A study of the binding of anti-aggregatory substances to platelet bound plasma proteins.

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A STUDY OF THE BINDING OF ANTI-AGGREGATORY SUBSTANCES TO PLATELET BOUND PLASMA PROTEINS AND TO PLATELETS.

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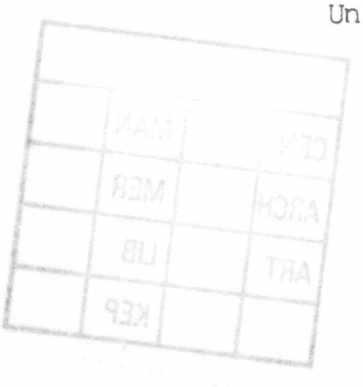

TO MY PARENTS

What one cannot repay
one can still acknowledge.

ABSTRACT

A Study of the Binding of Anti-aggregatory Substances to Platelet Bound Plasma Proteins and to Platelets

Christopher Simpson

The binding of a number of platelet active compounds to serum albumin was studied using several direct fluorescence methods and fluorescence polarisation. Particular emphasis was placed on the study of the binding of dipyridamole (DPD) and its analogues. The data obtained from the above experiments was compared with that obtained from the classical methods of equilibrium dialysis, dynamic dialysis, and continuous ultrafiltration and used to set up a number of nonlinear least squares regression analysis computer programmes to analyse binding isotherms. In the course of this study, the fluorescence methods were applied to a number of binding systems not previously investigated. Several binding systems showed no change in the fluorescence characteristics of the protein or ligand. This led to the development of a Sephadex batch method to be used in conjunction with fluorescence. The concentration of free ligand was measured directly in the Sephadex gel. Factors which effect the fluorescence from the Sephadex bed were extensively studied. To show the flexibility of this method it was applied to a number of binding systems involving both high and low affinity constants and to the binding of basic and acidic compounds. Fluorescence methods were, for the first time, applied to the binding of a number of ligands to α_1 acid glycoprotein (AGP). Using classical and fluorescence methods some of the characteristics of the binding site were established. Extensive use was made of competitive studies as part of this work.

Having established binding characteristics of a number of platelet active ligands to plasma proteins studies were conducted with platelets. A number of methods for washing platelets were investigated in order to find an efficient and reproducible method of washing platelets. The binding of DPD to platelets was then studied using direct fluorescence, fluorescence polarisation and a centrifuging difference method. Two classes of binding sites were observed and so the significance of both the primary and secondary sites were investigated. Possible correlation of the binding with a number of enzyme and uptake systems was attempted together with a comparison of the binding with that of AGP. It was shown that the primary site correlates with the inhibition of adenosine uptake and the secondary with the inhibition of phosphodiesterase activity. As part of this work the binding of DPD was compared with the binding of a number of other ligands. Finally the importance of the binding in terms of the inhibition of aggregation by DPD was discussed.

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CONTENTS

Figures

Tables

ABBREVIATIONS

AA	Arachidonic acid
AGP	α_1 acid glycoprotein
c-AMP	cyclic adenosine monophosphate
ADP	adenosine diphosphate
ASA	aspirin
ATP	adenosine triphosphate
BSA	bovine serum albumin
DBQ	dibenzo (f,h) quinoxaline
DMQ	2,3- dimethyl quinoxaline
DPD	dipyridamole
DPHT	diphenyl hexatriene
2,3 DPP	2,3- diphenyl pyrazine
2,5 DPP	2,5- diphenyl pyrazine
DPP2H	4,5- dihydro -2,3-diphenyl pyrazine
DPQ	2,3- diphenyl quinoxaline
c-GMP	cyclic guanosine monophosphate
5-HT	5- hydroxytryptamine
NCMIA	N-carboxymethyl isatoic anhyride
PPP	platelet poor plasma
PRP	platelet rich plasma
SA	salicylic acid

OBJECTIVES

Dipyridamole (DPD) is a potent vasodilator and an anti-platelet drug. DPD and related compounds bind to α_1 acid glycoprotein (AGP) and to platelets. The nature of, and factors effecting these binding interactions are largely unknown. Thus the objectives of this project will be :-

1) To develop and apply fluorescence methods appropriate to the study of such binding interactions. Basic feasibility studies will be carried out using albumin as the classical "binder" and platelet active compounds with known binding characteristics. When compared to the classical methods available for studying ligand protein interactions, fluorescence techniques have a number of distinct advantages

a) They are more rapid than procedures such as equilibrium dialysis and ultrafiltration.

b) They cannot only be used to measure the amount of bound and free drug in a given system but can yield additional information on the nature of the drug protein interaction such as the location of the binding region with respect to tryptophan residues and changes in the microenvironment of the ligand on binding.

c) No separation step is required to determine the free and bound concentration.

d) Fluorescence methods may specifically look at one site, usually the primary site.

e) They have no problems with binding of the ligand to membranes as encountered in equilibrium dialysis and ultrafiltration.

The binding data obtained from these experiments will allow a theoretical background to be established and the setting up of a

number of computer and graphical methods of analysis.

One of the major problems of the fluorescence methods is that they are limited to compounds whose intrinsic fluorescence change on binding or which change the intrinsic fluorescence of the protein. To try and make the fluorescence methods more generally applicable for measuring binding interactions of fluorescent ligands, a Sephadex batch method will be extensively studied. Sephadex batch methods have fallen into disuse because of the inability to measure directly, drug concentrations within the Sephadex gel bead. It has been observed in this laboratory that marked fluorescence enhancement occurs when a fluorophore passes into a gel bead. Thus an evaluation of the possibility of using the Sephadex batch method in conjunction with direct fluorescence measurements to quantify protein binding interactions will be carried out.

Many basic molecules bind significantly to the plasma AGP. Indeed it is considered that DPD action in vivo is limited by its binding to AGP. Fluorescence and fluorescence polarisation will be used with the objective of characterising the binding site (or sites) for DPD on AGP. Competitive studies with related compounds will also be carried out.

2) To compare various methods of washing platelets with the objective of perfecting a method which will reproducibly wash platelets. Subsequent binding and aggregation studies will then be conducted.

3) The binding and factors affecting the binding of various platelet active compounds to the platelet will be considered. Particular reference will be made to the phosphodiesterase inhibitors. It is

suggested that DPD owes its anti-aggregatory effect, to ADP, to its phosphodiesterase inhibitory activity. As a consequence of this c-AMP and possibly c-GMP levels are raised. However very high doses are required to produce this effect in vivo. Also no correlation exists between anti-aggregatory effect to ADP, and c-AMP and c-GMP levels. This suggests that the mechanism of action may not necessarily be in the main linked with phosphodiesterase activity. An attempt will be made to characterise the DPD binding sites on or in the platelet by using direct fluorescence methods, fluorescence polarisation, fluorescent probes and a difference centrifuging method. This will involve the use of whole platelets and platelet membranes. The significance of the binding of DPD to the platelet and plasma proteins will be considered in relation to inhibition of aggregation, c-AMP and c-GMP levels and adenosine uptake into the platelet. In the light of the results obtained a postulated mechanism of action of such drugs will be presented.

CHAPTER ONE

INTRODUCTION TO FLUORESCENCE

INTRODUCTION TO FLUORESCENCE

1.1 History of fluorescence

Luminescence is one of the oldest and most established analytical techniques, which was first observed by Monardes in 1565 from an extract of Ligirium Nephiticiem. Sir David Brewster noted the red emission from chlorophyll in 1833, and Sir G. G. Stokes described the mechanism of the absorption and emission in 1852. Stokes named fluorescence after the mineral fluorspar, which exhibits a blue white fluorescence. Busck (1906) first reported that serum altered the fluorescence and light absorption of certain dyes. The use of fluorescence to measure such binding interactions was not further investigated until the first commercial spectrophotofluorometer became available (Bowman et al., 1955). Chignell (1969, 1970c, 1972, 1973a and 1973b) has subsequently developed a number of fluorescence techniques for measuring ligand macromolecule interactions.

1.2 Fluorescence

When a quantum of light impinges on a molecule, it is absorbed in about 10^{-15} second, and a transition to a higher singlet electronic state takes place. This absorption of radiation is highly specific, and radiation of a particular wavelength is absorbed only by a characteristic structure. The absorption transition usually originates in the lowest vibrational level of the ground state. During the time the molecule can spend in the excited state, up to 10^{-4} second in fluorescence, the energy in excess of the lowest vibrational energy level is rapidly dissipated. The lowest vibrational level of the excited singlet state is attained. If all the excess energy is not further dissipated by collisions with other

molecules, the electron returns to the ground electronic state with emission of energy. This phenomena is called fluorescence. Because some energy is lost in the brief period before emission can occur, the emitted energy is of longer wavelength than the energy that was absorbed.

1.3 Characteristics of fluorescence

On binding to plasma proteins one or more of the fluorescence characteristics of the ligand may be altered. In order to understand such changes some of the important characteristics of fluorescence will be considered.

Unlike other spectroscopic methods a fluorescent compound has two characteristic spectra, excitation and emission. The shape of the excitation spectrum should be identical with that of the absorption spectrum and independent of the wavelength at which the fluorescence is measured. But this is not normally the case because the photomultiplier sensitivity changes with the radiation wavelength, the band width of the monochromator changes and the intensity of the source varies with wavelength. The emission wavelength of the compound results from the re-emission of of radiation absorbed by that molecule. The quantum yield and the shape of the emission spectrum are independent of the wavelength of the excitation radiation.

An important physical constant for a fluorescent compound which is dependent on the excitation and emission maximum of a molecule is the Stokes shift. Stokes shift indicates the energy dissipated during the life time of the excited state before return to the ground state, defined by

$$\text{Stokes shift} = 10^7 \quad (1/\lambda ex - 1/\lambda em) \quad (1.1)$$

where

λex and λem are corrected maximum wavelengths for excitation and emission respectively.

The Stokes shift is one of the fluorescent parameters which may change when a ligand binds to a protein. Changes in this parameter may allow information about the microenvironment of the binding site to be deduced.

As well as changes in the Stokes shift on binding the fluorescence intensity of the ligand may be changed. This may be due to either :-

1) A change in the fluorescence quantum yield of the molecule where the fluorescence quantum yield is defined by the relationship

$$\text{Quantum yield} = \frac{\text{number of quantum emitted}}{\text{number of quantum absorbed}} \quad (1.2)$$

2) A change in the average fluorescent life time of the molecule The fluorescence lifetime τ refers to the mean life time of the excited state, being related to the fluorescence intensity by

$$I = Io\, e^{-\frac{t}{\tau}} \quad (1.3)$$

where

I is the fluorescence intensity at any time t

Io is the maximum fluorescence intensity

τ is the average lifetime of the excited state

For the study of ligand macromolecule interactions fluorescence polarisation is a very useful technique. Polarisation of fluorescence in solution depends on the following :-

1) Life time of the excited state

2) Viscosity of the medium

3) Temperature

4) Molar volume

5) Shape of the molecule

6) Excitation wavelength

An approximate equation relating the degree of polarisation P, to the above parameters has been obtained by application of the laws of hydrodynamics and the theory of Brownian motion. Perrin and Levshin derived the following equation

$$(1/P - 1/3) = (1/Po - 1/3)(1 + 6R\tau) \qquad (1.4)$$

for rigid spheres

$$R = \frac{kT}{6\eta V} \qquad (1.5)$$

where

Po is limiting value of polarisation

τ is the life time of the excited state

R is the rate of rotation of the ligand

T is the absolute temperature

η is the viscosity of the solvent

V is the volume of the complex

k is Boltzman constant

Polarisation is a measure of the rotation of the fluorophore from the time of excitation to the time of emission. A small molecule will have a lower value of polarisation than a large macromolecule. It is

therefore possible to measure the binding of a ligand to the macromolecule by the change in the fluorescence polarisation on binding.

Probably the most important relationship when using fluorescence is the relationship between fluorescence intensity and concentration. This relationship is derived from the Beer -Lambert equation

$$I = Io\ 10^{-ecd} \qquad (1.6)$$

where

Io is the intensity of the incident light

I is the intensity of the transmitted light

e is the molar absorptivity

c is the concentration

d is the path length

Therefore the intensity of light absorbed

$$Ia = Io\ (1 - 10^{-ecd}) \qquad (1.7)$$

The intensity of fluorescence is proportional to the amount of radiation absorbed being linked by the quantum yield ϕ

$$F = Io\phi(1 - 10^{-ecd}) \qquad (1.8)$$

$$F = Io\phi(1 - (1 + (-2.3ecd)/1! + (-2.3ecd)^2/2! + ...)) \qquad (1.9)$$

$$F = Io\phi(2.3ecd - (2.3ecd)^2/2! + (2.3ecd)^3/3!....) \qquad (1.10)$$

From equation 1.10 it can be seen that a linear relationship of fluorescence intensity with concentration is only true when the absorbance of the solution is small when equation 1.10 can be simplified to give

$$F = Io\phi\ 2.3\ ecd \qquad (1.11)$$

Therefore there are three major factors other than concentration that

effect the fluorescence intensity

1) Fluorescence quantum yield

2) Intensity of incident radiation

3) Molar absorptivity of the compound

1.4 Protein binding

Various fluorescence methods have been developed for studying protein ligand interactions (Chignell, 1972), using changes in the intrinsic fluorescence of the protein or drug, fluorescence polarisation and fluorescent probes. The study of the binding of ligands to platelets although more complex can also be investigated by similar techniques. These topics will be introduced and discussed in subsequent sections.

CHAPTER TWO

A STUDY OF THE BINDING OF PLATELET ACTIVE COMPOUNDS TO SERUM ALBUMIN

A STUDY OF THE BINDING OF PLATELET ACTIVE COMPOUNDS TO SERUM ALBUMIN

2.1 INTRODUCTION

For many years the significance of the binding of drugs to serum albumin has been appreciated. The first review of the protein binding literature was by Goldstein in 1949, which further stimulated research into this subject. Subsequent reviews have been published by Meyer and Guttman (1968a), Steinhardt and Reynolds (1969), Bridges and Wilson (1976), Jusko and Gretch (1976), Vallner (1977) and Kragh-Hansen (1981). To introduce this section, the structure and function of albumin will be considered followed by the protein binding of platelet active compounds to albumin. Finally the use of fluorescence for investigating ligand macromolecule interactions will be discussed.

2.1.1 Serum albumin

Serum albumin is the most abundant plasma protein and serves as a depot protein and transport protein for numerous endogenous and exogenous compounds. These include fatty acids, bilirubin, salicylic acid and warfarin.

Serum albumin consists of a single polypeptide chain which contains only amino acid residues. Behrens et al. (1975) and Meloun et al. (1975) were the first to publish the complete amino acid sequence of human serum albumin (HSA). They found that HSA contains 585 amino acid residues. Only a few differences exists between the postulated structures from the two teams of workers. HSA contains 17 disulphide bonds which help stabilise the structure with a

lone cysteine group in position 34. Bovine serum albumin (BSA) has a very similar structure to HSA, the difference were discussed by Steinhardt et al. (1971).

The secondary structure of albumin contains 50-55% alpha helix, about 15% beta conformation and the remainder random coil. It has been suggested that serum albumin consists of three domains. Each domain can then be subdivided into two subdomains.

Many ligands bind to serum albumin with a high affinity site together with a number of lower affinity sites. Kragh-Hansen (1981) suggested six high affinity binding regions.

2.1.2 Binding of platelet active ligands to serum albumin

2.1.2.1 Cyclo-oxygenase inhibitors

Very little information has been reported about the binding of aspirin (ASA) to serum albumin. ASA and salicylic acid (SA) binding was studied by Andereasen (1973), who showed SA was bound to a greater extent than ASA. Hucker et al. (1972) also showed that ASA was bound to HSA. Neither of the above teams of workers quoted an affinity constant for ASA binding to serum albumin. Hawkins et al. (1969) reported that ASA acetylates HSA under physiological conditions. In plasma ASA has a very short half life (Harris and Riegelman, 1967; Morgan and Truit, 1965) due to hydrolysis to SA. The binding of SA to serum albumin has been extensively studied. One of the earliest reports of SA binding was that of Smith et al. in 1946 who used ultrafiltration. Other workers have subsequently measured the binding of SA to serum albumin including Keresztes-Nagy et al. (1972), Mais et al. (1974), Meyer and Guttman (1970), Kostenbauder et al. (1970),

Brown and Crookes (1976) and Hultmark et al. (1975)

Hultmark et al. (1975) measured the binding of indomethacin to HSA using equilbrium dialysis. They found two classes of binding sites where present on HSA. Similar results were obtained by Mason (1974) using ultrafiltration.

2.1.2.2 Anticoagulants

The binding of warfarin to serum albumin has been extensively studied using fluorescence (Chignell, 1970a, 1972, and 1974). Sudlow et al. (1979) applied a fluorescence method for investigating the binding of warfarin to serum albumin and also looked at competitive binding between warfarin and other anionic ligands. Otagiri (1979) exploited the effectiveness of this method of looking at the primary binding site, to investigate the binding of the enantiomers of warfarin. Chakrabarti et al. (1976) and Chakrabarti (1978) employed fluorescence polarisation in their study of the binding of warfarin to serum albumin in order to investigate changes in the conformation of the complex when titrated with fatty acids. Other workers have used classical methods including Mais et al. (1974) who used gel filtration, O'Reilly (1969) using equilibrium dialysis and Solomon et al. (1968) using ultrafiltration.

2.1.2.3 Phosphodiesterase inhibitors

Very little has been published giving data for the binding of DPD to HSA. Kopitar and Weissenberger (1971) showed that DPD was bound significantly less to serum albumin than to α_1 acid glycoprotein (AGP). He quoted a nk value for DPD binding to serum albumin of 5.7 X 10^4 M^{-1}. No reports exist concerning the binding of RA 233 or RA 433

to serum albumin.

2.1.3 The use of fluorescence for investigating albumin ligand interactions

Using direct fluorescence two methods are available for measuring ligand protein interactions

1) Changes in the intrinsic fluorescence of the protein

2) Changes in the intrinsic fluorescence of the ligand.

2.1.3.1 Changes in the the intrinsic fluorescence of serum albumin

Steinhardt et al. (1971) pointed out that HSA contains one tryptophan residue while BSA contains two. Kragh-Hansen (1981) added HSA contains one tryptophan residue in position 213 and 15 tyrosine residues while BSA contains two tryptophan residues in positions 134 and 212 and 17 tyrosine residues. The most important difference in the structure of BSA and HSA, as far as this study is concerned, is the difference in the number of tryptophan residues.

When native uncombined HSA or BSA is excited by radiation with an excitation wavelength of 280nm the emitted fluorescence contains emission from both the tyrosine and tryptophan residues. With BSA the tyrosine contribution is much less evident than with HSA. Tryptophan emission comes not only from side chains excited by the external radiation but also from tryptophan side chains excited by radiationless energy transfer from excited tyrosine side chains (Chen, 1967). However when either HSA or BSA is excited by a wavelength between 295 and 305nm all of the tryptophans which emit are excited by the exciting radiation. Tyrosine is not excited and thus neither emits nor transfers energy. Therefore by exciting with such

wavelengths only a pure tryptophan spectrum is obtained with the position of the emission maximum dependent on the properties of the tryptophan environment.

Since BSA contains twice as many tryptophan residues as HSA a large difference in emission intensity is to be expected. However the ratio of intensities actually found when the fluorescence of both proteins are measured at the same molar concentration is close to 2.7. Nevertheless the lifetimes of the excited states are about the same.

A classical example used to demonstrate the quenching of the tryptophan fluorescence on binding of a ligand is that which Chignell (1969) demonstrated when 4- butyl -1- (p- nitrophenyl) -2- phenyl -3,5- pyrazolidinedione, an analogue of phenylbutazone, binds to HSA. Forster (1951) has shown that fluorescence quenching of this type is the result of nonresonance transfer of energy from tryptophan to the ligand. It was shown that the degree of quenching was dependent upon the protein concentration

Fluorescence quenching has also been used to study the binding of thyroxine (Steiner et al., 1966), steroids (Attalan and Lata, 1968) and warfarin (Chignell, 1970b). Steinhardt et al. (1971) studied the influence of the binding of hexyl, octyl and dodecyl sulphate on the fluorescence of HSA and BSA. The ligands changed the wavelength of maximal emission of both HSA and BSA towards lower wavelengths. For BSA, one mole of ligand resulted in quenching of the fluorescence, HSA required 3 or 4 moles before an effect was seen.

2.1.3.2 Drugs that change their intrinsic fluorescence on binding to plasma albumin.

Chignell (1970b) reported that when warfarin binds to HSA the fluorescence quantum yield of the drug is increased eightfold. Solomon et al. (1968) reported using a similar method that warfarin was displaced by phenylbutazone. Guarino et al. (1973) showed that the fluorescence intensity of camptothecin was quenched on binding to serum albumin. This method has not been extensively exploited and so very few other reports exist.

2.1.3.3 Fluorescence polarisation

If the intrinsic fluorescence of the albumin or if the intrinsic fluorescence of the ligand is not changed on binding, binding may be able to be investigated by fluorescence polarisation (Udenfriend, 1962 and 1969; Weber, 1953; Haber and Bennett, 1962). Guarino et al. (1973) used this technique to characterise the binding of camptothecin to HSA. Chein and Weber (1973) used fluorescence polarisation to study the kinetics and binding of 1, N ethenoadenosine triphosphate to asparate transcarbamylase. Scandurra et al. (1974) looked at the binding of ditazol to plasma proteins using fluorescence polarisation.

2.1.4 Data analysis

Many problems are associated with the analysis of binding data which has lead to numerous papers being published on this subject. Before the advent and ready availability of high speed computers a number of graphical methods had been developed (Rosenthal, 1967; Thompson and Klotz, 1971; Klotz et al., 1971; Vallner et al., 1976).

Rosenthal's method of analysing data (1967) was particularly useful because it was arranged so that the graphical representation of the binding was independent of the protein concentration. This has found numerous applications where the concentration of binding proteins is not known.

Klotz et al. (1971) derived equations to represent the intercepts and slopes of graphical parameters for multiple classes of sites for the Scatchard, Klotz and Scot plots. Thompson and Klotz (1971) developed a method to calculate the binding parameters from a double logarithmic presentation of moles bound versus free ligand concentration. The graphical analysis is simple and unambiguous and probably not seriously biased if there is only a single class of binding sites. However usually there are at least one or two high affinity sites and one or more classes with less affinity. In these cases the graphical representation including the Scatchard and Klotz plot are curved and evaluation of the binding parameters by graphical analysis is impossible without involving numerous assumptions.

These problems have lead to the development of numerous computer fitting techniques using nonlinear regression analysis. The problems associated with nonlinear regression analysis are discussed by Daniel and Wood (1980). Perrin et al. (1974) presented a statistically unbiased method for estimating ligand macromolecule binding constants from experimental data. Madsen and Robertson (1974) and Weder et al. (1974) also presented a similar method. Klotz and Hunston (1979) discussed some of the problems associated with the method. Feldman (1972) and Vallner et al. (1976) compared various graphical methods with computer fitting methods.

Monot et al. (1983) suggested an alternative model to that of Scatchard to reduce the number of parameters estimated. The model presented helped to overcome some of the problems associated with nonlinear regression analysis when four or more constants need to be evaluated for a multiple binding system. But the constants do not have the same physical significance as the classical model and so are unlikely to be readily adopted.

2.1.5 Aims and objectives

Before studying the binding of ligands to platelets it is necessary to look at the binding of these compounds to plasma proteins in order to be able to estimate the free plasma concentrations. In this section therefore the binding of platelet active compounds to albumin and other plasma proteins were considered. Initially the binding of prednisolone to human serum was investigated using continuous ultrafiltration and equilibrium dialysis. This was followed by competitive studies between prednisolone and salicylic acid. Data obtained from the above experiments was used to set up and develop a number of computer programmes to analyse binding isotherms. A number of fluorescence methods were then perfected by the investigation of the binding of warfarin to serum albumin. The binding of a number of compounds not previously investigated by fluorescence methods were then conducted. The fluorescence methods have been compared with classical methods and with the Sephadex batch method.

2.2 MATERIALS

Visking dialysis membranes 18/32 and 32/36 were purchased from the Scientific Instrument Central Ltd.

Bovine serum albumin (BSA) was obtained from Sigma Chemical Co and Koch-Light Laboratories Ltd.

Warfarin and salicylic acid (SA), were obtained from Sigma Chemical Co.

Dipyridamole (DPD) Figure 2.1, RA 233 (2,6,- bis(diethanolamino) -4-piperidino pyrimido (5,4d) pyrimidine) Figure 2.1, RA 433 (2,4,6-trimorpholinopyrimido (5,4d) pyrimidine) Figure 2.1 and SH 1242 (5 -methyl -3- piparazinyl -1- (4- thiomorpholinyl) - isochinolin -s-oxide (Figure 2.2) were the kind gift of Boehringer Ingelhein Ltd.

Prazosin was the kind gift of Pfizer Ltd (Figure 2.2).

Diphenyl quinoxaline (DPQ) was synthesised by Dr. D. G. Durham.

Diaflo ultrafilters XM 50 membranes were purchased from Amicon.

All chemicals were used without further purification.

A M/15 phosphate buffer pH 7.4 was used for all solutions unless otherwise stated.

2.3 INSTRUMENTATION

Fluorescence measurements were made using a Baird Atomic Ratiometric spectrofluorimeter model RC 200 FP.

Continuous ultrafiltration was conducted using the Amicon MMC system.

Absorbance measurements were made using the Pye Unicam SP 1800 ultraviolet spectrophotometer and Cecil CE 588 microcomputer scanning spectrophotometer.

Equilibrium dialysis was conducted using the Dianorm equilibrium dialyser.

2.4 METHODS

2.4.1 Changes in the intrinsic fluorescence of the protein

Using an excitation wavelength between 295-305nm and an emission wavelength of 335nm, a fixed concentration of the ligand was titrated with the BSA or HSA solution. The fluorescence was compared with that of a protein solution with no ligand. From this the amount of ligand bound per unit fluorescence quenching was calculated. A BSA or HSA solution was then titrated with the ligand and the free and bound concentration calculated from the above relationship. All fluorescence measurements were corrected for self quenching using the formula of Chignell (1972) and Chen (1972).

$$x = \text{antilog } ([\, da + de]/2) \qquad (2.1)$$

where

x is the correction factor by which fluorescence intensities must be multiplied.

da is the absorbance at the exciting wavelength

de is the absorbance at the wavelength of emission.

2.4.2 Changes in the intrinsic fluorescence of the ligand on binding

For a ligand with a single binding site a fixed concentration of the protein solution was titrated with the ligand using the excitation and emission maxima of the ligand, with a 5mm path length in a 10 x 10mm quartz cuvette. A plot of the fluorescence enhancement against the molar ratio of protein to ligand was constructed. From this the fluorescence enhancement of the ligand on binding was calculated.

The bound concentration was given by

$$Db = Q\ Pt \qquad (2.2)$$

$$Q = f/Fmax \qquad (2.3)$$

where

Fmax is the fluorescence enhancement when all the binding sites are completely saturated.

f is the fluorescence at a given point in the titration

Pt is the total concentration of protein.

The concentration of free drug Df is given by

$$Df = Dt - Db \qquad (2.4)$$

A Scatchard plot could then be constructed.

For a ligand with multibinding sites, Chignell's method which did not assume any number of binding sites was employed. A fixed concentration of albumin was titrated with the ligand and the fluorescence enhancement calculated. The titration was continued until a maximum enhancement was observed. At this point it was assumed that the ligand was totally bound and therefore the concentration of ligand bound per unit fluorescence enhancement could be calculated. A second fluorescence titration was then carried out in which increments of the drug are added to a fixed concentration of the protein. If the increase in the fluorescence quantum yield of the ligand is the same at all binding sites then at any point in the titration where the mixture has a fluorescence enhancement f the concentration of bound ligand Db is given by

$$Db = p\ f \qquad (2.5)$$

where

p is the concentration bound per unit change in fluorescence.

2.4.3 Fluorescence polarisation

A 5 X 5mm quartz cuvette was used for all these experiments, care being taken to make all measurements from the same face of the cuvette. Both the excitation and emission slits were set at 10nm. An excitation spectrum was recorded using the maximum emission wavelength of the compound with both the excitation and emission polarisers set in the vertical mode. This was repeated but the emission polariser was changed to the horizontal position. Two further spectra were recorded with both the excitation and emission polarisers in the horizontal mode and with the excitation polariser in the horizontal mode and the emission polariser in the vertical position. These readings were used to calculate the grating correction factor G.

P the quantitive measurement of fluorescence polarisation was calculated from the formula

$$P = \frac{Ivv - G\ Ivh}{Ivv + G\ Ivh} \qquad (2.6)$$

$$G = \frac{Ihv}{Ihh} \qquad (2.7)$$

where

Ivv is the fluorescence intensity with both the excitation and emission polarisers in the vertical mode.

Ivh is the fluorescence intensity with the excitation polariser in the

vertical mode and the emission polariser horizontal.

Ihv is the fluorescence intensity when the excitation polariser is in the horizontal mode and the emission polariser in the vertical mode.

Ihh is the fluorescence intensity with both the excitation and emission polarisers in the horizontal mode.

Protein was then added to the system and the above repeated. For routine measurements a fixed excitation wavelength was employed at a value which gave the maximun change in polarisation with the addition of the protein. The saturation factor s is defined by

$$s = Db/Dt \qquad (2.8)$$

from the polarisation values

$$s = 1 - \frac{I\ (Pmax - P)}{Io\ (Pmax - Pmin)} \qquad (2.9)$$

where

I is the fluorescence intensity of the protein ligand solution.

Io is the fluorescence intensity of a standard ligand solution with no protein.

Pmax is the fluorescence polarisation when all the ligand is bound.

Pmin is the polarisation of a solution of the ligand with no protein.

P is the polarisation at any point in the titration

Pmax was calculated by titrating a fixed concentration of DPD with BSA. Pmax was taken to be the maximum value in a fluorescence polarisation against the ratio of BSA to DPD.

The saturation factor was then used to calculate the free and bound concentration of drug and a Rosenthal plot constructed.

2.4.4 Ultraviolet difference spectroscopy

A cell containing the protein solution and a cell containing the buffer were placed both in the reference and sample compartments. After a base line had been recorded the ligand solution was added to the protein cell in the sample compartment and the buffer solution in the reference compartment and the difference spectrum recorded. The titration with the ligand was continued, recording a spectrum after each addition.

2.4.5 Continuous ultrafiltration

The continuous ultrafiltration studies were conducted using Diaflo XM 50 membranes with a chamber volume of 4ml. An operating pressure of approximately 50 p.s.i. was used. All experiments were conducted at 37° C. The total drug concentration Dt, was calculated from the relationship

$$Dt = \frac{1}{V} \sum_{i=1}^{i=n} (D\,vi - Df\,vi) \qquad (2.10)$$

where

D is the concentration of drug in the reservoir

Df is the concentration of the free ligand

vi is the volume of the ultrafiltrate fraction i

V is the volume of the chamber

Df was the concentration of the drug in the ultrafiltrate, from this the concentration of drug bound was calculated

$$Db = Dt - Df \qquad (2.11)$$

The tritiated prednisolone was determined by liquid scintillation counting using the following scintillation fluid: toluene (750ml), Triton X-100 (249ml), diphenyloxazole (5.01g). SA was detected by Trinder's (1953) method and by fluorescence.

2.4.6 Equilibrium Dialysis

The dialysis membrane was prepared by washing the membrane in ethanol followed by soaking in distilled water over night. One hour prior to use, the distilled water was replaced by buffer. Initially buffer was placed one side of the membrane and the ligand solution on the other side. This allowed the time for the equilibrium to be established and membrane binding of the ligand to be determined. The protein solution was then placed on one side of the cell and the ligand on the other. The ligand concentration in the protein compartment at equilibrium is equal to Db + Df and the concentration in the protein free compartment Df. The free bound and total concentration being linked by

$$Dt = Db + 2Df \qquad (2.12)$$

When the chamber volumes were equal, the ligand was placed in one cell and the protein in the other. Db and Df was therefore determined and the binding isotherms analysed by nonlinear regression analysis.

2.4.7 Dynamic Dialysis

Using the method of dynamic dialysis (Meyer and Guttman, 1968b, 1970a and 1970b), the binding of platelet active ligands to serum albumin was investigated. 5ml of the ligand solution was placed in a 7cm long dialysis sac and tied at both ends. The sac was immersed in 150ml of the buffer solution in a jacketed beaker and the external solution stirred continuously. At predetermined time intervals 100ml of the external solution was removed and immediately replaced by 100ml of fresh buffer. The concentration of the ligand in the removed sample was determined using fluorescence and the concentration in the sac calculated from a knowledge of the internal concentration. The dialytic behaviour obeyed first order kinetics.

$$\frac{d(Dt)}{dT} = - k\ Df \qquad (2.13)$$

where

d(Dt)/dT is the rate of loss of the ligand from the dialysis sac

k is the first order rate constant

Df is the free ligand concentration

Therefore for a first order process

$$Ln\ (Dt) = - k\ T \qquad (2.14)$$

A plot of Ln (Dt) against time gives a straight line with a gradient of -k.

The above was repeated with the addition of the protein to the dialysis sac. A plot of Ln (Dt) against time was constructed again but now was not linear. The rate of dialysis is dependent on the free concentration of the ligand and not on the total concentration. The

free concentration of ligand in the dialysis sac was calculated from equation (2.13) with a knowledge of k. The instantaneous rate at a value of Dt in the presence of macromolecule was estimated graphically from the gradient at any point of a plot of Dt versus time. Alternatively the data was fitted more accurately using an iterative nonlinear curve fitting programme. Two equations were tried, a polynomial

$$Dt = a + bT + cT^2 + dT^3 + eT^4 + fT^5 \quad (2.15)$$

$$d(Dt)/dT = b + 2cT + 3dT^2 + 4eT^3 + 5fT^4 \quad (2.16)$$

and a six parameter triexponential (Appendix 1)

$$Dt = a\,e^{bT} + c\,e^{dT} + e\,e^{fT} \quad (2.17)$$

$$d(Dt)/dT = ab\,e^{bT} + cd\,e^{dT} + ef\,e^{fT} \quad (2.18)$$

where

a, b, c, d, e, and f are constants.

The instantaneous rates at various values of Dt can therefore be calculated and Df evaluated.

2.4.8 Analysis of the binding data

The following assumptions have been made in the analysis of the binding data :-

(1) Two distinct classes of reactant are initially present in solution namely ligand and binding protein. No species acts as both ligand and binding molecule. No other active reagents are present.

(2) The binding is reversible between the ligand and the binding molecule. No complex precipitates.

(3) Each binding reaction proceeds independently to equilibrium according to second order kinetics. The affinity constant is unaffected by the progression of the reaction. All binding sites are independent.

(4) The equilibrium composition of the solution can be measured accurately without disturbing the equilibrium.

(5) The activity of the ligand is equal to the concentration.

For one class of binding sites the equilibrium between the protein and the ligand can be represented by

$$P + Df \rightleftharpoons PD$$

where P is the free concentration of protein

D is the free concentration of drug

PD is the concentration of the complex

The association constant is therefore given by

$$k = \frac{PD}{P \; Df} \qquad (2.19)$$

$$PD = k \; P \; Df \qquad (2.20)$$

Defining r as

$$r = \frac{\text{moles of drug bound}}{\text{total moles of protein}} \qquad (2.21)$$

$$r = \frac{PD}{P + PD} \qquad (2.22)$$

$$r = \frac{k \; P \; Df}{k \; P \; Df + P} \qquad (2.23)$$

$$r = \frac{k\ Df}{1 + k\ Df} \qquad (2.24)$$

This can be transformed to give for n binding sites.

(1) Klotz plot

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{n\ k\ Df} \qquad (2.25)$$

(2) Scatchard plot

$$r/Df = nk - rk \qquad (2.26)$$

In terms of fraction of the drug molecule bound

$$r = \frac{Db}{Pt} = \frac{kDf\,n}{1 + k\ Df} \qquad (2.27)$$

$$\frac{Pt}{Db} = \frac{1 + k\ Df}{k\ Df\,n} \qquad (2.28)$$

$$\frac{Df}{Db} = \frac{1 + k\ Df}{k\ Pt\,n} \qquad (2.29)$$

$$Df = \frac{Db\ (\ 1/k + Df\ )}{n\ Pt} \qquad (2.30)$$

$$\text{Fraction bound} = \frac{Db}{Db + Df} \qquad (2.31)$$

$$\text{Fraction bound} = \frac{1}{1 + 1/n\ Pt\ k + Df/n\ Pt} \qquad (2.32)$$

The results were analysed by linear regression analysis using the Scatchard or Rosenthal linear transformation or using nonlinear regression analysis. Two computer programmes were used for the nonlinear regression analysis

1) The Decus programme Nonlin

2) A programme based on the method of Hooke (listings given in Appendix 2 - 4)

Three models were set up for each programme

1) A one site model

$$Db = \frac{Df\ k\ n P_1}{1 + k\ Df} \qquad (2.34)$$

2) A two site model

$$Db = \frac{Df\ k_1\ n P_1}{1 + k_1\ Df} + \frac{Df\ k_2\ n P_2}{1 + k_2\ Df} \qquad (2.35)$$

3) A one site model with partition (unsaturable binding)

$$Db = \frac{Df\ k\ n P_1}{1 + k\ Df} + nkp\ P_2\ Df \qquad (2.36)$$

Nonlin is a nonlinear least squares curve fitting programme. The programme was written by D. A. Meeter from the University of Wisconsin, using Marquarts (1963) maximum neighbourhood method. The programme was subsequently revised by a number of workers and is used in Fortran IV. To run the programme a data file was first constructed (Appendix 5) The first line is a control line stating the identification of the problem, the number of coefficients to be estimated, the number of independent variables, the number of the subroutine which the equation should read, the starting value of lambda the multiplier to scale the space or size steps taken, the

value of nu the divider and multiplier to change the size of lambda, and the stop criteria. This is followed by a format statement giving the format of the data which is to be read. A format of (A4, 8x, 2F6.0) was used in all the problems. The next line states the starting values of the coefficients, which is followed by information cards for the print out and the names of the coefficients. The data is then input following the above format statement. The final line is an end statement.

Hooke is also a least squares nonlinear curve fitting programme. Again the programme requires a data file to run (Appendix 6). First the interger number of pairs of experimental points is input followed by all the Db values and then the Df values. Six numbers are placed per line separated by commas using (6G) as a format statement. The Hooke programme generates a data file to be used with a graphics programme (Appendix 7). This constructs a Rosenthal plot drawing the theoretical line to the fitted data and puts the experimental points on the graph. This was drawn by a Benson plotter.

Both programmes, in common with all nonlinear regression analysis programmes, require initial estimates of the constants to be evaluated. The starting values for k1 and k2 were estimated from the slope of a Scatchard or Rosenthal plot at the two extremes of the plot. The values for np are calculated from the intercept of the Rosenthal plot.

Nonlin gives a more comprehensive printout than the Hooke programme. Nonlin estimates a value for the standard error of the coefficients and confidence intervals for each coefficient while Hooke performs analysis of variance. These values are only approximate

because the results obtained are not the theoretical minimum as in linear regression analysis, but are stopped by the programme when the residual sum of the squares changes by less than a preset value. With both the Hooke and the Nonlin programme the residual values of Db are one of the most important pieces of information given. The negative and positive values should be evenly distributed. Nonlin as well as calculating the residual values of Db, also plots the residuals against the fitted value of Db and the value of Df. These scatter diagrams quickly show if the residuals follow any pattern indicating a poor fit (Daniel and Wood, 1980). The residuals should form a sample from a normal distribution of mean 0. Nonlin in its output gives a cumulative distribution of the residuals. It plots the ranked residual (observed Db - fitted Db) on a cumulative frequency normal probability grid. If the residuals are normally distributed the points should fall approximately on a straight line with a mean of zero. The maximum negative and positive values should be approximately equal. A very high value was tested to see if it was within the 95% confidence interval using

test statistic = residual/residual root mean square.

If this value was greater than 1.96 the point was removed and the data rerun.

The iterations of Hooke were terminated when the residual sum of the squares changes less than a preset value. Nonlin can be terminated when the residual sum of the squares changes less than a preset value or when the ratio of the constants changes less than a preset value. The change in the residual sum of the squares was always used as the terminating factor.

The selection of a model which gave a random distribution of the scatter of observed data points about the theoretical curve and resulted in the residual sum of the squares being a minimum was conducted using the method of Boxenbaum et al. (1974). This F ratio test was used to test whether or not the residual sum of the squares had been sufficiently reduced to justify fitting the additional parameters.

The test statistic was given by

$$F = \frac{RSSa - RSSb}{RSSb} \times \frac{Dfb}{Dfa - Dfb} \qquad (2.37)$$

where

RSSa is the residual sum of the squares of model a

RSSb is the residual sum of the squares of model b

Dfa is the degrees of freedom for model a which is equal to the number of data points used minus the number of parameters fitted

Dfb is the degrees of freedom for model b

Dfa > Dfb

The calculated F value was compared to the critical values derived from a table (numerator has (Dfa -Dfb) degrees of freedom, denominator has Dfb degrees of freedom). If the F value was less than the 5% level of significance from the table, it was concluded that the RSS are not significantly different.

The values of the affinity constants evaluated from different experiments were compared using a T test. The test statistic was given by

$$t = \frac{kia - kib}{SDia - SDib} \qquad (2.38)$$

where

t is the test statistic

kia is the value of ki for experiment a

kib is the value of ki for experiment b

SDia is the standard deviation of ki form experiment a

SDib is the standard deviation of ki from experiment b

2.5 RESULTS AND DISCUSSION

2.5.1 Data analysis

The binding of prednisolone and SA to human serum has been studied in order to set up a number of computer programmes to analyse binding isotherms. SA was chosen because it has two classes of binding sites and prednisolone because it has been reported to bind with a primary site and partition. Both these compounds are of interest because they inhibit enzyme systems involved in platelet aggregation. One of prednisolone's actions is that it inhibits phospholipase therefore inhibiting the formation of arachidonic acid. Salicylic acid is the major decomposition product of ASA an inhibitor of cyclo-oxygenase. It has been reported that prednisolone has a high affinity site on transcortin and a lower affinity site on albumin (Agabeyoglu et al., 1979; Gambertoglio et al., 1980; Rocci and Jusko, 1981). It is unlikely that SA binds to the transcortin site but may compete for the albumin site. The binding of prednisolone and salicylic acid to human serum and possible competition between them was therefore investigated. Figure 2.3 shows a Rosenthal plot for the binding of prednisolone to human serum using ultrafiltration and Figure 2.4 using equilibrium dialysis. The data obtained from these experiments were analysed by nonlinear least squares regression

analysis using either the commercial programme Nonlin or a programme based on the method of Hooke.

Both the Nonlin programme and the Hooke programme had their advantages and disadvantages. Nonlin is a more efficient programme in terms of the central processor unit time. While Nonlin may take a few seconds central processor unit time Hooke may take several minutes. This is because Nonlin can change the value of lambda, the multiplier of the coefficients, so that the iterations can come to a speedy conclusion. In the Hooke programme these constants are fixed for each coefficient, but this makes Hooke a more flexible programme because of the control given over the increment of each constant. By making the increment for a given constant 0, that constant can be fixed to the starting value. One or more constants were fixed using this technique after a satisfactory constant had been evaluated, while the other constants were optimised. This proved to be useful when there were more than one class of binding sites where four or more unknown constants are to be evaluated. This technique was also used for the analysis of the competitive binding studies where mean values of the primary affinity constants could be fixed while the programme optimised the inhibition constant. The only way this could be achieved by Nonlin was by changing the subroutine of the main programme. These constants need to be reasonable estimates or the programmes may generate unrealistic values for the constants. With nonlinear analysis with several unknown constants, there may be a number of sets of constants that will give a statistically significant fit to the problem. The programme will try and reduce the residual sum of the squares value to a minimum. If the initial estimates of

the constants are too far from the 'physically significant' values of the constants, the programme may find a set of constants with no physical significance. These may include negative values for affinity or capacity constants or very high or low values. Nonlin is more efficient at using initial estimates further from the real values than Hooke.

For the case when only one class of binding sites existed, linear regression analysis was also employed and compared with the above methods. The results were in close agreement. Kruger-Thiemer (1967) observed that analysis of drug protein data using a nonlinear approach ensures more reproducible results than any of the linear transformations. Madsen and Robertson (1974) confirmed the above findings. Musulin (1973) has shown, in viscosity work, that linear and Nonlin estimates of a given problem may differ but that these converge with the precision of the data.

A theoretical problem exists when applying nonlinear regression analysis to the problem of evaluating the binding parameters of a system. The method assumes an independent variable with very small errors and a dependent variable with much larger errors. It has been assumed that Db is the dependent variable and Df is the independent. Both Db and Df will have errors in their determination. When Df is large the error in Df will be smaller than that of Db. When Db is large compared with Df, the error in Df will be much bigger than that of Db. No mathematical method has so far been developed which will take account of the above problem.

For prednisolone, the model which gave the best fit with both methods using equation 2.37 was a one site with partition (unsaturable binding) model. A primary affinity constant of 6.7×10^7 M^{-1} with a capacity of 1.65 μM and a partition constant kpp of 0.55. This example was of particular interest because one primary binding site was given with an unsaturable binding site. It was envisaged that binding of some ligands to platelets may follow a similar pattern. The binding of Salicylic acid to serum is shown in Figure 2.5. ($k1 = 8.145 \times 10^4$ M^{-1} and $k2 = 3.3 \times 10^3$ M^{-1})

Table 2.1 shows the results of the binding constants calculated with University of Aberdeen programme and also with Nonlin and Hooke. These results are in relatively close agreement. No competition was observed between prednisolone and SA either on the transcortin site or on albumin.

2.5.2 The binding of warfarin to BSA

The fluorescence of warfarin was enhanced on binding to BSA. Figure 2.6 shows the fluorescence of 1μM of warfarin when titrated with BSA using an excitation wavelength of 340nm and an emission wavelength of 380nm. The excitation wavelength was shifted from the maximum in order to minimise the effect of self quenching. All the fluorescence measurements were corrected for self quenching as previously described. Figure 2.7 shows the results of a typical titration of 0.01% BSA solution with warfarin. From this a Rosenthal plot was constructed (Figure 2.8). An affinity constant of 1.70×10^5 M^{-1} se 0.167 was calculated using nonlinear regression analysis. This is in close agreement with values quoted in the literature (Sudlow et al., 1979).

On binding to HSA or BSA the fluorescence polarisation of warfarin was increased. Using a 1.45 X 10^{-6} M solution of BSA a maximum polarisation of 0.170 was given. Figure 2.9 shows the effect on the fluorescence polarisation of titrating 1.0 X 10^{-6} M warfarin with BSA. The large enhancement of the fluorescence of warfarin on binding to BSA made this method imprecise for calculation of the affinity constants. The large enhancement of the fluorescence resulted in a slow decrease in the fluorescence polarisation of warfarin when a fixed concentration of BSA was titrated with warfarin.

2.5.3 the binding of cyclo-oxygenase inhibitors to BSA

2.5.3.1 Saliclyic acid and acetylsaliclyic acid

ASA is rapidly hydrolysed to SA at pH 7.4. Fluorescence is therefore an ideal method for investigating this protein binding interaction because of the speed that measurements can be made. It would be impossible to measure ASA binding using equilibrium dialysis because the prolonged equilibration times would allow marked decomposition of the ASA.

At pH 7.4 ASA has a very low fluorescence quantum yield. The intrinsic fluorescence of ASA is not changed in the presence of BSA. It is possible to measure the rate of hydrolysis of ASA by the appearance of SA. This rate is not increased in the presence of BSA.

The absorbance spectrum of ASA partially overlaps the emission spectrum of the BSA. Nonresonance transfer of energy can therefore theoretically take place if the ASA is binding near either of the tryptophan residues. No quenching of the intrinsic fluorescence of the BSA was observed when a protein concentration of between 1.45 X

10^{-6} to 1.45 X 10^{-5} M was used. Dynamic dialysis conducted over a short period of 15-30 minutes, to limit the hydrolysis of ASA gave no evidence to suggest ASA was significantly bound to BSA. Continuous ultrafiltration studies, determining ASA by hydrolysis to SA and measuring the fluorescence intensity gave no conclusive evidence to suggest ASA was bound.

The binding of SA to BSA and HSA has been investigated by numerous workers but no one has reported the use of fluorescence techniques to measure such interactions. SA has an excitation maximum at 305nm and an emission maximum at 410nm. It has a higher fluorescence quantum yield than ASA. In contrast to ASA the fluorescence quantum yield is enhanced on binding to BSA. When 7.25 X 10^{-6} M Sigma BSA was titrated with SA a maximum fluorescence was observed (Figure 2.10). Db and Df was calculated and a Scatchard plot constructed (Figure 2.11). Using Koch-Light BSA the fluorescence enhancement was less than with Sigma BSA (Figure 2.10). The affinity constants calculated from these two albumins were not significantly different. Sigma BSA gave a primary affinity constant of 8.4 X 10^4 M^{-1} se 2.2 X 10^4 . No information was given about the secondary sites using this method.

These studies indicate that ASA, if bound at all to plasma proteins, is significantly less bound than SA. The main problem with the determination of the binding of ASA was its instability. It is unlikely that the plasma binding of ASA will effect its binding to platelets. This will be investigated in the final section. SA is highly bound to albumin and plasma. This may therefore effect the binding of SA to the platelet.

2.5.3.2 Indomethacin

Like SA the binding of indomethacin to serum albumin has been reported by many workers but no reports exist for the study of this binding system using fluorescence. Indomethacin has a very low fluorescent quantum yield at pH 7.4. The intrinsic fluorescence of indomethacin is not enhanced in the presence of BSA in the concentration range of 1.45×10^{-6} to 1.45×10^{-5} M. The emission spectrum of BSA and HSA overlaps the absorption spectrum of indomethacin (Figure 2.12). Using an excitation wavelength of 300nm and an emission wavelength of 335nm the intrinsic fluorescence of the BSA was quenched by the indomethacin on binding. It was observed that the fluorescence was dependent on the protein concentration (Chignell, 1972). Figure 2.13 is a plot of the percentage fluorescence against the molar ratio of indomethacin/BSA. Similar results were given for the binding of indomethacin to HSA (Figure 2.14). Figure 2.15 is a Scatchard plot for the binding of indomethacin to HSA. A primary affinity constant of 4.85×10^{5} M^{-1} se 0.43 with 1.13 binding sites were evaluated. This is in close agreement to values quoted in the literature by Hultmark et al. (1975).

The protein concentration binding of indomethacin can partly be explained by reference to equation 2.32. This equation demonstrates that the fraction bound is dependent on the value of $1/(k\ n\ Pt)$ and Df/nPt.

Therefore if $k\ n\ Pt \ll 1$ the reciprocal will be large resulting in a low fraction bound.

Conversely if $k\ n\ Pt \gg 1$ the fraction bound will approach 1 at maximum binding. This indicates the importance of the experimental

condition for investigating protein binding.

When $1/k >> nPt$ then the fraction bound will be small.
For the case when 1/k is 100 times n Pt the maximum percentage bound will be approximately 1%. This shows that the protein concentration should be in the same order or greater than the value of 1/k. This may cause a problem when the association constant is relatively low. The only way such a binding interaction could be investigated would be by using a high protein concentration. Using fluorescence methods, this may give problems due to high absorbance values.

The above therefore explains why the fluorescence quenching at low ratios of indomethacin/HSA, for low protein concentrations was less than at high protein concentrations. At high drug concentrations the equation 2.32 predicts that the fraction bound will approach 0. At high ratios in Figure 2.13 and 2.14 the quenching at different ratios was markedly different. It was envisaged that if the ratio was increased further the quenching would continue to fall. Because of the low solubility of the indomethacin the ratios could not be taken higher.

The results drawn from equation 2.32 are important and not widely appreciated. The majority of binding studies are conducted using a fixed protein concentration usually plasma or serum. Using spectroscopic methods, high protein concentrations are not normally used so the choice of protein concentration becomes more important.

The fluorescence method as well as evaluating an affinity constant for the primary binding site has shown that the site is located in the region of the tryptophan residue thus helping to

further characterise the binding site.

2.5.4 Binding of phosphodiesterase inhibitors to BSA

2.5.4.1 Dipyridamole

In the presence of BSA the intrinsic fluorescence of DPD is not changed using any excitation or emission wavelength. A change in the Stokes shift does not occur. Possible changes in the intrinsic fluorescence of the BSA was investigated using an emission wavelength of 335nm and an excitation wavelength of either 295nm to specifically look at the tryptophan fluorescence or 285nm to look at both the tyrosine and tryptophan residues. No quenching of the protein fluorescence was observed at either excitation wavelength. HSA gave similar results.

The binding of DPD to BSA was also studied using fluorescence polarisation. Figure 2.16 shows the effect of titrating 1μM DPD with BSA. Very little change in the fluorescence polarisation was observed at low DPD/BSA ratios suggesting a low affinity constant. When 1.45 X 10^{-5} M BSA was titrated with DPD no change was observed in the fluorescence polarisation of the DPD. When the protein concentration was increased to 1.45 X 10^{-4} M the fluorescence polarisation also increased. Using the method of Chignell (1972) a Scatchard plot was constructed (Figure 2.17). Very low values for the number of binding sites were given. This may be due to the low DPD/BSA ratios used because of the low solubility of DPD. Alternatively DPD could have been binding to an impurity in the BSA. Peters (1975) stated that AGP is likely to be an impurity of fraction V.

Ultraviolet difference experiments using a 1% BSA solution with a 5mm path length gave a positive peak at approximately 310nm and a negative peak at 295nm. Two isobestic points were given at 305nm and 315nm. Other difference peaks were considerably less pronounced than the above peaks. Very little change in the UV spectrum was observed using 0.1 or 0.01% BSA solution.

Binding of DPD to 1.45×10^{-4} M BSA was demonstrated using equilibrium dialysis. The data was not analysed by a Scatchard or Rosenthal plot because of the low ratio of DPD/BSA employed. The binding of DPD to BSA can therefore be considered either to be partition as observed in the case of prednisolone or a classical model with $Db << Pt$. The percentage bound in the conditions of the experiment was concentration independent. For prednisolone it was observed that it bound with an high affinity site and partition. Data has been presented by a number of workers (Agabeyoglu et al., 1979) in the form of a high affinity with a partition constant. For DPD the complication of an high affinity site is not present. The data in this case is more usefully presented by making the approximation of Pearlman and Crepy (1967) so that a value of nk is evaluated. The two constants partition and nk represent the same unsaturated binding. The partition constant kpp is related to nk by the following equation

$$kpp = nk\ Pt$$

Using the method of Pearlman and Crepy (1967) nk was estimated to be $4.1 \times 10^{3}\ M^{-1}$ se 0.63.

Using dynamic dialysis it has been shown that the binding of DPD to plasma is significantly higher than that of DPD to albumin. Studies undertaken with continuous ultrafiltration were unsuccessful because very marked binding of DPD occurred to the membranes.

2.5.4.2 RA 233 and RA 433

RA 233 and RA 433 are both very fluorescent compounds with similar structures to DPD (Figure 2.1). In the presence of BSA the intrinsic fluorescence of RA 233 and RA 433 was unchanged. No change was seen in the intrinsic fluorescence of the BSA or HSA. The fluorescence polarisation of these two compounds was unchanged on binding. No data was obtained from continuous ultrafiltration studies due to membrane binding of RA 233 and RA 433. Equilibrium dialysis studies using 1.45×10^{-4} M BSA showed that both RA 233 and RA 433 were bound. Data was analysed using the approximation of Pearlman and Crepy (1967). For RA 233 a nk value of 4.0×10^{3} M^{-1} se 0.26 was estimated and for RA 433 nk = 6.25×10^{3} M^{-1} se 0.108.

The binding constants for DPD, RA 233 and RA 433 are in the same order. The constants are relatively low indicating non specific binding of these compounds to BSA. It has been reported by Kraugh-Hansen (1981) that albumin binds acidic molecules in preference to basic molecules so this may account for no specific binding.

2.5.4.3 Prazosin

The main biological function of prazosin is an alpha adrenoreceptor blocker (Grahnen et al., 1981). In high concentration it has been reported to inhibit phosphodiesterase (Moore, 1982). The absorbance spectrum of prazosin overlaps the emission spectrum of BSA

and HSA so it is theoretically possible to get nonresonance transfer of energy from the protein to the prazosin. No quenching of the fluorescence of the protein was observed. The intrinsic fluorescence of the prazosin did not change on binding. No change in the fluorescence polarisation was observed. Using equilibrium dialysis and the approximation of Pearlman and Crepy (1967), nk was estimated to be 3.3×10^{4} M^{-1} se 0.167.

2.5.4.4 SH 1242

The fluorescence of SH 1242 is enhanced in the presence of BSA. Figure 2.18 shows the effect of titrating 1μM SH 1242 with BSA. At the maximum fluorescence it is assumed that the SH 1242 is totally bound. From this graph the concentration of SH 1242 bound per unit change in fluorescence was calculated. A titration of 1.45×10^{-6} M BSA with SH 1242 was conducted and the free and bound concentration calculated using equation 2.5 (Figure 2.19). Figure 2.20 is a typical Rosenthal plot for this binding system. An affinity constant of 1.06×10^{5} M^{-1} se 0.01 was evaluated with 1.05 se 0.05 binding sites.

2.5.4.5 Diphenyl quinoxaline

On binding to BSA the intrinsic fluorescence of the DPQ is quenched. Figure 2.21 shows the fluorescence intensity against the DPQ concentration and Figure 2.22 is a Rosenthal plot for the binding assuming one binding site. An affinity constant of 8.89×10^{5} M^{-1} was evaluated with 1.08 binding sites. A study was made of the fluorescence of DPQ in various solvents to investigated the effect of the environment on the fluorescence. The fluorescence was reduced in a number of organic solvents (Table 2.2). Within the pH range 3-11 no

change in the fluorescence of DPQ was observed. These results therefore suggest that DPQ was binding in an hydrophobic region of the protein.

The fluorescence of the BSA was also quenched in the presence of DPQ.

2.5.5 Effect of calcium, ADP, and citrate on binding

The effect of Ca^{2+}, ADP, and citrate was studied to establish if these compounds which are commonly used in platelet studies effect the binding of platelet active compounds. No effect on the binding of any of the above compounds were observed. Figure 2.23 shows that calcium (2mM) has no effect on the binding of SA to BSA using dynamic dialysis. Similar experiments have been conducted with DPD and the above compounds.

2.5.6 Binding of ligands to fibrinogen

Fibrinogen is a platelet active protein which binds to the platelet membrane. Although very few substances have been shown to bind to fibrinogen it is important to show that the platelet active compounds investigated here do not bind to it. The binding of RA 233, RA 433, SH 1242, DPD, SA, ASA, indomethacin, 2,3 DPP, 2,5 DPP, DPQ, DMQ, and warfarin have been studied using, direct fluorescence, fluorescence polarisation and dynamic dialysis. No evidence was obtained to suggest that any of these compounds were bound to fibrinogen.

2.6 CONCLUSION

Fluorescence methods have been successfully applied to the binding of a number of platelet active compounds to serum albumin and other plasma proteins. The binding of a number of these compounds have not previously been reported to have been measured by the use of fluorescence. These include SA, indomethacin, SH 1242 and diphenyl quinoxaline, but the binding of a number of compounds have been investigated without observing any change in the fluorescence of the compounds. These included RA 233, RA 433, and DPD. This therefore demonstrates a limitation of fluorescence methods. The next section will therefore investigate the possibility of measuring protein ligand interactions using a Sephadex batch method in conjunction with fluorescence. This may provide a method for looking at ligand interactions for the molecules which do not change any of their fluorescence characteristics.

It has been shown that there is a optimum protein concentration were the fraction bound at low concentration can approach 1. When the affinity constant is small it is not possible to measure the affinity constant at low protein concentrations. The protein concentration should be > 1/k otherwise the fraction bound will be low.

Table 2.1

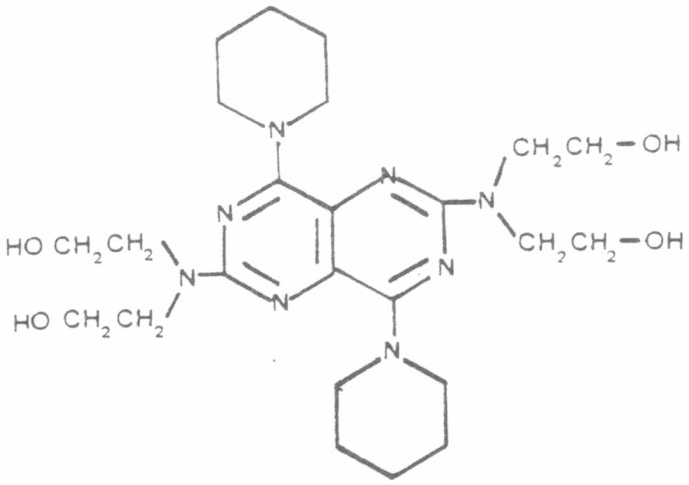
Comparison of the binding constants evaluate by the computer programmes Hooke and Nonlin for the binding of prednisolone to human serum.

	Nonlin	Hooke	Forresterhill
k	$66.9 \times 10^{6}\ M^{-1}$	$66.3 \times 10^{6}\ M^{-1}$	$67.12 \times 10^{6}\ M^{-1}$
np	0.55×10^{-6} M	0.55×10^{-6} M	0.55×10^{-6} M
kpp	1.65	1.64	1.65

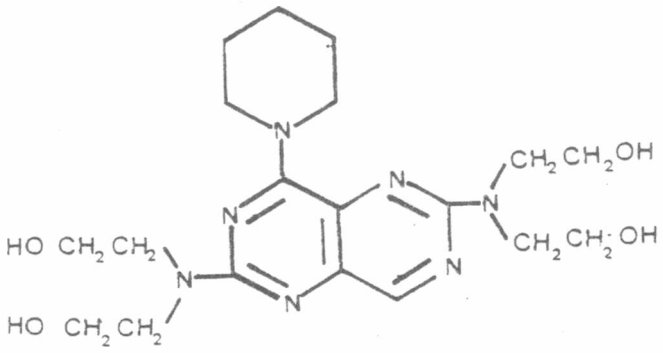
TABLE 2.2

A table showing the effect of organic solvents on the fluorescence intensity of diphenyl quinoxaline.

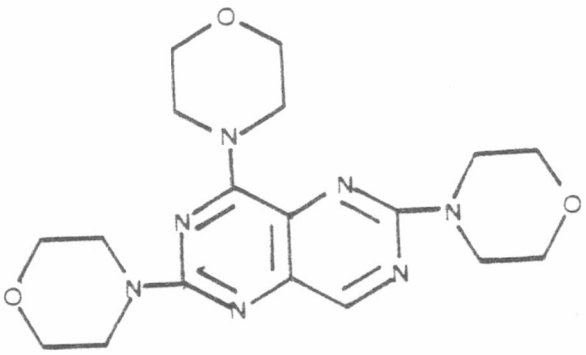
SOLVENT	FLUORESCENCE INTENSITY
M/15 phosphate buffer pH = 7.4	140
acetone	1
ethanol	2
methanol	7
acetic acid	30
pentane	1.5



DPD

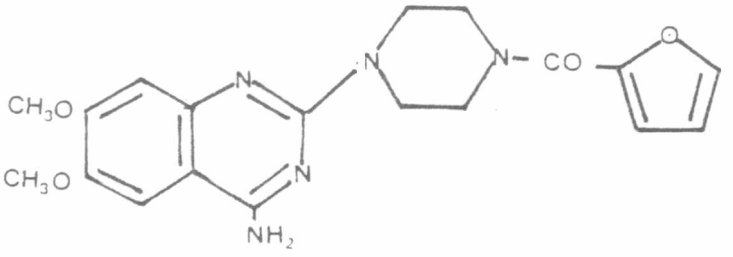


RA 233

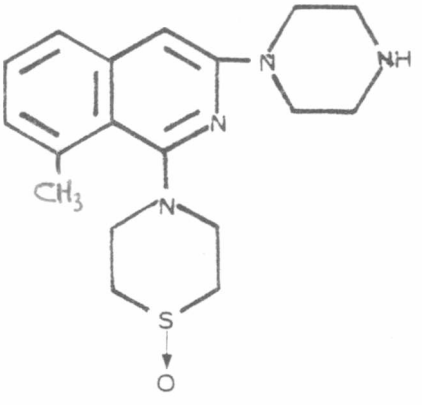


RA 433

Figure 2.1



PRAZOSIN



SH 1242

Figure 2.2

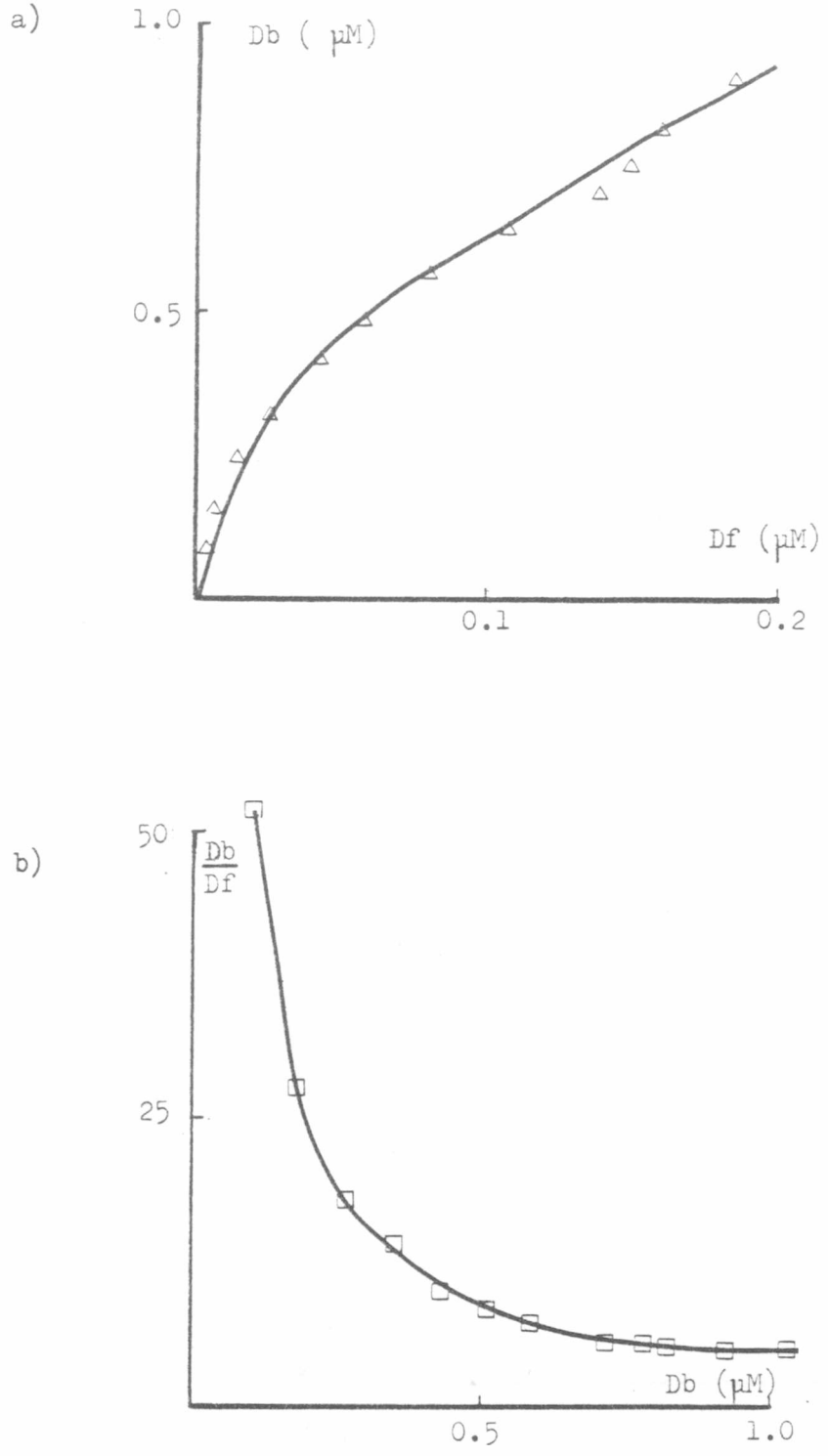


Figure 2.3

a) A graph of Db against Df for the binding of prednisolone to human serum. Data from continuous ultrafiltration.

b) A Rosenthal plot for the binding of prednisolone to human serum.

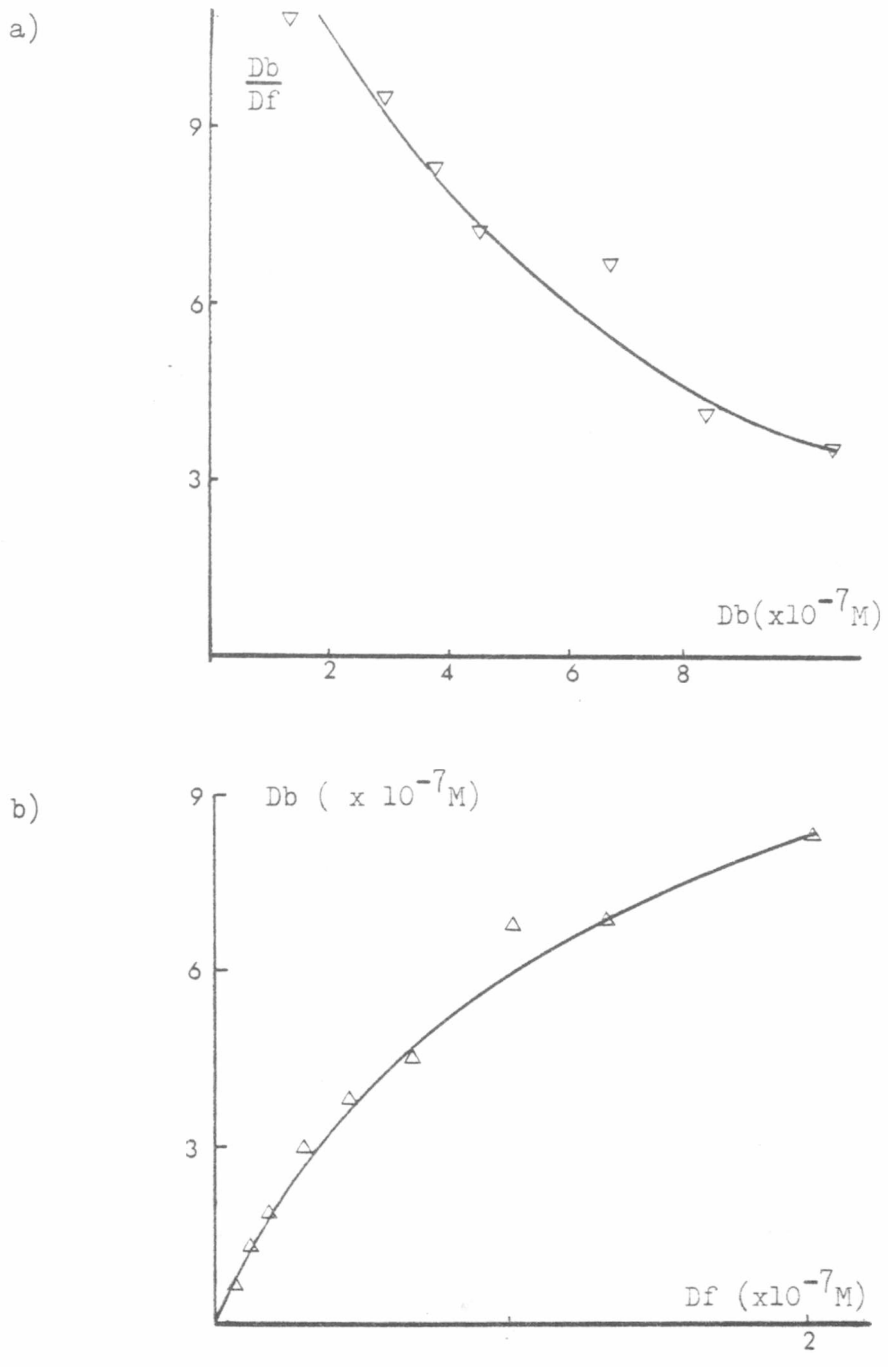


Figure 2.4

a) A Rosenthal plot for the binding of prednisolone to human serum. Data obtained from equilibrium dialysis.

b) A plot of Db against Df for the binding of prednisolone to human serum.

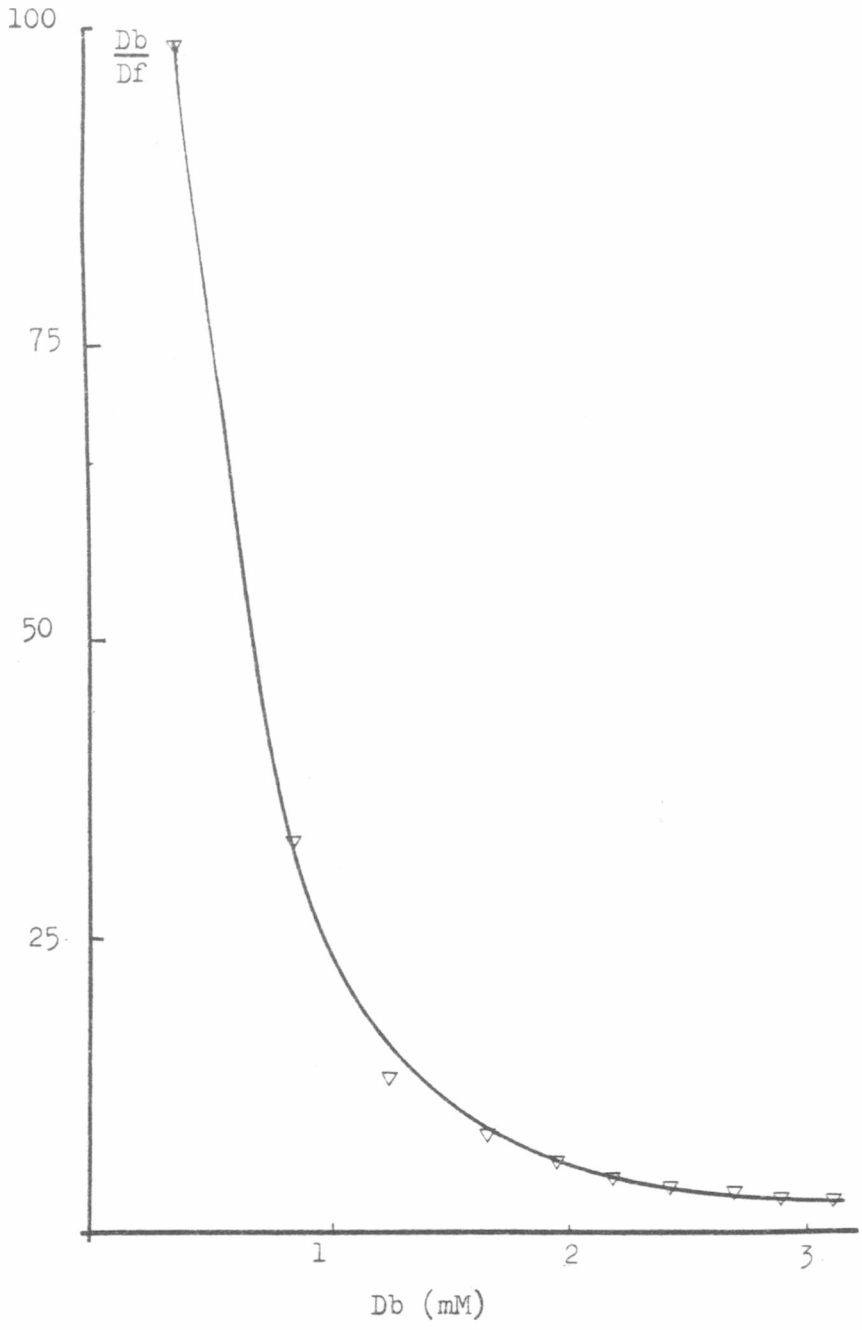


Figure 2.5

A Rosenthal plot for the binding of SA to human serum. Db and Df calculated from continuous ultrafiltration.

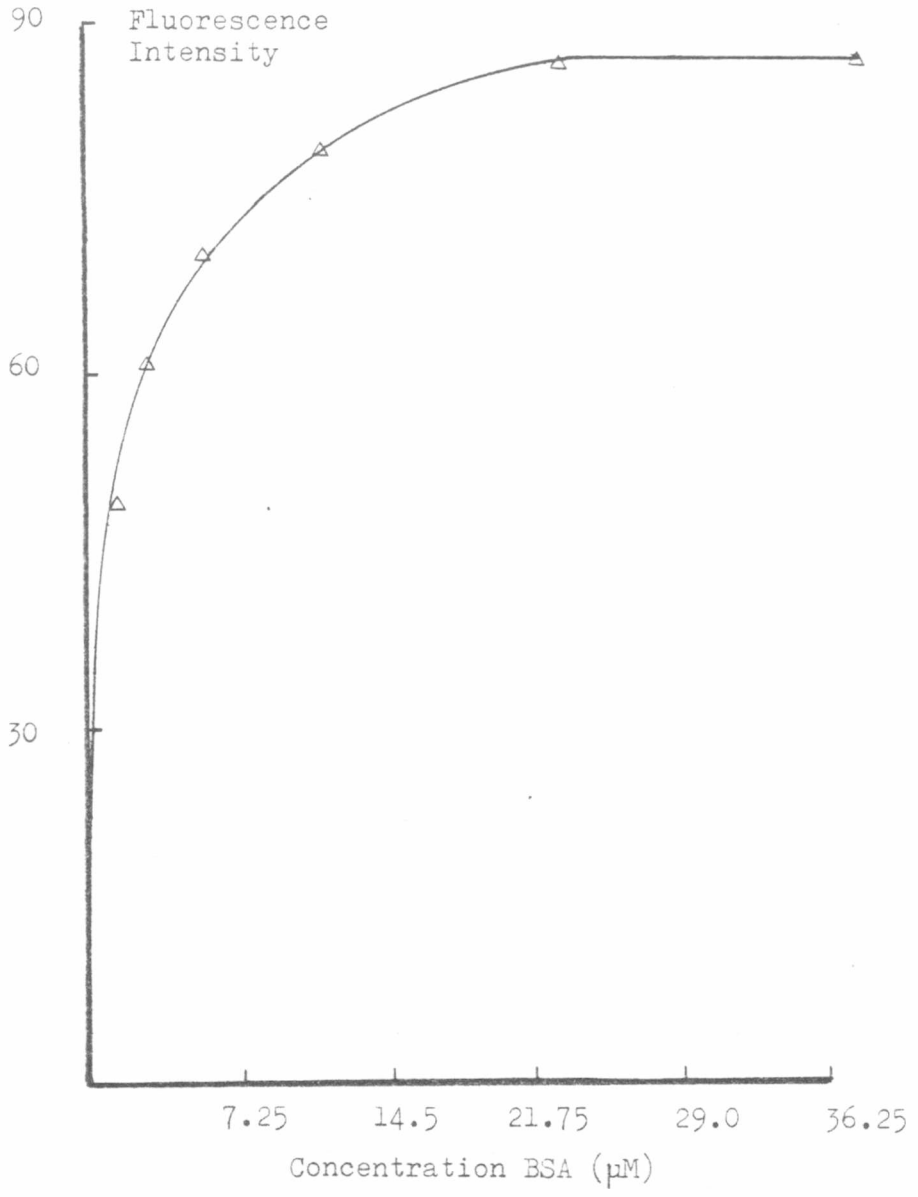


Figure 2.6

A graph showing the fluorescence enhancement of 1μM warfarin when titrated with BSA using an excitation wavelength of 340nm and emission wavelength of 490nm.

