the inhibition assay.

Table 3.1: Absorbance values for the standardization of the beta lipoprotein - HRP conjugate with different concentrations of sheep anti-beta-lipoprotein antibody.

Beta-lipo- protein HRP conjugate concentration	Absorbance values at 490 nm ($\times 10^{-3}$)					
	1% Casein	Antibody concentration $\mu\text{g/ml}$				
	Control	20	10	5	2.5	1
1/100	6	879	857	837	776	721
1/200	5	503	595	572	541	446
1/400	7	330	358	330	288	290
1/800	6	206	236	193	175	138
1/1,600	6	127	149	104	88	87
1/3,200	5	76	85	67	72	55
1/6,400	7	4	46	45	44	35
1/12,800	7	39	28	21	22	21
1/25,600	6	20	40	9	13	5
1/51,200	5	20	19	8	6	7

Figure 3.1: Standard Curve of beta-lipoprotein - HRP
conjugate with Sheep anti beta-lipoprotein
antibody



KEY

Antibody Concentration on sensitising layer

20 µg/ml	●
10 µg/ml	Δ
5 µg/ml	▲
2.5 µg/ml	□
1 µg/ml	○
Casein 1%	■

The first inhibition assay measured the effects of the different lipoprotein fractions and human serum. The absorbance values were recorded using the automatic ELISA reader. See Table 3.2a. Percentage inhibition was calculated by deviding the absorbance value of the inhibition antigen at any one concentration by the mean value of absorbance of no inhibitor, see the formula below.

$$\% \text{ Inhibition} = \frac{\begin{array}{l} \text{Value of no} \quad \text{Absorbance value} \\ \text{inhibitor} \quad - \quad \text{at certain inhibitor} \\ \quad \quad \quad \text{concentration} \end{array}}{\text{value of no inhibitor}} \times 100$$

The percentage inhibition figures are recorded in Table 3.2b. These values have been plotted on a graph in Figure 3.2. All the fractions of lipoprotein and human serum cause inhibition.

Table 3.2a: Competitive Inhibition ELISA, with beta lipoprotein -
 HRP conjugate and anti-beta lipoprotein antibodies.
 Testing lipoprotein fractions.

Dilution of	Absorbance value at 490 nm (x10 ⁻³)				
Fractions.					
Initial conc.	1% Casein	VLDL	LDL	IDL	Human
100 µg/ml	Control				Serum
1/1	871	091	655	230	312
1/2	883	124	654	239	343
1/4	1016	224	652	230	312
1/8	1125	199	714	267	320
1/16	1198	281	768	359	331
1/32	1222	364	762	386	369
1/128	1214	430	1007	496	517
1/256	1055	431	1018	561	534
0	1210	826	1119	919	821

VLDL = Very low density lipoprotein

LDL = Low density lipoprotein

IDL = Intermediate density lipoprotein

TABLE 3.2b: Competitive Inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing lipoprotein fractions.

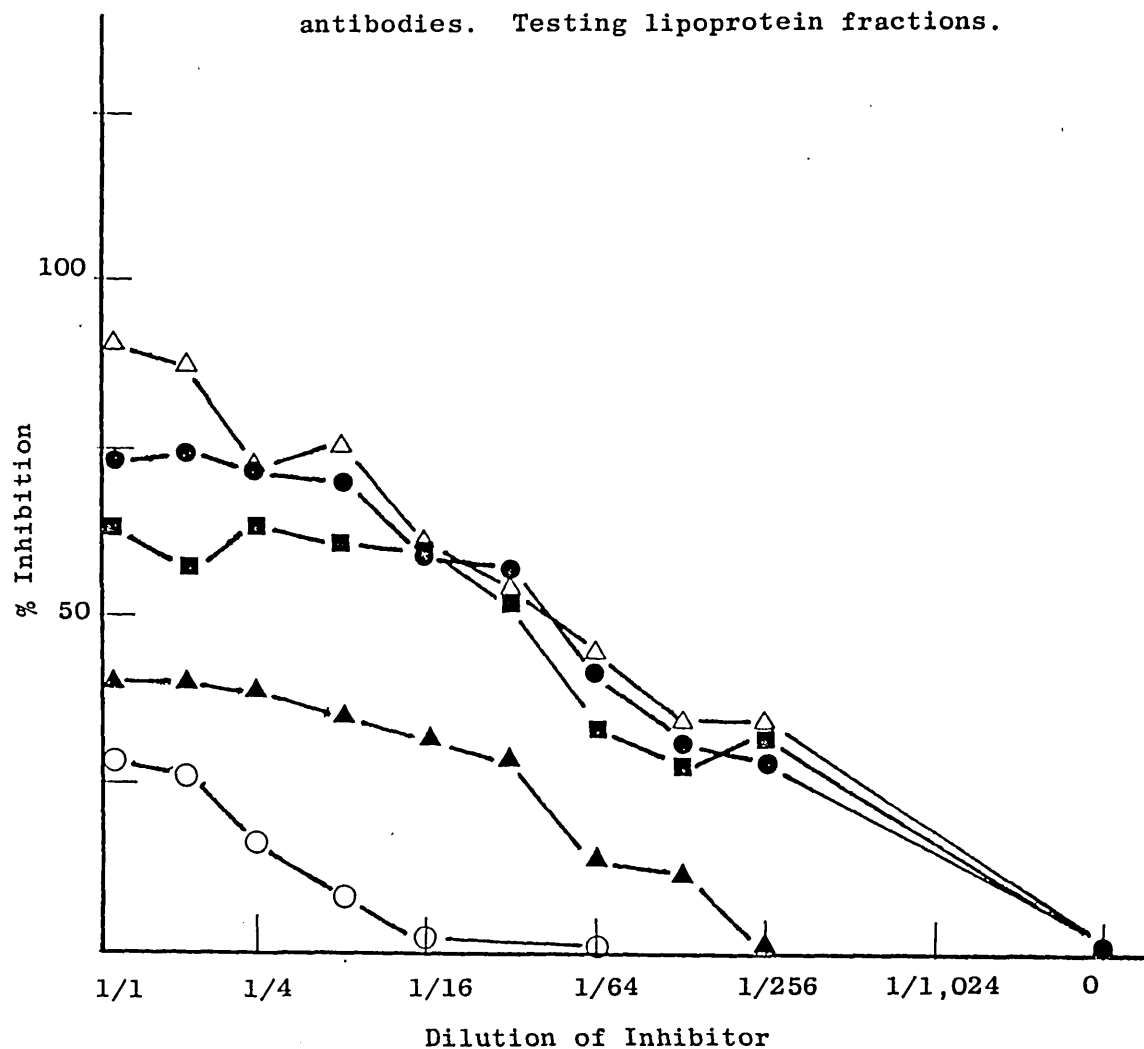
Dilution of		% Inhibition			
Fractions.					
Initial conc.	1% Casein	VLDL	LDL	IDL	Human
100 µg/ml					Serum
1/1	28	89	39	72	62
1/2	27	85	39	74	57
1/4	16	73	38	72	62
1/8	7	76	34	71	61
1/16	1	60	32	61	56
1/32	0	56	30	58	55
1/64	0	48	10	46	37
1/128	0	38	9	39	35
1/256	0	38	0	29	38
0	0	0	0	0	0

VLDL = Very low density lipoprotein

LDL = Low density lipoprotein

IDL = Intermediate density lipoprotein

Figure 3.2: Competitive Inhibition ELISA, with beta-lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing lipoprotein fractions.



Conjugate: beta lipoprotein - HRP 1/400

KEY

Inhibiting Antigen	Initial Concentration	
Casein	100 µg/ml	○
VLDL	" "	△
LDL	" "	●
IDL	" "	■
Human serum	1,000 µg/ml	▲

A similar inhibition ELISA was performed to test the specificity of the assay. Serum from five animals, dog, ox, horse, pig and goat were tested with human serum and the VLDL fraction of lipoproteins. The absorbance values of this assay are recorded in Table 3.3a. The percentage inhibition values are recorded in Table 3.3b and plotted in a graph in Figure 3.3. The assay demonstrates good specificity. None of the animal sera caused inhibition greater than 15% at their maximum concentration. Human serum caused 59% inhibition at the maximum concentration.

TABLE 3.3a: Specificity Test of competitive inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies.

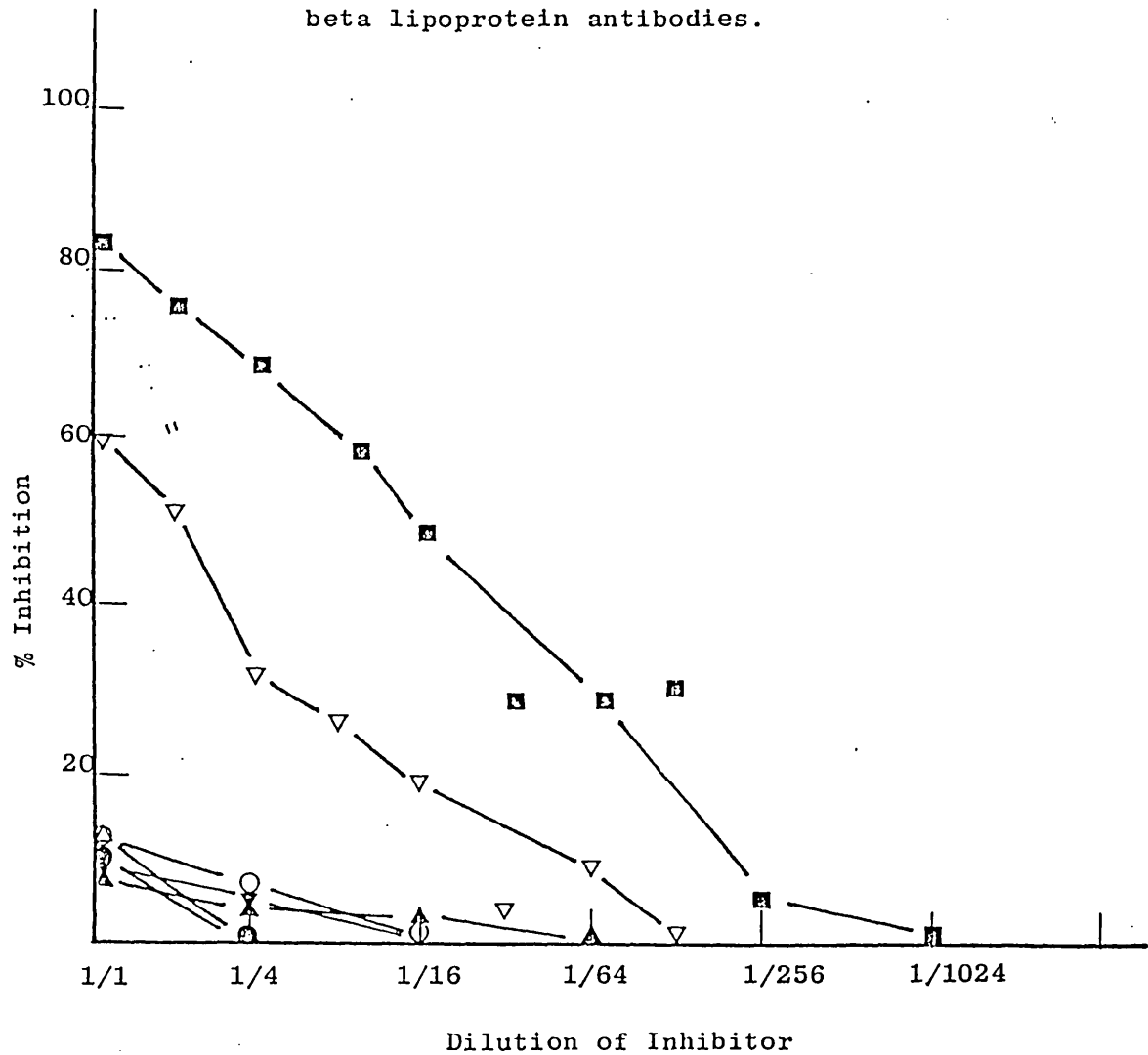
Absorbance values at 490 nm ($\times 10^{-3}$)								
Dilution of								
Inhibitor	VLDL	MAN	DOG	OX	HORSE	PIG	GOAT	No
	Inhibitor Initial Concentration ($\mu\text{g/ml}$)							Inhibitor
	100	1000	1000	1000	1000	1000	1000	Control
1/1	36	88	193	201	192	197	198	228
1/2	58	110	229	207	203	211	200	208
1/4	71	149	222	207	228	239	207	227
1/8	90	157	251	224	216	216	219	220
1/16	115	173	246	218	217	224	221	192
1/32	161	208	249	248	225	230	225	217
1/64	157	195	231	237	224	228	221	219
1/128	150	218	227	229	222	236	241	224
1/256	209	221	231	226	223	218	232	221
1/512	219	214	228	229	236	228	238	217

Mean value of no inhibitor control = 217

Table 3.3b: Specificity Test of Competitive Inhibition ELISA, with
beta lipoprotein - HRP conjugate and anti-beta
lipoprotein antibodies.

Dilution of Inhibitor	% Inhibition						
	VLDL	MAN	DOG	OX	HORSE	PIG	GOAT
	Initial inhibitor concentration ($\mu\text{g/ml}$)						
	100	1000	1000	1000	1000	1000	1000
1/1	83	59	11	7	9	9	5
1/2	73	49	0	5	7	0	4
1/4	67	31	0	5	0	0	5
1/8	59	28	0	0	0	0	0
1/16	47	20	0	0	0	0	0
1/32	26	4	0	0	0	0	0
1/64	31	10	0	0	0	0	0
1/128	5	0	0	0	0	0	0
1/256	0	0	0	0	0	0	0
1/512	0	0	0	0	0	0	0

FIGURE 3.3: Specificity Test of Competitive Inhibition
ELISA with beta lipoprotein HRP and anti
beta lipoprotein antibodies.



KEY

Inhibiting Antigen	Initial Concentration	
VLDL	100 µg/ml	■
Human serum	1,000 µg/ml	▽
Dog serum	" "	▲
Ox serum	" "	○
Horse serum	" "	▼
Pig serum	" "	△
Goat serum	" "	●

Applying the inhibition ELISA to insect bloodmeal identification, whole dried human blood and a mosquito bloodmeal on man were tested in the assay. A human VLDL fraction and immunoglobulin antigen was also included in the assay as positive and negative controls respectively. The absorbance values are recorded in Table 3.4a, the percentage inhibition values in Table 3.4b, these values being plotted in the graph in Figure 3.4. Both the whole dried blood and the mosquito bloodmeal inhibited the assay. Approximately 50% was seen at a dilution of 1/64 of both the mosquito bloodmeal and the 5 μ l whole dried blood sample. This represents 0.04 μ g of inhibiting antigen, B-lipoprotein.

TABLE 3.4a: Competitive Inhibition ELISA, with beta lipoprotein - HRP conjugate and anti-beta lipoprotein antibodies. Testing whole blood and mosquito bloodmeal samples.

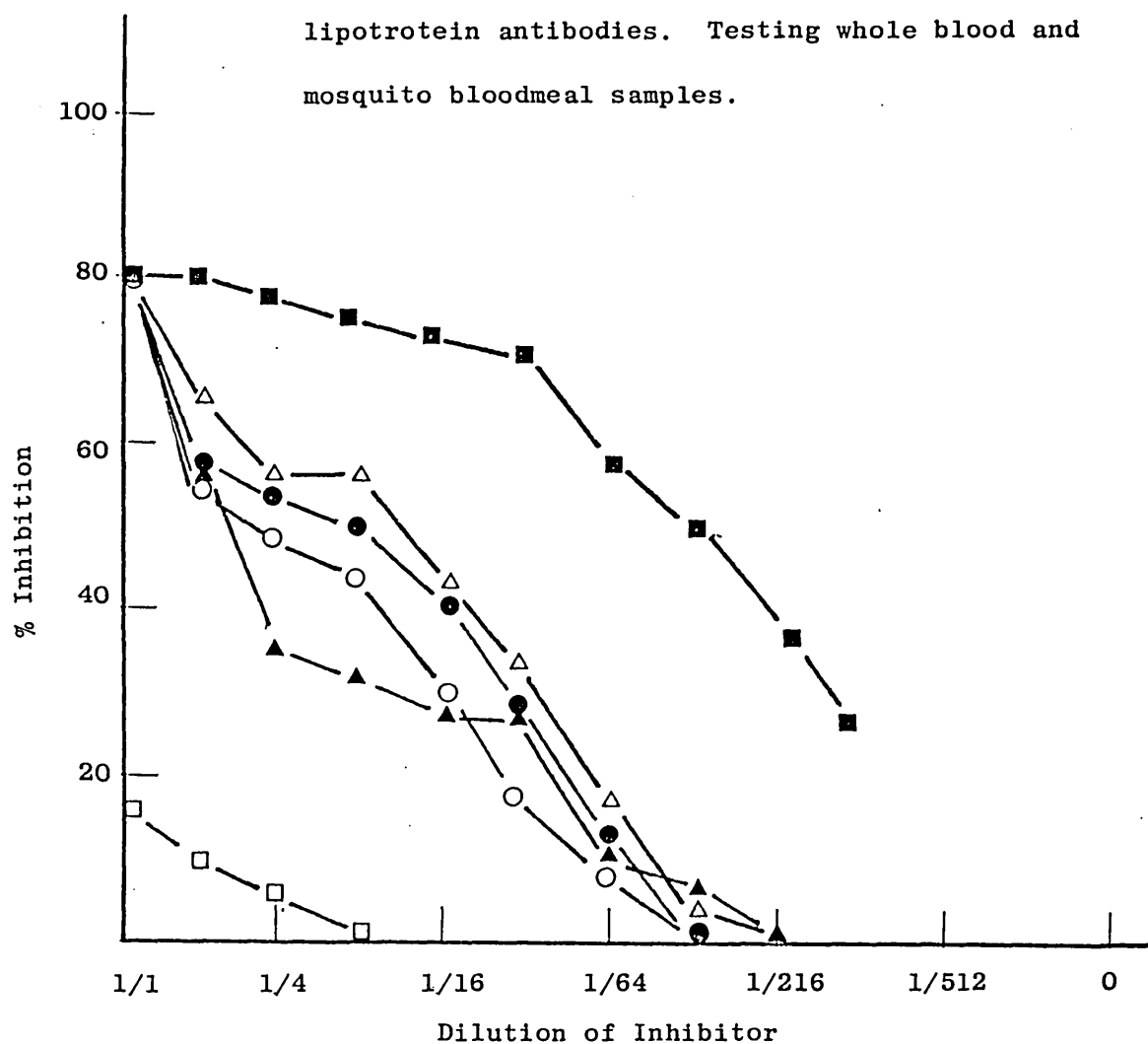
Absorbance values at 490 nm ($\times 10^{-3}$)							
Dilution							
of	Ig	VLDL	5 μ l	10 μ l	20 μ l	Mosquito	No
Inhibitor	Inhibitor Initial Concentration						Inhibitor
	100	100	Blood	Blood	Blood	Blood	Control
	μ g/ml	μ g/ml	spot	spot	spot	meal	
1/1	227	0	0	0	0	0	257
1/2	242	0	86	77	60	83	257
1/4	248	11	113	94	89	160	282
1/8	276	17	124	108	87	171	268
1/16	284	28	176	134	103	180	277
1/32	277	50	207	210	151	178	277
1/64	318	93	238	225	211	231	279
1/128	302	111	290	268	261	252	280
1/256	275	151	296	297	285	279	258
1/512	281	171	298	292	284	345	277

Mean value of No Inhibitor Control = 271

Table 3.4b: Competitive Inhibition ELISA, with beta lipoprotein -
 HRP conjugate and anti-beta lipoprotein antibodies.
 Testing whole blood and mosquito bloodmeal samples.

