A STUDY OF THE ASSOCIATION OF ORGANIC ACIDS WITH HUMAN SERUM ALBUMIN

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Science of the University of London,

by

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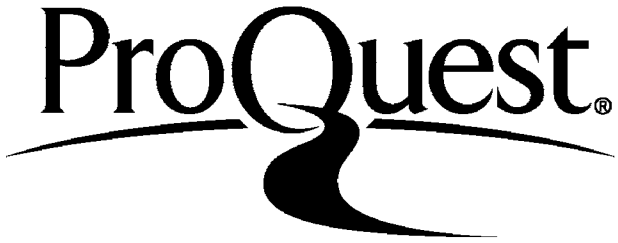
Bedford College, London.



This thesis comprises a report of full-time research undertaken by the author in the Physical Chemical Laboratories of Bedford College, University of London, from October, 1978, to September, 1981.

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ABSTRACT

U.V./Visible spectral changes accompanying the interactions of some organic acids with human serum albumin have been studied in detail.

The spectra have been analysed by means of matrix rank analysis to estimate the number of spectrophotometrically distinguishable species in solution. This may be used to estimate the number of distinct classes of binding sites on the albumin molecule.

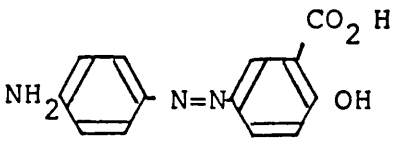
A new method has been developed to obtain equilibrium constants and numbers of binding sites per albumin molecule from spectrophotometric data.

The method involved the titration of a constant concentration dye solution with human serum albumin. The series of spectra thus obtained were analysed by means of a computer assisted data fitting routine. The routine was based on a model for the system, using two independent classes of binding sites on the albumin molecule. A series of derivatives of azobenzene were studied by the method in order to correlate structural features of the molecules with the extent to which they bound to human serum albumin.

The interactions of the azobenzene derivative series, with human serum albumin, were studied by an ultrafiltration technique. Projected ultrafiltration binding curves from the U.V./visible spectrophotometric experiments were found to be in agreement with those measured experimentally.

Spectral changes accompanying the competitive interactions between Bromophenol Blue, and the azobenzene

derivatives, with human serum albumin, have also been studied. An attempt has been made to correlate these results with the foregoing binding experiments.



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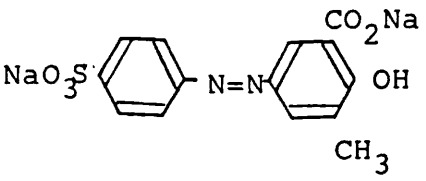

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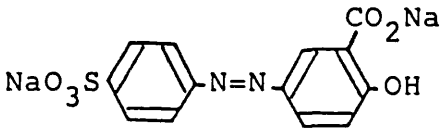

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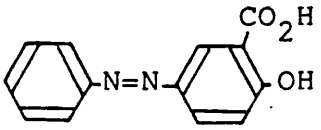

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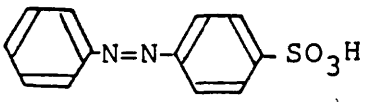
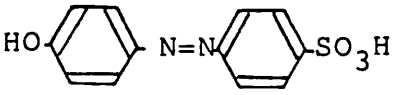
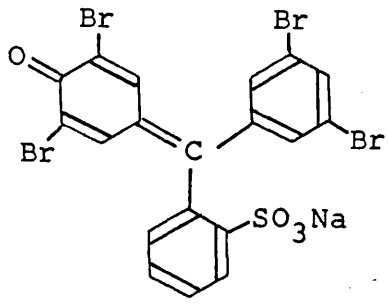