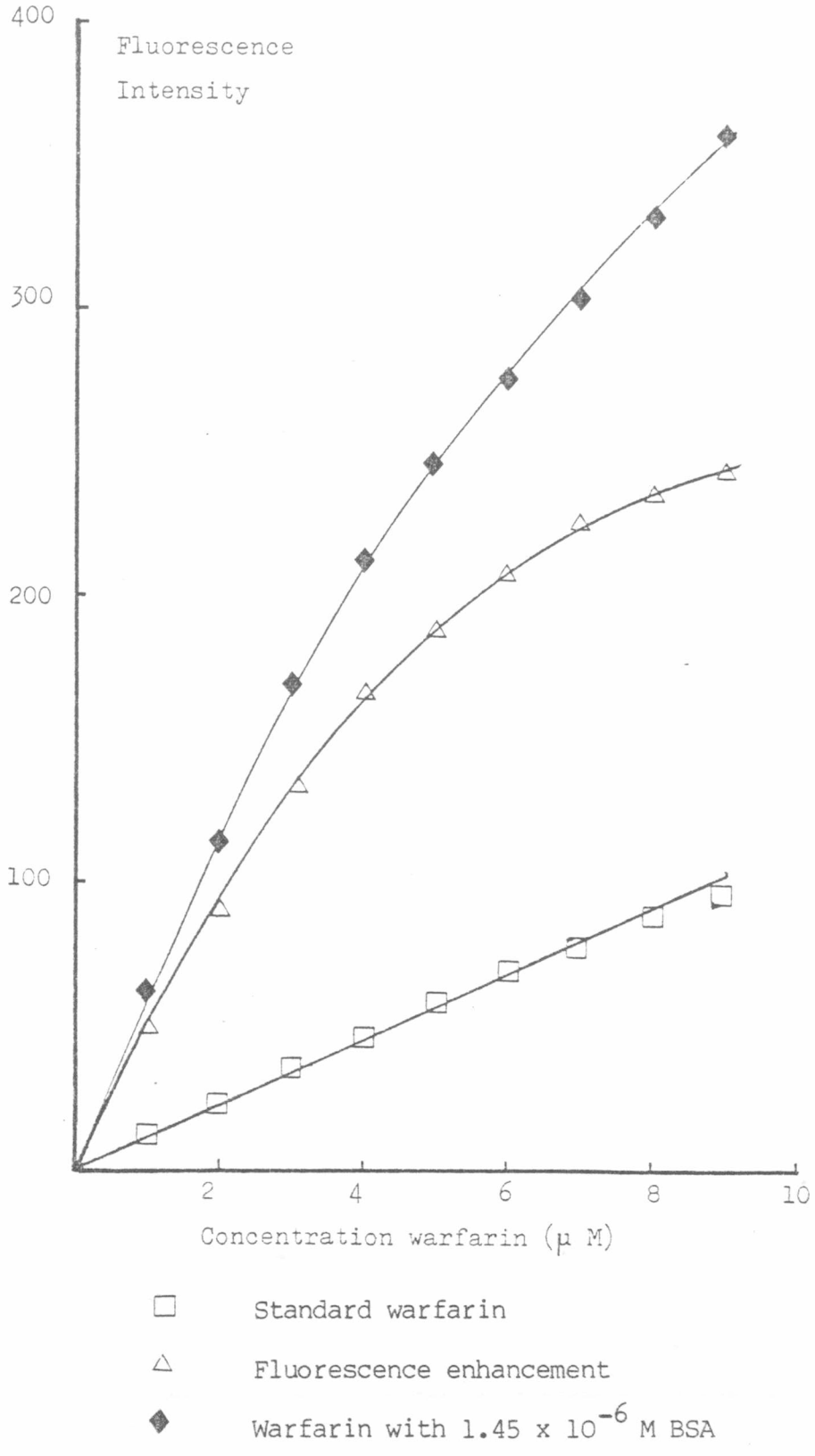


Figure 2.7

A graph showing the fluorescence enhancement of warfarin when a 1.45 x 10^{-6} M solution of BSA is titrated with warfarin.

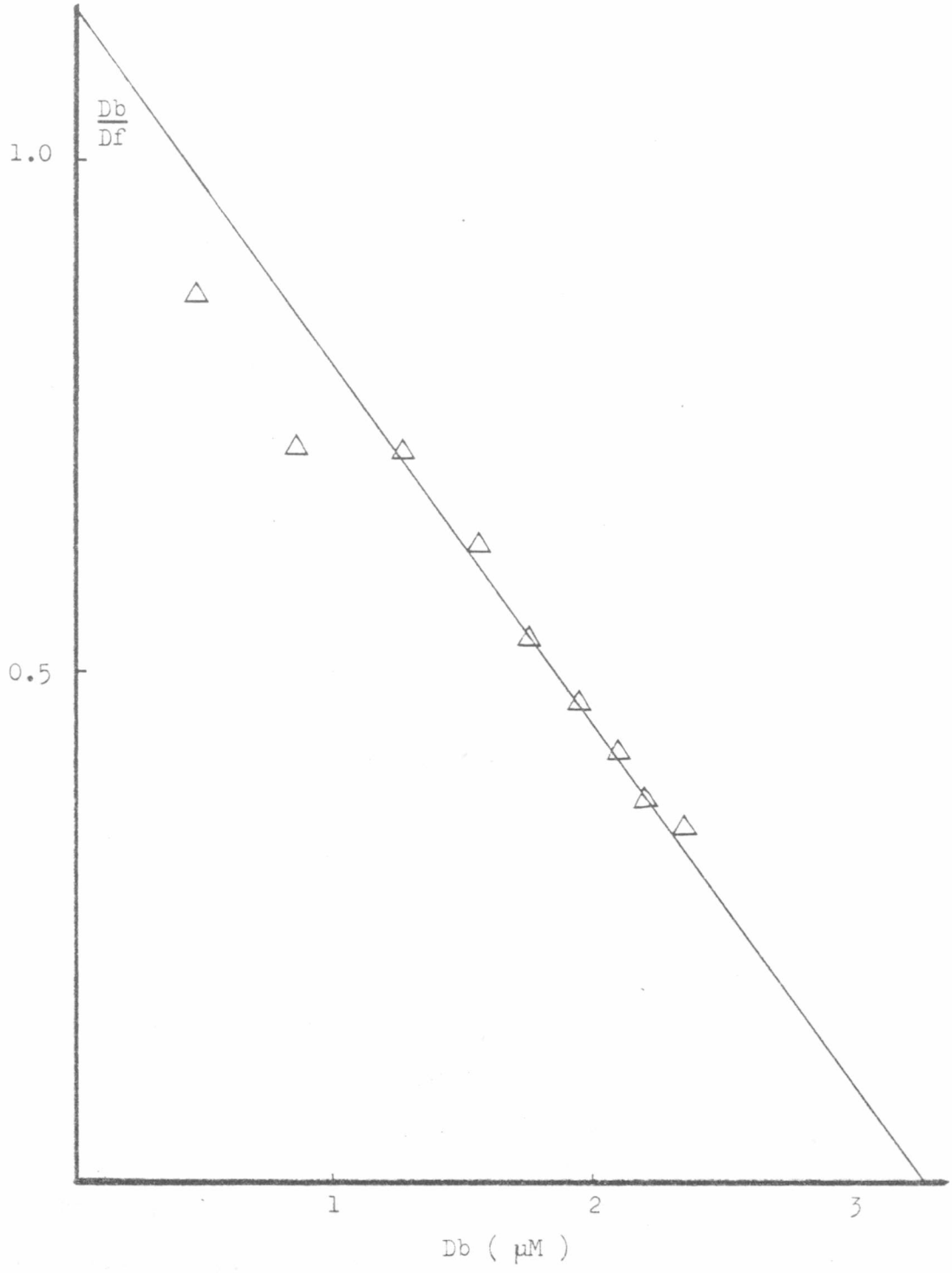


Figure 2.8

A Rosenthal plot for the binding of warfarin to 1.45×10^{-6}M BSA. Db and Df calculated from fluorescence enhancement data.

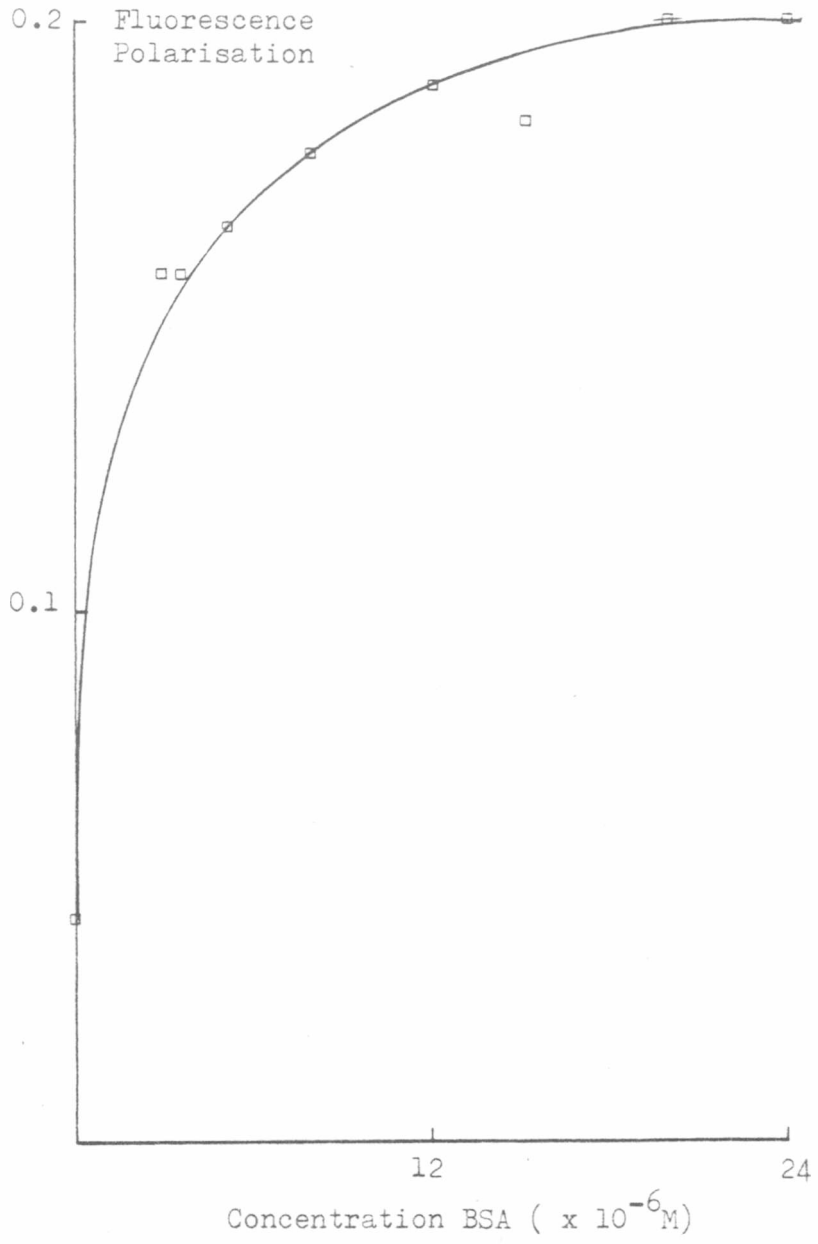


Figure 2.9

A graph showing the change in the fluorescence polarisation of 1 μM warfarin when titrated with BSA using an excitation wavelength of 320nm and emission of 380nm.

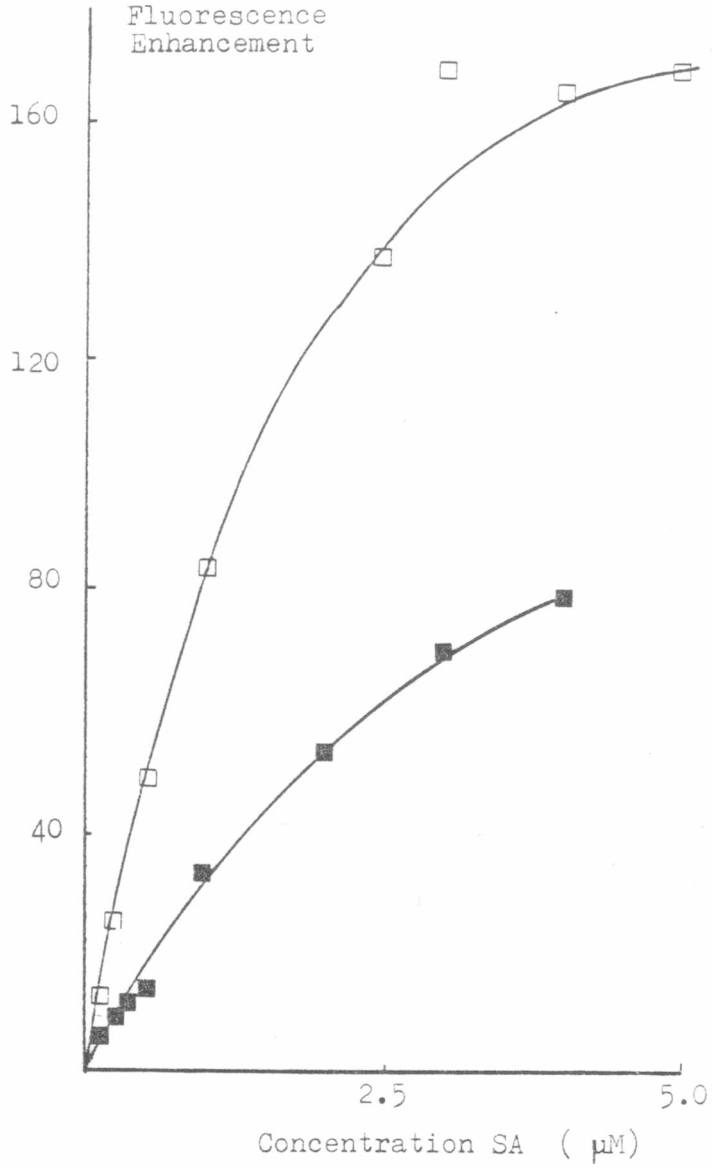


Figure 2.10

A graph showing the effect of titrating 7.25 x 10^{-6} M BSA with SA using Sigma and Koch light BSA. An excitation wavelength of 305nm and emission wavelength of 410nm were employed.

■ Koch BSA □ Sigma BSA

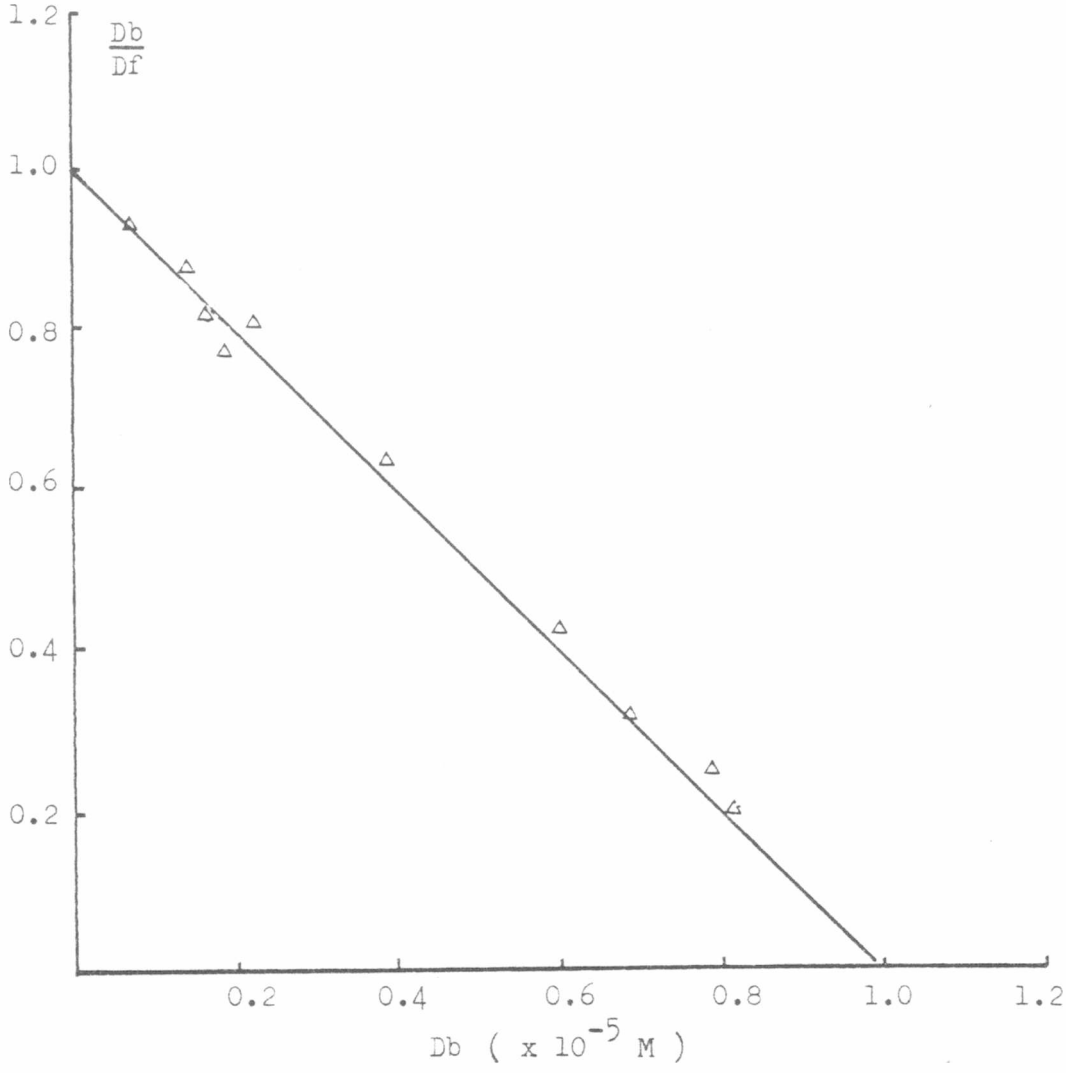


Figure 2.11

A Rosenthal plot for the binding of SA to 7.25 x 10^{-6}M BSA. Db and Df calculated from fluorescence intensity measurements.

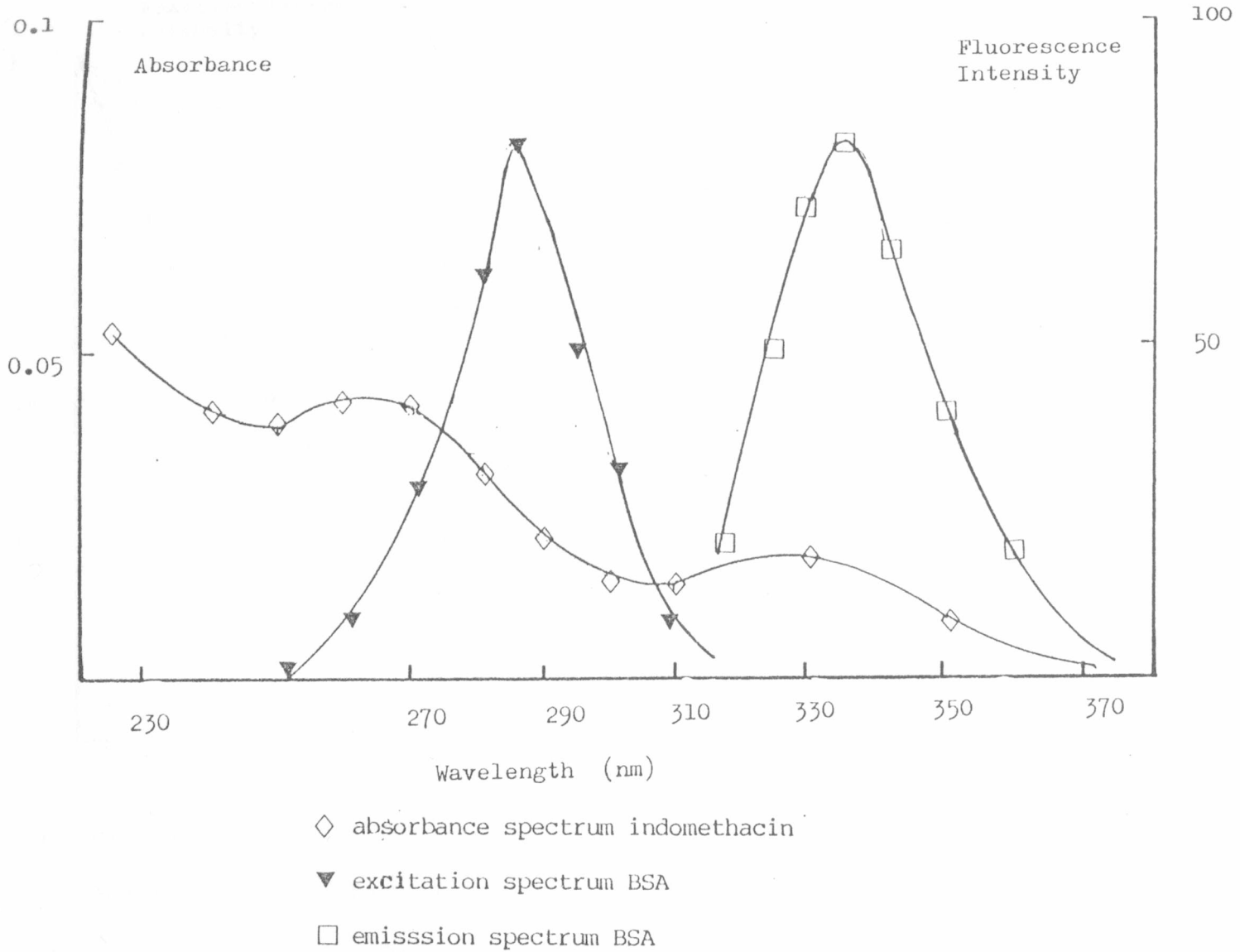


Figure 2.12

A graph showing the absorbance spectrum of indomethacin and BSA and the emission spectrum of BSA.

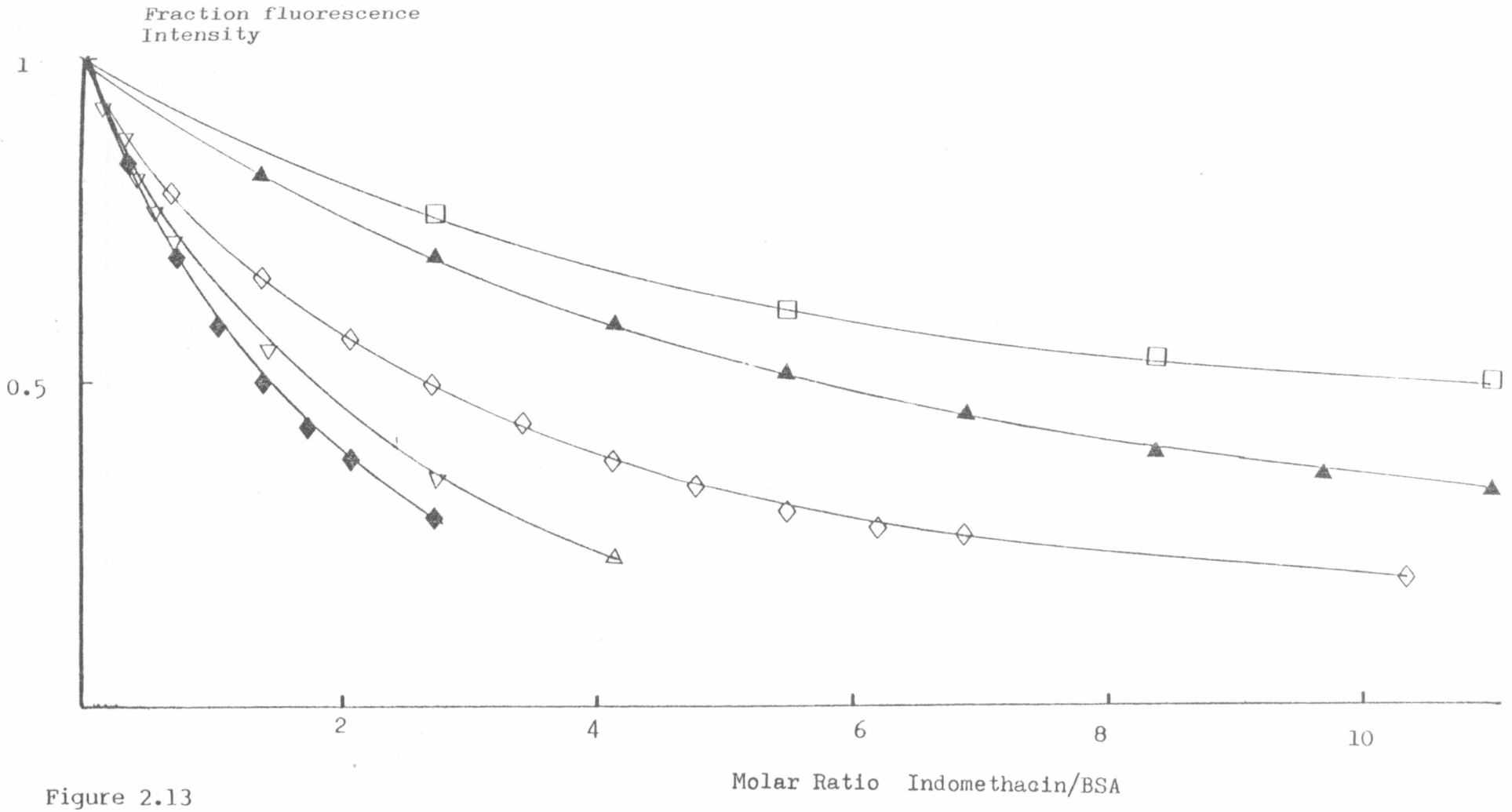


Figure 2.13

A graph showing the effect of protein concentration on the percentage fluorescence intensity of BSA against the ratio of indomethacin concentration to BSA using an excitation wavelength of 300nm and emission wavelength of 335nm.

◆ 14.5 ▽ 7.25 ◇ 1.45 ▲ 0.725 □ 0.36 μM BSA

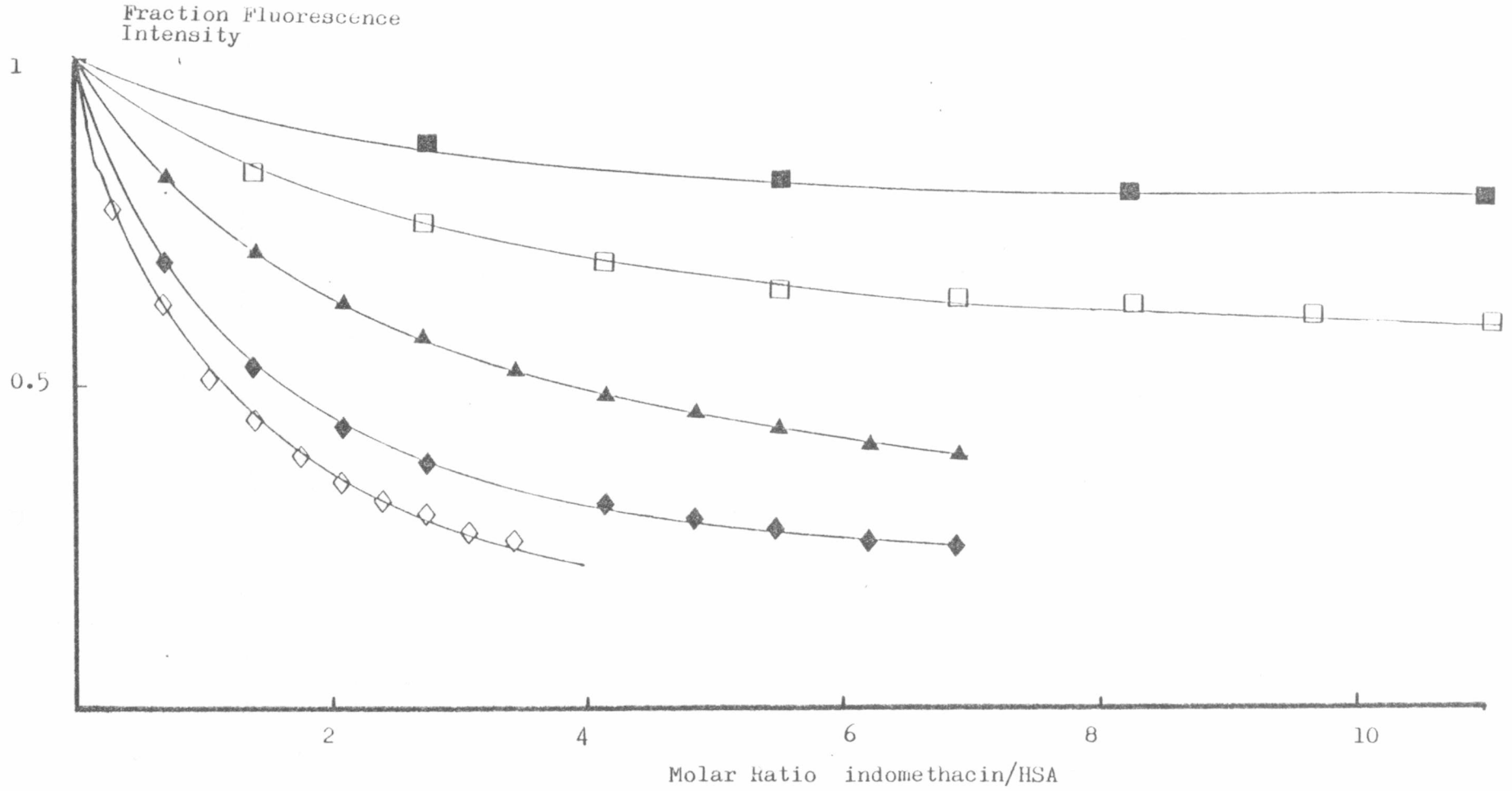


Figure 2.14

A graph showing the effect of protein concentration on the percentage fluorescence intensity of the HSA against the ratio of indomethacin to HSA using an excitation wavelength of 300nm and emission wavelength of 335nm.

◇ 14.5 ◆ 7.25 ▲ 1.45 □ 0.725 ■ 0.36 μM HSA

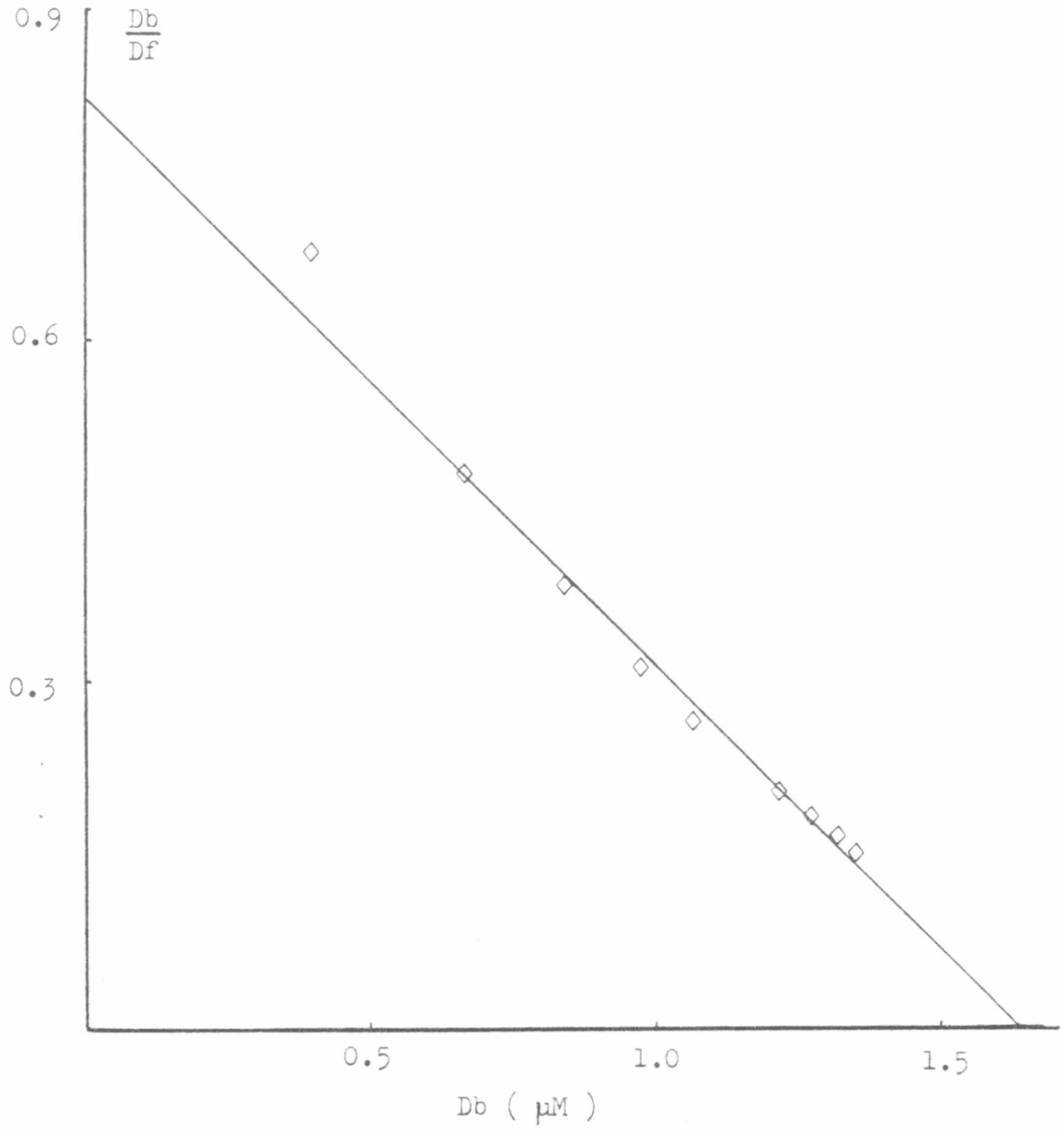


Figure 2.15

A Rosenthal plot showing the binding of indomethacin to 1.45 x 10^{-6} M HSA

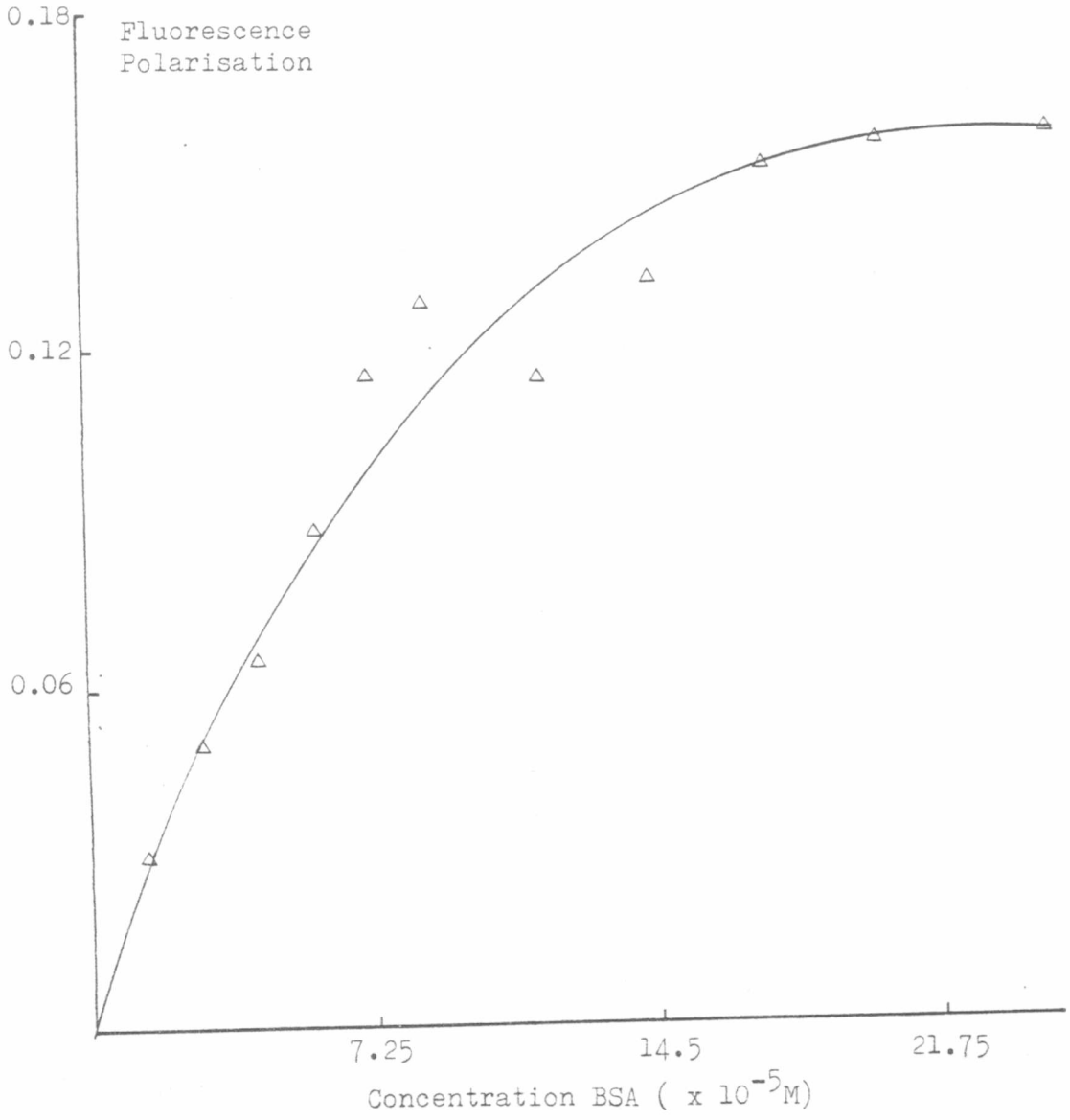


Figure 2.16

A graph showing the effect on the fluorescence polarisation of DPD when 1μM DPD is titrated with BSA using an excitation wavelength of 420nm and emission wavelength of 490nm.

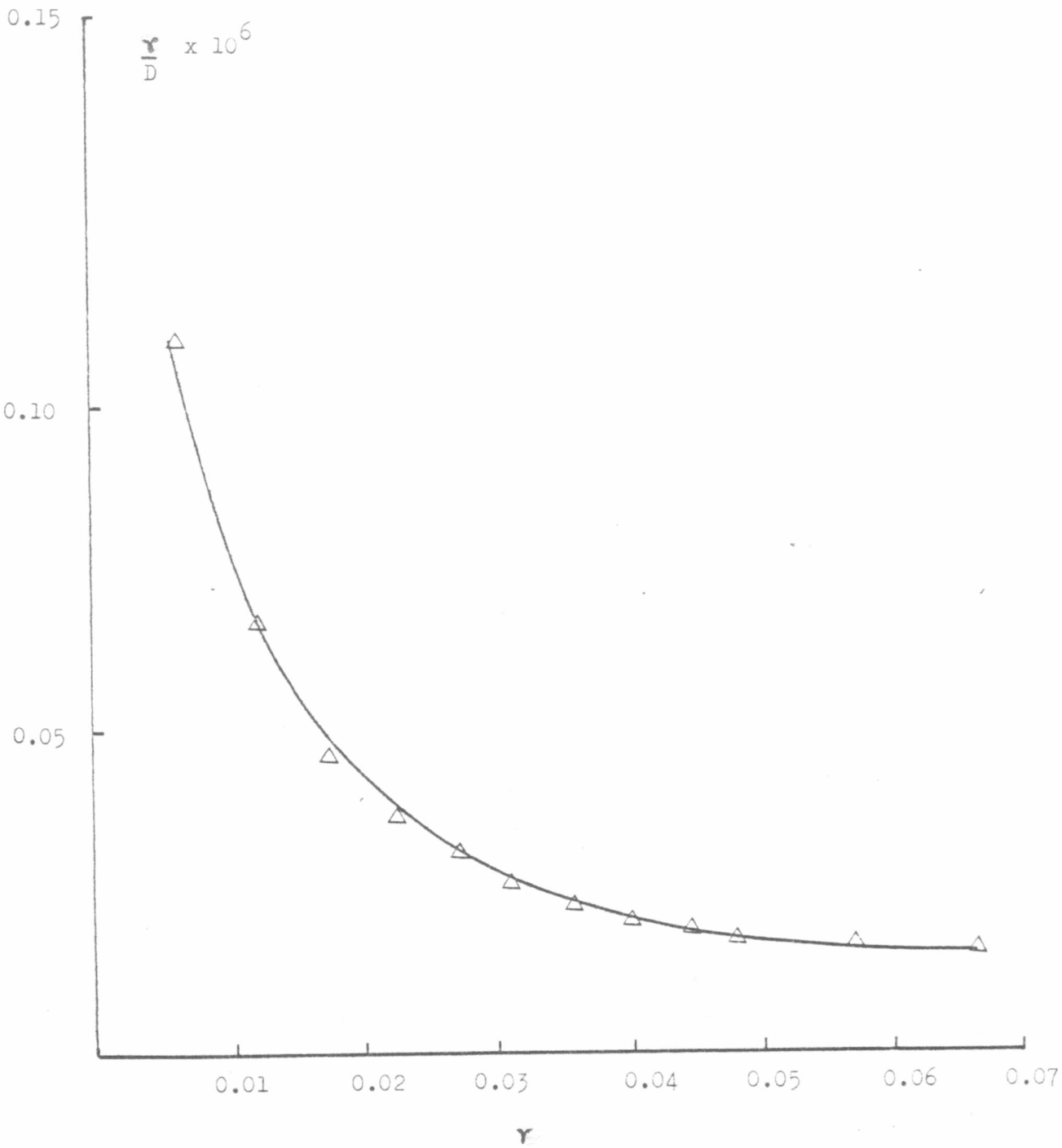


Figure 2.17

A Scatchard plot for the binding of DPD to BSA. Data calculated from fluorescence polarisation

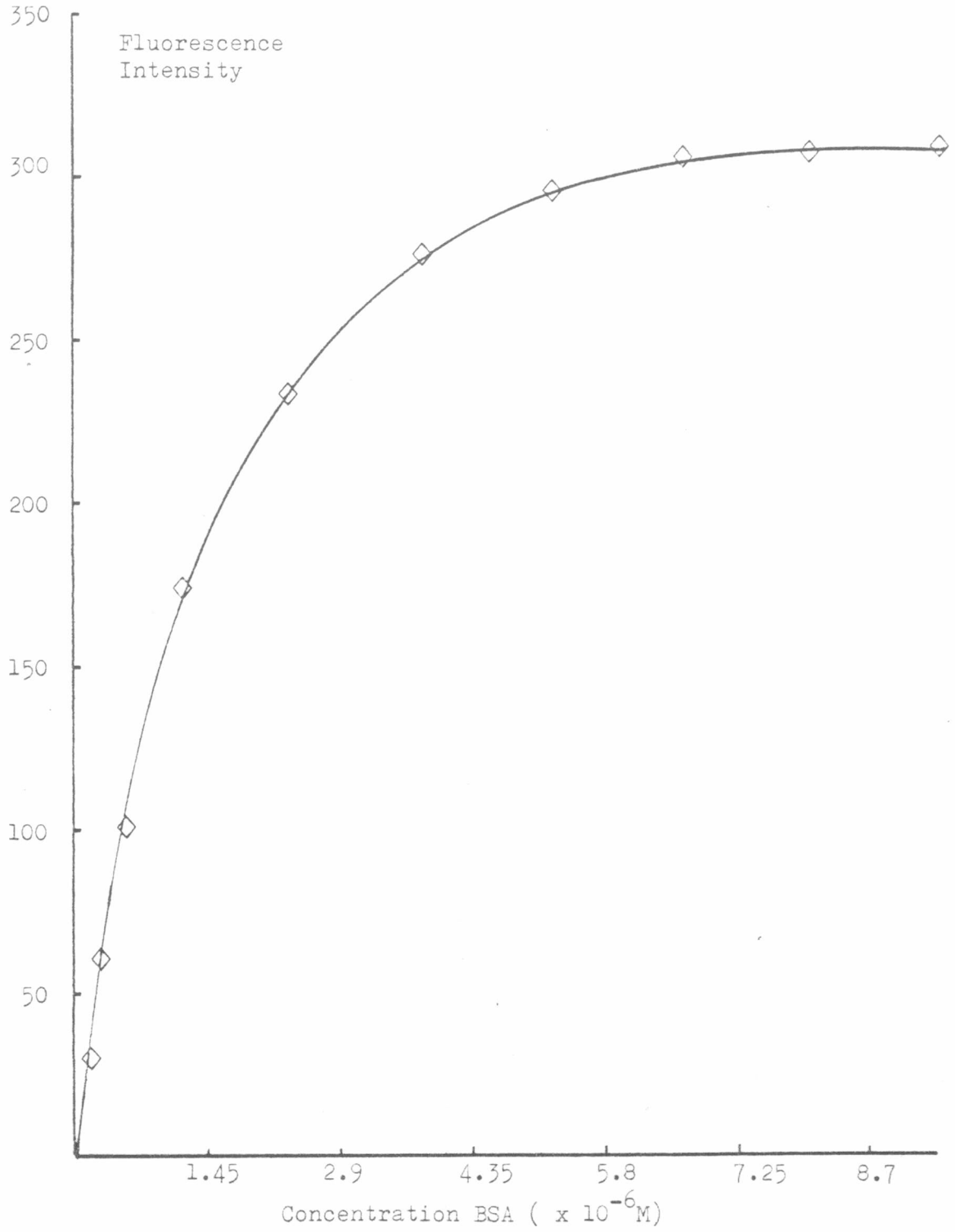


Figure 2.18

A graph showing the effect of titrating 1μM SH 1242 with BSA

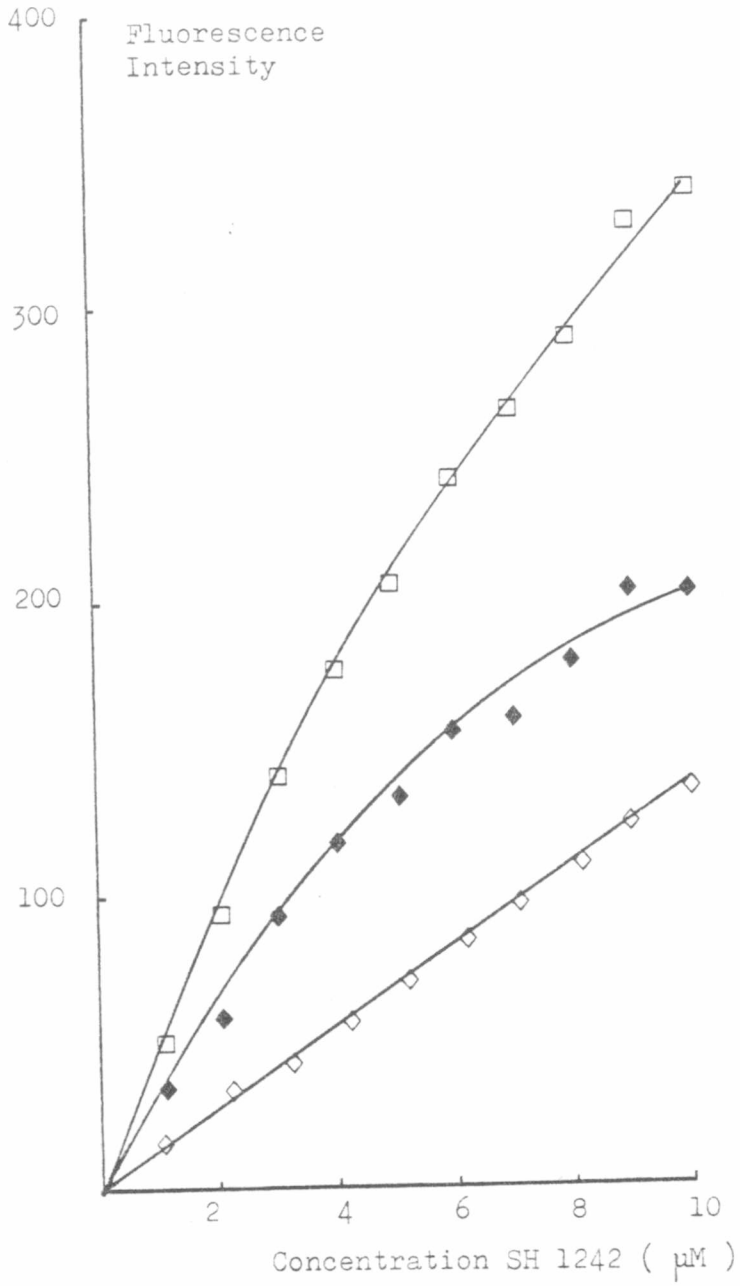


Figure 2.19

Effect of 1.45 x 10 $^{-6}$M BSA on the fluorescence intensity of SH 1242

◇ standard □ SH 1242 with BSA ♦ enhancement

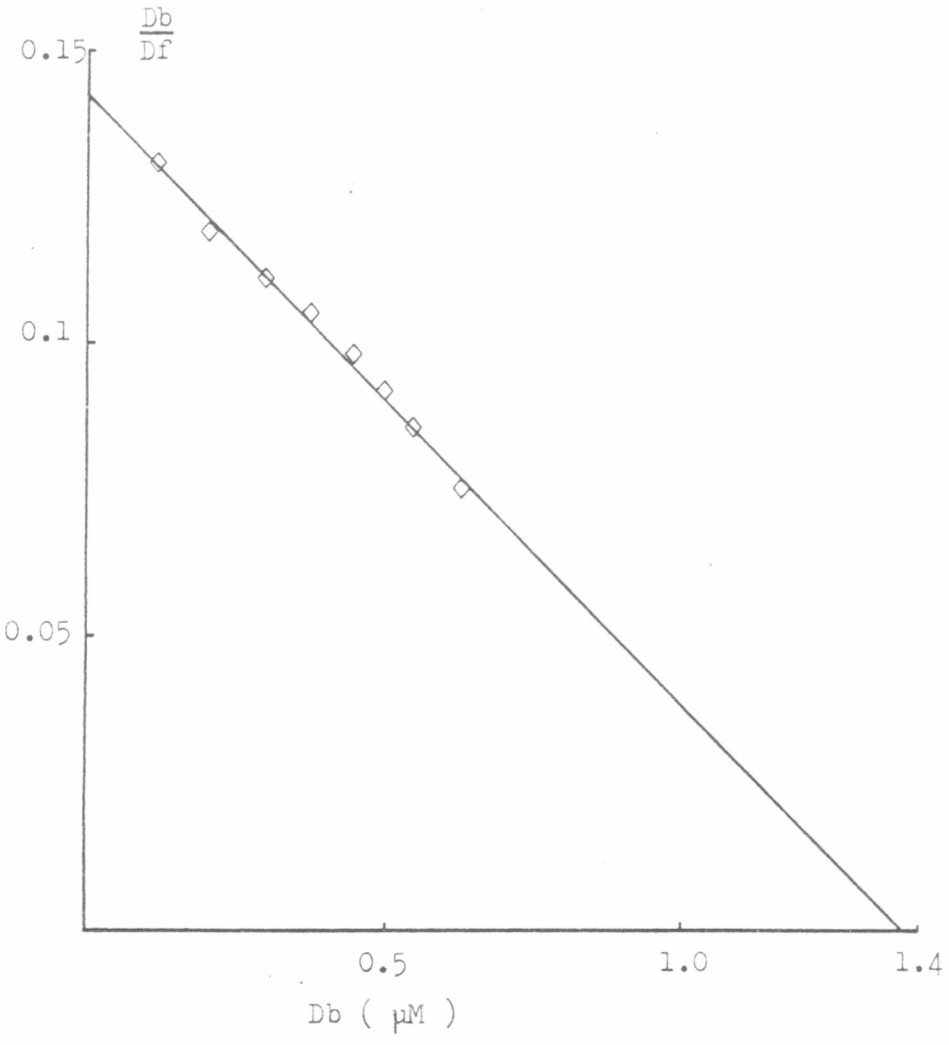


Figure 2.20

A Rosenthal plot for the binding of SH 1242 to 1.45×10^{-6} M BSA

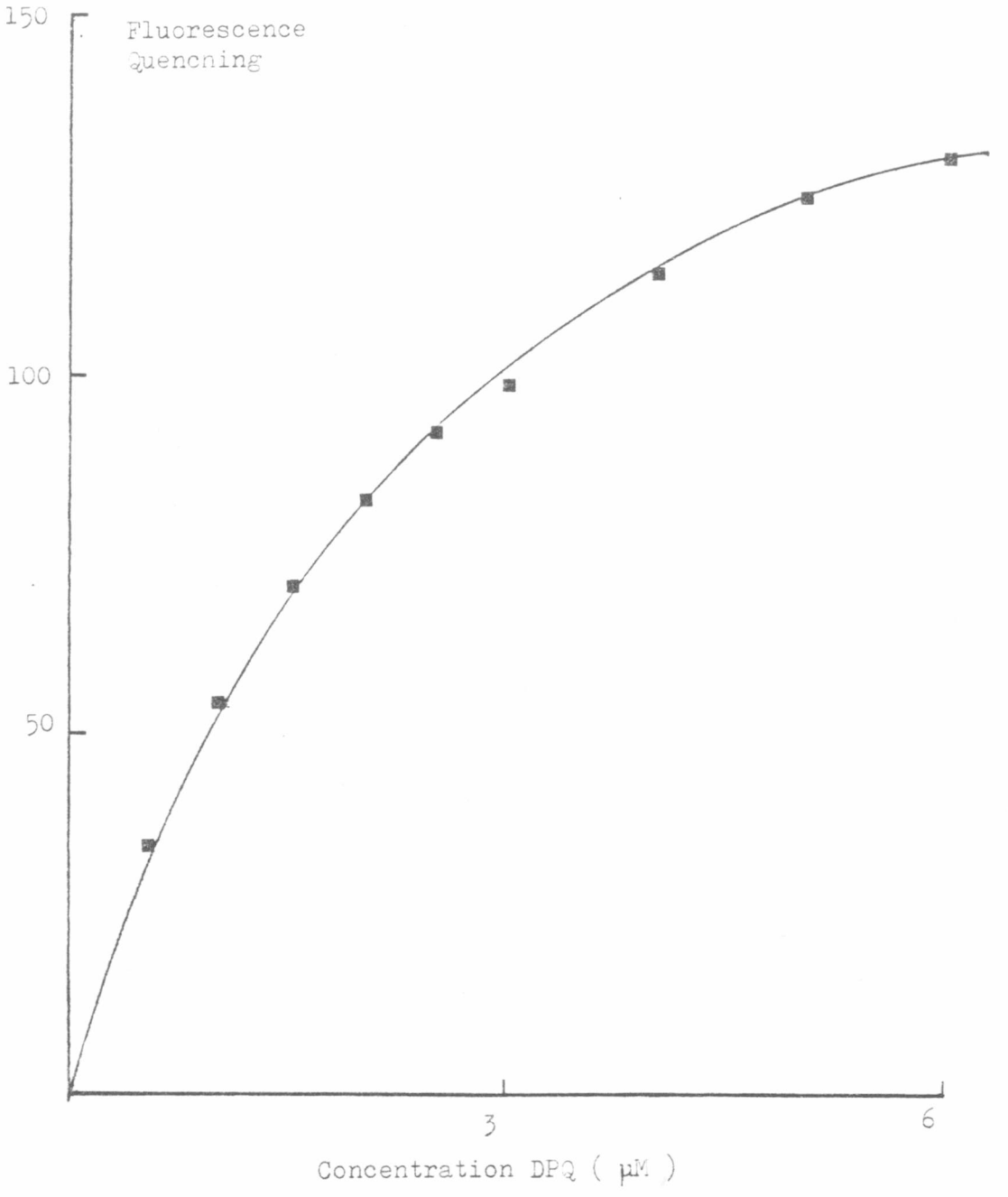


Figure 2.21

A graph showing the fluorescence quenching of DPQ on binding to 1.45 x 10^{-6} M BSA.

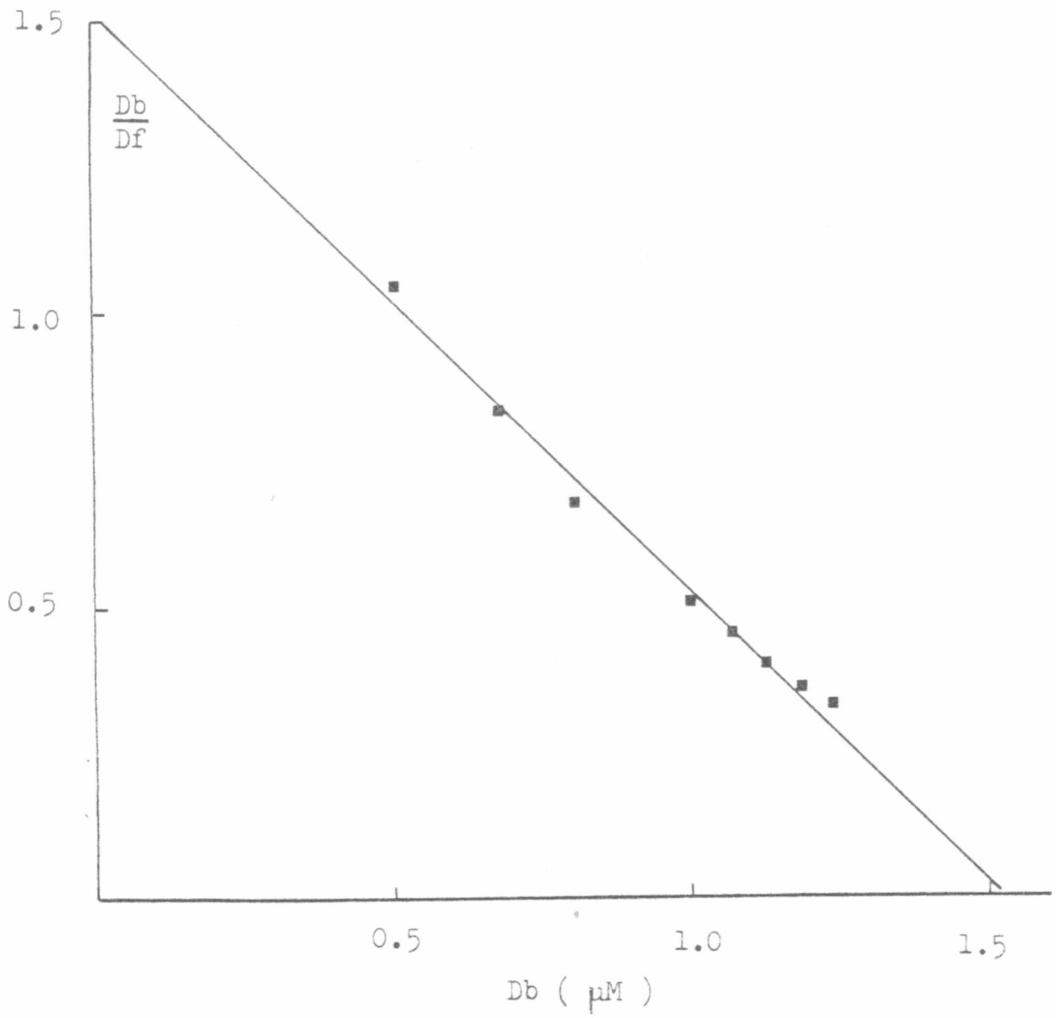


Figure 2.22

A Rosenthal plot for the binding of DPQ to 1.45 x 10^{-6} M BSA

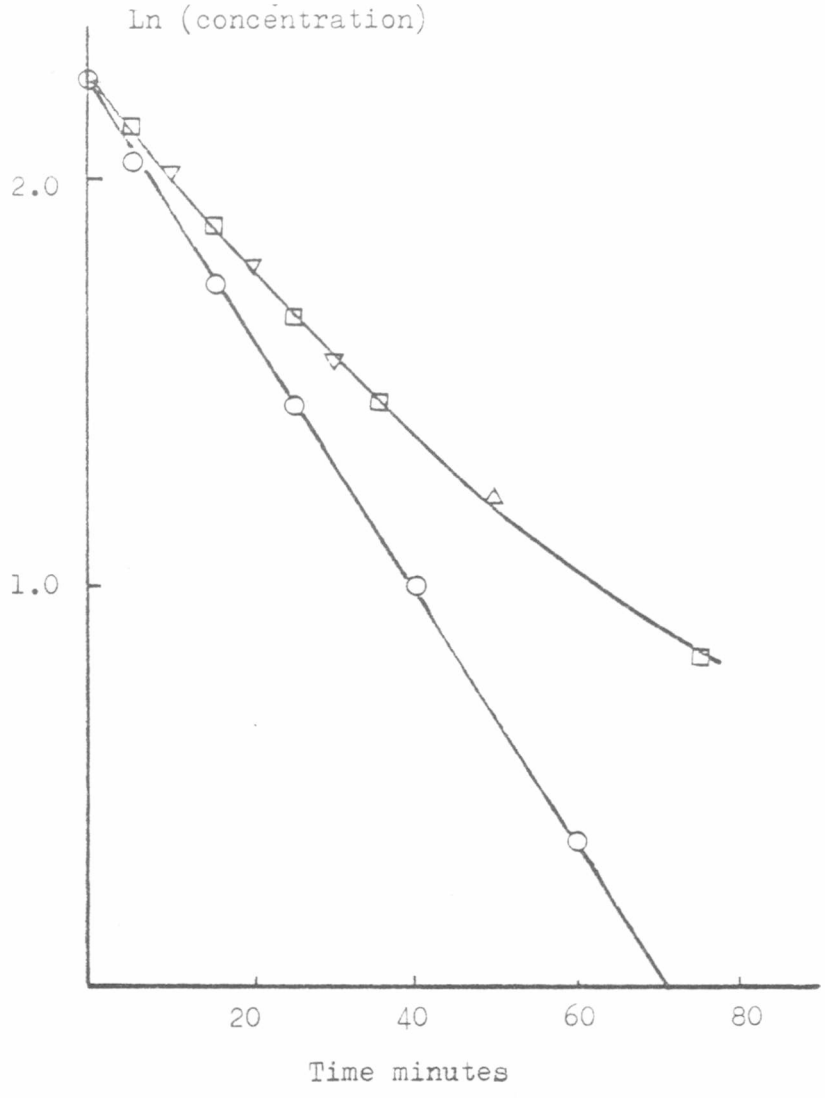


Figure 2.23

A plot of Ln (concentration) against time demonstrating that calcium ions have no effect on the binding of SA.

○ standard □ 1.45 x 10^{-5}M BSA △ 1.45 x 10^{-5}M BSA +2mM Ca^{2+}

CHAPTER THREE

THE DEVELOPMENT OF A NEW SEPHADEX BATCH METHOD FOR MEASURING PROTEIN LIGAND INTERACTIONS

THE DEVELOPMENT OF A NEW SEPHADEX BATCH METHOD FOR MEASURING PROTEIN LIGAND INTERACTIONS

3.1 INTRODUCTION

Dextran gels allow the separation of compounds with different molecular weights. One of the most widely used dextran gels is Sephadex. Sephadex is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin, the pore size depending on the degree of cross-linking. The large number of hydroxyl groups renders the gel extremely hydrophilic, consequently Sephadex swells in water and electrolyte solutions. It is stable in water, salt solutions, organic solvents, alkaline and weakly acidic solutions. Sephadex was first introduced in 1959 by Porath and Flodin, and since then numerous applications have been found, including the investigation of protein binding.

3.1.1 The use of dextran gels for measuring protein binding

The use of dextran gels for measuring macromolecule ligand interactions can be divided into two basic methods :-

1) Batch methods
2) Chromatographic methods

3.1.1.1 Batch Method

The batch method simply uses the Sephadex to estimate the free concentration of the ligand when a macromolecule ligand mixture is added to the gel. The protein and bound ligand are excluded from the gel matrix and internal volume while the free ligand partitions freely between the external and internal phases. The batch method in

principle is therefore very similar to equilibrium dialysis.

Fasella et al. (1965) first reported the use of a dextran gel batch method for determining macromolecule ligand interactions. In their study the interaction between ribonuclease and cytidine 3'-phosphate was investigated using Sephadex G-25. To show the validity of their method the results were compared with a difference spectroscopy and steady state kinetics method. One of the problems of the Sephadex batch method was brought to light , that of adsorption of some ligands onto the Sephadex.

Ashworth and Heard (1966) applied the batch method to determine the interaction between a preservative and a surfactant. They stated that this method has the advantage of a rapid equilibration time and ready availability of suitable gels of various pore sizes. Its main disadvantage is that the concentration of the ligand can only be determined in one phase, that containing the macromolecule. Consequently any error in this determination results in an error in the amount associated with the gel. It was reported that such errors are minimised if the volume of the external phase is chosen so that the quantity of ligand associated with this phase is approximately equal to the quantity associated with the gel.

Pearlman and Crepy (1967) used the batch method for studying the interaction between testosterone and human serum. Using Sephadex G-25 the affinity constant of testosterone to human serum was determined. A method for the evaluation of the data was presented. It was seen that it was critical to make a precise determination of the partition coefficient of the ligand between the gel's internal and external volume.

Ahtee et al. (1967) also used this method for measuring the binding of ligands to serum albumin. In their study the binding of phenothiazines to human serum were investigated. They stated that if the gel was allowed to swell in buffer for 60 minutes, this would minimise the protein adsorption.

Souliel and Nisonoff (1968) applied the method to the measurement of an antigen-antibody interaction. The main difficulty encountered was that of nonspecific adsorption to the gel of the protein at low concentrations. This is not normally a problem except when working with low ionic strengths. In his conclusion it was stated, 'the precision of the method is poor when a small percentage of ligand is bound because of adsorption of the ligand onto the gel.'

In more recent times Gueriguian (1976) exploited the rapid equilibration time in his investigation of the binding of prostaglandins to human serum albumin. Prolonged equilibration times would allow marked decomposition of the prostaglandins but using the batch method equilibrium was reached in under 30 minutes.

3.1.1.2 Chromatographic methods

Over the same period that the batch method was being developed chromatographic procedures were being investigated. A theory for gel filtration was first suggested by Laurent and Killander (1964). Their work was mainly applied to the separation of compounds of different molecular weight but equally applies to the investigation of binding interactions.

A method for measuring protein ligand interactions was suggested by Hardy and Mansford (1962), Quincey and Winzor (1964), Doe et al. (1964). For a complex which dissociates much slower than the rate of elution a small sample of the macromolecule ligand mixture can be added to the column which is eluted with buffer, This will separate the complex and free ligand. The problem with this method for investigating plasma protein binding is that the initial assumption that the dissociation rate is much slower than the rate of elution is not normally correct.

If the dissociation rate constant is not much less than the elution time, the column can be pre-equilibrated with the ligand at the same concentration as the ligand macromolecule mixture. The ligand macromolecule can then be added to the column and eluted with the ligand solution (Hummel and Dryer, 1962). This method requires large quantities of the ligand and equilibrating the column to each concentration of ligand is very time consuming. It may also be necessary to estimate the free concentration of the ligand before starting the experiment.

Cooper and Wood (1968) developed a frontal analysis method, which overcame many of the problems associated with the chromatographic method, in their investigation of the binding of sulphonamides to plasma. A large volume of BSA sulphonamide mixture was applied to the column and was then eluted with buffer. The concentration of ligand in the plateau regions of the chromatogram were used to calculate the binding of the sulphonamides to the BSA. But this method has several problems :-

1) It is very slow when a series of concentrations of ligand and

protein need to be investigated.

2) It is difficult to establish the conditions for the experiment so that three plateau regions are given in the chromatogram.

3) Large sample volumes are required.

Chen and Danon (1979) used an equilibrium gel filtration chromatographic method for their study of the binding of reserpine to albumin and lipoprotein. Their method again had the problem of estimating the free concentration of reserpine first, so that the column could be pre-equilibrated with that concentration.

Despite the problems associated with the gel filtration chromatographic method the batch method has fallen into disuse while the chromatographic method has been applied to numerous binding systems in recent times.

3.1.2 Adsorption of ligands onto the Sephadex

As shown above one of the major problems of the Sephadex batch method is adsorption of the ligand onto the Sephadex. Gelotte (1960) discussed the adsorption properties of Sephadex gel in his paper. He concluded for organic compounds, aromatic and heterocyclic substances have a greater tendency to be adsorbed than aliphatic substances. Basic groups in a molecule seems to increase, and acidic groups to decrease the adsorption. Jansen (1967) pointed out that electrostatic interactions are due to the fact that the cross-linked dextran chains contain a few carboxyl groups. The higher the matrix density of the Sephadex gel used, the more pronounced the adsorption.

3.1.3 Aims and objectives

The primary aim of this section was to develop a dextran gel batch method to investigate macromolecule ligand interactions using fluorescence to detect the ligand directly in the gel. By so doing it should be possible to overcome the criticism of this method, made by Cooper and Wood (1968) and Ashworth and Heard (1966), that only the composition of the external volume could be assayed with any precision, thus the free and bound concentration of the ligand cannot be determined directly. This method was used to quantify the binding of various fluorescent cationic and anionic aromatic and heterocyclic compounds to BSA, AGP, and plasma. Theoretical aspects of measuring the fluorescence intensity of a ligand in the gel were also considered. This included the study of the effect of path length, slit width, temperature, pore size of the gel, ratio of internal to external volume and ionic strength of the buffer.

3.2 MATERIALS

Sephadex G-10, G-15, G-25, G-50 and G-75 were purchased from Pharmacia, Uppsala, Sweden.

Chlorpromazine and propranolol were obtained from Sigma Chemical Co.

All chemicals were used without further purification.

A M/15 phosphate buffer pH 7.4, was used for all solutions unless otherwise stated.

3.3 METHODS

3.3.1 Water regain of the Sephadex gel

Determination of the water regain of the Sephadex was performed using three methods.

1) The void volume of the Sephadex was removed by filtration from Sephadex which had been allowed to swell in buffer for 24 hours. To 100mg of Filtered Sephadex 25ml of Karl-Fisher reagent was added and this was titrated with a standardised water solution in methanol using a dead stop end point.

2) 1g of dried Sephadex gel was weighed and 6ml of M/15 phosphate buffer was added. After 24 hours the void volume was removed by filtration through a sintered glass filter and the internal volume determined gravimetrically.

3) 6ml of 1.45×10^{-6} M BSA solution was added to 1g of dried gel. After 24 hours the concentration of BSA in the supernatant was determined using fluorescence, thus allowing the internal volume to be calculated.

3.3.2 Fluorescence

To 500mg of Sephadex G-25, 3ml of M/15 phosphate buffer was added and this was mixed for 5 minutes at room temperature. The gel was poured into a 10 X 10 mm quartz cuvette and allowed to settle under gravity. The Sephadex gel layer was placed directly in the fluorescence excitation beam and the excitation and emission spectra recorded. After determining the time for SA to equilibrate with the gel a calibration curve was determined using a series of SA solutions in the range of 10^{-6} to 2×10^{-5} M with an excitation wavelength of 305nm and an emission wavelength of 410nm. The fluorescence intensity

of the supernatant was also measured. The effect of temperature, grade of gel, path length, slit width, polarisation and ionic strength of the buffer were studied. The effect of a glass filter placed in the emission path was investigated. Using the Baird Atomic frontal scatter attachment the above experiments were repeated. The effect of preswelling the gel for 24 hours before titration with the ligand was studied. Various methods for packing the Sephadex gel in a cuvette were investigated including settling under gravity and packing by gentle vibration.

BSA was added to the system and a titration with SA was performed. The results were compared with a sample with no BSA. Various methods were investigated for removing the void volume of the gel. The most satisfactory method was to remove the bottom of a cuvette and adhere in its place a fine membrane. The Sephadex suspension could be poured into this cuvette and the void volume removed by the application of gentle suction. The fluorescence of the gel without the void volume could then be measured. The effect of changing the volume of external phase was investigated using 1g of dried gel with 6, 10, 25, 50, and 100ml of buffer.

3.3.3 Binding studies

Binding studies were conducted between ligands and BSA or plasma using 1g of dried Sephadex G-25 gel made up to 50ml volume with phosphate buffer. Either a fixed concentration of ligand was titrated with protein or protein titrated with ligand. The sample was well mixed after each addition in the titration and the free ligand allowed to equilibrate with the gel. For AGP binding studies 200mg of dried Sephadex G-25 gel was made up to 5ml volume with the buffer. A path

length of 5mm was used for the fluorescence measurements in both cases with 10nm excitation and emission slits. All readings were taken at 20 ^{o}C.

3.3.4 Data analysis

When Pt >> Db the binding data was analysed according to the method of Pearlman and Crepy (1967). The association constant k for the ligand protein interaction is given by the relationship

$$P + Df \rightleftharpoons PD$$

$$k = \frac{PD}{P \quad Df} \qquad -(3.1)$$

$$k = \frac{Db}{(nPt-Db) \quad Df} \qquad -(3.2)$$

where

Db= concentration of drug bound

Df= concentration of free drug

Pt= total concentration of protein

P= free concentration of protein

PD= concentration of complex

n= the number of binding sites with the affinity constant k

From this Pearlman and Crepy (1967) suggested two ways of analysing the data

When Pt >> Db

$$Pt-Db \Longrightarrow Pt$$

$$k = \frac{Db}{Df\ n\ Pt} \qquad -(3.3)$$

$$\frac{Df}{Db} = \frac{1}{k} \quad \frac{1}{n\ Pt} \qquad -(3.4)$$

A plot of Df/Db against 1/Pt gives a straight line with a gradient of 1/n k

alternatively

$$\frac{1}{Db} = \frac{1}{n\ k} \quad \frac{1}{Pt} \quad \frac{1}{Df} \qquad -(3.5)$$

Therefore a plot of 1/Db against 1/Df gives a linear response with a gradient of 1/n k Pt

An alternative approach is that which was developed by Blanchard (1982) for the binding of caffeine to plasma proteins.

Rearranging equation 3.2 gives

$$Db = \frac{nPt\ Df\ k}{1 + Df\ k} \qquad -(3.6)$$

$$Db = \frac{nPt\ Df}{1/k + Df} \qquad -(3.7)$$

When 1/ k >> Df

$$Db = \frac{n\ Pt\ Df}{1/k} \qquad -(3.8)$$

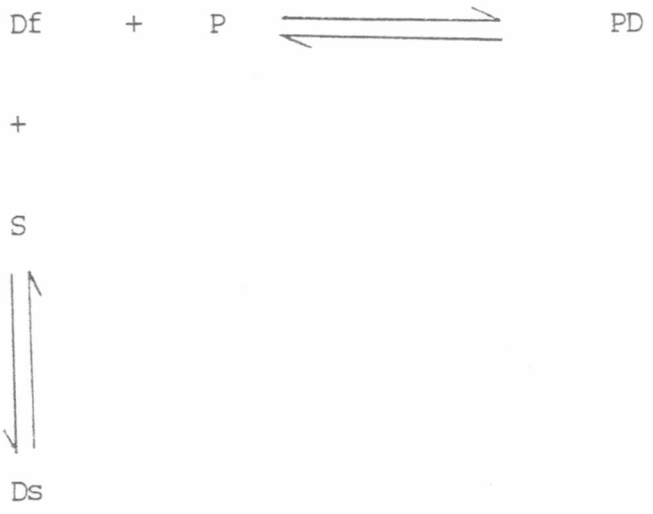
$$Db = n\ Pt\ k\ Df \qquad -(3.9)$$

The results from this approximation gives equivalent equations to that obtained from Pearlman and Crepy (1967) but obtained by making a different initial approximation.

When Pt was not very much greater than Db the data was analysed as previously discussed using either a one site model (equation 2.34), one site with partition (equation 2.36) or a two site model (equation 2.35) The data was represented graphically either by a Scatchard plot (1949) or a Rosenthal plot (1967).

3.3.4.1 Calculation of Db and Df from the Sephadex fluorescence

An equilibrium exists between the ligand and the protein and also between the ligand and the Sephadex.



Where

Ds is the concentration of ligand within the gel including the amount adsorbed

K' is the partition constant of the ligand between the gel and the supernatant

$$K' = \frac{Df}{Ds} \qquad -(3.11)$$

$$Df = K' Ds \qquad -(3.12)$$

(3.11) in (3.2)

$$k = \frac{Db}{K' Ds (nPt - Db)} \qquad -(3.13)$$

K' and Ds are not the most convenient constants to evaluate. It is more convenient to use the total moles of ligand added to the system and the concentration of ligand in the supernatant when no protein is added to the system.

A partition factor K'' can be defined as

$$K'' = \frac{Df}{Dt} \qquad -(3.14)$$

where

Dt is the concentration of ligand added to the system.

Df is the free concentration of ligand in the supernatant

When protein is added to the system the concentration of ligand in the gel is given by

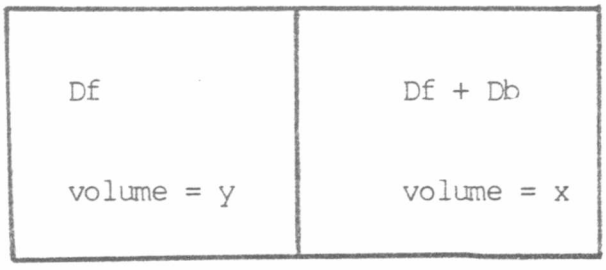
$$Dsp = \frac{Df}{K''} \qquad -(3.15)$$

where

Dsp is the concentration of ligand in the Sephadex when protein is added to the system

$$Df = K'' Dsp \quad -(3.16)$$

From this value of Df, Db can be calculated.



Internal volume External volume

When $K'' \Longrightarrow 1$

Total moles added to the system = moles in the internal volume + moles in the external volume

$$x\,Dt + y\,Dt = x\,Df + y\,Df + x\,Db \qquad (3.17)$$

$$Dt + \frac{(y\,Dt)}{x} = Df + \frac{(y\,Df)}{x} + Db \qquad (3.18)$$

$$Dt\,(1 + y/x) = Df\,(1 + y/x) + Db \qquad (3.19)$$

$$Db = (Dt - Df)(1 + y/x) \qquad (3.20)$$

when $x >> y$

$1 + y/x \Longrightarrow 1$

$$Dt - Df = Db \qquad (3.21)$$

where

x is the external volume

y is the internal volume

Comparing this with the results from equilibrium dialysis, when the

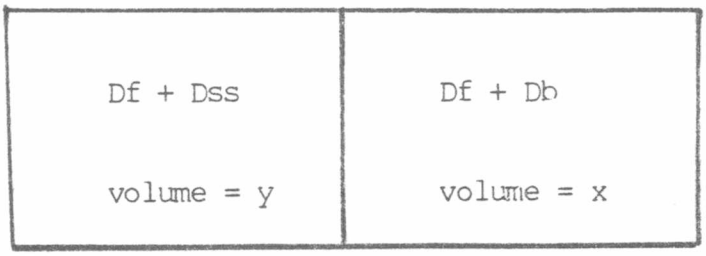
ligand is placed both sides of the membrane and the chamber volumes are equal.

$$2Dt = 2Df + Db \qquad (3.22)$$

$$Db = 2(Dt - Df) \qquad (3.23)$$

These equations are in a different form to the more frequently used equilibrium dialysis equations were the ligand is only placed one side of the membrane. This is the same as the Sephadex results when x=y

When K'' <1



Internal volume external volume

total moles added = moles in the internal + moles external

$$Dt\ x + Dt\ y = Df\ y + Dss\ y + Df\ x + Db\ x \qquad (3.24)$$

$$Dt + Dt\ (y/x) = Df\ (y/x) + Dss\ (y/x) + Df + Db \qquad (3.25)$$

$$Dt(1 + (y/x)) = Df\ (1 + (y/x)) + Dss\ (y/x) + Db \qquad (3.26)$$

$$Db = (Dt - Df)(1 + (y/x)) - Dss\ (y/x) \qquad (3.27)$$

when $y/x \Longrightarrow 0$

$$Db = (Dt - Df) \qquad (3.28)$$

where

Dss is the concentration adsorbed on the Sephadex

3.4 RESULTS AND DISCUSSION

3.4.1 Water regain of the Sephadex

Table 3.1 compares the results of the three methods used to determine the water regain of the Sephadex G-25. A titration of Sephadex with Karl-Fisher reagent gave an end point which was imprecise therefore a back titration was performed. Both the albumin and the gravimetric method gave similar results for the determination of the water regain but the Karl-Fisher back titration consistently gave low results. The water regain of the gel can be divided into two parts, the water of hydration which is firmly bound to the polysaccharide frame work in the gel and is inaccessible to the solute molecules and the internal volume which is accesssible to the solute molecules (Gelotte, 1960). The Karl-Fisher method may only determine the latter water content therefore accounting for the low values given. Haglund and Marsden (1974) pointed out that the complete removal of the void volume may be difficult so that the exclusion method is a more satisfactory technique. The results of the exclusion method of the albumin and the gravimetric method indicate the albumin is not adsorbed onto the Sephadex.

When the internal volume is in the same order as the external volume it is important to have an accurate measurement of the internal volume (Pearlman and Crepy, 1967) in order to determine Db and Df. As the external to internal volume ratio increases, any errors in the

determination of the internal volume will have a smaller effect on the values of Db and Df. When compared with the previous batch methods the precision of this determination is less critical because Df is being determined directly.

3.4.2 Equilibration time

The time for the SA to equilibrate with the gel was found to be less than 5 minutes. The equilibration times for all the other compounds investigated were of the same order as SA, thus demonstrating the clear advantage over equilibrium dialysis in this respect. Fasella et al. (1965) stated that the adsorption process is quite slow compared with the establishment of equilibrium between the internal and external phase. They stated that it took 3 minutes before any adsorption was seen. In this study it proved to be impossible to distinguish adsorption in this manner.