% Inhibition						
Dilution of Inhibitor	Inhibitor Initial Concentration					
	Ig	VLDL	5 μ l	10 μ l	20 μ l	Mosquito
	100	100	Blood	Blood	Blood	Bloodmeal
	μ g/ml	μ g/ml	Spot	Spot	Spot	
1/1	16	100	100	100	100	100
1/2	11	100	68	72	78	70
1/4	9	96	58	66	68	41
1/8	0	94	54	61	68	37
1/16	0	90	35	51	52	34
1/32	0	82	24	33	45	35
1/64	0	66	12	17	23	15
1/128	0	59	0	0	4	8
1/256	0	45	0	0	0	0
1/512	0	37	0	0	0	0

Figure 3.4: Competitive Inhibition ELISA, with beta lipoprotein - HRP conjugate and anti beta lipoprotein antibodies. Testing whole blood and mosquito bloodmeal samples.



KEY

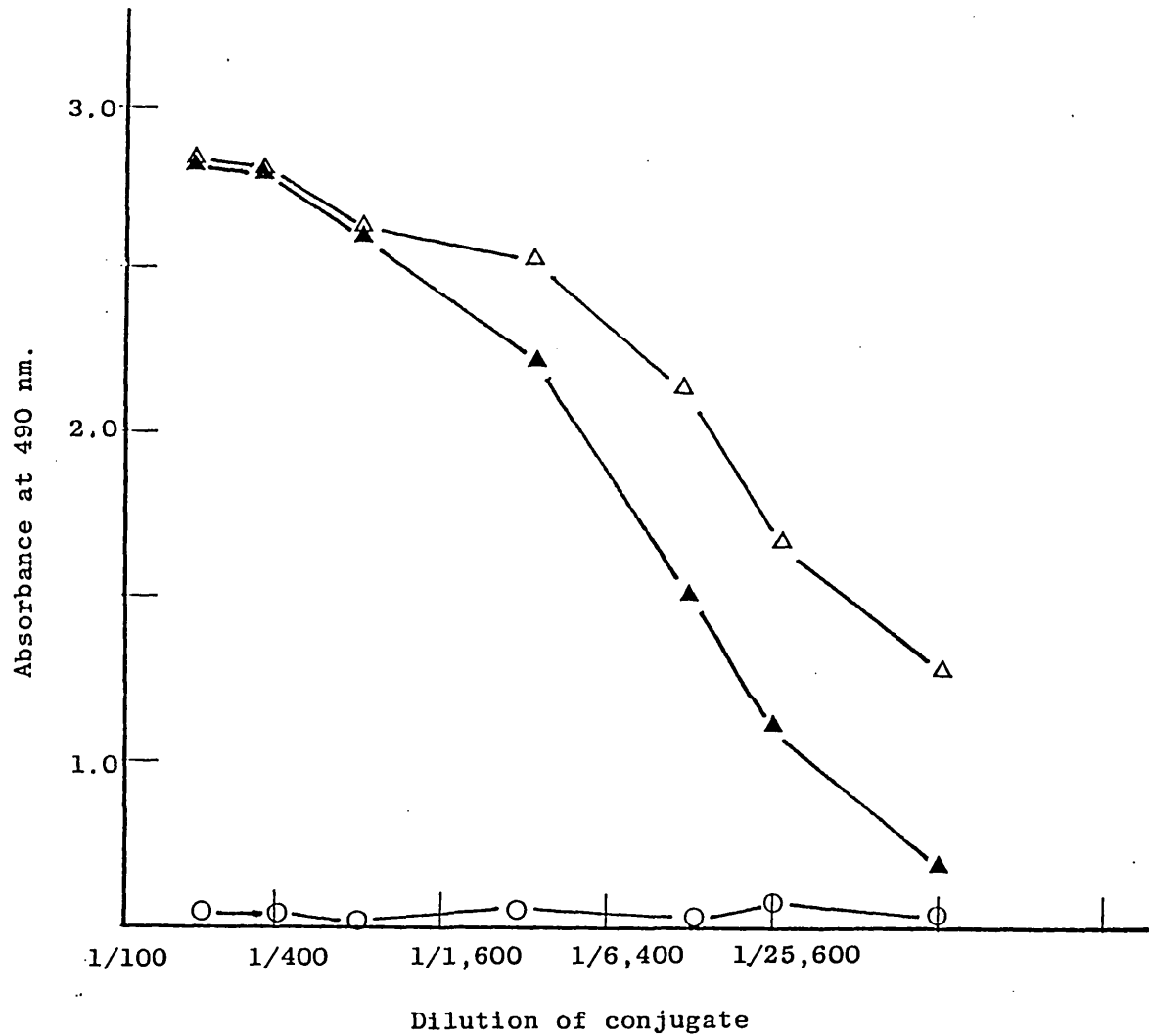
Inhibiting Antigen	Initial concentration	
Ig	100 µg/ml	□
VLDL	100 µg/ml	■
20 µl blood spot		△
10 µl blood spot		●
5 µl blood spot		○
Mosquito bloodmeal		▲

A similar inhibition ELISA was prepared using HRP conjugated DEAE purified IgG antigen from human serum. The antibody layer on the solid phase was the murine monoclonal antibody prepared against human IgG lambda light chains. This monoclonal antibody was prepared and supplied by Dr. Jefferies, Birmingham University. A standardising ELISA was performed, with four concentrations of coating antibody and seven dilutions of the conjugate. The absorbance values are recorded in Table 3.5 and the graph is plotted in Figure 3.5. An optimum antibody concentration, 2.5 µg/ml and conjugate dilution 1/10,000 was chosen for the inhibition assay.

Table 3.5: Absorbance values of the standardization of the IgG
HRP conjugate with the monoclonal antibody anti-IgG .

Absorbance values at 490 nm ($\times 10^{-3}$)					
Dilution					
of	1% Casein	Monoclonal Antibody Concentration			
Conjugate	Control	10	5	2.5	1 μ g/ml
1/500	47	2337	2396	2401	2340
1/1000	36	2410	2351	2342	2313
1/2000	32	2037	2027	2067	2104
1/8000	27	2162	2150	2103	1857
1/32,000	51	1709	1617	1513	1014
1/64,000	47	1266	1263	1257	650
1/256,000	40	875	903	853	247

Figure 3.5: Standard Curve of IgG - HRP conjugate
with the monoclonal antibody anti IgG .



KEY

Antibody concentration on sensitising layer

10 µg/ml	▲
5 µg/ml	△
2.5 µg/ml	
1 µg/ml	○
1% Casein	

The specificity of this antibody had been previously determined by Jefferies et al., (1982). The antibody was tested against the IgG molecules of 16 animal species, including new world and old world primates, canids, felids, rodents, equids, bovids, suids and avian groups. It was found to only bind to the light chain of IgG of human and gorilla.

The inhibition ELISA was tested with whole dried blood samples, which had proved to act the same in the beta lipoprotein inhibition assay as the mosquito bloodmeal. The results of this assay are recorded in Tables 3.6a and 3.6b and Figure 3.6. The results showed inhibition by the whole dried blood sample, almost identical to that of the standard inhibitor antigen, free IgG light chain.

This inhibition ELISA only identifies human or gorilla samples, to identify a broader spectrum of species, further polyclonal systems were developed. These assays were prepared in exactly the same way as those previously described.

The antisera was prepared in sheep from DEAE purified IgG separated from each animal sera except with anti-sheep prepared in rabbit. This purified IgG was also used to prepare the HRP antigen conjugate used in the assay. The animals to which antisera were prepared to were : Rabbit, Hamster, Rat, Ox, Chicken, Sheep, Pig, Guinea Pig, Horse, Dog and Mouse.

Table 3.6a: Competitive Inhibition ELISA, with IgG - HRP conjugate and the monoclonal antibody, anti-IgG. Testing with whole blood.

Dilution of Inhibitor.	Absorbance values at 490 nm ($\times 10^{-3}$)		
	Whole Blood	IgG	1% Casein
Initial conc.			
200 $\mu\text{g/ml}$			
1/1	4	0	264
1/2	6	6	320
1/4	36	28	304
1/8	43	44	289
1/16	71	85	269
1/32	103	132	266
1/64	112	138	265
1/128	157	183	281
1/256	211	204	292
0	286	269	276

Conjugate dilution: 1/10,000

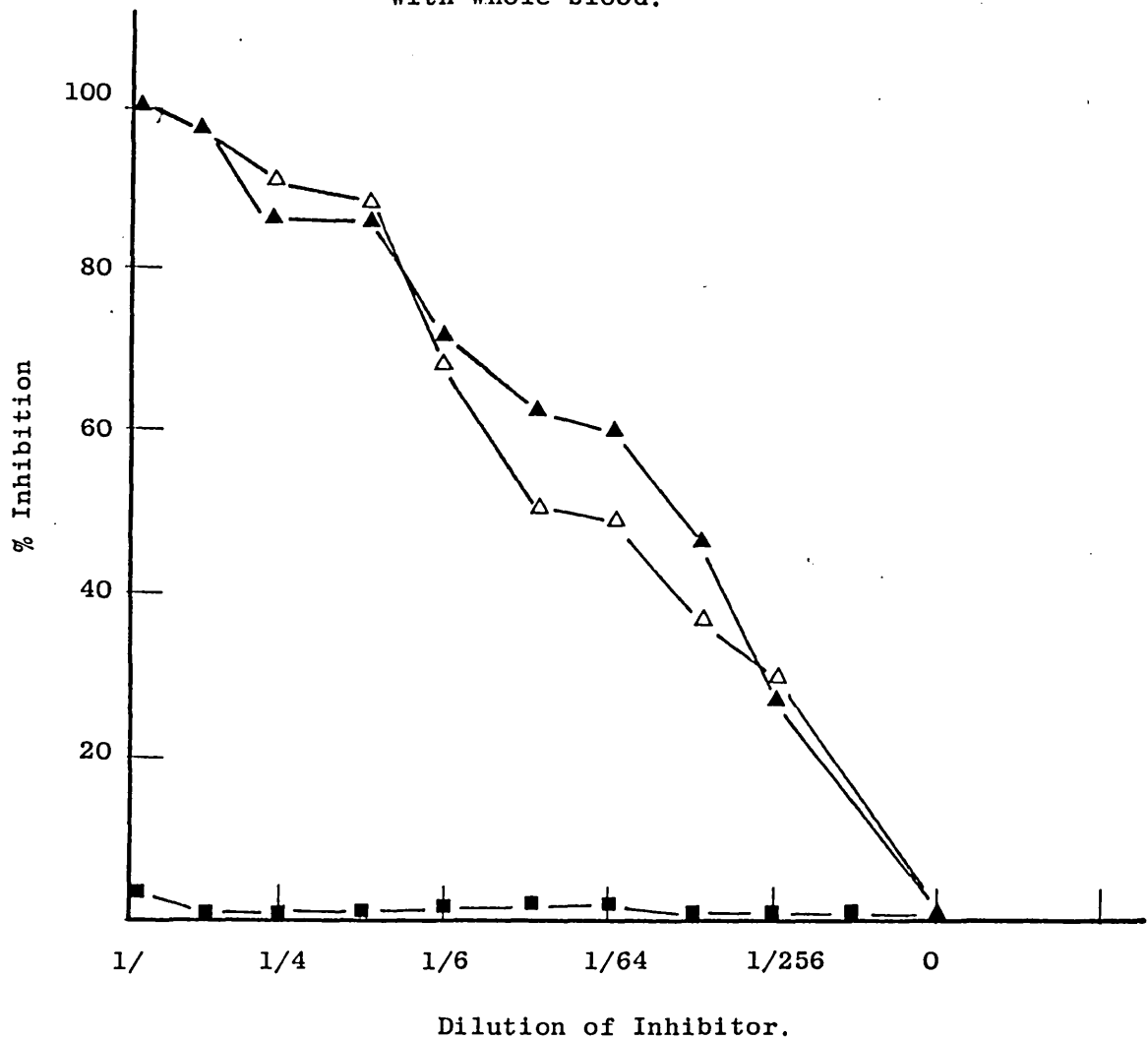
Monoclonal antibody concentration on ELISA plate: 2.5 $\mu\text{g/ml}$

Mean vaule of no inhibitor control = 283

Table 3.6b: Competitive Inhibition ELISA, with IgG - HRP conjugate and the monoclonal antibody, anti-IgG. Testing with whole blood.

Dilution of Inhibitors.	% Inhibition		
	Whole blood	IgG	1% Casein
Initial conc.			
200 µg/ml			
1/1	99	100	7
1/2	98	98	0
1/4	85	90	0
1/8	87	87	0
1/16	75	70	5
1/32	64	54	6
1/64	61	52	6
1/128	45	36	1
1/256	26	28	0

Figure 3.6: Competitive Inhibition ELISA, with IgG - HRP conjugate and the monoclonal antibody. Testing with whole blood.

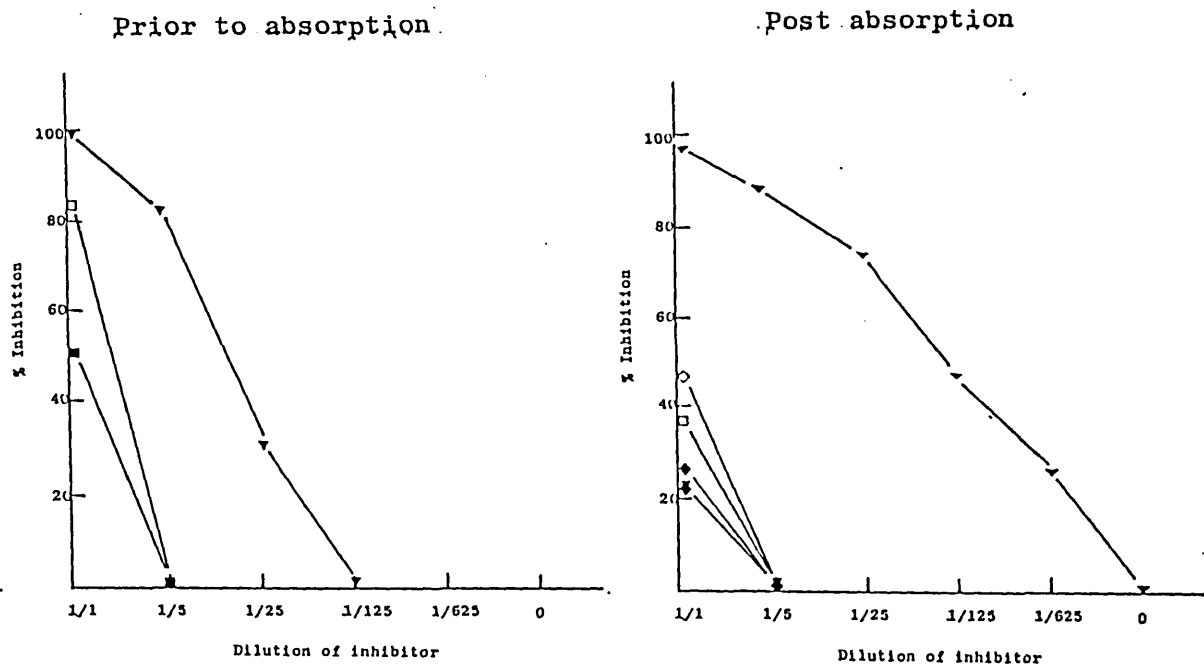


KEY

Inhibiting Antigen	Initial Concentration	
Whole blood	200 µg/ml	▲
Ig	" "	△
Casein	1%	■

Figures 3.7 to 3.17 show the results of testing each species conjugate against all the remaining species antigen before and after solid phase absorption with gluteraldehyde polymerised antigen. The absorbance and inhibition values have not been tabulated for reasons of clarity and economy.

Figure 3.7 Inhibition ELISA, testing specificity of Dog antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Dog antibodies



Antibody : Sheep anti-Dog IgG

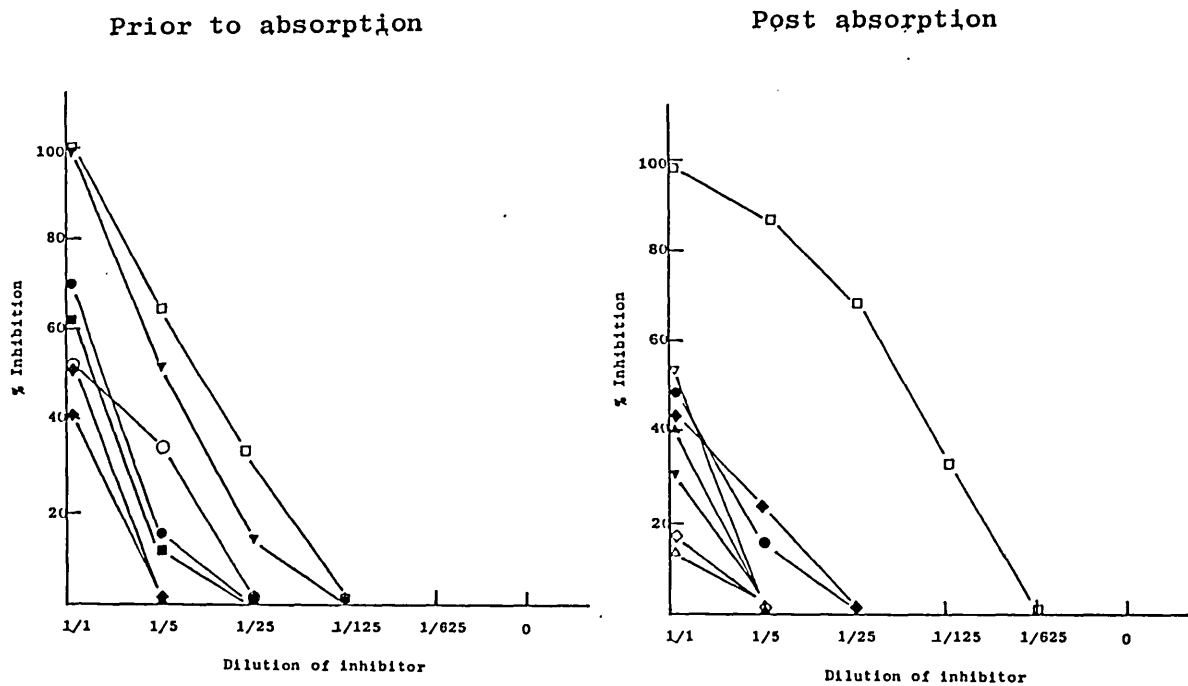
Conjugate: Dog IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	✚

Figure 3.8 Inhibition ELISA, testing specificity of Ox antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Ox antibodies



Antibody : Sheep anti-Ox IgG

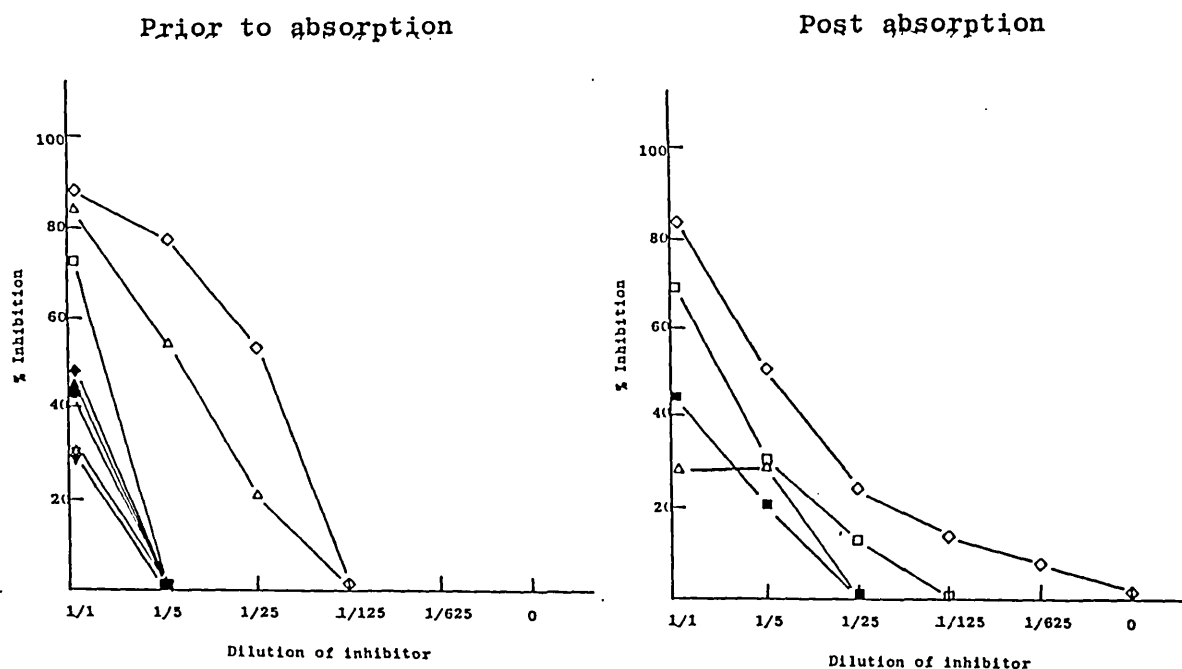
Conjugate: Ox IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	⚡

Figure 3.9 Inhibition ELISA, testing specificity of Hamster antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Hamster antibodies



Antibody : Sheep anti-Hamster IgG

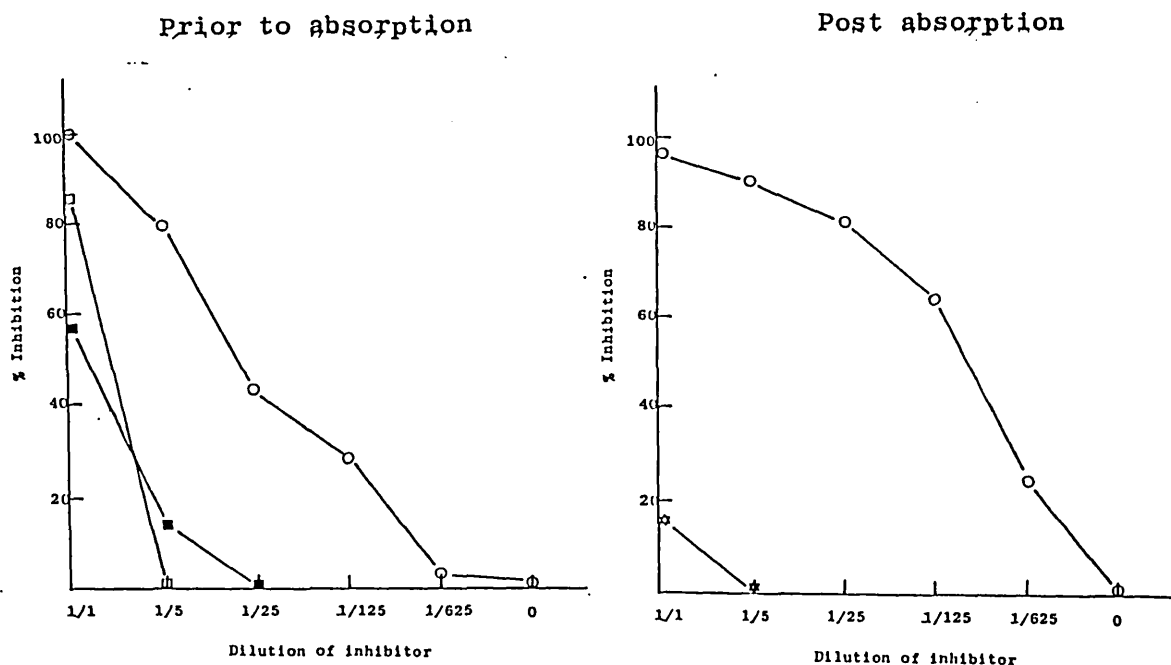
Conjugate: Hamster IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	◆

Figure 3.10 Inhibition ELISA, testing specificity of Pig antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Pig antibodies



Antibody : Sheep anti-Pig IgG

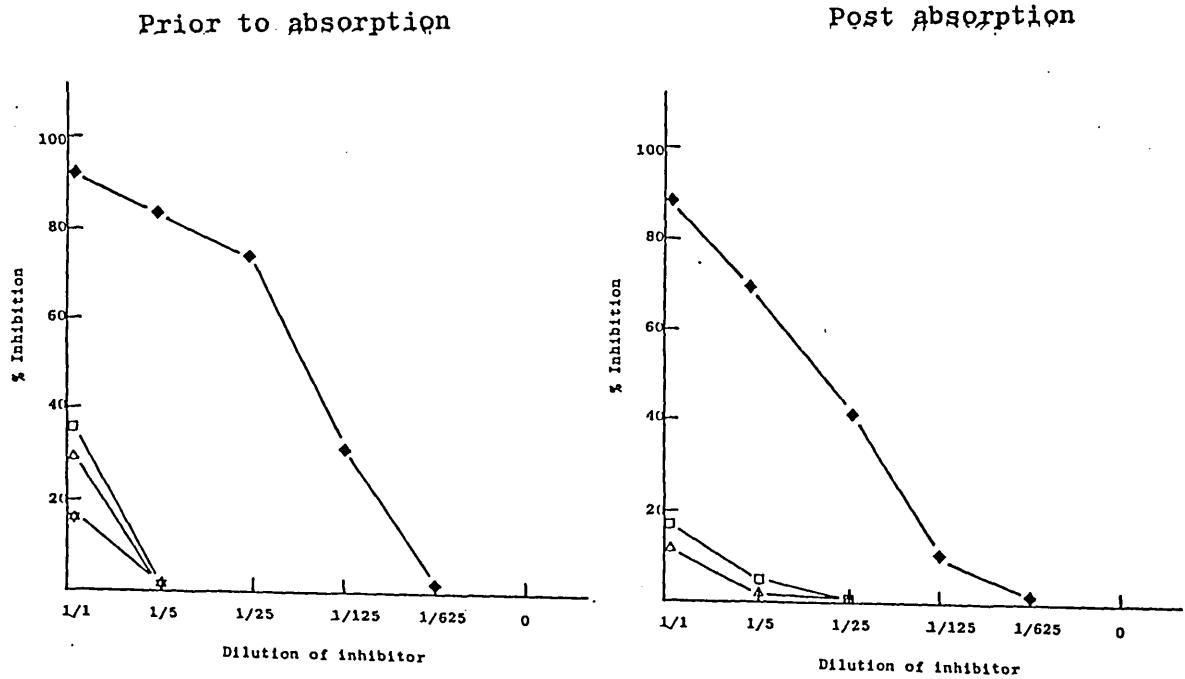
Conjugate: Pig IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	✕

Figure 3.11 Inhibition ELISA, testing specificity of Rat antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Rat antibodies



Antibody : Sheep anti-Rat IgG

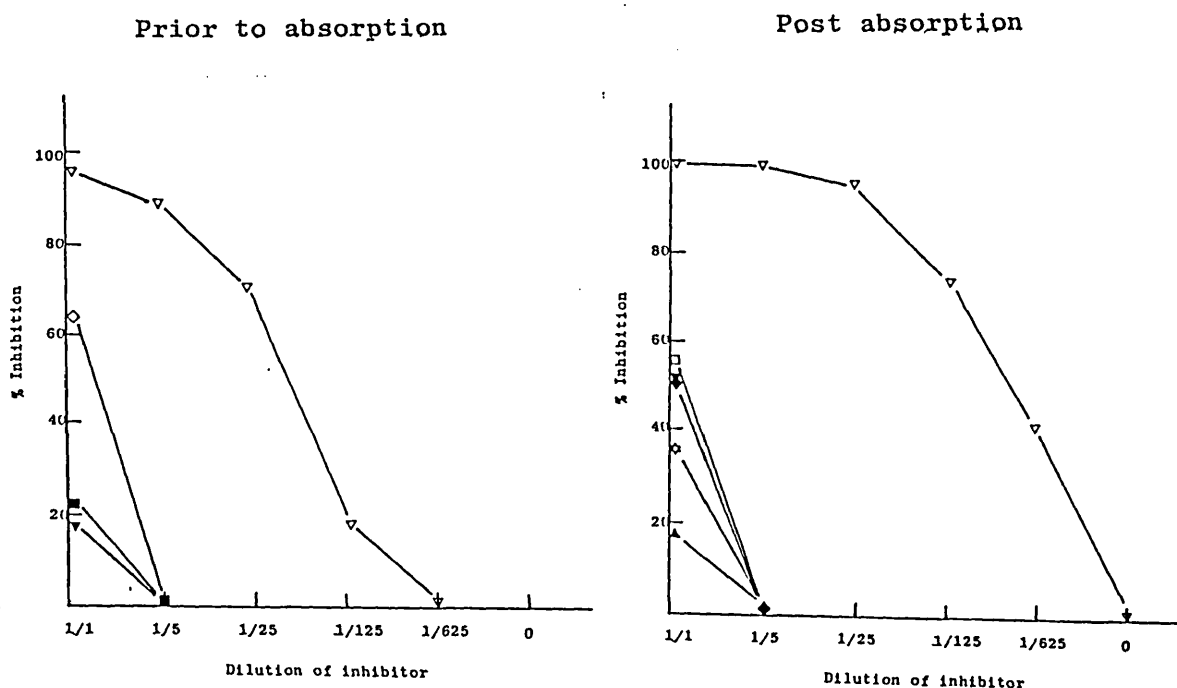
Conjugate: Rat IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	▼

Figure 3.12 Inhibition ELISA, testing specificity of Horse antigen conjugate, with all other species antigens, both prior and post absorption of the sheet anti-Horse antibodies



Antibody : Sheep anti-Horse IgG

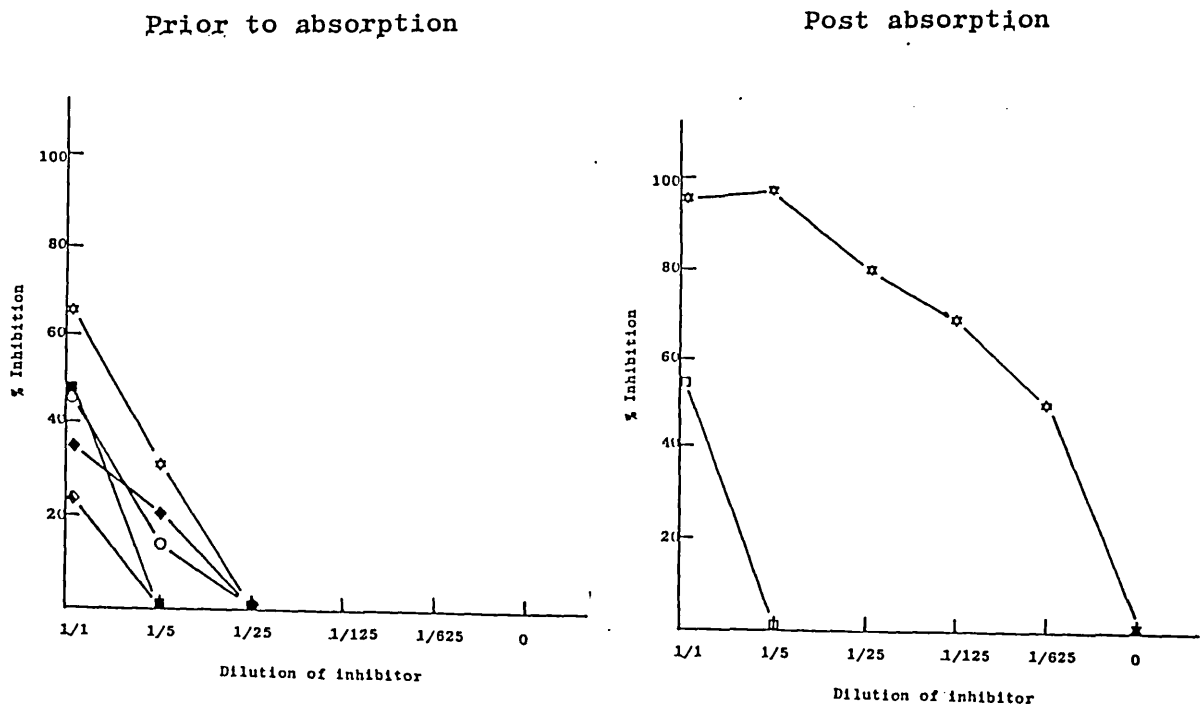
Conjugate: Horse IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	✚

Figure 3.13 Inhibition ELISA, testing specificity of Chicken antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Chicken antibodies



Antibody : Sheep anti-Chicken IgG

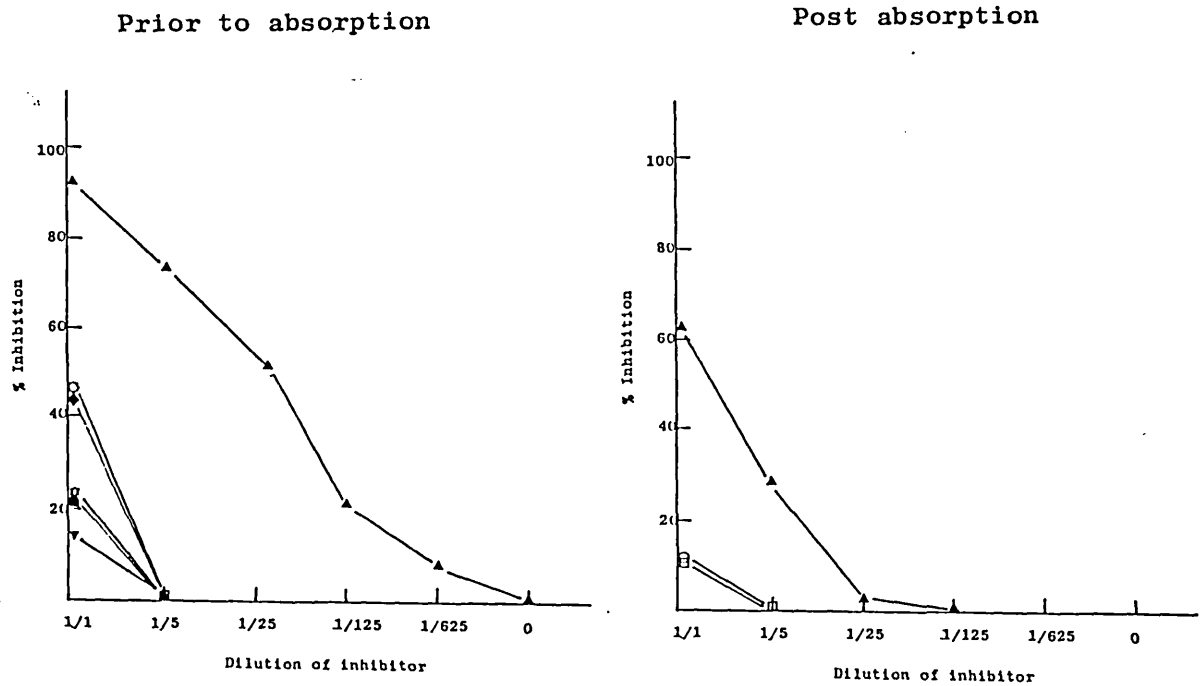
Conjugate: Chicken IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	✕

Figure 3.14 Inhibition ELISA, testing specificity of Guinea Pig antigen conjugate, with all other species antigens, both prior and post absorption of the sheet anti-Guinea Pig antibodies



Antibody : Sheep anti-Guinea Pig IgG

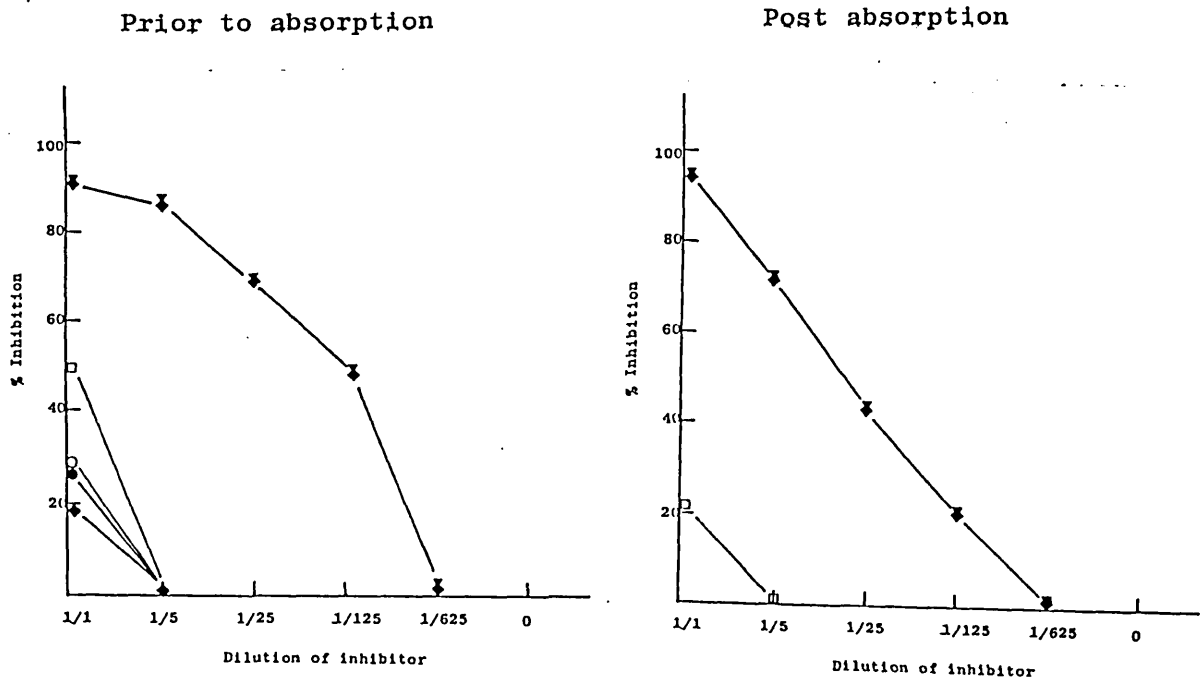
Conjugate: Guinea Pig IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	✕

Figure 3,15 Inhibition ELISA, testing specificity of Rabbit antigen conjugate, with all other species antigens, both prior and post absorption of the sheet anti-Rabbit antibodies