3.4.3 Fluorescence

3.4.3.1 Calibration curves

As shown in Figure 3.1 the fluorescence intensity of the Sephadex was directly proportional to the concentration of salicylic acid added. Similar results were also given for DPD, propranolol and warfarin (Figures 3.2, 3.3 and 3.4). This also proved to be the case with RA 233, RA 433, DPQ, SH 1242 and prazosin (calibration curves not shown). These results suggest that the measurement of the fluorescence of a compound within the Sephadex gel was not dependent on the nature of the fluorescent ligand. These compounds have excitation and emission maxima over a wide range (excitation maxima from 285-450nm and emission maxima from 335-530nm). The increase in

scatter which occurs at lower wavelength did not destroy the fluorescence of the Sephadex.

All the fluorescent compounds so far investigated have given an enhanced fluorescence intensity in the Sephadex gel. This enhancement was not constant for all of the ligands but depended on the characteristics of the ligand. Factors like the degree of adsorption of the ligand onto the gel effected the fluorescence enhancement. It was seen that the enhancement of warfarin in the Sephadex was markedly higher than the other ligands investigated. Warfarin has a fairly low quantum yield in an aqueous buffer pH 7.4 and so has the potential for a large increase in its fluorescence quantum yield due to restriction of movement in the Sephadex. Chlorpromazine, a compound which is only slightly fluorescent under the conditions of this experiment, did not have its fluorescence significantly changed, i.e. it still was not very fluorescent. This method will only affect the fluorescence of a molecule that was already fluorescent.

After correction for the Sephadex blank, the emission and excitation maxima remained the same as the standard solution, i.e. the Sephadex did not cause a change in the Stokes shift of the SA. Figure 3.5 shows the spectra of the excitation and emission peaks of the Sephadex after correction for the blank and a standard SA solution. This suggests that the microenvironment of the SA was not grossly changed in the Sephadex gel. No Stokes shift was seen in the excitation or emission spectra of DPD or the other compounds investigated. Changing the ionic strength of the buffer by addition of varying amounts of sodium chloride did not alter the fluorescence in the Sephadex layer.

Removal of the void volume from the Sephadex further increased the observed fluorescence enhancement in the Sephadex layer. But removing the void volume created several problems the major one being packing the Sephadex gel in the cuvette. A number of methods were investigated to remove the void volume. Centrifuging the gel and filtration through a sintered glass filter both subsequently required that the gel was packed into a cuvette. This was difficult to achieve so that a reproducible fluorescence intensity was given. The most satisfactory method for removing the void volume was by removing the bottom of a cuvette and adhering in its place a fine membrane. When the gel was poured into the cuvette, the void volume could be removed by the application of gentle pressure. Using this arrangement the fluorescence of the sample was followed while the void volume was removed. Within the first 30 seconds from the application of pressure a marked enhancement in the fluorescence intensity was observed. During this time the majority of the void volume was removed from the cuvette This would decrease the number of fluorescence ligand in the excitation path without a marked change in the bed volume. After about the first 30 seconds the fluorescence intensity of the sample increased much more slowly for some time. This slow increase may have been due to a gradual compression of the Sephadex bed or gradual evaporation of the buffer.

3.4.3.2 Optimisation of the Sephadex blank fluorescence

The fluorescence intensity of the SA in the Sephadex was enhanced but this does not lead to a decrease in the minimum detectable concentration, on the contrary this was markedly increased. This proved to be the major disadvantage in trying to determine low

concentrations (less than 5 X 10^{-7} M) SA in the Sephadex layer. The high background fluorescence readings were probably almost entirely due to scatter of the exciting radiation. Decreasing the interval between the excitation and emission wavelengths decreased the blank value in the characteristic fashion of scattered radiation. The scatter peak was very broad being much larger than the slit width of the monochromators. A glass filter reduced the scatter slightly when an excitation wavelentth below 350nm was used and an emission wavelength above 350nm.

The blank to signal ratio was reduced by changing both the emission and excitation slit width from 20nm to 10nm. Reducing the path length from 10mm to 5mm decreased further the blank to signal ratio and also had the advantage of further increasing the enhancement of the fluorescence in the Sephadex layer (Figure 3.6).

3.4.3.3 Effect of the pore size of the gel

For albumin to be completely excluded from the gel, Sephadex G-10 to G-50 may be used. Sephadex G-75 has a range of pore sizes between 3000-80000 Daltons so albumin would only be partially excluded. The gels with the smaller pore size, G-10 and G-15 showed a significantly higher blank to signal ratio than the gel with larger pore size, G-50 and G-75. Figure 3.7 shows the effect of different grades of gel on the fluorescence intensity of SA. The partition of SA into the Sephadex was greater with G-10 and G-15 but less with G-50 and G-75 when compared with G-25 thus confirming the findings of Jansen (1967). Sephadex G-10 also had the disadvantage of having a significant fluorescence peak in a region close to the fluorescence of SA.

Overall G-25 was considered to be the most satisfactory gel to use. The higher grade gels had the advantage of a lower background blank level with less adsorption of the SA onto the gel but had the disadvantage of being a less dense gel therefore the contribution of the void volume to the fluorescence was greater than with G-25. Also the observed enhancement was less with the higher grade gels. With the lower grade gels the background fluorescence was higher and the observed fluorescence was lower.

3.4.3.4 Polarisation and temperature

The use of polarisers in the excitation and emission beams did not alter the minimum detectable concentration of the SA. The most effective combination of the polarisers for reducing scatter was with the excitation polariser in the vertical mode and the emission polariser in the horizontal mode (Chen, 1966).

Increasing the temperature from 20 to 37^{o} C decreased the observed fluorescence intensity. Changing the ionic strength of the buffer, by addition of varying amount of NaCl, made no difference to the observed fluorescence intensity.

3.4.3.5 Frontal scatter

Looking at the fluorescence by frontal scatter from a cuvette with the Baird Atomic scatter attachment proved to have a number of disadvantages when compared with the direct measurements. The blank response was higher when compared with the response from the sample, thus increasing the blank to signal ratio. A standard solution gave no response so a direct comparison of the Sephadex fluorescence with a standard could not be made. This confirms that the direct method is

looking at the fluorescence in the Sephadex beads and not only from scatter from the surface of the bead.

3.4.3.6 The mechanism of the fluorescence from the Sephadex

Two important questions need to be answered, what causes the observed enhancement in the fluorescence of the Sephadex layer and what is being measured in the Sephadex?

3.4.3.6.1 The enhancement of the fluorescence

In a homogeneous solution the excitation beam passes through the centre of the cuvette as a parallel beam. The observed fluorescence therefore comes almost entirely form the centre of the cuvette (Figure 3.8). The Sephadex causes the excitation beam to be scattered. The number of molecules excited by the excitation beam will be increased and also the area of the cuvette where the fluorescence is actually being measured. It therefore follows that the observed fluorescence is enhanced. But the fluorescence intensity is directly proportional to the intensity of the excitation beam. Scatter will therefore reduce the intensity but because the Sephadex does not absorb the radiation this effect is less important than the increase in the number of molecules excited.

Removal of the void volume from the Sephadex bed increases the observed fluorescence enhancement, although about half the fluorescent molecules were removed with only a small change in the bed volume. Mie in 1908 elaborated a general theory for the scatter of light from large particles (i.e. particles greater in size than 1/20 of the wavelength of radiation). He stated that the intensity of light

scattered at various angles is related to m the ratio of the refractive index of the particle to that of the medium. This was subsequently verified by La Mer et al. (1946). In the above case it can be seen that removal of the void volume will increase m and therefore the scatter. An enhancement in the fluorescence on removal of the void volume is consistent with the idea that scatter has an effect on the fluorescence.

But it is universally believed that scatter has a detrimental effect on fluorescence measurements. Teller (1972) stated 'the primary reason for analytical sensitivity obtained with fluorescence procedures - that 'new' light appears in a dark portion of the spectrum - makes this system relatively more sensitive to light scatter effects' Lim (1976) came to the conclusion that scattering of the exciting and fluorescent light may cause considerable interference with, and hence errors in fluorimetric measurements. Light scattering may produce either an increase or a decrease in the apparent fluorescence. An increase in the apparent fluorescence may be caused by the overlapping of higher orders of scattered radiation with the fluorescence radiation. However Lim stated that the randomisation in the direction of the excitation beam caused by scatter will produce a reduced intensity of the excitation beam and therefore a reduction in the apparent fluorescence intensity. With the Sephadex system it was unlikely that the enhancement is due to overlap of the higher orders of scattered radiation. The Sephadex system has three main differences with the 'slight scatter' effects normally confronted in fluorescence work.

1) The gel particles are able to transmit the exciting radiation.

2) The gel is very dense and so will cause a greater degree of scatter

than in a homogeneous system.

3) The gel particles are large when compared with the wavelength of the exciting radiation.

Although a useful enhancement of the fluorescence was seen, in agreement to the findings of Teller (1972) and Lim (1976) a decrease in the minimum detectable concentration of SA was observed. The scatter which results from the gel was more of a theoretical problem than a practical one.

With DPD a model for the Sephadex was created using glass beads. Exciting at 305nm showed very little fluorescence at 490nm. Exciting at 420nm showed a very slight enhancement in the fluorescence of the DPD. At 305nm the exciting radiation was not transmitted through the glass beads therefore very little fluorescence was seen. At 420nm, where the glass no longer absorbs the radiation, fluorescence is observed. This was lower than that of a standard solution but after allowance has been made for the volume of the glass beads in the path of the beam a very slight enhancement was seen. Large increases in the fluorescence as seen with molecules that enter the Sephadex gel beads was not seen. This therefore indicates that the large enhancement in the fluorescence intensity was primarily due to the ligand in the internal volume of the bed and only a small enhancement due to the ligand in the void volume.

If the quantum yield of the SA was increased in the Sephadex gel due to restricted movement of the molecules, the fluorescence could not be enhanced above a certain value because the fluorescence quantum yield cannot be greater than 1. SA has a quantum yield of 0.28 at room temperature in water (Weber and Teale, 1957). After the removal

of the void volume, allowing for the partition of the SA into the gel, the observed fluorescence enhancement was greater than that allowed for by simply an increase of the quantum yield in the gel. This does not rule out that some of the observed enhancement is due to a change in the quantum yield. This enhancement can be explained by the fluorescent ligand being trapped in the gel matrix. This would restrict the movement of the fluorescent ligand in the gel. A decrease in the radiationless loss of energy from the excited molecule would occur because of a reduction in the collisional deactivation. Alternatively, it is possible that the excited radiation passes into the gel bead and is reflected around the gel bead before it is able to pass out, therefore exciting more molecules than if it passed directly through.

Evidence therefore exists to support the theory that part of the observed fluorescence enhancement is due to the scatter caused by the gel. This does not account for the large fluorescence enhancements seen by molecule which enters the gel. An increase in the quantum yield of the molecule when it enters the bead may account for this or the excitation beam being reflected back at the water bead interface and therefore exciting more molecules.

3.4.3.6.2 What is being measured in the Sephadex?

The second question which must be considered is what is being measured in the Sephadex layer. The Sephadex layer may be considered to be composed of two components, the internal phase and the void volume. The fluorescence of albumin a molecule which is total excluded from Sephadex G-25 can still be measured by this method. The observed fluorescence intensity is less than that of the supernatant

but after allowance is made for the volume of the Sephadex internal volume, a slight enhancement is seen. This was small when compared with that seen by SA and other ligands that pass into the internal volume of the gel. With SA as already stated removal of the void volume still allows the fluorescence of the SA to be determined. The exciting radiation must pass into the gel to excite the SA. It is unlikely that this is a surface effect because of the large enhancement.

Considering the glass bead model again with DPD. When the excitation wavelength was 305nm very little fluorescence of the DPD was observed. If the radiation was not passing through the Sephadex gel a similar effect would be expected when the excitation wavelength was 420nm but this was not the case. Therefore the exciting radiation must pass through the Sephadex gel as well as the void volume.

It can be concluded that what is being measured in the Sephadex is a combination of both the void volume and the internal volume. The contribution of the internal volume is much greater than that of the void volume. The contribution of the void volume can be expressed as a fraction of the fluorescence intensity of the supernatant, for Sephadex G-25 this works out to be about 0.6. Subtracting this from the total observed fluorescence gives the fluorescence intensity due to the ligand in the gel (i.e. the free concentration of the ligand). The addition of albumin to the system and making the above correction allows the determination of the free and bound concentration of the ligand.

3.4.3.7 The importance of the ratio of internal to external volume

With SA it was seen that the ratio of internal and external volume made a difference to the observed fluorescence intensity. Using 500mg of Sephadex and 3ml of buffer very little difference was seen in the fluorescence of SA with or without 1.45×10^{-5} M BSA. Removal of the void volume by filtration did show a difference between samples with and without albumin indicating the amount bound. Increasing the ratio of external volume to internal volume a difference was seen without the need to remove the void volume.

One explanation for the dependence of the fluorescence on the ratio of external to internal volume is that at the lower external internal volume ratio the adsorption sites are not saturated. But this is unlikely to be the case because a concentration independent adsorption was seen in the calibration curves of SA and all the other ligands so far investigated.

Looking at the case when the volume of the supernatant is zero. Before the protein is added to the system the ligand is distributed between the internal and external phases, adding the protein causes a redistribution of the SA into the external volume but this does not change the number of molecules in the excitation beam. The intrinsic fluorescence of SA is enhanced on binding so any difference between the fluorescence enhancement in the void volume and internal volume may be counteracted by this. With a small supernatant volume adding the protein to the system will cause the redistribution of the SA from the Sephadex bed volume to the supernatant. The number of molecule in the excitation beam will therefore be decreased, thus changing the observed fluorescence. The larger the ratio of external volume to

internal volume the greater will be the change in the fluorescence. 1g of Sephadex made up to 50ml was found to be satisfactory.

Several workers including Ashworth and Heard (1966) and Cooper and Wood (1968) stated that one of the problems with the batch method was that the concentration of ligand could only be determined in one phase that containing the protein. Therefore the free concentration or the bound concentration could not be determined directly but only (Db + Df). This results in Df being determined by the difference between the number of moles in the supernatant and the total number of moles of ligand added. Correction was also required for the partition of the ligand into the gel. The ratio of internal to external volume had to be kept fairly low in order to minimise any error. In this study Df can be determined directly. No problem therefore exists in calculating a value for Df from (Db + Df). Correction is still required for the partition but when the external to internal volume ratio is large any error in the calculation of Db will be small due to the small number of moles associated with the gel compared with the total number of moles in the system. With a large external to internal volume ratio any error in the calculation of this ratio will also be small. Under these conditions K'' may approach 1. This therefore simplifies the analysis of data.

3.4.3.8 Absorbance

In conventional fluorescence work it is well known that the absorbance of the solution must be kept below 0.04 (Chignell, 1972) for a linear response to concentration to be maintained. The absorbance of the Sephadex is clearly much greater than this value but a linear response was still given up to 10^{-5} M SA. Departure from the

linear response depends only on changes in the absorbance ,so although the Sephadex has a high absorbance it does not change significantly with the SA concentration. This also explains why changing the path length causes the observed fluorescence to change.

3.4.4 The problem of adsorption and partition

It is very difficult to distinguish between partition and adsorption onto the Sephadex in the conditions of this experiment. A classical view of adsorption would state that it was concentration dependent and could be saturated while partition was concentration independent and could not be saturated. In the concentration range being used in these experiments it may therefore be better to consider the adsorption/partition effect to be partition.
The partition coefficient was a characteristic of a given ligand. DPD had a larger K'' than SA. Over the concentration range used K'' remained constant for a given ligand.

In their study of the problems of adsorption, Cooper and Wood (1968) stated that adsorption of the ligand onto the Sephadex only causes problems under two circumstances.
1) When the adsorption is concentration dependent. This would cause an error in the determination of the concentration of ligand bound to the protein. It has been shown that the calibration curves of SA, propranolol and DPD (Figure 3.1, 3.2 and 3.3) are linear therefore the percentage adsorption is not dependent on the concentration of ligand. All the other ligands so far investigated have given similar results.
2) The second problem which may be associated with adsorption is if it was not reversible. The evidence obtained suggests that none of the ligands so far investigated are irreversibly adsorbed.

Figure 3.9 shows the effect of changing the void volume on the fluorescence of the supernatant and the Sephadex. Increasing the ratio of external to internal volume increases both the fluorescence of the supernatant and the Sephadex. This can be explained by the partition of the drug into the Sephadex gel. Figure 3.10 shows that as the ratio of external/internal volume increases the concentration of ligand in both the supernatant and Sephadex approaches a maximum. Considering the hypothetical situation when the partition coefficient is 0.5, the external to internal ratio 1:1, and the total concentration of SA added to the system 1mM.

Using these values the concentration of the ligand in the Sephadex and the supernatant was calculated from the relationship

fraction of ligand internal + fraction external ligand = 1

$$ax + (b/k)x = 1 \qquad (3.29)$$

where

a is the external volume

b is the internal volume

k = molar external concentration/molar internal concentration

x is a proportionality constant

The molar concentration of ligand in the gel (Cin) and the supernatant (Cex) is given by

$$Cex = (x\ Vt\ Ct) \qquad (3.30)$$

$$Cin = (x/k)\ Vt\ Ct \qquad (3.31)$$

where

Vt is the total volume of buffer added to the system.

Ct is the concentration of ligand added to the system

For the above example the concentration in the Sephadex internal volume will be 1.33mM and that in the external volume 0.67mM. When the external to internal ratio is 25:1 the concentration in the Sephadex internal volume will be 1.93mM and in the external volume 0.96mM. When the ratio was 100:1 these will increase to 1.98mM and 0.99mM respectively. It can therefore be seen that the effect of partition is to increase both the fluorescence intensity of the supernatant and the Sephadex. The concentration in the supernatant will approach the total concentration added and that of the Sephadex Ct/k.

To verify this the above was applied to the results of (Figure 3.11). The partition coefficient for SA into the gel was calculated from Figure 4 giving a value of 0.62. Table 3.2 compares the results of the theoretical treatment with the actual values

Increasing the external to internal volume will have a positive advantage on the measurement of protein ligand interactions when measured with this method. The value of K'' as defined in equation (3.14) will increase. In the cases where this value approaches 1 equation (3.27) will be simplified thus decreasing the error in the calculation of Db and Df.

3.4.5 Protein binding

3.4.5.1 The binding of SA to BSA

Using the excitation maximum at 305nm and emission maximum at 410nm (uncorrected) the binding of SA to BSA was studied. BSA has an absorbance maximum at 280nm, which is a fairly sharp peak. At 305nm using up to 1.45×10^{-5} M BSA the absorbance caused by the BSA does not need correction for the inner filter effect. Keeping the SA concentration constant and titrating with BSA using 1g of Sephadex G-25 to 50ml of buffer, with a 5mm path length at room temperature, with both the excitation and emission slits set at 10nm the binding of SA was investigated. Figure 3.11 shows how the BSA changes the fluorescence of the SA in the Sephadex layer. Using the method of Pearlman and Crepy (1967) Df/Db was plotted against 1/P (Figure 3.12). A nK value of 6.7×10^{4} M^{-1} was given. Alternatively the concentration of protein was kept constant and the SA concentration changed. Figure 3.13 shows a plot of 1/Db against 1/Df. This method still has the fundamental problem of adsorption of the ligand onto the gel matrix. It has been shown above that albumin is not adsorbed on the Sephadex so this presents no problem. For measuring low levels of binding using the fluorescence method the adsorption should be less of a problem because it is possible to determine the concentration of a fluorescent ligand directly in the Sephadex. A comparison can therefore be made between the fluorescence of a sample with and without the macromolecule.

As discussed in the previous section the binding was also investigated by a direct fluorescence method. Figure 2.11 is a Rosenthal plot for the binding of SA to BSA using the direct

fluorescence method. The results were also compared with a continuous ultrafiltration method. Table 3.3 compares the results of the three methods. These compare favourably with reported literature values (Cruze and Meyer, 1976; Meyer and Guttman, 1970b; Rudman et al., 1971; McArthur and Smith, 1969).

Because of the concentration range used only the primary affinity constant could be calculated with the Sephadex method. The direct fluorescence method was also only looking at the primary affinity constant.

To extend the linear range of the SA the wavelength of excitation can be moved away from the absorbance peak of 305nm. Both an excitation wavelength of 320nm and 330nm have been successfully used with 410nm emission. With an excitation wavelength of 330nm a linear fluorescence response is given up to 10^{-4} M. This will therefore allow the molar ratio of protein to SA to be increased (Figure 3.14). Figure 3.15 is a Rosenthal plot for the binding of SA to 1.45 $\times 10^{-5}$ M BSA using an excitation wavelength of 330nm. A primary affinity constant of 6.6×10^{4} M^{-1} with 0.9 binding site was given and k =900 M^{-1} n =3.7. The primary affinity constant given with this method was in the same order as nk evaluated by Pearlman and Crepy (1967) method. The method was not a satisfactory method for looking at secondary sites because the molar ratio of SA/BSA was too low. Further information about the secondary binding site could be obtained by increasing the linear range of the fluorescence response by displacing the excitation response. But in clinical terms the primary binding site is more important than the secondary sites. After a single oral dose of 650mg of aspirin a peak plasma level of 45 µg/ml (3.26 x

10^{-4} M). A person taking continuously 3g/day of aspirin has a peak salicylate concentration of 270 µg/ml (1.96×10^{-3} M). Plasma albumin concentrations range from $4.4 - 7.54 \times 10^{-4}$M. At these albumin SA ratios secondary binding would be relatively unimportant.

3.4.5.2 DPD and its analogues

The binding of DPD to BSA was also investigated using this method. DPD has two maxima in its excitation spectrum occurring at approximately 305nm and 420nm (uncorrected). One emission maximum is given at 490nm. For measuring protein binding the 420nm peak was chosen for the excitation wavelength and 490nm for the emission wavelength. The 420nm peak has the advantage of being well clear of the absorption spectrum of serum albumin and AGP. This means that high concentrations of these proteins, i.e 1.45×10^{-4}M BSA could be used without creating any absorbance problems. Their was no need at this protein concentration to correct for the inner filter effect. When a protein concentration of less than 1.45×10^{-5}M BSA was employed no binding was observed. This was due to the low affinity of DPD for BSA. Under such conditions of low affinity and low concentration of protein the percentage bound would be low. Increasing the protein concentration increased the percentage bound. Figure 3.16 shows how various concentrations of BSA affects the binding of DPD. Because of the low solubility of DPD at pH 7.4 only low ratios of DPD/BSA could be used. Using the method of Pearlman and Crepy (1967), nk was estimated to be 5.67×10^{3} se 0.50×10^{3} M^{-1} (Figure 3.17). This was in relatively close agreement with the value obtained using equilibrium dialysis were $nk=4.1 \times 10^{3}$ se 0.63×10^{3} M^{-1}. These values were lower than the values quoted by Kopitar

and Weissenberger (1971) for the binding of DPD to HSA thus suggesting a significant difference between BSA and HSA in the binding of DPD. The technique of displacing the wavelength of the excitation and emission radiation was not as useful as in the case of SA, due to the low solubility of DPD. Using the same method the binding of RA 233 and RA 433 to 1.45×10^{-4}M BSA was studied. For RA 233 nk was calculated to be 1.9×10^{4} M^{-1} and for RA 433 1.5×10^{4} M^{-1}.

The binding of DPD to plasma was significantly higher than the binding of DPD to albumin. This is probably due to the binding of DPD to AGP in the plasma.

RA 223 has a more complex excitation spectrum than DPD with a maximum at 295nm and inflexion at 330nm and further maxima at 440, 455 and 470nm. It has an emission maximum at 515nm. Although exciting at 295nm gave a more intense fluorescence than at 470nm using 515nm as the emission wavelength, the 470nm peak was used to look at the binding of RA 233 to plasma in order to cut down the effect of the absorbance of the plasma proteins. When a compound had more than one excitation peak it was found advantageous to use the one which overlapped the protein spectrum the least. Figure 3.18 shows how the fluorescence of the Sephadex changed with the concentration of RA 233 with and without rabbit plasma. The binding of RA 433 to plasma (using and excitation wavelength of 420nm and emission wavelength of 520nm) was also investigated (Figure 3.19). A comparison of the binding of DPD, RA 223 and RA 433 to rabbit plasma was made (Niewiarowski et al., 1975). It was seen that RA 433 and RA 233 binds significantly less than DPD (Figure 3.20).

The Sephadex method is most efficient when the P >> Db thus resulting in a large percentage bound. While P >> Db we are justified in making the assumption of Pearlman and Crepy (1967) as previously described. Alternatively the approximation of 1/K>> Df can be made to give the same final answer. A value can be determined for nK when the concentration of the binding protein is known. The system is more complex when plasma is used. Pearlman and Crepy (1967) suggested that the binding could be expressed in litre/mg of protein. This has the disadvantage of making it difficult to compare this method with other published results. An alternative way of expressing the results is simply as a percentage bound.

Using 200mg of Sephadex G-25 made up to a total volume of 5ml, the binding of DPD to AGP has been studied. In this work the assumption that Pt >>Db was not made. Using an AGP concentration above 2.5×10^{-5} M the percentage of DPD bound approached 100%. When 2.5×10^{-6} M AGP was used a wide range of DPD /AGP ratios could be employed. Figure 3.21 shows how the fluorescence of the Sephadex changed with the concentration of the DPD with and without 2.5×10^{-6} M AGP.* Figure 3.22 is a Rosenthal plot for the binding of DPD to 2.5×10^{-6} M AGP. The data was analysed using nonlinear regression analysis k $=3.15 \times 10^{6}$ M^{-1} se 0.310×10^{6} with 0.4 binding sites. This value for an affinity constant was in close agreement with the values quoted by Kopitar and Weissenberger (1971) and Subbarao et al. (1977a) but lower than that of Gamel et al. (1982).

* The Df values are calculated as shown in Figure 3.21, eg the Df value at point A on the binding curve is given by drawing the line AB and reading off the free concentration at point C.

3.4.5.3 Prazosin

Prazosin binding to plasma and albumin has been studied with this method, using an excitation wavelength of 345nm and emission wavelength of 400nm (Figure 3.23). It was shown that prazosin binds significantly more to plasma than albumin (Figure 3.24). This is probably due to the binding of prazosin to AGP (Grahnen et al., 1981). Using the approximation of Blanchard (1982) nk was calculated to be 4.39×10^{4} M^{-1} to BSA.

Displacing the excitation wavelength to 360nm the binding of prazosin to AGP was investigated. The results were analysed by nonlinear regression analysis. Using 2.5×10^{-6} M AGP an affinity constant of 3.2×10^{5} M^{-1} was evaluated with 0.936 binding sites. Figure 3.25 is a Rosenthal plot for this system.

3.5 CONCLUSION

Using the Sephadex batch method, in conjunction with fluorescence it has proved possible to measure the interaction of a ligand with a protein. The experimental conditions to measure such binding interactions were optimised in the course of this study. Unlike previous studies the free concentration was determined directly in the gel so overcoming the criticism of Cooper and Wood (1968) and Ashworth and Heard (1966) who could only measure the ligand concentration in the external phase. In this study it was advantageous to have a high external/internal volume ratio. Unlike previous studies this did not reduce the precision of the method because the free concentration was determined directly and not from the difference between the number of moles added and the number of moles in the external phase. The high

external/internal ratio also meant that the precision of the determination of the external and internal volume was less critical. The effect of partition into the gel was also reduced by using a high ratio.

The fluorescence of a ligand was enhanced when it passed into the Sephadex gel. This enhancement was increased by reducing the path length and the signal to blank ratio minimised by a reduction in the slit width. It has been postulated that this fluorescence enhancement was due both to scatter and an increase in the fluorescence quantum yield of the compound by a reduction in collisional deactivation. The increase in the fluorescence of the ligand increased the sensitivity of detection.

The errors with this method, like equilibrium dialysis and ultrafiltration, are lower when the fraction bound is high. It is limited to ligands which are fluorescent. Like all fluorescence methods the protein concentration must be limited when the excitation and emission spectra of the ligand overlap that of the protein. This means that when the spectra of the protein and ligand overlap only a high affinity association can be determined. When the spectra of the ligand and the protein are well separated it is possible to measure the binding of ligands with lower association constants provided $1/k \simeq Pt$. It may be necessary to make the approximation of Pearlman and Crepy (1967) in the evaluation of the binding data because the ligand/protein ratio is too low as in the case of the binding of DPD, RA 233 and RA 433 to BSA. When a ligand binds with a large fluorescence enhancement the use of this method is not practical. Although this method does not have membrane binding

problems as encountered with equilibrium dialysis and ultrafiltration, partition of a ligand into the Sephadex may be a problem. Such problems are reduced by using a large external/internal ratio.

This method is of limited use for studying plasma ligand interactions. The protein concentration of plasma will be too high to use with this method without prior dilution. The range of ligand/protein ratios used may also be more limited than other methods.

To conclude although the batch Sephadex method has not been reported to have been used in recent times it is still a viable method for investigating ligand macromolecule interactions. It has allowed the quantification of ligand macromolecule interactions in a number of cases when the direct fluorescence methods gave no information.

Table 3.1

A table comparing the water regain of the Sephadex

Method	Water regain (ml/g dried gel)
gravimetric	2.46
Karl Fisher	2.24
albumin exclusion	2.51

TABLE 3.2

A table showing the actual concentration of ligand in the supernatant and the calculated values

Total volume (ml)	Actual concentration in supernatant (μM)	Calculated concentration in supernatant (μM)
10	0.838	0.850
25	0.911	0.943
100	0.941	0.988

Table 3.3

Table showing the primary affinity constant for the binding of SA to 1.45×10^{-5} M BSA evaluated using different methods.

Method	Affinity constant (M^{-1})
Continuous ultrafiltration	1.0×10^{5} se 3.1×10^{4}
Direct fluorescence	8.4×10^{4} se 2.2×10^{4}
Sephadex	6.7×10^{4} se 2.5×10^{4}

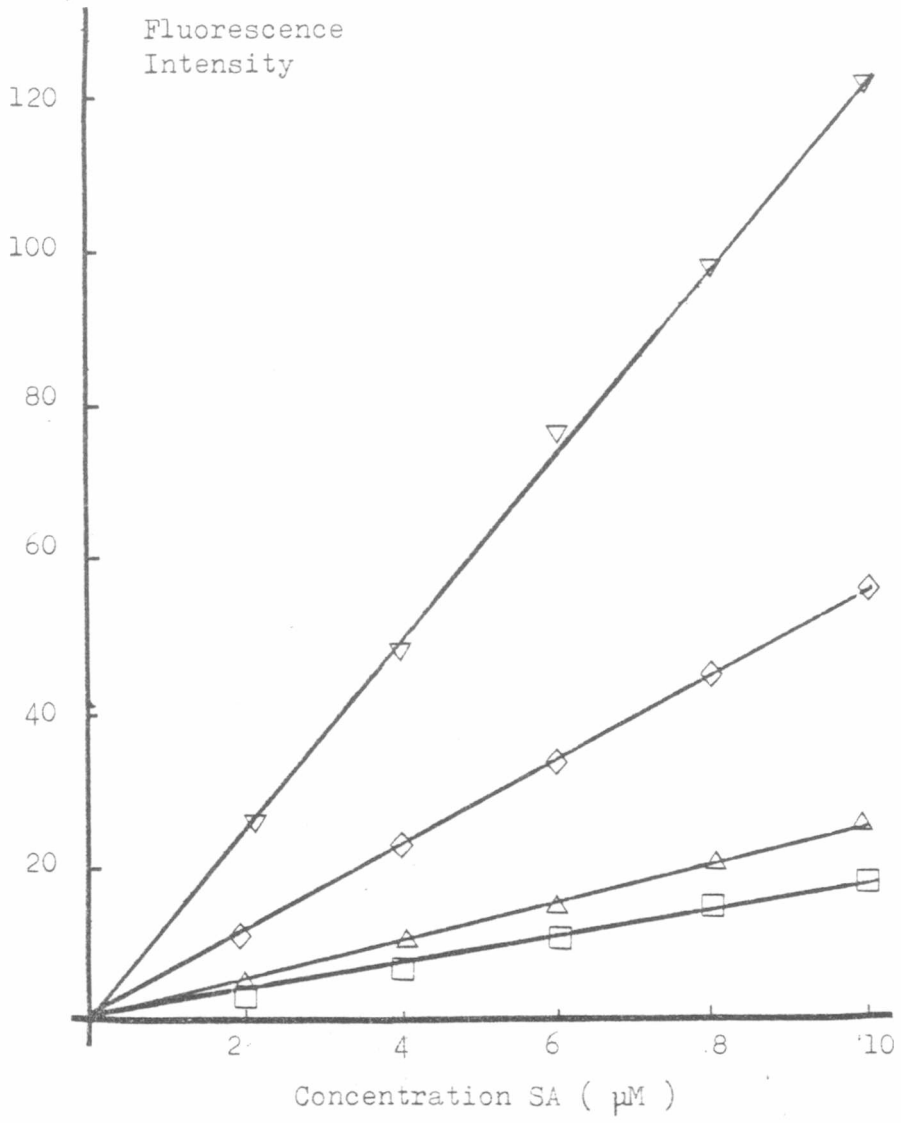


Figure 3.1

A graph showing the effect of Sephadex on the fluorescence intensity of salicylic acid using 0.5g of Sephadex with 3ml phosphate buffer. excitation wavelength 305nm emission 410nm

▽ Sephadex (no void volume) ◇ Sephadex

△ standard □ supernatant

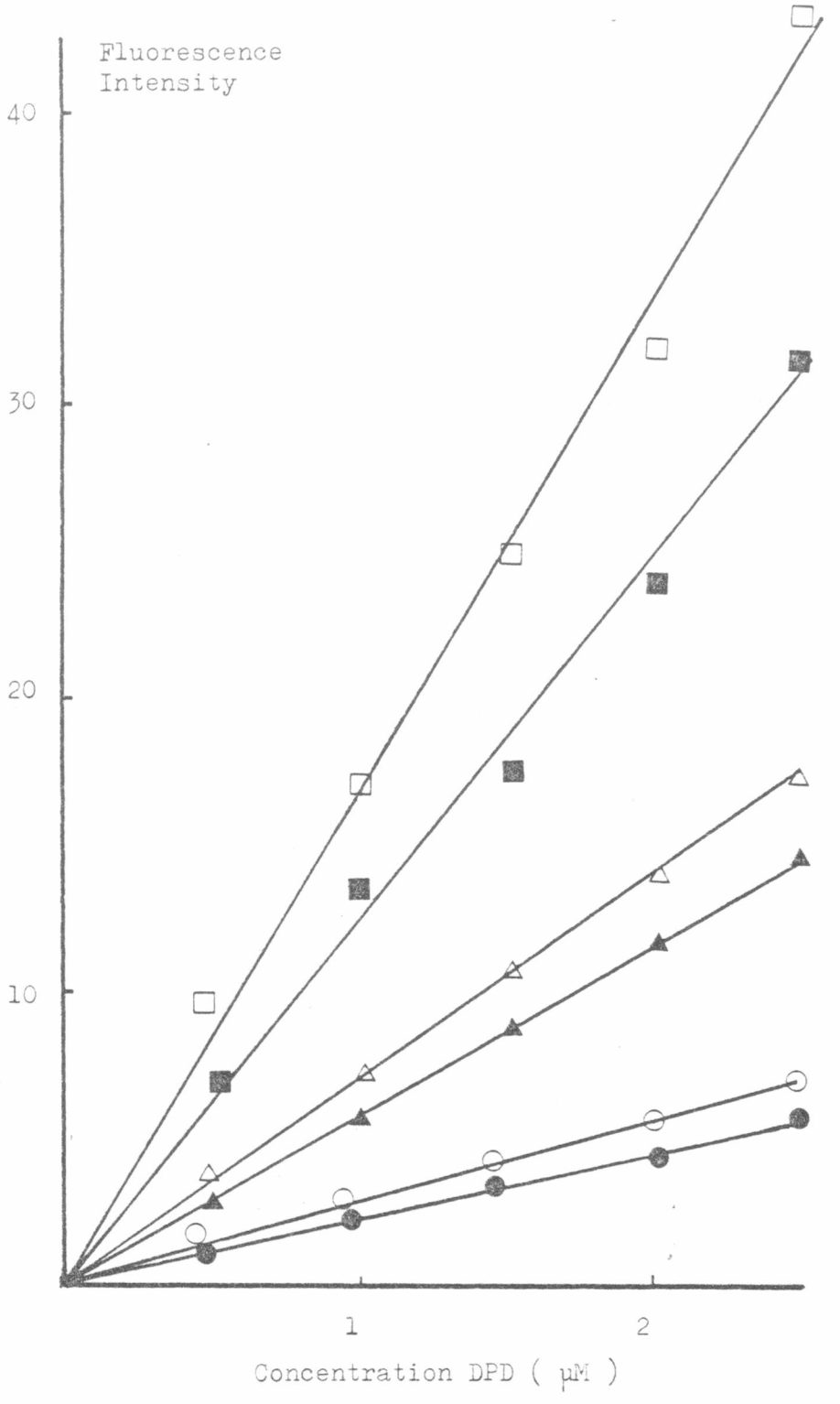


Figure 3.2

A graph showing the effect of Sephadex G-25 on the fluorescence intensity of DPD using 0.5g Sephadex to 3ml buffer and an excitation wavelength of either 305 or 420nm and an emission wavelength of 490nm.

● supernatant (ex = 310nm) ○ supernatant (ex=420nm)

▲ Sephadex (ex = 310nm) △ Sephadex (ex =420nm)

■ Sephadex (no void volume) □ Sephadex (no void volume)

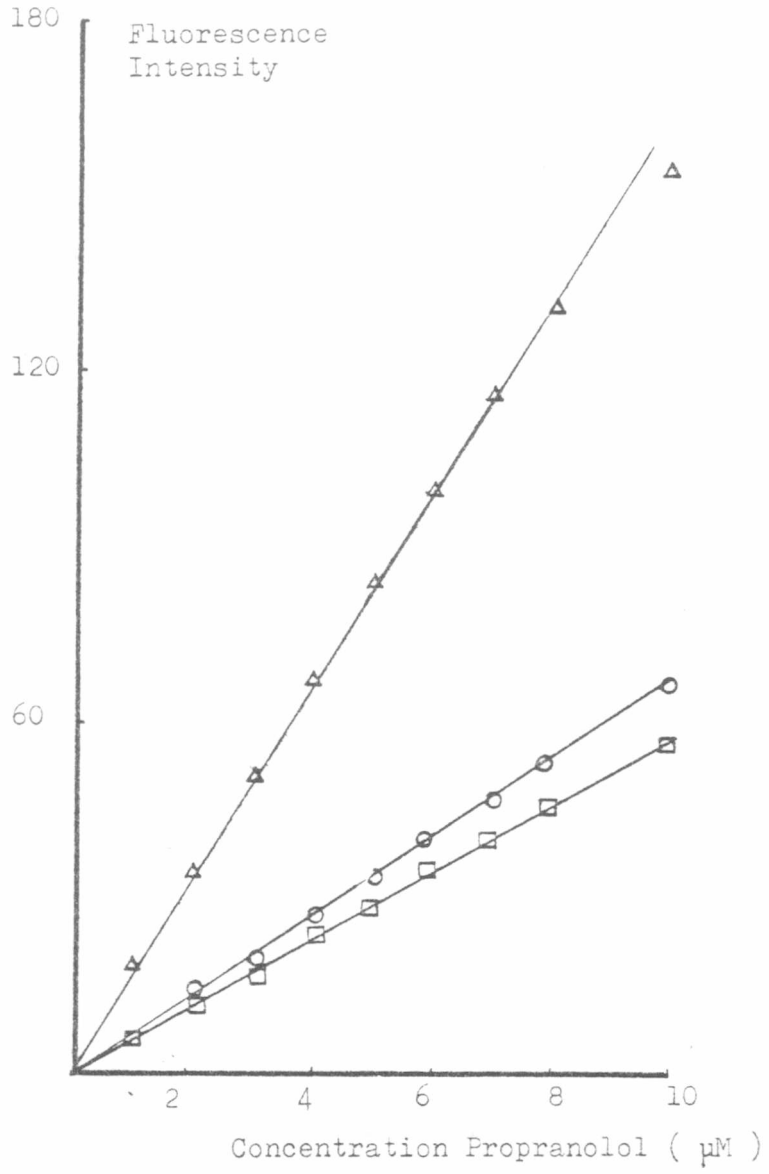


Figure 3.3

A graph showing the effect of Sephadex G-25 on the fluorescence intensity of propranolol

△ fluorescence of Sephadex

○ fluorescence of standard solution

□ fluorescence of supernatant

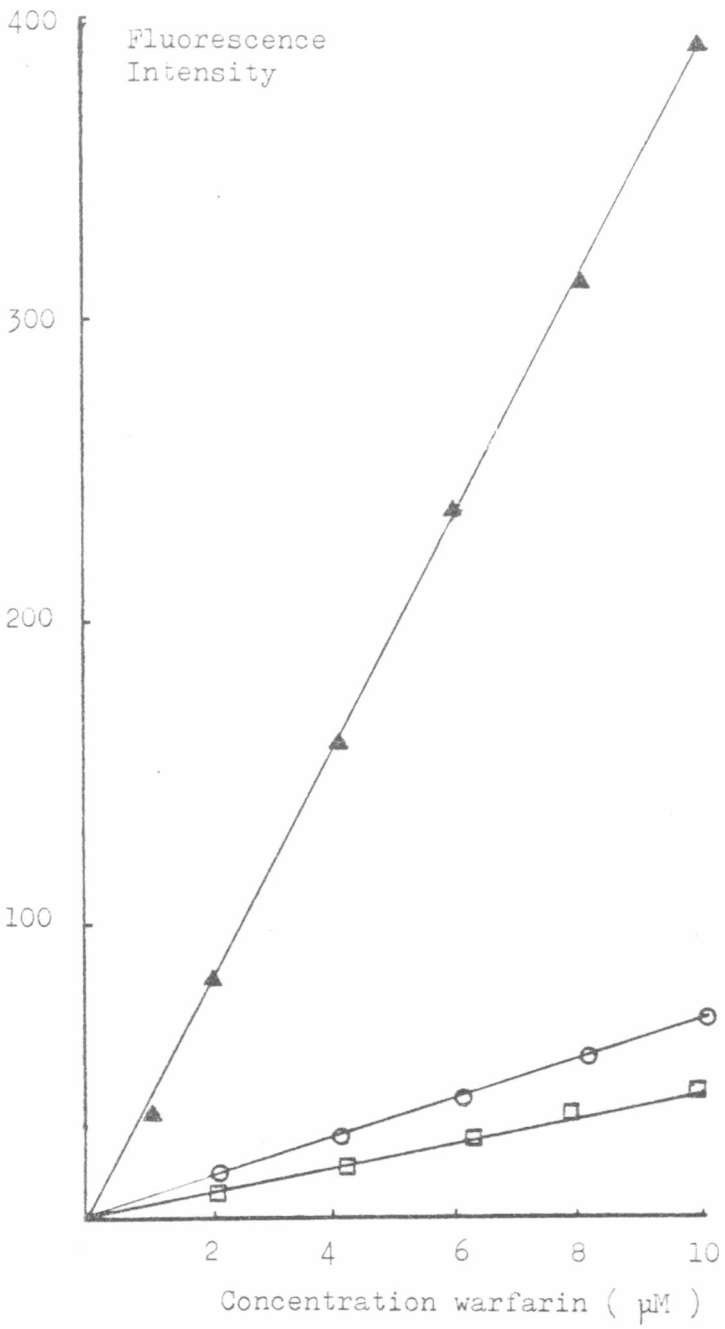


Figure 3.4

A graph showing the effect of Sephadex G-25 on the fluorescence intensity of warfarin

▲ fluorescence of Sephadex

○ fluorescence of standard solution

□ fluorescence of supernatant

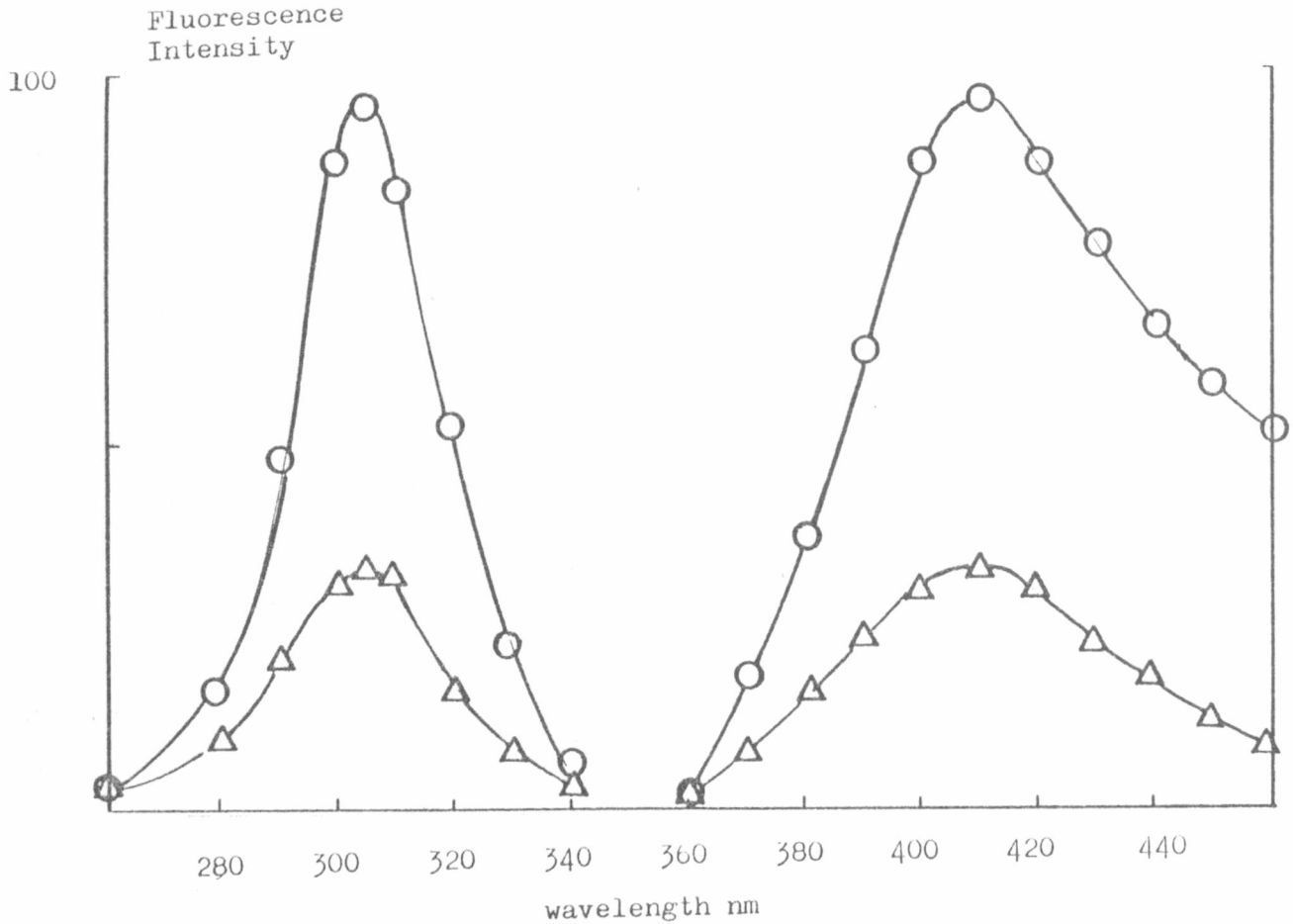


Figure 3.5

A graph showing the effect of Sephadex on the excitation and emission Spectra of SA (10^{-6} M)

O fluorescence of Sephadex

Δ fluorescence of standard solution

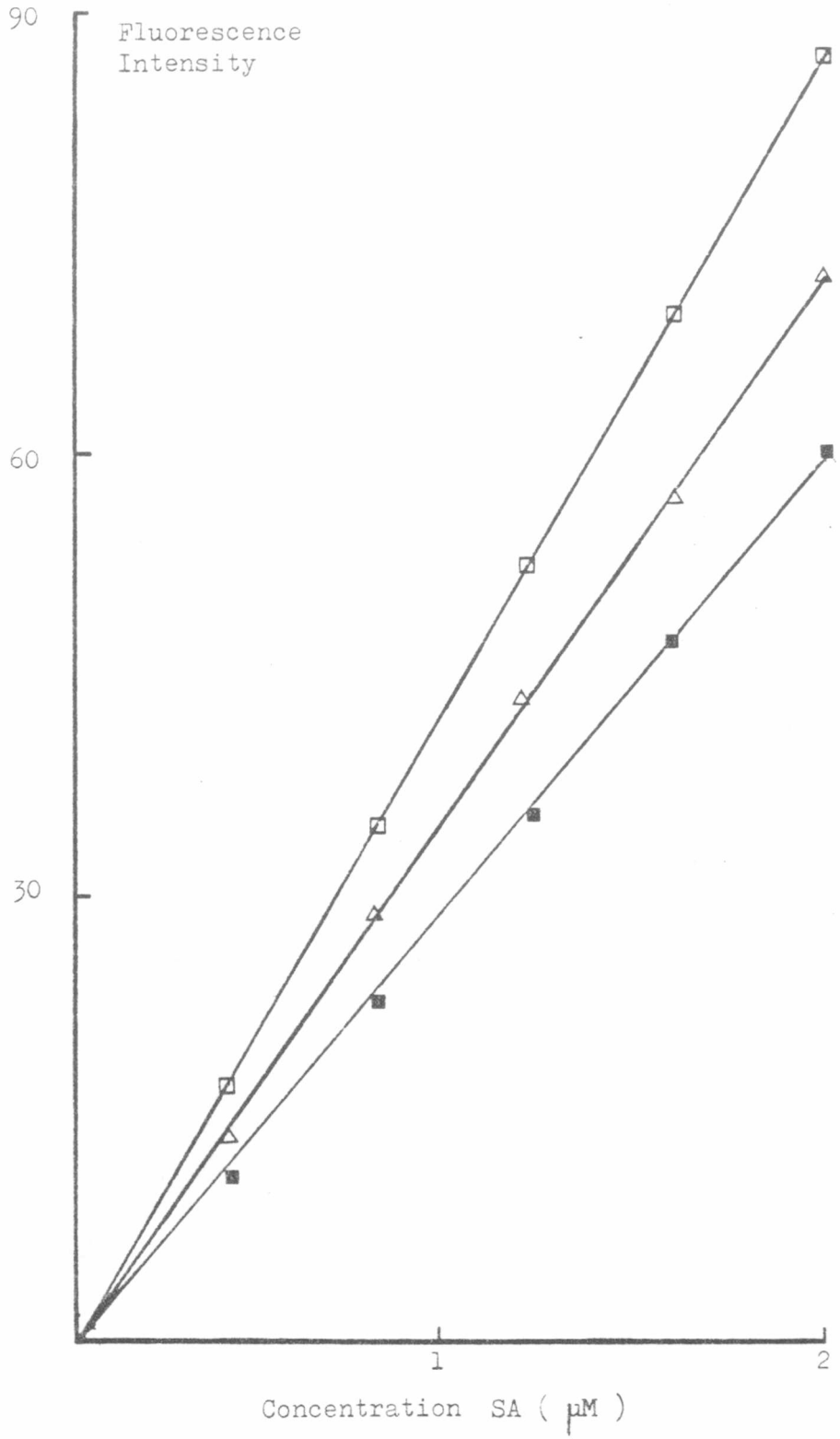


Figure 3.6

A graph showing the effect of path length on the fluorescence intensity of SA in the Sephadex gel.

■ 10mm △ 7.5mm □ 5mm

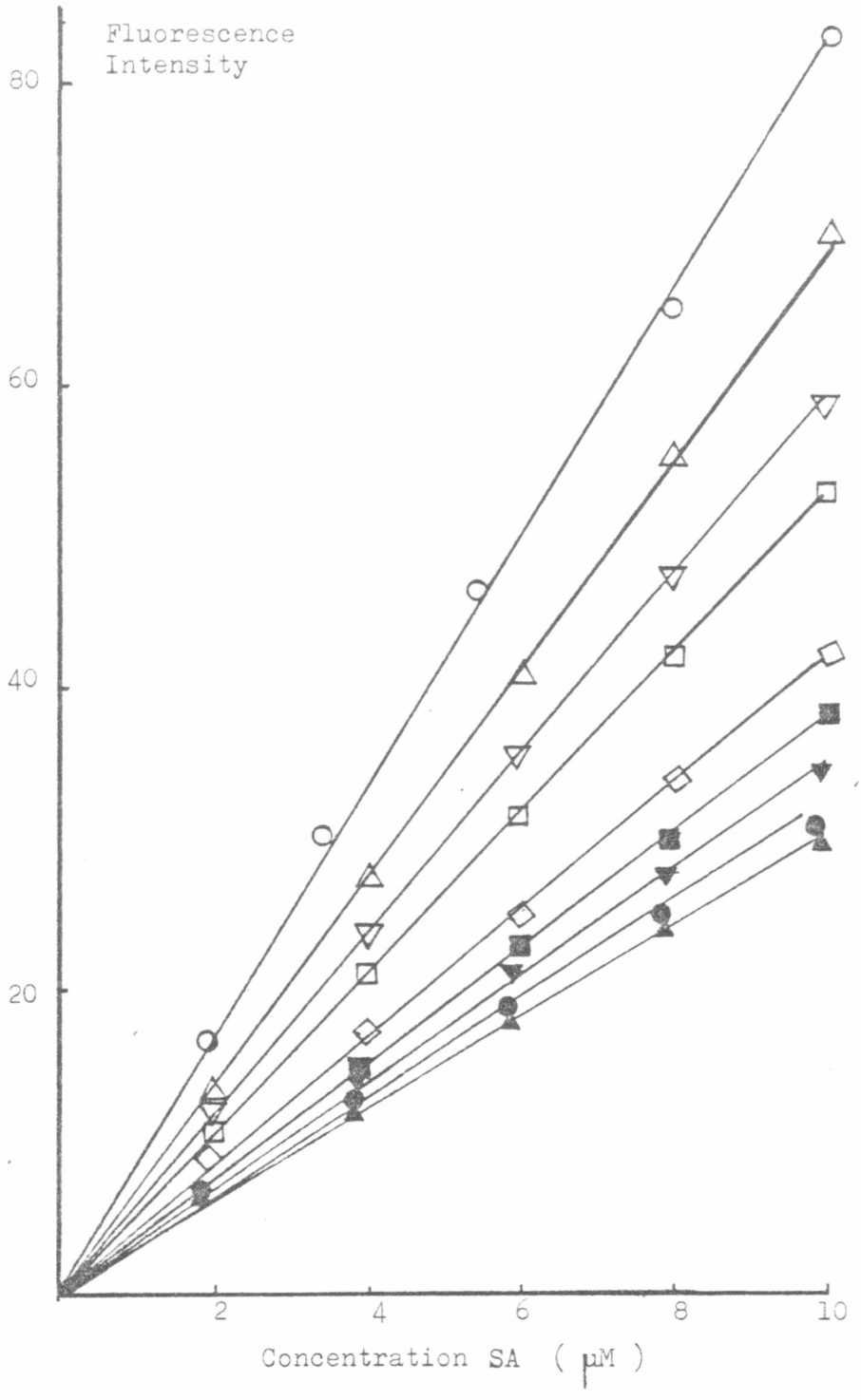


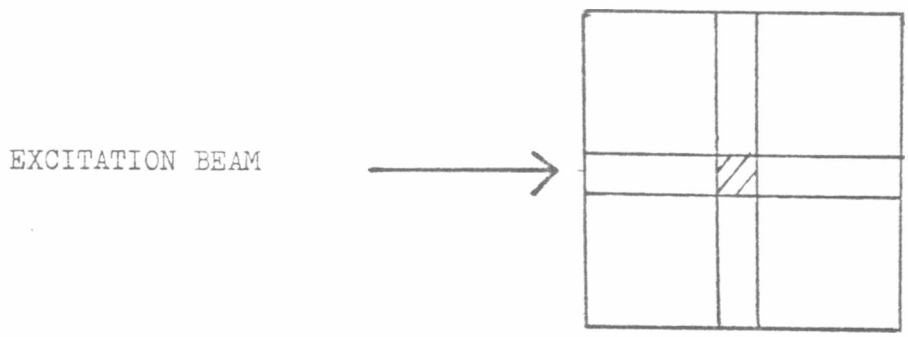
Figure 3.7

A graph showing the effect of different grades of gel on the fluorescence intensity of salicylic acid

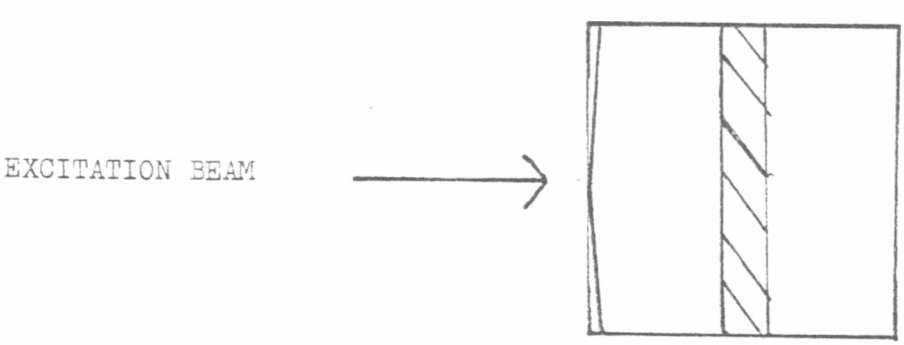
Sephadex G-25 ○ G-15 △ G-75 ▽ G-100 □

standard ◇

Supernatant G-25 ● G-15 ▲ G-75 ▼ G100 ■



(A) Fluorescence of a conventional solution



(B) Fluorescence of the Sephadex

Figure 3.8

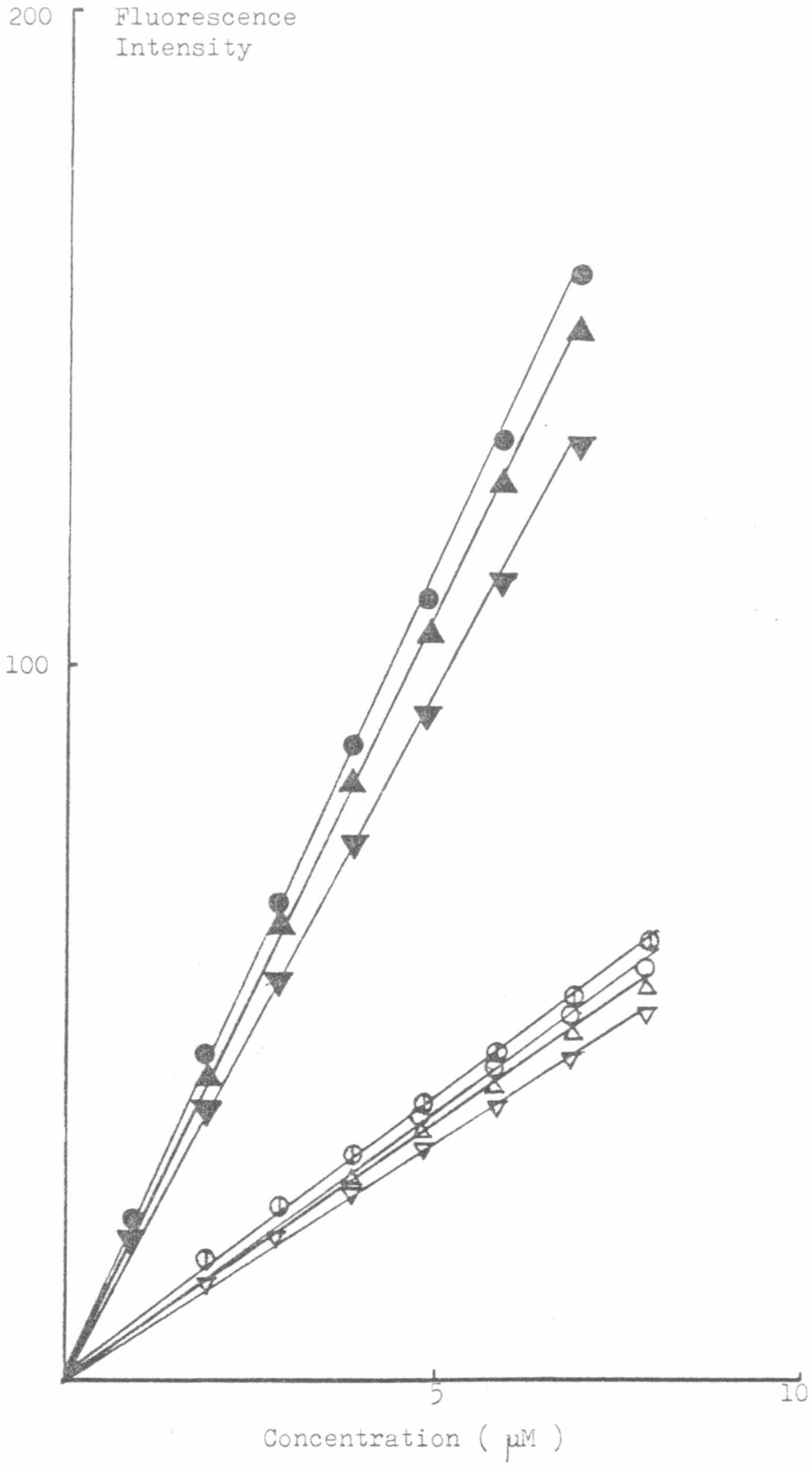


Figure 3.9

A graph showing the effect of supernatant volume on the fluorescence intensity of the SA in the Sephadex layer and supernatant.

Sephadex ● 100ml ▲ 25ml ▼ 10ml

standard ⊕

supernatant 100ml ○ 25ml △ 10ml ▽

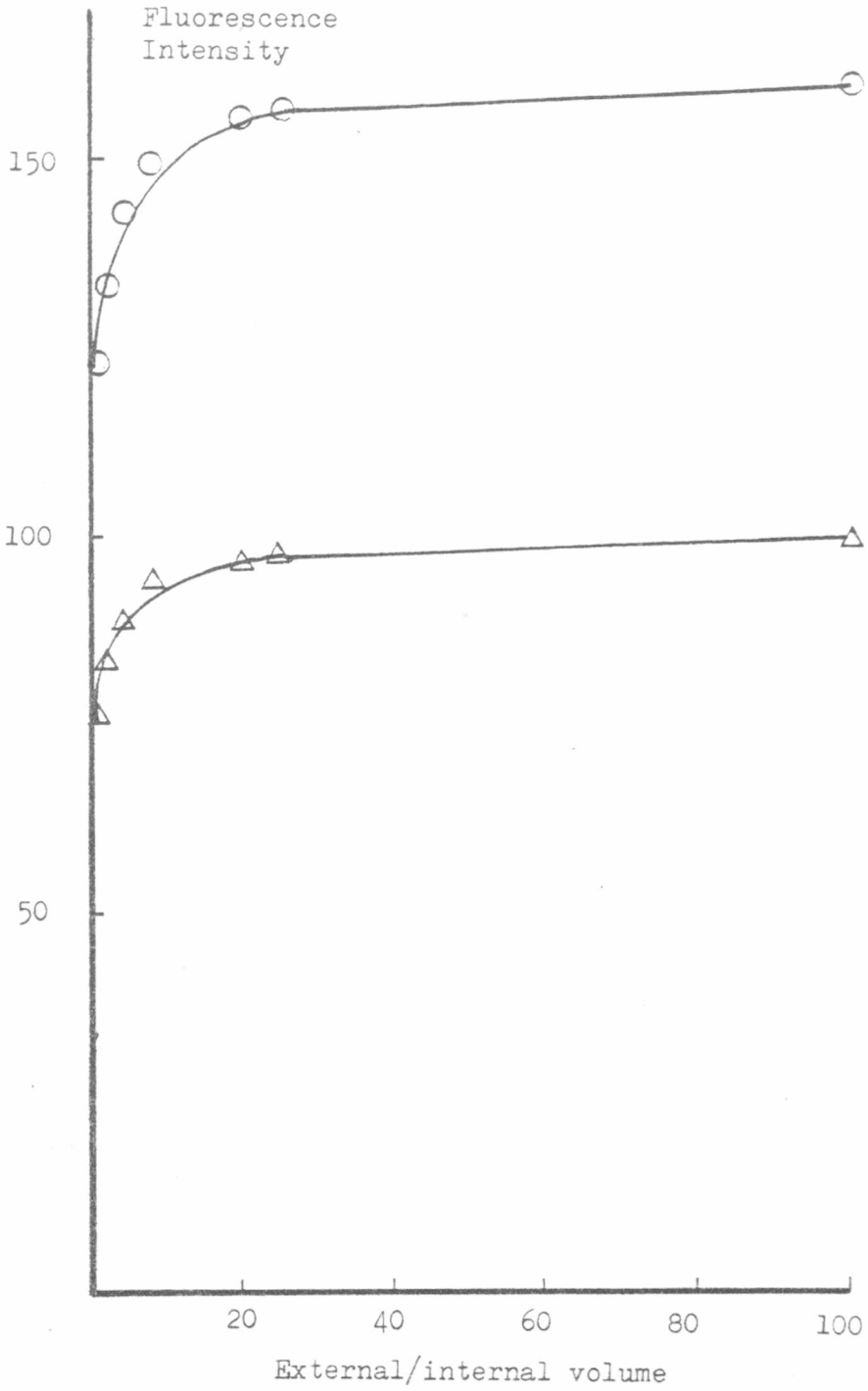


Figure 3.10

A graph showing how the ratio of external/internal volume changes the Fluorescence intensity of 2 x 10^{-6}M SA in the supernatant and Sephadex

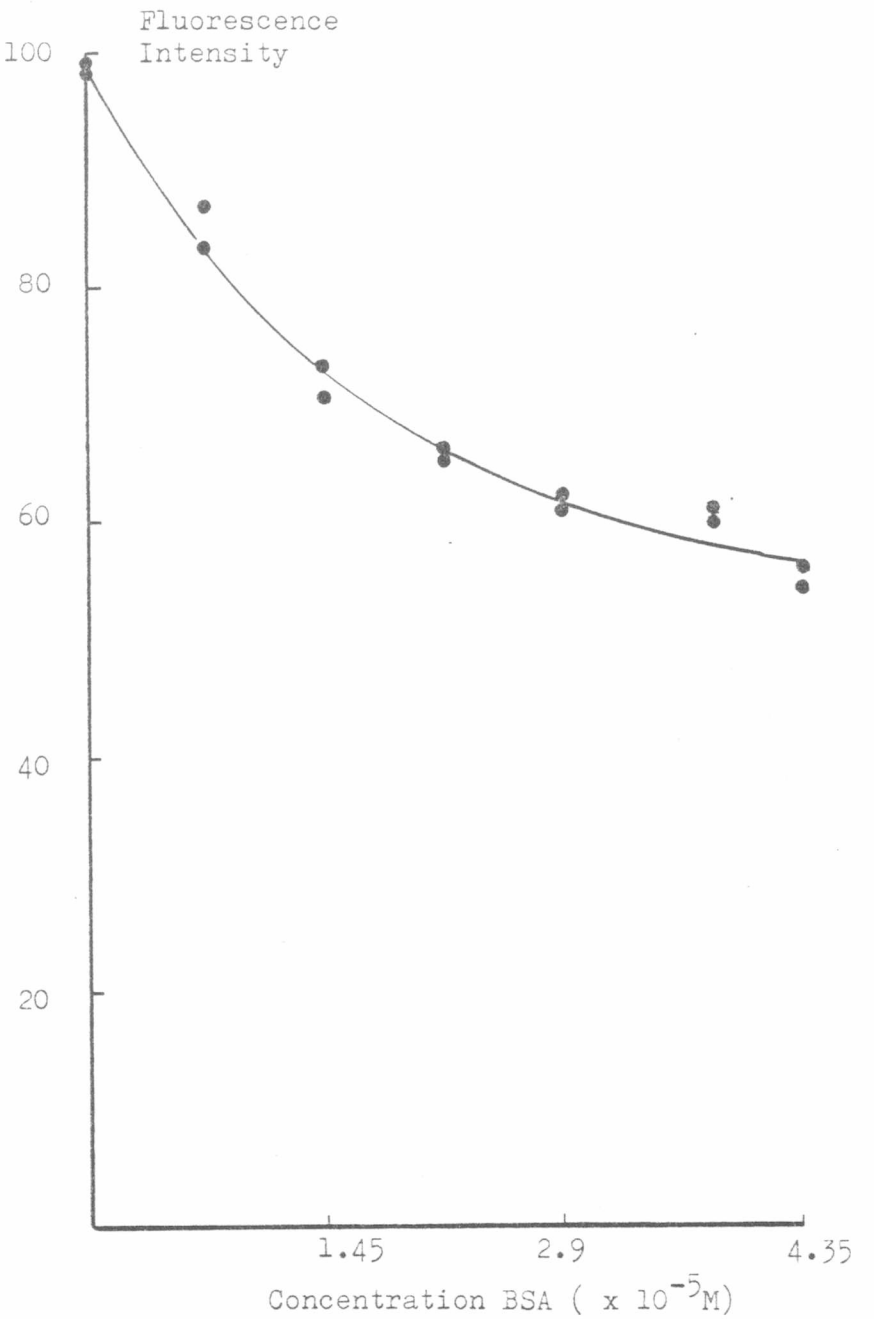


Figure 3.11

A graph showing the effect of BSA concentration on the fluorescence intensity of 1μM SA in the Sephadex

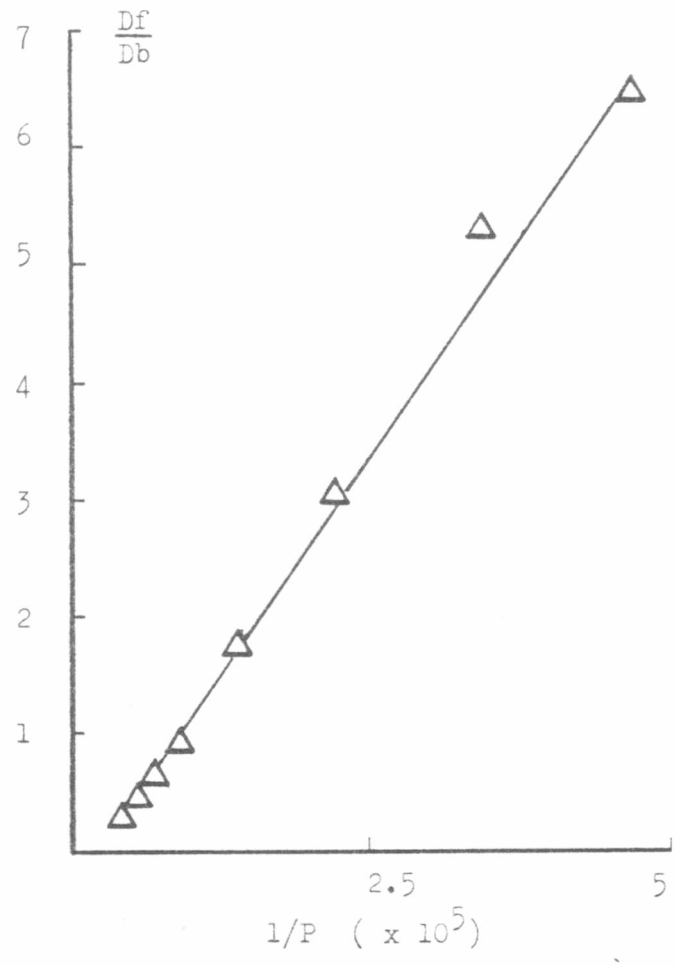


Figure 3.12

A plot of Df/Db against 1/P for the binding of 1μM SA to BSA using the batch Sephadex method

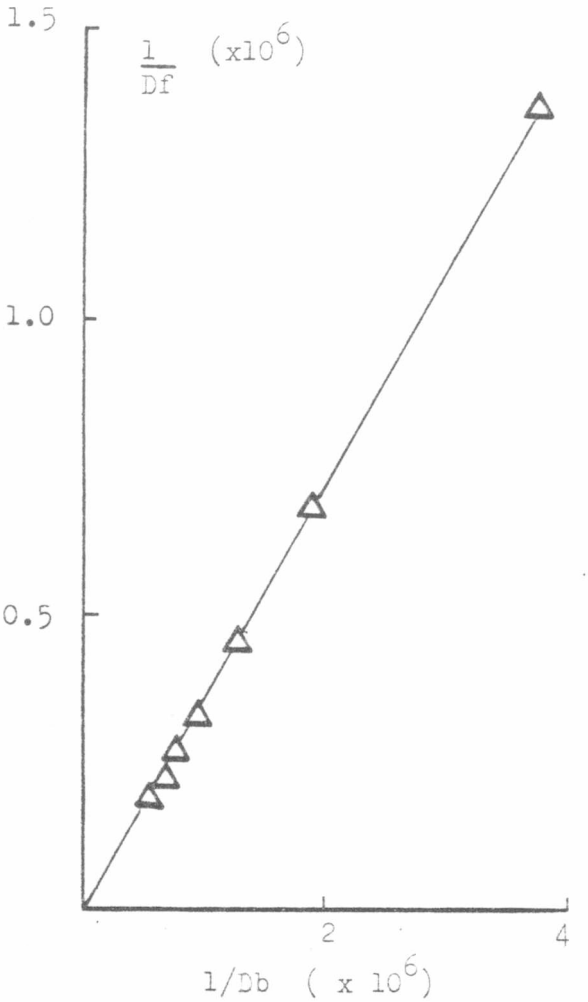


Figure 3.13

A plot of 1/Df against 1/Db for the binding of SA to 1.45 x 10^{-6}M BSA

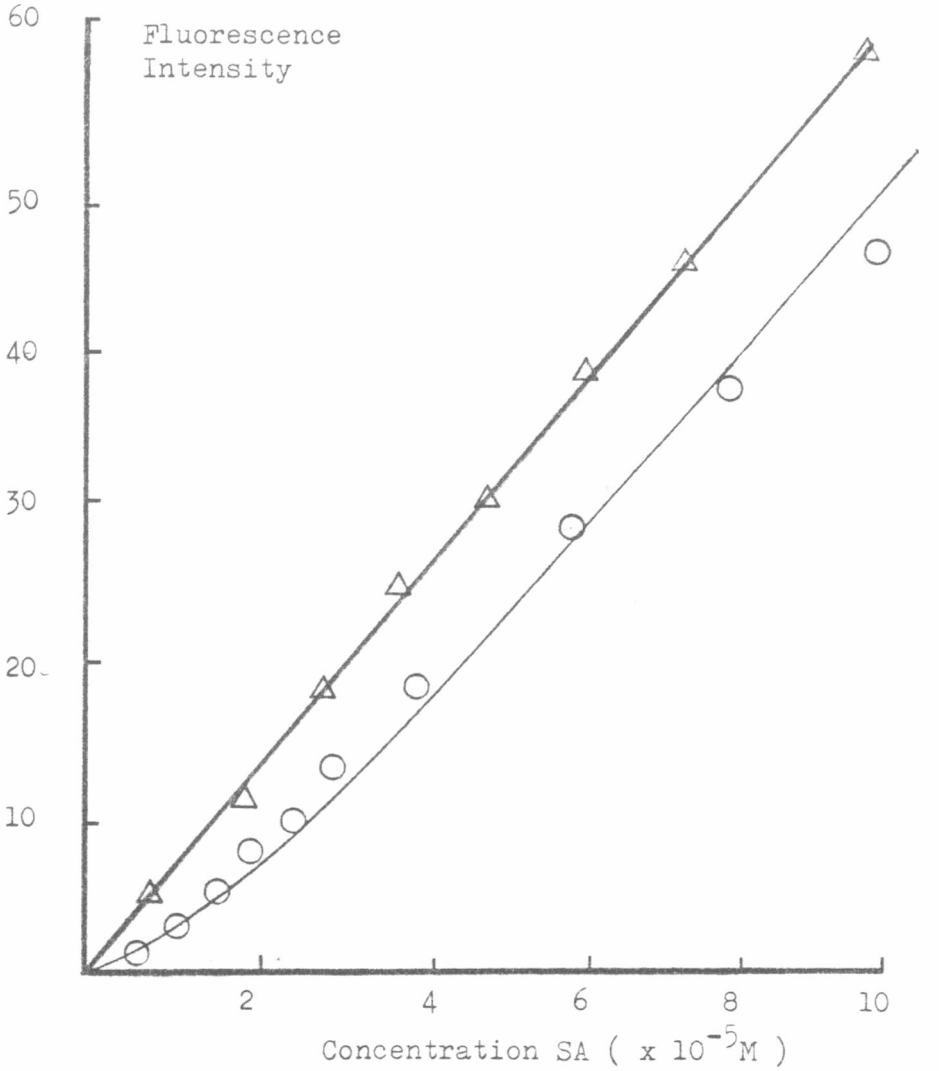


Figure 3.14

A graph showing the fluorescence intensity of the Sephadex layer with ○ and without △ 1.45 x 10^{-5}M BSA using an excitation wavelength of 330nm and an emission wavelength of 410nm.