Antibody : Sheep anti-Rabbit IgG

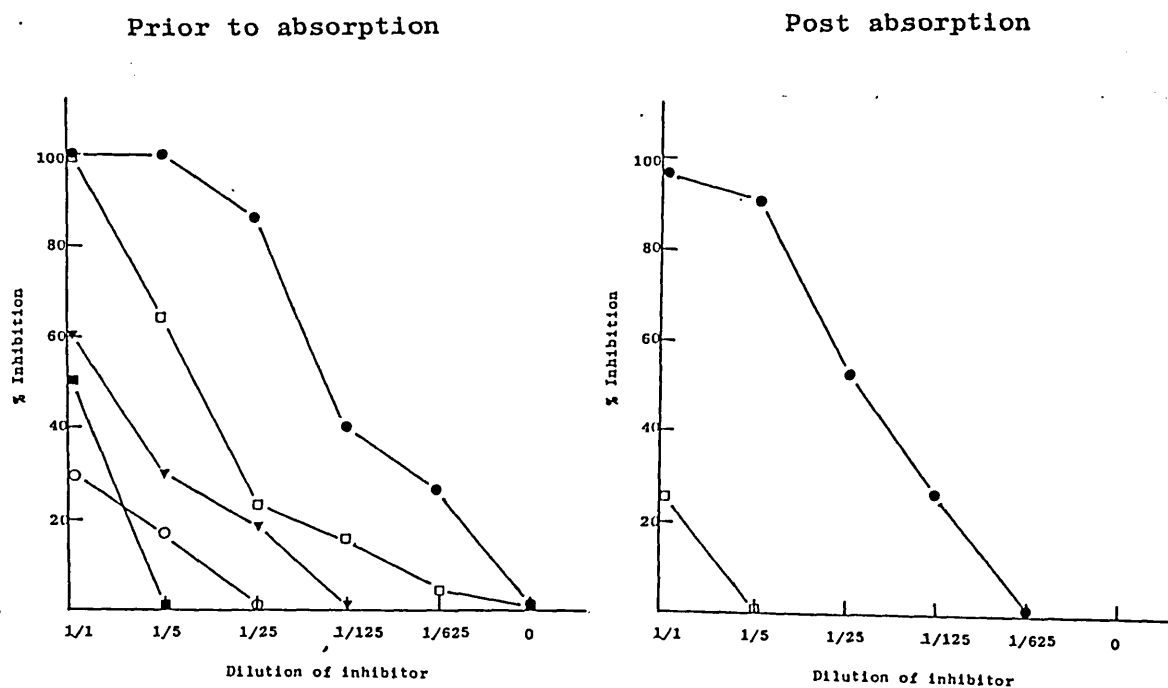
Conjugate: Rabbit IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	◆

Figure 3.16 Inhibition ELISA, testing specificity of Sheep antigen conjugate, with all other species antigens, both prior and post absorption of the Rabbit anti-Sheep antibodies



Antibody : Rabbit anti-Sheep IgG

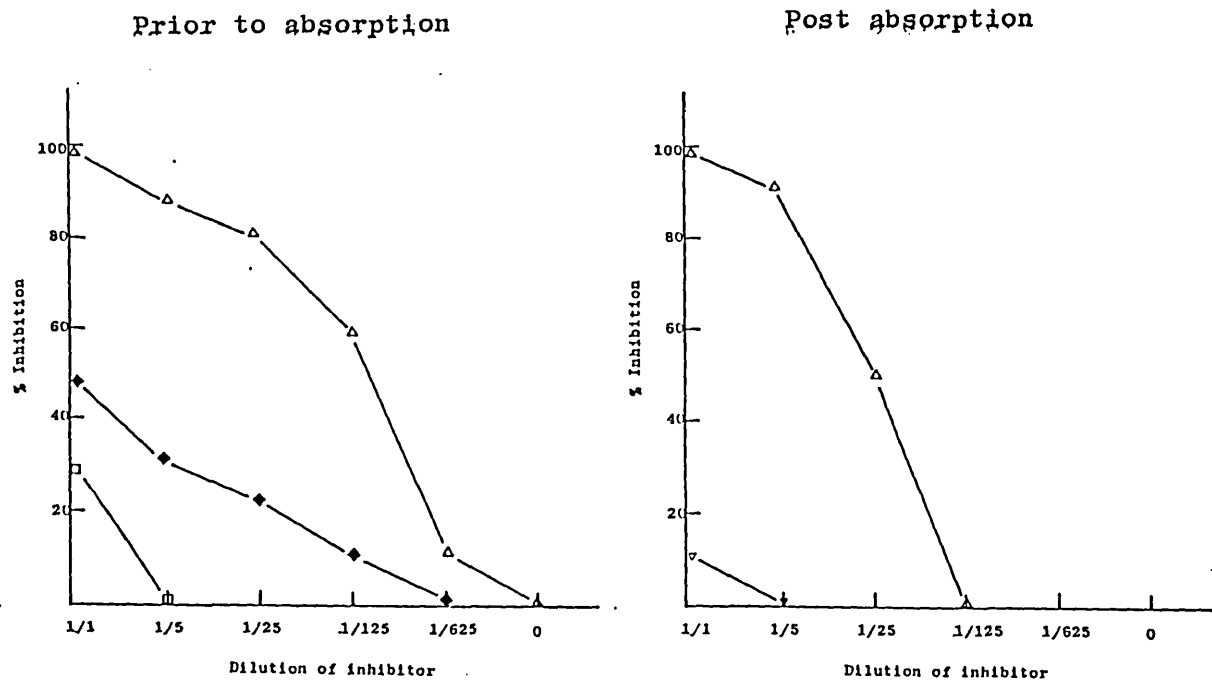
Conjugate: Sheep IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	△
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	◆

Figure 3.17 Inhibition ELISA, testing specificity of Mouse antigen conjugate, with all other species antigens, both prior and post absorption of the sheep anti-Mouse antibodies



Antibody : Sheep anti-Mouse IgG

Conjugate: Mouse IgG - HRP

Key.

Cross reacting animal Immunoglobulin G (100 µg/ml)

Chicken	☆	Sheep	●
Horse	▽	Hamster	◇
Rat	◆	Ox	□
Pig	○	Mouse	Δ
Human	■	Guinea Pig	▲
Dog	▼	Rabbit	⬇

The monoclonal antibody anti-IgG (human) inhibition ELISA has already previously been shown to be specific. For this reason a polyclonal inhibition ELISA was not developed and is thus not represented in figures 3.7 to 3.17

2. Dot blot Inhibition ELISA

The inhibition ELISA described earlier in this chapter was converted to a dot blot inhibition assay, with nitrocellulose acetate paper serving as the solid phase.

In order to reach the highest degree of sensitivity a variety of substrates were tested with the two enzymes horse radish peroxidase (HRP) and alkaline phosphatase (AP). The first attempt was using the HRP enzyme with the substrate di amino benzidine, this substrate proved to have a very high non-specific background reaction which rendered the result almost impossible to read. This result is not shown here.

Figures 3.18 to 3.23 show each experiment using the various substrates and enzymes.

Figure 3,18

Enzyme: Alkaline phosphatase

Substrate: Napthol AS-MX phosphate and Fast blue BB base.

Antibody concentration on solid phase: 12.5 ng/dot

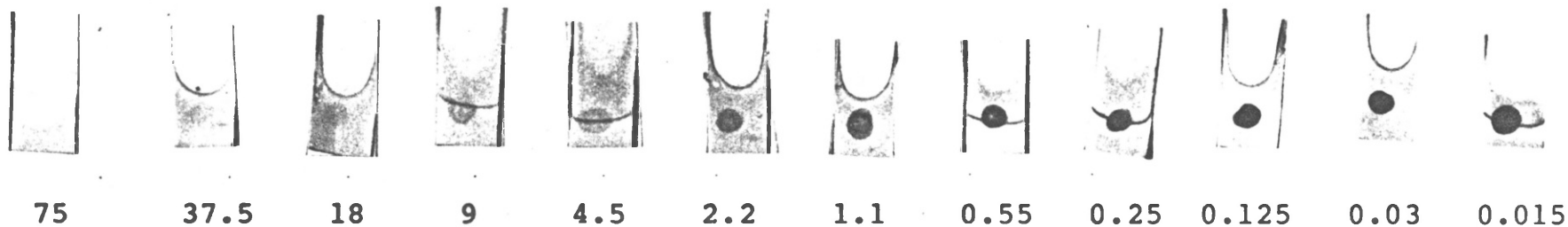
Amount of standard inhibitor (human IgG) where inhibition is still clearly visible: 2.2 µg/ml, i.e. 0.22 µg as only 100 µl of inhibitor solution was used in the assay.

Dilution of fresh human serum where similar inhibition is visible:
1/100,000

The contrast of the dot is good, although there is some non-specific background reaction. Inhibition, ie. a positive result can be clearly seen. The sensitivity of this substrate is quite high, 2 µg giving approximately 50% inhibition.

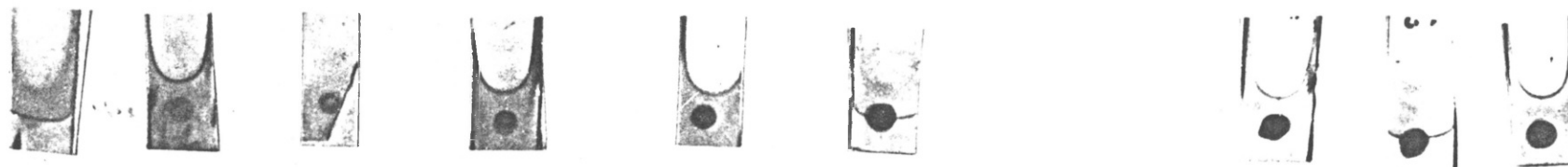
Figure 3.18: Dot Blot Inhibition ELISA (IgG λ - AP)

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Normal human serum

No Inhibitor



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - AP): 1/2000

Substrate : Naphthol AS MX phosphate and Fast blue B.B. base.

Figure 3.19

Enzyme: Alkaline phosphatase.

Substrate: Napthol AS-MX phosphate and Fast Red.

Antibody concentration on solid phase: 12.5 ng/dot.

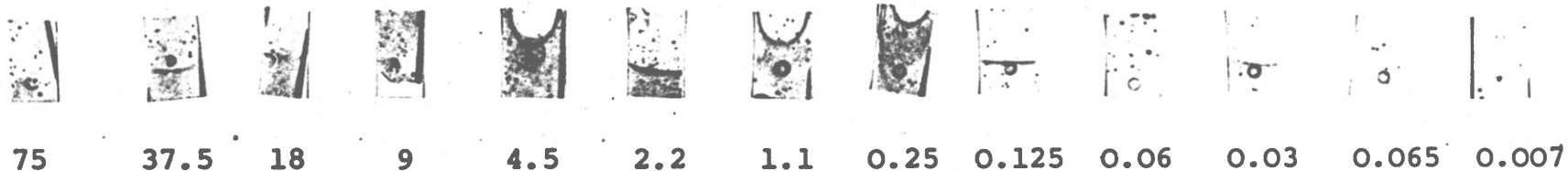
Amount of standard inhibitor (human IgG) where inhibition is still clearly visible: 2.2 µg/ml, i.e. 0.22 µg as only 100 µl of inhibitor solution was used in the assay.

Dilution of fresh human serum where similar inhibition is visible: 1/100,000.

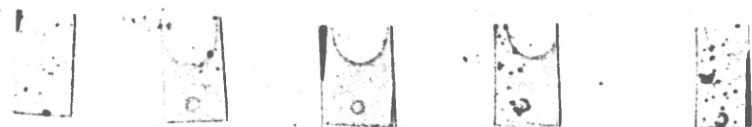
Resolution of the dot is very poor, there is a lot of non-specific background reaction and inhibition is difficult to determine.

Figure 3.19 : Dot Blot Inhibition ELISA (IgG λ - AP)

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).

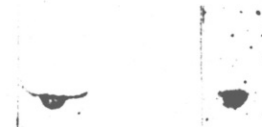


Normal human serum



1/200 1/1000 1/2000 1/10,000 1/100,000

No Inhibitor



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - AP): 1/2000

Substrate : Napthol AS-MX phosphate and Fast Red.

Figure 3.20

Enzyme: Alkaline phosphatase.

Substrate: 5-Bromo, 4-Chloro, 3-indolyl phosphate. (BCIP)

Antibody concentration on solid phase: 12.5 ng/dot.

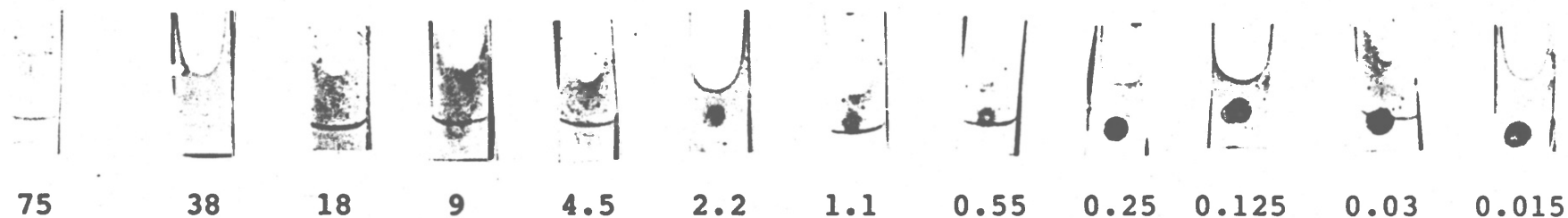
Amount of standard inhibitor (human IgG) where inhibition is still clearly visible: 1.1 µg/ml, i.e. 0.11 µg as only 100 µl of inhibitor solution was used in the assay.

Dilution of fresh human serum where similar inhibition is visible: 1/10,000.

There is a degree of non-specific background reaction, yet the dots are still clearly resolved. Inhibition is clearly visible and the sensitivity of this substrate is reasonably high

Figure 3.20 : Dot Blot Inhibition ELISA (IgG λ - AP)

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Normal human serum

No Inhibitor



1/200 1/1000 1/2000 1/10,000 1/20,000 1/100,000

Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - AP): 1/2000

Substrate : 5 - Bromo, 4 - Chloro, 3 - Indolyl phosphate.

Figure 3.21

Enzyme: Alkaline phosphatase.

Substrate: BCIP and nitroblue tetrazoleum (NBT)

Antibody concentration on solid phase: 12.5 ng/dot.

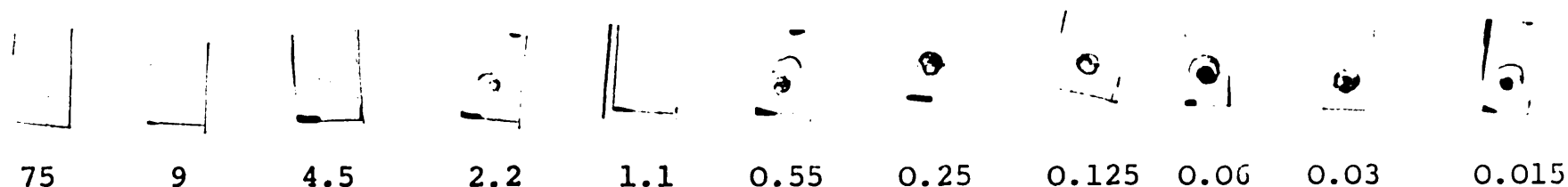
Amount of standard inhibitor(human IgG) where inhibition is still clearly visible: 0.25 µg/ml, i.e. 0.025 µg as only 100 µl of inhibitor solution was used in the assay.

Dilution of fresh human serum where similar inhibition is visible:
1/100,000.

Not a very clear contrasting dot, the background non-specific reaction is low. The sensitivity is quite high. This result might be improved with a more concentrated antibody solution, ie. to focus the dots more.

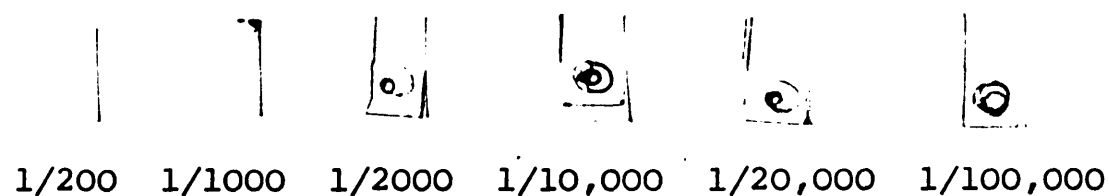
Figure 3.21 : Dot Blot Inhibition ELISA (IgG λ - AP)

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Normal human serum

No Inhibitor



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - AP): 1/2000

Substrate : BCIP followed by enhancement with Nitro blue Tetrazoleum.

Figure 3.22

Enzyme: Horse radish peroxidase.

Substrate: 3 Amino - 9 Ethyl Carbazole.

Antibody concentration on solid phase: 12.5 ng/dot.

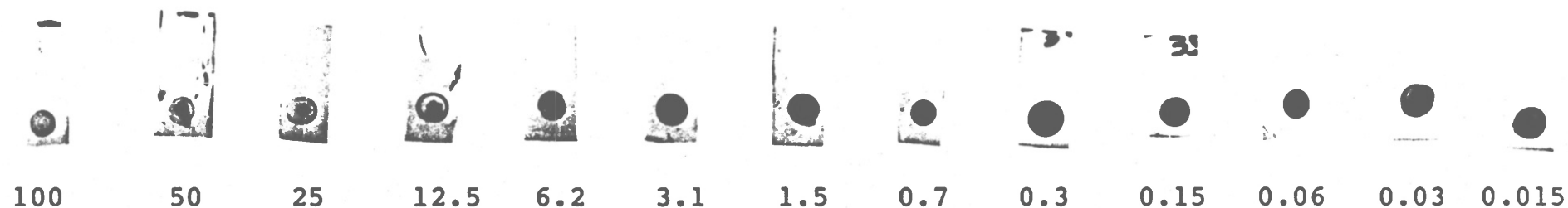
Amount of standard inhibitor (human IgG) where inhibition is still clearly visible: 12.5 $\mu\text{g/ml}$, i.e. 1.25 μg as only 100 μl of inhibitor solution was used in the assay.

Dilution of fresh human serum where similar inhibition is visible: 1/100.

No background reaction whatsoever, and a very clear contrasting dot. The sensitivity of this substrate is a little lower than the others.

Figure 3.22 : Dot Blot Inhibition ELISA (IgG λ - HRP)

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Normal human serum



No Inhibitor



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - HRP): 1/2000

Substrate : 3 Amino - 9 Ethyl carbazole

Samples were freshly prepared.

Figure 3.23

Enzyme: Horse radish peroxidase.

Substrate: 4 alpha chloro 1 Naphthol.

Antibody concentration on solid phase: 12.5 ng/dot.

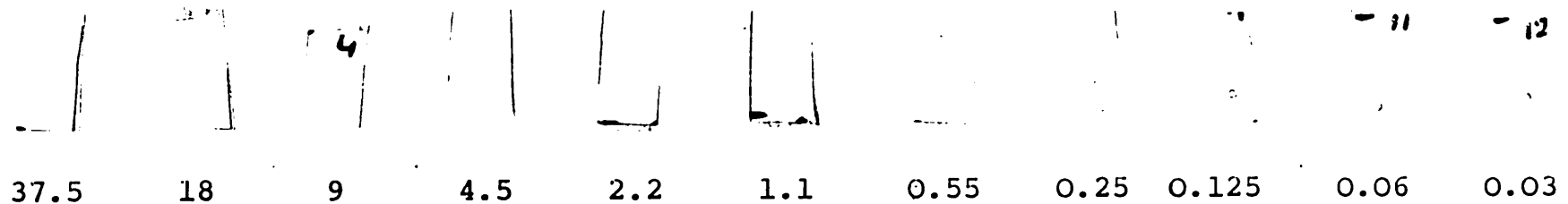
Amount of standard inhibitor (human IgG) where inhibition is still clearly visible: 0.55 $\mu\text{g/ml}$, i.e. 0.055 μg as only 100 μl of inhibitor solution was used in the assay.

Dilution of fresh human serum where similar inhibition is visible:
1/100,000.

Resolution of the dots is not very sharp, but there is little or no non-specific background reaction. The sensitivity of this substrate is high with a 1/100,000 dilution of human serum giving almost a 100% inhibition.

Figure 3.23 : Dot Blot Inhibition ELISA (IgG λ - HRP)

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Normal human serum

No Inhibitor



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - HRP): 1/2000

Substrate : 4 alpha chloro 1 Naphthol.

Figures 3.24 to 3.27 show the results of the effects of temperature and storage on efficacy of the dot blot assay.

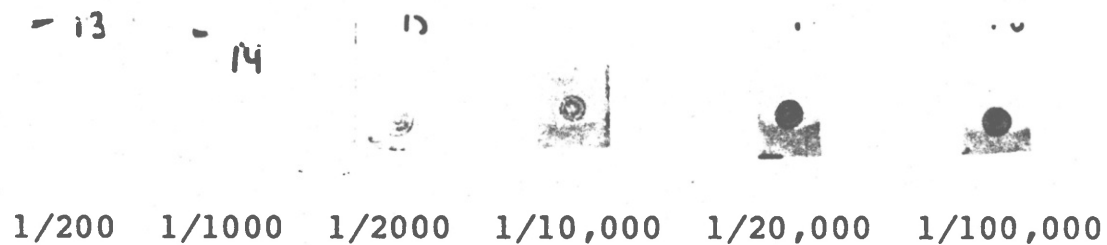
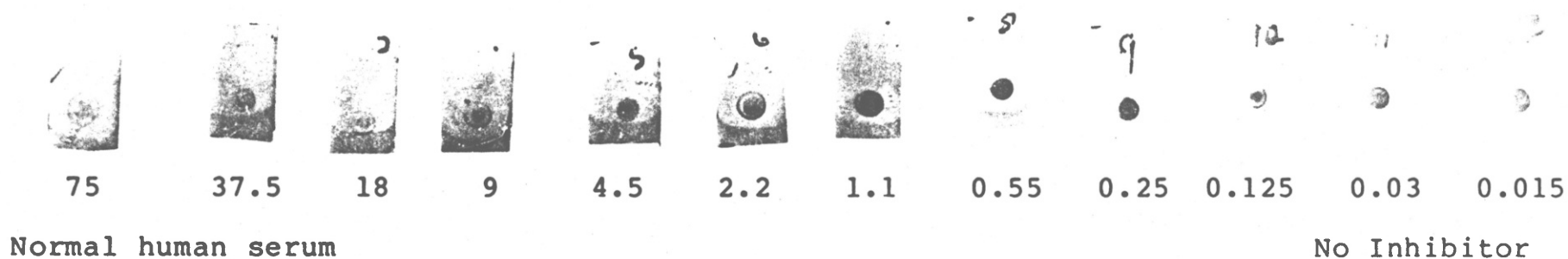
There are two overall effects, an increase in the background non-specific reaction, leading to a reduction in sensitivity because distinction cannot be made visually; the other is an actual drop in sensitivity that is not related to the resolution of the dots. These reductions in performance occur in all conditions of storage.

Figure 3.24: Dot Blot Inhibition ELISA (IgG λ - HRP)

Effects of temperature and storage

1. Kept for 15 days at +4°C

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - HRP): 1/2000

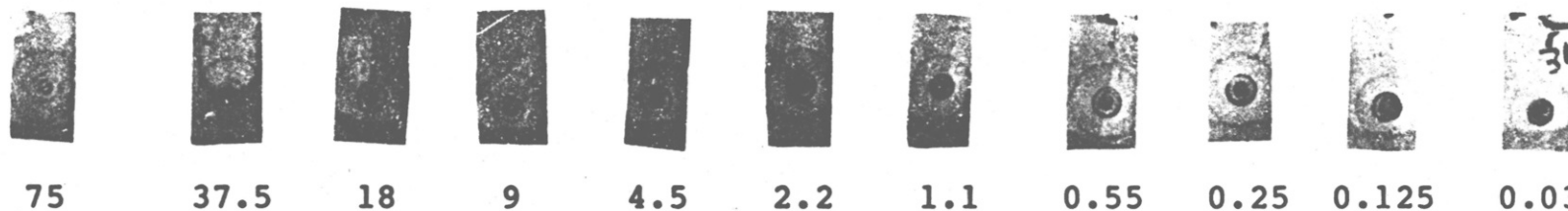
Substrate : 3 Amino - 9 Ethyl carbazole

Figure 3.25 : Dot Blot Inhibition ELISA (IgG λ - HRP)

Effects of temperature and storage

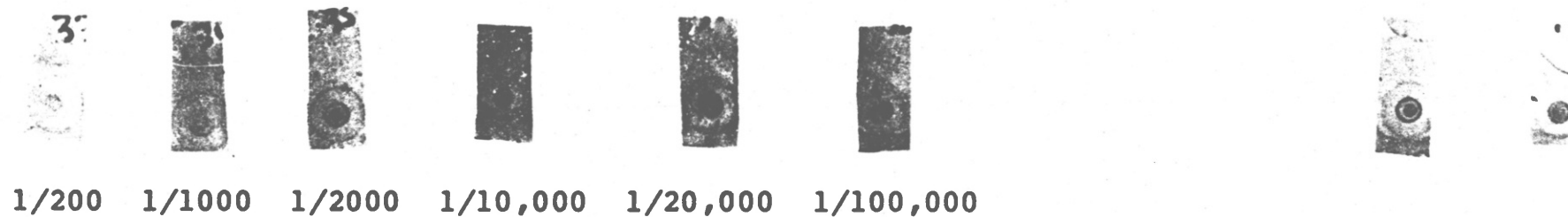
2. Kept for 15 days at +25°C

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Normal human serum

No Inhibitor



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

Conjugate concentration (IgG λ - HRP): 1/2000

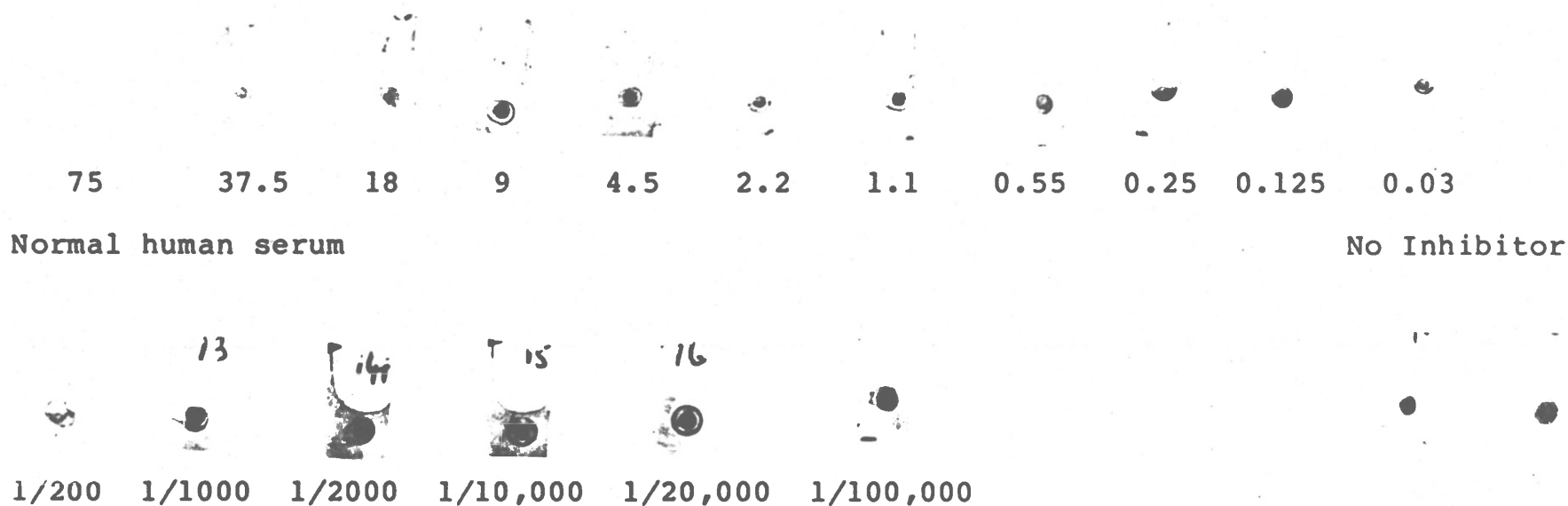
Substrate : 3 Amino - 9 Ethyl carbazole.

Figure 3.26 : Dot Blot Inhibition ELISA (IgG λ - HRP)

Effects of temperature and storage.

3. Kept for 30 days at +4°C

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



Antibody concentration (C4) : 2.5 $\mu\text{g/ml}$, 5 $\mu\text{l/dot}$.

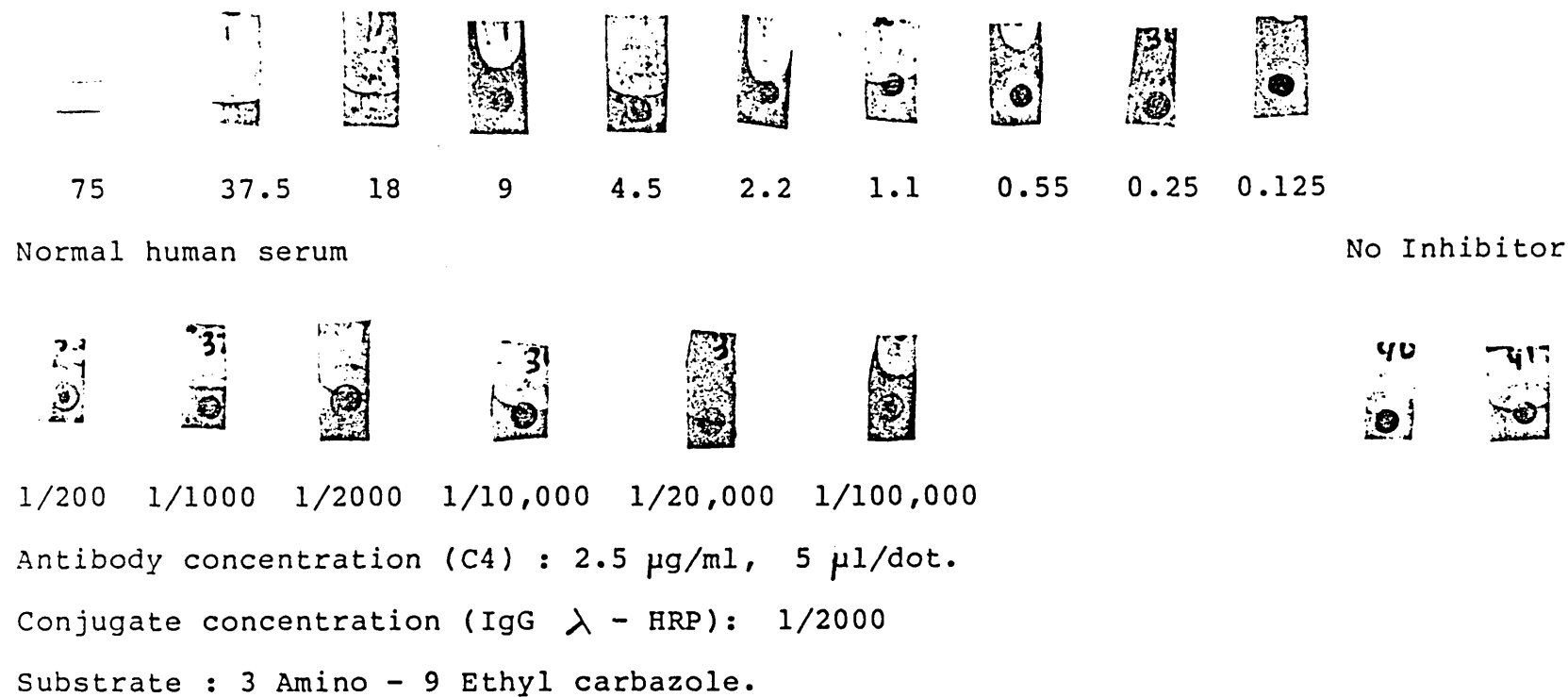
Conjugate concentration (IgG λ - HRP): 1/2000

Substrate : 3 Amino - 9 Ethyl carbazole.

Figure 3.27 : Dot Blot Inhibition ELISA (IgG λ - HRP)
Effects of temperature and storage.

4. Kept for 30 days at +25°C

Standard Inhibitor IgG λ ($\mu\text{g/ml}$).



3.4 DISCUSSION

All the arthropod bloodmeal identification assays that have been developed, the traditional methods and more recently the ELISA (Burkot, 1982) have used antiserum that has been prepared with whole animal sera as antigen. Efforts to improve specificity have been bulk liquid absorption, with limited success, the use of albumin as antigen and the use of more sensitive methods, such as ELISA. The use of a more specific target antigen, like beta lipoprotein has not been employed until now. Solid phase absorption has improved the specificity of the antisera, the inhibition ELISA using target antigens has increased it further.

The sensitivity of the immunoglobulin system (i.e. the immunoglobulin conjugated inhibition assay) is greater than any previously recorded. Burkot et al., (1981) stated that 232 ug of serum was needed for specific immunopositive identification. With the immunoglobulin system, 50% inhibition is observed with only 0.032 ug of serum. This sensitivity is extended in the dot blot assay, where only 12.5 ng of antibody are used to coat the solid phase and, in some cases, 0.025 ug of antigen inhibitor gave clear distinguishable results.

It is not possible to make a comparison with the assay described by Edrissian and Hafizi (1982), these workers just diluted a whole bloodmeal in 100 ul of coating buffer which was used to coat one well of a microtitre plate. It is not possible to estimate how much of the soluble antigen bound to the surface of the well, however, it is clear though that the present method is more sensitive, allowing many more tests to be performed with one bloodmeal. This is only important when one is attempting to identify animal feeds rather than just human feeds, for which the Edrissian and Hafizi ELISA was designed.

The beta lipoprotein inhibition assay as described is suitable for identifying human feeds only, similar systems could as easily be prepared for any choice of animal, providing enough serum was available to be able to isolate, purify and conjugate the beta lipoprotein.

Immunoglobulin occurs at a 3 fold higher serum concentration than beta lipoprotein so less serum is needed to prepare enough antigen for conjugation. The other advantage is that there is more antigen available in the bloodmeal, thus giving the assay, using immunoglobulin, a longer immunological half life, which improves the post digestion analysis (see Chapter 4).

The simplicity of the inhibition assay is an advantage in field use. With minor modifications a kit could be prepared, such a kit would contain a comb previously coated with the specific antibody. The effects of storage on the sensitivity of the assay were not very encouraging in this regard. Progress should be possible with alternative conditions of storage and the use of alternative quenching proteins. The bloodmeal under examination would be eluted and placed into a well, already containing the conjugated antigen, the comb can then be simply dipped into this mixture, followed by a wash. The comb then would be immersed in the substrate solution for the result. Positive controls could be included for cross reference and check on the result. The whole process would only take 30 mins. and the only requirement would be tap water.

The results from the dot blot inhibition assay using a variety of enzymes and substrates show a number of suitable combinations that could be used. Those substrates that give a clear, sensitive result without non-specific reaction, and are cheap, non-mutagenic and non-degradable.

For example, BCIP, with NBT and the enzyme alkaline phosphatase or 3-aminoethylcarbazole and 4-aminobenzothiazol with the enzyme horse radish peroxidase which also gave sensitive results.

The use of alkaline phosphatase as the enzyme is preferable, the substrates exhibit less non-specific reactions and are non-mutagenic. This is an obvious plus when considering that some field staff might not be very sophisticated. This enzyme does not require the use of H_2O_2 with its substrates, unlike the peroxidase enzyme. H_2O_2 might be difficult to obtain and maintain in the field. The only disadvantage of using alkaline phosphatase enzyme compared with horseradish peroxidase is the cost as it is a lot more expensive.

The inhibition assay could be made more sensitive, although it may not be really necessary, with the use of cascade enzymes, as mentioned in the introduction or with use of chemiluminescent substrates such as luminol or luciferase. However, such measures would not be an advantage for a field kit, involving considerably more cost and complexity of instrumentation.

In summary, using DEAE separated immunoglobulin fractions from a range of animal sera, a comprehensive simple diagnostic assay for insect bloodmeal identification has been developed. The assays' sensitivity surpasses that of any recorded previously and the use of alkaline phosphatase and the chromogen BCIP enhanced with NBT gives a sensitive result (Figure 3.22).

CHAPTER 4DEGRADATION STUDIES ON THE EFFECTS OF DIGESTION ON
INSECT BLOODMEALS

4.1 INTRODUCTION

The successful identification of arthropod bloodmeals depends not only on the quality of the antisera used and the sensitivity of the assay employed, but also on the nature and quantity of bloodmeal available in the gut of the arthropod at the time of capture. These conditions are mostly dependent on the original volume of the bloodmeal and on the rate of digestion of the bloodmeal. The volume of the bloodmeal obviously depends upon the size of the various species of arthropod and their particular feeding methods. Also involved is the tolerance of the host to the infliction of the bites and the natural palatability of the host to the arthropods that feed upon them.

The rate of digestion of the bloodmeal of a given volume will in turn vary considerably between different haematophagous insects and different environmental conditions. West and Eligh (1952) showed that temperature had a critical effect upon the rate of digestion.

Nearly all the literature concerned concentrates upon the effect of the rate of digestion upon identification. In fact many rates of digestion for various species of haematophagous arthropods are measured in this way. Weitz and Buxton (1953) studied the different rates of digestion of 10 species of arthropod under standard laboratory conditions. The results varied enormously between species, mosquito digesting the bloodmeals beyond identification in a matter of hours, while tsetse flies took days, with triatomid bugs and lice taking weeks.

Many comparative studies have been performed between different species and these are clearly reviewed by Gooding (1972). The author also compares blood volumes, ovarian development and gut physiology.

Very little work has been done to investigate the effects of the rate of digestion upon the degradation of specific antigenic components of a bloodmeal. Some workers have studied the alpha and gamma globulins.

Mattern et al., (1967) working with laboratory reared Culex pipiens quinquefasciatus (Say) and using the precipitin test showed albumin could be detected up to 48 hours post ingestion and IgG up to 4 to 5 days. The authors then caught wild mosquitoes (while they were feeding on volunteers) and repeated the analysis. The results were confusing, if the flies were caged in similar small cages as the laboratory specimens were, the same result was achieved. If the flies were released into a large room then recaptured the reverse result was found. It was found with IgG that after only 5 hours post ingestion a second precipitin band occurred, thus indicating digestion had commenced.

Zaman and Chellappah (1967) working with the mosquito, Armigeres subalbatus (Say) using gel diffusion and immuno electrophoresis showed the presence in the mid gut of albumin was longer (48 hours) than IgG (12 hours). These results confirmed similar studies performed by Williams (1953) with Aedes aegypti (Linnaeus) fed upon guinea pigs.

Herndon and Ringle (1967) working with Anopheles quadrimaculatus (Say) and Culex pipiens (Linnaeus) confirmed the work of West and Eligh (1952) on the importance of temperature on the rate of digestion of the bloodmeal. With the flies kept between 6°C and 9°C bloodmeal antigen was detectable for a week post ingestion when the temperature was raised to between 25°C and 28°C, detection was impossible after only 1 day.

Edman (1970a & b) working with A. aegypti found that complete digestion of the bloodmeal protein occurred between 60 - 66 hours post ingestion, with no difference between the alpha and gamma globulins.

In this chapter 26 different molecular components of the bloodmeal were studied to investigate the effect of digestion on the degradation of these molecules.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of mosquito bloodmeals

Dr. Sinden generously supplied 200 unfed female Anopheles stephensi (Liston) mosquitoes from his culture stock. These were then fed upon a volunteer and placed in a humidified incubator at 25°C, with sugar water on cotton wool. Subsequently after feeding a sample of mosquitoes were killed, by refrigeration, every three hours until 70 hours post feeding. Each mosquito was carefully squashed onto Whatman No.1 filter paper, which was labelled in pencil and stored in a desiccator containing silica gel at room temperature. The bloodmeals were kept in this way until required.

When required the bloodmeals were cut out from the filter paper and eluted for 1 hour into 500 µl of PBS - Tween 20 (0.05%). After elution the soluble bloodmeal was stored at -20°C.

4.2.2 SDS-PAGE and Western transfer

The materials and methods for both the SDS-PAGE and Western transfer are identical to those described in the section on characterisation of the monoclonal antibodies with SDS-PAGE and Western transfer Chapter 1.