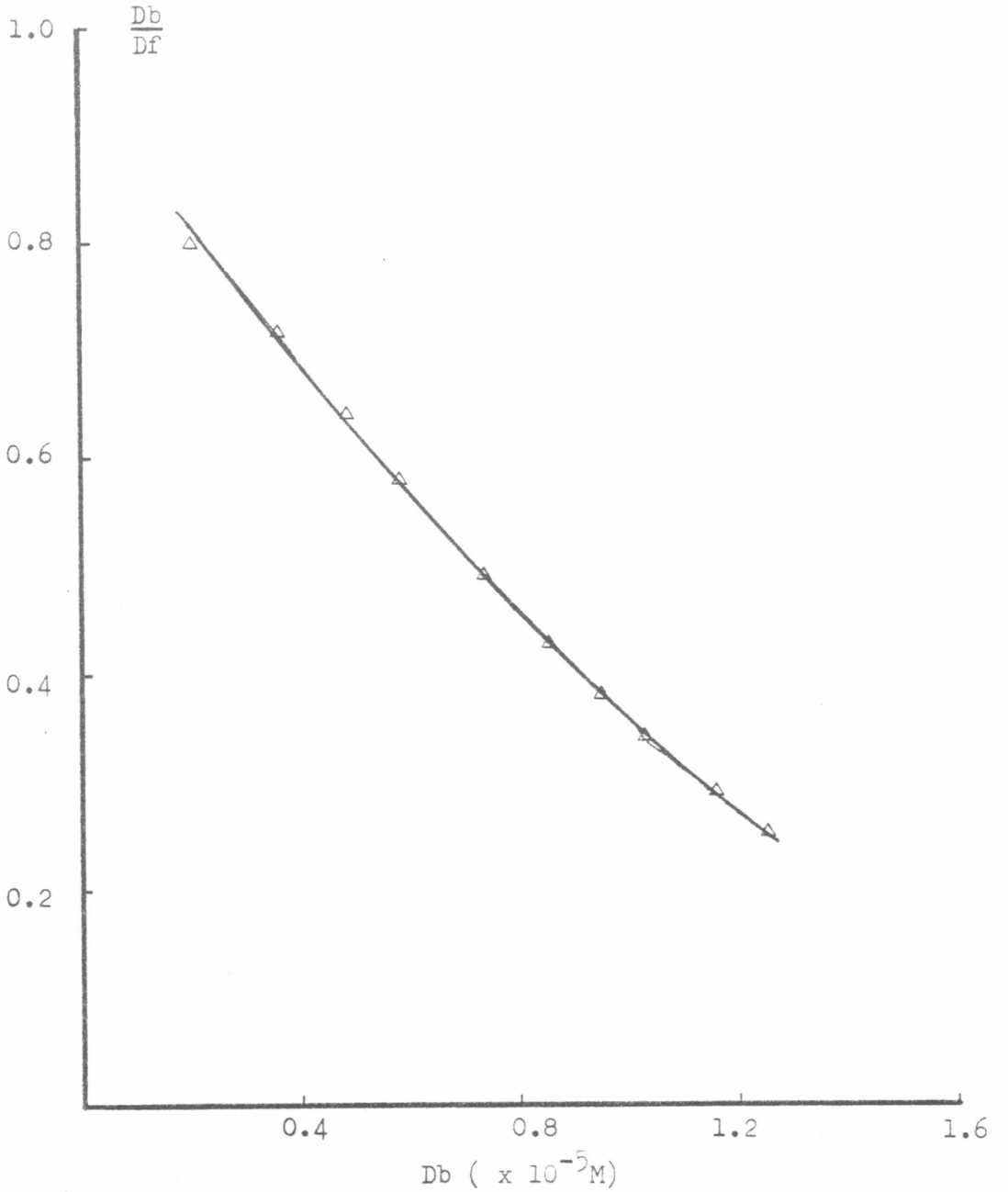


Figure 3.15

A Rosenthal plot for the binding of SA to 1.45 x 10^{-5} M BSA Db and Df were calculated from the Sephadex batch method

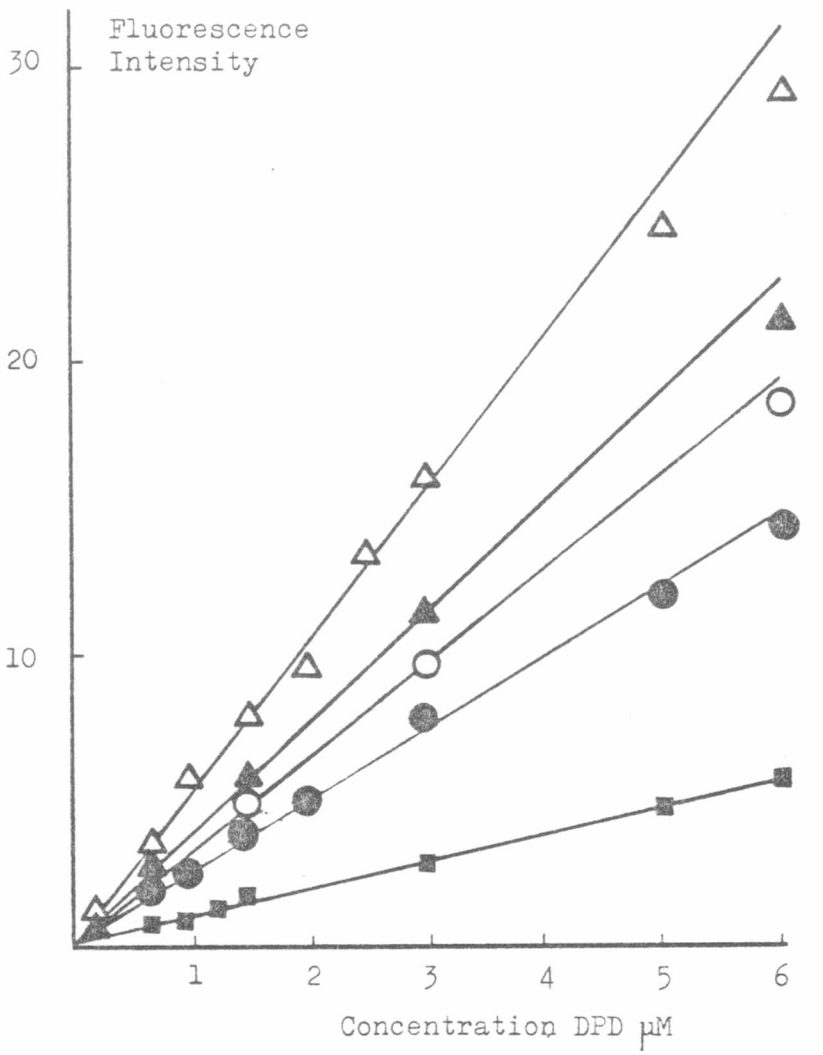


Figure 3.16

A graph showing the effect of varying the protein concentration on the fluorescence intensity of DPD

△ Standard Sephadex ▲ Sephadex 1% plasma

○ Sephadex 2% plasma ● Sephadex 4% plasma

■ standard

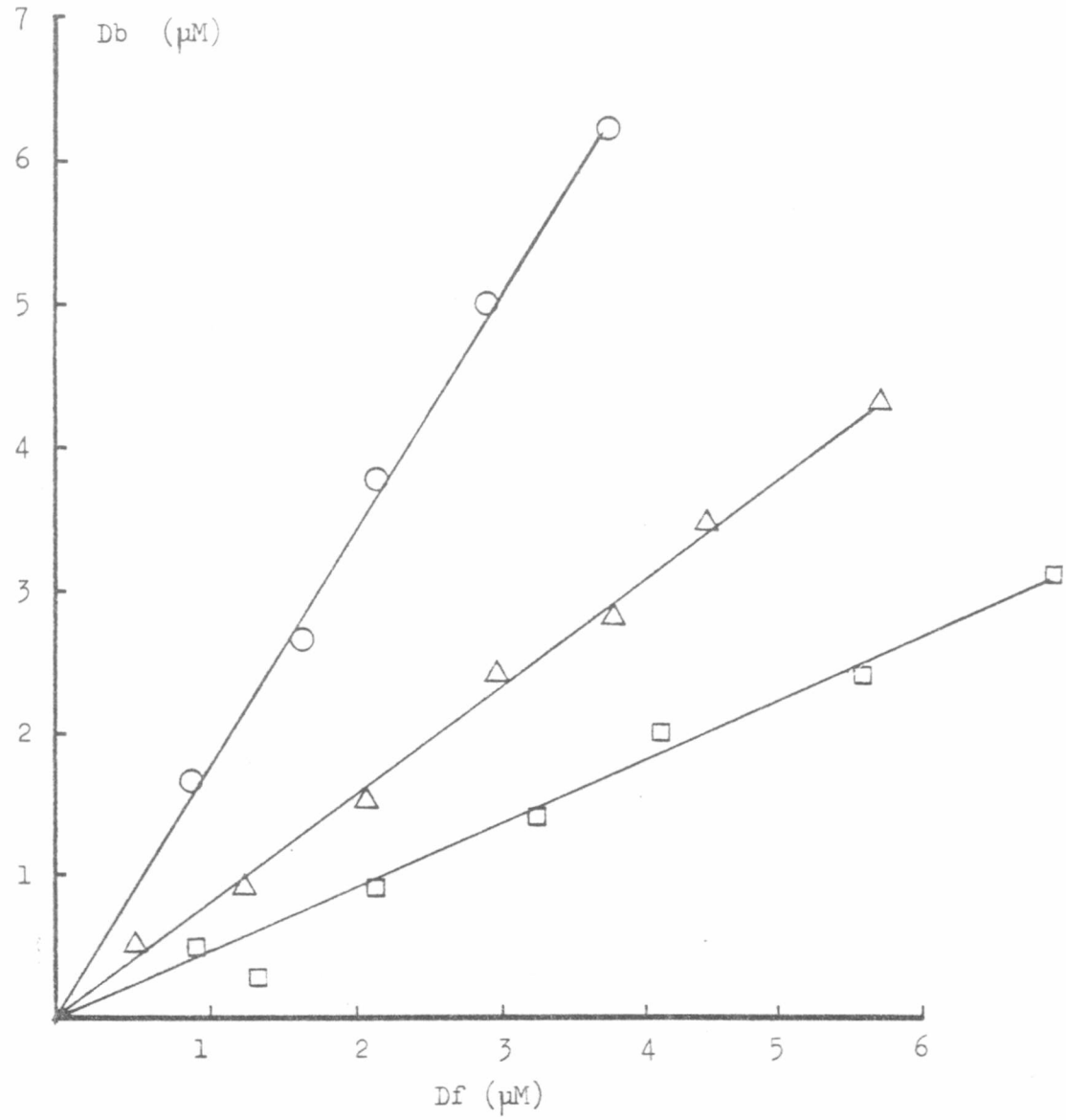


Figure 3.17

A graph showing a plot of Db vs Df for the binding of DPD to BSA

○ 2.9×10^{-4}M △ 1.45×10^{-4}M □ 7.25×10^{-5}M BSA

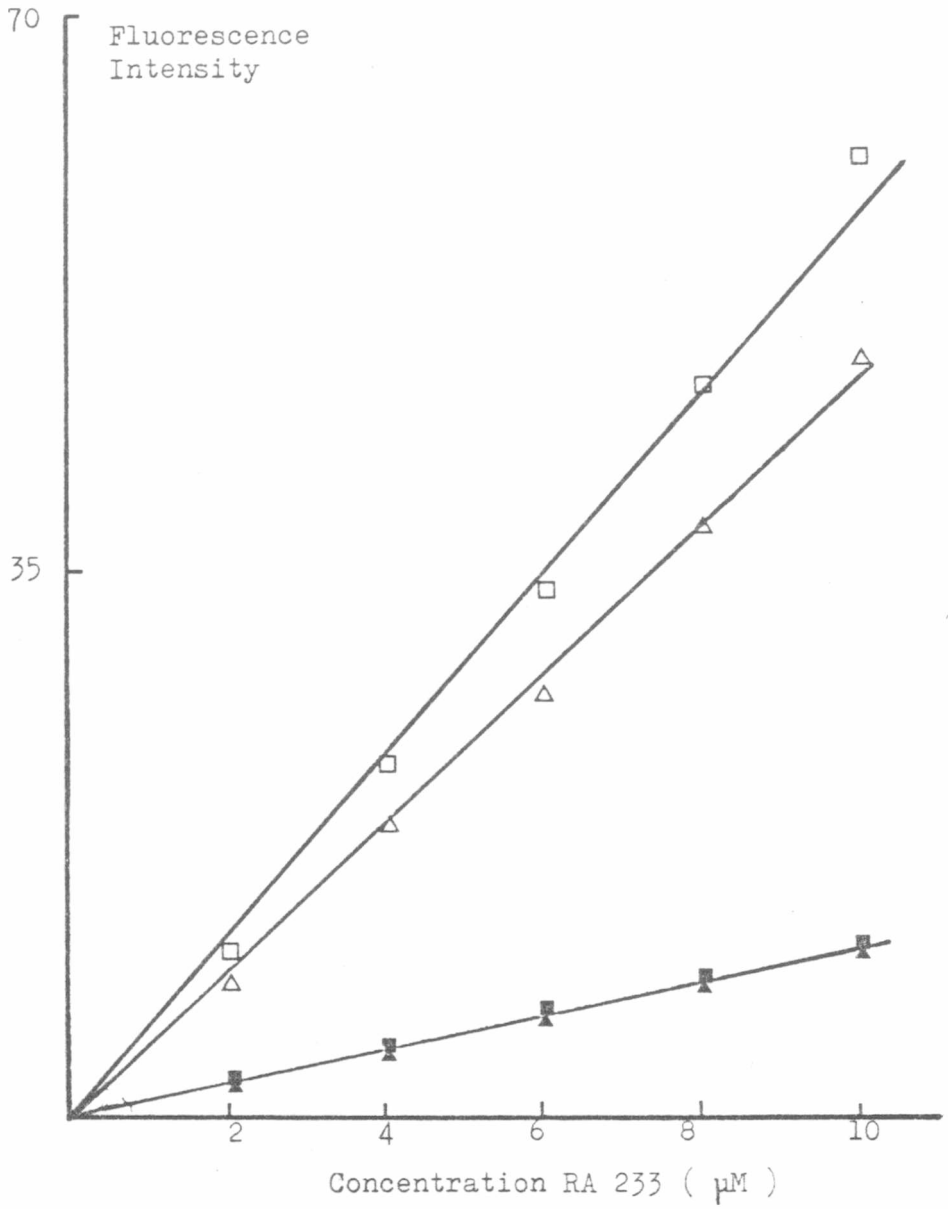


Figure 3.18

A graph showing the effect of plasma on the fluorescence intensity of RA 233 in the presence of the Sephadex

□ standard Sephadex △ Sephadex with 10% plasma

■ supernatant with 10% plasma

▲ standard

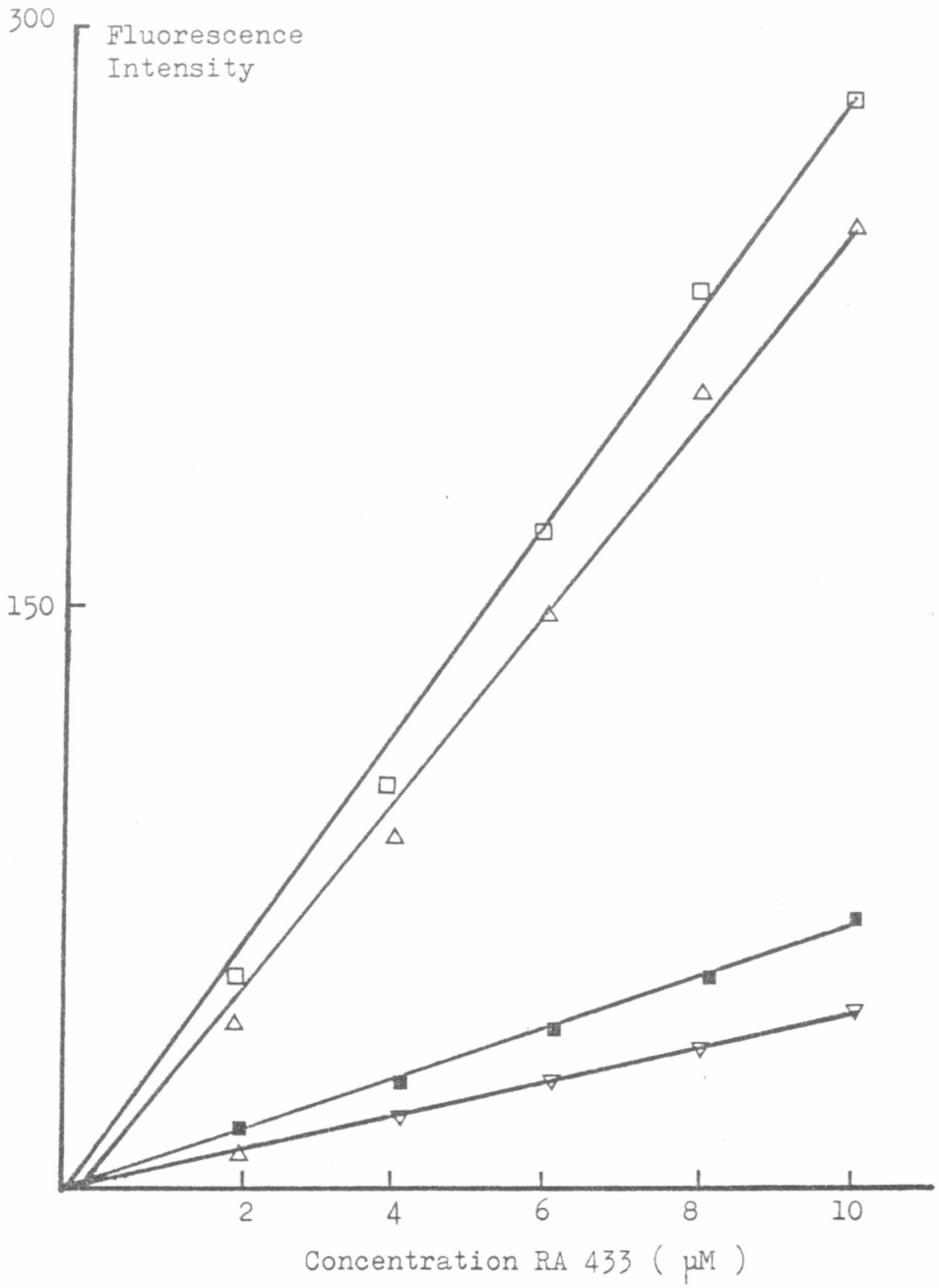


Figure 3.19

A graph showing the effect of plasma on the fluorescence of RA 433 in the presence of Sephadex

□ standard Sephadex △ Sephadex with 10% plasma

■ supernatant with 10% plasma

▽ standard

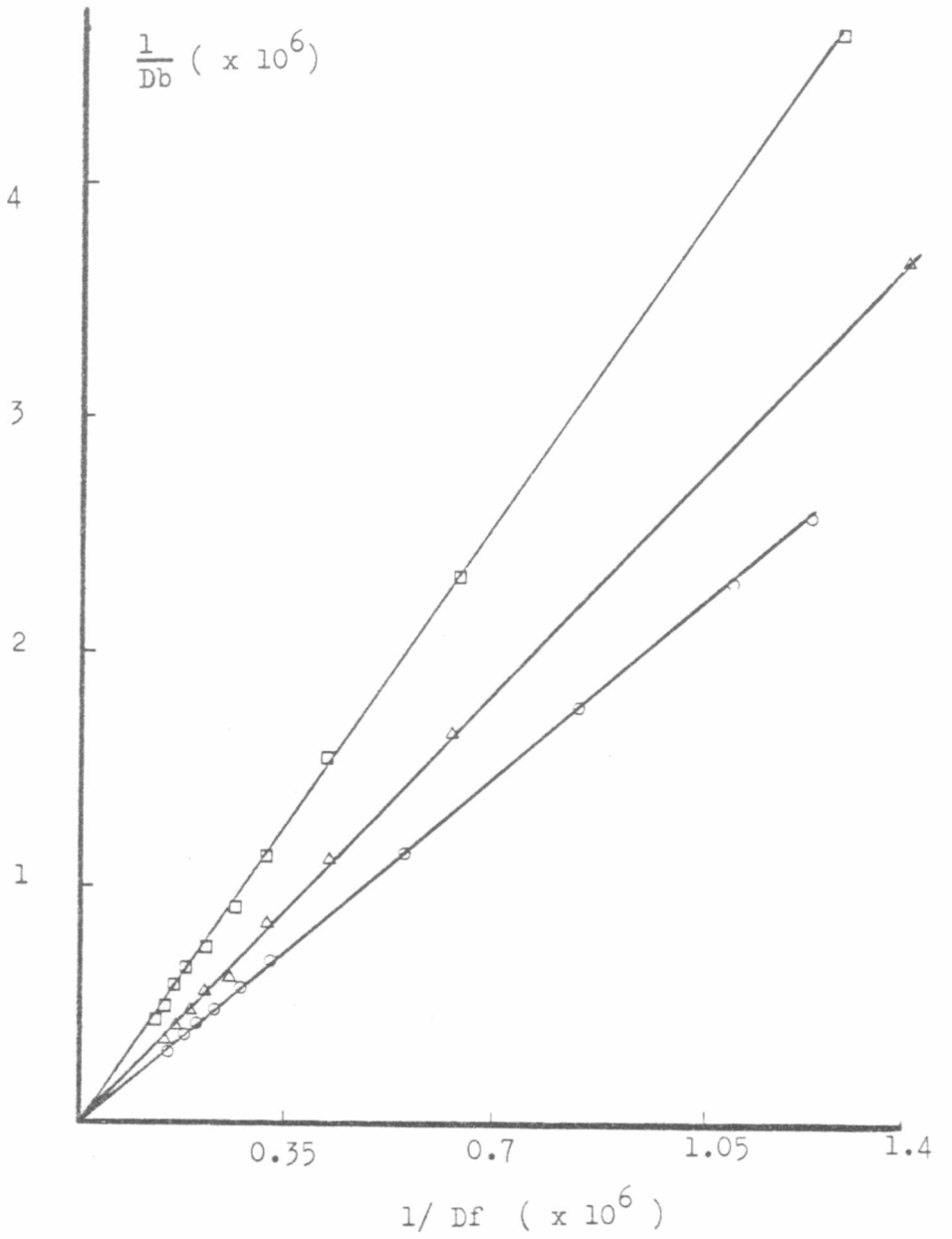


Figure 3.20

A graph comparing the binding of RA 233 and RA 433 to 10% plasma and DPD to 1% plasma. Data from the Sephadex batch method.

△ RA 233 □ RA 433 ○ DPD

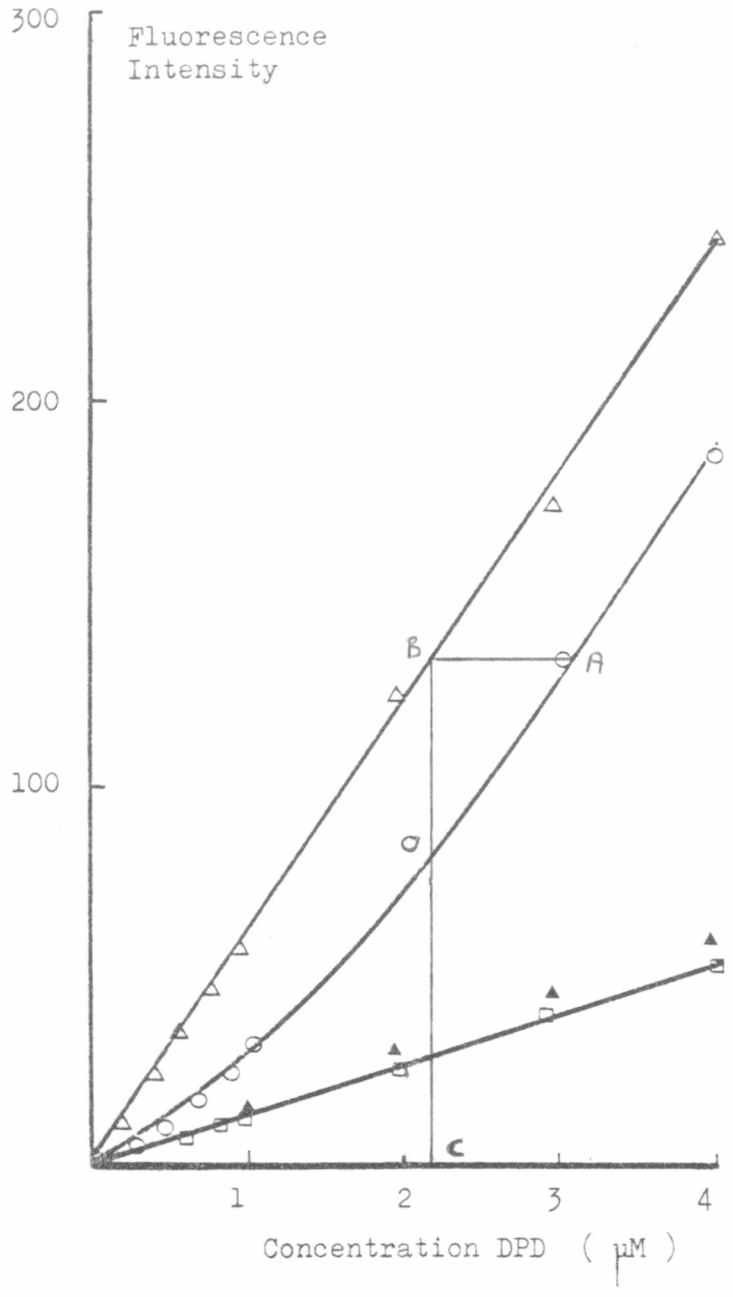


Figure 3.21

A graph of the fluorescence intensity of DPD against DPD concentration with ○ and without △ 2.5×10^{-6} M AGP

□ standard ▲ supernatant

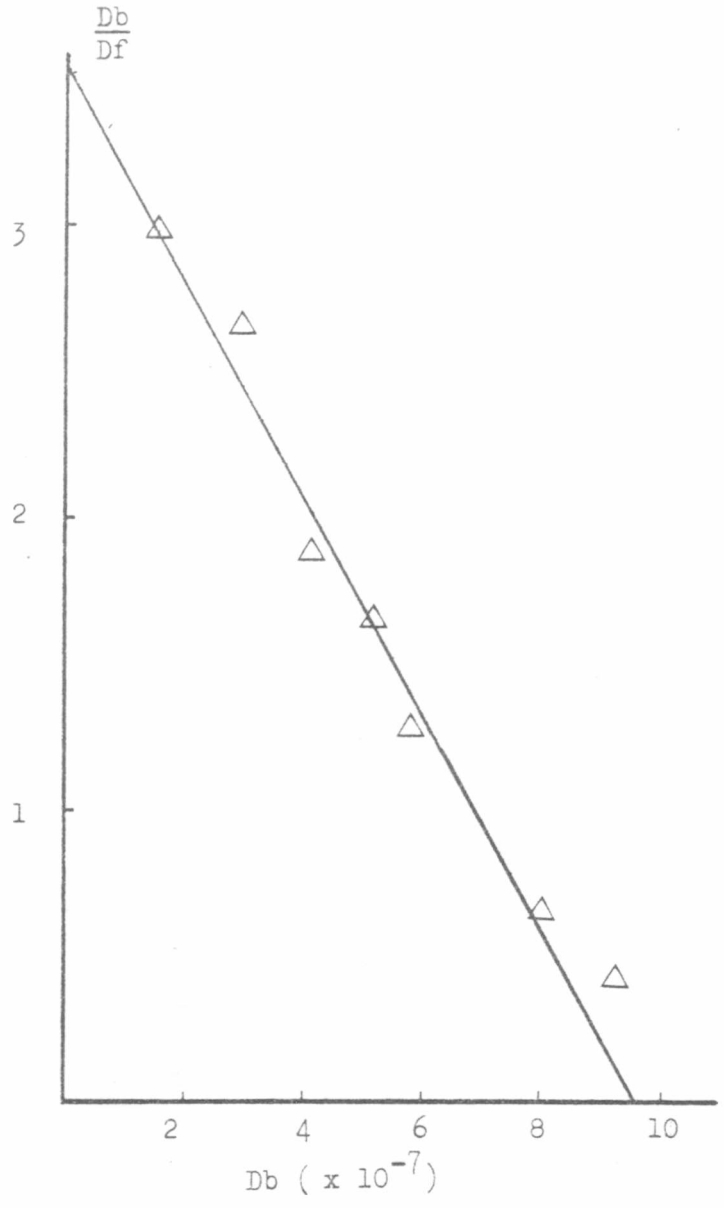


Figure 3.22

A Rosenthal plot for the binding of DPD to 0.01% AGP using the Sephadex batch method.

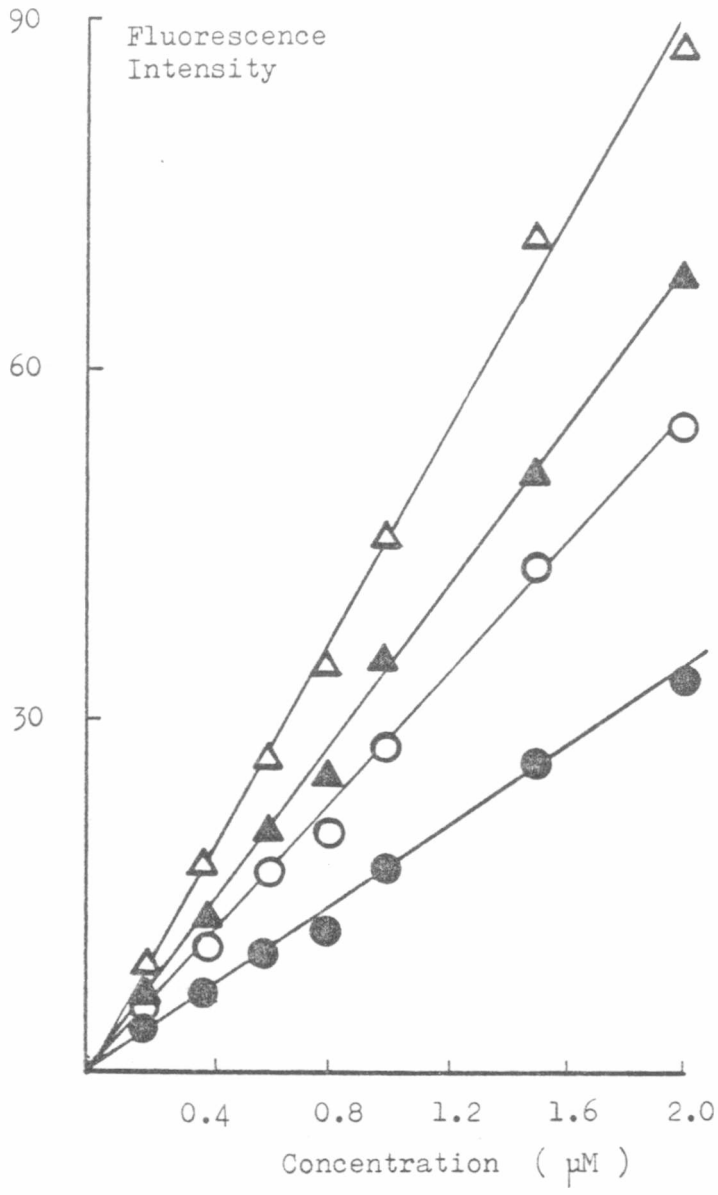


Figure 3.23

The binding of prazosin to plasma and albumin using Sephadex

△ standard Sephadex

▲ 1.45 x 10 M BSA

○ 10% plasma

● standard prazosin

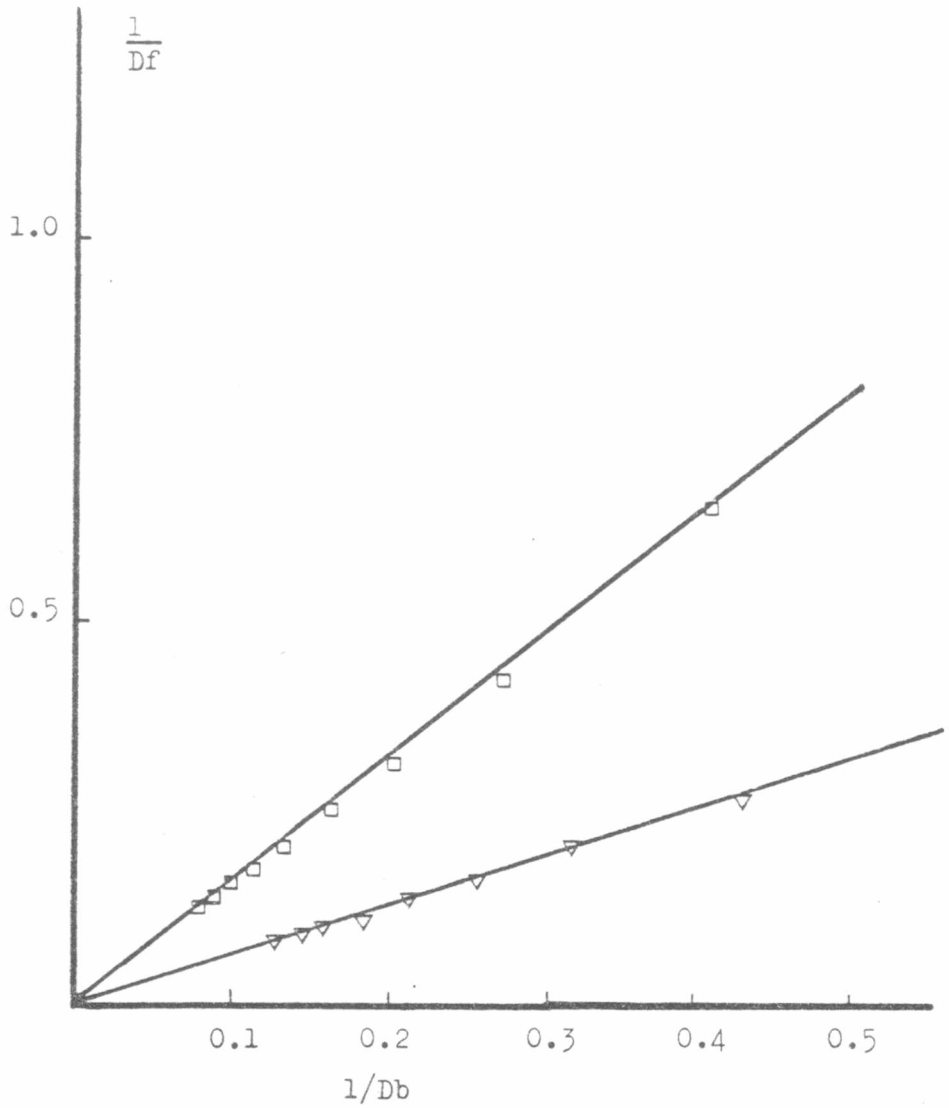


Figure 3.24

A graph of 1/Df against 1/Db for the binding of prazosin to albumin ▽ and plasma □

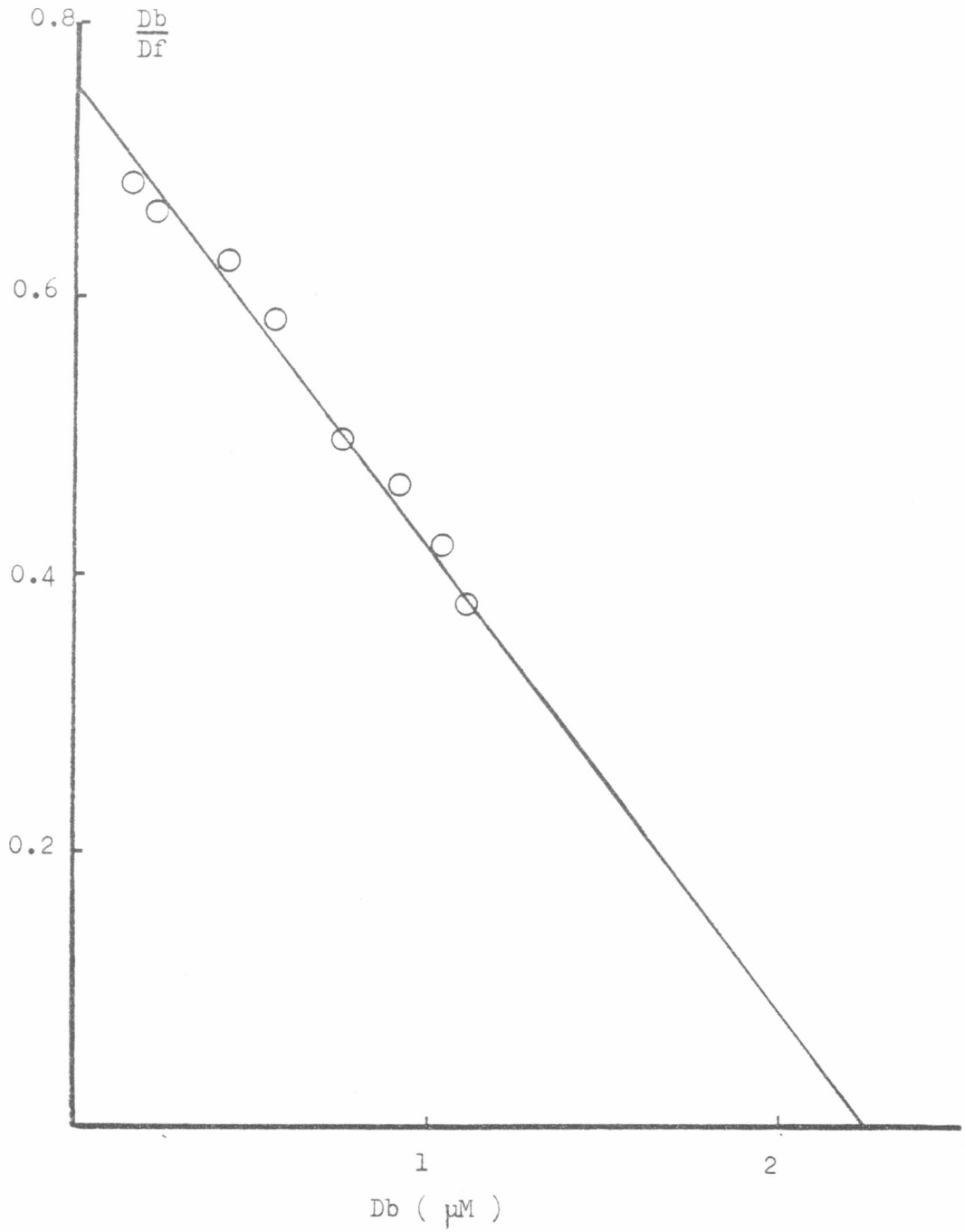


Figure 3.25

A Rosenthal plot for the binding of prazosin to 2.5×10^{-6} M AGP.

CHAPTER FOUR

A STUDY OF THE BINDING OF PLATELET ACTIVE COMPOUNDS TO α_1 ACID GLYCOPROTEIN

A STUDY OF THE BINDING OF PLATELET ACTIVE COMPOUNDS TO α_1 ACID GLYCOPROTEIN.

4.1 INTRODUCTION.

AGP is one of the best characterised glycoproteins, and it is the major binding protein for many cationic drugs in plasma. Thus the binding of propranolol, dipyridamole, and prazosin correlates with AGP concentration in plasma but not to albumin concentration. The function of AGP is not fully understood but the blood levels of it are above normal in a large number of conditions characterised by stress. These include inflammation (Jamieson et al., 1972a), myocardial infarction (Synder et al., 1975), ulcerative colitis, pregnancy (Adams and Wacher, 1968), cancer, pneumonia and rheumatoid arthritis. The parameters common to these states appear to be cell proliferation. In order to understand fully the binding of ligands to AGP it is necessary first to consider the structure of AGP. The binding of ligands to AGP can then be considered not only as a simple equilibrium but also in structural terms.

4.1.1 Physical characteristics.

Schmid (1975) suggested the following characteristic which distinguish AGP from other plasma proteins :-

1) A very high carbohydrate content
2) A large number of sialyl residues
3) A very acidic isoelectric point
4) A high solubility in water and certain polar organic solvents even when the protein is grossly denatured
5) A large number of amino acid substitutions

6) A significant degree of homology with the immunoglobulins

These physical characteristics help in the isolation and separation of AGP particularly :-

1) The electrostatic net charge which at pH values above 4 is always negative because of the very high acidic isoelectric point of the protein.

2) The solubility of the protein which is largely as a consequence of the electrostatic properties, is extremely high near neutrality.

Numerous methods have been published for the isolation of AGP including that of Weimer et al. (1950, 1955) and Marcais et al. (1970).

4.1.2 Molecular composition.

AGP is approximately composed of 45% carbohydrate and 55% polypeptide. The carbohydrate moiety has been reported to have a range of sugar constituents (Yamashina, 1956; Bezkorovainy and Winzler, 1961; Schultze, 1962; Hughes and Jeanloz, 1964; Isemura and Schmid, 1971). Differences reported by the above workers are probably due to the analysis of different preparations. Five different monosaccharides exist on the AGP according to Jeanloz (1972). The carbohydrate units are linked N-glycosidically to the asparagine residues in positions 15, 38, 54, 75 and 85 of the polypeptide chain (Schmid, 1975). No enzyme has yet been isolated that is known to specifically cleave the carbohydrate units from native or desialyzed AGP.

The reported amino acid composition of AGP has differed from investigation to investigation. Reports of the molecular weight have varied in the range of 40000 to 44000. The molecular weight of the polypeptide chain is about 21270 which consists of 181 residues (Schmid et al., 1973). Assuming the polypeptide residue moiety accounts for 55%

of the molecular weight the total molecular weight would be 39500. This is in close agreement of the now accepted molecular weight of 40000. Two disulphide bridges link residue 5 to 147 and 72 to 164. It contains three tryptophan residues in positions 25, 122, and 160. Multiple amino acid substitution has been shown.

Although the secondary structure of AGP has not yet been established using X-ray diffraction pattern analysis, preliminary assessment of the conformation present in AGP has been arrived at from optical rotatory dispersion and more recently circular dichroism measurements. The former determination (Schmid and Kamiguama, 1963) demonstrated that while the content of alpha helices is negligible a considerable proportion (Approximately 77%) of the polypeptide chain of the native protein has assumed a beta conformation. Circular dichroism data evaluated according to Greenfield and Fasman (1969) confirmed this conclusion and revealed the presence of approximately 70% of beta conformation and again negligible amount of alpha helices. The protein was transformed into a random coil by 4M guanidine, 2-chloroethanol and urea. Ganguly and Westphal (1968) noted that the intrinsic viscosity of AGP in 4M sodium chloride is smaller than in distilled water thus demonstrating a change in the conformation of the protein.

4.1.3 The binding of ligands to AGP

During the past decade AGP has attracted the attention of pharmacologists since it could be demonstrated that it represents the major binding protein of several basic drugs. However only a few observations have been made concerning the molecular aspects of the interactions of drugs with AGP (i.e number of sites, affinity constants, location and structure of the binding site).

De Leve and Piafsky (1981) stated that the plasma binding of propranolol, alprenolol, lidocaine, methocaine, methadone and quinidine correlates with AGP concentration in plasma and not to albumin concentration. Kerkay and Westphal (1969) showed that progesterone bound to AGP using equilibrium dialysis. Their data was analysed according to the method of Scatchard which gave one primary site with an association constant of 3.5×10^{7} M^{-1} at 37 °C and also a number of secondary binding sites. The progesterone binding was inhibited by magnesium ions. Ganguly and Westphal (1968) noted that the apparent binding affinity between progesterone and AGP is increased manifoldly by neutral salts such as disodium sulphate and ammonium sulphate. These salts generally stabilise the conformation of AGP. Lithium bromide, urea and calcium chloride, which destabilise the AGP, decreases the apparent stability of the complex.

Brinkschulte (1980) studied the contribution of AGP on the plasma binding of perazine, amitriptyline and nortriptyline in man using equilibrium dialysis. Piafsky and Borga (1977) showed that the binding of alprenolol and imipramine correlated with the plasma concentration of AGP. No attempt was made to evaluate association constants. Borga et al. (1977) demonstrated that there was a significant negative correlation between the plasma concentration of AGP and the free fraction of propranolol in plasma. Sager et al. (1979) showed that propranolol has a primary affinity site with a association constant of 1.3×10^{6} M^{-1}. The capacity of the site was shown to correlate with the AGP concentration. Hobbs and Twomey (1979) demonstrated the protein binding of prazosin by equilibrium dialysis.

Kopitar and Weissenberger (1971), using equilibrium dialysis found that DPD binds to AGP with one site and calculated a nK value of $8 \times 10^5 M^{-1}$. Niewiarowski et al. (1975) demonstrated using Sephadex G-25 a complex between DPD and AGP but no attempt was made to investigate the characteristics of the binding site. Subbarao et al. (1975) showed that AGP inhibited the binding of DPD to platelets. Further studies by Subbarao et al. (1977a) using equilibrium gel filtration evaluated a dissociation constant of 1.6μM. The molar ratio of the components in the complex was estimated according to the method of Hummel and Dryer (1962). It was suggested that one mole of DPD binds with one mole of AGP to form a 1:1 complex. Gamel et al. (1982) investigated the binding of DPD using circular dichroism, UV absorbance and equilibrium dialysis. Results obtained from equilibrium dialysis using 2.5×10^{-5} M AGP showed two classes of binding sites. Characteristics of the binding sites were investigated by changing the ionic strength of the buffer and adding urea.

4.1.4 Aims and objectives

Very little data is available for the binding of drugs to AGP. The literature search revealed that

1) The majority of the binding data has been obtained using plasma rather than AGP alone

2) Very little data is available which used pure AGP

3) The majority of studies used equilibrium dialysis

4) The majority of studies have used radiolabelled ligands

5) Very little data has beeen reported where an association constant has been evaluated

6) Very little competitive binding data is available

7) Very few studies have tried to characterise the binding site although AGP is the best characterised glycoprotein

DPD is probably the best characterised site on AGP but no competitive studies have been conducted. No one has used fluorescence or fluorescence polarisation for studying the binding of ligands to AGP. The aim of this study is to apply direct fluorescence methods and fluorescence polarisation to investigate the binding of DPD to AGP. As far as possible, using these two methods, the characteristics of the binding site will be evaluated. Competitive studies were also undertaken in order to further characterise the binding site..

4.2 Materials and Instrumentation

Materials and instrumentation were as previously described with the addition of the following.

Human AGP was purchased from Sigma. A molecular weight of 40000 for AGP was used throughout this work.

2,3- diphenyl pyrazine (2,3 DPP) Figure 4.1, 2,5- diphenyl pyrazine (2,5 DPP) Figure 4.2, 2,3- diphenyl quinoxaline (DPQ) Figure 4.1, 2,3- dimethyl quinoxaline (DMQ) Figure 4.1, 4,5- dihydro -2,3- diphenyl pyrazine (DPP2H) Figure 4.2, and dibenzo (f,h) quinoxaline (DBQ) Figure 4.2 were synthesised by Dr. D. G. Durham.

4.3 Methods

4.3.1 Direct fluorescence

4.3.1.1 Changes in the intrinsic fluorescence of AGP

Using an excitation wavelength of 295nm and an emission wavelength of 335nm the change in the intrinsic fluorescence of AGP was investigated in the presence of DPD. A 5 X 5mm quartz cuvette with both

the excitation and emission slit width set at 10nm, were used for all the measurements.

4.3.1.2 Titration assuming one binding site

A 2.5 X 10^{-6} M solution of AGP was titrated with DPD until no further quenching of the fluorescence of the AGP was observed. The concentration of free and bound DPD was then calculated

$$Db = q\ Pt \qquad (4.1)$$

where

$$q = (Fi - F)/(Fi - Fmin) \qquad (4.2)$$

Fi is the initial fluorescence intensity

Fmin is the minimum fluorescence intensity when all the binding sites are saturated

F is the fluorescence intensity at any point in the titration.

Pt is the total concentration of AGP

4.3.1.3 Titration not assuming a 1:1 complex but assuming that one DPD molecule effects only one tryptophan molecule

A 5 x 10^{-7} M solution of DPD was titrated with AGP. The fluorescence intensity was compared with that of a standard and the fluorescence quenching calculated by the difference. When a maximum was reached it was assumed that the DPD was totally bound. It was therefore possible to calculate the concentration of DPD bound per unit of the fluorescence quenching. 2.5 x 10^{-6} M AGP was then titrated with DPD. The concentration bound was calculated by the relationship

$$Db = Q\ F' \qquad (4.3)$$

where

Db is the concentration of DPD bound at any total DPD concentration Dt.

Q is the fluorescence quenching of the AGP at this concentration.

F' is the concentration of DPD bound per unit change in the fluorescence.

The free concentration Df could then be calculated. From this data a Rosenthal or Scatchard plot could be constructed.

4.3.1.4 Titration not assuming any fixed number of binding site or that the quenching is directly proportional to amount bound.

This method assumes that one tryptophan molecule is quenched by two DPD molecules or that the quenching is different for the different tryptophans. A number of fluorescence titrations were conducted using a range of protein concentrations. The fluorescence intensity at any point in the titration was expressed as a percentage of the initial fluorescence and plotted against the DPD/AGP ratio. The concentration of the AGP was increased until the titration curves were superimposable. It was assumed that the drug was then totally bound. The inner filter effect was corrected for, using Chignell's (1972) correction formula.

The concentration of bound drug can then be calculated. The fraction of drug bound at any point in the titration is given by

$$Db = x/(x + y) \qquad (4.4)$$

where

x and y are defined in Figure 4.3.

4.3.1.5 Changes in the intrinsic fluorescence of DPD

Using an emission wavelength of 490nm, an excitation spectrum of a 5×10^{-7} M solution of DPD was recorded from 250nm to 470nm. This was repeated in the presence of 2.5×10^{-6} M solution of AGP. A series of spectra were recorded for DPD solutions over the concentration range of 5×10^{-7} M to 10^{-5} M.

4.3.1.6 Fluorescence polarisation

Fluorescence polarisation measurements were made as previously described. Using a 1 μM solution of DPD with the emission wavelength fixed at 490nm an excitation spectrum was recorded first with both the excitation and emission polarisers set in the vertical mode. This was repeated but the emission polariser was changed to the horizontal position. Two further spectra were recorded with both the excitation and emission polarisers in the horizontal mode and with the excitation polariser in the horizontal mode and the emission polariser in the vertical position. These readings were used to calculate the grating correction factor G.

2.5×10^{-6} M AGP was added to the DPD solution and the above repeated. For routine polarisation measurements an excitation wavelength of 420nm and emission wavelength of 490nm were employed using a 2.5×10^{-6} M AGP solution.

The saturation factor was then used to calculate the free and bound concentration of drug and a Rosenthal plot constructed.

4.3.2 Competitive studies

For competitive studies a fixed concentration of the competitor was added to the AGP. This was titrated with DPD. Alternatively a fixed concentration of DPD was added and this titrated with the competitor. The equilibrium can be considered as

$$P + D + I \rightleftharpoons PD + PI$$

This involves two affinity constants, one for the drug and inhibitor

$$P + D \rightleftharpoons PD$$

$$P + I \rightleftharpoons PI$$

$$k = \frac{[PD]}{[P]\ [D]} \qquad (4.5)$$

$$[PD] = k\ [P]\ [D] \qquad (4.6)$$

$$Ki = \frac{[PI]}{[P]\ [I]} \qquad (4.7)$$

$$[PI] = Ki\ [P]\ [I] \qquad (4.8)$$

$$r = \frac{\text{moles of drug bound}}{\text{total moles of protein}}$$

$$r = \frac{[PD]}{[P] + [PD] + [PI]} \qquad (4.9)$$

$$r = \frac{k\ [P]\ [D]}{[P] + k\ [P]\ [D] + Ki\ [P]\ [D]} \qquad (4.10)$$

$$r = \frac{K\ [D]}{1 + k\ [D] + Ki\ [I]} \qquad (4.11)$$

$$\frac{1}{r} = 1 + \frac{1}{k\,[D]} + \frac{Ki\,[I]}{k\,[D]} \qquad (4.12)$$

$$\frac{1}{r} = 1 + \frac{1}{k\,[D]}\,(1 + Ki\,[I]) \qquad (4.13)$$

The results were analysed by non linear regression analysis. The listing of the programme is given in Appendix 8.

4.4 Results and discussion

4.4.1 Direct fluorescence

4.4.1.1 Changes in the intrinsic fluorescence of AGP

AGP contains both tyrosine and tryptophan residues. As previously described by using an excitation wavelength of 295nm or above only the tryptophan residues will be excited (Chignell, 1970). This therefore allows the three tryptophan residues to be investigated specifically. The fluorescence of the AGP was quenched by DPD. The emission spectrum of AGP overlaps the excitation spectrum of DPD. It was envisaged that nonresonance transfer of energy from the excited AGP molecule to the DPD was taking place. Using an excitation wavelength of 295nm therefore means that nonresonance transfer of energy is occuring from one of the tryptophan residues to the DPD molecule. This can only occur if DPD is binding in the region of one of the tryptophan residues (Forster, 1948; Forster, 1959; Chignell, 1970a and 1970b). Because AGP contains more than one tryptophan unit, the mean effective transfer distance between the tryptophan residue and the DPD cannot be calculated.

4.4.1.2 Evaluation of results assuming one binding site

Evaluating the data making the assumption of one binding site resulted in several problems. When the protein concentration was higher than 2×10^{-6} M, the concentration of DPD which was calculated to be bound was greater than the concentration present at low DPD/AGP ratios. At lower concentration of AGP this was not a problem but a Scatchard plot or Rosenthal plot was not linear. This therefore indicates that the initial assumption of one binding site per molecule of AGP was incorrect. The quenching indicates that more than one tryptophan residue is being effected by the DPD. In the next section the analysis of the binding data not assuming fixed number of binding sites has been described.

4.4.1.3 Evaluation of data not assuming any fixed number of binding sites

This method assumes that if there is more than one binding site the quenching of the tryptophan at both sites is the same. The quenching is assumed to be proportional to the concentration of DPD bound. Figure 4.4 shows the effect of titrating 5×10^{-7} M DPD with AGP. This was compared with a standard AGP solution. The concentration of DPD bound per unit change in the fluorescence intensity was calculated from this graph. Figure 4.5 shows the results of a typical titration of 2.5×10^{-6} M AGP with DPD. From this the free and bound concentration of DPD was calculated and a Rosenthal plot constructed, Figure 4.6. Using nonlinear regression analysis an affinity constant of 1.06×10^{6} M^{-1} se 0.065 with 0.5 se 0.02 binding sites.

4.4.1.4 Evaluation of data not assuming the quenching is directly proportional to the amount bound

The previous method used to calculate Db and Df assumes that only one DPD molecule is causing the quenching of any given tryptophan residue. If the protein has several drug binding sites each located at a distance equal to the average transfer distance from the tryptophan group, then the binding of a single drug molecule to the protein will result in a 50% decrease in the fluorescence intensity of the tryptophan. However a 50% decrease in the fluorescence quantum yield of tryptophan must mean that the fluorescence lifetime of the amino acid has also been shortened by 50%. Since the probability of energy transfer is dependent on the fluorescence lifetime of the tryptophan, it follows that when a second drug molecule binds to the protein the efficiency of transfer will be less than 50%. Thus the observed quenching of the tryptophan fluorescence will not be proportional to the number of drug molecules bound. In this study it is not envisaged that two drug molecules are affecting the fluorescence of one tryptophan but that two tryptophan residues are involved.

Figure 4.7 shows a plot of the percentage fluorescence intensity against the ratio of DPD to AGP. It was found that the binding of DPD to AGP was dependent on the concentration of protein. This was similar to the results of the binding of indomethacin to HSA and BSA (Figure 2.13 and 2.14), and may be explained similarly. Increasing the protein concentration increased the quenching of the tryptophan at any given molar ratio. Chignell (1973a) found similar results in his investigation. The calculation of Db and Df again presented problems indicating a low level of binding.

A fundamental problem exist with Chignell's method. The assumption is made that when the plot of percentage fluorescence intensity against DPD ratio are superimposed at different protein concentrations, the ligand is totally bound (Chignell, 1972). This does not necessarily follow. From equation 2.32, when the fluorescence titration curves are superimposed the ligand need not be totally bound but $1/(nkPt) \longrightarrow 0$. The fraction bound is therefore given by

$$\text{fraction bound} = 1/\ (1 + (Df/nPt))$$

Only when $Df \ll nPt$ will the fraction bound approach 1.

as $Dt \Longrightarrow Pt$

$Df/nPt \gg 0$

The fraction bound will therefore be less than 1 at high ligand/drug ratios. The dependence of the fluorescence quenching on protein concentration and the existence of a limiting protein concentration above which the fluorescence quenching curves are superimposable does not indicate that above this concentration the drug is totally bound. Chignell's method is therefore not valid.

4.4.1.5 Changes in the intrinsic fluorescence of DPD

At low concentrations of DPD the 305nm peak is effected more markedly than the 420nm peak, resulting in an enhanced fluorescence. As the concentration of DPD is increased the enhancement of the 305nm peak becomes smaller while that of the 420nm peak is quenched. These effects are probably small because of the already high fluorescence quantum yield of DPD. The fluorescence of DPD is not markedly effected by a number of hydrophobic solvents. Changes in the fluorescence due to the

change of the microenvironment of DPD on binding are therefore only likely to be small. The enhancement of the 305nm peak may be due to the nonresonance transfer of energy from the tryptophan residues to the DPD. In agreement with these results this would be most marked at low DPD/AGP ratios.

4.4.1.6 Fluorescence polarisation

The fluorescence polarisation of DPD was calculated at a number of excitation wavelengths using an emission wavelength of 490nm, with and without 2.5 X 10^{-6}M AGP. At the 305nm peak very little change was seen in the fluorescence polarisation in the presence of AGP. At the 420nm peak a marked fluorescence polarisation was seen. Figure 4.8 is a typical plot of the fluorescence polarisation of DPD against the ratio of AGP to DPD. 5 x 10^{-7} M DPD was titrated with AGP until a maximum fluorescence polarisation was reached. This value was used in subsequent experiments to determine the saturation factor of DPD. The results of the titration of 2.5 X 10^{-6}M AGP with DPD is shown in Figure 4.9. From this a free and bound concentration of DPD could be calculated. Figure 4.10 is a typical Rosenthal plot for this data. A mean value of 4.06 x 10^{6} M^{-1} se 0.425 x 10^{6} was evaluated for the affinity constant with 0.49 se 0.026 binding sites. The high polarisation value seen on the binding of DPD to AGP suggests that the freedom of movement is markedly restricted, assuming that the lifetime of the excited state remains approximately constant. This therefore indicates that DPD is bound with two or more regions of attachment.

The change in polarisation on binding of DPD was seen only at the 420nm peak. No change in the polarisation of the 305nm peak was observed under the conditions of these experiments. This suggests that

the central nucleus and the side chains of the DPD are involved in the binding.

Figure 4.11 shows the effect of changing the protein concentration on the fluorescence polarisation of DPD. The increase in the fluorescence polarisation with increasing AGP concentration is similar to the increase in the tryptophan quenching with increasing protein concentration (Figure 4.7). These results can be explained in a similar way with the help of equation 2.32. The fraction bound depends on the protein and drug concentration, the association constant of the drug protein complex and the number of sites on each protein molecule.

therefore as $1/(n\ k\ Pt) \Longrightarrow 0$

The fraction bound approaches 1

Dilution of the system containing bound drug will result in dissociation of the complex because the value of $1/(n\ k\ Pt)$ will increase thus decreasing the fraction bound. The maximum fraction bound is not necessarily unity as $Df/(n\ Pt)$ approaches 0 but is represented by the expression

$$1/(\ 1 + (1/(n\ k\ Pt)))$$

The polarisation values reflect this in that with dilution of the protein the maximum polarisation observed and therefore fraction bound decreases.

4.4.1.7 Sephadex

As shown in the previous chapter the binding of DPD to AGP can be demonstrated using a Sephadex batch method (Figure 3.22). One class of binding sites were observed with an affinity constant of $3.15 \times 10^{6} M^{-1}$

se 0.310 and 0.4 binding sites.

4.4.2 Ultraviolet difference

A 2.5 x 10^{-6} M solution of AGP when titrated with DPD gave a positive band at 295 and 315nm and a negative band at 275nm with isobestic points at 250 and 285nm. A smaller positive peak was also given at 420nm (Figure 4.12). The peaks between 280 and 340nm are probably related to the nonresonance transfer of energy from the tryptophan residues to the DPD. The peak at 420nm suggests that the DPD is binding to a hydrophobic region of the protein resulting in a characteristic red shift of its spectrum.

4.4.3 Equilibrium dialysis

Using a protein concentration of 2.5 x 10^{-6} M AGP, this method indicated that AGP had one class of binding sites (Figure 4.13). Using nonlinear regression analysis an affinity constant of 2.2 x 10^{6} M^{-1} se 1.25 x 10^{6} was evaluated with 0.4 se 0.04 binding sites. This is consistent with all the other methods which have been used to investigate the binding of DPD to AGP in this study. A theoretical problem exists in the low binding capacity that is estimated with all the methods of measuring protein binding. Bridges and Wilson (1976) reported that even low concentrations of contaminating ions may modify a drug protein binding characteristics. In plasma it was pointed out that n and k values can be considered to give an overall measure of the affinity and capacity of binding. Although the AGP was 95% pure impurities may effect the binding capacity. Costello et al. (1979) and Schmid (1975) noted that AGP exists in several polymorphic or altered forms. The DPD may only be binding to one of these forms. Other

workers have shown that the method of preparation of AGP may have a significant effect on the binding capacity of the protein. Borga et al. (1977a), Cotham and Shand (1975) and Piafsky (1980) noted that the plasticizer tris (2-butoxyethyl) phosphate inhibited the binding of various drugs to AGP. In this study it is unlikely that this is the reason for the low binding capacity. This would only fit the quenching data if the plasticizer quenched the fluorescence of the AGP. This was not expected because the absorbance spectrum of the plasticizer does not overlap the emission spectrum of AGP.

The number of binding sites evaluated with all the methods used to measure binding is approximately 0.5. An alternative explanation could be a complex forming between one molecule of DPD and two AGP. This would appear possible because of the symmetrical nature of DPD but such a complex would suffer from steric hinderance problems. When the concentration of DPD was therefore much greater than that of AGP a 1:1 complex would be more likely to be observed. This was not the case so this alternative was rejected. Luger and Roch (1983) determined the crystal structure of DPD and identified two distinct conformations. If similar conformations of DPD exist in solution the binding to AGP may only involve one of them. This would not account for the low number of binding sites per AGP molecule determined.

The binding data evaluated in this study has been compared with that reported in the literature. Subbarao et al. (1977a) found one class of sites with an affinity constant of $6.25 \times 10^{5}\ M^{-1}$. Kopitar and Weissenberger (1971) using equilibrium dialysis evaluated $nk = 8 \times 10^{5} M.^{-1}$ In agreement with the above workers this study has also found one class of binding sites with all the methods used to determine the binding.

The primary affinity constant was in the same order as the above reported values. Gamel et al. (1982) in their study using equilibrium dialysis showed two classes of binding sites for DPD. The difference between this work and that of Gamel et al. (1982) may have been due to the higher protein concentration that Gamel et al. (1982) employed. The higher protein concentration would favour the identification of a low affinity class of sites but would also create additional problems due to the low solubility of DPD at pH 7.4. Using the same protein concentration (25μM) as Gamel et al. (1982), the ratio of DPD to AGP could only be used in a limited range. The fraction of DPD bound did not change markedly over the ratios employed and therefore a value for n and k could not be evaluated but the approximation of Pearlman and Crepy (1967) was made to give a nk value. This is probably the reason why Kopitar presented his data in this way. The ratios could not be further increased due to the low solubility of DPD. Gamel et al. (1982) must have experienced similar problems in his work. The second class of binding sites that he identified may have been due to a decrease in pH at high DPD concentration. It has been show in this study that the binding of DPD is pH dependent.

Gamel et al. (1982) went some way in characterising the DPD binding sites. This study is in agreement with their findings that the site is in an hydrophobic region of the protein located near a tryptophan residue. This study has shown that the maximum quenching of the protein was approximately 2/3 thus suggesting two tryptophan residues were involved in the binding. Polarisation indicates a marked decrease in the rotational movement of the DPD on binding thus giving more evidence that it is binding to the central hydrophobic region of the protein. More than one point of attachment was envisaged because of

the high polarisation. It was thought that DPD was cross linking between the protein chain in the region of two of the tryptophans. The involvement of both the central nucleus and side chains of the DPD was predicted due to the sensitivity of the site to ligand structure.

4.4.4 Effect of urea and ionic strength

Urea abolished the increase in fluorescence polarisation of DPD on binding to AGP. This was taken to mean that urea abolished the binding of DPD to AGP. Urea causes the polypeptide chain of the AGP to form a random coil. This confirms that the DPD is binding to the polypeptide chain and not to the carbohydrate regions of the AGP.

Increasing the ionic strength of the buffer by the addition of varying amounts of NaCl did not effect the polarisation of DPD. NaCl has the effect of stabilising the protein conformation by making the protein a more compacted and ordered structure. This suggests that the site is already in a very compacted region of the AGP in contrast to progesterone's binding. (Ganguly and Westphal (1968) found that the affinity of progesterone was increased by the addition of NaCl.)

4.4.5 Effect of pH on the binding of DPD to AGP

AGP is a very stable protein, able to withstand a wide range of pH. The ionisation of DPD is also dependent on the pH of the buffer. Using a fluorescence method a pKa value of 6.4 was given (Figure 4.14) which is in close agreement with the value quoted in Martindale (1983). At pH 7.4 the DPD exists in an equilibrium between the base and the salt. At this pH the base concentration is approximately 10 times higher than that of the salt. A study was made of the binding over a range of pH values so that the binding of the base and the salt could be

investigated separately. Using the tryptophan quenching method the effect of the pH of the buffer over the range of 4-9.25 was investigated. Figure 4.15 shows the effect of the pH on the quenching. A quenching of the intrinsic fluorescence of AGP was observed at all pH values investigated. The quenching was less at low pH values than at higher values. The absorbances of DPD was shifted by a reduction in the pH but the emission spectrum of AGP still overlapped the absorbance spectrum of DPD. The fluorescence of AGP is marginally enhanced at low pH values. The above suggest that either the lowering of the pH is affecting the binding site on the AGP or the salt binds considerably less than the base.

The effect of pH was also investigated using fluorescence polarisation. The pH range used was more limited than the tryptophan quenching because of the reduced fluorescence of DPD at low pH's. Very little change in the binding was observed from pH 7.4 to 9.

4.4.6 The binding of analogues of DPD to AGP

The binding of RA 233 and RA 433 have been investigated using direct fluorescence and fluorescence polarisation. The intrinsic fluorescence of RA 233 and RA 433 was neither enhanced nor quenched in the presence of AGP. No shift in the maximum of the fluorescence peaks were observed. In marked contrast to DPD, the intrinsic fluorescence of AGP was not quenched by either of the analogues. The absorption spectra of these two compounds were very similar to that of DPD. The absorption spectra of both analogues overlapped the emission spectrum of AGP, therefore nonresonance transfer of energy could theoretically take place. This indicates a very specific binding site for DPD. RA 233 and RA 433 have only minor structural differences to DPD but show no

quenching.

Fluorescence polarisation studies showed that the fluorescence polarisation of these compounds did not change in the presence of AGP at any wavelength in their excitation spectra. Again this points to the specificity of the DPD site. No fluorescent compound other than DPD, which was investigated showed an increase in fluorescence polarisation or quenching in the AGP fluorescence. Compounds investigated included prazosin, 2,3 DPP, 2,5 DPP, DPQ, DMQ, DPP2H, SH 1242 and propranolol.

Binding studies were conducted using a number of classical methods. Studies conducted using continuous ultrafiltration were unsuccessful due to marked binding of RA 233 and RA 433 to the membrane. Using equilibrium dialysis with a protein concentration of 2.5×10^{-6} M, very little binding, if any, of RA 233 and RA 433 was observed. No evidence was obtained from the Sephadex batch method using the same concentration of protein to indicate binding of these compounds. No evidence was obtained from dynamic dialysis to show that these compounds were bound. It therefore must be concluded that the binding of these compounds to AGP is significantly less than DPD although these compounds are structurally similar. This is in contrast to the binding of these compounds to albumin.

4.4.7 Competitive binding studies between DPD and competitors for AGP

The aim of this section was to try to further characterise the DPD binding sites and try and determine which regions of the DPD molecule were important in the binding to AGP. The first problem to be considered was whether or not the side chains of DPD are important in the binding or whether the central nucleus was important.

Triethanolamine, diethanolamine, and ethanolamine did not compete for the DPD site in the concentration range investigated (up to 10 M). Piperazine and piperidine did not compete for the DPD site. This suggests that the primary points of attachment of the DPD to the AGP does not involve the side chains.

Competitive studies with various nucleotides have been investigated ATP, ADP, AMP, c-AMP, adenosine and adenine did not compete with DPD. Other nucleotides cytosine and guanosine showed no competition. The hydrophilic regions of these molecules may have inhibited their binding to AGP and competition with DPD. The vitamins riboflavin, folic acid and pyridoxine did not compete. These molecules contained acidic groups. It has been widely reported that AGP binds basic molecules in preference to acidic compounds, so the above molecules probably did not compete because of acidic groups even though the central nuclei were similar.

Competitive studies have been conducted between DPD and various analogues of DPD. No competition was seen between RA 433 and DPD. Figure 4.16 shows the effect of SH 1242 in the concentration range 2×10^{-5} M to 2×10^{-4} M on the fluorescence polarisation of DPD using 2.5×10^{-6} M AGP. From this the free and bound concentration of the DPD were calculated and a series of Klotz plots constructed (Figure 4.17). The Klotz plot indicates that the SH 1242 competitively inhibits the binding of DPD to AGP. Using the method of Segel (1975) an inhibition constant of 1×10^{5} M^{-1} was calculated. Data was also fitted using nonlinear regression analysis which gave an inhibition constant of $1.43 \times 10^{5} M^{-1}$ se 6.90×10^{4} .

2,3 DPP, 2,5 DPP, DMQ, DPQ, DPP2H and DBQ are related to known platelet active compounds and therefore of interest to this study. 2,3 DPP, DPQ, and DPP2H, showed similar results to SH1242 using fluorescence polarisation. Figure 4.18 to 4.20 shows Klotz plots for the competition of these compounds with DPD.

Competitive studies have also been conducted between DPD and a number of basic drugs. Figure 4.21 shows a Klotz plot for the competition of prazosin with DPD. An inhibition constant of 7.23 x 10^4 M^{-1} was evaluated using nonlinear regression analysis. Figure 4.22 shows a Klotz plot for the competition of papaverine with DPD, giving an inhibition constant of 4.23 x 10^4 M^{-1}. Figure 4.23 is a Klotz plot for the competition of propranolol with DPD. An inhibition constant of 6.0 x 10^4 M^{-1} was evaluated. The results of the above work is summarised in Table 4.1.

These competitive studies throw further light on the characteristics of the DPD site. It has been widely quoted De Leve and Piafsky (1981) that AGP binds preferentially basic molecules. DPD and the majority of the analogues investigated here contain a number of nitrogens but at pH 7.4 are not ionised. (The pKa of the analogues were determined using a fluorescence method). As already discussed, evidence exists which indicates that pH effects the binding of DPD. The binding of the cationic form of DPD quenched the tryptophan fluorescence less than the unionised form. The evidence obtained here suggest that DPD is binding to a hydrophobic region of the protein. A number of nitrogens in the molecule appear to be important in the binding but the majority of compounds investigated showed that these were not ionised.

The DPD site was shown to be very dependent on its structure. The apparently minor structural changes seen in the analogues RA 233 and RA 433, compared with DPD, markedly decreased the binding to AGP. This indicates that the DPD site involves both the central nucleus and the side chains. The piperidino group removed from DPD in RA233 markedly decreased the binding, thus indicating the importance of this group.

No hydrophilic or anionic molecules were shown to compete with DPD for the AGP site. The majority of competitors were planar or partially planar structures.

Kragh-Hansen (1981) criticised the use of fluorescence for the investigation of competitive binding because the free and bound concentration of the competitor could not be determined. In this investigation no attempt was made to determine the free and bound concentration of the competitor. The conditions of the experiments were arranged to have a high concentration of competitor when compared with the protein concentration. The assumption could then be made that the free concentration of the competitor was approximately equal to the total concentration of the competitor. This arrangement would not be practical unless the affinity constant of the inhibitor was markedly less than the drug. Making the above assumption allowed the calculation of the inhibition constants of the inhibitors.

No claim is being made that the above competitive binding is of clinical importance. It is likely that at clinical concentrations, due to the low concentrations and high affinity of DPD and low concentrations of competitors, no significant displacement of DPD from AGP occurs.

4.5 CONCLUSION

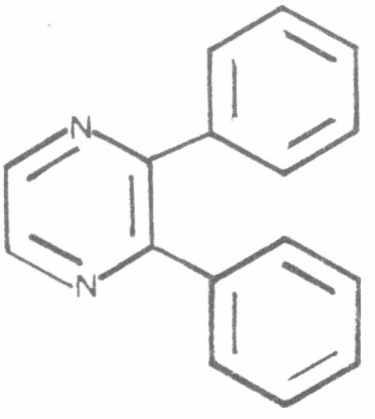
DPD on binding to AGP caused a quenching of the tryptophan fluorescence indicating that it is binding in the regions of one of the tryptophans. The quenching suggested that more than one of the tryptophans were involved. Very little change in the intrinsic fluorescence of the DPD was observed. On binding to AGP the fluorescence polarisation of the DPD was markedly increased at the 420nm excitation peak with very little change in the 305nm peak. This suggests that the freedom of rotation of DPD was decreased on binding and the binding site probably involves the central nucleus and the side chains of DPD. It was envisaged that DPD bridged the polypeptide chain being located near two of the tryptophan residues in a hydrophobic region of the protein. This was possibly between tryptophan 25 and 160 (Figure 4.24). Increasing the ionic strength of the buffer gave no significant change in the tryptophan quenching or the change in polarisation suggesting that the binding site is located in a compact region of the protein. Urea abolished the polarisation effect indicating that the binding site is in the polypeptide chain and not in the hydrophilic carbohydrate residues.

Competitive studies allowed further characterisation of the binding site. No hydrophilic or anionic compounds competed with DPD. These studies indicated that the DPD site was very specific to DPD, slight structural changes affecting the binding. Although it has been widely reported that AGP binds cationic molecules, the majority of compounds investigated in this study were uncharged. This gives further evidence that the site is in a hydrophobic region of the protein.

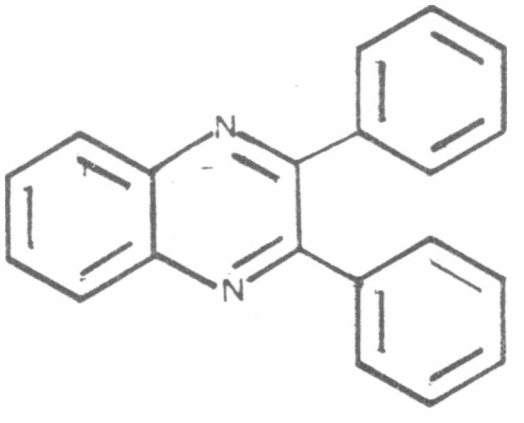
Table 4.1

Summary of inhibition constants for competition with DPD for AGP

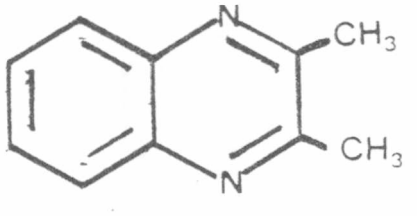
Competitor	Inhibition constant (M^{-1})
2,3 DPP	1.56×10^4
DPQ	2.88×10^4
prazosin	7.23×10^4
papaverine	4.61×10^4
propranolol	6.00×10^4
SH 1242	1.43×10^5
warfarin	———
salicylic acid	———
acetyl salicylic acid	———
indomethacin	———



DPP

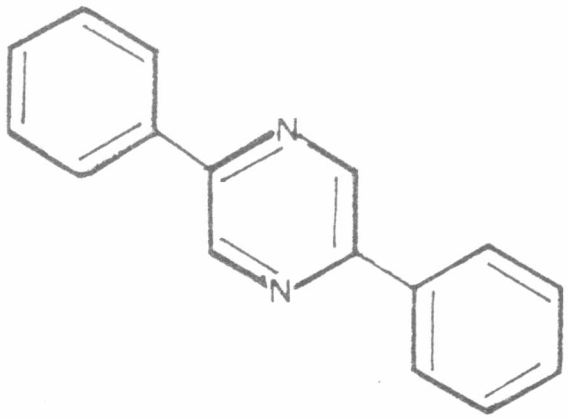


DPQ

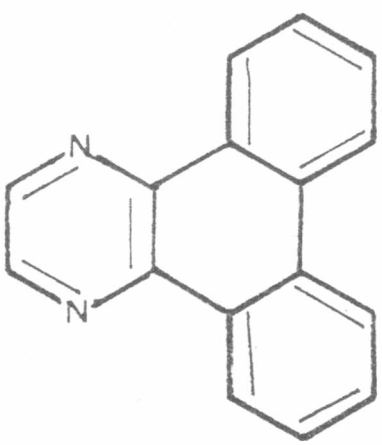


DMQ

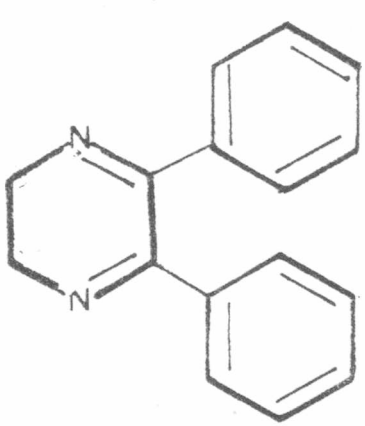
Figure 4.1



2,5-DPP



DBQ



DPP2H

Figure 4.2

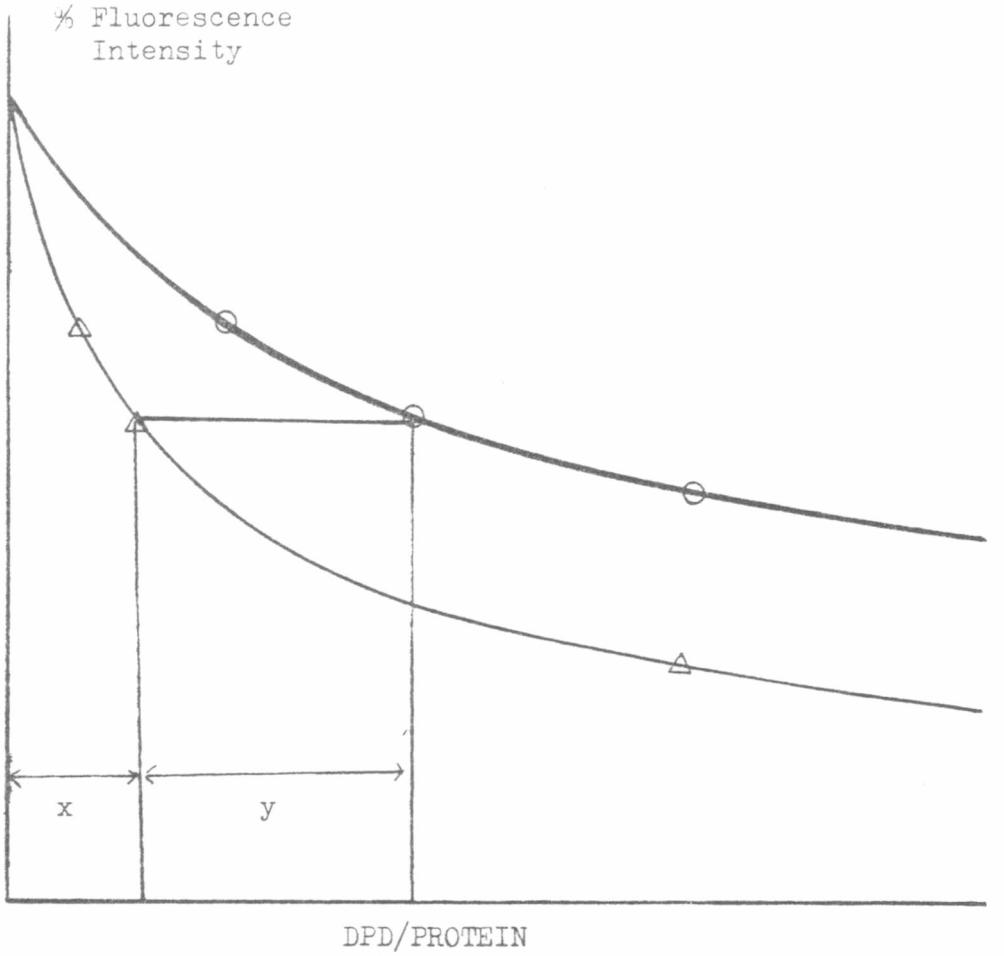


Figure 4.3

A graph defining x and y for the calculation of Db and Df for multiple binding sites using equation 4.4

Δ 100% bound ○ fractional bound

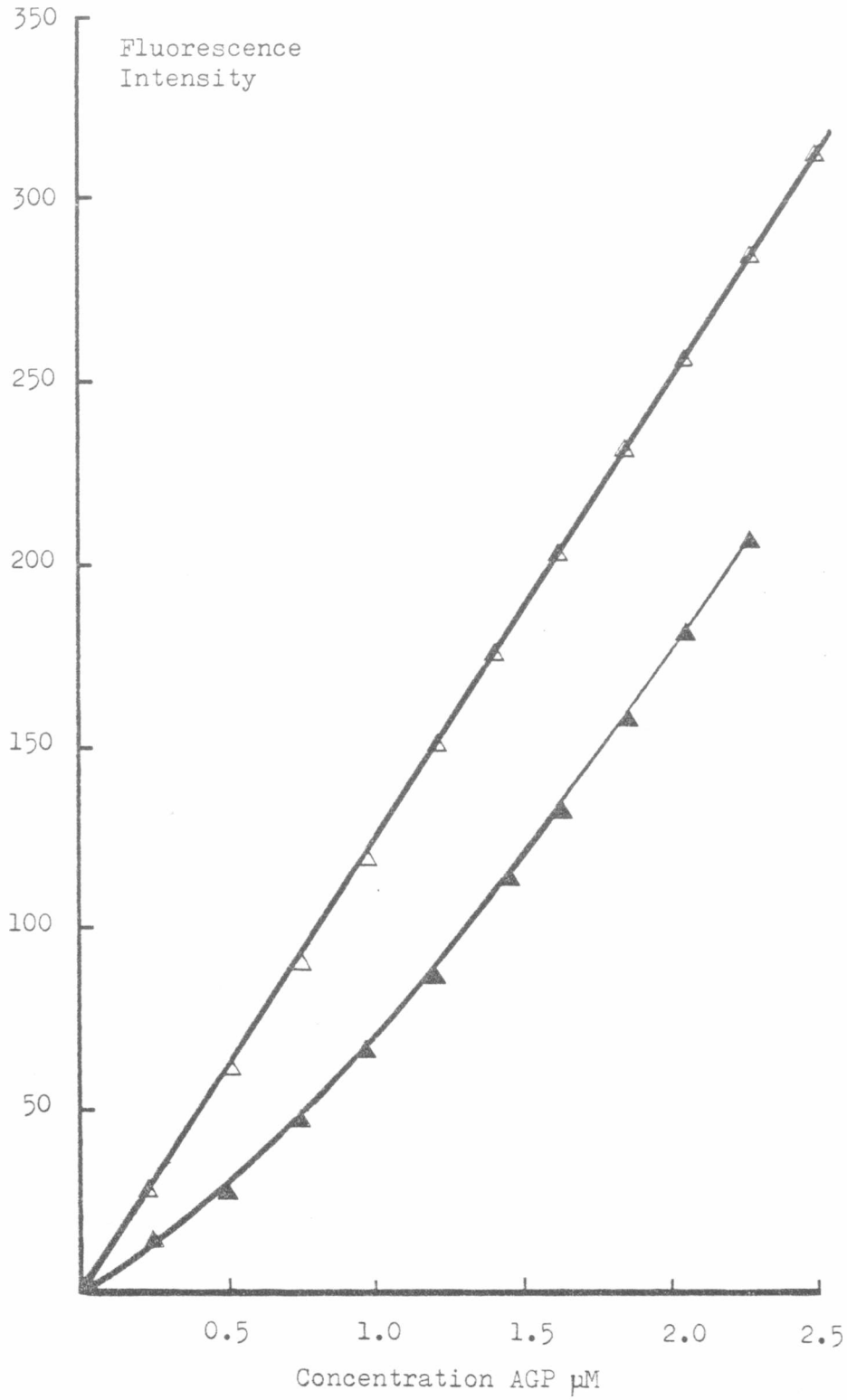


Figure 4.4

A graph showing the effect of titrating 5 x 10^{-7}M DPD with AGP using an excitation wavelength of 295nm and an emission wavelength of 335nm.