4.2.3 Immunoprobng after Western transfer

In order to study the effects of digestion on the contents of the mosquito bloodmeals, immunoprobng with antisera and enzyme conjugated antibodies was performed on the nitrocellulose paper after Western transfer. the immunological probes, i.e. a series of sheep antisera raised to components of human serum were supplied by the Immuno

diagnostic Research Laboratories, Birmingham (IDRL) at Birmingham University and are listed below.

Anti albumin

- " Immunoglobulin IgG
- " Beta lipoprotein
- " Complement component C1
- " " " C3
- " " " C4
- " " " C5
- " " " C9
- " KAF (C3b inhibitor)
- " BIH
- " C1 inactivator
- " Haptoglobin
- " Inter Alpha 1 Trypsin Inhibitor
- " Alpha 2 macroglobulin
- " Alpha 1 Antitrypsin
- " Fibronectin
- " Fibrin
- " Orosomucoid (Alpha 1, acid glycoprotein)
- " Factor B (C3 proactivator)
- " Alpha 1 antichymotrypsin
- " Thromboplastin III
- " Prothrombin II
- " Factor II
- " Plasminogen
- " Transferrin
- " Ferritin

After blotting the nitrocellulose paper sheet was placed between two pieces of polythene which with a heat sealer was made into a plastic bag. The use of this bag reduced the volume of reagents used, which was important considering the cost of some of the antisera. The bag was first filled with 30 ml of 1% casein PBS - Tween 20 (0.05%) and the paper quenched for 1 hour at 37°C. It was then washed in physiological saline - Tween 20 (0.05%) three times.

Then a 30 ml volume of a 1/200 of one of the above antisera diluted in 1% casein PBS - Tween 20 (0.05%) was incubated with the paper for 2 hours in saline - Tween 20 (0.05%) three times.

The blot was then placed into another bag containing 30 ml of donkey anti sheep IgG - HRP conjugate solution. This conjugate was diluted to 1/3000 in 1% casein PBS - Tween 20 (0.05%). The blot was then washed three times in saline Tween 20 (0.05%).

The next stage was the developing stage. Initially the substrate, diaminobenzidine (DAB) was used, this is prepared with 100 ml of 0.08 M Tris buffer with 25 μ l of H_2O_2 . The colour soon developed and was stopped with tap water.

Another substrate was also used, 3-amino-6-ethyl carbazole. 80 mg was dissolved in a minimum quantity of dimethyl - formaldehyde and added to 190 mls of distilled water. The pH was adjusted to 3.7 using formic acid. 820 mg sodium acetate was added raising the pH to 5.0. The slurry formed was filtered through Whatman No.1 paper and 40 μ l H_2O_2 added immediately prior to use. The colour reaction was red. The reaction was stopped by rinsing in tap water and being left in the dark to dry.

4.3 RESULTS

The product of SDS-PAGE, western transfer and immuno- probing results in many sheets of nitrocellulose paper with coloured bands. For reasons of clarity all the results have been tabulated together with additional information concerning the molecular weight, serum concentration and approximate bloodmeal concentration. The results can be seen in Table 4.1.

A human serum sample was always included in every gel that was prepared. This was used as indicator, as to whether the antigens being studied were detectable or not. Of the 26 antigens searched for, 9 were detectable in the human serum sample, these were:

	Hours still detectable post ingestion
Albumin	36
Immunoglobulin	42
Beta lipoprotein	39
Alpha ₁ antitrypsin	24
Transferrin	18
Haptoglobin	6
Alpha ₁ antichymotrypsin	30
Complement component C ₃	-
Complement component C ₄	-

Of these two were not detectable within the fresh bloodmeal, these were the two complement components C₃ and C₄.

Table 4.1: Summary table of western transfer and immunoprobng results of Anopheles stephensi digested bloodmeals

Molecule	Molecular Weight (Daltons)	Serum Concentration mg/ml	Approximate Bloodmeal Concentration mg	Positive Result with Human Serum	Positive Result with Bloodmeal	Hours post ingestion still positive	Positive Activity after Solid Phase Adsorption	Candidate Target Antigens Post 35 hours Digestion
Albumin	66,000	40	0.4	Yes	Yes	36	No	No
Immuno-globulin	160,000	13	0.13	Yes	Yes	42	Yes	Yes
Beta-lipoprotein	2,400,000	4	0.004	Yes	Yes	39	Yes	Yes
Alpha 1 Antitrypsin	54,000	3.0	0.03	Yes	Yes	24	No	No
Transferrin	76,500	2.5	0.025	Yes	Yes	18	Yes	No

Table 4.1: (continuation sheet 1)

Molecule	Molecular Weight (Daltons)	Serum Concentration mg/ml	Approximate Bloodmeal Concentration mg	Positive Result with Human Serum	Positive Result with Bloodmeal	Hours post ingestion still positive	Positive Activity after Solid Phase Adsorption	Candidate Target Antigens Post 35 hours Digestion
Alpha 2 macro-globulin	725,000	2.5	0.025	No	No	No	No	No
Haptoglobin	200,000	2.2	0.022	Yes	Yes	6	Yes	No
Complement Component C ₃	185,000	0.90	0.009	Yes	No	No	No	No
Orosomucoid	44,000	0.75	0.0075	No	No	No	No	No
Inter alpha 1 trypsin inhibitor	160,000	0.55	0.0005	No	No	No	No	No

Table 4.1: (continuation sheet 2)

Molecule	Molecular Weight (Daltons)	Serum Concentration mg/ml	Approximate Bloodmeal Concentration mg	Positive Result with Human Serum	Positive Result with Bloodmeal	Hours post ingestion still positive	Positive Activity after Solid Phase Adsorption	Candidate Target Antigens Post 35 hours Digestion
Alpha 1 anti-chymotrypsin	68,000	0.5	0.0005	Yes	Yes	30	No	No
Complement Component C ₄	205,000	0.4	0.004	Yes	No	No	Yes	No
Fibronectin	440,000	0.33	0.0004	No	No	No	No	No
Plasminogen	91,000	0.25	0.0025	No	No	No	No	No
C ₁ Inhibitor	104,000	0.25	0.0025	No	No	No	No	No

Table 4.1: (continuation sheet 3)

Molecule	Molecular Weight (Daltons)	Serum Concentration mg/ml	Approximate Bloodmeal Concentration mg	Positive Result with Human Serum	Positive Result with Bloodmeal	Hours post ingestion still positive	Positive Activity after Solid Phase Adsorption	Candidate Target Antigens Post 35 hours Digestion
Factor B	93,000	0.25	0.0025	No	No	No	No	No
Complement Component C ₉	79,000	0.20	0.002	No	No	No	No	No
Complement Component C ₁₂	400,000	0.15	0.0015	No	No	No	No	No
B.H.	150,000	0.13	0.0004	No	No	No	No	No
Factor II	68,700	0.075	0.00075	No	No	No	No	No

Table 4.1: (continuation sheet 4)

Molecule	Molecular Weight (Daltons)	Serum Concentration mg/ml	Approximate Bloodmeal Concentration mg	Positive Result with Human Serum	Positive Result with Bloodmeal	Hours post ingestion still positive	Positive Activity after Solid Phase Adsorption	Candidate Target Antigens Post 35 hours Digestion
Complement Component C ₅	195,000	0.07	0.0007	No	No	No	No	No
KAF C ₃ Inhibitor	90,000	0.04	0.0004	No	No	No	No	No
Thyroxine binding globulin	57,000	0.035	0.00035	No	No	No	No	No
Ferritin	450,000	3×10^{-5}	3×10^{-7}	No	No	No	No	No
Thrombin III	65,000	No	No	No	No	No	No	No
Fibrin	No	No	No	No	No	No	No	No

4.4 DISCUSSION

The technique of immuno probing after western transfer is very similar to that of the dot blot ELISA, it can be compared both for its specificity and sensitivity, which both surpass that of the precipitin test as used by Mattern et al., (1967) in similar studies; these authors only considered the albumin and immunoglobulin content of the bloodmeal.

These studies show that 7 molecules can be detected in the bloodmeal for various times post ingestion. The obvious comment to make is that serum concentration is an important factor, those molecules such as albumin, immunoglobulin and beta lipoprotein having the highest concentration lasted immunologically intact the longest.

It has been demonstrated (Howard, 1962) that the peritrophic membrane completely surrounds and seals the bloodmeal and that the process of digestion starts and occurs at the surface of the peritrophic membrane working inwards as digestion progresses (Huff, 1934). Those molecules of higher concentration will remain undigested longer at high enough concentrations to still be detectable, some time after digestion has been initiated.

There are a number of examples of a form of molecular sieving that takes place across the peritrophic membrane. Molecules of a particular size have been found to diffuse through the peritrophic membrane undigested and pass into the haemolymph. Nogge & Giannetti (1979) working with G. morsitans (Westwood), found that albumin and partially digested immunoglobulin, Fab & Fc, passed through the peritrophic membrane into the haemolymph. Using FITC labelled dextrans, they showed the upper size limit to be approximately 50,000 d. There has also been confirmed a similar phenomenon in Hypoderma bovis (De Geer).

Wigglesworth (1943) demonstrated that haemoglobin was taken up into the haemolymph by the triatomid Rhodnius prolixus (Stahl).

More recently Schlein et al., (1976) demonstrated the passage of serum immunoglobulins through the gut of Sarcophaga falculata (Pand). Smith et al., (1969) suggested that this occurrence was due to temporary absorption by the gut epithelial cells through pinocytosis. It could also be that the immunoglobulin molecules are being cleaved into Fab & Fc fragments, diffusing through the peritrophic membrane and reforming as whole molecules.

Serum concentration of these molecules is an important factor in the rate of diffusion across the peritrophic membrane.

Considering the molecular size limit of the sieving properties of the peritrophic membrane only a few of the molecules studied could actually transfer into the haemolymph undigested. Out of a total of 8 molecules, which might do so, four were detected for sometime post ingestion. These were transferrin, albumin, alpha antichymotrypsin and alpha antitrypsin. The other four were thrombin III, thyroxine binding globulin, factor II and orosomucoid. Of these, the first three either occur at very low concentration in human serum or not at all until triggered, e.g. thrombin III. Orosomucoid occurs at a similar concentration to that of alpha 1 antichymotrypsin but was not detected.

The other three molecules that were detected in the bloodmeal are of a molecular weight too large for this passage into the haemolymph to occur. There are beta lipoprotein, haptoglobin and immunoglobulins. One fact here needs stressing the system for detecting immunoglobulin not only detected whole molecule IgG but also Fab fractions as well. So after the initial cleavage Fab fragments could pass into the haemolymph

without further digestion. This molecular sieving affords these molecules a longer half life as regards immunological detection. As the contents of the gut are disseminated, some molecules that are able will move into the haemolymph avoiding digestion, until subsequently most of the molecules are eventually digested. This phenomenon perhaps explains the faster digestion of the haptoglobins compared with other molecules that occur at a similar serum concentration such as alpha 1 antitrypsin.

The molecular weight of the haptoglobin is 200,000 Kd and thus it is impossible for this molecule to pass into the haemolymph which might save it from faster digestion. The other molecules that were detected but could not pass through the haemolymph due to their molecular size, beta lipoprotein and immunoglobulin occur at much higher concentration than any other molecules in serum apart from albumin.

In conclusion, seven molecules were detected in the mosquito post ingestion. The molecules occurring at the highest concentrations in human serum were detectable the longest, albumin, immunoglobulin and beta lipoprotein. Alpha₁ antichymotrypsin, alpha 1 antitrypsin, transferrin and haptoglobin were also detected; the first three of these molecules were all of a sufficiently low molecular size to experience diffusion into the haemolymph, thus avoiding some digestion and increasing the immunological detectable half life provided haemolymph accompanies the blood in the specimen. The last, haptoglobin has a high molecular weight and does not experience such diffusion and was quite rapidly digested within 6 hours post ingestion.

CHAPTER 5

GENERAL DISCUSSION

In Chapter 1 the results show that the ELISA is by far the most appropriate assay for use in bloodmeal identification. The superiority of its sensitivity and specificity to that of the more traditional methods, together with the ease with which the assay can be performed lends itself to development as a field assay. This would require a central laboratory only to prepare ingredients and thus overcome the problems and expense associated with such a coordinated set up.

Revealing the specificities of the absorbed antisera has allowed a more sensitive and more simple assay to be developed, the dot blot inhibition ELISA. This assay shows great promise for adaptation to the field, combining quick sensitive methods with low costs and simplicity. The results concerning durability of reagents under storage conditions were not altogether encouraging. The effects of storage seemed to reduce the advantages of the assay. This problem though should be easily overcome, proteins both immunoglobulins and enzymes are successfully stored in dried conditions with almost no adverse effects upon their function. The important criteria is how such molecules are processed for storage, developments in such area should overcome these initial teething problems.

The results from Chapter 4 show promise of an assay that will perform successfully for much longer as regards post digestion of the bloodmeals. Both immunoglobulin and beta lipoprotein lasted immunologically intact the longest i.e. 42 and 39 hours respectively. This combined with the fact that both these molecules were shown to

contain species specific antigenic determinants, suggests these to be the most likely candidate antigens for incorporation into an inhibition ELISA. More work would need to be done using the beta lipoprotein molecule, as so far this system is only suitable for identifying human feeds.

Adaptation of this inhibition assay to large scale laboratory testing holds no problems, the assay could easily be installed and running in any reasonable laboratory. On the other hand field kit testing is not as straight forward. Theoretically the assay will perform well in the field but as yet the manufacture and general production of such an assay needs further investigation. Initially, a simpler human feeds only assay could be tested as a pilot study, this would be of great value all over the world to those workers studying the epidemiology of malaria and some virus diseases. Animal feeds and mixed feed assays could developed in due course. Considering wider applications, there are obvious forensic implications and use in the identification of freshly killed meat.

There is room for a further detailed study of the mechanisms of digestion and the effects upon the immunological competence of the antigens in the bloodmeal. Perhaps a more careful dissection of the bloodmeal from the abdomen of the insect, successfully separating the gut contents in the peritrophic membrane from the haemolymph, followed by similar SDS-PAGE and western transfer analysis on both components could yield more precise information.

The adaptation of this assay system to a sufficiently specific degree that individuals within species could be identified would be desirable but at the moment, there is little prospect of any success.

The pursuit of a monoclonal library might move some way towards such an eventuality. The consequences of a progression of this kind would be very exciting epidemiologically. Experiments could be performed to test individual host preferences and the implications such preferences might have in the dynamics of the transmission of disease would be considerable.

Other developments in bloodmeal analysis, such as isoelectric focussing and DNA probing show little promise of success as yet, the differences are not big enough for the former, and there is barely enough DNA available in a bloodmeal for the latter as it is presently seen. More interest lies with the idea that artificial antigens could be produced for use in an inhibition assay. Such antigens might improve sensitivity and specificity even further. Two approaches could be taken, firstly the production of anti-idiotypic antigens or genetic engineering with Escherichia coli, swapping genes about so that specific antigen products can be harvested simply and cheaply.

Another possibility, when considering the use of anti-idiotypic antibodies is the detection of host antibody in a bloodmeal that would prove diagnostic for some parasitic infections. Such an assay could measure the contact the vectors are having with presently or previously infected vertebrate hosts.

REFERENCES

- Abercrombie, M., Hickman, G.J. & Johnson, M.L. (1975) 6th Ed. A Dictionary of Biology. Penguin Books, London.
- Allison, A.C. & Monton, J.A. (1953). Species specificity in the inhibition of antiglobulin sera. J. Clin. Pathol., 6, 314-319.
- Albers, J.J. & Dray, S. (1968). Identification and Genetic control of two rabbit low density lipoprotein allotypes. Biochemical Genetics. 2, 25-35.
- Albers, J.J. & Hazzard, W.R. (1973). Immunochemical quantification of human plasma Lp(a). Lipoprotein. Lipids, 9, No.1, 15-26.
- Ascoli, M. (1902). Über der Mechanismus der Albumincine durch Eiereiweiss. Munch Med. Wochschr, 49, 398-401.
- Averameas, S. & Ternyck, T. (1971). Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration (letter). Immunochemistry, 8, 1145-1179.
- Averameas, S., Ternyuck, T. & Guesdon, J.L. (1978). Coupling of Enzymes to antibodies and antigens. Scand. J.Immunol. 8, Suppl. 7, 7-23.
- Bauer, K. (1970). Cross-reactions between human and animal plasma proteins. I. The immunological evolution groups (IEG) I and III. Humangenetik, 8, 325.
- Bauer, K. (1971a). The different phylogenetic age of antigenic determinants on human L chains. Humangenetik, 12, 261-279.
- Bauer, K. (1971b). Cross-reactions between human and animal plasma proteins. IV. Non-eutherian mammalia. Humangenetik, 13, 49.
- Bauer, K. (1974). Cross-reactions between human and animal plasma proteins. V. The role of antiserum and immunological specificty to the phenomenon of cross-reactions. Humangenetik, 21, 179-181.

- Belanger, L., Hamel, D., Dufour, D. & Pouliot, M. (1976). Double-antibody enzyme immunoassay applied to human - Fetoprotein. Clin. Chem. 22, 198-204.
- Bellrose, F.C. and Anderson, H.G. (1943). Preferential rating of duck food plants. Illinois Natural History Survey Bulletin, 22, 5, 417-433.
- Berg, K., Boman, M., Torsvik, H. & Walker, S.M. (1971). Allotypy of high density lipoproteins of rabbit serum. Proc. Nat. Acad. Sci., 68, 905 - 909.
- Berger, P.H., Thornbury, D.W. & Pirone, T.P. (1985). Detection of picogram quantities of potyviruses using a dot blot immunobinding assay. J. Virol. Meth., 12, 31-39.
- Biocca, E. (1950). Le ricerche del "fenomeno di gelificazione" e dei "gruppi cristallografici sangui di Amantea" quali prove orientative per la identificazione del sangue ottenuto dallo stomaco delle zanzare. Nuoviann igine microbiol., 1, 10-23.
- Bode, L., Bentin, L. & Kohler, H. (1984). Nitro cellulose-enzyme linked immunosorbent assay (NC - ELISA) - A sensitive technique for the rapid visual detection of both viral antigens and antibodies. J. Virol. Meth., 8, 111-121.
- Boreham, P.F.L. (1972). Serological identification of arthropod blood-meals and its applications. PANS, 18, 205-211.
- Boreham, P.F.L. & Gill, G.S. (1973). Serological identification of reptile feeds of Glossina. Acta tropica, 30, 356.
- Bordet, J. (1899). Agglutination et dissolution des globules rouges par le serum. Ann. Inst. Pasteur., 13, 273-297.
- Boyd, W.C. (1946). Fundamentals of Immunology, London, p.365.

- Boyd, M.F. (1949). Malariology. Saunders, Philadelphia & London, p.653.
- Boyden, A. (1942). Systematic serology : a critical appreciation. Physiol. Zool., 15, 109-145.
- Boyden, S.V. (1951). The absorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by anti protein sera. J. Exp. Med. 93, 107.
- Bruce, D. (1910). The natural food of Glossina palpalis. Proc. Roy. Soc. (London) B, 82, 490-497.
- Bruce-Chwatt, L.J. (1974). Resurgence of malaria and its control. J. Trop. Med. Hyg., 77, (Suppl.), 62.
- Bull, C.G. & King, W.V. (1923). The identification of bloodmeals of mosquitoes by means of the precipitin test. Am. J. Hyg., 3, 491-496.
- Bullock, S.L. & Walls, K.W. (1977). Evaluation of some of the parameters of the enzyme-linked immunospecific assay. J. Infect. Dis., 136, (Suppl.), S 279-285.
- Burkot, T.R. & De Foliart, G.R. (1982). Bloodmeal sources of Aedes triseriatus and A. vexans in a Southern Wisconsin forest endemic for la crosse encephalitis virus. Am. J. Trop. Med. Hyg., 31, C21, 376-381.
- Burkot, T.R., Goodman, W.G. & De Foliart, G.R. (1981). Identification of mosquito blood meals by enzyme linked immunosorbent assay. Am. J. Trop. Med. Hyg., 30, 1336-1341.
- Burkot, T.R., Zavala, F., Gwadz, R.W., Collins, F.M., Nussenzweig, R.S. and Roberts, D.R. (1984). Identification of malaria infected mosquitoes by a two site ELISA. Am. J. Trop. Med. Hyg., 33, 227-231.