△ AGP no DPD

▲ AGP with 5 x 10^{-7} M DPD

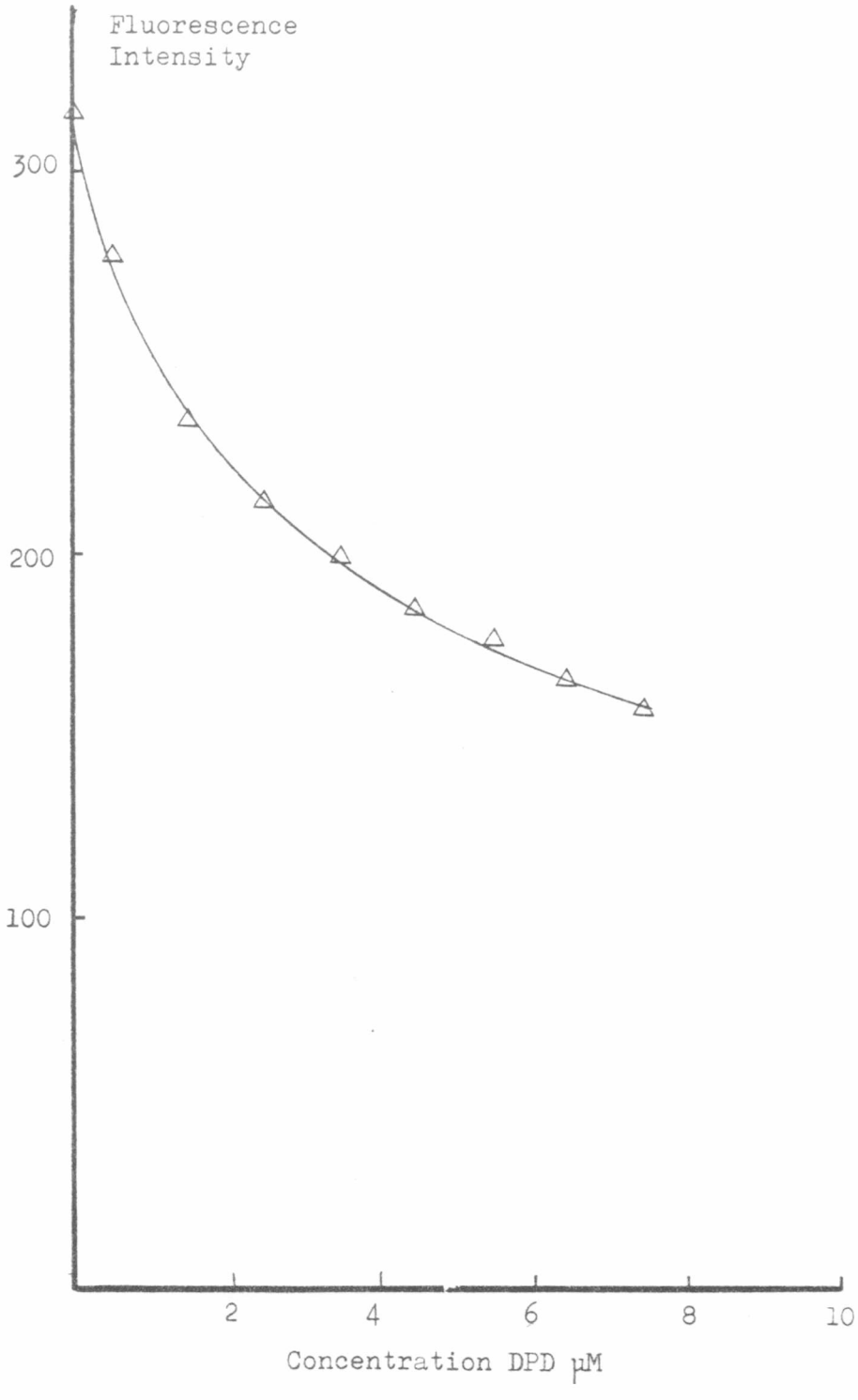


Figure 4.5

A graph showing the quenching of 2.5×10^{-6} M AGP with DPD using an excitation wavelength of 295nm and emission wavelength of 335nm.

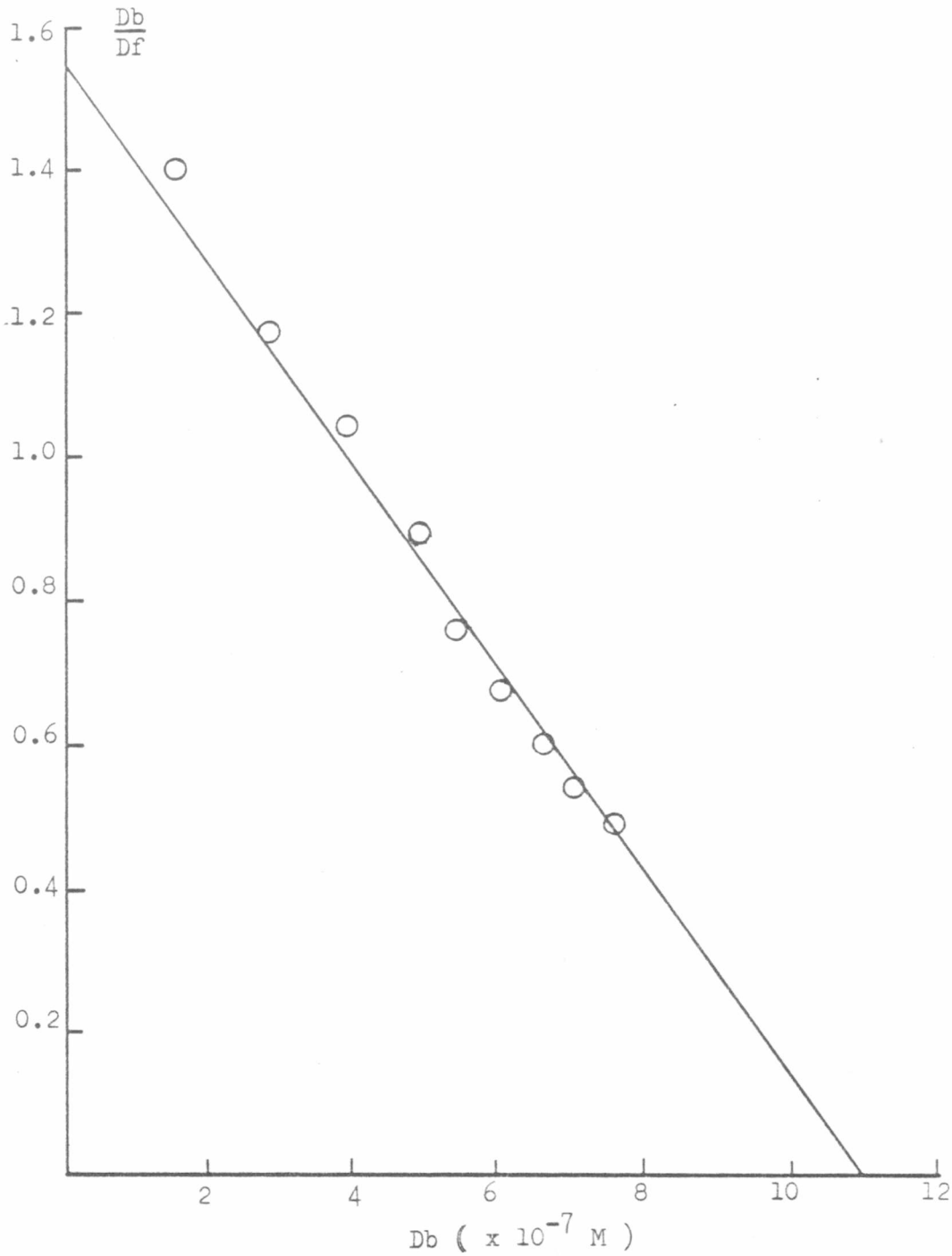


Figure 4.6

A Rosenthal plot for the binding of DPD to 2.5 x 10^{-6} M AGP.

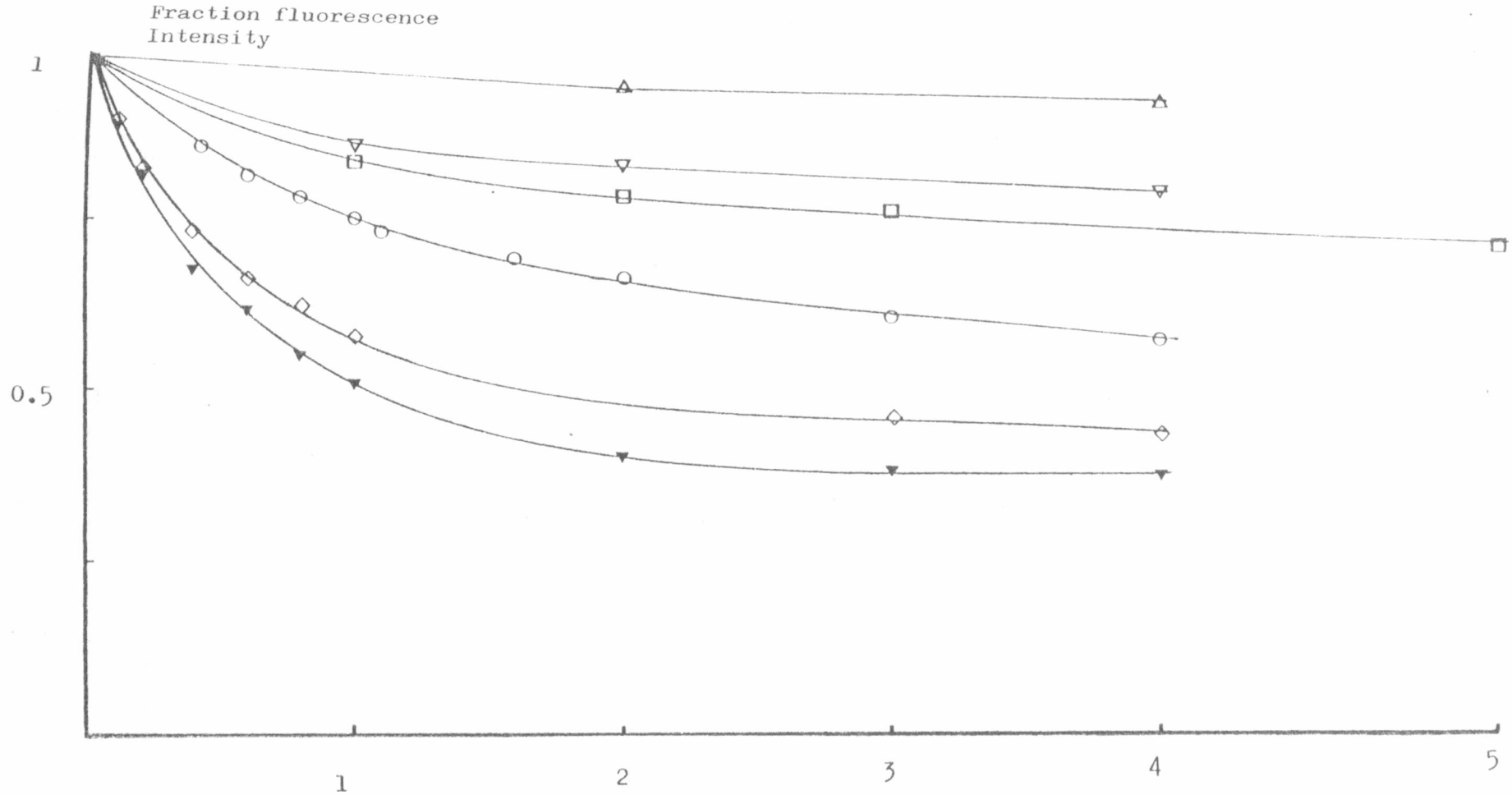


Figure 4.7

A graph of the percentage fluorescecne intensity of AGP against the ratio DPD/AGP using different protein concentrations with an excitation wavelength of 295nm and an emission wavelength of 335nm.

△ 0.25 ▽ 0.5 □ 1.0 ○ 2.5 ◇ 10.0 ▲ 15.0 μM AGP

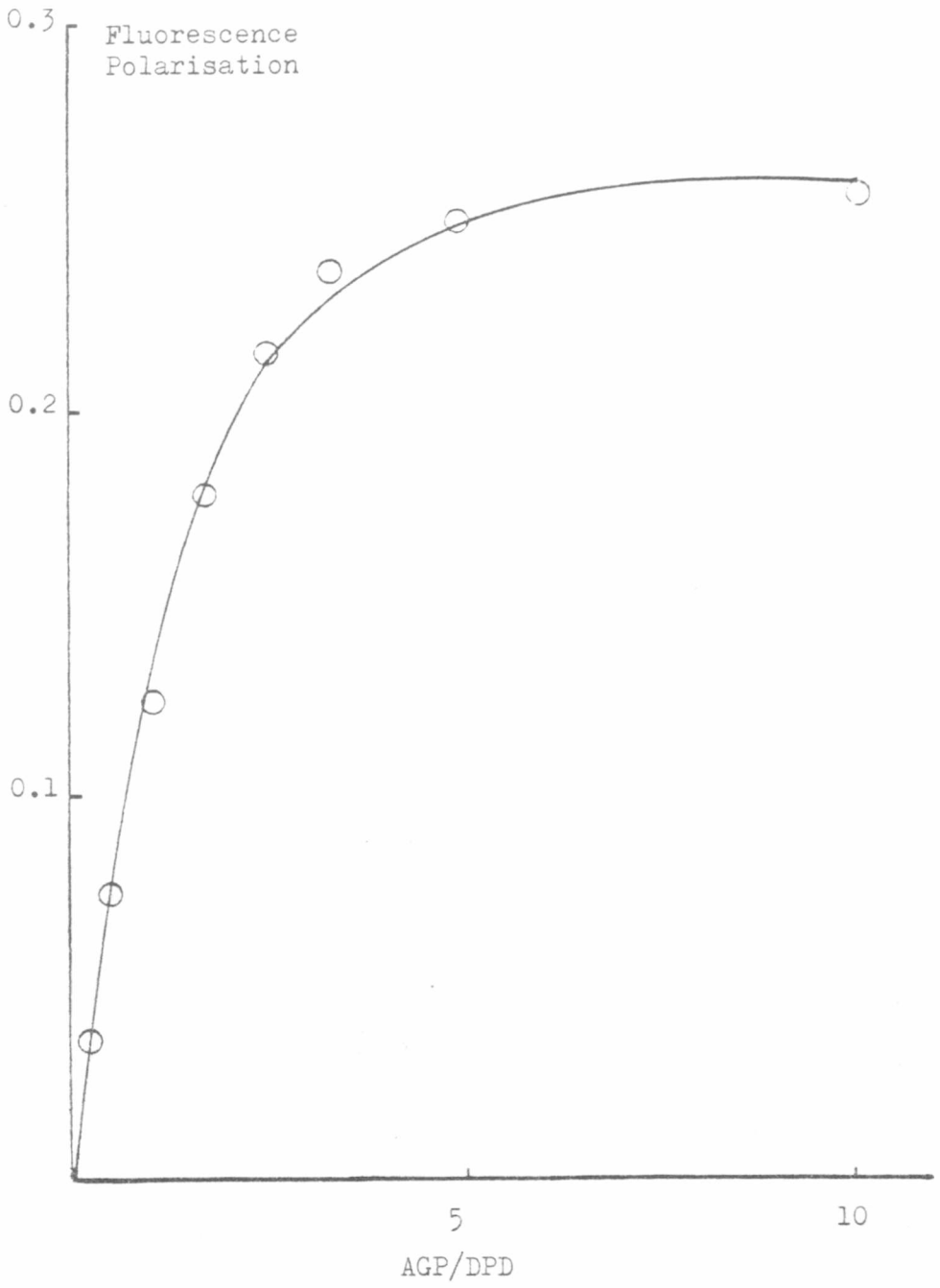


Figure 4.8

A graph showing the fluorescence polarisation of 5 x 10^{-7} M DPD against the AGP/DPD ratio using an excitation wavelength of 420nm and an emission wavelength of 490nm.

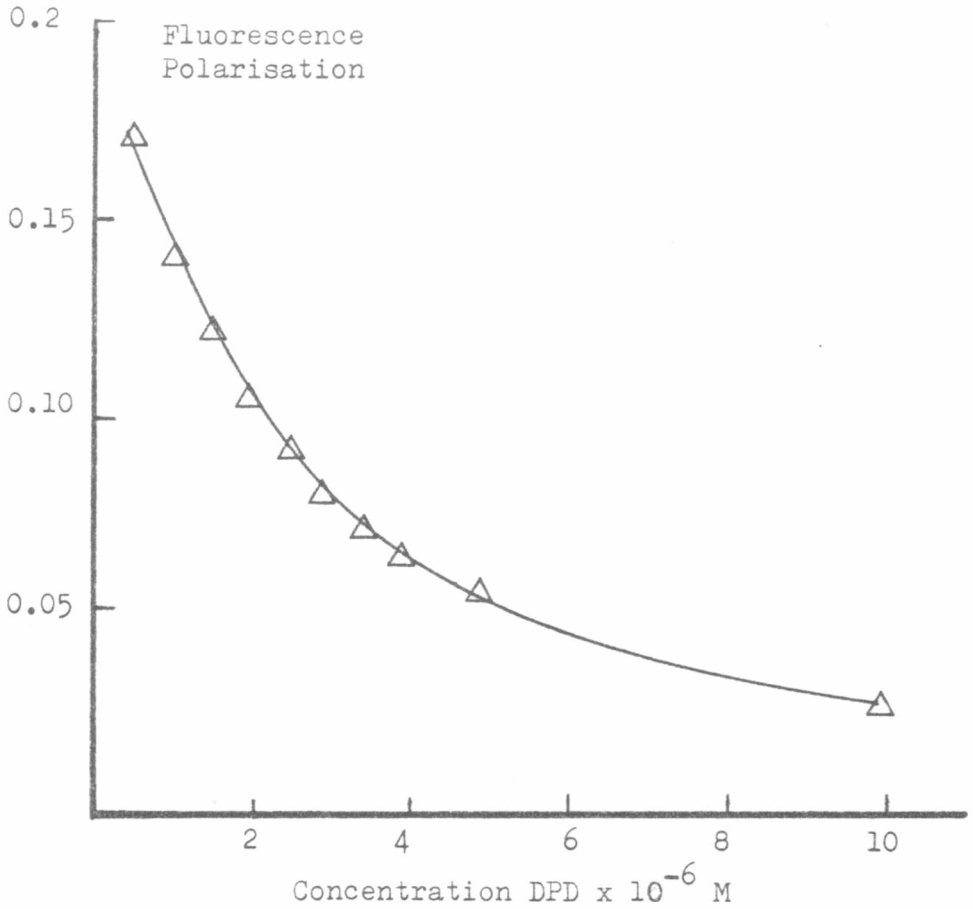


Figure 4.9

A graph showing the fluorescence polarisation against the concentration of DPD using an excitation wavelength of 420nm and an emission wavelength of 490nm with 2.5 x 10^{-6}M AGP.

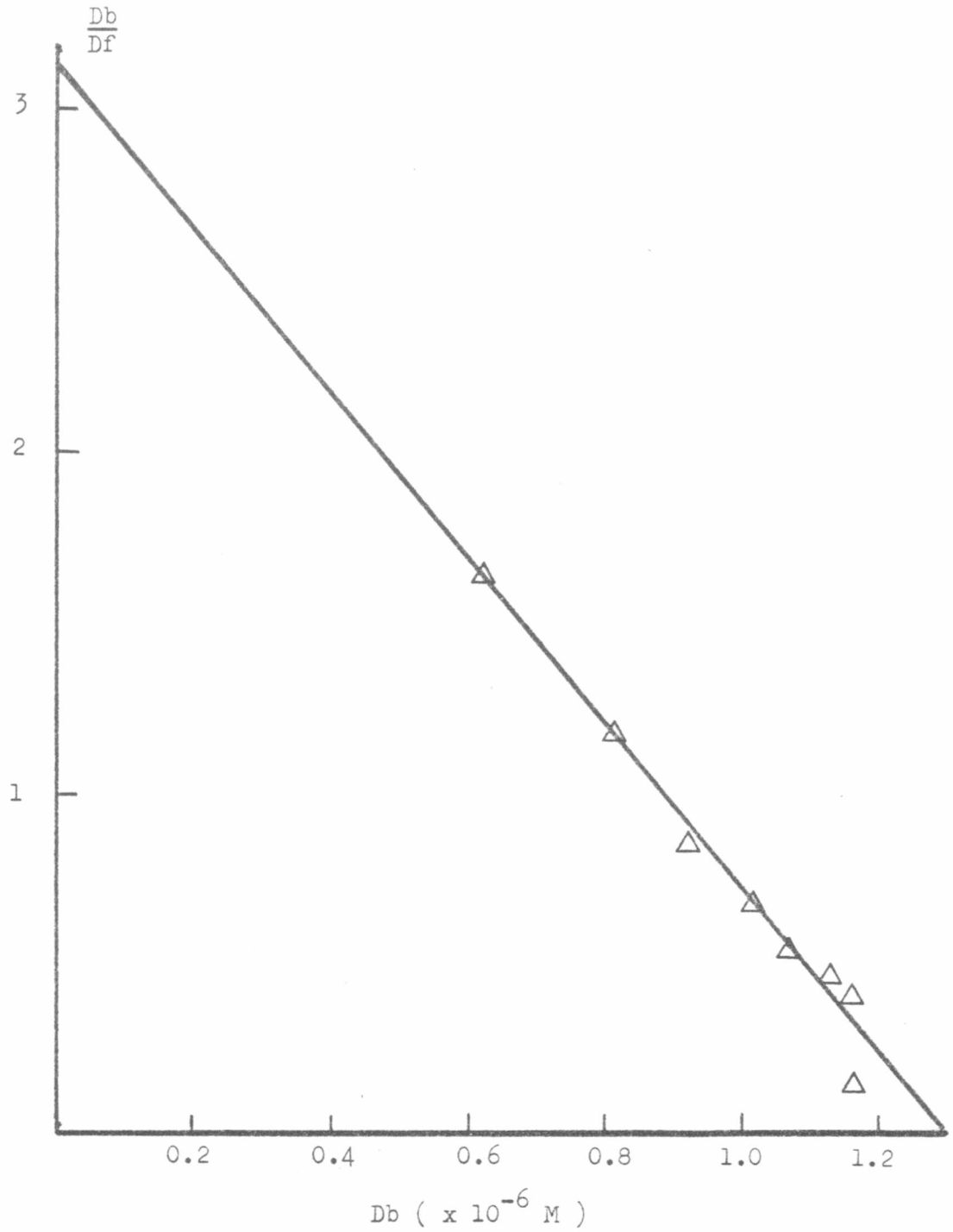


Figure 4.10

A Rosenthal plot for the binding of DPD to 2.5 x 10^{-6}M AGP.

Db and Df calculated from the polarisation data.

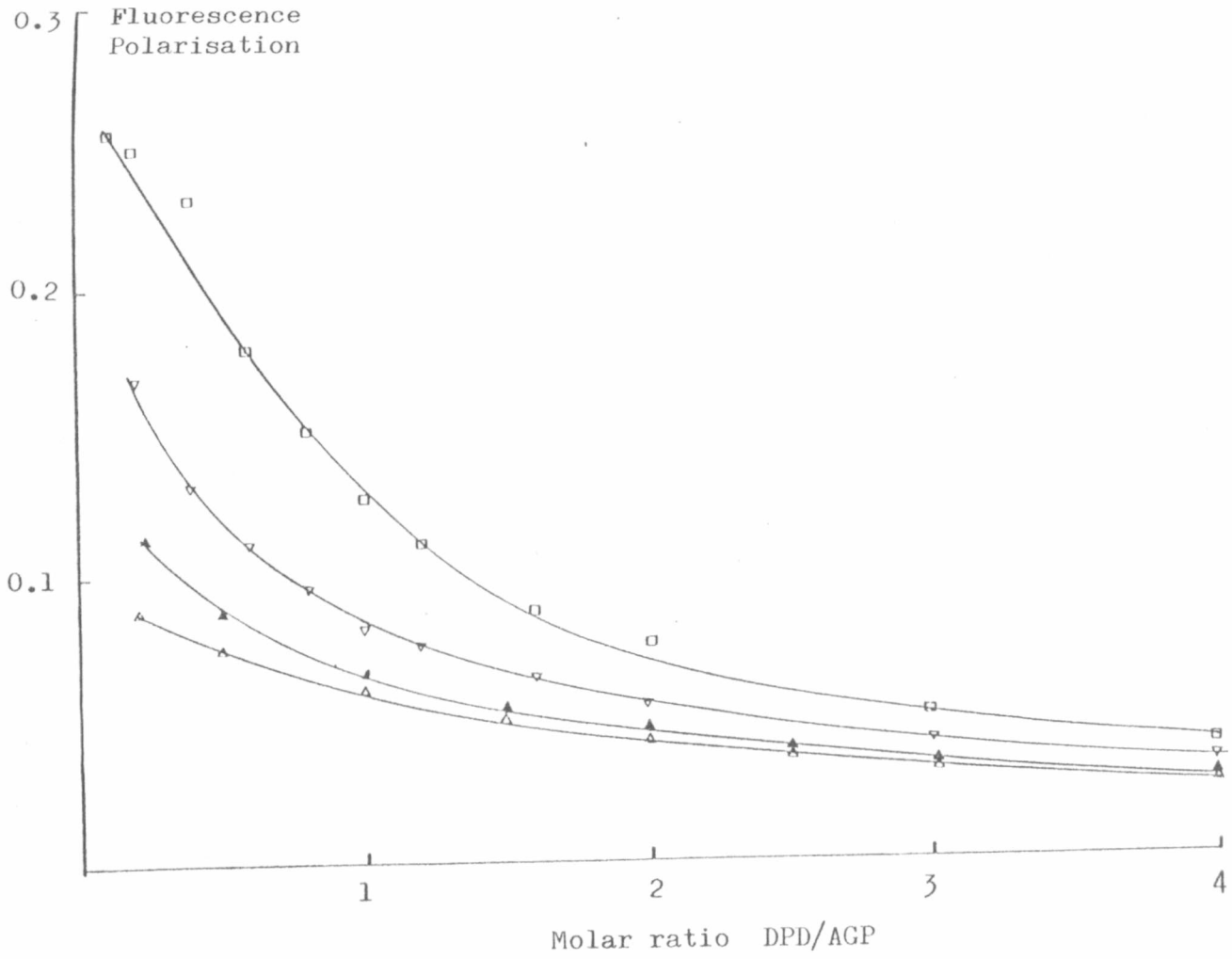


Figure 4.11

A graph showing the effect of varying the protein concentration on the fluorescence polarisation of DPD using an excitation wavelength of 420nm and emission of 490nm.

□ 10 ▽ 2.5 ▲ 1.0 △ 0.5 μM AGP

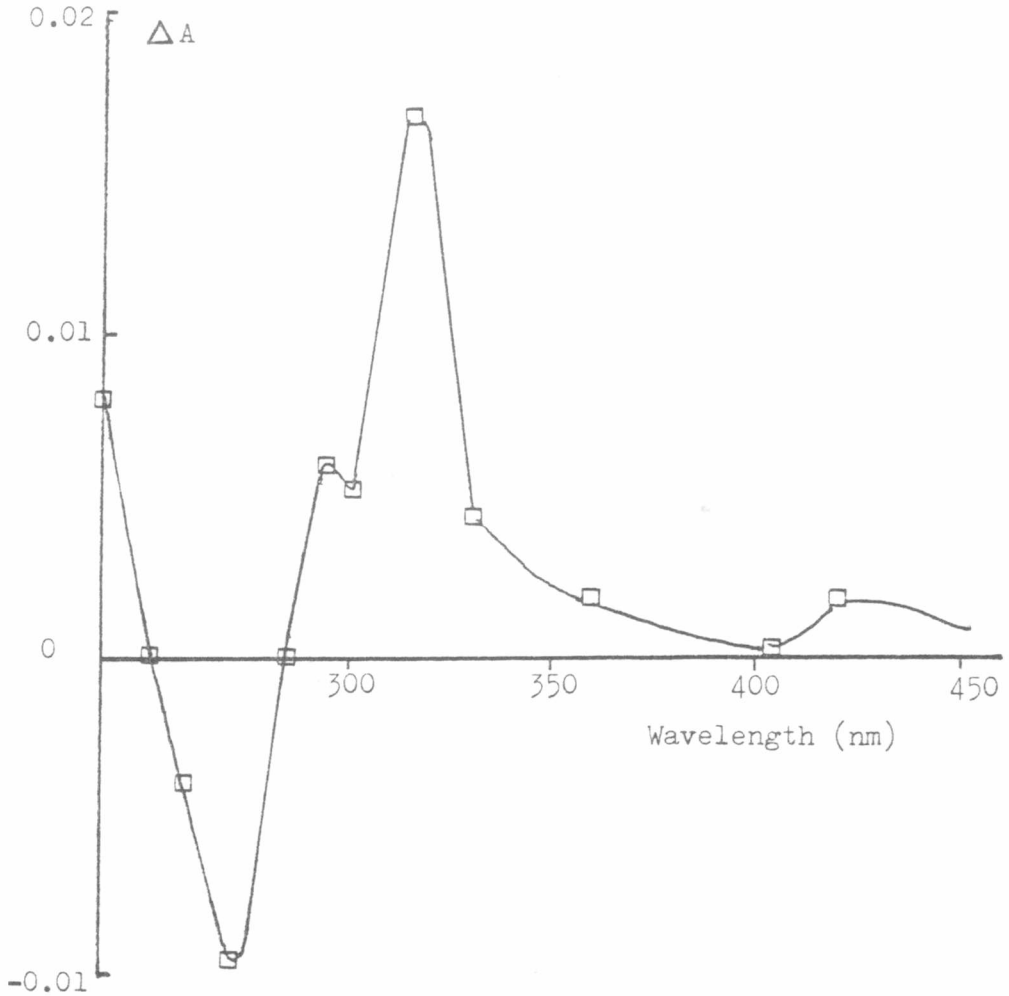


Figure 4.12

A graph showing the ultraviolet difference spectrum for the binding of DPD to 2.5×10^{-6} M AGP.

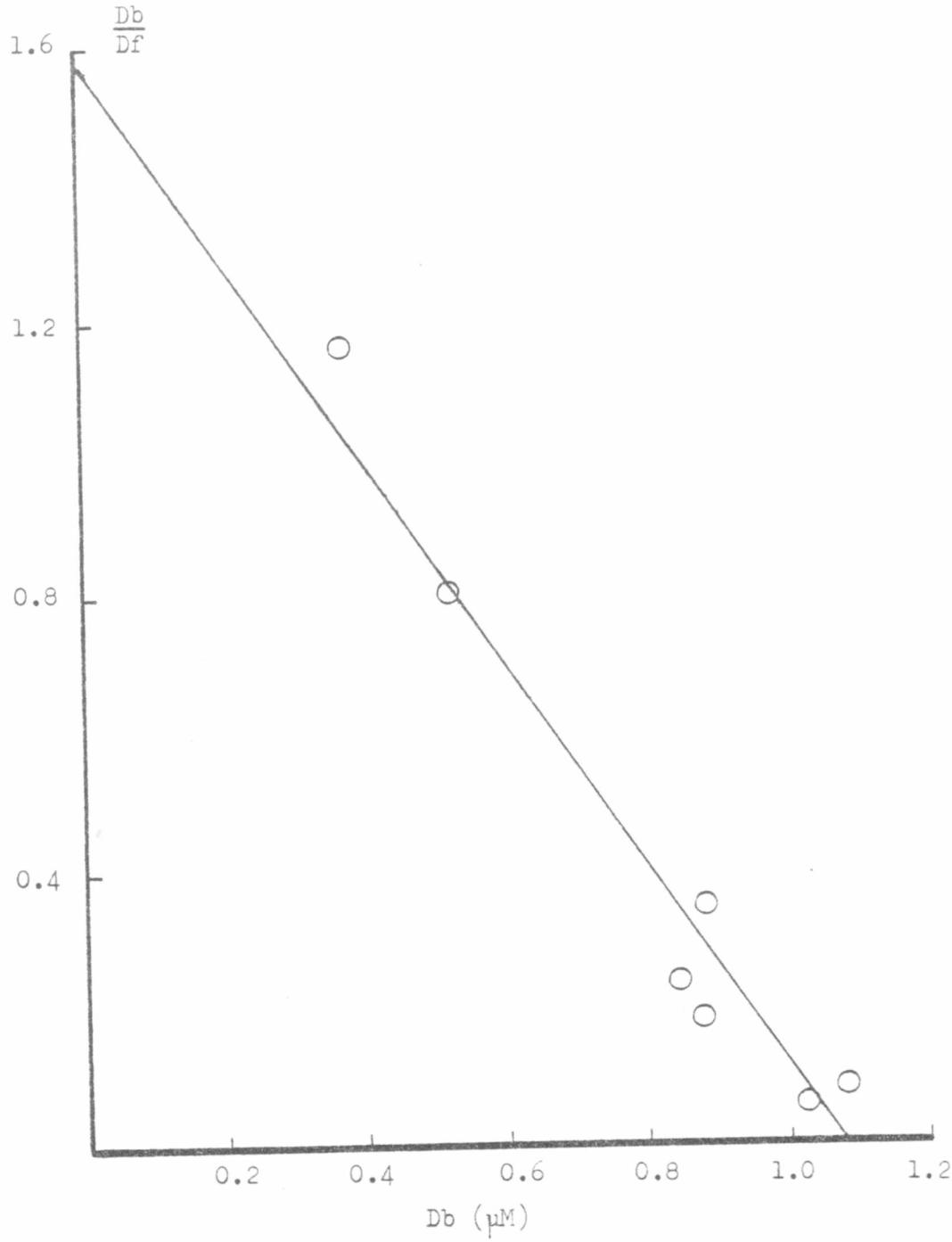


Figure 4.13

A Rosenthal plot showing the binding of DPD to 2.5 μM AGP. Data from equilibrium dialysis

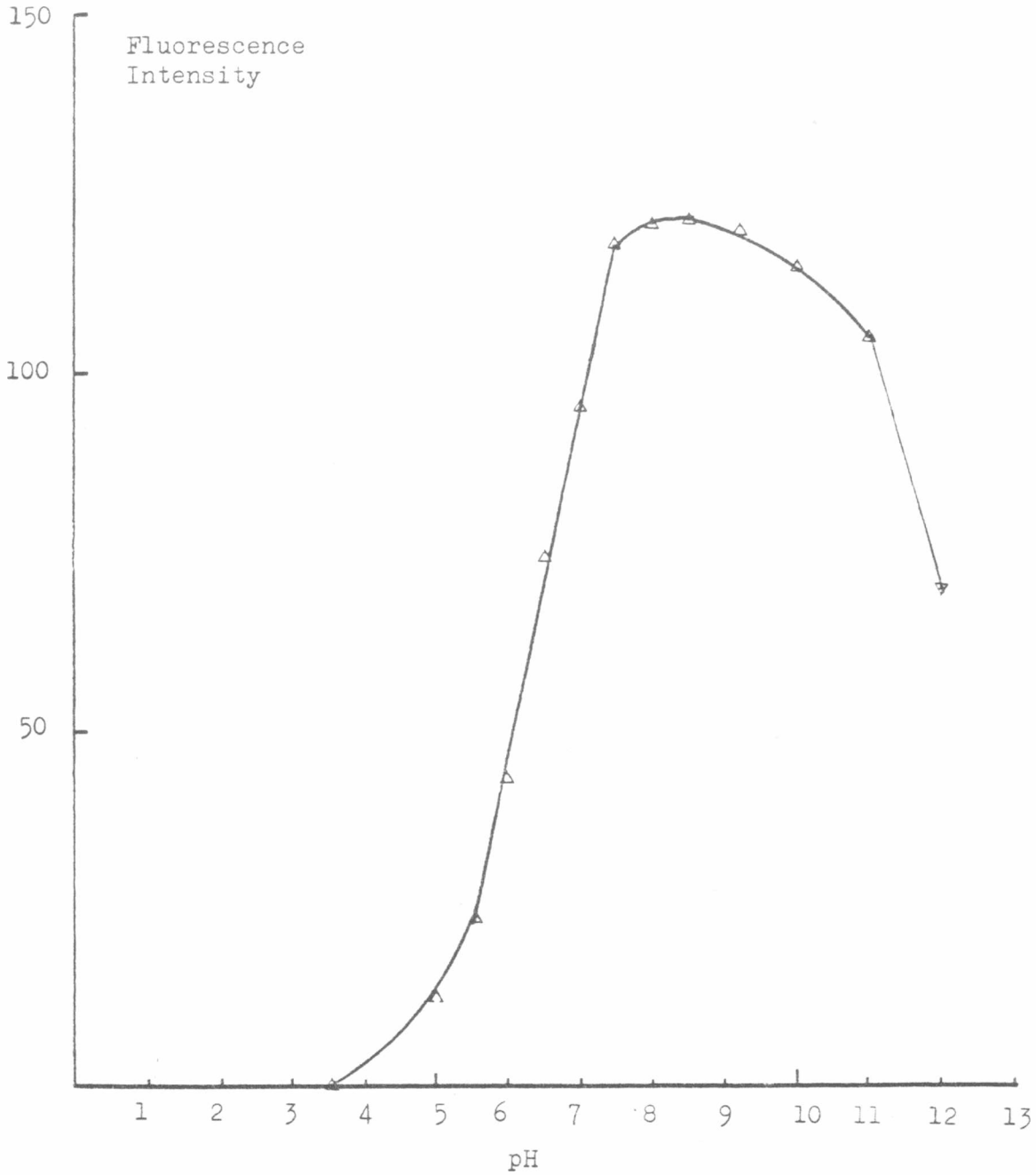


Figure 4.14

A graph showing the effect of pH on the fluorescence intensity of 1μM of DPD using an excitation wavelength of 420nm and emission of 490nm.

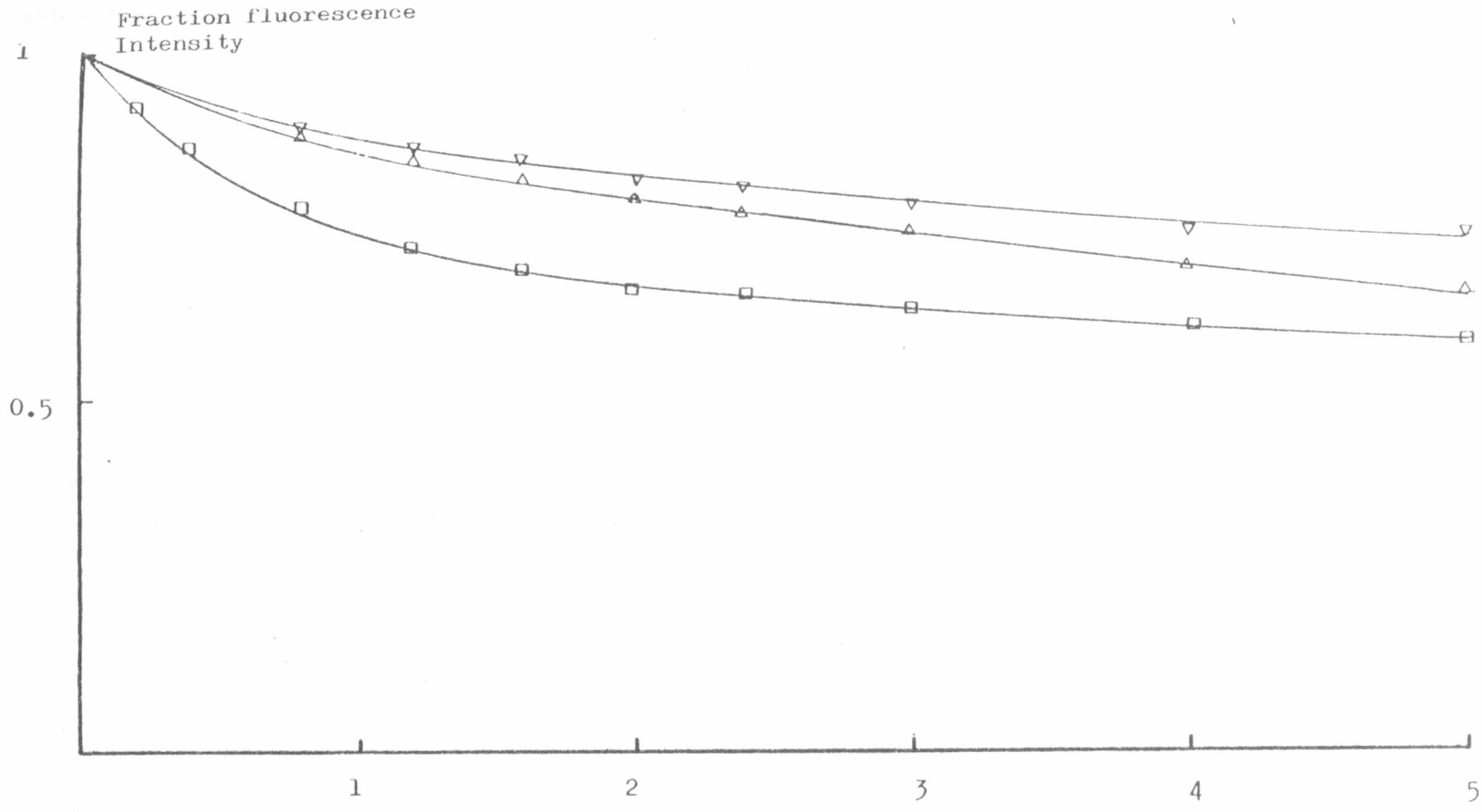


Figure 4.15

A graph showing the effect of pH on the fluorescence intensity of AGP when titrated with DPD using an excitation wavelength of 420nm and an emission wavelength of 490nm.

pH = 4 ▽ pH = 6.5 △ pH = 9.25 □

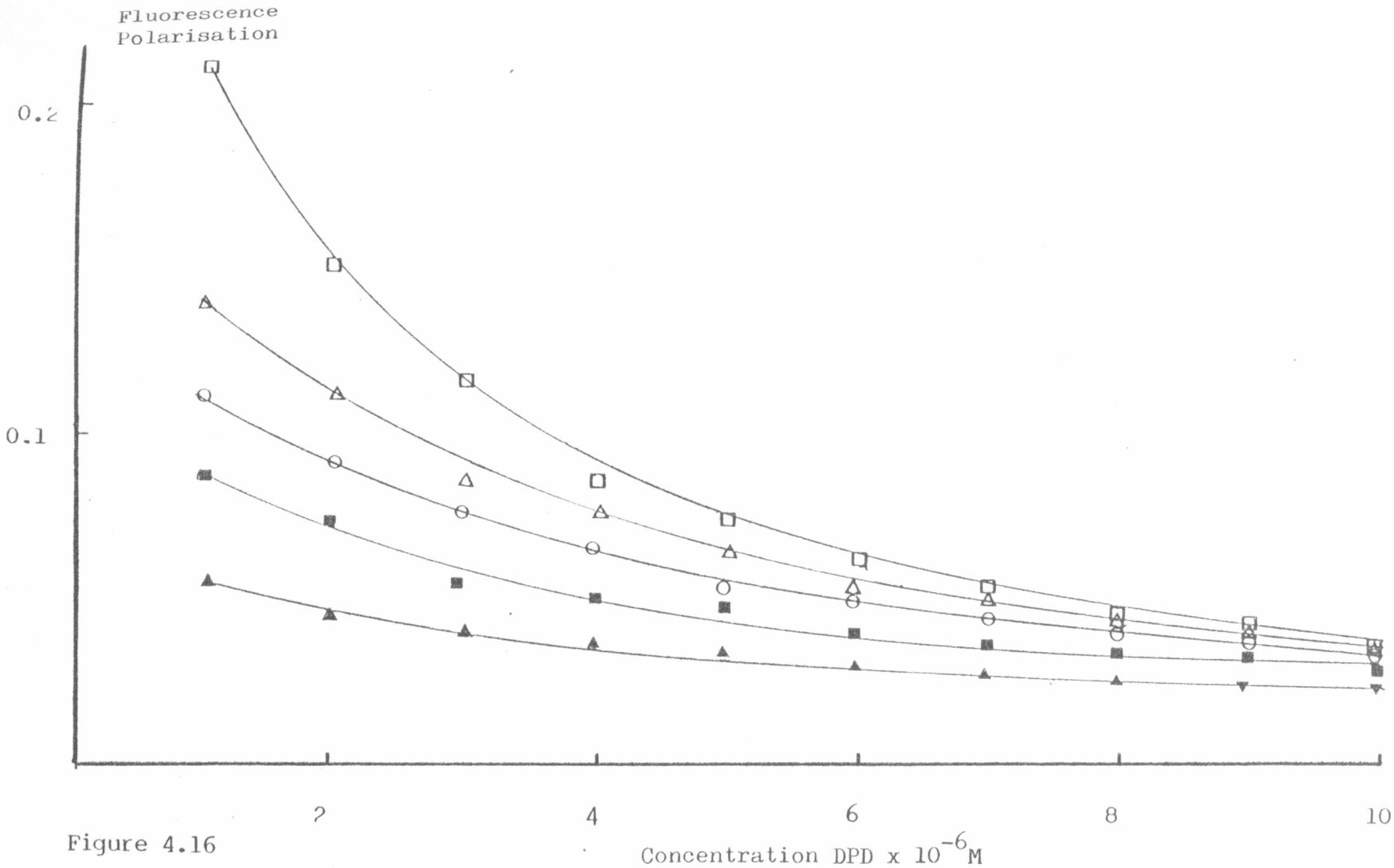


Figure 4.16

A graph showing the effect of SH 1242 on the fluorescence polarisation of DPD using an excitation wavelength of 420nm and emission of 490nm.

□ 0 △ 20 ○ 50 ■ 100 ▲ 200 μM SH 1242

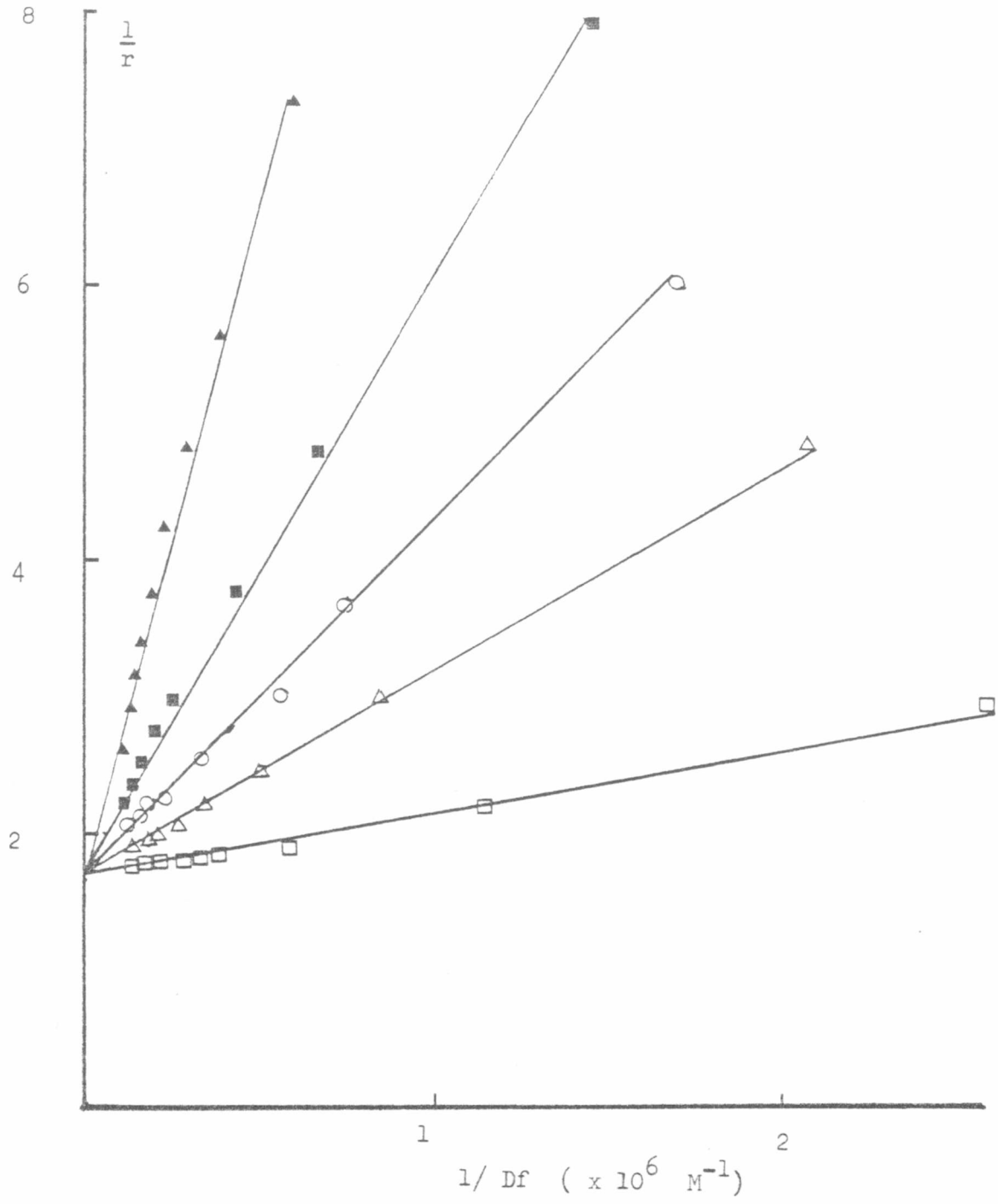


Figure 4.17

A Klotz plot for the binding of DPD to 2.5×10^{-6} M AGP and competition with SH 1242

□ 0 △ 20 ○ 50 ■ 100 ▲ 200μM SH 1242

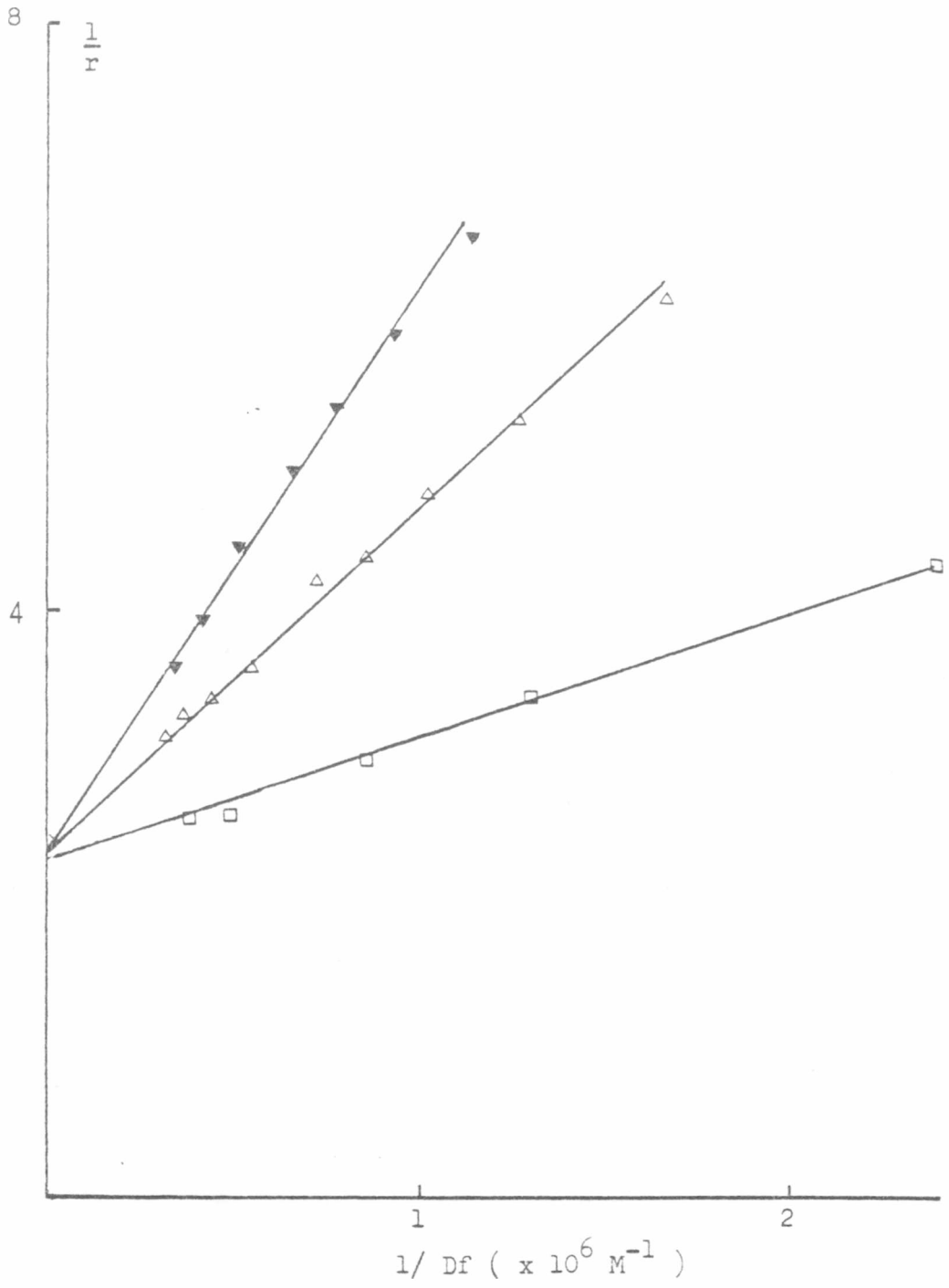


Figure 4.18

A Klotz plot showing competition between DPD and 2,3 DPP

□ 0 △ 100 ▼ 200 μM 2,3 DPP

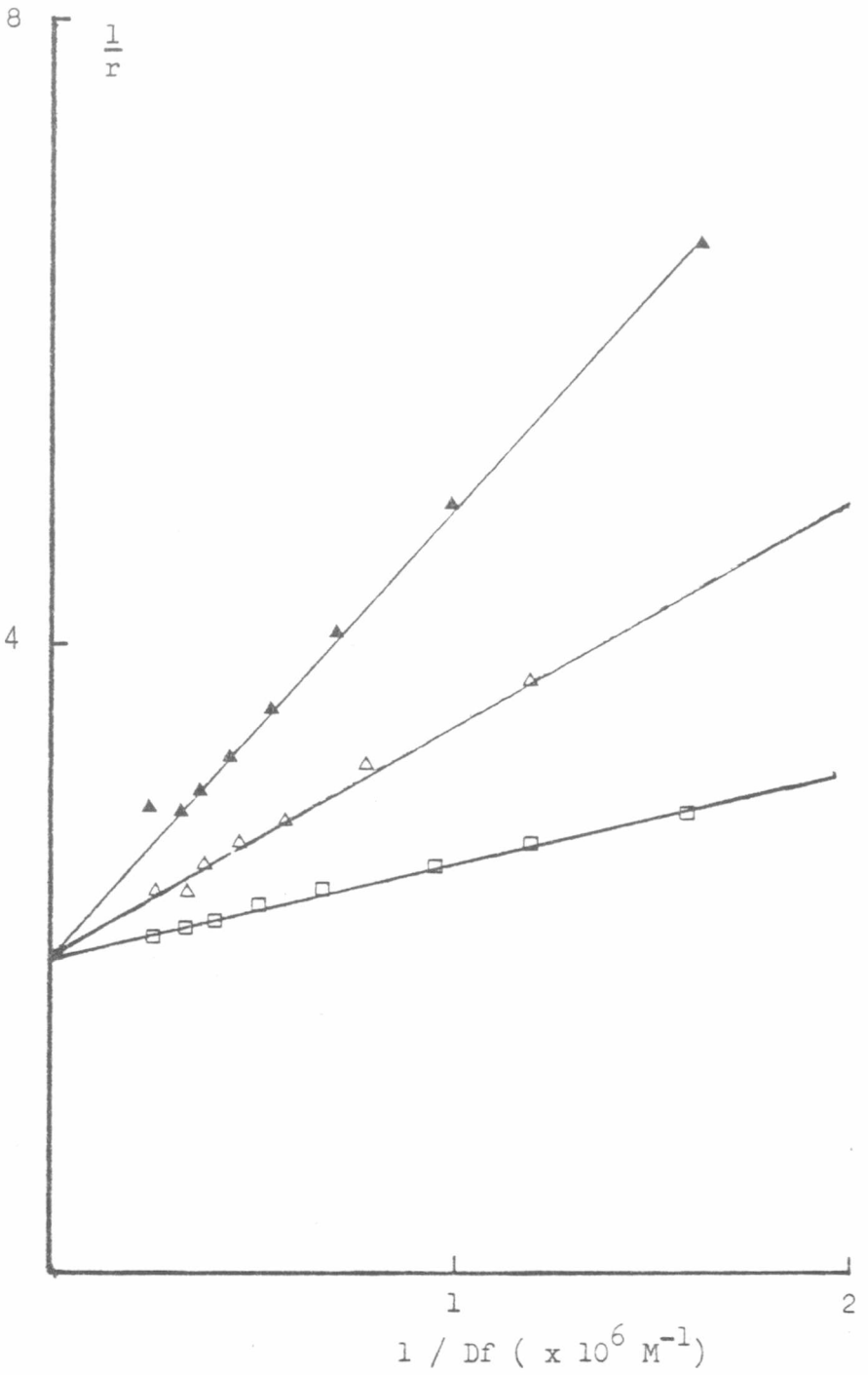


Figure 4.19

A Klotz plot showing competition between DPD and DPQ

□ 0 △ 10 ▲ 20μM DPQ

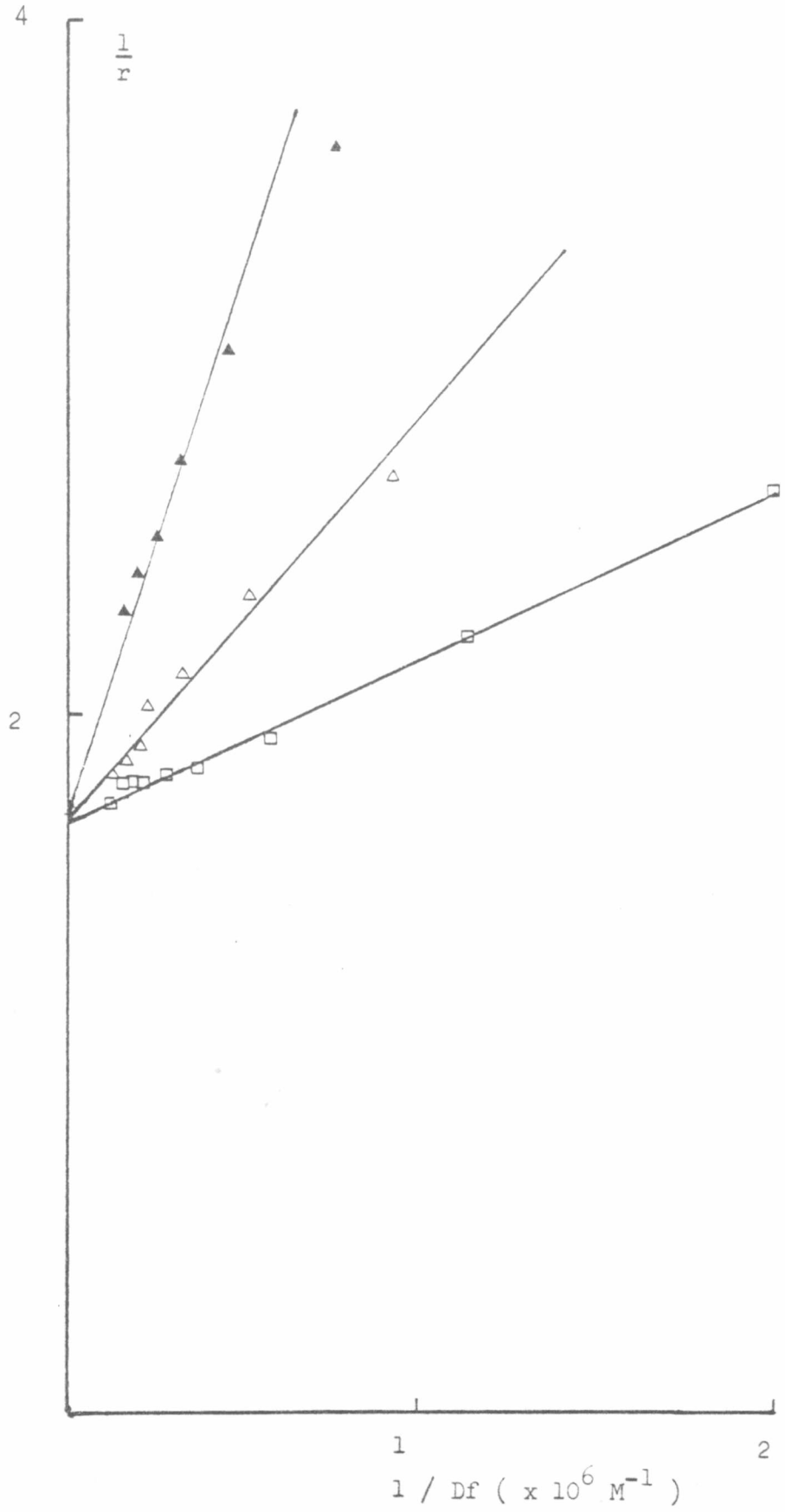


Figure 4.20

A Klotz plot showing competition between DPD and DPP2H

□ 0 △ 42.7 ▲ 214μM DPP2H

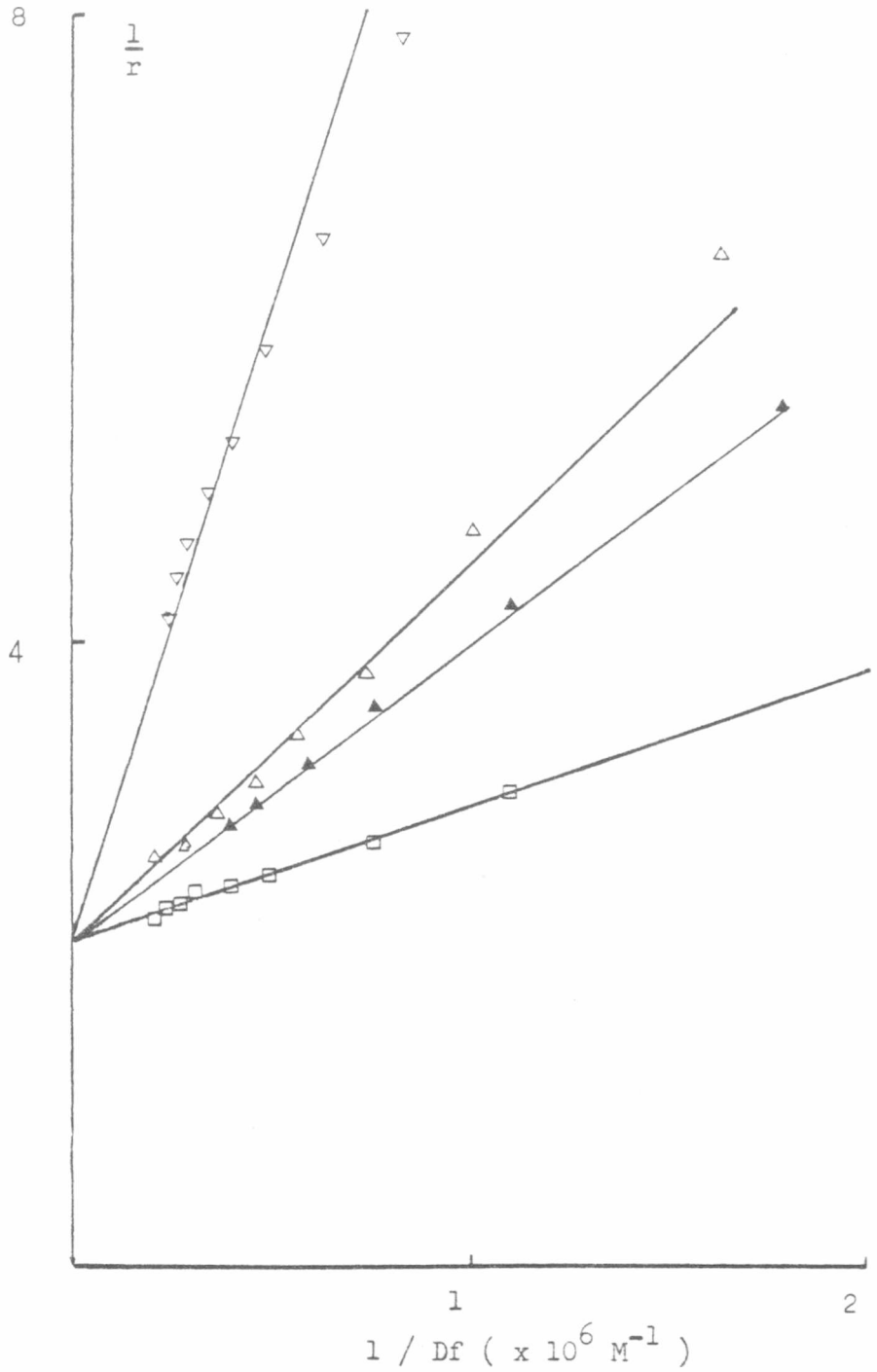


Figure 4.21

A Klotz plot showing competition between DPD and prazosin

□ 0 ▲ 10 △ 20 ▽ 100 μM prazosin

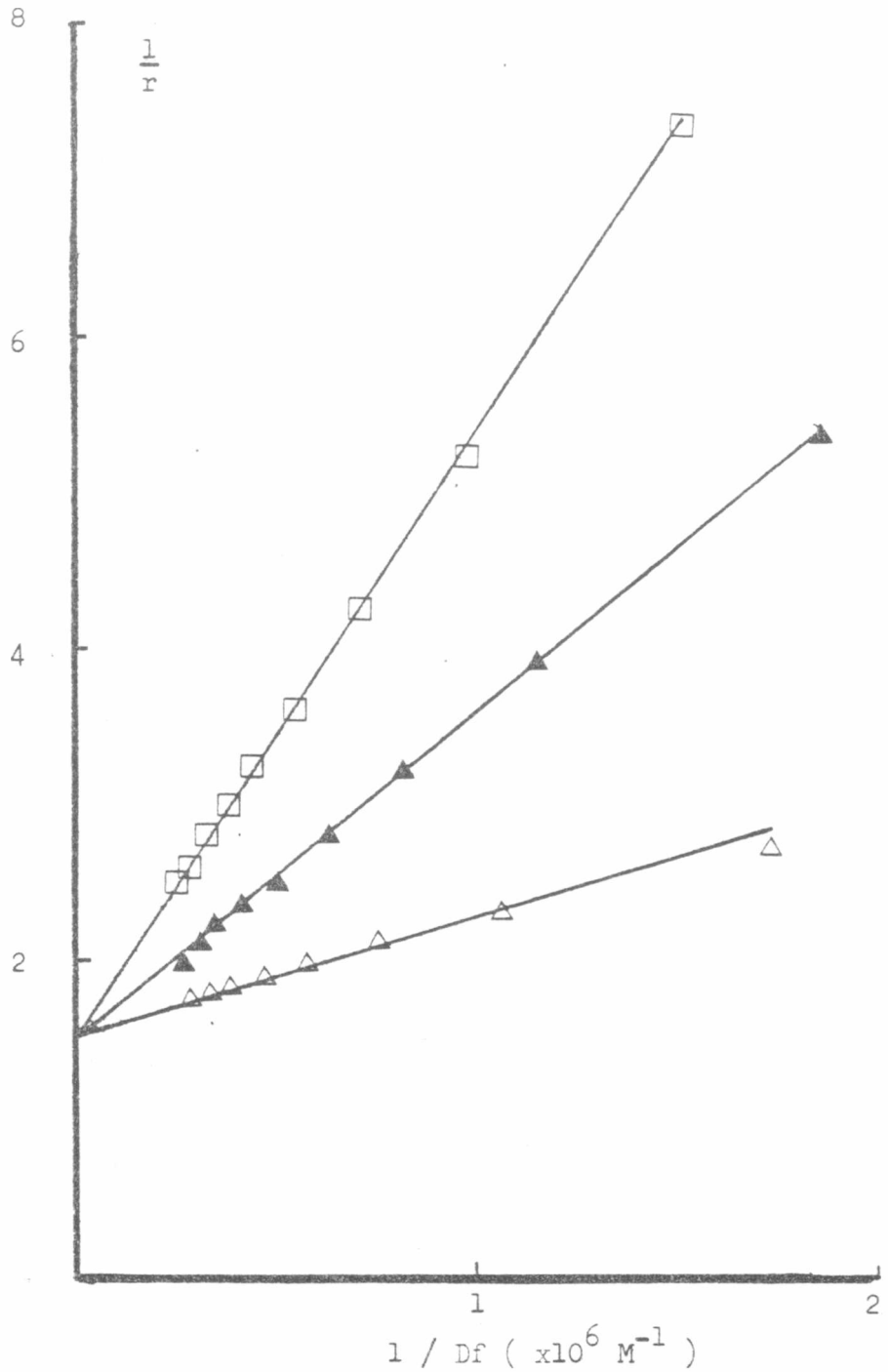


Figure 4.22

A Klotz plot showing competition between DPD and papaverine for AGP

△ 0 ▲ 50 □ 100 μM papaverine

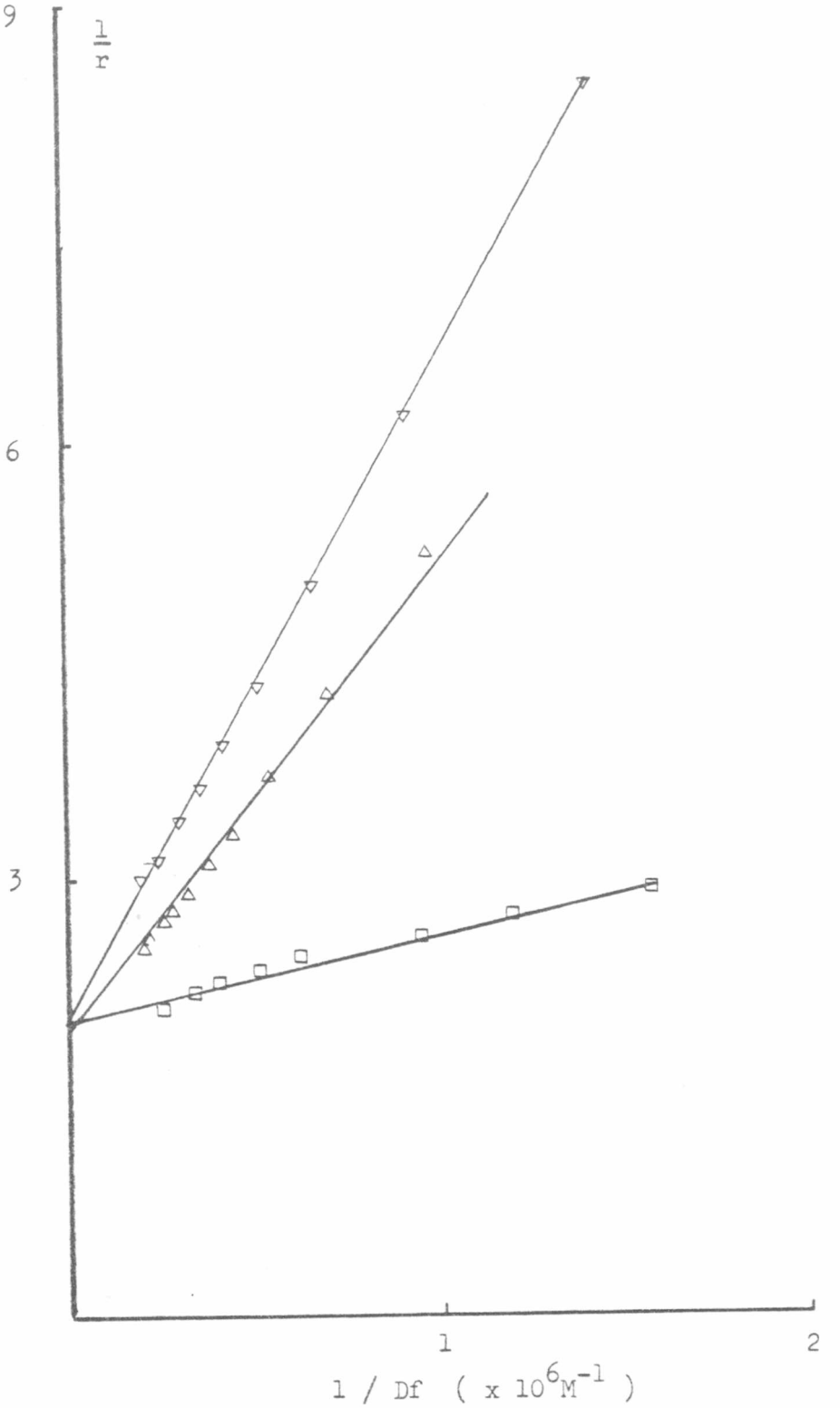


Figure 4.23

A Klotz plot showing competition between DPD and propranolol for AGP

□ 0 △ 50 ▽ 100μM propranolol

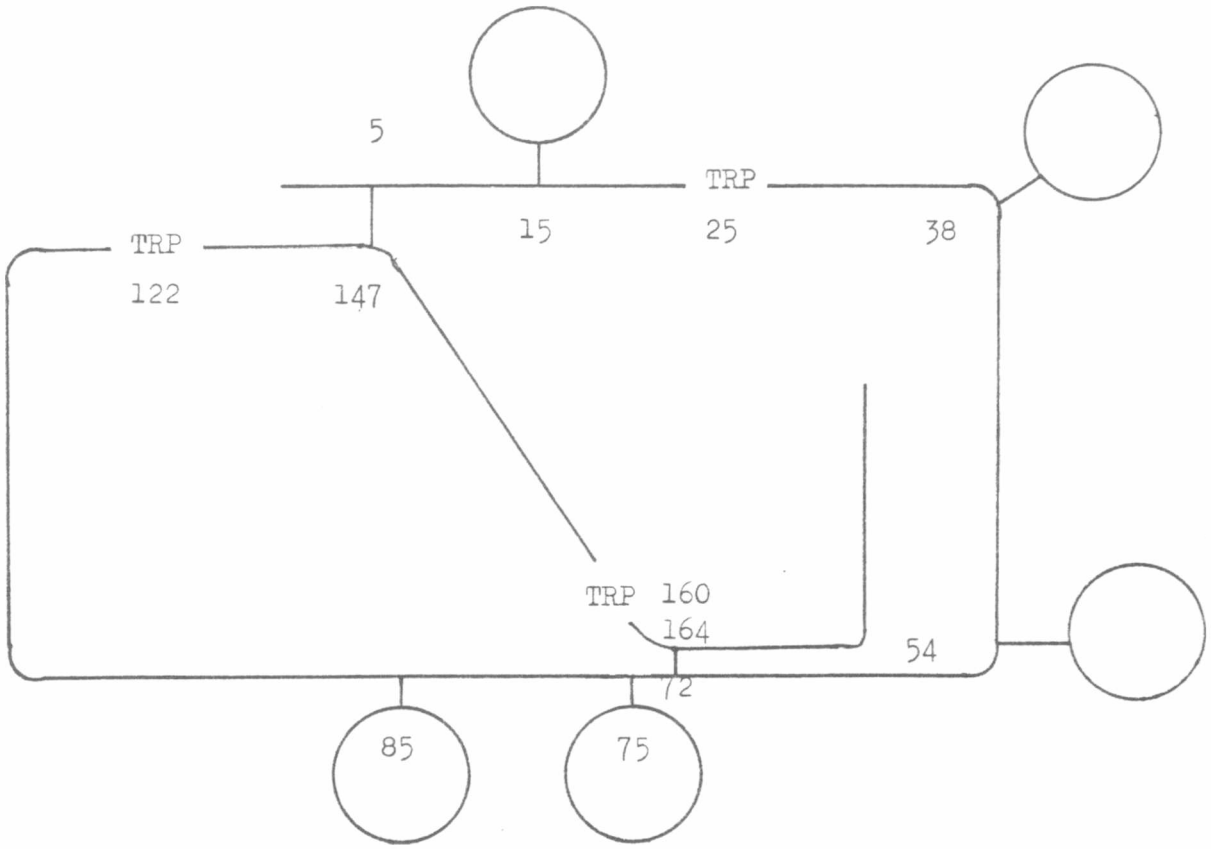


Figure 4.24

A schematic presentation of AGP showing the important structural features to this study. Two disulphide bonds link residue 5 to 147 and 72 to 164. Five carbohydrate units are linked N-glycosidically to the asparagine residues 15, 38, 54, 75 and 85. It contains three tryptophan residues in positions 25, 122 and 160.

CHAPTER FIVE

A STUDY OF VARIOUS METHODS FOR WASHING PLATELETS

A STUDY OF VARIOUS METHODS FOR WASHING PLATELETS

5.1 INTRODUCTION

Platelets are the smallest formed elements of the blood. When the blood vessels are injured an accumulation of the platelets is formed at the point of injury and this mass is stabilised by the formation of fibrin from fibrinogen. This apparently simple response has been investigated by many researchers but the mechanism of platelet aggregation has yet to be fully discovered.

In order to clearly control, define and analyse the responses of platelets, platelet rich plasma (PRP) may be prepared for subsequent study. PRP is still a very complex system and to further simplify this system the platelets may be suspended in an artifical medium. This has the advantages of excluding plasma enzymes and allows the manipulation of the constituents of the suspension medium (Rathbone et al., 1971)

5.1.1 Methods of washing platelets

The methods for washing platelets may be divided into three basic groups:

(1) Gel filtration

(2) Differential Centrifuging

(3) Density gradient centrifuging

Each method has its advantages and limitations and therefore the use of a particular method depends on the subsequent use of the platelets. The following section will discuss the different methods of washing platelets.

5.1.1.1 Gel filtration

The first reports of the use of gel filtration for the separation of blood platelets from plasma was that of Tangen et al. (1969 and 1971). A Sepharose 2B column was employed with an elution buffer of 0.154M NaCl or 0.145M NaCl buffered to pH 7.4 with 0.05M Trizma base containing 5 X 10^{-5} M $CaCl_2$ and 1% glucose. Tangen et al. (1973a) reported a modification of this method using a more complex buffer and the addition of albumin to inhibit the release of ADP and ATP from the platelet. Zucker et al. (1974) found, using Tangen's method (1971), that ultrastructural changes were only minor and included irregularities of cytoplasmic outline, formation of a few short pseudopods and slight dilatation of the surface connected canalicular system. Tangen et al. (1973b) reported similar findings showing that gel filtration produced more platelets with three or more pseudopods than did leaving the platelets undisturbed in citrated plasma, but less than those subjected to differential centrifugation. Lindon and Waugh (1974) reported that during gel filtration the platelets did not exhibit spontaneous release of serotonin and adenine nucleotides but significant release occurred during two hours of storage. An increase in prostaglandin E_2 synthesis occurred during and after filtration. Mason and McQueen (1974), making an investigation of various methods of washing platelets, stated that Tangen's method proved to be generally dependable and reproducible.

Lages et al. (1975) developed a method for washing platelets by gel filtration in a more physiologically acceptable elution buffer. Studies were made of the effect of the buffer system on the

aggregation response. With a system buffered with Tris, an inconsistent aggregation reponse was observed on addition of ADP, adrenaline or collagen. Such variability in the aggregation response was not seen when a modified Tyrode's solution was used. It was suggested that Tris based buffers are unlikely to prove satisfactory for gel filtration of human platelets.

5.1.1.2 Differential centrifuging

One of the earliest reports of a method for washing platelets was that of Brinkhous et al. (1958). Ethylene diamine tetra acetic acid (EDTA) was used as the anticoagulant during the collection of blood. PRP was prepared by centrifuging at 225g for 10 minutes and then a platelet plug was prepared by centrifuging at 2075g for 10 minutes. The resuspension buffer was a saline EDTA solution or a saline citrate buffer.

From this early method a more complex resuspension medium has been developed to give a final resuspension medium of similar ionic composition to plasma. Ardlie et al. (1970) studied the conditions necessary for washing platelets which would aggregate normally. During the washing steps the conditions were maintained to inhibit platelet aggregation. The first resuspending solution was a modified Tyrode's solution, pH 6.5, containing 2.0mM of magnesium and 0.2mM of EGTA with no calcium. The second and subsequent resuspending solution was a Tyrode's solution, again with no calcium. Finally the platelets were suspended in a Tyrode's solution pH 7.4. All solution contained 0.35% BSA.

A modification of this method was presented by Mustard et al. (1972). The resuspending solutions were the same as Ardlie et al. (1970) but with the addition of 50-100mg/l of apyrase. Mason and McQueen (1974) in their study reported difficulty in using Mustards method. They stated that

> "Despite careful attention to detail particular care with pH of buffers, 17 of 23 of these platelet preparations were of such low yield as to be useless. Failures were due to massive clumping of platelets upon attempted resuspension following the second washing step or to massive adhesion of platelets to container walls during centrifuging."

Zucker et al. (1974) reported that platelets in plasma undergoing the differential centrifugation procedure of Mustard et al. (1975), showed the least ultrastructural changes compared to all the methods they investigated.

Bang et al. (1972) developed a method for centrifuging the platelets onto a cushion of red blood cells to reduce any damage to the platelets. Prostaglandin E_2 was added by Rotman and Heldman (1980) to inhibit platelet activation during centrifuging. Moncada et al. (1982) and Vargas et al. (1982) further developed the use of prostaglandins by using prostacyclin to inhibit aggregation during centrifuging.

Numerous other reports of using the differential centrifuging method for washing platelets have been reported. The main difference between them was the composition of the resuspending solutions. These include those of Anderson et al. (1980), Dutilh et al. (1980) Davey and Luscher (1968), Haslam (1964) and Born and Cross (1964).

5.1.1.3 Density gradient separation.

Walsh (1972) developed a method of washing platelets using density gradient separation. 9ml of PRP was layered over 1ml of 40% BSA. The interface between the albumin and the PRP was gently mixed and followed by centrifugation. A development of this was made by Walsh et al. (1977). A 40% solution of BSA was diluted with calcium free Tyrode to create a discontinuous density gradient. PRP was then gently layered over this and centrifuged at 1500g. The supernatant platelet poor plasma (PPP) was removed and the platelets resuspended. Nicholls and Hampton (1972) also developed a method for washing platelets by a density gradient method. A discontinuous albumin gradient was set up using a calcium free Tyrode's solution pH 6.5. It was claimed that this method removed the need to add a chelating agent which may irreversibly modify the platelet membrane. The major advantage of this method over other centrifugation methods is that the platelets do not have to be resuspended from a platelet plug. Ganguly and Sonnichson (1973) developed a method using sodium metrizoate as a cushion for the platelets.

Mason and McQueen (1974) claimed that the platelets prepared by density gradient methods tended to be hypoactive. They stated

> "Both methods for platelet preparation by albumin density gradient centrifugation were unsatisfactory. The method of Nicholls and Hampton (1972) despite careful attention to published instructions consistently gave a low yield of platelets which tended to aggregate poorly. The method of Walsh (1972) gave an acceptable yield of platelets but ultrastructural studies revealed marked alteration and some preparations were hypoactive."

Zucker et al. (1974) found that the density gradient centrifuging methods showed the most marked alterations of ultrastructure of the platelet when compared with gel filtration and differential centrifugation methods.

5.1.2 Protein requirements of platelets

The concept of the platelet atmosphere of protein necessary for optimum platelet function was formulated by Roskan (1923). Bang et al. (1972) suggested the following in regards to the platelet atmosphere

(1) Three cationic proteins - fibrinogen, Hageman factor and gamma globulin - are all capable of restoring the aggregation capability of washed platelets.

(2) The aggregation restoring effect of gamma globulins is inhibited by the acidic proteins in subfractions of Cohn fractions IV and fractions V and VI

(3) The rate of aggregation of platelets is regulated in part by the equilibrium between cationic and anionic proteins in plasma.

Hansen and Bang (1979) developed this further in proposing that proteins of higher positive charge density enhanced platelet aggregation whereas proteins of higher negative charge density inhibited platelet aggregation. In their study they found that oxygen consumption of washed platelets was lower than in PRP while the lactate production was unchanged. It was found that plasma albumin restored the oxygen consumption. It was also reported that c-AMP levels are considerably lower in washed platelets than in PRP.

Experiments conducted with washed platelets have shown that they adhere and aggregate more slowly than PRP. Born and Cross (1964) identified a heat labile plasma protein concentrated in the euglobulin fraction which significantly restored to washed platelets the ability to aggregate to ADP. Deykin et al. (1965) found that fibrinogen was necessary to help restore platelet function but also noted that additional heat stable plasma proteins were required.

Rossi (1972) found that suspensions of human platelets in isotonic protein free artifical media contained lactate dehydrogenase, adenosine triphosphatase (ATP ase) and adenyl kinase activities in both the platelet free ambient media and the platelet suspension. When platelets were repeatedly centrifuged these enzyme activities increased. The addition of 5mg/ml of BSA to the resuspension fluids diminished enzyme loss from the platelets. Zingg et al. (1981) also studied the effect of albumin concentration and storage time on the adhesion of washed platelets to glass. It was found that the initial rate of platelet adhesion was inversely related to albumin concentration.

5.1.3 Aggregation

The most commonly used in vitro method to investigate platelet aggregation is to measure changes in light transmission through platelet rich plasma or a suspension of washed platelets (Born, 1962). When an adenosine diphosphate (ADP) solution of low concentration is added to stirred PRP or washed platelets at 37^{0} C there is an increase in light transmission because of dilution. Then as the platelets change from discs to spheres with pseudopods, light transmission decreases. This is followed by a rapid increase in light

transmission which reaches a maximum after one or two minutes due to the formation of platelet aggregates. The light transmission then decreases due to platelet disaggregation.

Born (1962) and O'Brien (1962) observed that the rate of stirring is important because the rate and extent of aggregation increases with the speed of stirring. Born and Cross (1963) showed that if all conditions remained constant the rate of increase in light transmission during the first 30 seconds is proportional to the log [ADP]. Marcus and Zucker (1965) showed that the minimum concentration of ADP to cause platelet aggregation was 10^{-7} M. McLean and Velso (1967) showed that platelet aggregation was inhibited by low pH. At pH 6.5 ADP only caused shape change of the platelets.

5.1.4 Aims and objectives

The aim of this section was to find a reproducible and reliable method for washing platelets which will not alter the functions or characteristics of the platelets when compared with PRP. The ability of the platelet to aggregate was used as a test of platelet function. Binding studies will be conducted using these methods of washing platelets in the next section. It is therefore desirable that BSA and AGP are not added to the platelet suspension so that the binding of the ligand to the platelet is not masked by the binding of the ligand to these plasma proteins.

5.2 Materials and instrumentation

Ethyleneglycol-bis-(β-amino-ethyl ether) n,n'-tetra acetic acid (EGTA), adenosine diphosphate (ADP), fibrinogen, Collagen type II, thrombin, and prostacyclin were purchased from Sigma Chemical Company.

Surfasil was obtained from Pierce Chemical Company.

Ethylene diamine tetra acetic acid was purchased from Fisons.

Brillant cresyl green was purchased from BDH.

Aggregation studies were conducted using an ADG instrument Model 1002 aggregometer and a Lumi aggro-meter Model 400.

5.3 Methods

5.3.1 Preparation of PRP

The ear of a New Zealand white rabbit (of either sex) was shaved and xylene was applied as a vasodilator to the ear. The marginal ear vein of the rabbit was cut and the blood collected into a 15ml siliconised plastic centrifuge tube containing the anticoagulant.

Anticoagulants used were :-

(1) 3.8% sodium citrate - 1ml to 9ml of blood.

(2) Acid citrate dextrose (ACD) containing 2.5g sodium citrate, 1.5g citric acid, 1.25g glucose and water to 100ml. Final pH 4.5.

1.5ml to 8.5ml of blood.

(3) EDTA. 2g EDTA, 0.505g sodium chloride, distilled water to 100ml. Final pH 7.3.

1ml to 9ml of blood.

The anticoagulant and blood was mixed gently during and after collection.

Platelet rich plasma (PRP) was then prepared by centrifuging the blood at room temperature, for 15 minutes at 120g. The supernatant PRP was removed using a siliconised plastic pipette and stored at room temperature until used. Platelet poor plasma was obtained by centrifuging the remaining blood at 2000g for 15 minutes.

5.3.2 Platelet count.

Platelet counts were performed by diluting the PRP 1 in 20 with the stain.

Sodium citrate 3.8g
formaldehyde 37% 0.126ml
brilliant cresyl blue 0.1g
distilled water to 100ml

The platelet suspension and stain were mixed for 5 minutes. A sample of the resulting mixture was placed in a counting chamber (improved Neubauer haemocytometer B.S. 748) and 16 squares counted.
The number of platelets per mm was 80000 N, were N was the average number of platelets per square.

5.3.3 Preparation of washed platelets

5.3.3.1 Preparation of washed platelets by the method of Livsey.

Using 3.8% sodium citrate as the anticoagulant PRP was prepared as described above. Undiluted PRP was pipetted into a plastic centrifuge tubes containing a 10% sucrose solution in the ratio of 5 volumes of PRP to 1 volume of the sucrose solution. After gentle agitation the mixture was centrifuged at room temperature for 15 minutes at 1200g. The supernatant plasma was removed by decantation leaving a platelet plug. Modified Tyrode (Table 5.1) was then added to the platelet plug using a volume equivalent to half the volume of the PRP. The platelet plug was resuspended using a siliconised wide bore pipette to gently blow the buffer over the plug.

5.3.3.2 Preparation of washed platelets by Ardlie and Mustard method

PRP was prepared by centrifuging whole blood for 15 minutes at 190g. The PRP was removed and centrifuged at 1100g for 15 minutes. The platelet plug was resuspended in a modified Tyrode's solution, either that of Mustard et al. (1972), (Table 5.2), or Ardlie et al. (1970) (Table 5.3). The resuspended platelets were centrifuged at 1100g for 15 minutes as above and the supernatant removed. The platelet plug was resuspended in the second modified Tyrode and the above repeated. The final resuspending buffer was then used.

5.3.3.3 Preparation of washed platelets using prostacyclin

Washed platelets were prepared according to the method of Moncada et al. (1982) and Vargas et al. (1982). Rabbit blood was collected as described previously using 3.8% sodium citrate as the anticoagulant. PRP was prepared by centrifuging at 190g for 15 minutes. The PRP was removed with a siliconized plastic pipette and after adding 2ng/ml of prostacyclin recentrifuged at 110g for 10 minutes to sediment red and white blood cells remaining in the PRP. The resulting PRP was separated, 300ng/ml of prostacyclin added and the PRP was recentrifuged at 800g for 10 minutes. The plasma was decanted and the platelets resuspended in Tyrode's solution. The platelets were resuspended by gentle sucking and blowing with a plastic pipette. After further additions of prostacyclin the suspension was recentrifuged at 600g for 10 minutes and the process repeated. The final resuspension involved no prostacyclin. Table (5.4) shows the composition of the suspending solutions at each stage.

5.3.3.4 Preparation of washed platelets by gel filtration

Sephadex G-100 and G-200 and Sepharose 2B were used to prepare a washed platelet suspension. Sephadex G-100 has an exclusion range of 4000-150000 Daltons so would not fully separate the platelets from all the plasma proteins. Sephadex G-200 has an exclusion range of 5000-600000 Daltons. This would separate the platelets from all the plasma proteins apart from the lipoproteins. Sepharose 2B has an exclusion range of 70000-40000000. Sepharose 2B columns and Sephadex columns were prepared according to the method of Larges et al. (1975). Two size of columns have been employed, 1cm X 25cm and 2.5cm X 25cm. Columns were siliconized with a 1% solution of surfacil, rinsed and dried. Both the G-100 and G-200 was allowed to swell in buffer for 48 hours before packing the column. After de-aeration of the gel under vacuum, the suspension was poured into the column in one step ensuring that no air bubbles were trapped. The gel was allowed to pack by gravity flow to a height of approximately 20cm. PRP was prepared as described previously. To a column that had previously been equilibrated with the elution buffer (Table 5.1) a sample of PRP was added to the column and eluted. Fractions were collected at fixed time intervals. The initial sample which came off the column containing the platelets were rejected and the platelet fractions in the middle were pooled and used in subsequent experiments. For aggregation experiments 0.3 mg/ml of BSA and 2mM of calcium was added to the platelet suspension.

5.4 Results and discussion

5.4.1 Anticoagulant

Three anticoagulants have been used, 3.8% citrate, ACD and EDTA. Using EDTA and ACD the PRP would not aggregate to ADP. EDTA lowers the Ca^{2+} content of the buffer so that the platelets cannot aggregate. It has been reported by Day et al. (1975) that even when Ca^{2+} is added to the system the platelets change shape and aggregate poorly to ADP.

ACD lowers the pH of the buffer so that the platelets do not aggregate to ADP. When the pH is raised to 7.4 the platelets aggregate normally. ACD was therefore used by Mustard et al. (1972) and Ardlie et al. (1970) in their method of washing platelets to inhibit platelet aggregation during the first centrifuging step. It was not used by Moncada because the low pH would accelerate the rate of hydrolysis of the prostacyclin.

Using a trisodium citrate as the anticoagulant the concentration of free Ca^{2+} in the plasma is sufficient to allow platelet aggregation but too low to support coagulation. Trisodium citrate was therefore used as the anticoagulant for all aggregation studies using PRP.

5.4.2 Differential centrifuging

5.4.2.1 Livsey and Smith method

Livsey's and Smith's (1975) method was not an efficient method of washing platelets. The method only removed the plasma and did not go on to wash the platelets. Significant amounts of plasma proteins were carried over into the resuspending solution. Difficulty was experienced in resuspending the platelets from the platelet plug. On

several occasions the platelet suspension aggregated spontaneously on stirring without the addition of an aggregating agent.

The conditions of this method were not arranged so as to inhibit aggregation during the preparation of the platelet plug. A citrate anticoagulant was used with a pH of 7.4. If an ACD anticoagulant had been employed with a pH of 4.5 the platelets would have been inhibited from aggregating during the centrifuging step by the low pH. The tyrode contained 2mM of calcium without any anticoagulant to prevent the formation of thrombin. Any prothrombin carried over in the plasma will therefore be able to be converted to thrombin causing the platelets to aggregate. This method also contained no step to remove any red blood cells (RBC) carried over in the PRP. If the red blood cells are damaged during the centrifuging they will release ADP thus causing the platelets to aggregate. Any RBC in the final suspension will also decrease the sensitivity of the measurement of the platelets to aggregate by the turbidometric method.

5.4.2.2 Ardlie and Mustard methods

These two methods are identical apart from the addition of apyrase in Mustard's method. Both these methods gave problems with the resuspension of the platelet plug. The resuspension of the platelets in the final resuspension solution was particularly difficult, the platelets aggregated on several occasions. It would be desirable if the final resuspension buffer did not contain albumin but when albumin was omitted from the method the platelets aggregated spontaneously when stirred. The platelet yield with these methods was consistently low. The advantages with these methods were the control allowed of the final platelet numbers and that the composition of the

buffer could easily be modified.

5.4.2.3 Prostacyclin method

This method proved to be the most reproducible and reliable of the differential centrifuging methods. Addition of prostacyclin at each centrifuging stage inhibited platelet aggregation during centrifuging. The platelet plug would resuspend with no difficulty. The major problem with this method was that the platelets would not aggregate for several hours after the last washing step. The platelets would then respond normally for a considerable period. The method was unsuitable for platelets which were subsequently used for assay of c-AMP and c-GMP. It was possible to prepare a washed platelet suspension when the final suspending buffers were modified so as not to contain any BSA.

The platelet yield with this method was high when compared with Ardlie's or Mustard's method. The preparation of the buffers were also considerably simplified, with all the buffers at the same pH and containing both calcium and magnesium ions.

5.4.3 Gel fitration

Fractions were collected as the column was eluted and the absorbance at 285nm was measured together with the fluorescence scatter using an excitation and emission wavelength of 600nm. Figure 5.1, 5.2 and 5.3 show the chromatograms of the eluant using these two methods of detection for Sephadex G-100, G-200 and Sepharose 2B.

Sephadex G-100 separated the platelets from the majority of the plasma proteins but very poor resolution between the peaks was observed. Sephadex G-200 showed adequate resolution between the protein peak and the platelets. The resolution was considerably less than that of Sepharose 2B. Sepharose 2B gave the best resolution between the platelets and the plasma proteins. This allowed the sample size of PRP added to the column to be increased without losing the resolution between the platelets and the plasma proteins.

A number of eluants were used including saline, modified Tyrode (Table 5.1) and a saline/glucose/citrate solution (Table 5.1). All these buffer systems gave a satisfactory washed platelet suspension. For routine preparations the modified Tyrode's solution was employed because its ionic composition was the most similar to plasma using Sepharose 2B gel. A platelet yield of approximately 95% was given but the first and last fractions containing platelets were rejected because of their low platelet numbers.

A inconsistent aggregation response was given when Tris was included in the buffer. On a number of occasions the platelets would not aggregate. This method proved to be reproducible and reliable when a modified Tyrode eluant was used. The platelets would aggregate normally if 2mM of calcium and 0.35% BSA was added to the suspension. It was possible to prepare a platelet suspension without the addition of BSA. Two problems were associated with this method:
Only a comparatively small quantity of PRP could be applied to the column in one run therefore limiting the number of platelets that could be prepared in one step.
Platelet numbers varied from about 100000 to 300000/mm^3. The method

allowed very little control of the platelet numbers.
Its main advantage was that it was relatively quick, from the addition of the PRP to the column the platelets were all eluted within 45 minutes.

5.4.4 Density Gradient centrifugation

The density gradient methods of Walsh et al. (1977) and Nicholls and Hampton (1972) left the platelets suspended in a medium containing up to 4% albumin. For subsequent experiments involving binding of ligands and fluorescence this concentration of albumin was far to high. These methods were therefore not used.

5.4.5 Aggregation

Aggregation studies were conducted using both ADP and collagen. Washed platelets prepared by gel filtration and with prostacyclin would aggregate, to ADP, without the addition of fibrinogen. When the platelets would not aggregate to ADP on a number of occasions, they would aggregate to collagen. This was thought to be due to the platelets becoming refractory to ADP released from RBC and damaged platelets.

5.4.6 Light Microscopy

Platelets prepared by the methods of Livsey and Smith (1975), Mustard et al. (1972), and Ardlie et al. (1970), on a number of occasions showed a degree of platelet aggregation when viewed by a light microscope. The prostacyclin method and gel filtration platelets very rarely showed such aggregates. No difference could be seen in the ultrastructure of the platelets due to the low

magnification of light microscopy.

5.5 Conclusion

The differential centrifuging method of Moncada et al. (1982) and the gel filtration method proved to be reliable and reproducible. These two methods have been used for the routine preparation of platelets for aggregation and binding studies.

Table 5.1

1) A table showing the composition of the eluant used for gel filtration and the suspending solution for Livsey's method.

Composition	g/l
NaCl	8.0
KCl	0.2
$NaHCO_3$	1.0
$NaH_2PO_4.H_2O$.	0.05
$CaCl_2.6H_2O$	-
$MgCl_2.6H_2O$	0.406
glucose	1.0
BSA	3.5

2) An alternative eluant for gel filtration.

Composition	g/l
NaCl	8.18
Trisodium citrate	4.12
Glucose	1

Table 5.2

A table showing the composition of the suspending fluids for Mustard's method of washing platelets

Composition	First washing fluid g/l	Second washing fluid g/l	Final suspennding fluid g/l
NaCl	8.0	8.0	8.0
KCl	0.2	0.2	0.2
$NaHCO_3$	1.0	1.0	1.0
$NaH_2PO_4.H_2O$	0.05	0.05	0.05
$CaCl_2.6H_2O$	0.438	0.438	0.438
$MgCl_2.6H_2O$	0.203	0.203	0.203
glucose	1.0	1.0	1.0
BSA	3.5	3.5	3.5
heparin (u/l)	25000	-	-
apyrase (mg/l)	50	50	50
pH	7.35	7.35	7.35

TABLE 5.3

A table showing the composition of the suspending fluids for Ardlies method of washing platelets

Composition	First washing solution g/l	Second washing solution g/l	Final suspending solution g/l
NaCl	8.0	8.0	8.0
KCl	0.2	0.2	0.2
$NaHCO_3$	1.0	1.0	1.0
$Na\ H_2PO_4.H_2O$	0.05	0.05	0.05
$CaCl_2.6H_2O$	-	-	0.438
$MgCl_2.6H_2O$	0.406	0.406	0.203
glucose	1.0	1.0	1.0
BSA	3.5	3.5	3.5
EGTA	0.076	-	-
pH	6.5	6.5	7.4

Table 5.4

A table showing the composition of the buffers for preparing washed platelets using Moncada's method.

Step	Object	Prostacyclin ng/ml	Heparin u/ml	Tyrode	Albumin g/l	Centrifugal force G	Time minutes
1	Preparation PRP	-	-	-	-	220	20
2	Sediment red and white cells	2	-	-	-	110	10
3	Sediment platelets	300	-	-	-	800	10
4	Wash platelets	300	25	+	3	600	10
5	Wash platelets	300	-	+	3	600	10
6	Final Resuspension	-	-	+	3	-	-

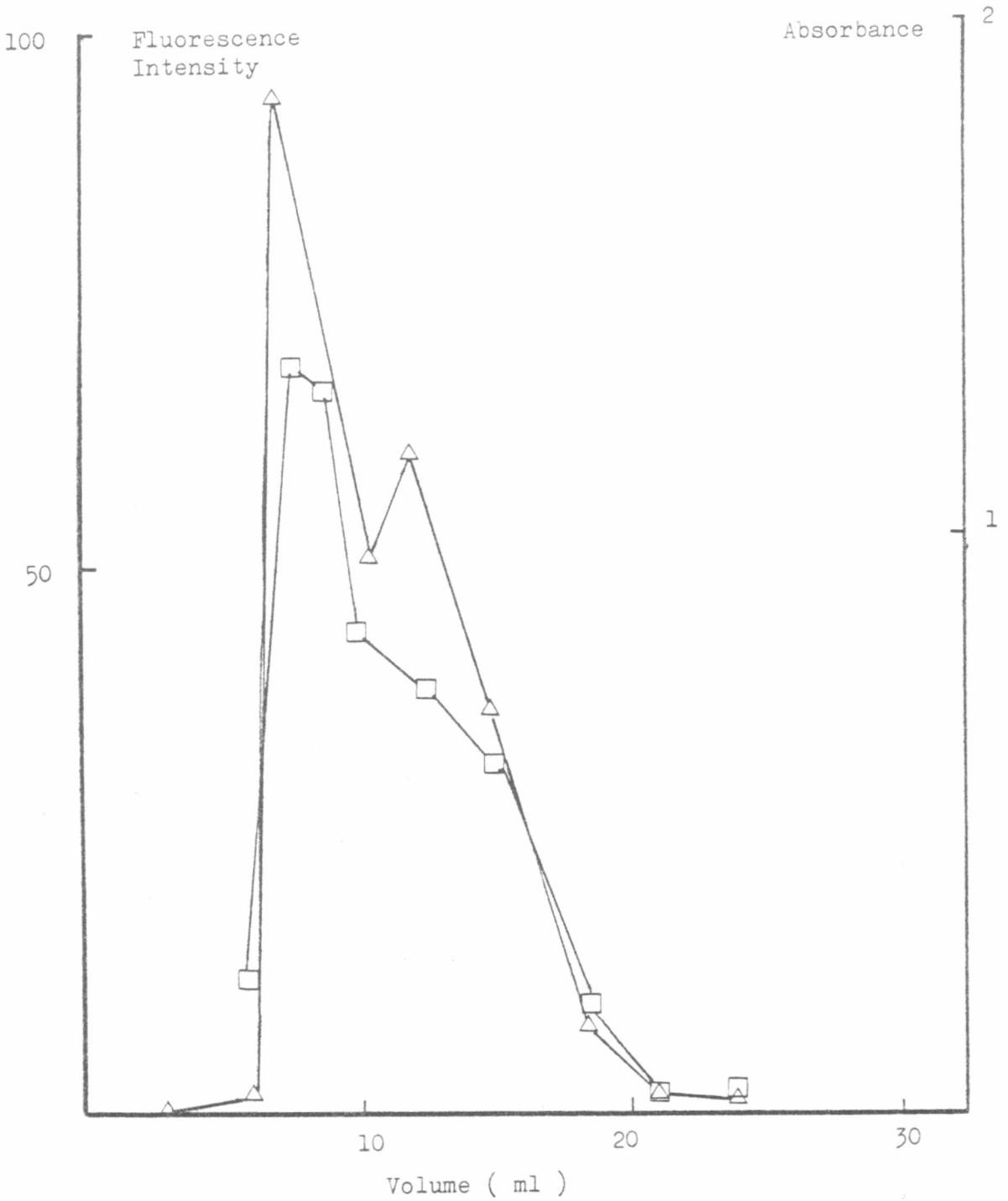


Figure 5.1

A chromatogram for the elution of PRP from a Sephadex G-100 column.

□ absorbance

△ 600nm scatter

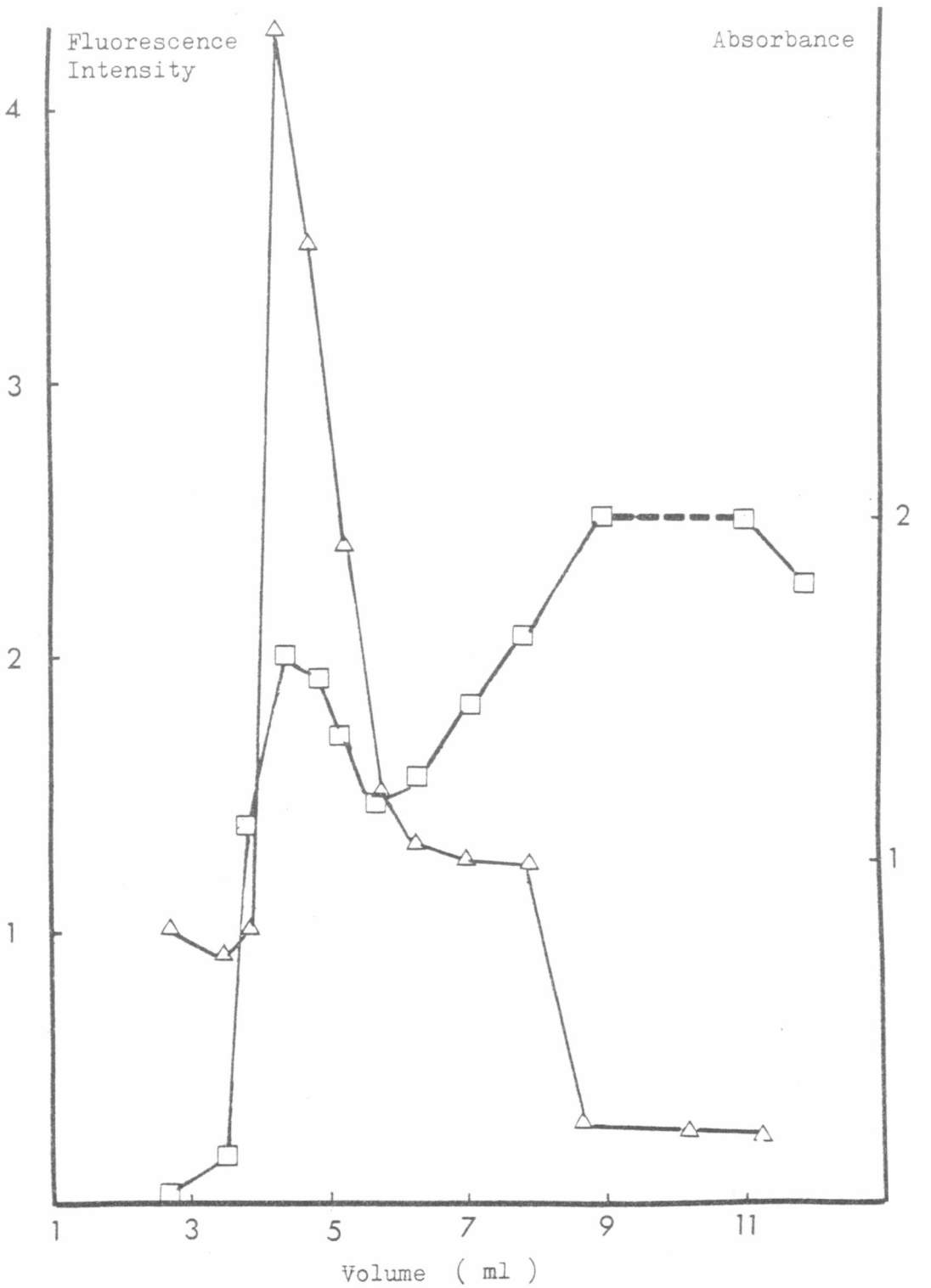


Figure 5.2

A chromatogram for the elution of PRP from a Sephadex G-200 column

□ absorbance

△ 600nm scatter

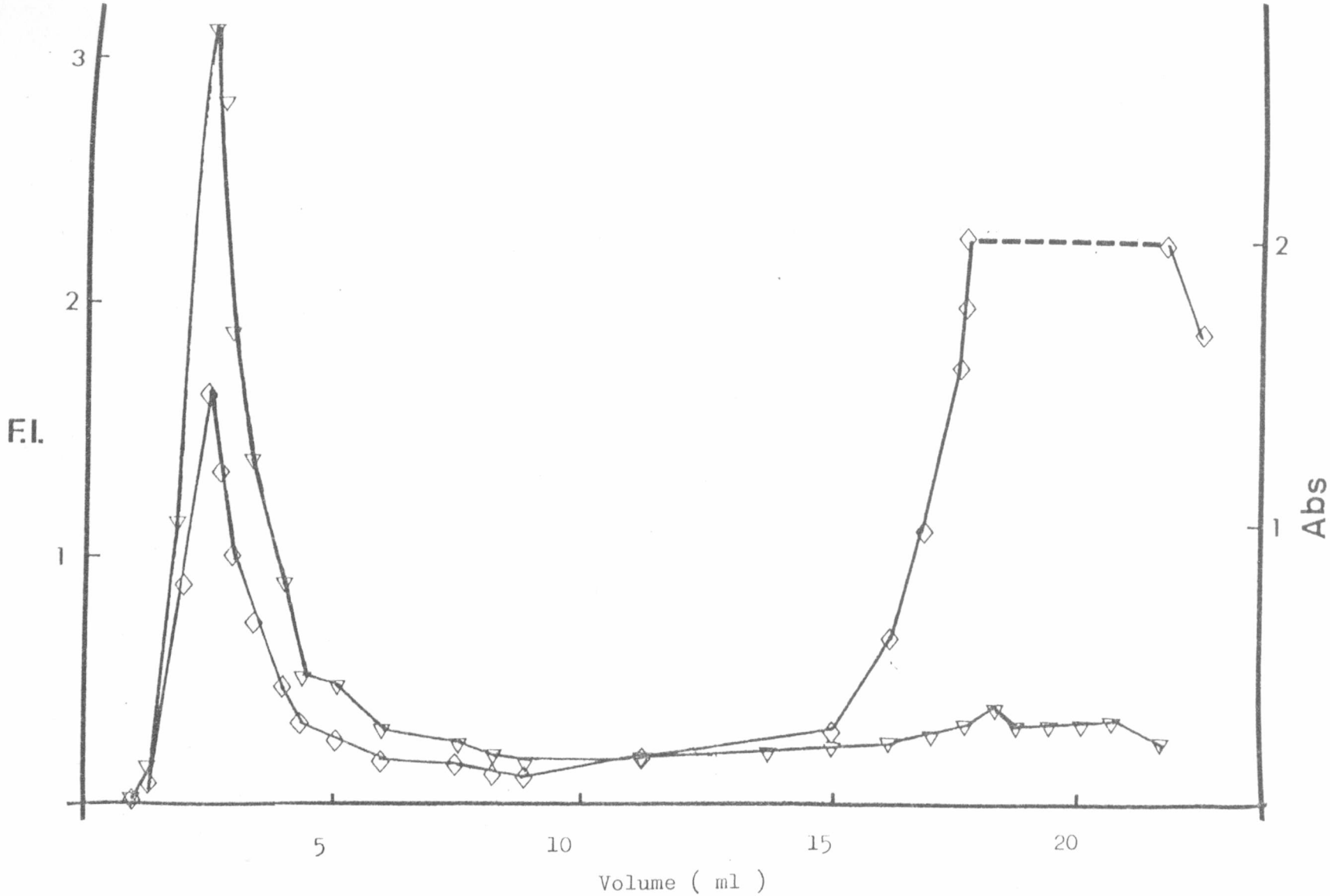


Figure 5.3

A chromatogram for the elution of PRP from a Sepharose 2B column.

▽ absorbance ◇ 600nm scatter

CHAPTER SIX

A STUDY OF THE BINDING OF ANTI-AGGREGATORY COMPOUNDS TO WASHED RABBIT PLATELETS

A STUDY OF THE BINDING OF ANTI-AGGREGATORY COMPOUNDS TO WASHED RABBIT PLATELETS

6.1 INTRODUCTION

The binding of DPD has been studied in relation to the already characterised binding sites and enzyme systems, and with the possibility of it having its own independent sites. Therefore to introduce this section the binding of various ligands to platelets has been considered. This has been followed by an introduction to fluorescence for investigating ligand platelet interactions.

6.1.1 Binding of ligand to platelets

The study of the binding of ligands to platelets can be divided into two groups-

(1) The binding of endogenous ligands

(2) The binding of exogenous ligands

6.1.1.1 The binding of endogenous ligands

6.1.1.1.1 5-Hydroxytryptamine

Rand and Reid (1951) first showed that human platelets contained 5-HT in comparatively high concentrations. Born and Gillson (1959) and Born and Brinknell (1959) suggested that platelets have a mechanism for the active transport of 5-HT through their membrane and also by passive diffusion. Born and Michal (1975) made a study of the binding of 5-HT to platelets. They found evidence that indicated the existence of high affinity binding sites, presumably on the membrane. Kim et al. (1980) looked at 5-HT binding to formaldehyde fixed human platelets. They demonstrated two classes of binding sites. Malmgren

et al. (1981) made a study of the effect of various inhibitors on the 5-HT binding and uptake of human platelets using platelet rich plasma.

6.1.1.1.2 Adenosine and adenosine diphosphate

Born (1965) first studied the uptake of adenosine and ADP in whole platelets. It was shown that ADP binds to a specific receptor on the platelet surface membrane. It was pointed out that because ADP is a nucleotide containing ionised phosphate groups, it is unable to penetrate through the surface membrane. Adenosine, a neutral molecule, is taken up into the platelets. Cusack and Hourani (1982) observed that adenosine competitively inhibited the effect of ADP on adenyl cyclase and platelet aggregation. Boullin et al. (1972) reported that bound labelled ADP was in washed platelet aggregates after the addition of ADP to platelet rich plasma. Mustard et al. (1975) looked into the enzyme activities on the platelet surface in relation to the action of ADP. It was concluded that with washed rabbit platelets ADP reacts with nucleotide diphosphate kinase on the platelet surface. Nachman (1975) demonstrated binding of ADP to human platelet membranes. He showed that the process was reversible, temperature and divalent cation dependent and related to membrane protein thiol groups. Binding data was presented giving a primary affinity constant of $6.5 \times 10^{6}\ M^{-1}$.

6.1.1.1.3 Fibrinogen

Linked with the ADP site is the fibrinogen binding site. Castaldi and Caen (1965) showed that platelet fibrinogen may be differentiated into adsorbed (plasma) and extractable (intraplatelet) fractions. Marguerie et al. (1979) studied the binding of fibrinogen

and the effect of ADP on the binding. It was shown that fibrinogen does not specifically associate with human platelets. Following the addition of ADP, binding of fibrinogen was observed. Analysis of the saturable binding by Scatchard plots indicated a maximum of 4700 molecules of fibrinogen can be bound per platelet with an estimated association constant of $7.7 \times 10^{6} M^{-1}$. Hawiger et al. (1980) showed that prostacyclin inhibited the mobilisation of specific binding sites for fibrinogen on human platelets. Zucker and Peerschke (1979) studied the effect of shape change and aggregation on the binding of fibrinogen to platelets. They demonstrated that fibrinogen binding could occur without shape change.

6.1.1.1.4 Prostaglandins

The latest class of endogenous compounds which have been shown to bind to platelets are the prostaglandins. Prostaglandins have two distinct receptors on the platelet membrane (Whittle et al., 1978; Miller and Gorman, 1979). It was show that PGE_2 and prostacyclin acts on one of these receptors whereas PGD_2 acts on the other. Town et al. (1982) went some way in the characterisation of the prostacyclin receptor using ciloprost a stable prostacyclin analogue.

6.1.1.1.5 AGP

Tripodi et al. (1971) suggested that AGP was either bound to the platelet membrane or part of the platelet membrane. Steel et al. (1982) also noted that AGP was associated with the platelet membrane. It has previously been shown in this study that a number of platelet active compounds are bound to AGP. It is therefore possible that these platelet active drugs do not bind directly to the platelet

membrane but to AGP which is then bound to the platelet.

6.1.1.2 The binding of exogenous ligands

6.1.1.2.1 Dipyridamole

The binding of DPD to washed platelets was first investigated by Horch et al. (1970) who found that in the presence of plasma proteins there was, if at all, only a very small fraction (<1%) of the DPD bound. Subbarao et al. (1975), in a study of the binding of DPD to washed platelets, showed that 1μM DPD binds about 20-30% to the platelet, reaching a maximum within one minute. Factors affecting such binding were also investigated. A more detailed study was completed by Subbarao et al.(1977a). It was reported that washed platelets contain two populations of binding sites. The binding of DPD to the high affinity site had a dissociation constant of 0.04 μM with approximately 20000 binding sites per platelet. The lower dissociation constant was 4 μM. AGP significantly decreased the binding of DPD to the platelet. DPD decreased the uptake of adenosine but did not compete for the binding site.

6.1.1.2.2 Prazosin and others

Kerry et al. (1983) studied the binding of tritium labelled prazosin to washed human platelets. It was shown that prazosin bound specifically to the platelet at very low concentrations. Competitive studies were also conducted.

A large number of other endogenous ligands have been reported to bind to platelets. Most of these are cationic molecules and include propranolol (Weksler et al. 1977) and mepacrine (Dise et al. 1982).

6.1.2 The use of fluorescence in platelet binding studies

Scandurra et al. (1974) reported a fluorescence method for studying the binding of ditazol to plasma proteins and human platelets. They suggested that platelet aggregation may be inhibited by drugs through one of three different mechanisms :-

(1) Direct interaction of the drug with the platelet.

(2) Indirect interaction of the drug with the platelet mediated by a plasma component adsorbed on the platelet mantle.

(3) Interference of the drug with the production and or metabolism of substances inducing platelet aggregation.

No change in the fluorescence polarisation of the drug was seen in a washed platelet suspension, but a marked change in the fluorescence polarisation was seen in the presence of plasma. They concluded that a direct interaction of the drug with the platelet did not occur but claimed that the drug bound to a macromolecule serum component adsorbed on the platelet mantle.

Horne et al. (1975) used noncovalently bound fluorescent probes to study changes in the platelet which may occur during platelet aggregation. N-phenyl - naphthylamine and 8- anilino -1- naphthalene sulphonic acid were bound to the platelet. Platelet aggregation by collagen or thrombin was unaffected by the presence of the label. No change in the fluorescence intensity or wavelength of maximum intensity was observed during platelet aggregation. Mely-Goubert et al. (1979) conducted a study of platelet membrane proteins through fluorescence polarisation of diphenyl hexatriene (DPHT) labelled platelets. A small change in the fluorescence polarisation was observed during aggregation. Fleischer et al. (1980) used two

fluorescent probes, N-carboxymethylisatoic anhydride, which binds to membrane glycoproteins, and DPHT. The use of fluorescent probes to investigate binding will be useful only when the ligand binds in the proximity of the probe or on binding, causes a change in the environment of the probe.

6.1.3 Aims and objectives

The binding of various platelet active ligands to albumin, AGP, fibrinogen and plasma has already been considered. In this section the binding of the above compounds to washed rabbit platelets was investigated. Very few studies have been conducted using fluorescence or fluorescence polarisation techniques to measure the binding of clinically useful compounds to washed platelets. Fluorescence methods have a number of advantages over corresponding radio-isotope procedures:-

(1) No separation step is required to determine the free and bound concentration of ligands.

(2) The rate of uptake of the ligand into the platelet can be measured directly.

(3) The method does not depend on the purity of the radiolabelled ligand.

The major problem with the method is that the minimum detectable concentration is much higher than with radio isotopes.

In this study the characteristics of the binding sites of DPD on the platelet were more extensively investigated than in any previous study. Competitive studies were conducted. The characteristics of the binding sites on the platelet were compared with that of AGP. Finally the mechanism of action of DPD was discussed.

6.2 MATERIALS AND INSTRUMENTATION

Adenosine, cyclic adenosine monophosphate (c-AMP), 5-hydroxytryptamine (5-HT), valinomycin, gramicidin-D, adrenaline, ouabain and phosphodiesterase (bovine heart) were obtained from Sigma Pure Chemical Ltd.

1,6 diphenylhexatriene (DPHT) was purchased from Aldrich Chemical Co.

$^{14}_{8}C$ adenosine (54mCi/mM), cyclic adenosine monophosphate (c-AMP) protein binding assay and the c-GMP radioimmunoassay were purcahsed from Amersham Radiochemicals.

N carboxymethyl isatoic anhydride (NCMIA) was supplied by Polysciences Inc.

Packard Tri-Carb liquid scintillation spectrometer Model 2425 was used to detect the tritium and ^{14}C labels.

6.3 METHODS

6.3.1 Fixed platelets

The washed platelets were fixed with 247mM formaldehyde solution (0.2 volume of 3.7% formaldehyde solution in phosphate buffer) for 30 minutes at room temperature (Kim et al., 1980).

6.3.2 Preparation of platelet membranes.

PRP was centrifuged to eliminate red blood cells and the supernatant centrifuged at 1900g for 15 minutes to form a platelet pellet. The pellet was resuspended in ice cold 50mM Tris HCl buffer (pH 8.0), containing 0.11M sodium chloride and 0.02M disodium EDTA and centrifuged. After a further resuspension in the same buffer, the platelets were resuspended in 50mM Tris buffer (pH 8.0) containing 5mM disodium EDTA and disrupted ultrasonically. This lysate was then

centrifuged at 14000g for 30 minutes and the platelet membrane plug resuspended (Daiguji et al., 1981; Newman et al., 1978).

6.3.3 Binding studies

6.3.3.1 Direct fluorescence methods

0.5ml of the platelet suspension was placed in a 5mm x5mm siliconised quartz cuvette and this was titrated with DPD. An excitation wavelength of 420nm and emission wavelength of 490nm were used with both the emission and excitation slit set at 10nm. The fluorescence and fluorescence polarisation were measured in the concentration range $5 \times 10^{-8} - 10^{-6}$ M DPD. A study of how the fluorescence intensity of the DPD changed in the platelet suspension with time was also made. In order to set up a standard system to compare the fluorescence of normal platelets, platelets were heat treated for 15 minutes at 80 ^{o}C (Subbarao et al., 1977a) or fixed with formaldehyde.

6.3.3.2 Difference method

DPD was added to a series of 0.5ml samples of the platelet suspension to give a concentration range of 5×10^{-8} to 10^{-6}M. The platelets were allowed to equilibrate with the DPD for 30 minutes either at room temperature or 37 ^{o}C and then centrifuged at 2000g for 15 minutes. The supernatant was removed and the fluorescence of the ligand measured using an excitation wavelength of 420nm and an emission wavelength of 490nm. The blank reading was taken from the supernatant with no DPD and this sample titrated with DPD to construct a standard curve.

Binding studies were also conducted using RA 233, RA 433, SH 1242, DMQ, DPQ, propranolol, indomethacin, SA and ASA. ASA was hydrolysed to SA and the SA assayed using an excitation wavelength of 305nm and an emission wavelength of 410nm. Indomethacin was assayed by adding sodium hydroxide to give a final concentration of 0.1 M. This was allowed to stand for 5 minutes. The fluorescence of the sample was then measured using an excitation wavelength of 300nm and emission of 385nm.

6.3.3.3 Sephadex batch method

200mg of dried Sephadex was preswelled for 24 hours in 1ml of the final Tyrode resuspension solution. The volume was made up to 5ml with the washed platelet suspension. This was then titrated with DPD in the concentration range of 10^{-7} to 10^{-6} M. The results were analysed as previously described.

6.3.4 Assay of c-AMP and c-GMP

c-AMP was assayed using a protein binding procedure (Lam et al., 1982 and 1980; Tovey et al., 1974; Steiner et al., 1972). Suspensions of washed platelets were treated with the c-AMP phosphodiesterase inhibitors followed one minute later by either the addition of 0.5μM prostacyclin or saline. One minute after this addition, the platelets were lysed by the addition of cold perchloric acid. This was then neutralised with potassium hydroxide. The precipitate was removed by centrifuging. To 50 μl of the supernatant, tritiated c-AMP was added followed by the binding protein. This was mixed and allowed to stand for two hours in a refrigerator. 0.1ml of an ice cold charcoal suspension was added and the samples were allowed

to stand for 1 minute. They were then centrifuged and the supernatant added to a scintillation vial using a toluene triton X-100 scintillant.

For c-GMP the samples were prepared as for the c-AMP. The sample was mixed with the tritiated c-GMP and the antiserum. This was allowed to equilibrate for 1.5 hours in a refrigerator. 1ml of ice cold 60% saturated ammonium sulphate was added to each sample and they were allowed to stand for 10 minutes. They were then centrifuged; the supernatant was discarded and the precipitate dissolved in distilled water. This was then added to a scintillation vial using a toluene triton X-100 scintillant.

6.3.5 Adenosine uptake

The platelet suspension was incubated with the drug for ten minutes at 37 ^{o}C. Between 10^{-7} to 10^{-6} M ^{14}C radiolabelled adenosine was added to a 0.25ml samples of the platelet suspension containing 500000 platelets /mm^{3}. The suspension was incubated at 37 ^{o} C for ten minutes and then centrifuged at 2000g for 5 minutes and the supernatant removed. The concentration of adenosine in the supernatant was determined by liquid scintillation counting.

6.3.6 Platelet membrane labels

To label platelets with DPHT, the platelets were first washed as described previously (section 5.3.3.3). To 3ml of the washed platelet suspension 0.35% of gelatin was added and 3 µl of a 10^{-4} M solution of the DPHT in tetrahydrofuran. This was left at room temperature for 30 minutes. The suspension was then centrifuged at 1000g for 10 minutes and resuspended in a phosphate buffer (pH 8).

To label platelets with NCMIA, platelets were washed as above, but the final resuspension buffer contained no albumin. 0.2mg of NCMIA was added per ml of washed platelet suspension and allowed to stand at room temperature for one hour. The suspension was then centrifuged at 1000g for ten minutes and the platelets resuspended in a fresh Tyrode's solution. The label was detected using an excitation wavelength of 350nm and an emission wavelength of 430nm

6.3.7 Aggregation

Aggregation of PRP and washed platelets were studied using the turbidimetric method of Born and Cross (1963). For both PRP and washed platelets a 0.4ml platelet sample was used at 37 ^{o} C. The drug under test was first added to the platelet suspension, followed one minute later by the aggregating agent. The percentage inhibition of aggregation could then be determined.

6.4 RESULTS AND DISCUSSION

6.4.1 A comparison of the binding of DPD to washed platelets prepared by different methods using difference centrifuging

When studying the binding of ligands to platelets, it is desirable in the first instance to remove the plasma proteins so that the binding of the ligand to the plasma proteins does not mask the binding of the ligand to the platelets. Both albumin and AGP may preferentially bind the ligand, thus reducing the amount bound to the platelet. The plasma proteins can be eliminated by washing the platelets and resuspending in an artifical medium. Washing the platelets may alter their binding characteristics or damage them and so effect their binding affinity or capacity. It has also been

reported that the platelet has a plasmatic atmosphere (Roskam, 1923; Adelson, 1961; Bang et al., 1970 and 1972). Washing the platelet will disrupt this atmosphere and therefore the platelet may show an uncharacteristic binding pattern. The binding of ligands to platelets washed by various methods have therefore been investigated to look at what effect the method of washing has on the binding.

The method of Mustard et al. (1972) and Ardlie et al. (1970) for washing platelets proved to be unreliable. With platelets prepared by these methods, no binding of DPD to the platelet was observed. Livsey and Smith's (1975) method had problems with the resuspension of the platelets. Spontaneous aggregation upon resuspension was common. The binding of DPD to washed platelets prepared by this method was not observed.

Binding of DPD to washed platelets was demonstrated using platelets prepared by gel filtration and by the prostacyclin method. The binding was measured by the difference in the concentration of DPD between a standard and the supernatant of the platelet suspension after centrifuging. The major factor that effected the ability to measure binding was the minimum detectable concentration of DPD in the platelet supernatant. This was dependent on the intensity of the platelet blank fluorescence. For routine measurements an excitation wavelength of 420nm and emission wavelength of 490nm were employed. A protein fluorescence peak could be detected in the blank but this was relatively small. Using the 420nm excitation peak gave a lower blank than the 305nm peak. The 420nm peak also had the advantage of very little interference from proteins added to the system. As the platelets aged, the size of the blank increased. The results obtained

from these methods indicated a multibinding site system which were analysed using Nonlin. Three models were tested.

(1) One class of binding site model.

(2) One class of binding sites with partition.

(3) Two classes of binding site model.

Figures 6.1 and 6.2 show a typical binding plot obtained from platelets washed by these two methods. The shape of the curve suggests that the binding did not fit a one site model. Satisfactory fits were obtained with a two site model and a one site model with partition. A comparison was made between the two site model and the one site model with partition, using the method of Boxenbaum et al. (1974), (equation 2.36). The test showed that the residual sum of the squares was not sufficiently reduced to justify fitting the additional parameter in the two site model. The binding of DPD to platelets can therefore be interpreted to have a primary class of binding sites together with either a true partition or a second class of binding sites which are not saturated. The binding data obtained from the two methods were not significantly different. A primary affinity constant of $1.9 \times 10^{7} M^{-1}$ was evaluated with 96000 binding sites per platelet and a partition constant of 0.035.

The binding of DPD to washed platelets was reversible. DPD could be detected in the supernatant after a platelet plug previously incubated with DPD was resuspended in fresh buffer and centrifuged.

The difference centrifuging method is the most widely used method in platelet binding studies. However it does have its problems. The centrifuging step may alter the binding characteristics of the platelets, or the platelets may be activated which may alter the

binding. The concentration of the binding protein is also changing during the centrifuging step. Equation 2.32 predicts that the fraction bound is dependent on the protein concentration, thus a greater fraction bound may be calculated after centrifuging than is actually correct. Direct fluorescence, which requires no separation step, will have no such problems.

6.4.2 An investigation of the binding of DPD to washed platelets using direct fluorescence methods

6.4.2.1 Fluorescence intensity

It has already been shown that the binding of a number of fluorescent ligands to plasma proteins can be measured using direct fluorescence methods. Similar techniques have been applied to the binding of ligands to platelets, but a number of additional complications have resulted. Scatter of the excitation beam and the emitted fluorescence were major considerations. The rate of binding of a ligand to plasma proteins is relatively rapid. Thus the rate of binding cannot readily be studied. With the platelet system, it is not only possible to calculate the binding affinity and capacity constants, but also the rate of uptake into the platelet. The platelet may also show a true partition of the ligand into the platelet.

Using the prostacyclin method for washing the platelets, where the buffer contained no albumin, no change in the fluorescence intensity was observed when less than 1μM DPD was added. This indicates that the fluorescence of DPD does not change when it binds to the primary site. When a concentration of greater than 10μM DPD was added, uptake of DPD could be followed as a function of time

(Figure 6.3). The fluorescence intensity of the DPD first declined and after reaching a minimum value increased to a constant level which was lower than the initial response. This change is therefore associated with the secondary binding. The size of the change was too large to be connected with the primary binding site. It was observed that with different concentrations of DPD, using the same number of platelets, the percentage decrease in the DPD fluorescence was always of the same order. Also in general, increasing the number of platelets led to an increase in the percentage decrease in the fluorescence. This suggests that at high dose levels DPD is partitioning into the platelet.

The binding and uptake of a number of other compounds were investigated to compare with that of DPD. When 10μM 5-HT was added to a washed platelet suspension its uptake into the platelet could be followed by the decrease in the fluorescence of the 5-HT (Figure 6.4a). This was taken to be the passive diffusion of the 5-HT into the platelet and not primarily associated with the active transport due to the high dose. The fluorescence intensity of the lipophilic label DPHT showed an initial increase in its fluorescence followed by a gradual decrease (Figure 6.4b). The initial increase was due to the label partitioning into the lipid of the platelet membrane. The fluorescence of this label was higher in hydrophobic solvents than in water. The gradual decrease was then caused by the redistribution of the label into the intracellular component of the platelet. Other compounds which had no platelet activity including riboflavin, folic acid and SA showed no change in their fluorescence when added to the platelet suspension. It is therefore likely that the platelet can be considered to be composed of two compartments. In one compartment the

fluorescence from a ligand cannot be detected thus accounting for the response seen by prazosin and 5-HT. In the other compartment the fluorescence can be observed and corresponds to that of DPHT. This may correspond to two different parts of the membrane or to the membrane and intracellular components. For DPD the initial sharp decrease in the fluorescence intensity was therefore thought to be due to the partition of DPD into the platelet which caused a quenching of the fluorescence. This step was then followed by the DPD binding possibly to the phosphodiesterase on the inside of the platelet membrane which causes the fluorescence to increase. It is unlikely that the fluorescence intensity of DPD changed because of a change in its environment, because it is very fluorescent both in hydrophilic and hydrophobic solvents. This also helps to confirm that the primary site is associated with the membrane because no change in the fluorescence intensity was observed.

Competitive studies were conducted using this method. Adenosine, 5-HT, and c-AMP did not cause any change in the two phase uptake of DPD. Competition with a number of phosphodiesterase inhibitors was also investigated. Caffeine, theophylline, aminophylline, and prazosin did not effect this response. No competition was observed between DPD and ASA, indomethacin, warfarin, heparin, or ticlopidine. Activation of the platelet by ADP did not effect the uptake of DPD. All such evidence tends to support the theory that DPD, in terms of secondary binding, does not bind to the surface of the membrane, but requires to partition into the platelet.

Fibrinogen inhibited the changes in the fluorescence of DPD when added prior to DPD (Figure 6.5). This effect was not dependent on the presence of ADP to mobilise the fibrinogen receptor. It has been previously shown that DPD does not bind directly to fibrinogen. It was not envisaged that DPD was competing directly for the fibrinogen binding site because the response was not dependent on ADP. This may be due to a change in the platelet surface charge.

When prostacyclin was added prior to DPD the decrease in the fluorescence response was inhibited (Figure 6.6). When it was added after the DPD had equilibrated with the platelet suspension, the DPD was not displaced. If a further dose was given the decrease in the fluorescence was inhibited. Again it was not envisaged that DPD was competing directly for the prostcyclin receptor, but that the change was due to a change in the membrane caused by the prostacyclin. If as postulated above the primary change in the fluorescence intensity of DPD was due to the partition of the DPD into the platelet, prostacyclin may be changing the permeability of the membrane. Packham (1983) noted that prostacyclin inhibited the exposure of receptors for activated coagulation factors on the surface of stimulated platelets, thus indicating a membrane effect of prostacyclin. Prostacyclin does not effect the uptake of prazosin so this indicates that the uptake of DPD is more complex than prazosin.

A model was created using an octan-1-ol/water emulsion to study in isolation the effect of partition on the fluorescence of DPD. Uptake of DPD into the octan-1-ol could be observed and followed a first order relationship (Figure 6.8). This uptake corresponded more closely to the uptake of prazosin into the platelet than that of DPD.

The rate constants for the uptake of prazosin and 5-HT into the platelet and that of DPD into the emulsion were in the same order. Again this indicated that the uptake of DPD into the platelet is more complex than simple partition of the drug into the platelet.

It has been reported by Subbarao et al. (1977a) and confirmed by centrifuging, that heat treated platelets do not bind DPD. Using the direct fluorescence methods no uptake of DPD was observed either with heat treated platelets or fixed platelets. No change in the fluorescence polarisation of DPD was observed in platelet suspensions so treated.

6.4.2.2 Sephadex batch method

The Sephadex batch method has been applied to the investigation of the binding of DPD to washed platelets. With the direct fluorescence methods no change in the intrinsic fluorescence of DPD was observed below 1μM of DPD. This may indicate that the fluorescence of DPD does not change on binding to washed platelets or that DPD was not binding. The results obtained from the centrifuging method may have resulted from damaged caused to the platelets while centrifuging. Using the Sephadex batch method, in the concentration range of 0.1 to 1 μM DPD, binding of DPD to the washed platelets was observed. Figure 6.9 is a Rosenthal plot for this binding interaction. A satisfactory fit was given to a one site model with partition and a twosite model. Using the F test of Boxenbaum et al. (1974) the residual sum of the squares was not sufficiently reduced to justify the additional parameter in the two site model. A primary affinity constant of 3.475×10^7 M^{-1} se 1.37×10^7 was evaluated with kpp=0.367 se 0.163. It was observed in the course of such

studies that the fraction bound was higher after the first washing step than after the second washing. This can be explained simply by the presence of plasma proteins which are removed after the second washing step. Because this method is looking at the total binding of DPD and not specifically the binding to the platelet this method was able to see a difference after a second washing.

6.4.2.3 Fluorescence polarisation

It has already been observed that the fluorescence polarisation of DPD was markedly increased on binding to AGP. Similar binding studies have been conducted here but with the additional problem of making the fluorescence polarisation measurements in a suspension.

Using a concentration range of 5×10^{-8} to 10^{-6} M DPD, the binding to washed platelets was investigated using fluorescence polarisation. Over this concentration range using a platelet suspension of between 100000-1000000 platelets per mm^3, prepared by both gel filtration and the prostacyclin method, no change was observed in the fluorescence polarisation of the DPD using an excitation wavelength of between 220-470nm and an emission wavelength of 490nm. Prostacyclin did not alter this response or activation of the platelets with ADP. This could be due to four reasons:-

1) DPD does not bind to the platelet. It has previously been shown by the difference method that DPD does bind to the platelet in confirmation of the results obtained by Subbarao et al. (1975 and 1977a). The conclusion of Scandurra et al. (1974), that because there is no change in the fluorescence polarisation the compound was not binding was not accepted.

2) The scatter caused by the platelet suspension destroys the

polarisation of the DPD on binding. Teale (1969) in his paper discussed the fluorescence depolarisation by light scattering in turbid solutions. He reported that scatter would depolarise fluorescence therefore there exists a possibility that the scatter caused by the platelets may destroy the polarisation of the DPD. But Fleischer et al. (1980) and Mely-Goubert et al. (1979) observed changes in the fluorescence polarisation of labelled platelets. In this study it was observed that the fluorescence polarisation of NCMIA increases on binding to the platelet. Also when AGP was added to the washed platelet suspension, the polarisation of DPD increased. Fleischer et al. (1980) stated that secondary scatter caused a reduction in the observed fluorescence polarisation. The reason why no polarisation of DPD was observed may have been a combination of the depolarising effect of secondary scatter and the low capacity of the DPD for the platelet.

3) When DPD binds to the platelet its freedom of movement is not grossly changed therefore no polarisation was observed. The high affinity constant of DPD for the primary site of the platelet would make this seem unlikely.

4) The DPD binds with quenching to the platelet therefore no fluorescence polarisation could be measured. Experiments conducted with direct methods showed that DPD did not bind with quenching at the primary site. This therefore does not explain why no fluorescence polarisation of DPD was observed on binding to platelets.

6.4.3 The effect of platelet ageing on binding

It was observed that platelets washed by the method of Moncada took several hours after the last washing stage, before they would aggregate to ADP. The platelets would then aggregate for a considerable period sometimes up to 12 hours later. An experiment was therefore conducted to investigate how the binding and uptake varied with the time after the last washing step. The blank fluorescence of the washed platelet suspension changed considerably over a period of 72 hours (Figure 6.7). Several distinct peaks appeared in its excitation spectrum when an emission wavelength of 490nm was employed. A fluorescence peak with an excitation maximum of 405nm and emission maximum of 460nm developed with the ageing of the platelets. A peak with a maximum of 360nm excitation and 490nm emission also formed but this had a much lower intensity than the former one.

Experiments were undertaken to try and identify the peaks in the fluorescence spectrum of the blank. The peak at 295nm was due to protein and 5-HT. An emission maximum was observed at 335-340nm with this excitation wavelength. Decreasing the pH of the supernatant by addition of 10M HCl caused an additional emission maximum at approximately 550nm to appear. This is characteristic of 5-HT due to excited state protonation. A major decomposition product of 5-HT is 5- hydroxyindole acetic acid. This compounds is also fluorescent but does not account for the 360, 405, or 460nm excitation peaks. Because of the high excitation wavelengths the fluorescence of the blank was compared with some of the B vitamins but none of the fluorescence spectra of these compounds corresponded to any of the peaks in the blank. The platelet blank may contain significant concentrations of

adenine nucleotides but none of these compounds were fluorescent at pH 7.4. The addition of albumin to the final resuspension buffer reduced the rate of appearance of the 405nm peak. The addition of perchloric acid caused a shift in the 405nm peak to 370nm excitation and 425nm emission. The absorbance spectrum showed peaks at 235, 275 and 415nm. An attempt was made to correlate the appearance and growth of the 405, 460nm peak with any changes in the uptake of the platelet suspension. It was noted that with ageing the change in the fluorescence of DPD with time increased. Also an effect was given at 2×10^{-6} M DPD which was only seen at ten times this concentration previously. At this concentration the primary binding site would be saturated. It was therefore unlikely that the response was due to binding at the primary site. No correlation was seen between the appearance of the 405, 460nm peak and increase in uptake. The above would suggest that the deterioration of the platelet surface would appear to favour DPD's diffusion into the platelet. Hence it is important to standardise the timing of binding experiments to ensure that the platelets are all in approximately the same condition. All experiments were conducted between two to five hours of the last washing step.

6.4.4 Binding of DPD to washed platelet membranes

In order to characterise the binding site further, binding studies were conducted to a platelet membrane suspension. It is possible that DPD is binding to a membrane component or to intracellular components. By using a difference centrifuging method the binding of DPD to the platelet membrane was demonstrated giving a $k = 8.1 \times 10^{6} M^{-1}$ se 1.87×10^{6} and Kpp = 0.207 (Figure 6.10). Barber and Jamieson (1970), Amer et al. (1973) and Asano (1977) found

at least two pools of c-AMP phosphodiesterases in the platelet, one soluble and one membrane bound. The DPD may therefore be binding to the membrane bound phosphodiesterase. When 10^{-5} M c-AMP was added to the system, before or after DPD, the binding of DPD was not inhibited. This may indicate that the DPD must first penetrate the membrane before binding. Since c-AMP cannot penetrate the membrane, it showed no competition with DPD.

Using direct fluorescence measurements and fluorescence polarisation the binding of DPD to the soluble platelet fractions in the supernatant of the platelet lysate was investigated. No evidence was obtained to suggest that DPD was binding to any of the components of the supernatant. The intrinsic fluorescence of DPD did not change at any point in its excitation or emission spectra. A high polarisation value of DPD was observed when the supernatant was titrated with DPD. Using an excitation wavelength of 420nm and an emission wavelength of 490nm. This value did not change over the concentration range 10^{-7}M to 5×10^{-6} M. When the supernatant was diluted the fluorescence polarisation decreased but did not change when titrated with DPD over the same concentration range. The high polarisation was thought to be due to scatter and not to indicate binding.

6.4.5 Binding of DPD to PRP

It proved impossible using direct fluorescence techniques to measure the binding of DPD to platelets in PRP because only a small fraction of DPD was bound together with a high fluorescent blank. The capacity of the albumin and AGP masked the binding of DPD to the platelet.

6.4.6 DPD as an inhibitor of phosphodiesterase

It has been widely reported that DPD inhibits platelet aggregation by inhibiting c-AMP phosphodiesterase in the platelet (Mills and Smith, 1971; Moncada and Korbut, 1978; Best et al., 1979). But recent studies (Tsien et al., 1981; Lam et al., 1982) have shown that there is lack of correlation between the inhibition of c-AMP levels and platelet aggregation.

Using the difference and direct fluorescence methods competitive binding studies between c-AMP and DPD have shown that c-AMP does not inhibit the binding of DPD even when present in a vast excess (10^{-4} M). This suggests that the DPD must first pass into the platelet membrane before binding to the phosphodiesterase. The charge on the c-AMP would inhibit the transport of c-AMP across the membrane and thus no competition would be seen with DPD. No inhibition of the binding of DPD would therefore be observed with c-AMP because it is not in the correct position. It is envisaged that the phosphodiesterase is bound within the platelet membrane. Amer and Mayol (1973) characterised the phosphodiesterases into two c-AMP and one c-GMP phosphodiesterases in human platelets. The c-AMP phosphodiesterase with the lower Km was found to be associated with the platelet membrane and may play the more significant role in controlling the intracellular c-AMP levels in the platelet. The other c-AMP phosphodiesterase was found to be a soluble form. Amer and Mayol (1973) also stated that collagen exposed the membrane bound phosphodiesterase. Stimulation of the platelets with collagen did not increase the binding of DPD.

Using direct fluorescence and fluorescence polarisation methods the interaction between bovine heart phosphodiesterase and DPD was investigated. The fluorescence polarisation of DPD was not increased in the presence of the phosphodiesterase. No change in the intrinsic fluorescence of DPD or in the intrinsic fluorescence of the phosphodiesterase was seen when a 1mg/ml solution of phosphodiesterase was titrated with DPD. This suggests that when DPD binds to the phosphodiesterase its freedom of movement is not grossly changed or its microenvironment. It is likely that DPD does not bind near any of the tryptophan residues of of the phosphodiesterase.

6.4.7 Effect of AGP on the binding of DPD to washed platelets

AGP reduces the binding of DPD to the washed platelets when added before or after the DPD (Figure 6.11). It was envisaged that AGP was causing a redistribution of the DPD and not inhibiting the binding by competitive inhibition. This point was proved by fluorescence polarisation measurements. The fluorescence polarisation of DPD was markedly increased by the addition of AGP to the platelet supension. It could also be shown that the fluorescence polarisation changed when a platelet AGP mixture was titrated with DPD, indicating that the DPD was bound to the AGP.

Tripodi et al. (1971) showed that AGP was either bound to the platelet membrane or it was a integral part of the platelet membrane. Steel et al. (1982) also noted that the platelet membrane contains AGP and suggested that vincristine was binding to the glycoprotein on the platelet membrane. It has already been observed that DPD binds to AGP. It was therefore possible that DPD was binding to AGP on the platelet membrane and not directly to the platelet. The primary

affinity constant for the binding of DPD to the platelets is higher than the binding of DPD to AGP. No polarisation of DPD was seen on binding to the platelets. This has already been discussed and may not necessarily indicate that DPD does not bind without a change in the polarisation. It was not possible to observe any change in the fluorescence intensity of the protein peak on binding, due to the excess of non-binding proteins. Competitive studies have shown that the platelet sites have different characteristics to that of AGP e.g. propranolol and prazosin do not compete for the DPD on the platelet. It must therefore be concluded that the AGP and platelet site for DPD are different. If DPD is binding to AGP bound to the platelet, the capacity of this site is too low to be detected with these methods. Tripodi et al. (1971) only resuspended plaletets in a buffer and did not go on to wash them. It is therefore possible that the AGP detected was carried over from the plasma or was associated with the platelet atmosphere (Bang et al., 1972). In these studies where the platelets were washed three times, it is likely that this AGP was removed. Although the affinity constant of the primary site on the platelet is higher than that of the AGP site the high capacity of the AGP site reduces the binding to the platelet.

Costello et al. (1979) showed that AGP and desialised AGP in the concentration range 5-15mg/ml inhibited platelet aggregation to ADP and collagen. Desialised AGP was more potent than AGP. In this investigation a study was made of the inhibitory effect of AGP on platelet aggregation in the concentration range 0.01 - 1 mg/ml. Over this concentration range AGP, did not inhibit the aggregation of washed rabbit platelets, to ADP. This indicates that at an *in vivo* concentration, AGP does not directly inhibit platelet aggregation.

6.4.8 Assay of c-AMP and c-GMP

c-AMP and c-GMP levels in washed platelets were determined to try and correlate the binding of DPD to the platelet with increases in c-AMP and c-GMP.

c-AMP levels were determined in PRP with DPD concentrations between 1-200 μM with and without stimulation of adenyl cyclase by prostacyclin. Table 6.1 shows the results of these investigations. DPD without the stimulation of adenyl cyclase did not increase c-AMP. This is probably due to the low basal activity of adenyl cyclase and phosphodiesterase in unstimulated platelets. This also indicates that DPD in the concentration range of 1-200μM does not stimulate adenyl cyclase. Prostacyclin increased the c-AMP levels of the platelet by the stimulation of adenyl cyclase. When 100-200μM DPD was added in combination with prostacyclin a potentiation of the increase in the c-AMP levels was observed.

c-GMP was assayed in PRP using 2mM of arachidonic acid (AA) to stimulate c-GMP synthesis (Best et al., 1979), using the same concentration range of DPD. The results are shown in Table 6.2. DPD did not increase the c-GMP levels. No effect was observed with AA or DPD and AA. This may have been because the AA was bound to the albumin in plasma.

In a washed platelet suspension when the DPD concentration was below 0.5 μM it was envisaged from the binding experiments that the majority of the DPD bound would be bound to the primary site. No change in c-AMP concentrations were observed at these concentrations. Stimulation of the adenyl cyclase with 0.5 μM prostacyclin showed an

increase in c-AMP levels and these levels were not potentiated with DPD (Table 6.3). Using a DPD concentration above 1 μM, where the primary site would be saturated and so the majority of the DPD would be bound to the secondary site, no change in the c-AMP levels of the platelets were observed. A combination of prostacyclin and DPD potentiated the c-AMP levels. Again these results indicate that DPD does not stimulate adenyl cyclase in the concentration range used. The stimulation of adenyl cyclase by prostacyclin increased the c-AMP levels of the platelet. In combination with DPD the break down of c-AMP was inhibited by the inhibition of the phosphodiesterase which leads to a further increase in the c-AMP platelet levels. These results suggest that phosphodiesterase inhibition is not dependent on the primary class of binding sites but on the secondary class.

Titrating a washed platelet suspension with adenosine increased the c-AMP levels of the platelet. This has been reported to be due to stimulation of adenyl cyclase (Haslam and Rosson, 1975). The addition of 200μM DPD potentiated this increase. Adenosine is a less active stimulator of adenyl cyclase than prostacyclin but an increase in c-AMP was still observed. This helps to confirm that DPD does not stimulate adenyl cyclase activity. The potentiation of c-AMP levels shows that DPD does not compete with adenosine for the adenyl cyclase site.

In contrast to DPD, papaverine increased the platelet c-AMP levels of a washed platelet suspension without stimulation of adenyl cyclase. When 1-100μM papaverine was added in combination with prostacyclin a potentiation of c-AMP levels were observed (Table 6.4).

6.4.9 Uptake of adenosine

The uptake of 10^{-7}M adenosine was inhibited by DPD over the concentration range 10^{-9} -10^{-6} M. Prostacyclin (300ng/ml) had no effect on the uptake of adenosine and did not potentiate or inhibit the effect of DPD. Figure 6.12 shows how DPD effects the uptake of adenosine into the platelet. It can clearly be seen that the inhibition of adenosine uptake was dependent on the concentration of DPD. RA 233, RA 433 and papaverine also inhibited the uptake of adenosine. Table 6.5 compares the inhibitory effect of these compounds. It is envisaged that the adenosine binding site responsible for stimulation of adenyl cyclase and the adenosine transport mechanism are independent because DPD inhibits the uptake of adenosine without effecting the adenosine action of stimulating adenyl cyclase. In agreement with the finding of Subbarao et al. (1977a and 1977b), it was found that adenosine does not inhibit the binding of DPD. This suggests that DPD does not inhibit adenosine uptake by competitive inhibition but indicates that the two sites are distinct. But the inhibition of adenosine uptake was dependent on DPD concentration. DPD did not totally block the uptake of adenosine into the platelet even at high DPD concentrations. This can be explained by the observation of Sixma et al. (1976) who showed that platelets contained two different transport systems with a high and a low Km. DPD is inhibiting the low Km transport system which is most important at low adenosine levels. Formaldehyde-fixed platelets did not totally block the uptake of adenosine. The high Km adenosine transport system may have only been diffusion of adenosine into the platelet and therefore not effected by fixing. Using 10μM DPD and titrating the platelet suspension with adenosine showed that DPD did not totally

inhibit the uptake of adenosine. The inhibition of adenosine uptake was greater at low concentrations of adenosine. This therefore demonstrates the two uptake sites of adenosine.

6.4.10 Aggregatory studies

Having established that DPD binds to washed rabbit platelets and that this binding did not directly effect c-AMP levels in the platelet, anti-aggregatory studies were conducted to try and correlate binding with the inhibition of aggregation.

PRP was titrated with ADP in order to find a concentration which caused submaximal aggregation. The concentration of drug to cause 50% inhibition of aggregation was then determined (Table 6.6).

Aggregatory studies were also conducted with washed platelets. The platelets were left for two hours after the final washing step to allow the prostacyclin to hydrolyse. ADP (5×10^{-6}M) was used to aggregate the platelets. This concentration was found to be a submaximal does. Figure 6.13 shows a graph of the percentage inhibition against the log (DPD concentration). It was found that 7.1×10^{-7} M DPD was required to inhibit aggregation by 50%. This was higher than the value quoted by Niewarowski et al. (1975) and less than that quoted by Subbarao et al. (1977b) and Cucuianu et al. (1971). Table 6.6 shows the concentration of inhibitors required to inhibit aggregation induced by ADP, by 50% in PRP and washed platelets to ADP.

Figure 6.14 shows the correlation of anti-aggregatory activity of DPD with the binding to the primary and secondary sites. Subbarao et al. (1975), working with human and rabbit platelets stated that DPD

binding correlated with the inhibition of ADP induced aggregation. Looking at the primary binding site only, in this study correlation with the binding of DPD to this site and inhibition of aggregation was not observed.

The inhibition of ADP aggregation of platelets by DPD was potentiated by prostacyclin. This has been explained by Moncada and Korbut (1978), who stated that prostacyclin stimulated adenyl cyclase thus increasing platelet c-AMP, this increase being potentiated by DPD due to its inhibitory action on phosphodiesterase activity. This suggests that the inhibition of aggregation involves c-AMP though this may not account for all its activity. Thus one can conclude, as far as the anti-aggregatory activity of DPD to ADP is concerned in vitro, that secondary site binding is involved with inhibition of platelet phosphodiesterase. This is in agreement with other workers observations (Lam et al., 1982).

The clinical levels of DPD in vivo are lower than that necessary to inhibit platelet aggregation in vitro. This study has shown that in a washed platelet suspension the inhibition of aggregation by adenosine is potentiated by DPD. It has been shown by a number of workers that DPD increases blood levels of adenosine by the inhibition of the uptake of adenosine into red blood cells (Roos and Pfleger, 1972). Sollevi (1982) demonstrated a doubling of the basal plasma levels of adenosine by 2.5 μM DPD in 15-60 minutes. This inhibition of adenosine uptake occurs at clinically active concentrations. In vivo, because of the high affinity of DPD for AGP, very little DPD will have the opportunity to bind to the secondary class of sites on the platelet. DPD will therefore have very little inhibitory effect

on the platelet phosphodiesterase. A possible explanation of the mode of action of DPD is through the increased plasma levels of adenosine which will stimulate adenyl cyclase.

Collagen and thrombin induced platelet aggregation was also inhibited by DPD. The mechanism of collagen and thrombin induced aggregation is more complex than that of ADP. Packham (1983) showed that it involved the formation of thromboxane A_2, a potent aggregatory agent, followed by the release of ADP. In a washed platelet suspension, with a physiological concentration of ionised calcium, thromboxane A_2 is not released during ADP induced aggregation. ADP aggregation was therefore employed routinely to simplify the system, so that the aggregation was solely due to ADP.

6.4.11 Competitive binding between DPD and other exogenous ligands

6.4.11.1 Prazosin

Using the centrifuging difference method the binding and uptake of prazosin to washed platelets was studied using an excitation wavelength of 350nm and an emission wavelength of 400nm. Figure 6.15 is a Rosenthal plot for the binding of prazosin to platelets. The data fitted a partition model and not a classical one or two binding site model. This indicates that the "binding" of prazosin, in contrast to DPD, is nonspecific. 2.5×10^{-6} M AGP reduced the "binding" of prazosin into the platelet. Competitive binding studies with DPD showed that prazosin did not compete for the primary or secondary binding sites.

A marked difference exist between the binding capacity ot the DPD binding sites and the uptake of prazosin. The partition coefficients of prazosin between octan-1-ol and a phosphate buffer pH 7.4, was greater than that of DPD. The uptake of prazosin into the platelet did not inhibit the aggregation in the concentration range 10^{-7} to 10^{-5}M. In both PRP and a washed platelet suspension prazosin did not inhibit platelet aggregation to ADP. It has been reported by Moore (1982) that prazosin inhibits phosphodiesterase. The phosphodiesterase inhibitors papaverine, RA 233, RA 433 SH 1242 and caffeine did not compete with prazosin. Prazosin did not increase the basal levels of c-GMP or c-AMP in washed platelets or PRP using a concentration range of 10^{-7} to 10^{-5} M. No potentiation of c-AMP levels were observed when the platelets were stimulated with prostacyclin or in the c-GMP when stimulated with arachidonic acid.

The evidence indicates that the uptake of prazosin into the platelet is nonspecific and not related to any specific biological activity. The higher partition coefficient of prazosin between water and octan-1-ol than DPD may account for the higher uptake of prazosin.

Using the direct method the uptake of prazosin was followed with time. From the addition of prazosin where the fluorescence intensity was a maximum a gradual decrease of the fluorescence was seen. The results were analysed according to zero and first order kinetics. A satisfactory fit was given to a first order model (Figure 6.16). The uptake was dependent on the initial concentration of the prazosin and on the number of platelets. Using this method DPD did not effect the uptake of prazosin (Figure 6.17) and prazosin did not effect the binding of DPD. Papaverine had no effect on the uptake of prazosin

(Figure 6.18). A similar pattern was observed with the uptake of a high concentration of 5-HT. The uptake of prazosin with this method was more reproducible than the similar work with DPD. No batch of platelets investigated failed to show uptake of prazosin. Batches of platelets which did not respond to DPD responded to prazosin. The work of Kerry et al. (1983) demonstrated the binding of prazosin to platelets at very low concentration (fM). The concentration range used in this study is in the micro mole region. It is likely that this study is only looking at partition or diffusion of the prazosin into the platelet.

Platelets which had been fixed with formaldehyde still showed uptake of prazosin. This suggests that prazosin's uptake did not involve active transport but was a simple partition of prazosin into the platelet. The uptake of prazosin is in marked contrast to that of DPD. This suggests that prazosin simply partitions or diffuses into the platelet and is not dependent on the condition of the platelets. DPD is much more sensitive to the condition of the platelets and is easily blocked. The block of the binding of DPD by formaldehyde shows that the membrane binding is involved but it is not thought that DPD is actively taken up into the platelet because of the low capacity. DPD appears to have difficulty in crossing the membrane when compared with prazosin or DPHT.

6.4.11.2 Papaverine

The direct binding of papaverine to washed platelets was not investigated because the minimum detectable concentration of papaverine was too high. Competitive studies between DPD and papaverine have been conducted using both the direct method and the

difference centrifuging method.

As shown in Table 6.6 papaverine is a more active inhibitor of platelet aggregation in PRP than DPD. This can be accounted for quite simply by the binding of DPD to AGP in PRP. In a washed platelet suspension DPD is more active than papaverine. It has been reported by Lam et al. (1982) that two pools of c-AMP are present in the platelet, one associated with the membrane and the other in the intracellular region of the platelet. Two c-AMP phosphodiesterases have also been identified, a membrane bound low Km and soluble high Km phosphodiesterase (Mill and Smith, 1971; Amer and Mayol, 1973). Amer and Mayol (1973) suggested that the low Km membrane bound form was the most important form in the control of intracellular c-AMP levels. DPD is more efficient at inhibiting the low Km phosphodiesterase while papaverine preferentially inhibits the high Km form. The difference in the activity of these two compounds may be explained by inhibition of different phosphodiesterases. But at a concentration which DPD or papaverine inhibits platelet aggregation no change in the platelet c-AMP levels were observed. This together with the fact that prazosin, a phosphodiesterase inhibitor, does not inhibit platelet aggregation suggests an additional mechanism of action. DPD is a potent inhibitor of adenosine uptake while papaverine is markedly less active. This is likely to be more important in vivo than in vitro since adenosine originates in vivo from other tissues.