- Butler, R. (1967). Polymorphisms of the human low density lipoproteins. Vox. Sang., 12, 2-17.
- Buxton, P.A. (1948). Trypanosomiasis in Eastern Africa, 1947. London H.M. Stationery Office, p.44.
- Buxton, T.B. & Rissing, J.P. (1980). The antibody and antigen ELISA : Future applications. Quality line, 2, 4-6.
- Cabannes, R. & Serain, C. (1955). Etude electrophoretique des hemoglobines des mammiferes domestiques d'Algerie. Compt. Redn. Soc. Biol., 149, 1193-1197.
- Carlsson, H.E., Lindberg, A.A. & Hammarstrom, S. (1972). Titration of antibodies to Salmonella O antigen by enzyme-linked immuno-sorbent assay. Infect. Immun., 6, 703-708.
- Carpenter, P.L. (1975). In : Immunology and Serology, 3rd ed., W.B. Saunders Co., Philadelphia, pp. 82-83.
- Catt, K. & Tregear, G.W. (1967). Solid-phase radioimmunoassay in antibody coated tubes. Science, 158, 1570-1572.
- Chafin, L. & Williams, K. (1978). Monoclonal antibodies production and applications. Curr. Top. Microbiol., 81, 107.
- Chamberlain, R.W. & Sudia, W.D. (1967). Methods for the study of mosquitoes as virus hosts and vectors. In: Methods in Virology. Vol.1., Chap.3. Edited by Maramorosch, K. and Koprowski, M. Academic Press, New York & London.
- Chow, B.F. (1947). The determination of plasma or serum albumin by means of a precipitin reaction. J. Biol. Chem., 164, 757-763.
- Chow, L., Yen, L. & Bourgeois, A.L. (1980). Identification of mosquito bloodmeals by cellulose acetate and starch gel electrophoresis. Chi. J. Micro. Immunol., 13, 386-394.

- Clarke, F.C. (1914). Forensic value of the precipitin test in the enforcement of the game laws in California. Univ. California. Publ. Pathol., 2, (1), 131-138.
- Collins, R.T., Dash, B.K., Agarwala, R.S. & Dhal, K.B. (1983). An adaption of the gel diffusion technique for identifying the source of mosquito bloodmeals. WHO/MAL/83.992
- Crans, W.J. (1969). An agar gel diffusion method for the identification of mosquito bloodmeals. Mosq. News, 29, 563-566.
- Cupp, E.W. & Stokes, G.M. (1976). Feeding patterns of Culex salinarius Coquillett in Jefferson Parish, Louisiana. Mosq. News, 36, 332-335.
- Darling, S.T. (1925). Entomological research in malaria. Southern Medical Journal, 18, (No.6), 446.
- Davis, G.E. & Philip, C.B. (1931). The identification of the bloodmeal in West African mosquitoes by means of the precipitin test. A preliminary paper. Am. J. Hyg., 14, 130-141.
- Davis, N.C. & Shannon, R.C. (1928). The blood feeding habits of Anopheles pseudopunctipennis in northern Argentina. Am. J. Trop. Med., 8, 443.
- De Lalla, O.F. & Gofman, J.W. (1954). Ultra centrifugal analysis of serum lipoproteins. Methods Biochem. Anal. 1, 459-478.
- Diamond, L.K. (1950). Mitchell-Nelson Text book of Pediatrics. W.B. Saunders Co., Philadelphia, London.
- Druguet, M. & Pepys, M.B. (1977). Enumeration of lymphocyte populations in whole peripheral blood with alkaline phosphatase labelled reagents. Clin. Exp. Immunol., 29, 162.
- Edman, J.D. (1970a). Rate of digestion of vertebrate blood in Aedes aegypti (L) Effect of age, humidity and temperature. Am. J. trop. Med. Hyg. 19, 1031-1033.

- Edman, J.D. (1970b). Rate of digestion of three human blood fractions in Aedes aegypti (Diptera : Culicidae). Ann. Ent. Soc. Am. 63, 1778-1779.
- Edman, J.D. (1971). Host feeding patterns of Florida mosquitoes. I. Aedes, Anopheles, Coquillettidia, Mansonia and Psorophora. J. Med. Ent. 8, 687-695.
- Edman, J.D. (1974). Host feeding patterns of Florida mosquitoes. III. Culex (Culex) and Culex (Neoculex). J. Med. Ent., 11, 95.
- Edman, J.D. & Kale, H.W. (1971). Host behavior : Its influence on the feeding success of mosquitoes. Ann. Entomol. Soc. Am., 64, 513-516.
- Edrissian, Gh.H. & Hafizi, A. (1982). Application of enzyme-linked immunosorbent assay (ELISA) to identification of Anopheles mosquito bloodmeals. Trans. Roy. Soc. Trop. Med. Hyg., 76, 54-56.
- Ekins, R. (1980). More sensitive immunoassays. Nature, 284, 14-15.
- Eliason, D.A. (1971). Identification of mosquito bloodmeals: A comparison of selected procedures. Dr. P.H. dissertation. Univ. NC at Chapel Hill. Univ. Microfilms, Ann. Arbor. Mich. pp.91.
- Engvall, E. & Perlmann, P. (1971). Enzyme linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry, 8, 871.
- Esteves, M.B. and Binaghi, R.A. (1972). Antigenic similarities among mammalian immunoglobulins. Immunology, 23, 137.
- Farrah, S.R., Shah, D.O. & Ingram, L.O. (1981). Effects of chaotropic and antichaotropic agents on elution of polio virus adsorbed on membrane filters. Proc. Natl. Acad. Sci., U.S.A. 78, 1229.
- Fine, J-M. (1957). Appont des methodes immunologiques recentes au probleme de l'identification du sang humain. Ann. Inst. Pasteur, 93, 592-601.

- Fiske, W.F. (1920). Investigations into the bionomics of Glossina palpalis. Bull. Entomol. Res., 10, 347-363.
- Fornet, W. & Muller, M. (1910). Praktische und theoretische prazipitinunter suchungen. Ztschr. f. Hyg., 66, 215.
- Freund, J., Casals-ariet, J. and Schaefer-Genghof, D. (1940). The synergistic effect of paraffin oil combined with heat killed tubercle bacilli. J. Immunol., 38, 67-99.
- Friend, W.G. & Smith, J.J.B. (1977). Factors affecting feeding by blood-sucking insects. Ann. Rev. Entomol., 22, 309-331.
- Garrett-Jones, C. (1964). The human blood index of malaria vectors in relation to epidemiological assessment. Bull. W.H.O., 30, 241.
- Garrett-Jones, C., Boreham, P.F.L. and Pant, C.P. (1980). Feeding habits of anophelines (Diptera : Culicidae) in 1971-1978, with reference to the human blood index : a review. Bull. Ent. Res., 70, 165-185.
- Gentry, J.W., Moore, C.G. & Hayes, D.E. (1967). Preliminary report on soluble antigen fluorescent antibody technique for identification of host source of mosquito bloodmeals. Mosq. News, 27, 141-143.
- Gershoni, J.M. & Palade, G.E. (1983). Protein blotting. Principles of application. Anal. Biochem, 131, 1-15.
- Gillies, M.T. (1956). The problem of exophily in Anopheles gambiae. Bull. W.H.O., 15, 437-449.
- Goding, J.W. (1980). Antibody production by hybridomas. J. Immunol. Meth., 39, 285-308.
- Goettsch, E. & Kendall, F.E. (1935). Analysis of albumin and globin in biological fluids by the precipitin reaction. J. Biol. Chem., 109, 221-231.

- Gooding, R.H. (1972). Digestive processes of haemotophagus insects. I. A literature review. Quaestiones Ent., 8, 5-60.
- Gozony, L., Hindle, E. & Ross, P.M. (1914). Serological Tests. I. On the persistence of precipitins in sera stored in vitro. II. On the reaction obtained with (a) complement fixation and (b) precipitin test, with the gut contents of blood-sucking arthropods. J. Hyg., 14, 354-359.
- Grjebine, A., Eyquem, A. & Fine, J. (1954). Utilisation des hemagglutins pour l'identification de l'origine specifique des hematies ingrees par les moustiques hematophages. Ann. Inst. Pasteur, 86, 741.
- Guedson, J.L. & Averameas, S. (1977). Magnetic solid phase enzyme-immunoassay. Immunocytochemistry, 14, 443-447.
- Guedson, J.L., Ternynck, T. & Averameas, S. (1979). The use of avidin-biotin interaction in immuno-enzymatic techniques. J. Histochem. Cytochem. 26, 1131-1139.
- Gushaw, J.B., Hu, M.W., Singh, P., Miller, J.G. & Schneider, R.S. (1977). Homogenous enzyme immunoassay for theophylline in serum (abstract). Clin. Chem., 23, 1144.
- Handman, E. & Jarvis, H.M. (1985). Nitro-cellulose based assays for the detection of glycolipids and other antigens. Mechanism of binding to nitro-cellulose. J. Immunol. Meth., 83, 113-123.
- Harrison, H. (1940). The game experiment. Tsetse Research Report 1935-38. Govt. Printers, Tanganyika. p.27. Dar-es-Salaam.
- Harvie, N.R. & Schultz, J.S. (1970). Studies of Lp-lipoprotein as a quantitative genetic trait. Proc. Nat. Acad. Sci. 66 No.1, 99-103
- Havely, J.M.B. (1958). Host relationships of Glossina sw In: Lumsden, W.H.R. East African Trypanosomiasis Research Organisation Report. East Africa High Commission, Nairobi. 1956/57. p.69.

- Hawkes, R.H., Niday, E. & Gordon, J. (1982). A dot immunobinding assay for monoclonal and other antibodies. Anal. Biochem., 119, 142-147.
- Hayes, R.O., Tempelis, C.H., Hess, A.D. and Reeves, W.C. (1973). Mosquito hosts preference studies in Hale County, Texas. Am. J. Trop. Med. Hyg., 22, 270-277.
- Hektoen, L. (1918). The precipitin test for Blood. J. Am. Med. Assoc., 70 (18), 1273-1278.
- Herbert, G.A. (1974). Ammonium sulphate fractionation of sera : Mouse, hamster, guinea pig, monkey, chimpanzee, swine, chicken and cattle. Appl. Microbiol., 27, 389-393.
- Herbert, G.A., Pelham, P.L. & Pittman, B. (1973). Determination of the optimal ammonium sulphate concentration for the fractionation of rabbit, sheep, horse and goat antisera. Appl. Microbiol., 25, 26-36.
- Herndon, B.L. & Ringle, D.A. (1967). Identification of host and host antibodies from mosquito bloodmeals. Nature Lond., 213, 624-625.
- Herrmann, J.E. & Collins, M.F. (1976). Quantitation of immunoglobulin absorption to plastic. J. Immunol. Meth. 14, 183-199.
- Herzenberg, L.A., Herzenberg, L. & Milstein, C. (1979). Cell hybrids of myelomas with antibody forming cells and T-lymphomas with T cells. In: D.M. Weir (ed). Handbook of Experimental Immunology. 3rd ed. Blackwell, Oxford, pp.25.1-25.7.
- Hess, A.D., Hayes, R.O. & Tempelis, C.H. (1968). The use of the forage ratio technique in mosquito host preference studies. Mosquito News, 28, No.3, 386-389
- Hess, A.D. & Rainwater, J.M. (1959). A method for measuring the food preferences of trout. Copeia, 3, 154-157.

- Hewitt, L.F. (1937). The antigenic behaviour of serum proteins with special reference to crystalalbumin and sero glycoïd. Biochem. J., 31, 1047-1052.
- Hill, H.R. & Matsell, J.M. (1983). Enzyme linked immuno sorbent assay and radio-immunoassay in the serologic diagnosis of infectious diseases. J. Infect. Dis., 147, 258-262.
- Holland, V.R., Saunders, B.C., Rose, F.L. & Walpole, A.L. (1974). The carcinogenic potential of O-toluidine. Tetrahedron, 30, 3229-3302.
- Holmquist, L. (1980). Quantitation of human serum very low density apo-lipoproteins C-1, C-11, C-111 and E, by enzyme immunoassay. J. Immunol. Meth. 34, 243-251.
- Hommel, M.W., Peters, W., Ranque, J., Quilici, M. & Lanotte, G. (1978). The micro ELISA technique in the serodiagnosis of visceral leishmaniasis. Ann. Trop. Med. Parasitol., 72, 213.
- Hoogstraal, M. (1956). African Ixodoidea. Vol. 1. Ticks of the Sudan. U.S. Naval Medical Research Unit No.3, Research Report pp. 1101.
- Horejri, V. & Hilgert, I. (1983). Nitro-cellulose membrane as an antigen or antibody carrier for screening hybridoma cultures. J. Immunol. Meth. 62, 325-329.
- Howard, L.M. (1962). Studies on the mechanisms of infection of the mosquito midgut by Plasmodium gallinaceum. Am. J. Hyg., 75, 287-300.
- Huff, G.G. (1934). Comparative studies on susceptible and unsusceptible Culex pipiens in relation to infections with Plasmodium cathemerium and P. relictum. Am. J. Hyg. 19, 123.
- Iacona, A., Piri, C. & Vicari, G. (1980). Enzyme-linked immunosorbent assay (ELISA) in the serodiagnosis of hydatid disease, Am. J. trop. Med. Hyg. 29, 95-102.

- Jackson, C.H.N. & Vanderplank, F.L. (1941). Diameters of mammalian erythrocytes. Proc. Zool. Soc. London, A 112, 57-60.
- Jeffery, G.M. (1956). Bloodmeal volume in Anopheles quadrimaculatus, A. gambiae and Aedes aegypti. Exptl. Parasitol., 5, 371-375.
- Jefferies, R., Lowe, J., Ling, N.R., Porter, P. & Senior, S. (1982). Immunogenic and antigenic epitopes of Igs. I. Cross reactivity of murine monoclonal antibodies to human IgG with the immunoglobulins of certain animal species. Immunology, 45, 71-77.
- Johnson, W.B. & Lloyd, L. (1923). First Report of the tsetse fly investigations in the northern provinces of Nigeria. Bull. Ent. Res., 13, 373-376.
- Johnson, W.D. & Rawson, P.H. (1927). Use of the precipitin test to determine the food supply of tsetse flies; a preliminary note. Trans. Roy. Soc. Trop. Med. Hyg., 21, 133-149.
- Jonas, A. (1972). Physiochemical properties of bovine serum high density lipoprotein. J. Biol. Chem., 247, 7767-7772.
- Kalica, A.R., Purcell, R.M., Sereno, M.M., Wyatt, R.G., Kim, H.W., Chanock, R.M. & Kapikian, A.Z. (1974). A microtitre solid phase radio-immunoassay for detection of the human reovirus like agent in stools. J. Immunol. 118, 1275-1279.
- Kafatos, F.C., Jones, C.W. & Efstradiadis, F.C. (1979). Determination of nucleic acid sequence homologies and relative concentration by a dot hybridization procedure. Nucl. Acids. Res., 7, 1541-1552.
- Kaminski, M., Mauger, J.P. & Sykiotis, M. (1978). Immunological relationship and chemical similarities between HDL lipoproteins from various mammal and avian species. Genetics A3, 179-181.

- Kennett, R.H. (1980). Monoclonal antibodies : a new dimension in biological analyses. ed: R.H.Kennett, T.J. McKearn & K.B.Beihtol. Plenum Press, New York.
- Kennett, R.H., Denis, K.A., Tung, A.S., Klinman, N.R. (1978). Hybrid plasmacytoma production fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Curr. Top. Microbiol. Immunol., 81, 77-91.
- Keogh, E.V., North, E.A. & Warburton, M.F. (1948). Adsorption of bacterial polysaccharides to erythrocytes. Nature, Lond. 161, 687.
- King, W.V. & Bull, C.G. (1923). The blood feeding habits of malaria carrying mosquitoes. Am. J. Hyg., 3, 497-513.
- Kirsch, J.A.W. & Murray, M.D. (1969). A scheme for the identification of the blood of Australasian marsupials. Austal. J. Zool., 17, 799-816.
- Köhler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, Lond. 256, 495-497.
- Kominami, G., Fujisaka, I., Yamauchi, A. & Kono, M. (1980). A sensitive enzyme immunoassay for plasma control. Clin. Chem. Acta., 103, 281-291.
- Kraus, R. (1897). Über spezifische Reactionen in Keimfreien Filtraten aus Cholera, Typhus und Pestbouillon culturen, erzeugt durch homologes serum. Wein klin. Wochischr., 10, 736-738.
- Kumar, S., Band, A.M., Samantaray, J.C., Dang, N. & Talwar, G.P. (1985). A dot enzyme-linked immunosorbent assay for detection of antibodies against E. histolytica. J. Immunol. Meth., 83, 125-133.

- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, Lond. 227, 680-685.
- Laurel, A.G. (1931). Feeding Activities of some Philippine Anopheles. Rev. filipina Med. y Farm. 22, No.11.
- Leary, J.L. Brigati, D.J. & Ward, D.C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labelled DNA probes hybridized to DNA or RNA immunobilized on nitro-cellulose : Bio-blots. Proc. Nat. Acad. Sci. U.S.A., 80, 4045-4052.
- Leone, C.A. (1952). Effect of multiple injections of antigen upon the specificity of antisera. J. Immunol., 69, 285-295.
- Lems-Van Kan, P., Verspaget, H.W. & Pena, A.S. (1983). ELISA assay for quantitative measurement of human immunoglobulins IgA, IgG and IgM in nanograms. J. Immunol. Meth. 57, 51.
- Lloyd, L., Johnson, W.B., Young, W.A. & Morrison, H. (1924). Second Report of the tsetse fly investigation in the Northern Provinces of Nigeria. Bull. Ent. Res., 15, 1-27.
- Lloyd, R.B., Napier, L.E. & Smith, R.O.A. (1924). The 'bloodmeal' of Phlebotomus argentipes identified by precipitin antisera. Indian J. Med. Research, 12, 811-817.
- Lombardi, S. & Esposito, F. (1986). A new method for identification of the animal origin of mosquito bloodmeals by the immunobinding of peroxidase anti peroxidase complexes on nitro cellulose. J. Immunol. Meth., 86, 1-5.
- Lunde, M.N., Ottesen, E.A. & Cheever, A.W. (1979). Serological differences between acute and chronic Schistosomiasis detected by enzyme linked immunosorbent assay (ELISA). Am. J. Trop. Med. Hyg., 28, 87-95.

- Macdonald, G. (1957). The epidemiology and control of malaria. Oxford University Press, London.
- Masseyeff, R. (1978). Assay of tumor-associated antigens. Scand. J. Immunol. 8, (Suppl.7), 83-90.
- Mason, D.Y. & Sammons, R. (1978). Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. J. Clin. Pathol., 31, 454-460.
- Mattern, P., Masseyeff, R., Taufflieb, R. & Cornet, M. (1967). Degradation of human serum proteins in the digestive tract of mosquitoes. Nature, Lond. 215, 1193-1194.
- McClelland, G.A.H. & Weitz, B. (1963). Serological identification of the natural hosts of Aedes aegypti (L.) and some other mosquitoes (Diptera, Culicidae) caught resting in vegetation in Kenya and Uganda. Ann. Trop. Med. Parasitol., 57, 214-224.
- McCrae, A.W.R., Boreham, P.F.L. & Ssenkubuge, Y. (1976). The behavioural ecology of host selection in Anopheles implexus (Theobald) (Diptera, Culicidae). Bull. Ent. Res., 66, 587-631.
- McFarlane, A.S. (1942). Behaviour of lipoids in human serum. Nature, London, 149, 439.
- McKinney, R.M., Spillane, J.T. & Holden, P. (1972). Mosquito bloodmeals: identification by a fluorescent antibody method. Am. J. Trop. Med. Hyg., 21, 999-1003.
- Mills, G.L. & Taylaur, C.E. (1971). The distribution and composition of serum lipoproteins in eighteen animals. Comp. Biochem. Physiol., 40B, 489-591.
- Munro-Fox, H. (1945). Individual haemoglobins. Nature, Lond. 156, 18.

- Murray, M.D. (1970). The identification of bloodmeals in biting midges (Culicoides Ceratopogonidae). Ann. Trop. Med. Parasitol., 64, 115-122.
- Nakane, P.K. & Kawaoi, A. (1974). Peroxidase labelled antibody: A new method of conjugation. J. Histochem. Cytochem., 22, 1084-1091.
- Neoh, S.H., Jahoda, D.M., Rowe, D.S. & Voller, A. (1974). Immunoglobulin classes in mammalian species identified by cross-reactivity with antisera to human immunoglobulin. Immunochemistry, 10, 805-810.
- Nogge, G. & Giannetti, M. (1979). Midgut absorption of undigested albumin and other proteins by tsetse, Glossina m. morsitans (Diptera : Glossinidae). J. Med. Entomol. 16 No.3, 263-265.
- Nuttal, G.H.F. (1904). Blood immunity and blood relationships. Cambridge University Press, Lond.
- O'Connor, C.G. & Ashman, L.K. (1982). Application of the nitrocellulose transfer technique and alkaline phosphatase conjugated anti-immunoglobulin for determination of the specificity of monoclonal antibodies to protein mixtures. J. Immunol. Meth., 54, 267.
- Ouchterlony, O. (1948). In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta. path et microbiol. Scandinav., 25, 186-191.
- Pal, S.B. (1978). Enzyme labelled immunoassay of hormones and drugs. Walter de Gruyter, Berlin.
- Pappas, M.G., Hajkowski, R., Cannon, L.T. & Hockmeyer, W.T. (1984). DOT enzyme linked immunosorbent assay (Dot-ELISA): comparison with standard ELISA and complement fixation assays for the diagnosis of human visceral leishmaniasis. Vet. Parasit., 14, 239-249.

- Pappas, M.G., Hajkowski, R. & Hockmeyer, W.T. (1983). Dot Enzyme-linked immunosorbent assay (Dot-ELISA): a micro technique for the rapid diagnosis of visceral Leishmaniasis. J. Immunol. Meth., 64, 205-214.
- Pearson, T & Anderson, N.L. (1980). XXVIII. Dissection of complex antigenic mixture using monoclonal antibodies and two dimensional gel electrophoresis. Anal. Biochem., 101, 377-386.
- Pennington, N.E. & Phelps, C.A. (1968). Identification of the host range of Culex tritaeniorhynchus mosquitoes on Okinawa, Kyukyu Islands. J. Med. Entomol., 5, 483-487.
- Pepple, J., Moxon, E.R. & Yolken, R.H. (1980). Indirect ELISA for the quantification of the type specific antigen of Hemophilus influenzae b: a preliminary report. J. Paediatr. 97, 233-237.
- Pesce, A.J., Modesto, R.R., Ford, D.J., Settri, K., Clyne, D.N. & Pollak, V.E. (1976). Preparation and analysis of peroxidase antibody and alkaline phosphatase antibody conjugates. In: G. Feldmann, P. Dreutt, J. Bignon & S. Averameas (ed), Immunoenzymatic Techniques. N. Holland, Amsterdam, 1976, p.7-23.
- Pesce, A.J., Ford, d.J. & Gizutis, A. (1978). Qualititative and quantitative aspects of immunoassays. Scand. J. Immunol. 8 (Suppl.7), 1-7.
- Phelps, D.S. (1984). Electrophoretic transfer of proteins from fixed and stained gels. Anal. Biochem., 141, 409-412.
- Porter, P. (1979). Structural and functional characteristics of immunoglobulins of common species. In: Advances in Veterinary Science and Comparative Medicine, Vol. 23. ed. B.I.Osburn & R.O.Schultz. Academic Press, New York.

- Prates, M.M. (1928). Precipitin test applied to the blood in the alimentary canal of Glossinae. Final report of the League of Nations International Commission on Human Trypanosomiasis, Geneva, C.H. 629, 179-226.
- Price-Jones, C. (1933). Red blood cell diameter. Oxford University Press, London.
- Proom, H. (1943). The preparation of precipitating sera for the identification of animal species. J. Path. Bact., 55, 419-426.
- Rao, U.S.B. (1984). A rapid method for identification of mosquito blood meals. Ind. J. Med. Res., 79, 836-840.
- Rapacz, J., Grunmer, R.H., Haslet, J. & Shackelford, R.M. (1969). Allotype polymorphism of low density B-lipoprotein in Pig serum (LDLppl, LDLpp2). Nature, Lond. 225, 941-942.
- Reeves, W.C. & Hammon, W. Mc.D. (1962). II. Infection in other vertebrate hosts. III. The role of arthropod vectors. In: Epidemiology of the arthropod borne viral encephalitides in Kern County, California. 1943-52. Univ. Calif. Pub. Public Health. Vol.4, p. 46-108. Univ. of Calif. Press, Berkeley.
- Reid, J.A. & Weitz, B. (1961). Anopheline mosquitoes as vectors of animal malaria in Malaya. Ann. Trop. Med. Parasit., 55, 180.
- Rempel, J.G., Riddell, W.A. & McNelly, E.M. (1946). Multiple feeding habits of Saskatchewan mosquitoes. Can. J. Res., 24, 71-78.
- Rice, J.B. & Barber, M.A. (1934). Malaria studies in Greece. A modification of the Uhlenhuth - Weidanz precipitin test for determining the source of blood meals in mosquitoes and other insects. J. Lab. Clin. Med., 201, 876-883.

- Riddell, W.A., Rempel, J.G. & McNelly, E. (1947). The specificity of the precipitin reaction, as used in the study of mosquito feeding habits. Can. J. Res., 25, 210-215.
- Roitt, I. (1984). Essential Immunology. 5th ed. Blackwell Scientific Publications, Oxford, London.
- Romana, C. (1939). Utilization de la methode des precipitines pour l'identification du sang ingere par certains reduvides. Bull. Soc. Pathol. Exotique, 32, 625-628.
- Ross, R. (1911). The Prevention of Malaria. John Murray, London.
- Roubaud, E. (1921). La differentiation des races zootropiques d'anopatekes et la regression spontanee paludisme. Bull. Soc. Path. Exot., 14, 577-595.
- Roy, D.N. & Ganguli, S.K. (1943). The precipitin test and the production of precipitating sera. Indian Med. Gaz., 78, 81-89.
- Sack, D.A., Neogi, P.K.B. & Alam, K. Md. (1980). Immunobead enzyme-linked immunosorbent assay for quantitating Immunoglobulin A in human secretions and serum. Infect. Immun., 29, 281.
- Salonen, E.M. & Valien, A. (1979). Immobilization of viral and mycoplasma antigens and of immunoglobulins on polystyrene surface for immunoassays. J. Immunol Methods, 30, 209.
- Scharpe, S.L., Cooreman, W.M., Bloome, W.J. & Laekeman, G.M. (1976). Quantitative enzyme immunoassay - current status. Clin. Chem. 22, 733-738.
- Schlein, Y., Spira, D.T. & Jacobson, R.L. (1976). The passage of serum immunoglobulins through the gut of Sarcophaga falculata (Pand). Ann. Trop. Med. Parasitol. 70, No.2. 227-230.

- Schneider, Z. (1981). A micro method for estimation of adenosine deaminase and adenosine nucleosidase with modified cellulose nitrate membranes. Anal. Biochem., 108, 96-111.
- Schonbaum, G.R. (1973). New complexes of peroxidase with hydroxamic acids, hydrazides, and amides. J. Biol. Chem. 248, 502-511.
- Schubert, J.H. & Holdeman, L.V. (1956). A modified precipitin technique for determining the source of mosquito bloodmeals. Am. J. Trop. Med. Hyg., 5, 272-273.
- Segal, E., Berg, R.A., Pizzo, P.A. & Bennett, J.E. (1979). Detection of Candida antigen in sera of patients with candidiasis by an enzyme linked immunosorbent assay inhibition technique. J. Clin. Microbiol., 10, 116-118.
- Self, C.H. (1985). Enzyme application. A general method applied to provide an immuno-assisted assay for placental alkaline phosphatase. J. Immunol. Methods. 76, 389.
- Senior-White, R. (1947). On the anthropophilic indices of some Anopheles found in East Central India. Indian J. Malariol., 1, 111-122.
- Service, M.W. (1970). A battery-operated light-trap for sampling mosquito populations. Bull. W.H.O., 43, 635-641.
- Service, M.W. (1976). Mosquito Ecology : Field Sampling Methods. Applied Science Publishers Ltd., London.
- Smith, D.S., Compmer, K., Janners, M., Lipton, C. & Wittle, L. (1969). Cellular organization in the mid gut epithelium of a moth Ephestia kuhniella. J. Morphol. 127, 44-72.
- Solyom, A., Bradford, R.M. & Furman, R.M. (1971). Apo lipoproteins and lipid distribution in canine serum lipoprotein. Biochem. Biophys. Acta., 229, 471-483.

- Spriggs, A.I. & Sladden, R.A. (1958). The influence of age on the red cell diameter. J. Clin. Pathol., 2, 53-55.
- Stirling, W. & Brito, P.S. (1882). On the digestion of blood by the common leech, and on the formation of haemoglobin crystals. Anat. Physiol., 16, 446-457.
- Storiko, K. & Fisher, G.B. (1966). Immunological investigations on LDL. Protides Biol. Fluids, Pix. Colloq., 13, 291-306.
- Sudia, W.D. & Chamberlain, R. W. (1962). Battery-operated light trap, an improved model. Mosquito News, 22, 126.
- Sutherland, W.D. & Mitra, G.C. (1914). Misleading reactions obtained with precipitating antisera and how to avoid them. Ind. J. Med. Res., I, 4, 707-711.
- Sutherland, W.D. & Mitra, G.C. (1920). A note on the best method of obtaining precipitating antisera. Ind. J. Med. Res., VII, 4, 669-673.
- Swynnerton, C.F.M. (1936). The tsetse flies of East Africa. A first study of their ecology, with a view to their control. Trans. Roy. Entomol. Soc. London, 84, 1-579.
- Symes, C.B. & McMahon, J.P. (1937). The food of tsetse flies (Glossina swynnertonii and G. palpalis) as determined by the precipitin test. Bull. Ent. Res., 28, 31-42.
- Taylor, A.W. (1930). Glossina palpalis and sleeping sickness at Ganawuri Plateau Province, Northern Nigeria. Bull. Ent. Res. 21, 333-340.
- Tchistovitch, T. (1899). Etudes sur l'immunisation contre le serum d'anguilles. Am. J. Trop. Med. Hyg., 13, 406-425.

- Tempelis, C.H. (1970). Host preferences of mosquitoes. Proc. and Papers 38th annual Conference, Calif. Mosquito Control Assoc. Inc., 25, 29-37.
- Tempelis, C.H. (1975). Host feeding patterns of mosquitoes with a review of advances in analysis of bloodmeals by serology. J. Med. Entomol., 11, 635-653.
- Tempelis, C.H. & Lofy, M.F. (1963). A modified precipitin method for identification of mosquito bloodmeals. Am. J. Trop. Med., 12, 825-831.
- Tempelis, C.H. & Reeves, W.C. (1962). The production of a specific antiserum to bird serum. Am. J. Trop. Med. Hyg., 11, 294-306.
- Tempelis, C.H. & Rodrick, M.L. (1972). Passive haemagglutination inhibition technique for the identification of arthropod bloodmeals. Am. J. Trop. Med. Hyg., 21, 238-245.
- Tempelis, C.H. & Washino, R.K. (1967). Host feeding patterns of Culex tarsalis in the Sacramento Valley, California, with notes on other species. J. Med. Ent., 4, 315-320.
- Tesh, R.B., Chaniotis, B.N., Aronson, M.D. & Johnson, K.M. (1971). Natural host preferences of Panamanian phlebotomine sandflies as determined by precipitin test. Am. J. Trop. Med. Hyg., 20, 150-156.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: Procedure and some applications. Proc. Nat. Acad. Sci., 76, No.9, 4350-4354.
- Uhlenhuth, P. (1901). Eine methode zur Unterscheidung der verschiedenen Blutarten im besonderen zur differential - diagnostischen Nachweise des Menschenblutes. Dtsch. Med. Wschr., 27, 82-83.

- Uhlenhuth, P., Weidanz, O. & Angeloff, A. (1908). Uber den biologischen Nachweis der Herkunft von Blut in blutsan genden in sekten. Arb. Gesundh - Amte (Bevl.), 28, 595.
- Vaerman, J.P., Heremans, J.F. & Van Kerckhoven, G. (1969). Identification of IgA in several mammalian species. J. Immunol., 103, 1421.
- Venitt, S. & Searle, C.E. (1976). Mutagenicity and possible carcinogenicity of hair colourants and constituents. In: C. Rosenfield & W. Davis (ed). Inserm symposium series, Vo.52, IARC Scientific publication No.13, Inserm. Paris, p.263-271.
- Voller, A., Bartlett, A. & Bidwell, D.E. (1976). Enzyme immunoassays for parasitic diseases. Trans. Roy. Soc. trop. Med. Hyg., 70, 98-106.
- Voller, A., Bidwell, D.E. & Bartlett, A. (1977). The Enzyme linked immunosorbent Assay (ELISA). Flowline Publications, Germany.
- Voller, A., Bidwell, B. & Bartlett, A. (1980). Enzyme linked immunosorbent assay. In : Manual of Clinical Immunology, ed. N.R. Rose, H. Friedman. pp 359-371. Washington D.C. : Am. Soc. Microbiol. Publ. 2nd ed.
- Waant, M.R.D., Snelting, A., Cichy, J., Wolters, G. & Schnurs, A. (1978). Enzyme immunoassay in the diagnosis of Hepatitis with emphasis on the detection of 'e' antigen (HBe Ag). J. Med. Virol., 3, 43-49.
- Warren, R.C., Bartlett, A., Bidwell, D.E., Richardson, M.D., Voller, A. & White, L.O. (1977). Diagnosis of invasive conditions by enzyme immunoassay of serum antigen. Br. Med. J., 1, 1183-1185.

- Washino, R.K. & Else, J.G. (1972). Identification of bloodmeals of haematophagus arthropods by the haemoglobin crystallization method. Am. J. Trop. Med. Hyg., 21, 120-122.
- Weitz, B. (1952). The antigenicity of sera of man and animals in relation to the preparation of specific precipitating antisera. J. Hyg., 50, 275-294.
- Weitz, B. (1956). Identification of bloodmeals of blood sucking arthropods. Bull. W.H.O., 15, 473-490.
- Weitz, B. (1957). An automatic dispenser for multiple serological titrations. J. Clin. Pathol., 10, 200-207.
- Weitz, B. (1960). Feeding habits of blood-sucking arthropods. Exp. Parasitol., 9, 63-82.
- Weitz, B. (1963). The feeding habits of Glossina. Bull. W.H.O., 28, 711-729.
- Weitz, B. & Buxton, P.A. (1953). The rate of digestion of bloodmeals of various haematophagous arthropods as determined by the precipitin test. Bull. Ent. Res., 44, 445-450.
- Weitz, B. & Jackson, C.H.N. (1956). The host animals of Glossina morsitans at Daga-Ilol. Bull. Ent. Res., 46, 531-538.
- West, A.S. (1950). The precipitin test as an entomological tool. Can. Ent., 82, 241-244.
- West, A.S. & Eligh, G.S. (1952). The rate of digestion of blood in mosquitoes. Precipitin test studies. Can. J. Zool., 30, 267-272.
- Wigglesworth, V.B. (1943). 'The fate of haemoglobin in Rhodinus prolixus (Hemiptera) and other blood sucking arthropods. Proc. Roy. Soc. London, B. 131, 313-339.

- Williams, C.A.Jr. (1953). Digestion of serum proteins by Aedes aegypti.
14th Int.Congr.Zool.Proc. p.278
- Wilson, M.B. & Nakane, O.P. (1980). Recent developments in the
periodate method of conjugation of horse radish peroxidase to
antibodies. J. Histochem. Cytochem
- Wolfe, H.R. (1935). The effect of injection method on the species
specificity of serum precipitins. J. Immunol., 29, 1-11.
- Wolfe, H.R. (1939). Standardization of the precipitin technique and its
application to studies of relationships in mammals, birds and
reptiles. Biol. Bull. Woods Hole, 76, 108-120.
- Wolfe, H.R. & Dilks, E. (1946). Precipitin production in chickens. II.
Studies on the in vitro rise of the interfacial titres and the
formation of precipitins. J. Immunol., 52, 331-341.
- Yolken, R.H. (1980). ELISA: a practical test for rapid diagnosis of
viruses and other infectious agents. Yale. J. Biol. Med. 53,
85-92.
- Yolken, R.H., Kim, H.W., Clem, T., Wyatt, R.G., Kailca, A.R., Chanock,
R.M. & Kapikian, A.Z. (1977). .Enzyme linked immunosorbent assay
(ELISA) for detection of retrovirus - the agent of infantile
gastroenteritis. Lancet. 2, 263-267.
- Zaman, V. & Chellappah, W.T. (1967). The assessment of the rate of
digestion in mosquito by gel-diffusion and immunoelectrophoresis.
Experientia, 23, 378-379.

APPENDIX 1

BUFFERS

- 1) Coating Carbonate buffer 0.05M, pH 9.6.
1.59g Na_2CO_3 anhydrous (M.Wt. 105.99)
2.93g NaHCO_3 (M.Wt. 84.01)
Dissolved in 1L of distilled water (dH_2O)
containing merthiolate (0.001%).

- 2) PBS pH 7.4
8g NaCl
0.2g KH_2PO_4
2.8g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
0.2g KCL
Dissolved in 1L of dH_2O .

- 3) PBS - Tween
0.5ml Tween-20 added to 1L of PBS.

- 4) Substrate buffer - Citrate pH 5.0 (FOR OPD).
7.3g Citric acid
11.86g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
Dissolved in 1L of dH_2O .

- 5) Barbitone buffer pH 8.8.
0.92g Barbitone
5.15g Sodium barbitone
Dissolved in 500ml of dH₂O.
- 6) Coomassie blue destain solution for gel diffusion.
water; acetic acid and methanol (5:1:5)
- 7) Electrode buffer
6g Tris
28.8g Glycine
10 ml 20% SDS
Dissolved in 2L of dH₂O.
- 8) Sample buffer
2ml 20% SDS.
1ml 1M Tris HCL pH 7.0
1ml 60% Sucrose
15ml H₂O
4 drops Bromophenol blue
- 9) Triethanolamine buffered saline.
25 g of - Triethanolamine dissolved in 1
litre of 0.1 M PBS. pH 7.5

- 10) Coomassie blue Stain for SDS-PAGE.
- 0.12g Coomassie blue in 400ml of solvent
- Stain solvent
- 260ml H_2O
- 100ml Ethanol
- 40ml Acetic acid
- 11) Coomassie blue Destain solution for SDS-PAGE
- 425ml H_2O
- 50ml Ethanol
- 25ml Acetic acid
- 12) Western transfer buffer
- 15g Tris
- 72g Glycine
- 1L Methanol
- 4L dH_2O
- 13) Quenching solution
- 1% Casein in PBS pH 7.4
- or
- 1% Casein in Carbonate buffer pH 9.6