It has been shown that 10^{-4} M papaverine significantly decreased the binding of DPD to a washed platelet suspension using the centrifuging difference method Figure 6.19. The binding at the primary site was blocked. This confirms that the direct method is

only looking at the secondary sites because papaverine had no effect with this method.

6.4.11.3 RA 233 and RA 433

The binding of RA 233 was demonstrated by the difference method. Figure 6.20 is a Rosenthal plot for the binding of RA 233 to washed platelets. A primary affinity constant of 1.9×10^{6} M^{-1} se 0.727 was evaluated. No change in the fluorescence polarisation of RA 233 was observed on binding. Direct fluorescence studies showed that RA 233 behaved like DPD. A marked uptake like that seen with prazosin was not observed.

Competitive studies resulted in similar problems as the competitive studies with AGP.

The binding of RA 433 to platelets was shown using the difference centrifuging method giving a primary affinity constant of 2.6×10^{6} M^{-1} se 0.759. Figure 6.21 is a Rosenthal plot for this binding. No change in the fluorescence polarisation was observed. Direct fluorescence showed that it behaved similar to DPD.

RA 233 and RA 433 are more active than DPD at inhibiting platelet aggregation in PRP but less active in a washed platelet suspension. This can simply be explained, as in the case of papaverine, by the difference in the binding of these compounds to AGP as already demonstrated. No potentiation of the inhibition of aggregation was observed when these compounds were added together suggesting that they are working by the same mechanism.

6.4.11.4 DPQ

Using the difference method the binding of DPQ to platelets has been demonstrated. Figure 6.22 is a Rosenthal plot for this binding. Using nonlinear regression analysis an affinity constant of $2.8 \times 10^{5} M^{-1}$ se 0.133 was evaluated. This binding was not as specific as that of DPD because of the high capacity of this site.

6.4.11.5 Warfarin

It has been shown that the fluorescence intensity of the anticoagulant warfarin is enhanced on binding to serum albumin with an increase in its fluorescence polarisation. In a washed platelet suspension no change in the fluorescence intensity of the warfarin was observed or change in its fluorescence polarisation in the concentration range of 1 to 10μM. No binding was observed using the difference method. Warfarin did not inhibit the binding of DPD to the platelet. Platelet aggregation in PRP and washed platelets were not effected by the presence of warfarin.

6.4.11.6 Salicylic acid and acetyl salicylic acid

In a washed platelet suspension no change in the intrinsic fluorescence of SA was observed or change in its fluorescence polarisation. The difference method showed that SA did not binding to the platelet in the concentration range of 1-10μM SA had no effect on the binding of DPD to washed platelets.

ASA has a low intrinsic fluorescence at pH 7.4. The use of fluorescence to conduct direct binding studies was not possilbe.

6.4.11.7 Indomethacin

Indomethacin has a low fluorescence at pH 7.4 so no direct fluorescence studies could be conducted. The difference method indicated that indomethacin was bound to platelets (Figure 6.23). An affinity constant of 1.65×10^6 M^{-1} was evaluated. No competition was seen between indomethacin and DPD for washed platelets.

6.4.12 Membrane labels

When 10 M diphenyl hexatriene (DPHT) was added to a washed platelet suspension using an excitation wavelength of 360nm and an emission wavelength of 430nm a rapid increase in the fluorescence intensity was observed. This peaked after about one minute and was followed by a gradual reduction in the fluorescence intensity (Figure 6.4b. DPHT has a higher fluorescence in non-polar medium than in a polar medium. It was therefore envisaged that the initial increase in the fluorescence intensity was due to the partition of the probe from the aqueous solution to the lipid of the membrane. This was followed by a redistribution of the probe into the intracellular components causing a reduction in the fluorescence intensity.

DPHT has a high fluorescence polarisation in an aqueous solution. When added to a platelet suspension the fluorescence polarisation is not markedly altered.

The fluorescence intensity of the label was quenched on addition of DPD at fairly high doses. DPHT is a lipophilic label which has been reported to be associated with the platelet membrane lipid (Mely-Goubert et al., 1979). It was therefore envisaged that the binding was fairly nonspecific with a high capacity. It was therefore

unlikely that DPHT was being displaced from the membrane lipid by DPD. The emission spectrum of DPHT overlaps the absorption spectrum of DPD. If the DPD binding site was in close proximity to the DPHT, nonresonance transfer of energy could occur. The quenching of DPHT fluorescence only occurred at relatively high DPD concentrations. No effect was seen with concentrations below 1μM. The quenching was therefore associated with the secondary binding of the DPD.

NCMIA is a covalent label specific to glycoproteins in intact platelets (Fleischer et al., 1980). On binding to the platelet the polarisation was increased from approximately 0 to 0.06. This probe helps to confirm that fluorescence polarisation measurement can be made in the presence of a large degree of scatter and therefore that DPD binds with no change in its fluorescence intensity or fluorescence polarisation. Titration of the labelled platelets with DPD did not effect the fluorescence polarisation of the label. This indicates that DPD does not change the conformation of the label's binding site on the glycoprotein. When the label was added to the washed platelets the fluorescence intensity increases slowly over a period of 30 minutes. This was much slower than the increase in the fluorescence of the noncovalent label DPHT or the decrease seen with 5-HT or prazosin.

Valinomycin does not change the fluorescence intensity of the label. The addition of K^+ had no effect. This indicates that NCMIA is uneffected by membrane potential. Gramicidin had no effect.

In order to observe what effect prostacyclin had on the platelet membrane, prostacyclin was added to labelled platelets. No effect was observed in the fluorescence of the label or in its fluorescence polarisation.

Titration of the NCMIA labelled platelets label with DPD had no effect on the fluorescence intensity of the label at low concentrations of DPD (below 1μM). Above 1μM DPD a reduction of the fluorescence intensity of the label was observed. NCMIA cannot be displaced from the membrane because it is covalently bound. The change in the fluorescence of the label must therefore be interpreted in terms of either a change in the membrane conformation or transfer of energy from the NCMIA to the DPD. During shape change and aggregation of the platelet, Fleischer et al. (1980) reported an increase in the fluorescence intensity of the label.

These two labels have shown that DPD can be located both in the lipophilic parts of the membrane and with the glycoproteins. DPD may therefore bind at the interface between the membrane protein and lipid thus stabilising the membrane in a similar manner to propranolol (Dachary-Prigent, 1979). This may involve cross linking between glycoproteins within the membrane in a similar manner to it's binding to AGP.

6.4.13 Effect of membrane potential on ligand binding

The platelet maintains a large transmembrane gradient of Na^+ (MacIntyre and Rink, 1982) and also has a negative membrane potential (MacIntyre et al., 1978). The effect of this membrane potential and concentration gradient on the binding of DPD and prazosin has been

investigated

Valinomycin adds an additional selective K^+ permeability (Harris et al., 1967). Valinomycin added prior to DPD inhibited the uptake of DPD. Valinomycin added after DPD had equilibrated with the platelets increased the fluorescence response, apparently causing a redistribution of the DPD. Addition of varying concentrations of K^+ to the platelet suspension did not reverse the effect of valinomycin. Gramicidin-D adds an additional Na^+ and K^+ permeability to the membrane by forming a channel. Gramicidin- D had no effect on the uptake or binding of DPD. It did not reverse the effect of valinomycin. Replacing the Na in the buffer by K^+ had no effect on the uptake of DPD. Valinomycin still blocked the uptake in the K^+ buffer.

These results suggest that the binding of DPD is dependent on the membrane potential of the platelet. No effect was seen with the addition of K^+ but this may have been due to experiments being carried out at room temperature and not 37 ^{o} C or above. Valinomycin cannot freely cross the membrane of the platelets below 41 ^{o} C. It is therefore possible the valinomycin K^+ complex altered the membrane charge by remaining in the membrane thus reducing the binding of DPD.

Valinomycin did not inhibit the uptake and binding of prazosin. The addition of K^+ made no difference to the observed uptake. When the Na in the Tyrode's solution was replaced by K^+ the uptake was still observed.

Ouabain inhibits ATPase which controls Na^+ /K^+ exchange. This compound did not effect the binding of DPD to washed platelets or the uptake of prazosin.

6.5 CONCLUSION

Since most drugs bind to a lesser or greater extent to plasma proteins it is essential to have a reliable method for preparing washed platelets from PRP (as discussed in the previous section). A comparison of the various methods available has shown that the method of Moncada et al. (1982) is the most reliable. In this study a fluorescence method has been developed which will differentiate between :-

1) Surface binding

2) Partition or diffusion into the platelet

Thus it is now possible to determine the importance of such parameters in terms of antiaggregatory effects to, for example, ADP.

It has been shown that DPD binds to washed platelets by a number of methods in this study with one class of binding sites and partition (or unsaturable binding). Unlike previous investigations attempts have been made to locate the binding within the platelet and characterise it. Studies conducted with a membrane fraction of platelets have shown that DPD binds with a primary affinity constant in the same order as that of whole platelets and with a secondary class of sites. This therefore demonstrates that DPD is binding to the platelet membrane. In confirmation of this, no evidence was obtained using direct fluorescence, fluorescence polarisation, or the Sephadex batch method, to indicate that DPD was binding to the soluble platelet fraction. DPHT, a lipophilic membrane label, and NCMIA, a

glycoprotein membrane label, were employed to further characterise the binding site. DPD in a concentration above 10μM quenched the fluorescence of DPHT. This therefore indicated that the lipophilic region of the protein was associated with the secondary binding. The fluorescence of the covalent glycoprotein label was quenched above 1μM DPD. This therefore further confirmed that the binding of DPD is associated with the platelet membrane and involves both the glycoprotein and lipophilic regions of the membrane.

The effect of membrane potential on the binding of DPD has been studied. Valinomycin inhibited the binding of DPD when added before or after DPD. The addition of high concentration of K^+ did not effect the binding. The binding of DPD is therefore affected by the charge on or in the membrane. Gramicidin-D had no effect on the binding of DPD. Ouabain, which specifically inhibits the ATPase which controls Na^+ K^+ exchange had no effect on the binding. Fibrinogen and prostacyclin inhibited the binding of DPD. It was thought that this was not specific competition with the fibrinogen and prostacyclin binding sites but a non specific membrane effect.

It has been widely reported that DPD acts as as inhibitor of phosphodiesterase. No competition was observed between c-AMP and DPD for the primary site on the platelet. This suggests that the primary site is not the membrane bound phosphodiesterase. Confirmation of this was found in studies of c-AMP levels in platelets. DPD had no effect on the c-AMP levels of washed platelets but this may be due to the low basal activity of the adenyl cyclase. DPD at high concentrations had no adenyl cyclase activity. When the adenyl cyclase was stimulated by prostacyclin using a concentration of DPD of

less than 10^{-6}M, so the majority of the binding would be to the primary site, no potentiation of c-AMP levels were observed. Potentiation of c-AMP levels occurred when 100μM DPD was used. The phosphodiesterase activity is therefore related to the secondary binding. It is envisaged that DPD must first pass into the membrane because no competition was observed between DPD and c-AMP for the secondary site. The phosphodiesterase must therefore be located within the platelet membrane.

The binding of DPD to the primary site of the platelet did not correlate with the inhibition of platelet aggregation. The inhibition of platelet aggregation occurred at a concentration when the primary site was saturated. These studies suggest that it was the secondary binding sites that are more important in the inhibition of aggregation in Vitro.

In order to identify the primary binding site, a comparison of the primary binding site with AGP's binding site for DPD has been conducted. It has been reported by Tripodi et al. (1971) and Steel et al. (1982) that platelet membranes contain AGP. The possibility exists that DPD could exert its antiaggregatory action through binding to AGP in the membrane. However evidence presented in this study would suggest this is highly unlikely. In particular DPD shows a high polarisation which should be of sufficient magnitude to be measurable in a platelet suspension. No polarisation change was observed.

It has been shown that DPD inhibits the uptake of adenosine at low concentrations. This correlates with the binding at the primary site. No direct competition was observed between adenosine or ADP and DPD. It was envisaged that DPD was only effecting the uptake of

adenosine and had no effect on the adenyl cyclase activity. Evidence for this was obtained from aggregation studies. DPD potentiated the inhibition of aggregation observed by adenosine. The potentiation was similar to that observed with prostacyclin and DPD. This suggests that the mode of action of DPD is a result of two independent actions. At clinically active concentrations adenosine uptake is inhibited by the binding of DPD to the primary class of binding sites. In vivo this will increase the adenosine concentration in plasma, accounting for the vasodilation produced by DPD. The increased adenosine levels will also stimulate the platelet membrane adenyl cyclase thus inhibiting platelet aggregation. This effect at high DPD concentrations will be potentiated by the inhibition of the platelet phosphodiesterase. Further evidence is given to this arguement by the fact that DPD is a more potent inhibitor of platelet aggregation than papaverine. DPD is a less active inhibitor of phosphodiesterase but is many times more potent inhibitor of adenosine uptake than papaverine in PRP and washed platelets. If the phosphodiesterase activity was the only mechanism of action the opposite would be predicted. No significant lag time was observed from the addition of DPD to the onset of the inhibition of aggregation. The theory suggested by Cucuianu et al. (1971) that the effect of inhibiting platelet aggregation was due to a decrease in platelet ATP levels and thus available metabolic energy does not fit in with the above finding.

In addition to the above mode of actions Blass et al. (1980) showed that DPD increased the biosynthesis of prostacyclin. The concentration range of DPD used to demonstrate this was higher than clinical levels. Platelets do not contain any prostacyclin synthetase

so this mode of action will not account for the in vitro activity of DPD. Best et al. (1979) showed that DPD had a dose dependent inhibitory effect on thromboxane synthesis. In the condition of the aggregation experiments carried out in this study with ADP thromboxane A_2 is not involved so it is unlikely that this accounts for the mode of action of DPD.

In terms of anti-aggregatory activity , RA 233 and RA 433 are more potent, in PRP, than DPD because DPD has a high affinity for AGP. In washed platelets DPD is the most active. However, it is envisaged that these compounds have the same mechanism of action.

As a summary Figure 6.24 further expands these biochemical pathways indicating the importance of the platelet membrane.

It has been reported that prazosin inhibits phosphodiesterase (Moore, 1982) but this does not lead to a direct inhibition of platelet aggregation. Here, in contrast to DPD, binding studies with prazosin demonstrated a very marked uptake into platelets which followed first order kinetics. Prazosin had no specific low capacity binding site as far as this study could observe, but was taken up into the platelet to a much greater extent than DPD. The significance of this uptake remains to be elucidated.

TABLE 6.1

A table showing c-AMP levels in PRP and the effect of DPD and prostacyclin on these levels

Concentration DPD µM	Concentration prostacyclin µM	Concentration c-AMP pM
0	0	2.4 se 0.50
0	0.5	5.6 se 0.26
200	0	2.4 se 0.39
200	0.5	11.8 se 0.43
100	0	2.4 se 0.42
100	0.5	8.7 se 0.28
10	0	2.42 se 0.50
10	0.5	5.9 se 0.99
1	0	2.6 se 0.14
1	0.5	5.6 se 0.49

Table 6.2

A table showing the effect of DPD on c-GMP levels in PRP

Concentration DPD µM	Concentration AA mM	Concentration c-GMP pM
0	0	1.6 se 0.7
0	2	1.5 se 0.8
200	0	1.6 se 0.7
200	2	1.6 se 0.3
100	0	1.7 se 0.1
100	2	1.8 se 0.7
10	0	1.7 se 0.3
10	2	1.6 se 0.1
1	0	1.8 se 0.7
1	2	1.6 se 0.7

Table 6.3

The effect of DPD on c-AMP levels in washed platelets.

Concentration DPD µM	Concentration prostacyclin µM	Concentration c-AMP pM
0	0	1.5 se 0.33
0	0.5	2.9 se 0.51
0.1	0	1.4 se 0.27
0.1	0.5	2.7 se 0.72
1	0	1.6 se 0.30
1	0.5	3.2 se 0.47
10	0	1.3 se 0.19
10	0.5	3.1 se 0.41
100	0	1.6 se 0.28
100	0.5	4.6 se 0.45

Table 6.4

A table showing the effect of papaverine on the c-AMP levels of washed platelets before and after stimulation with prostacyclin.

Concentration papaverine μM	Concentration prostacyclin μM	Concentration c-AMP pM	
0	0	0.8	se 0.11
0	0.5	1.9	se 0.09
1	0	0.8	se 0.29
1	0.5	2.3	se 0.19
10	0	0.9	se 0.08
10	0.5	2.4	se 0.13
100	0	1.4	se 0.09
100	0.5	8.4	se 0.36

Table 6.5

A table comparing the percentage inhibition of adenosine uptake of a number of platelet active compounds.

Compound	% Inhibition
0	0
DPD (1μM)	44
RA 233 (1μM)	29
RA 433 (1μM)	30
papaverine (10μM)	15

TABLE 6.6

A table comparing the concentration of a number of platelet active compounds which inhibit aggregation by 50% in washed platelets and PRP.

Compound	Washed platelets μM	PRP μM
DPD	0.71	190
prazosin	———	———
RA 233	1.00	3.80
RA 433	1.40	3.40
SH 1242	0.79	0.750
papaverine	4.50	27.0
propranolol	———	———

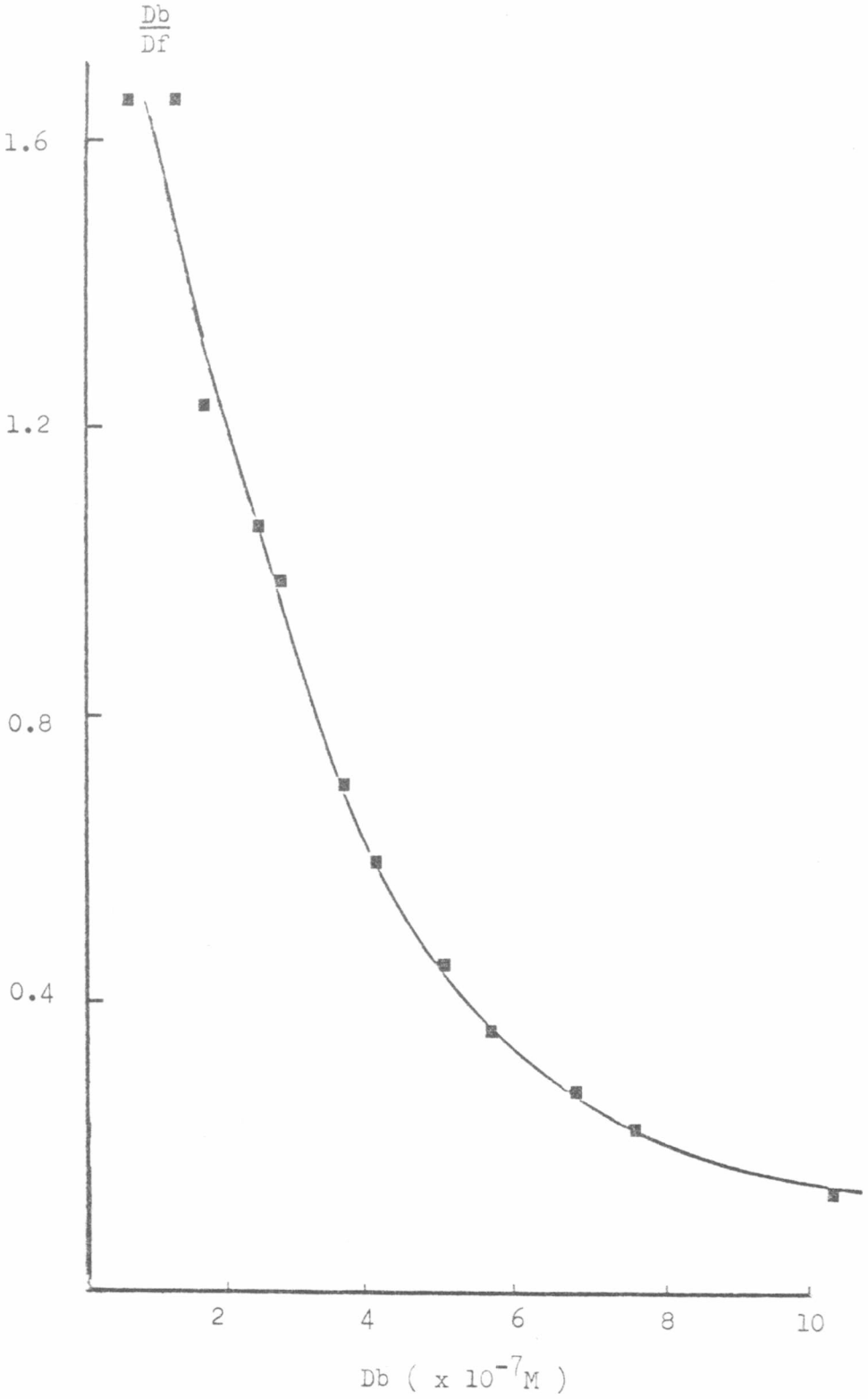


Figure 6.1

A Rosenthal plot for the binding of DPD to a washed platelet suspension (500000/mm ^{3}) prepared by Moncada's method.

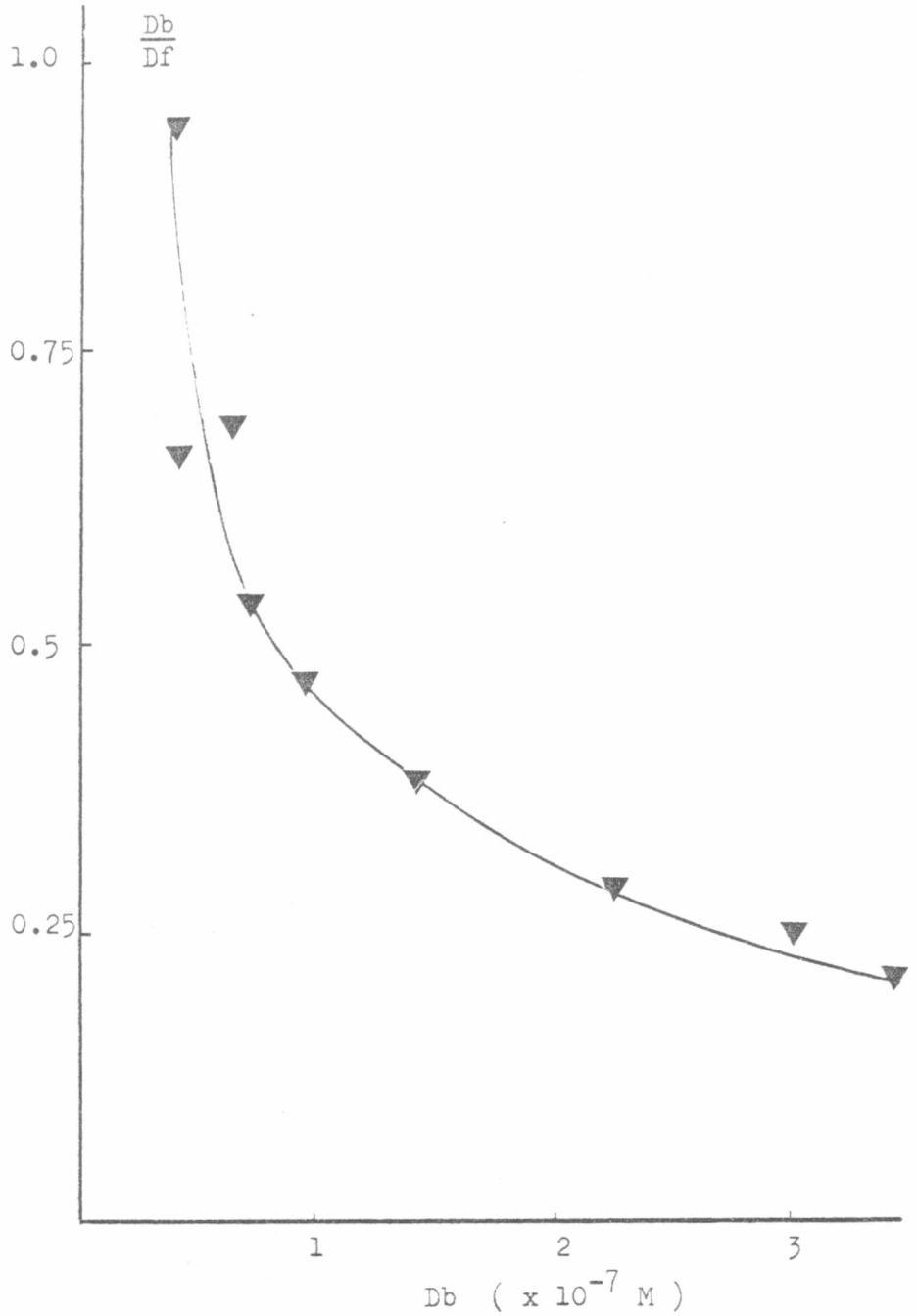


Figure 6.2

A Rosenthal plot for the binding of DPD to a washed platelet suspension (150000/mm ^{3}) prepared by gel filtration.

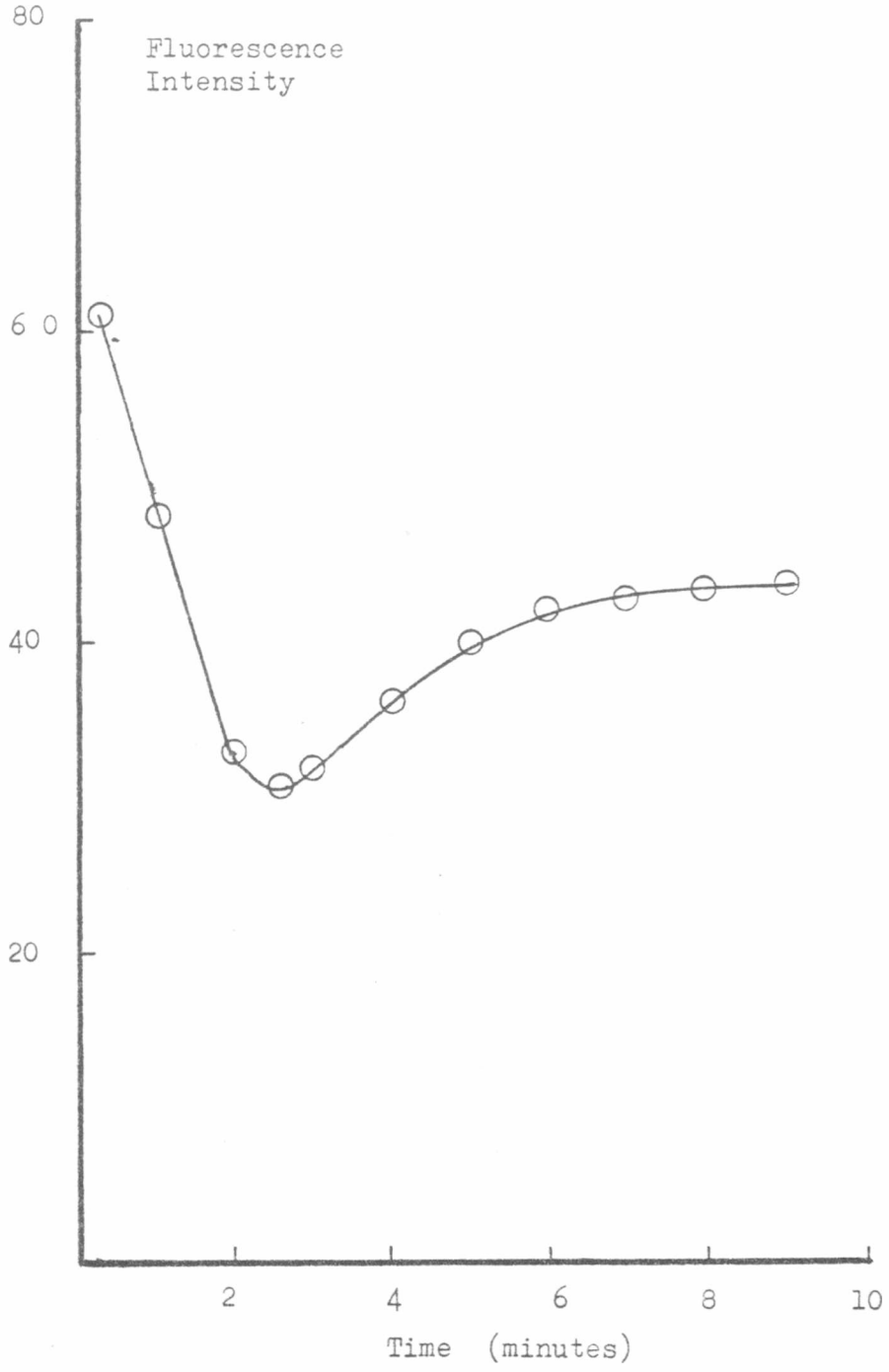


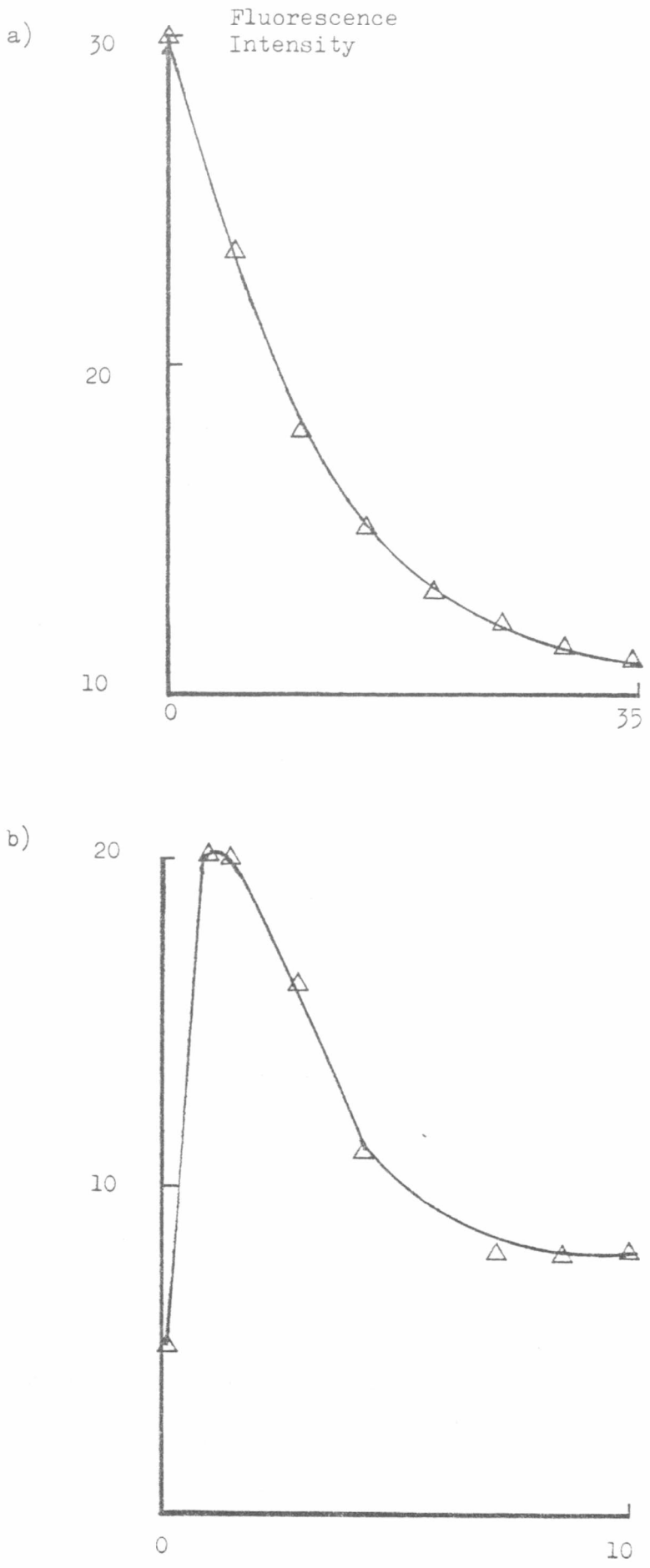
Figure 6.3

A graph showing the change in the fluorescence intensity of 10^{-5}M DPD with time in a platelet suspension washed by the method of Moncada.

Figure 6.4

a) A graph showing the change in the fluorescence intensity of 10μM 5-HT with time in a washed platelet suspension.

b) A graph showing the change in the fluorescence intensity of 1μM DPHT with time in a washed platelet suspension.


a)
Fluorescence
Intensity
30
20
10
0
35
b)
20
10
0
10
Time (minutes)

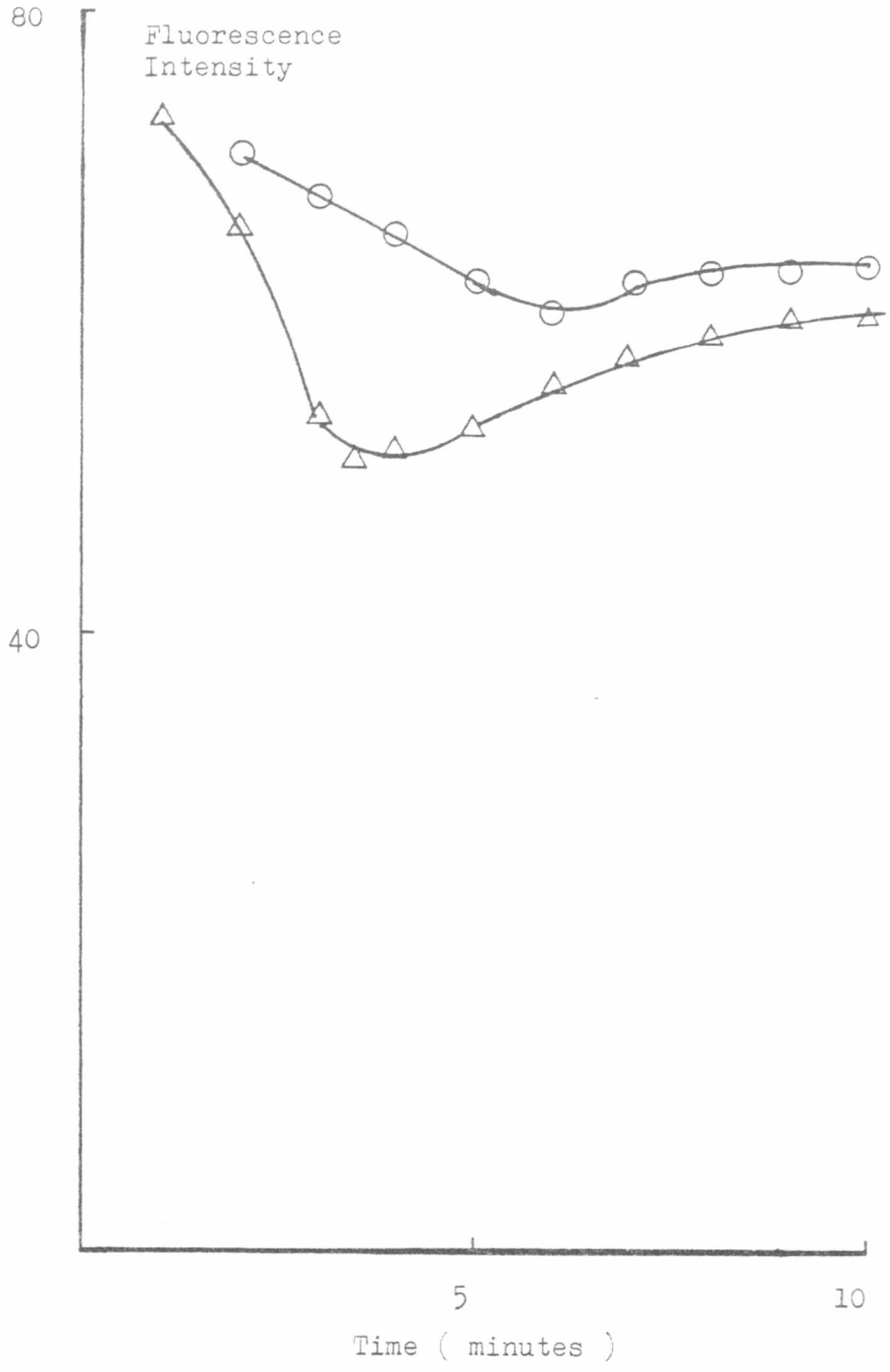


Figure 6.5

A graph showing the effect of fibrinogen (0.5%) on the uptake of DPD into a washed platelet suspension

△ no fibrinogen ○ 0.5% fibrinogen

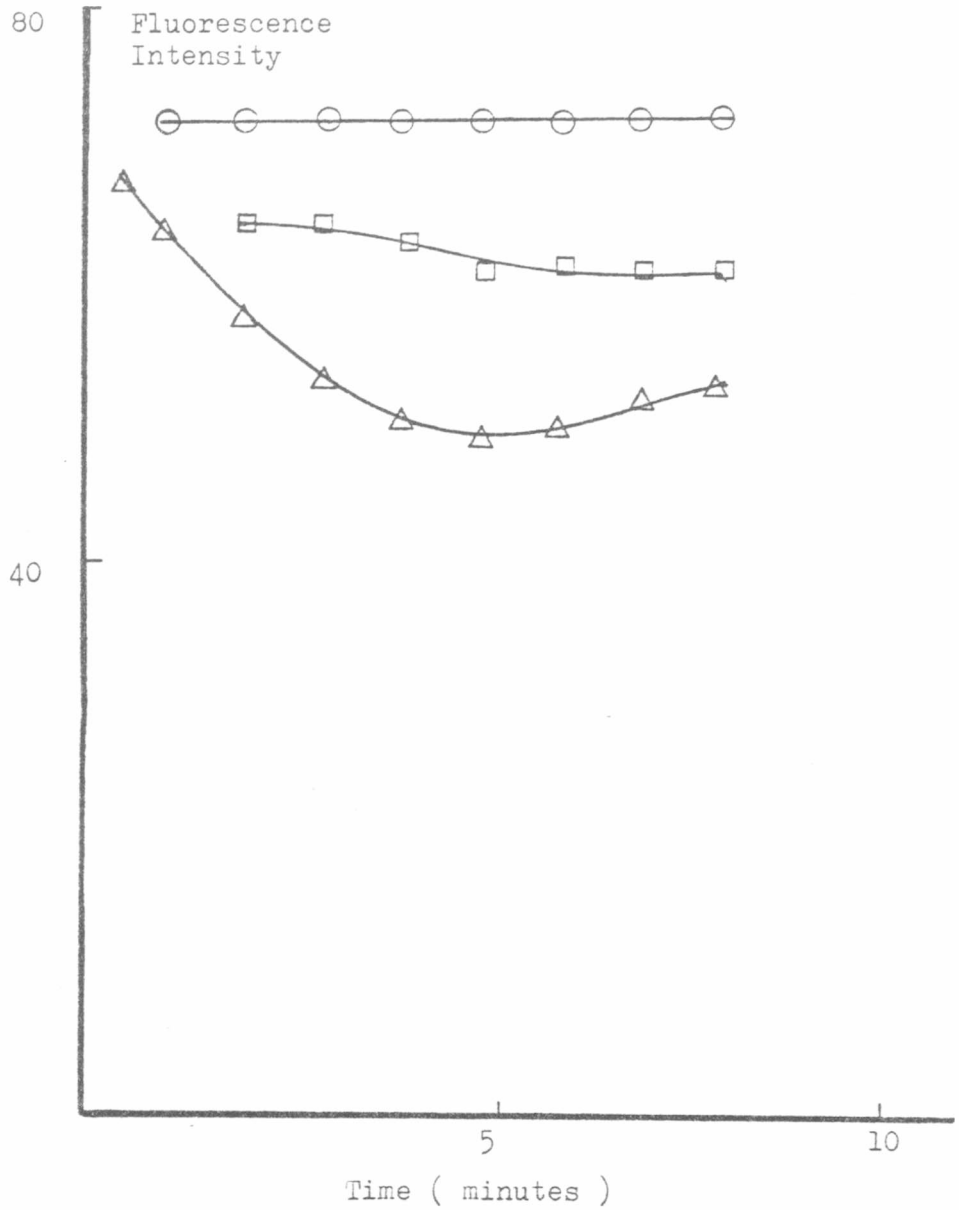


Figure 6.6

A graph showing the effect of prostacyclin on the uptake of 10^{-5} M DPD in a washed platelet suspension.

△ no prostacyclin □ 1μM ○ 5μM prostacyclin

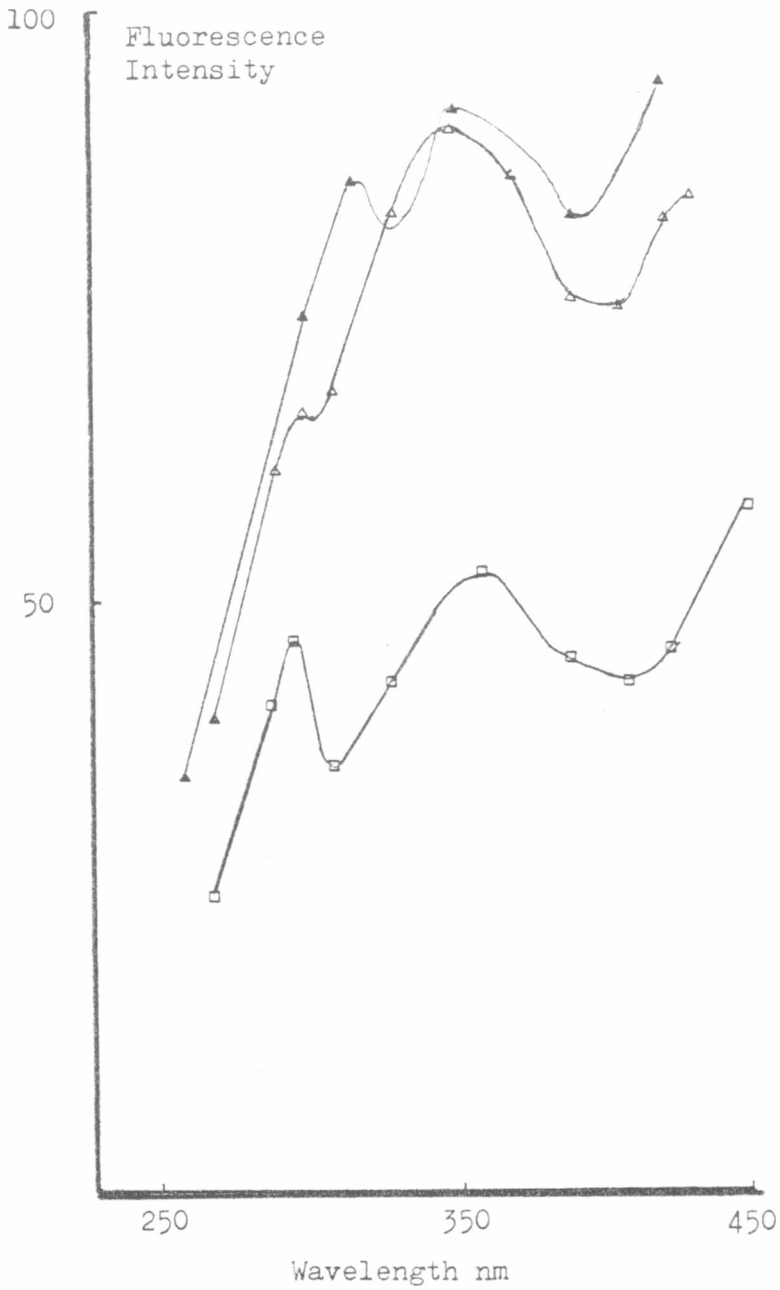


Figure 6.7

A graph showing how the platelet blank fluorescence changes with time after the final washing step.

▫ 1hour △ 24hours ▲ 48hours

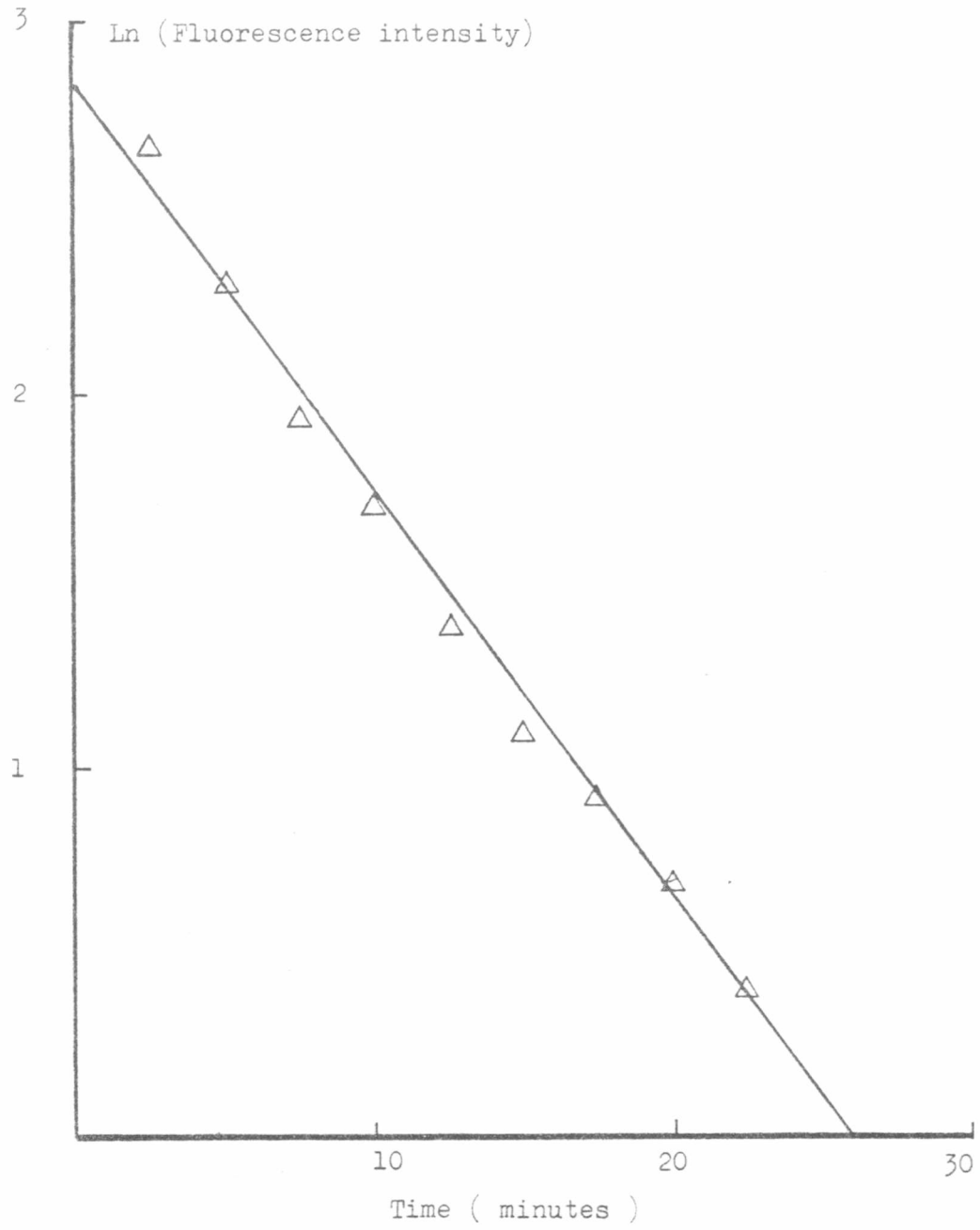


Figure 6.8

A graph of Ln (fluorescence intensity) against time for the uptake of 10^{-5}M DPD into a octan-1-ol water emulsion.

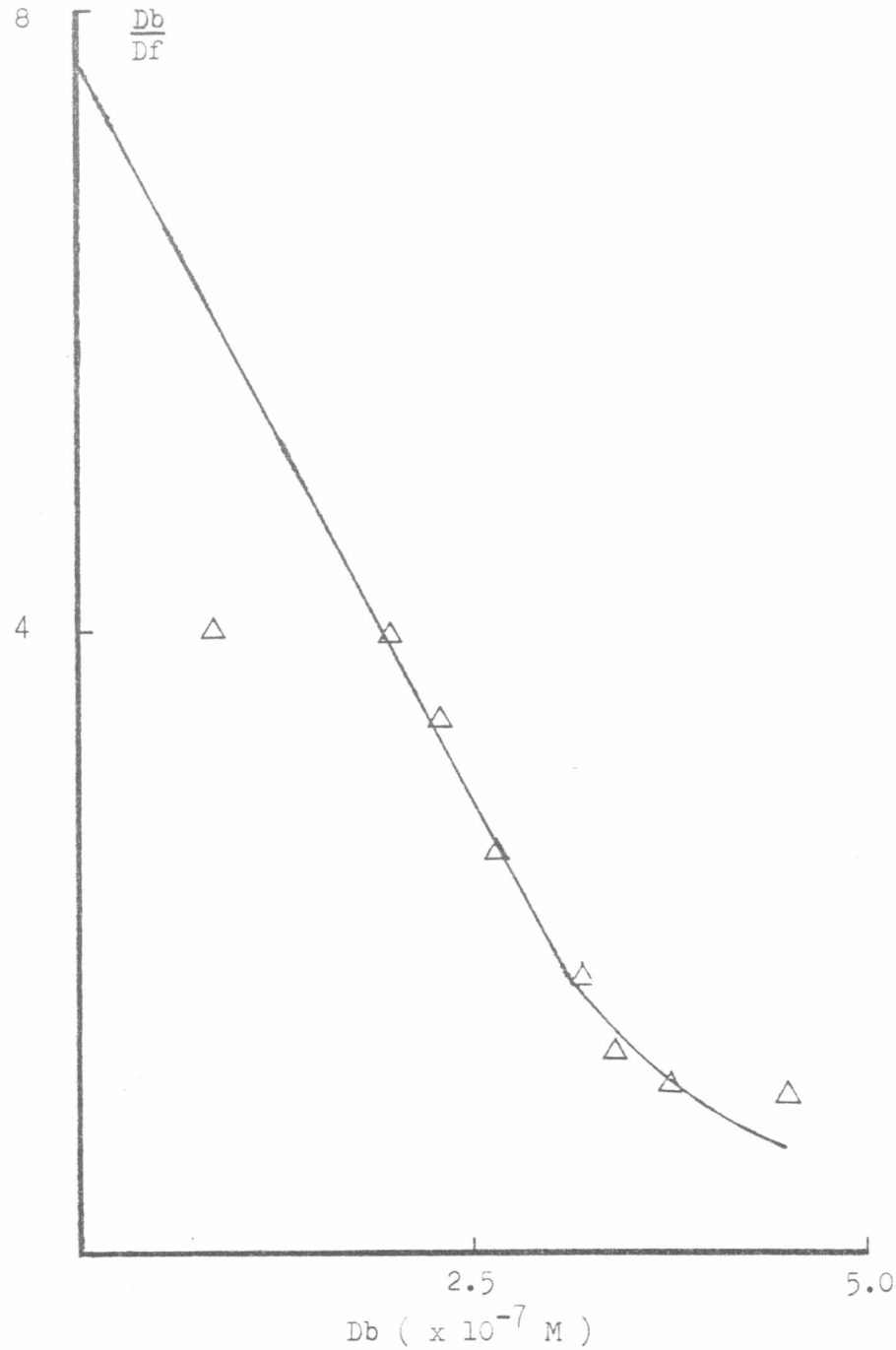


Figure 6.9

A Rosenthal plot for the binding of DPD to a washed platelet suspension. Data from the Sephadex batch method.

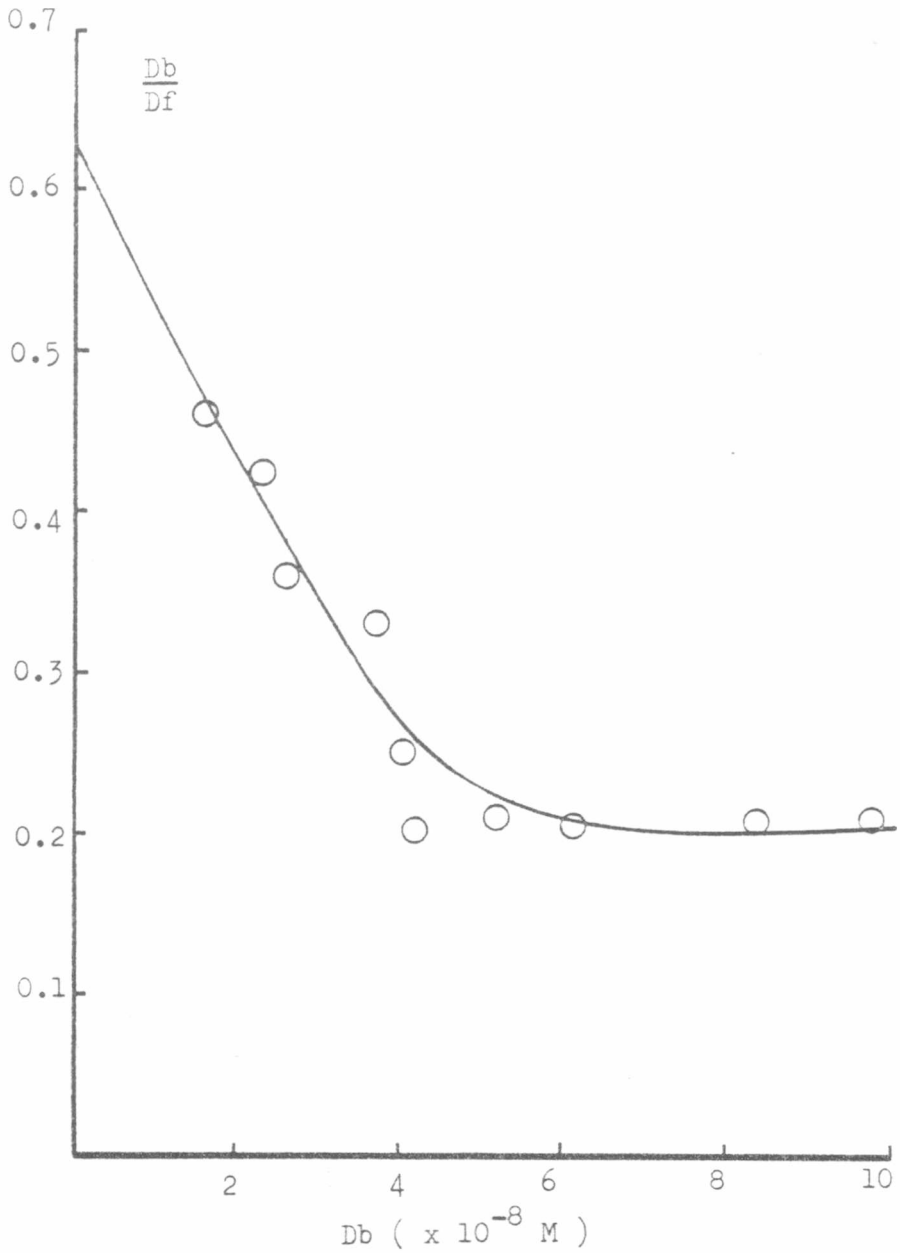


Figure 6.10

A Rosenthal plot showing the binding of DPD to washed platelet membranes.

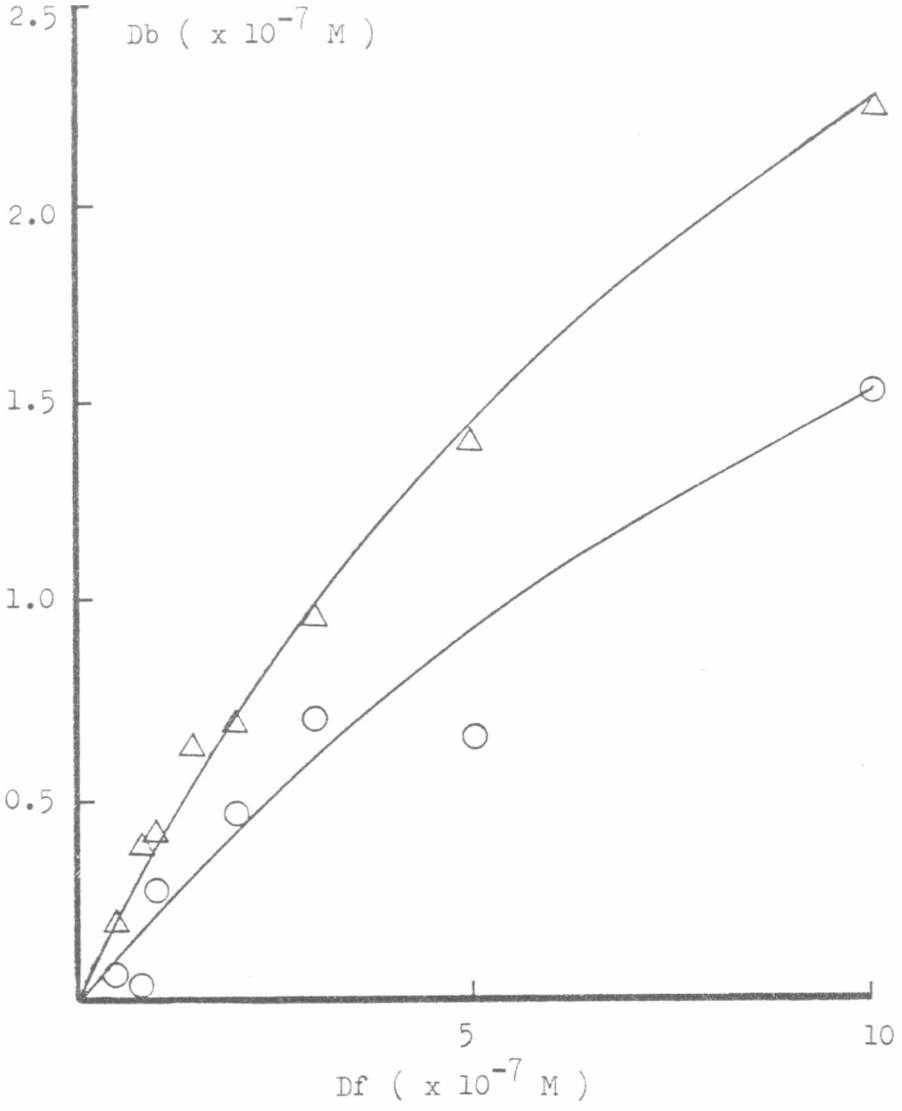


Figure 6.11

A graph showing the effect of 5 x 10^{-6} M AGP on the binding of DPD to a washed platelet suspension.

△ No AGP ○ 5 x 10^{-6}M AGP

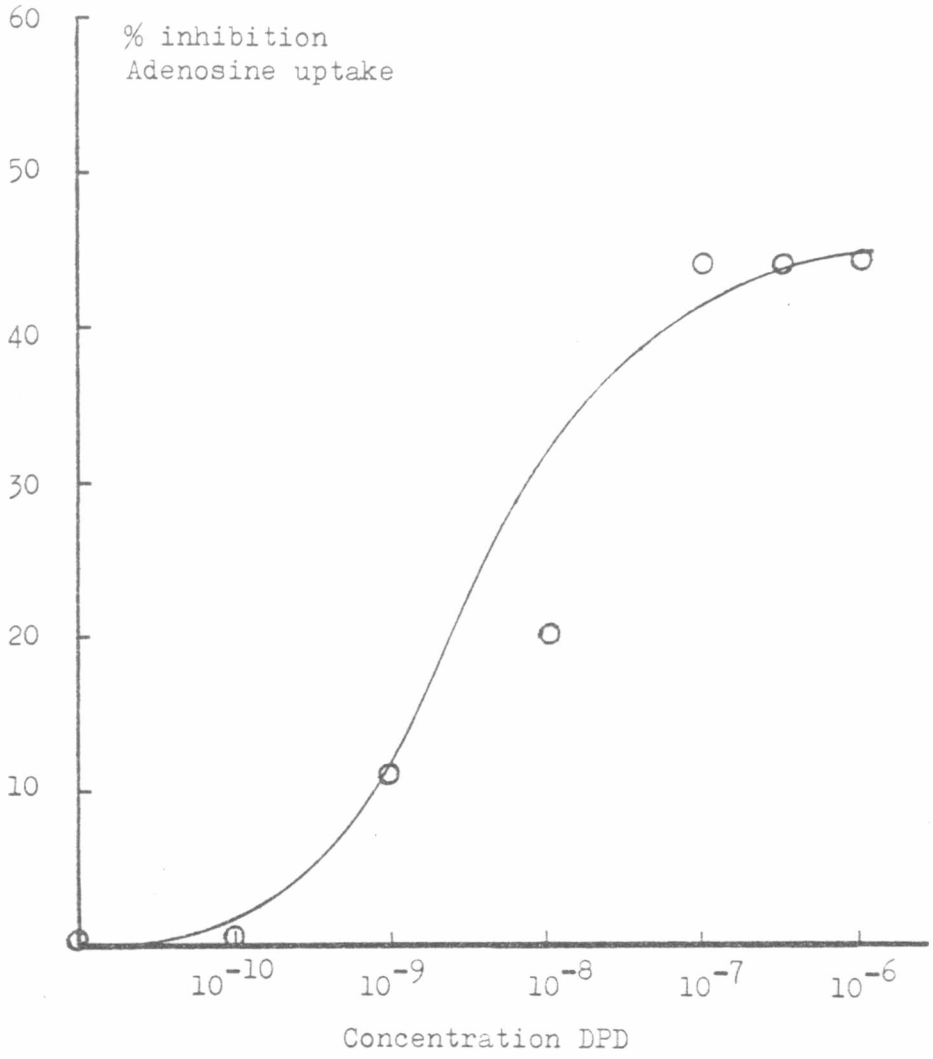
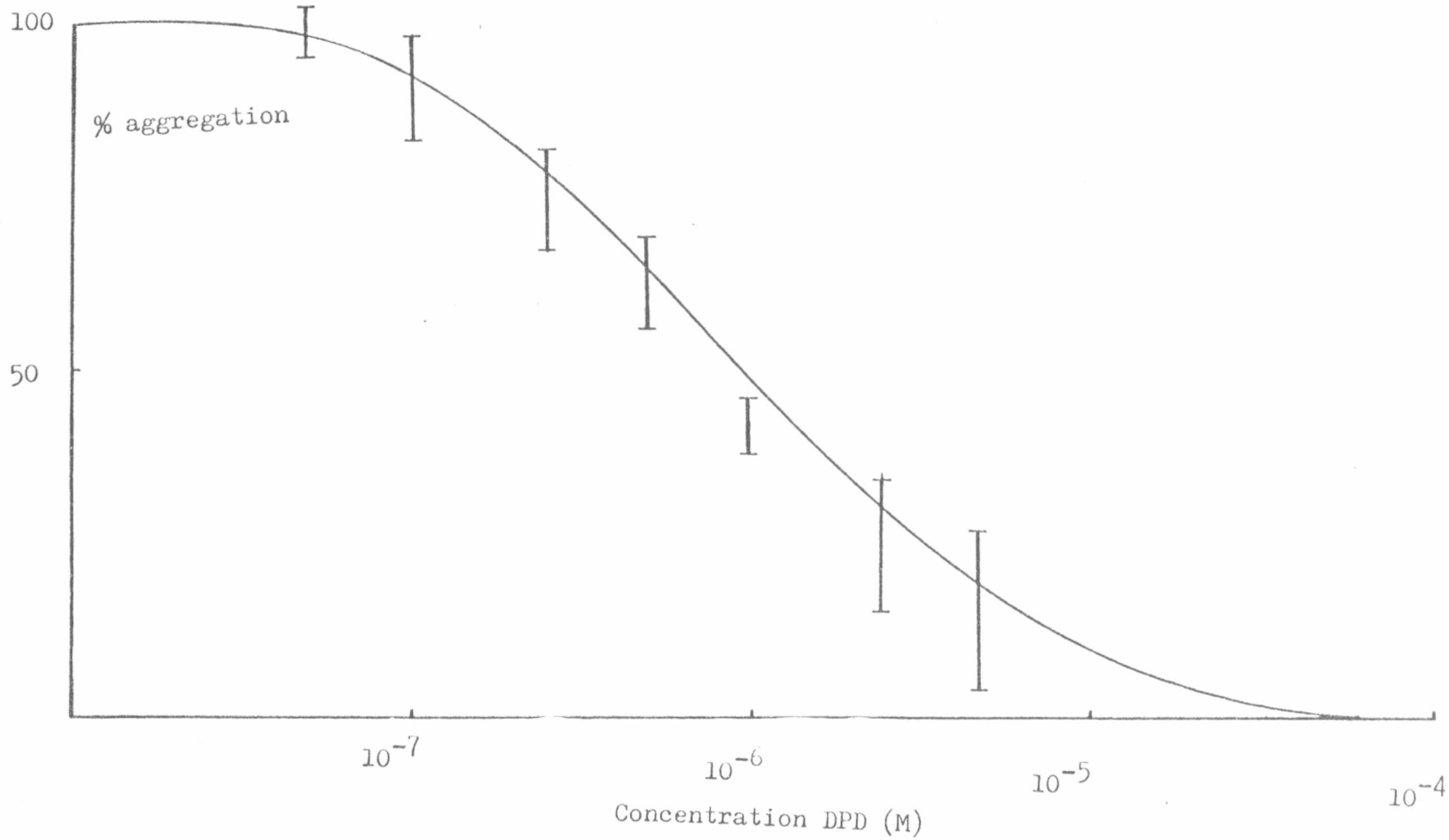


Figure 6.12

A graph showing the effect of DPD on the uptake of adenosine

Figure 6.13

A graph showing the percentage inhibition of aggregation against the log DPD concentration in a washed platelet suspension


100
% aggregation
50
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration DPD (M)

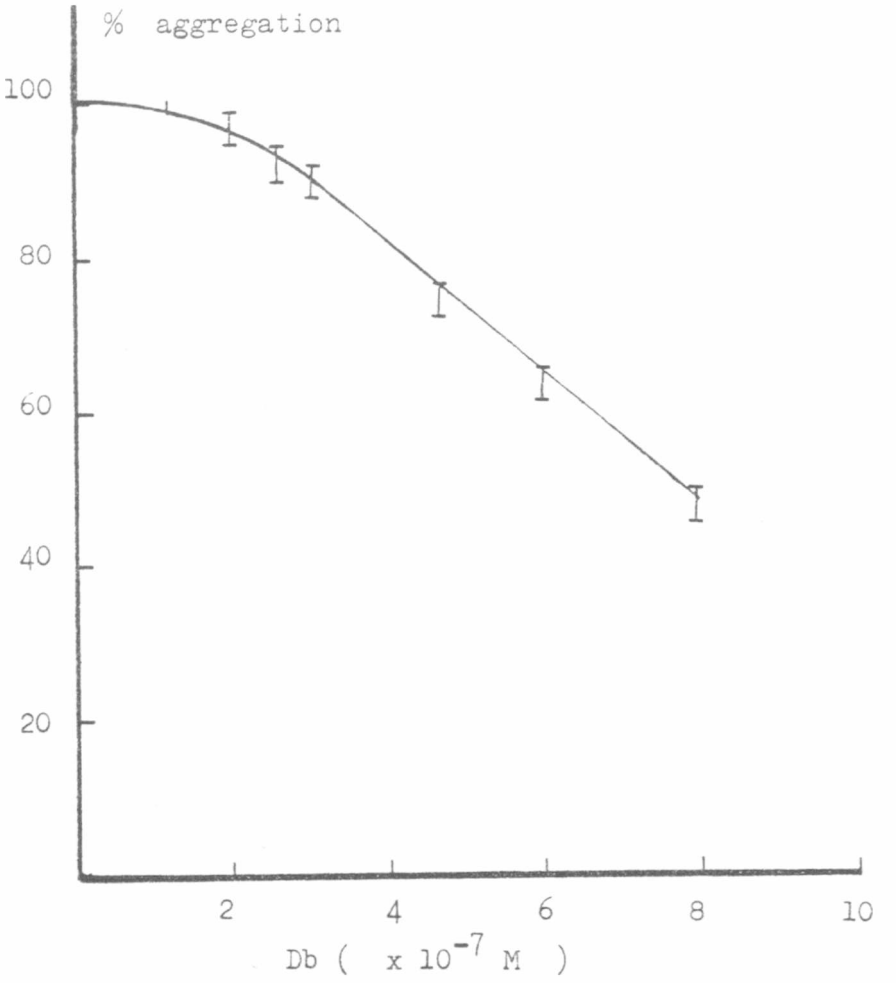


Figure 6.14

A graph showing the correlation of the inhibition of aggregation with the binding of DPD to a washed platelet suspension.

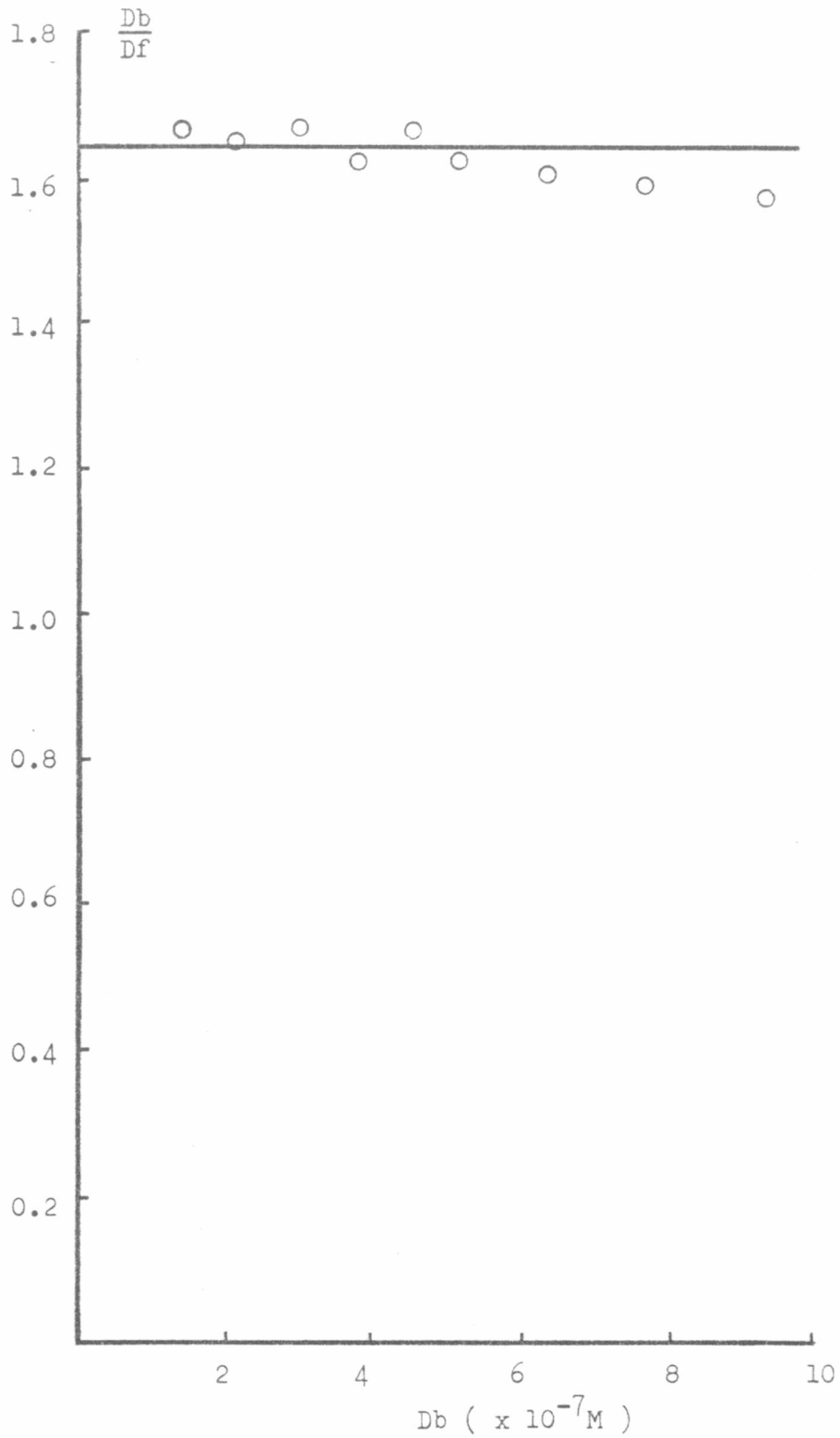


Figure 6.15

A Rosenthal plot for the binding of prazosin to a washed platelet suspension (500000). Data from the centrifuging difference method

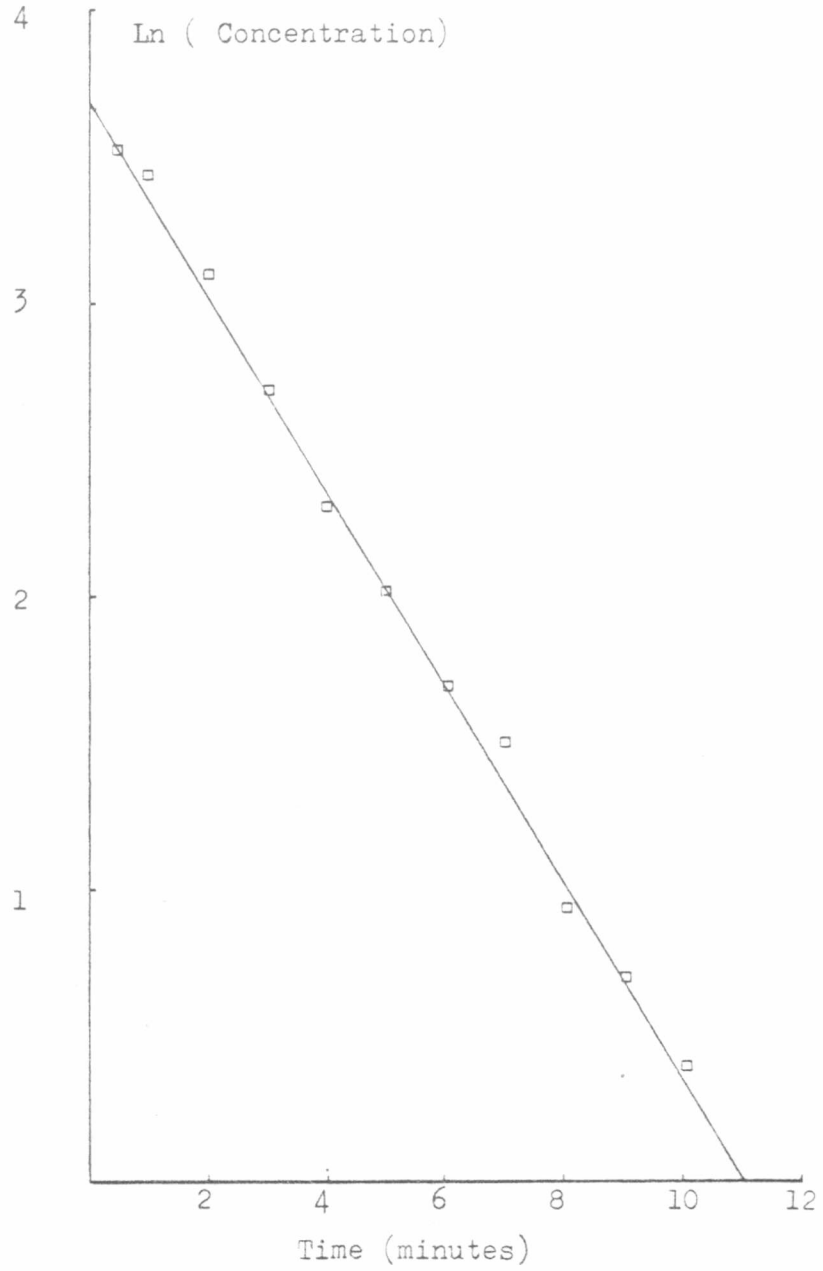


Figure 6.16

A graph showing the kinetics of uptake of 10μM prazosin into a washed platelet suspension.

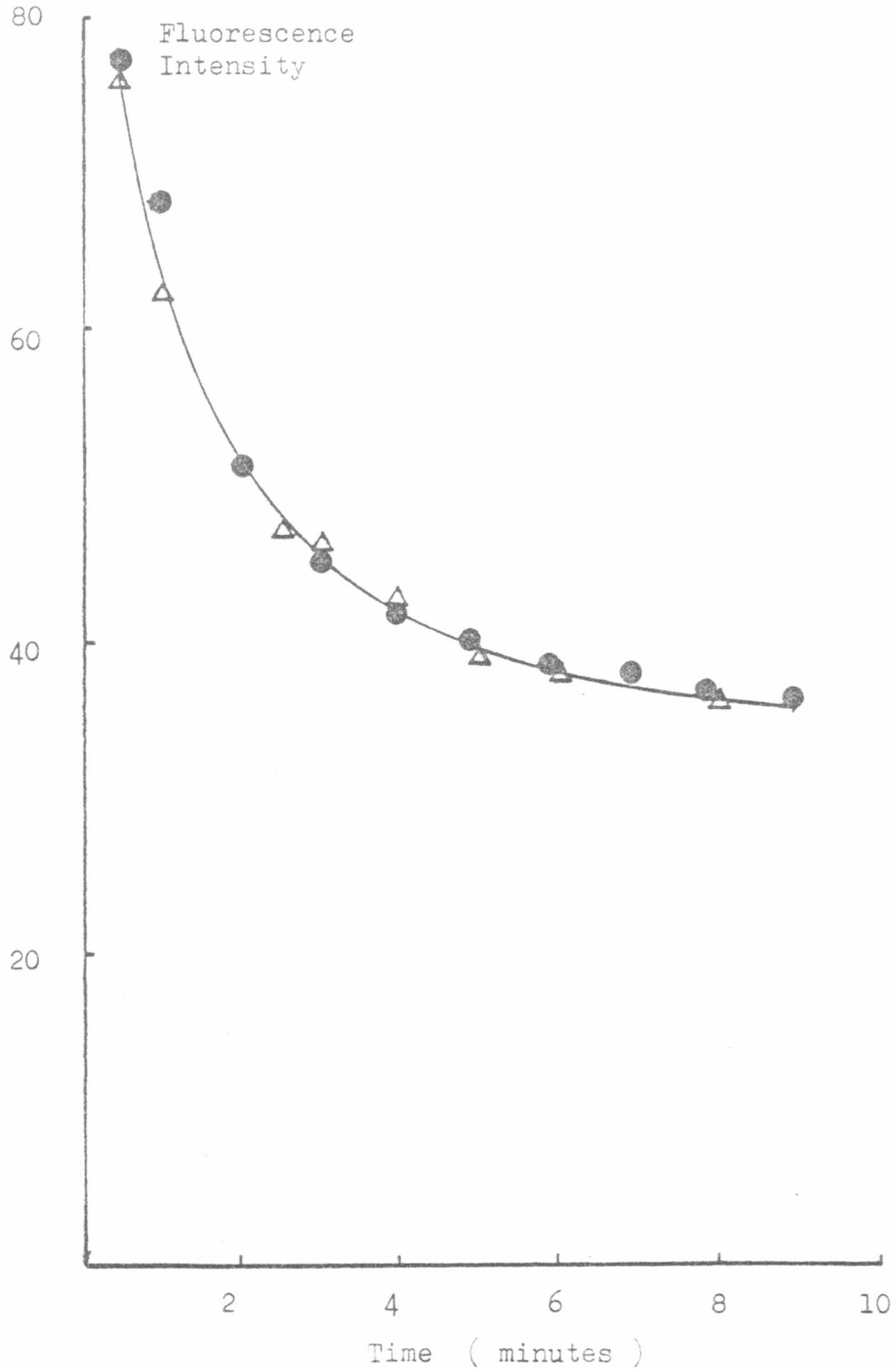


Figure 6.17

A graph showing the effect of DPD on the uptake of prazosin(10^{-5} M)

△ No DPD ● 10^{-5}M DPD

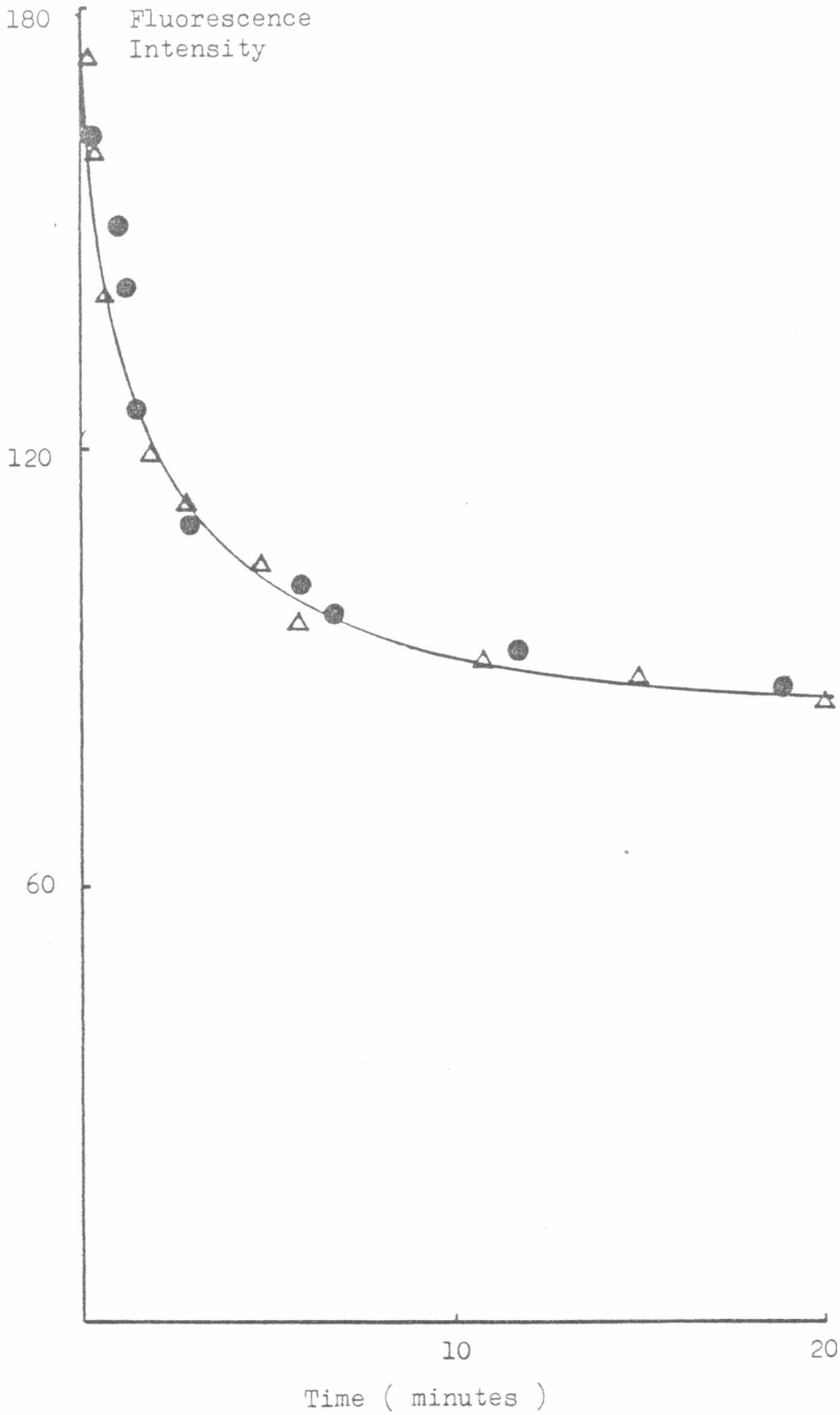


Figure 6.18

A graph showing the effect of papaverine on the uptake of prazosin (10^{-5}) in a washed platelet suspension.

△ No papaverine ● 10^{-5}M papaverine

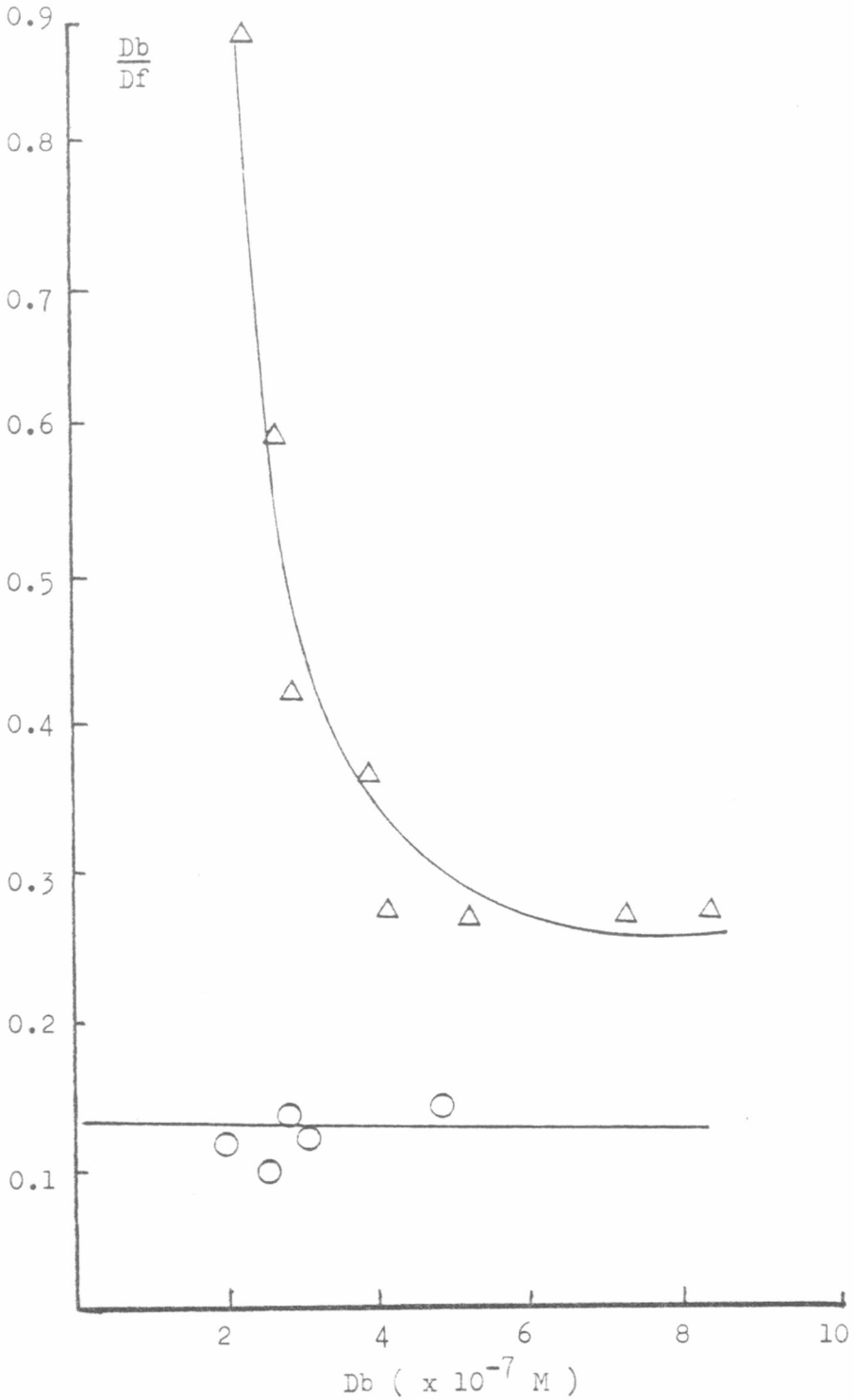


Figure 6.19

A graph showing the effect of papaverine on the binding of DPD to a washed platelet suspension.

△ No papaverine ○ 10^{-4}M papaverine

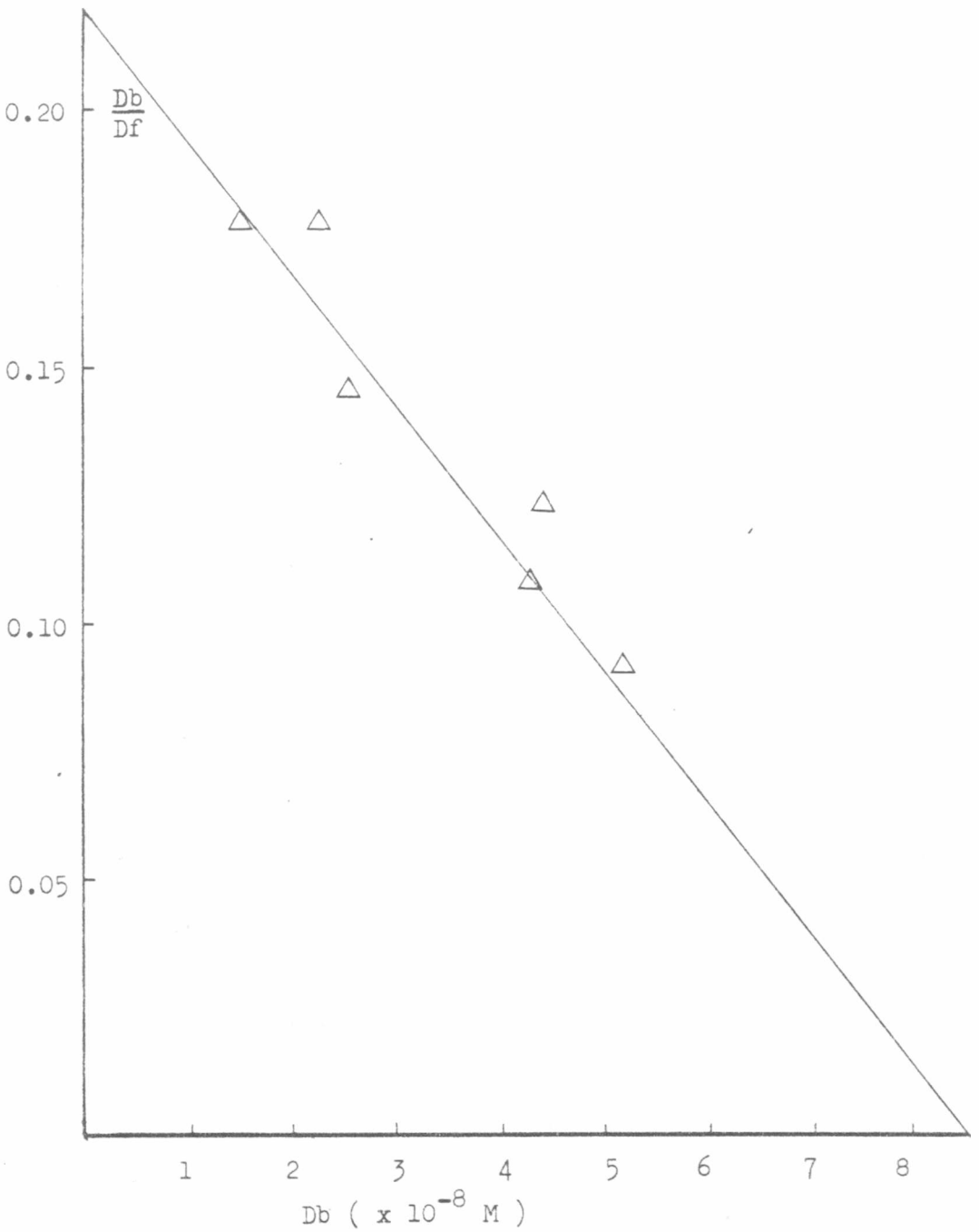


Figure 6.20

A Rosenthal plot for the binding of RA 233 to washed platelets

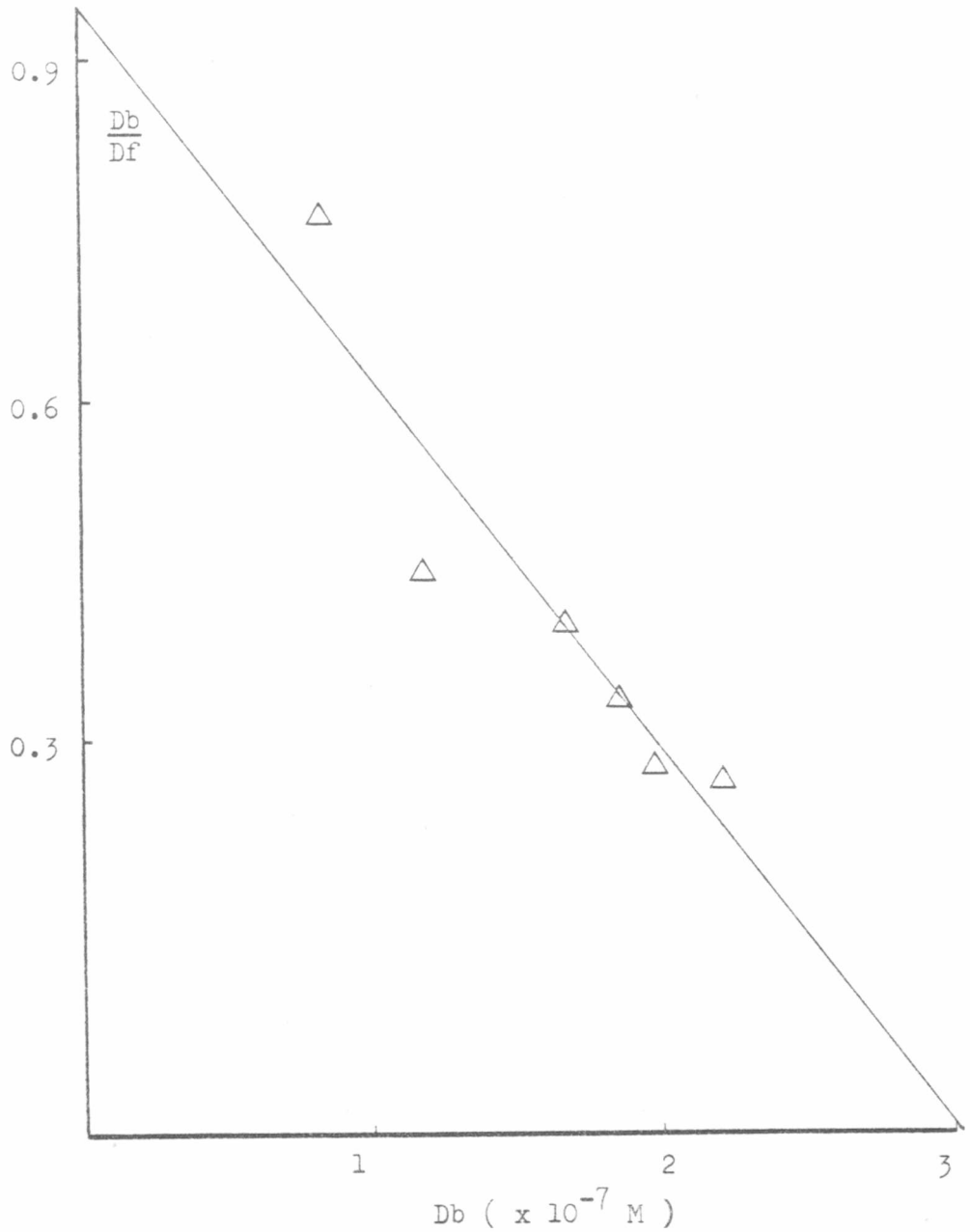


Figure 6.21

A Rosenthal plot for the binding of RA433 to washed platelets

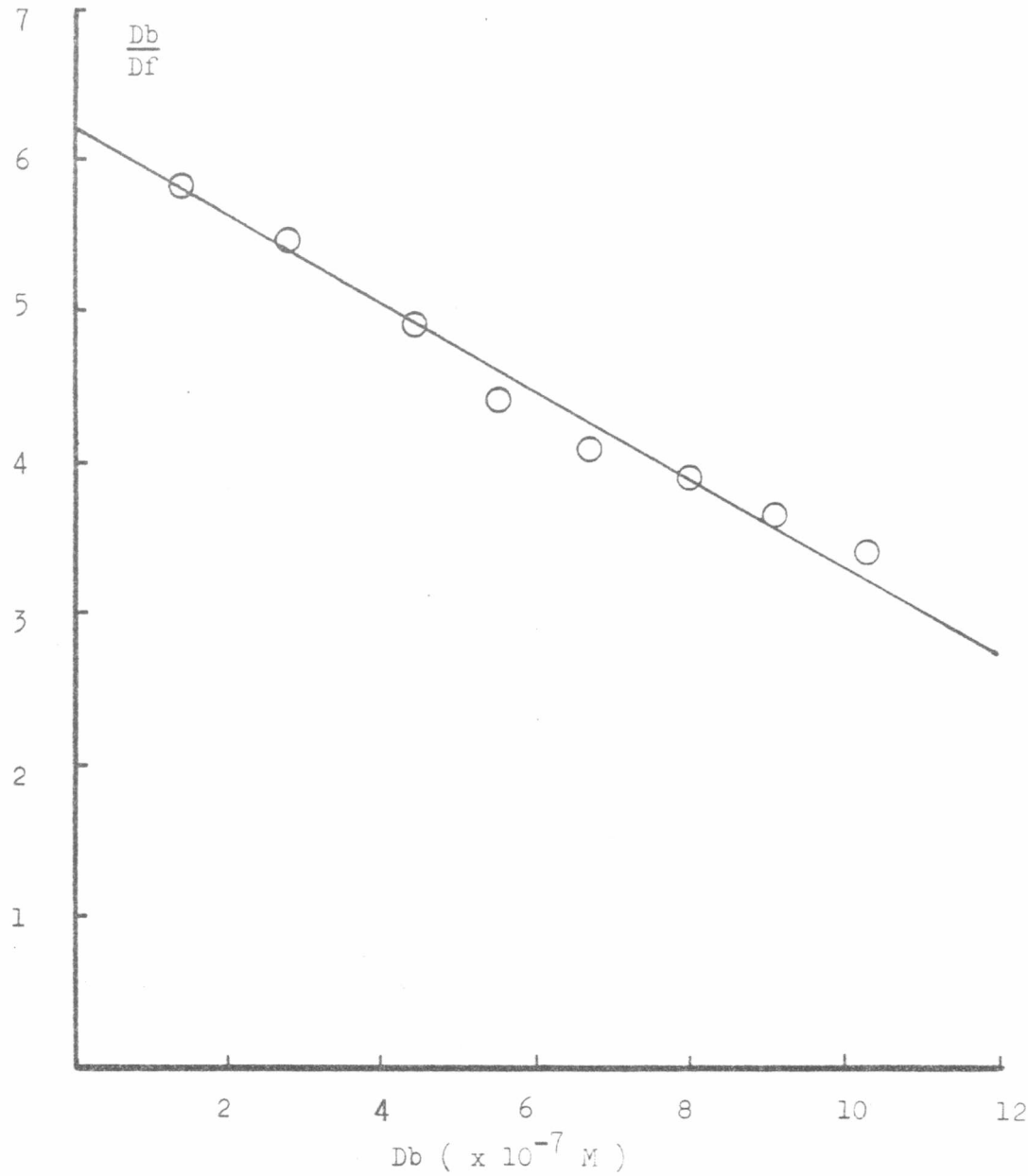


Figure 6.22

A Rosenthal plot for the binding of DPQ to washed platelets.

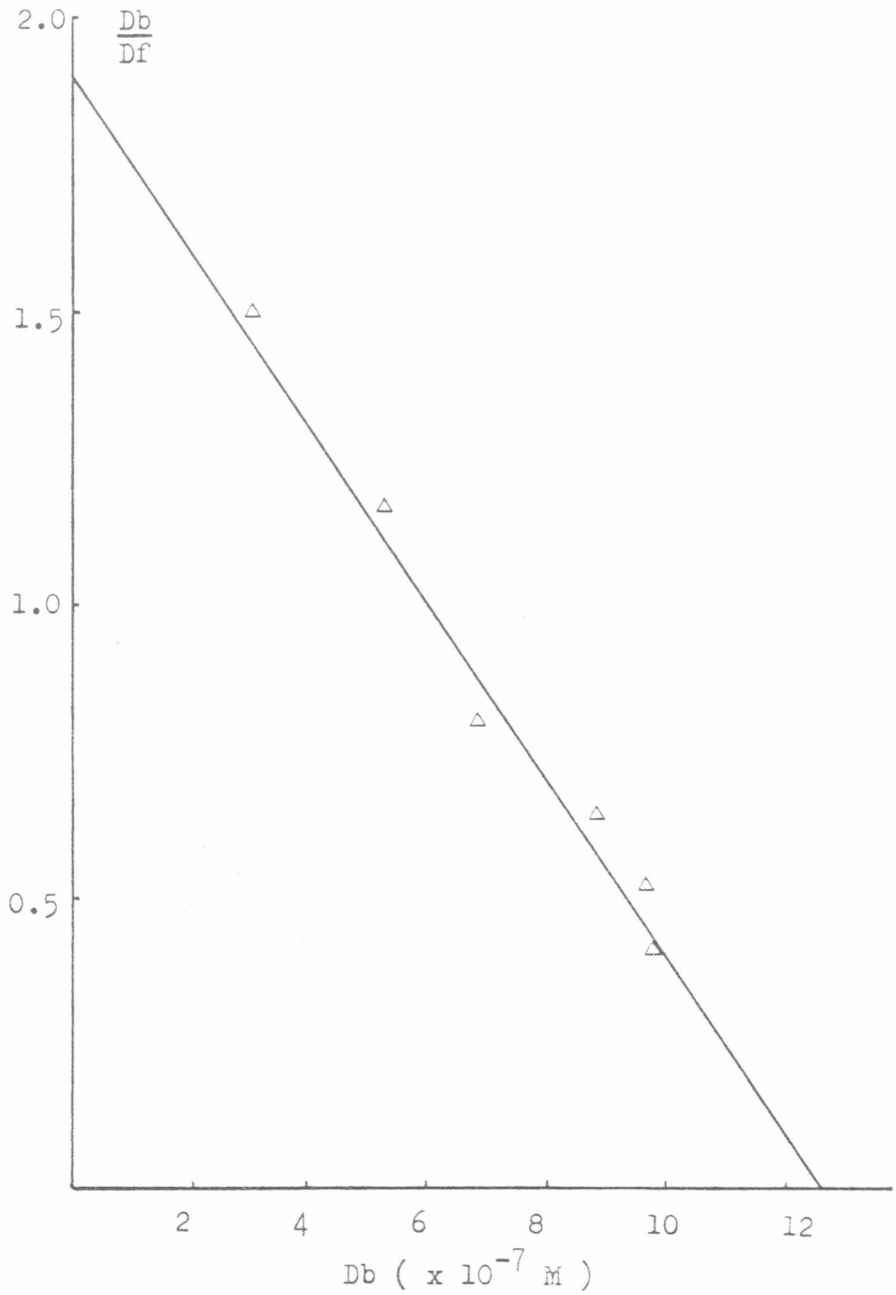
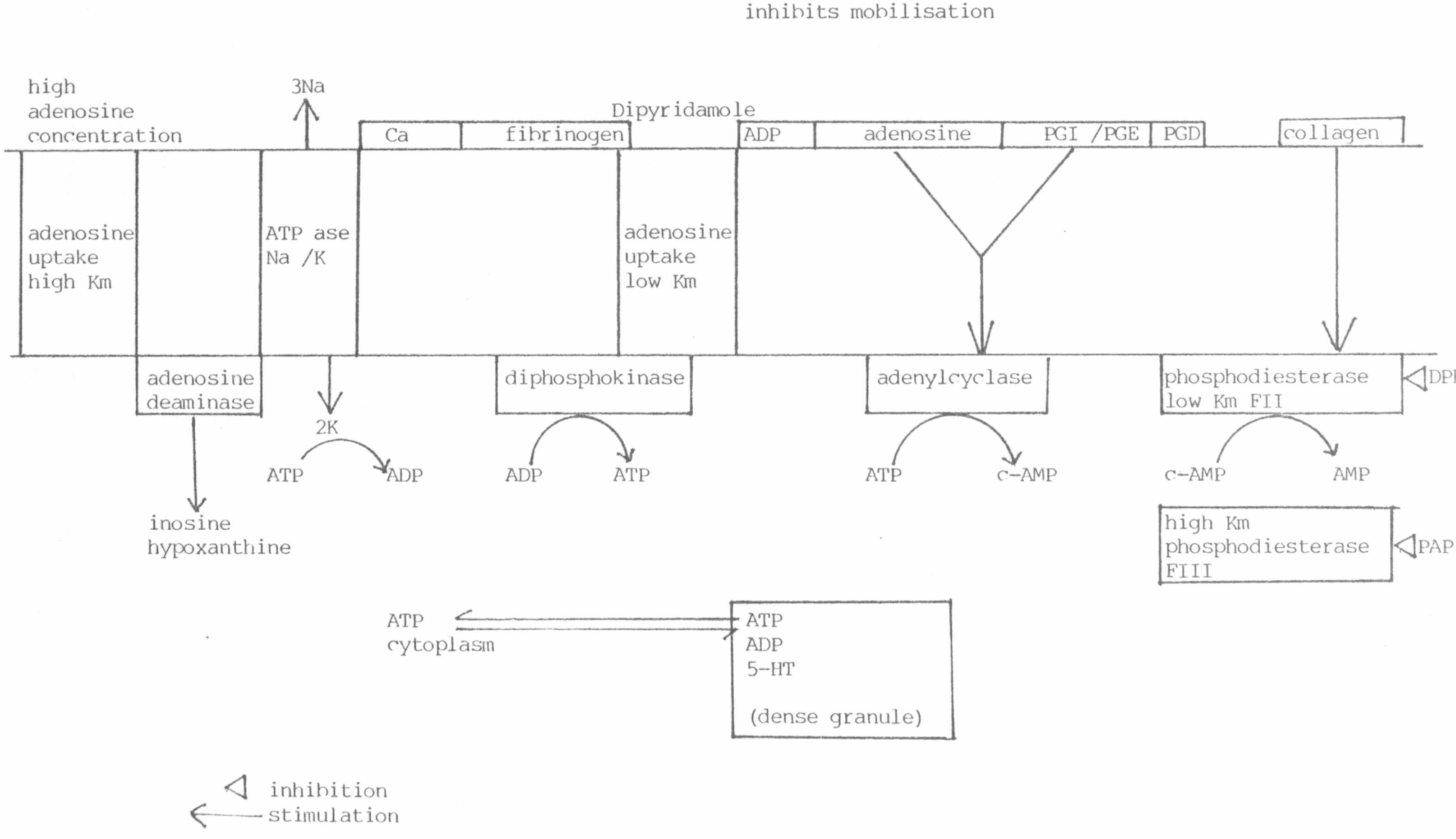


Figure 6.23

A Rosenthal plot for the binding of indomethacin to washed platelets

Figure 6.24

A diagram indicating the importance of membrane bound enzymes on the biochemical pathways of the platelet.



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APPENDIX 1

The listing of the programme used to fit data obtained from dynamic dialysis to a triexpoential function and to differentiate this function to give the free and bound concentration of ligand.

```
      COMMON DT,TIME,R,M
      DOUBLE PRECISION A3,A4,A5,A6,A7,A8,A9,A10,A11
      DOUBLE PRECISION DT(30),TIME(30),R(30),DTC(30),DIFF(30)
      DOUBLE PRECISION RC(30), DB(30), DF(30), DX(30)
      DOUBLE PRECISION EPS,XK,X0,Y0,X11,Y11,X1,Y1,X,Y,F,W
      DIMENSION X0(10),X(10),X11(10),X1(10),XK(10)
      DIMENSION DFEST(101),DBEST(101),REST(101)
   29 OPEN (UNIT=1,FILE='HOOKE.DAT')
      READ(1,39)M,(DT(I),I=1,M),(TIME(I),I=1,M),AB
   39 FORMAT(6G)
   59 N=6
      WRITE(5,1)
 1    FORMAT (/,1H,'INPUT NUMBER OF CONSTANTS')
      READ(5,20)M2
   69 WRITE(5,47)
   47 FORMAT(/,1H,'INPUT STEP SIZES A, B, C, D, E, F, AND EPS,SEP
RET')
      READ(5,20)(XK(I),I=1,N),EPS
      WRITE(5,95)
   95 FORMAT (/,1H,'INPUT INITIAL VALUES OF A, B, C, D, E, F,')
      READ(5,20)(X0(K),K=1,N)
   20 FORMAT(G)
  410 FORMAT(1F12.5)
C**** COMPUTING PART********
      Y0=F(X0)
      DO 30 I=1,N
   30 X11(I)=X0(I)
      Y11=Y0
  111 DO 40 I=1,N
   40 X1(I)=X11(I)
      Y1=Y11
      I=0
  222 I=I+1
      DO 50 K=1,N
      IF(K.EQ.I) GOTO 60
      W=0.0D0; GOTO 70
   60 W=1.0D0
   70 X(K)=X1(K)+XK(K)*W
   50 CONTINUE
      Y=F(X)
      IF(Y.LT.Y1) GOTO 80
      DO 51 K=1,N
      IF(K.EQ.I) GOTO 61
      W=0.0D0; GOTO 71
   61 W=1.0D0
   71 X(K)=X1(K)-XK(K)*W
   51 CONTINUE
      Y=F(X)
      IF(Y.GE.Y1)GOTO 90
   80 DO 100 K=1,N
  100 X1(K)=X(K)
```

```
      Y1=Y
   90 IF(I.NE.N) GOTO 222
      IF(Y1.LT.Y0) GOTO 110
      DO 120 K=1,N
      IF(X11(K).NE.X0(K))GOTO 130
  120 CONTINUE
      DO 555 K=1,N
  555 XK(K)=XK(K)/10.0D0
      DO 666 K=1,N
      IF(DABS(XK(K)).GE.EPS)GOTO 111
  666 CONTINUE
      GOTO 333
  130 DO 140 K=1,N
  140 X11(K)=X0(K)
      Y11=Y0
      GOTO 111
C****** COMPUTING FINISHED *****
  333 WRITE(5,150)
  150 FORMAT (1H ,'SOLUTION VECTOR-A')
      WRITE(5,94)X0(1)
   94 FORMAT(1H ,G)
      WRITE(5,151)
  151 FORMAT(1H ,'SOLUTION VECTOR-B')
      WRITE(5,94)X0(2)
      WRITE(5,152)
  152 FORMAT(1H ,'SOLUTION VECTOR-C')
      WRITE(5,94)X0(3)
      WRITE(5,160)
  160 FORMAT(1H ,'SOLUTION VECTOR-D, E, AND F')
      WRITE(5,940)X0(4),X0(5),X0(6)
 940  FORMAT(1H ,3G)
      WRITE(5,660)Y0
  660 FORMAT(1H ,'FUNCTION VALUE=',G)
C     X0(1) IS K1 X0(2) IS NP1 X0(3) IS K2 X0(4) IS NP2
      DO 17 I=1,M
      DTC(I)=((X0(1)*EXP(X0(2)*TIME(I))))+(X0(3)*EXP(X0(4)*TIME(I)))
     1+(X0(5)*EXP(X0(6)*TIME(I)))
      DIFF(I)=DTC(I)-DT(I)
  17  CONTINUE
      WRITE(5,24)
   24 FORMAT(/,1H,6X,'TIME',6X,'DT',10X,'DTC',10X,'DIFF')
      DO 54 I=1,M
   44 FORMAT(4F12.5)
      WRITE(5,44)TIME(I),DT(I),DTC(I),DIFF(I)
      DFES=0
   54 CONTINUE
      WRITE (5,55)
   55 FORMAT( /,1H,6X,'DY/DX',6X,'DF',10X,'DB',10X,'R')
      DO 56 I=1,M
      DX(I)= X0(1)*X0(2)*EXP(X0(2)*TIME(I))
     1+X0(3)*X0(4)*EXP(X0(4)*TIME(I))
     2+ X0(5)*X0(6)*EXP(X0(6)*TIME(I))
      DF(I)=DX(I)/AB
      DB(I)=DT(I)-DF(I)
      R(I)=DB(I)/DF(I)
      WRITE(5,44) DX(I),DF(I),DB(I),R(I)
   56 CONTINUE
```

```
C   ****** CREATES PLOT FILE *****
      DO 420 I=1,101
      DFEST(I)=DFES+B

DBEST(I)=((X0(1)*EXP(X0(2)*DFEST(I))))+(X0(3)*EXP(X0(4)*DFEST(I)))
     1+(X0(5)*EXP(X0(6)*DFEST(I)))
      DFES=DFEST(I)
  420 CONTINUE
      NSET=2
      NINPUT=M*2
      OPEN(UNIT=1,FILE='FOR07.DAT')
      WRITE(1,400)NSET,NINPUT,(DBEST(I),I=1,101),(DFEST(I),I=1,101),
     1(DT(I),I=1,M),(TIME(I),I=1,M)
  400 FORMAT(2I,30(/10F7.3))
C     *********ANOVA*********
      N1=M-M2
      N2=M2-1
      N3=M-1
      WRITE(5,163)
  163  FORMAT(1H ,'NUMBER OF OBSERVATIONS')
      WRITE(5,94)M
      WRITE(5,164)
  164  FORMAT(1H ,'NUMBER OF CONSTANTS')
      WRITE(5,94)M2
      WRITE(5,165)
  165  FORMAT(1H ,'D.F. OF TOTAL')
      WRITE (5,94)N3
      WRITE(5,153)
  153  FORMAT(1H ,'D.F. ABOUT REGRESSION')
      WRITE(5,94)N1
      WRITE(5,154)
  154  FORMAT(1H ,'D.F. DUE TO REGRESSION')
      WRITE(5,94)N2
      A3=0
      DO 155 I=1,M
      A3=A3+DT(I)
  155  CONTINUE
      A4=A3/FLOAT(M)
      A5=0
      A7=0
      DO 156 I=1,M
      A5=A5+(DT(I)-A4)**2.
      A7=A7+(DT(I)-DTC(I))**2.
  156  CONTINUE
      A6=A5-A7
      A8=A7/FLOAT(N1)
      A9=A6/FLOAT(N2)
      A11=A6/A5
      WRITE(5,157)
  157  FORMAT(1H ,'SS DUE TO REGRESSION')
      WRITE(5,94)A6
      WRITE(5,158)
  158  FORMAT(1H ,'SS ABOUT REGRESSION')
      WRITE(5,94)A7
      WRITE(5,159)
  159  FORMAT(1H ,'SS TOTAL CORRECTED FOR MEAN')
      WRITE(5,94)A5
```

```
      WRITE(5,166)
  166 FORMAT(1H ,'MS DUE TO REGRESSION')
      WRITE(5,94)A9
      WRITE(5,161)
  161 FORMAT(1H ,'MS ABOUT REGRESSION')
      WRITE(5,94)A8
      A1Ø=A9/A8
      WRITE(5,162)
  162 FORMAT(1H ,' F STATISTIC')
      WRITE(5,94)A1Ø
      WRITE(5,168)
  168 FORMAT(1H ,'R**2.')
      WRITE(5,94)A11
      GOTO 444
  11Ø DO 17Ø K=1,N
      X11(K)=2.ØDØ*X1(K)-XØ(K)
  17Ø XØ(K)=X1(K)
      YØ=Y1;Y11=F(X11)
      GOTO 111
  444 STOP;END
      DOUBLE PRECISION FUNCTION F(X)
      DOUBLE PRECISION X(1Ø)
      DOUBLE PRECISION DT(3Ø),TIME(3Ø),R(3Ø)
      DOUBLE PRECISION SUM,TERM
      COMMON DT,DF,R,M
      SUM=Ø.ØDØ
      DO 1Ø I=1,M
      TERM=DT(I)-((X(1)*EXP(X(2)*TIME(I)))+(X(3)*EXP(X(4)*TIME(I)))
     1+(X(5)*EXP(X(6)*TIME(I))))
      SUM=SUM+TERM*TERM
   1Ø CONTINUE
      F=SUM
      RETURN;END
```

APPENDIX 2

The listing of the Hooke computer programme used for the analysis of a one class of binding site system.

```
C     MODEL IS DB=((K1*NP1*DF)/(1.+K1*DF))
      COMMON DB,DF,R,M
      DOUBLE PRECISION A3,A4,A5,A6,A7,A8,A9,A10,A11
      DOUBLE PRECISION DB(30),DF(30),R(30),DBC(30),DIFF(30)
      DOUBLE PRECISION RC(30)
      DOUBLE PRECISION EPS,XK,X0,Y0,X11,Y11,X1,Y1,X,Y,F,W
      DIMENSION X0(10),X(10),X11(10),X1(10),XK(10)
      DIMENSION DFEST(101),DBEST(101),REST(101)
   29 OPEN (UNIT=1,FILE='HOOKE.DAT')
      READ(1,39)M,(DB(I),I=1,M),(DF(I),I=1,M)
   39 FORMAT(6G)
   59 N=2
      WRITE(5,1)
 1    FORMAT (/,1H,'INPUT NUMBER OF CONSTANTS')
      READ(5,20)M2
   69 WRITE(5,47)
   47 FORMAT(/,1H,'INPUT STEP SIZES K1,NP1,EPS,SEP RET')
      READ(5,20)(XK(I),I=1,N),EPS
      WRITE(5,95)
   95 FORMAT (/,1H,'INPUT INITIAL VALUES OF K1,NP1')
      READ(5,20)(X0(K),K=1,N)
   20 FORMAT(G)
      DO 89 I=1,M
      R(I)=DB(I)/DF(I)
  89  CONTINUE
      DO 411 I=M
      DFMAX=DF(M)
      B=(DFMAX+DFMAX/50)/101
 411  CONTINUE
      WRITE(5,409)
 409  FORMAT (/,1H,'MAX DF')
      WRITE (5,410)DFMAX
 410  FORMAT(1F12.5)
C**** COMPUTING PART********
      Y0=F(X0)
      DO 30 I=1,N
  30  X11(I)=X0(I)
      Y11=Y0
 111  DO 40 I=1,N
  40  X1(I)=X11(I)
      Y1=Y11
      I=0
 222  I=I+1
      DO 50 K=1,N
      IF(K.EQ.I) GOTO 60
      W=0.0D0; GOTO 70
  60  W=1.0D0
  70  X(K)=X1(K)+XK(K)*W
  50  CONTINUE
      Y=F(X)
      IF(Y.LT.Y1) GOTO 80
      DO 51 K=1,N
```

```
      IF(K.EQ.I) GOTO 61
      W=0.0D0; GOTO 71
   61 W=1.0D0
   71 X(K)=X1(K)-XK(K)*W
   51 CONTINUE
      Y=F(X)
      IF(Y.GE.Y1)GOTO 90
   80 DO 100 K=1,N
  100 X1(K)=X(K)
      Y1=Y
   90 IF(I.NE.N) GOTO 222
      IF(Y1.LT.Y0) GOTO 110
      DO 120 K=1,N
      IF(X11(K).NE.X0(K))GOTO 130
  120 CONTINUE
      DO 555 K=1,N
  555 XK(K)=XK(K)/10.0D0
      DO 666 K=1,N
      IF(DABS(XK(K)).GE.EPS)GOTO 111
  666 CONTINUE
      GOTO 333
  130 DO 140 K=1,N
  140 X11(K)=X0(K)
      Y11=Y0
      GOTO 111
C****** COMPUTING FINISHED *****
  333 WRITE(5,150)
  150 FORMAT (1H ,'SOLUTION VECTOR-K1')
      WRITE(5,94)X0(1)
   94 FORMAT(1H ,G)
      WRITE(5,151)
  151 FORMAT(1H ,'SOLUTION VECTOR-NP1')
      WRITE(5,94)X0(2)
      WRITE(5,660)Y0
  660 FORMAT(1H ,'FUNCTION VALUE=',G)
C     XO(1) IS K1 X0(2) IS NP1
      DO 17 I=1,M
      DBC(I)=((X0(1)*X0(2)*DF(I))/(1.+X0(1)*DF(I)))
      RC(I)=DBC(I)/DF(I)
      DIFF(I)=DBC(I)-DB(I)
   17 CONTINUE
      WRITE(5,24)
   24 FORMAT(/,1H,6X,'DF',10X,'DB',10X,'DBC',10X,'DIFF',
     110X,'R',10X,'RC')
      DO 54 I=1,M
   44 FORMAT(6F12.5)
      WRITE(5,44)DF(I),DB(I),DBC(I),DIFF(I),R(I),RC(I)
      DFES=0
   54 CONTINUE
C   ****** CREATES PLOT FILE *****
      DO 420 I=1,101
      DFEST(I)=DFES+B
      DBEST(I)=((X0(1)*X0(2)*DFEST(I))/(1.+X0(1)*DFEST(I)))
      REST(I)=DBEST(I)/DFEST(I)
      DFES=DFEST(I)
  420 CONTINUE
      NSET=2
```

```
      NINPUT=M*2
      OPEN(UNIT=1,FILE='FORØ7.DAT')
      WRITE(1,4ØØ)NSET,NINPUT,(REST(I),I=1,1Ø1),(DBEST(I),I=1,1Ø1),
     1(R(I),I=1,M),(DB(I),I=1,M)
  4ØØ FORMAT(2I,3Ø(/1ØF7.3))
C     CREATES FILE FOR NONLIN
      OPEN (UNIT=1,FILE='NONLIN.DAT')
      DO 115 I=1,M
      WRITE (1,11ØØ) DB(I),DF(I)
  115 CONTINUE
 11ØØ FORMAT (12X, 2F6.3)
C     *********ANOVA*********
      N1=M-M2
      N2=M2-1
      N3=M-1
      WRITE(5,163)
 163  FORMAT(1H ,'NUMBER OF OBSERVATIONS')
      WRITE(5,94)M
      WRITE(5,164)
 164  FORMAT(1H ,'NUMBER OF CONSTANTS')
      WRITE(5,94)M2
      WRITE(5,165)
 165  FORMAT(1H ,'D.F. OF TOTAL')
      WRITE (5,94)N3
      WRITE(5,153)
 153  FORMAT(1H ,'D.F. ABOUT REGRESSION')
      WRITE(5,94)N1
      WRITE(5,154)
 154  FORMAT(1H ,'D.F. DUE TO REGRESSION')
      WRITE(5,94)N2
      A3=Ø
      DO 155 I=1,M
      A3=A3+DB(I)
 155  CONTINUE
      A4=A3/FLOAT(M)
      A5=Ø
      A7=Ø
      DO 156 I=1,M
      A5=A5+(DB(I)-A4)**2.
      A7=A7+(DB(I)-DBC(I))**2.
 156  CONTINUE
      A6=A5-A7
      A8=A7/FLOAT(N1)
      A9=A6/FLOAT(N2)
      A11=A6/A5
      WRITE(5,157)
 157  FORMAT(1H ,'SS DUE TO REGRESSION')
      WRITE(5,94)A6
      WRITE(5,158)
 158  FORMAT(1H ,'SS ABOUT REGRESSION')
      WRITE(5,94)A7
      WRITE(5,159)
 159  FORMAT(1H ,'SS TOTAL CORRECTED FOR MEAN')
      WRITE(5,94)A5
      WRITE(5,166)
 166  FORMAT(1H ,'MS DUE TO REGRESSION')
      WRITE(5,94)A9
```

```
      WRITE(5,161)
  161 FORMAT(1H ,'MS ABOUT REGRESSION')
      WRITE(5,94)A8
      A10=A9/A8
      WRITE(5,162)
  162 FORMAT(1H ,' F STATISTIC')
      WRITE(5,94)A10
      WRITE(5,168)
  168 FORMAT(1H ,'R**2.')
      WRITE(5,94)A11
      GOTO 444
  110 DO 170 K=1,N
      X11(K)=2.0D0*X1(K)-X0(K)
  170 X0(K)=X1(K)
      Y0=Y1;Y11=F(X11)
      GOTO 111
  444 STOP;END
      DOUBLE PRECISION FUNCTION F(X)
      DOUBLE PRECISION X(10)
      DOUBLE PRECISION DB(30),DF(30),R(30)
      DOUBLE PRECISION SUM,TERM
      COMMON DB,DF,R,M
      SUM=0.0D0
      DO 10 I=1,M
      TERM=DB(I)-((X(1)*X(2)*DF(I))/(1.+X(1)*DF(I)))
      SUM=SUM+TERM*TERM
   10 CONTINUE
      F=SUM
      RETURN;END
```

APPENDIX 3

The listing of the Hooke computer programme used for the analysis of a one site with partition system.

```
C     MODEL IS DB=((K1*NP1*DF)/(1.+K1*DF))
C          + KNP
C      ANALYSIS FOR ONE CLASSES OF BINDING SITES
C           + PARTITION
      COMMON DB,DF,R,M
      DOUBLE PRECISION DB(18),DF(18),R(18),DBC(18),DIFF(18)
      DOUBLE PRECISION RC(18)
      DOUBLE PRECISION EPS,XK,XØ,YØ,X11,Y11,X1,Y1,X,Y,F,W
      DIMENSION XØ(1Ø),X(1Ø),X11(1Ø),X1(1Ø),XK(1Ø)
      DIMENSION DFEST(1Ø1),DBEST(1Ø1),REST(1Ø1)
   29 OPEN (UNIT=1,FILE='HOOKE.DAT')
      READ(1,39)M,(DB(I),I=1,M),(DF(I),I=1,M)
   39 FORMAT(6G)
   59 N=3
   69 WRITE(5,47)
   47 FORMAT(/,1H,'INPUT STEP SIZES K1,NP1,KNP,EPS,SEP RET')
      READ(5,2Ø)(XK(I),I=1,N),EPS
      WRITE(5,95)
   95 FORMAT (/,1H,'INPUT INITIAL VALUES OF K1,NP1,KNP,')
      READ(5,2Ø)(XØ(K),K=1,N)
   2Ø FORMAT(G)
      DO 89 I=1,M
      R(I)=DB(I)/DF(I)
  89  CONTINUE
      DO 411 I=M
      DFMAX=DF(M)
      B=(DFMAX+DFMAX/5Ø)/1Ø1
 411  CONTINUE
      WRITE(5,4Ø9)
 4Ø9 FORMAT (/,1H,'MAX DF')
      WRITE (5,41Ø)DFMAX
 41Ø FORMAT(1F12.5)
C**** COMPUTING PART********
      YØ=F(XØ)
      DO 3Ø I=1,N
   3Ø X11(I)=XØ(I)
      Y11=YØ
 111 DO 4Ø I=1,N
   4Ø X1(I)=X11(I)
      Y1=Y11
      I=Ø
 222 I=I+1
      DO 5Ø K=1,N
      IF(K.EQ.I) GOTO 6Ø
      W=Ø.ØDØ; GOTO 7Ø
   6Ø W=1.ØDØ
   7Ø X(K)=X1(K)+XK(K)*W
   5Ø CONTINUE
      Y=F(X)
      IF(Y.LT.Y1) GOTO 8Ø
      DO 51 K=1,N
      IF(K.EQ.I) GOTO 61
```

```
      W=Ø.ØDØ; GOTO 71
   61 W=1.ØDØ
   71 X(K)=X1(K)-XK(K)*W
   51 CONTINUE
      Y=F(X)
      IF(Y.GE.Y1)GOTO 9Ø
   8Ø DO 1ØØ K=1,N
  1ØØ X1(K)=X(K)
      Y1=Y
   9Ø IF(I.NE.N) GOTO 222
      IF(Y1.LT.YØ) GOTO 11Ø
      DO 12Ø K=1,N
      IF(X11(K).NE.XØ(K))GOTO 13Ø
  12Ø CONTINUE
      DO 555 K=1,N
  555 XK(K)=XK(K)/1Ø.ØDØ
      DO 666 K=1,N
      IF(DABS(XK(K)).GE.EPS)GOTO 111
  666 CONTINUE
      GOTO 333
  13Ø DO 14Ø K=1,N
  14Ø X11(K)=XØ(K)
      Y11=YØ
      GOTO 111
C****** COMPUTING FINISHED *****
  333 WRITE(5,15Ø)
  15Ø FORMAT (1H ,'SOLUTION VECTOR-K1')
      WRITE(5,94)XØ(1)
   94 FORMAT(1H ,G)
      WRITE(5,151)
  151 FORMAT(1H ,'SOLUTION VECTOR-NP1')
      WRITE(5,94)XØ(2)
      WRITE(5,152)
  152 FORMAT(1H ,'SOLUTION VECTOR-KNP')
      WRITE(5,94)XØ(3)
      WRITE(5,66Ø)YØ
  66Ø FORMAT(1H ,'FUNCTION VALUE=',G)
C     XO(1) IS K1 XØ(2) IS NP1 XØ(3) IS KNP
      DO 17 I=1,M
      DBC(I)=((XØ(1)*XØ(2)*DF(I))/(1+XØ(1)*DF(I)))
     1+(XØ(3)*DF(I))
      RC(I)=DBC(I)/DF(I)
      DIFF(I)=DBC(I)-DB(I)
  17  CONTINUE
      WRITE(5,24)
   24 FORMAT(/,1H,6X,'DF',1ØX,'DB',1ØX,'DBC',1ØX,'DIFF',
     1 11ØX,'R',1ØX,'RC')
      DO 54 I=1,M
   44 FORMAT(6F12.5)
      WRITE(5,44)DF(I),DB(I),DBC(I),DIFF(I),R(I),RC(I)
      DFES=Ø
   54 CONTINUE
      DO 42Ø I=1,1Ø1
      DFEST(I)=DFES+B
      DBEST(I)=((XØ(1)*XØ(2)*DFEST(I))/(1+XØ(1)*DFEST(I)))
     1+(XØ(3)*DFEST(I))
      REST(I)=DBEST(I)/DFEST(I)
```

```
      DFES=DFEST(I)
  420 CONTINUE
      NSET=2
      NINPUT=M*2
      OPEN(UNIT=1,FILE='FOR07.DAT')
      WRITE(1,400)NSET,NINPUT,(REST(I),I=1,101),(DBEST(I),I=1,101),
     1(R(I),I=1,M),(DB(I),I=1,M)
  400 FORMAT(2I,30(/10F7.3))
      GOTO 444
  110 DO 170 K=1,N
      X11(K)=2.0D0*X1(K)-X0(K)
  170 X0(K)=X1(K)
      Y0=Y1;Y11=F(X11)
      GOTO 111
  444 STOP;END
      DOUBLE PRECISION FUNCTION F(X)
      DOUBLE PRECISION X(10)
      DOUBLE PRECISION DB(18),DF(18),R(18)
      DOUBLE PRECISION SUM,TERM
      COMMON DB,DF,R,M
      SUM=0.0D0
      DO 10 I=1,M
      TERM=DB(I)-((X(1)*X(2)*DF(I))/(1+X(1)*DF(I)))
     1-X(3)*DF(I)
      SUM=SUM+TERM*TERM
   10 CONTINUE
      F=SUM
      RETURN;END
```

APPENDIX 4

The listing of the Hooke computer programme for the analysis of a two class system.

```
C     MODEL IS DB=((K1*NP1*DF)/(1.+K1*DF))
C                +((K2*NP2*DF)/(1.+K2*DF))
C      ANALYSIS FOR TWO CLASSES OF BINDING SITES
      COMMON DB,DF,R,M
      DOUBLE PRECISION A3,A4,A5,A6,A7,A8,A9,A10,A11
      DOUBLE PRECISION DB(30),DF(30),R(30),DBC(30),DIFF(30)
      DOUBLE PRECISION RC(30)
      DOUBLE PRECISION EPS,XK,X0,Y0,X11,Y11,X1,Y1,X,Y,F,W
      DIMENSION X0(10),X(10),X11(10),X1(10),XK(10)
      DIMENSION DFEST(101),DBEST(101),R1
   29 OPEN (UNIT=1,FILE='HOOKE.DAT')
      READ(1,39)M,(DB(I),I=1,M),(DF(I),I=1,M)
   39 FORMAT(6G)
   59 N=4
      WRITE(5,1)
 1    FORMAT (/,1H,'INPUT NUMBER OF CONSTANTS')
      READ(5,20)M2
   69 WRITE(5,47)
   47 FORMAT(/,1H,'INPUT STEP SIZES K1,NP1,K2,NP2,EPS,SEP RET')
      READ(5,20)(XK(I),I=1,N),EPS
      WRITE(5,95)
   95 FORMAT (/,1H,'INPUT INITIAL VALUES OF K1,NP1,K2,NP2,')
      READ(5,20)(X0(K),K=1,N)
   20 FORMAT(G)
      DO 89 I=1,M
      R(I)=DB(I)/DF(I)
  89  CONTINUE
      DO 411 I=M
      DFMAX=DF(M)
      B=(DFMAX+DFMAX/50)/101
 411  CONTINUE
      WRITE(5,409)
  409 FORMAT (/,1H,'MAX DF')
      WRITE (5,410)DFMAX
  410 FORMAT(1F12.5)
C**** COMPUTING PART********
      Y0=F(X0)
      DO 30 I=1,N
   30 X11(I)=X0(I)
      Y11=Y0
  111 DO 40 I=1,N
   40 X1(I)=X11(I)
      Y1=Y11
      I=0
  222 I=I+1
      DO 50 K=1,N
      IF(K.EQ.I) GOTO 60
      W=0.0D0; GOTO 70
   60 W=1.0D0
   70 X(K)=X1(K)+XK(K)*W
   50 CONTINUE
      Y=F(X)
```

```
      IF(Y.LT.Y1) GOTO 8Ø
      DO 51 K=1,N
      IF(K.EQ.I) GOTO 61
      W=Ø.ØDØ; GOTO 71
   61 W=1.ØDØ
   71 X(K)=X1(K)-XK(K)*W
   51 CONTINUE
      Y=F(X)
      IF(Y.GE.Y1)GOTO 9Ø
   8Ø DO 1ØØ K=1,N
  1ØØ X1(K)=X(K)
      Y1=Y
   9Ø IF(I.NE.N) GOTO 222
      IF(Y1.LT.YØ) GOTO 11Ø
      DO 12Ø K=1,N
      IF(X11(K).NE.XØ(K))GOTO 13Ø
  12Ø CONTINUE
      DO 555 K=1,N
  555 XK(K)=XK(K)/1Ø.ØDØ
      DO 666 K=1,N
      IF(DABS(XK(K)).GE.EPS)GOTO 111
  666 CONTINUE
      GOTO 333
  13Ø DO 14Ø K=1,N
  14Ø X11(K)=XØ(K)
      Y11=YØ
      GOTO 111
C****** COMPUTING FINISHED *****
  333 WRITE(5,15Ø)
  15Ø FORMAT (1H ,'SOLUTION VECTOR-K1')
      WRITE(5,94)XØ(1)
   94 FORMAT(1H ,G)
      WRITE(5,151)
  151 FORMAT(1H ,'SOLUTION VECTOR-NP1')
      WRITE(5,94)XØ(2)
      WRITE(5,152)
  152 FORMAT(1H ,'SOLUTION VECTOR-K2')
      WRITE(5,94)XØ(3)
      WRITE(5,16Ø)
  16Ø FORMAT(1H ,'SOLUTION VECTOR-NP2')
      WRITE(5,94)XØ(4)
      WRITE(5,66Ø)YØ
  66Ø FORMAT(1H ,'FUNCTION VALUE=',G)
C     XO(1) IS K1 XØ(2) IS NP1 XØ(3) IS K2 XØ(4) IS NP2
      DO 17 I=1,M
      DBC(I)=((XØ(1)*XØ(2)*DF(I))/(1.+XØ(1)*DF(I)))
     1+((XØ(3)*XØ(4)*DF(I))/(1.+XØ(3)*DF(I)))
      RC(I)=DBC(I)/DF(I)
      DIFF(I)=DBC(I)-DB(I)
   17 CONTINUE
      WRITE(5,24)
   24 FORMAT(/,1H,6X,'DF',1ØX,'DB',1ØX,'DBC',1ØX,'DIFF',
     1 11ØX,'R',1ØX,'RC')
      DO 54 I=1,M
   44 FORMAT(6F12.5)
      WRITE(5,44)DF(I),DB(I),DBC(I),DIFF(I),R(I),RC(I)
      DFES=Ø
```

```
   54 CONTINUE
C   ****** CREATES PLOT FILE *****
      DO 420 I=1,101
      DFEST(I)=DFES+B
      DBEST(I)=((X0(1)*X0(2)*DFEST(I))/(1.+X0(1)*DFEST(I)))
     1+((X0(3)*X0(4)*DFEST(I))/(1.+X0(3)*DFEST(I)))
      REST(I)=DBEST(I)/DFEST(I)
      DFES=DFEST(I)
  420 CONTINUE
      NSET=2
      NINPUT=M*2
      OPEN(UNIT=1,FILE='FOR07.DAT')
      WRITE(1,400)NSET,NINPUT,(REST(I),I=1,101),(DBEST(I),I=1,101),
     1(R(I),I=1,M),(DB(I),I=1,M)
  400 FORMAT(2I,30(/10F7.3))
C    CREATES FILE FOR NONLIN
      OPEN (UNIT=1,FILE='NONLIN.DAT')
      DO 115 I=1,M
      WRITE (1,1100) DB(I),DF(I)
  115 CONTINUE
 1100 FORMAT (12X, 2F6.3)
C    *********ANOVA*********
      N1=M-M2
      N2=M2-1
      N3=M-1
      WRITE(5,163)
 163  FORMAT(1H ,'NUMBER OF OBSERVATIONS')
      WRITE(5,94)M
      WRITE(5,164)
 164  FORMAT(1H ,'NUMBER OF CONSTANTS')
      WRITE(5,94)M2
      WRITE(5,165)
 165  FORMAT(1H ,'D.F. OF TOTAL')
      WRITE (5,94)N3
      WRITE(5,153)
 153  FORMAT(1H ,'D.F. ABOUT REGRESSION')
      WRITE(5,94)N1
      WRITE(5,154)
 154  FORMAT(1H ,'D.F. DUE TO REGRESSION')
      WRITE(5,94)N2
      A3=0
      DO 155 I=1,M
      A3=A3+DB(I)
 155  CONTINUE
      A4=A3/FLOAT(M)
      A5=0
      A7=0
      DO 156 I=1,M
      A5=A5+(DB(I)-A4)**2.
      A7=A7+(DB(I)-DBC(I))**2.
 156  CONTINUE
      A6=A5-A7
      A8=A7/FLOAT(N1)
      A9=A6/FLOAT(N2)
      A11=A6/A5
      WRITE(5,157)
 157  FORMAT(1H ,'SS DUE TO REGRESSION')
```

```
      WRITE(5,94)A6
      WRITE(5,158)
  158 FORMAT(1H ,'SS ABOUT REGRESSION')
      WRITE(5,94)A7
      WRITE(5,159)
  159 FORMAT(1H ,'SS TOTAL CORRECTED FOR MEAN')
      WRITE(5,94)A5
      WRITE(5,166)
  166 FORMAT(1H ,'MS DUE TO REGRESSION')
      WRITE(5,94)A9
      WRITE(5,161)
  161 FORMAT(1H ,'MS ABOUT REGRESSION')
      WRITE(5,94)A8
      A1Ø=A9/A8
      WRITE(5,162)
  162 FORMAT(1H ,' F STATISTIC')
      WRITE(5,94)A1Ø
      WRITE(5,168)
  168 FORMAT(1H ,'R**2.')
      WRITE(5,94)A11
      GOTO 444
  11Ø DO 17Ø K=1,N
      X11(K)=2.ØDØ*X1(K)-XØ(K)
  17Ø XØ(K)=X1(K)
      YØ=Y1;Y11=F(X11)
      GOTO 111
  444 STOP;END
      DOUBLE PRECISION FUNCTION F(X)
      DOUBLE PRECISION X(1Ø)
      DOUBLE PRECISION DB(3Ø),DF(3Ø),R(3Ø)
      DOUBLE PRECISION SUM,TERM
      COMMON DB,DF,R,M
      SUM=Ø.ØDØ
      DO 1Ø I=1,M
      TERM=DB(I)-((X(1)*X(2)*DF(I))/(1.+X(1)*DF(I)))
     1-((X(3)*X(4)*DF(I))/(1.+X(3)*DF(I)))
      SUM=SUM+TERM*TERM
   1Ø CONTINUE
      F=SUM
      RETURN;END
```

APPENDIX 5

An example of a data file used for Nonlin.

```
TWO BINDING SITES AN0  4   1   1      1Ø  99      Ø.ØØØ1     Ø.Ø 5 13
( A4, 8X, 2F6.Ø )
1.45      1.Ø       14.5       12.
Y=K1*N1*X/(1.+K1*X) + K2*N2*X/(1.+K2*X)
WHERE X=DF
      Y=DB
      K1,N1 ARE THE PRIMARY SITE CONSTANTS
      K2,N2 ARE THE SECONDARY SITE CONSTANTS
NP1       K1        NP2       K2
             Ø.4Ø6 Ø.594
             Ø.659 1.34Ø
             Ø.838 2.162
             Ø.97Ø 3.Ø3Ø
             1.Ø67 3.933
             1.ØØØ 4.919
             1.211 5.789
             1.272 6.728
             1.318 7.682
             1.355 8.644
END
```

APPENDIX 6

An example of a data file used with any of the Hooke programmes.

```
10,0.0777,0.156,0.233,0.311,0.389,
0.467,0.544,0.622,0.700,0.788,0.122,
0.224,0.367,0.489,0.661,0.773,0.855,
0.978,1.099,1.222,
```

APPENDIX 7

The listing of the graphics programme used to plot binding isotherms.

```
      DIMENSION YEST(101),XEST(101),ICHAR(5),AUHELP(14)
      DIMENSION YACT(20),XACT(20)
      OPEN(UNIT=1,FILE='FOR07.DAT')
      READ(1,5)NSET,NINPUT,YEST,XEST,(YACT(I),I=1,NINPUT/2)
     1,(XACT(I),I=1,NINPUT/2)
5     FORMAT(2I,30(/10F))
      WRITE(5,3)
3     FORMAT(////,1H.'IF YOU WANT HELP TYPE H. ?  ',$)
      READ(5,6)HELP
6     FORMAT(A1)
      IF (HELP .EQ. 'H') GOTO 9
      GOTO 1
9     OPEN(UNIT=1,FILE='AUHELP.DAT')
      DO 97 K2=1,32
      READ(1,99)(AUHELP(K1),K1=1,14)
99    FORMAT(14A5)
98    FORMAT(1H ,14A5)
97    WRITE(5,98)(AUHELP(K1),K1=1,14)
1     DO 110 I=1,101
      IF (YEST(I)-YEST(I+1))110,110,100
100   JMAX=I
      YMAX=YEST(I)
      GOTO 66
110   CONTINUE
66    WRITE(5,133)
133   FORMAT(1H ,'DO YOU WANT ANY MODIFICATION OF THE GRAPH')
      WRITE(5,134)
134   FORMAT(1H ,'READ THE HELP FILE BEFORE ANSWER YES OR NO.?',$)
      READ(5,135)DMODI
135   FORMAT(A3)
      IF (DMODI .EQ. 'YES') GOTO 120
      GOTO 137
120   DO 200 I=1,JMAX
      IF (YEST(I) .EQ. YMAX) GOTO 210
      IF (YEST(I+1)-YEST(I))180,180,200
180   YEST(I+1)=YEST(I)+0.003
200   CONTINUE
210   DO 300 I=JMAX,101
      IF (YEST(I) .EQ. YMAX) GOTO 300
      IF (YEST(I+1)-YEST(I)) 300,290,290
290   YEST(I+1)=YEST(I)-0.002
300   CONTINUE
      OPEN(UNIT=1,FILE='FOR08.DAT')
      WRITE(1,400)NSET,NINPUT,YEST,XEST,YACT,XACT
400   FORMAT(2I,30(/10F7.3))
137   WRITE(5,7)
7     FORMAT(//,1H,'INPUT LENGTH OF X-AXIS,DEFAULT VALUE=20')
      READ(5,8) XLEN
8     FORMAT(F)
      IF (XLEN .EQ. 0) XLEN=20.0
11    WRITE(5,13)
13    FORMAT(1H,'INPUT LENGTH OF Y-AXIS,MAX.= 20,DEFAULT VALUE=20')
      READ(5,15) YLEN
```

```
15      FORMAT(F)
        IF (YLEN .EQ. Ø) YLEN=2Ø.Ø
19      WRITE(5,21)
21      FORMAT(1H,'DO YOU WANT AUTO-SCALE,YES OR NO ?',$)
        READ(5,23) KYX
23      FORMAT(A3)
        IF (KYX .EQ. 'NO ' ) GOTO 22
        GOTO 4Ø
22      WRITE(5,24)YMAX
24      FORMAT(1H ,'MAX. ESTIMATED DB/DF.=',F)
25      WRITE(5,1Ø)
1Ø      FORMAT(1H ,'MAXIMUM VALUE OF DB/DF ? ',$)
        READ(5,2Ø) DY
2Ø      FORMAT(F)
        WRITE(5,3Ø)
3Ø      FORMAT(1H ,'MAXIMUM DB LIMIT ? ',$)
        READ(5,2Ø) DX
        DX=DX*1.1/XLEN
        DY=DY*1.1/YLEN
        GOTO 5Ø
4Ø      CALL SCAL2Ø(XEST,XLEN,1Ø1,1,FX,DX)
        CALL SCAL2Ø(YEST,YLEN,1Ø1,1,FY,DY)
5Ø      CALL PLTS2Ø(2Ø,'AUTOAN.PLT')
        CALL PLOT2Ø(2.Ø,2.Ø,-3)
        WRITE(5,55)
55      FORMAT(1H ,'LABEL OF THE X-AXIS, MAX. 25 CHARACTERS.')
        WRITE(5,6Ø)
6Ø      FORMAT($,1H ,'(',25X,')'/1H+,'(')
        READ(5,65)ICHAR(1),ICHAR(2),ICHAR(3),ICHAR(4),ICHAR(5)
65      FORMAT(5A5)
        CALL AXIS2Ø(Ø.Ø,Ø.Ø,ICHAR,-25,XLEN,Ø.Ø,FX,DX)
        WRITE(5,7Ø)
7Ø      FORMAT(1H ,'LABEL OF Y-AXIS,MAX. 25 CHARACTERS')
        WRITE(5,6Ø)
        READ(5,65)ICHAR(1),ICHAR(2),ICHAR(3),ICHAR(4),ICHAR(5)
        CALL AXIS2Ø(Ø.Ø,Ø.Ø,ICHAR,25,YLEN,9Ø.Ø,FY,DY)
        WRITE(5,8Ø)
        CALL LINE2Ø(XEST,FX,DX,YEST,FY,DY,1Ø1,1,Ø,Ø)
        CALL LINE2Ø(XACT,FX,DX,YACT,FY,DY,NINPUT/2,1,-1,11)
8Ø      FORMAT(1H ,'LABEL OF THE GRAPH MAX.5Ø CHARACTER')
        WRITE (5,6Ø)
        READ(5,65)ICHAR(1),ICHAR(2),ICHAR(3),ICHAR(4),ICHAR(5)
        CALL SYMB2Ø(4.Ø,(YLEN-Ø.5),Ø.28,ICHAR,Ø.Ø,25)
        WRITE(5,6Ø)
        READ(5,65)ICHAR(1),ICHAR(2),ICHAR(3),ICHAR(4),ICHAR(5)
        CALL SYMB2Ø(4.Ø,(YLEN-1.Ø),Ø.28,ICHAR,Ø.Ø,25)
        CALL SYMB2Ø(4.Ø,(YLEN+1),Ø.2,11,Ø.Ø,-1)
        ICHAR(1)=' ARE '
        ICHAR(2)='OBSER'
        ICHAR(3)='VED P'
        ICHAR(4)='OINTS'
        CALL SYMB2Ø(5.Ø,(YLEN+1.Ø),Ø.28,ICHAR,Ø.Ø,2Ø)
        CALL SYMB2Ø(4.Ø,(YLEN+Ø.5),1.Ø,28,Ø.Ø,-1)
        ICHAR(1)=' ARE '
        ICHAR(2)='PREDI'
        ICHAR(3)='CTED '
        ICHAR(4)='POINT'
```

```
      ICHAR(5)='S'
      CALL SYMB20(5.0,(YLEN+0.5),0.28,ICHAR,0.0,21)
      CALL PLTF20
      WRITE(5,33)
33    FORMAT(//,1H ,'THE PLOT FILE IS *AUTOAN.PLT*')
      END
```

APPENDIX 8

The listing of the computer programme used for the analysis of competitive binding data.

```
C     MODEL IS DB=((K1*NP1*DF)/(1.+DI*KI+K1*DF))
C                +((K2*NP2*DF)/(1.+K2*DF))
C     WITH COMPETITION
C      ANALYSIS FOR TWO CLASSES OF BINDING SITES
      COMMON DB,DF,R,M,DI
      DOUBLE PRECISION A3,A4,A5,A6,A7,A8,A9,A10,A11
      DOUBLE PRECISION DB(30),DF(30),R(30),DBC(30),DIFF(30)
      DOUBLE PRECISION RC(30),DI,KI
      DOUBLE PRECISION EPS,XK,X0,Y0,X11,Y11,X1,Y1,X,Y,F,W
      DIMENSION X0(10),X(10),X11(10),X1(10),XK(10)
      DIMENSION DFEST(101),DBEST(101),REST(101)
   29 OPEN (UNIT=1,FILE='HOOKE1.DAT')
      READ(1,39)M,(DB(I),I=1,M),(DF(I),I=1,M),DI
   39 FORMAT(6G)
   59 N=5
      WRITE(5,1)
 1    FORMAT (/,1H,'INPUT NUMBER OF CONSTANTS')
      READ(5,20)M2
   69 WRITE(5,47)
   47 FORMAT(/,1H,'INPUT STEP SIZES K1,NP1,K2,NP2,KI,EPS,SEP RET')
      READ(5,20)(XK(I),I=1,N),EPS
      WRITE(5,95)
   95 FORMAT (/,1H,'INPUT INITIAL VALUES OF K1,NP1,K2,NP2,KI')
      READ(5,20)(X0(K),K=1,N)
   20 FORMAT(G)
      DO 89 I=1,M
      R(I)=DB(I)/DF(I)
  89  CONTINUE
      DO 411 I=M
      DFMAX=DF(M)
      B=(DFMAX+DFMAX/50)/101
 411  CONTINUE
      WRITE(5,409)
  409 FORMAT (/,1H,'MAX DF')
      WRITE (5,410)DFMAX
  410 FORMAT(1F12.5)
C**** COMPUTING PART********
      Y0=F(X0)
      DO 30 I=1,N
   30 X11(I)=X0(I)
      Y11=Y0
  111 DO 40 I=1,N
   40 X1(I)=X11(I)
      Y1=Y11
      I=0
  222 I=I+1
      DO 50 K=1,N
      IF(K.EQ.I) GOTO 60
      W=0.0D0; GOTO 70
   60 W=1.0D0
   70 X(K)=X1(K)+XK(K)*W
   50 CONTINUE
```

```
      Y=F(X)
      IF(Y.LT.Y1) GOTO 80
      DO 51 K=1,N
      IF(K.EQ.I) GOTO 61
      W=0.0D0; GOTO 71
   61 W=1.0D0
   71 X(K)=X1(K)-XK(K)*W
   51 CONTINUE
      Y=F(X)
      IF(Y.GE.Y1)GOTO 90
   80 DO 100 K=1,N
  100 X1(K)=X(K)
      Y1=Y
   90 IF(I.NE.N) GOTO 222
      IF(Y1.LT.Y0) GOTO 110
      DO 120 K=1,N
      IF(X11(K).NE.X0(K))GOTO 130
  120 CONTINUE
      DO 555 K=1,N
  555 XK(K)=XK(K)/10.0D0
      DO 666 K=1,N
      IF(DABS(XK(K)).GE.EPS)GOTO 111
  666 CONTINUE
      GOTO 333
  130 DO 140 K=1,N
  140 X11(K)=X0(K)
      Y11=Y0
      GOTO 111
C****** COMPUTING FINISHED *****
  333 WRITE(5,150)
  150 FORMAT (1H ,'SOLUTION VECTOR-K1')
      WRITE(5,94)X0(1)
   94 FORMAT(1H ,G)
      WRITE(5,151)
  151 FORMAT(1H ,'SOLUTION VECTOR-NP1')
      WRITE(5,94)X0(2)
      WRITE(5,152)
  152 FORMAT(1H ,'SOLUTION VECTOR-K2')
      WRITE(5,94)X0(3)
      WRITE(5,160)
  160 FORMAT(1H ,'SOLUTION VECTOR-NP2')
      WRITE(5,94)X0(4)
      WRITE(5,1611)
 1611 FORMAT(1H ,'SOLUTION VECTOR KI1')
      WRITE(5,94)X0(5)
      WRITE(5,660)Y0
  660 FORMAT(1H ,'FUNCTION VALUE=',G)
C     XO(1) IS K1 X0(2) IS NP1 X0(3) IS K2 X0(4) IS NP2
      DO 17 I=1,M
      DBC(I)=((X0(1)*X0(2)*DF(I))/(1.+(DI*X0(5))+X0(1)*DF(I)))
     1+((X0(3)*X0(4)*DF(I))/(1.+X0(3)*DF(I)))
      RC(I)=DBC(I)/DF(I)
      DIFF(I)=DBC(I)-DB(I)
   17 CONTINUE
      WRITE(5,24)
   24 FORMAT(/,1H,6X,'DF',10X,'DB',10X,'DBC',10X,'DIFF',
     110X,'R',10X,'RC')
```

```
      DO 54 I=1,M
   44 FORMAT(6F12.5)
      WRITE(5,44)DF(I),DB(I),DBC(I),DIFF(I),R(I),RC(I)
      DFES=Ø
   54 CONTINUE
C   ****** CREATES PLOT FILE *****
      DO 42Ø I=1,1Ø1
      DFEST(I)=DFES+B
      DBEST(I)=((XØ(1)*XØ(2)*DFEST(I))/(1.+(DI*XØ(5))+XØ(1)*DFEST(I)))
     1+((XØ(3)*XØ(4)*DFEST(I))/(1.+XØ(3)*DFEST(I)))
      REST(I)=DBEST(I)/DFEST(I)
      DFES=DFEST(I)
  42Ø CONTINUE
      NSET=2
      NINPUT=M*2
      OPEN(UNIT=1,FILE='FORØ7.DAT')
      WRITE(1,4ØØ)NSET,NINPUT,(REST(I),I=1,1Ø1),(DBEST(I),I=1,1Ø1),
     1(R(I),I=1,M),(DB(I),I=1,M)
  4ØØ FORMAT(2I,3Ø(/1ØF7.3))
C    CREATES FILE FOR NONLIN
      OPEN (UNIT=1,FILE='NONLIN.DAT')
      DO 115 I=1,M
      WRITE (1,11ØØ) DB(I),DF(I)
  115 CONTINUE
 11ØØ FORMAT (12X, 2F6.3)
C    *********ANOVA*********
      N1=M-M2
      N2=M2-1
      N3=M-1
      WRITE(5,163)
 163  FORMAT(1H ,'NUMBER OF OBSERVATIONS')
      WRITE(5,94)M
      WRITE(5,164)
 164  FORMAT(1H ,'NUMBER OF CONSTANTS')
      WRITE(5,94)M2
      WRITE(5,165)
 165  FORMAT(1H ,'D.F. OF TOTAL')
      WRITE (5,94)N3
      WRITE(5,153)
 153  FORMAT(1H ,'D.F. ABOUT REGRESSION')
      WRITE(5,94)N1
      WRITE(5,154)
 154  FORMAT(1H ,'D.F. DUE TO REGRESSION')
      WRITE(5,94)N2
      A3=Ø
      DO 155 I=1,M
      A3=A3+DB(I)
 155  CONTINUE
      A4=A3/FLOAT(M)
      A5=Ø
      A7=Ø
      DO 156 I=1,M
      A5=A5+(DB(I)-A4)**2.
      A7=A7+(DB(I)-DBC(I))**2.
 156  CONTINUE
      A6=A5-A7
      A8=A7/FLOAT(N1)
```

```
      A9=A6/FLOAT(N2)
      A11=A6/A5
      WRITE(5,157)
  157 FORMAT(1H ,'SS DUE TO REGRESSION')
      WRITE(5,94)A6
      WRITE(5,158)
  158 FORMAT(1H ,'SS ABOUT REGRESSION')
      WRITE(5,94)A7
      WRITE(5,159)
  159 FORMAT(1H ,'SS TOTAL CORRECTED FOR MEAN')
      WRITE(5,94)A5
      WRITE(5,166)
  166 FORMAT(1H ,'MS DUE TO REGRESSION')
      WRITE(5,94)A9
      WRITE(5,161)
  161 FORMAT(1H ,'MS ABOUT REGRESSION')
      WRITE(5,94)A8
      A10=A9/A8
      WRITE(5,162)
  162 FORMAT(1H ,' F STATISTIC')
      WRITE(5,94)A10
      WRITE(5,168)
  168 FORMAT(1H ,'R**2.')
      WRITE(5,94)A11
      GOTO 444
  110 DO 170 K=1,N
      X11(K)=2.0D0*X1(K)-X0(K)
  170 X0(K)=X1(K)
      Y0=Y1;Y11=F(X11)
      GOTO 111
  444 STOP;END
      DOUBLE PRECISION FUNCTION F(X)
      DOUBLE PRECISION X(10)
      DOUBLE PRECISION DB(30),DF(30),R(30)
      DOUBLE PRECISION SUM,TERM
      COMMON DB,DF,R,M,DI
      SUM=0.0D0
      DO 10 I=1,M
      TERM=DB(I)-((X(1)*X(2)*DF(I))/(1.+(DI*X(5))+X(1)*DF(I)))
     1-((X(3)*X(4)*DF(I))/(1.+X(3)*DF(I)))
      SUM=SUM+TERM*TERM
   10 CONTINUE
      F=SUM
      RETURN;END
```

APPENDIX 9

COURSES AND CONFERENCES

The following courses and scientific meeting were attended in connection with this programme of research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

1) A residential course in fluorescence at the University of Technology, Loughborough, in the Department of Chemistry.

2) A statistic course held in the Department of Mathematics, Robert Gordons Institute of Technology, Aberdeen.

3) A postgraduate course in statistics and computing held in the Department of Mathematics, Robert Gordon's Institute of Technology, Aberdeen.

4) A meeting of the Scottish Pharmaceutical Society group in Edinburgh 1981

5) The British Pharmacological Society meetings in London, January 1983.

6) The British Pharmacological Society meetings in Cambridge, April 1983.

7) The British Pharmaceutical Society Conference in London September 1983.

COMPETITIVE BINDING STUDIES WITH DIPYRIDAMOLE AND α_1-ACID GLYCOPROTEIN

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Dipyridamole (DPD) is a clinically effective vasodilator and platelet anti-aggregatory agent. Its effectiveness is limited because of its marked binding affinity for plasma α_1 acid glycoprotein (AGP) (Subbarao et al (1977)).

All fluorescence measurements were made using a Baird Atomic Ratiometric spectrofluorimeter model RC200. The polarisation readings were made using the Baird polariser in this instrument. DPD is a highly fluorescent compound with two excitation maxima at 305 nm and 420 nm and an emission maximum at 490 nm. Using an excitation wavelength of 295 nm, a wavelength which will only excite the tryptophan residues on the AGP (Chignell (1970)), and an emission wavelength of 335 nm, it was found that DPD binding quenched the fluorescence of the protein, this being associated with an increase in the fluorescence of the DPD thus indicating resonance transfer of energy. Using the method of analysis suggested by Chignell et al (1970) for binding with quenching, association constants $k = 4.07 \pm 0.4 \times 10^7\ M^{-1}$ and $k_2 = 5.70 \pm 0.35 \times 10^5\ M^{-1}$ were obtained. The quenching effect was paralleled by a marked fluorescence polarisation at the 420 nm peak. The polarisation data was analysed using the method of Guarino et al (1973) and gave association constants $k_1 = 2.88 \pm 0.15 \times 10^7\ M^{-1}$ and $k_2 = 6.98 \pm 0.3 \times 10^5\ M^{-1}$. The data obtained suggested that one DPD molecule binds to two sites on the AGP with different association constants. Urea which causes the protein to unfold and form a random coil abolished both the quenching and the polarisation effect. The extent of quenching suggested that more than one tryptophan residue is involved in the binding. A cross linking of DPD between strands of the chain in the protein molecule is envisaged. These findings confirm and extend the observations of El-Gamel (1982) who used optical rotation measurements to study the interaction between DPD and AGP.

For competitive studies fluorescence polarisation was used because it was specific for DPD binding. The specificity of this binding was demonstrated by the fact that RA233 (2,6-bis(diethanolamino)-4-piperidino-pyrimido-(5,4-d) pyrimidine), structurally very similar to DPD, did not quench the protein's fluorescence or show a change in polarisation on binding. We found that hydrophobic planar molecules compete most efficiently for the DPD primary binding site. Examples of competitors given in order of inhibition constants (Segal (1975)are RA233 $K_1 = 1.5 \times 10^{-5}$ M, papaverine $K_1 = 2.5 \times 10^{-5}$ M, S-H1242 (5-methyl-3 piparazinyl-1-(4-thiomorpholinyl) isochinolin-s-oxide) $K_1 = 3.1 \times 10^{-5}$ M and propranolol $K_1 = 3.7 \times 10^{-5}$ M. Compounds that did not compete include adenosine, caffeine and methotrexate.

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