4-azobenzenesulphonic acid

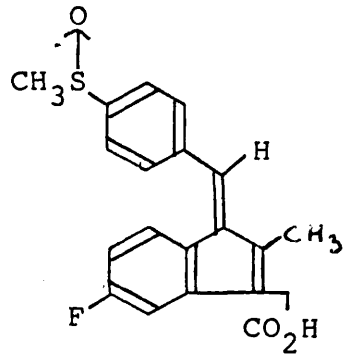


4-hydroxyazobenzene-4'-sulphonic acid

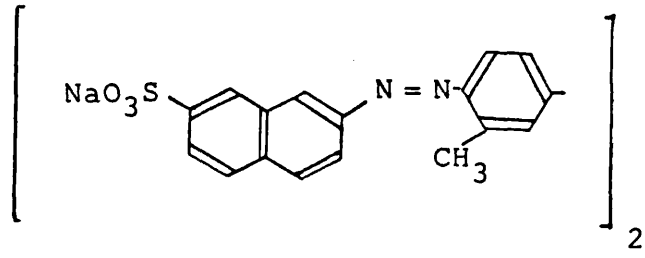
Compounds used in this study



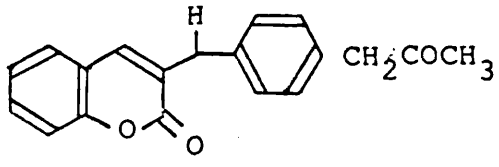
Bromophenol Blue



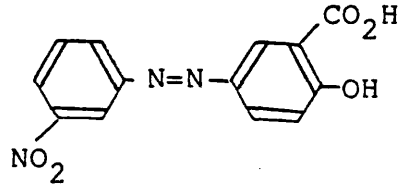
Sulindac



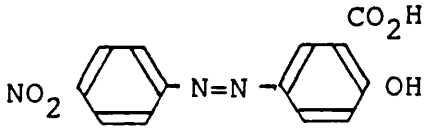
Evans Blue



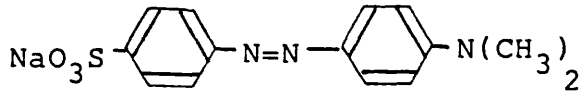
Warfarin



Alizarin Yellow GG



Mordant Orange 1



Methyl Orange

Compounds used in this study

CHAPTER 1

INTRODUCTION

1.1. General aspects of drug-albumin interactions

The interactions between drugs (pharmacologically active molecules) and blood proteins have been extensively studied in recent years. Binding of drug molecules to plasma proteins is important because drugs will not normally be biologically active when bound to a plasma protein unless they displace pharmacologically active substances from their binding sites.

Drugs, other xenobiotics, and endogenous compounds may be transported in the blood stream in simple solution as a suspension or bound to plasma proteins or blood cells. If a drug is bound in this way to one of the blood constituents its potential for interaction with receptor sites may be limited. Similarly its availability to the drug metabolism and excretory systems may be reduced. If these binding processes are weak and reversible it is likely that they are of little therapeutic significance. However, for strongly bound drugs, plasma binding may strongly influence a drug's potential bioavailability.

Plasma binding may also be of importance in the case of the displacement of a drug by a more strongly bound competitor. Thus a strongly plasma bound compound, such as 'Warfarin', can be displaced by the administration of other plasma bound drugs such as acetyl salicylic acid (Aspirin). Such effects will seriously increase the levels of free drug biologically available.

1.2. Human serum albumin -

1.2.1. Structure and function

The principal components of human blood are as follows:[1]

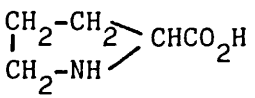
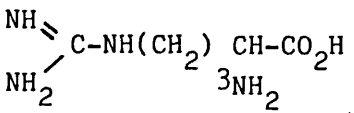
Component	Concentration/g dm^{-3}
Albumin	40
α-Globulin	3.1
α_2-Globulin	4.8
β-Globulin	8.1
Fibrinogen	3.4
γ-Globulin	7.4
Erythocytes	150

As can be seen, albumin is the principal plasma protein and it makes the largest contribution towards the plasma protein binding of most drugs, as well as interacting with other endogenous materials such as fatty acids and bilirubin. The unique binding capacity of albumin is thought to be necessary for the transport of endogenous materials in the blood, and possibly across cell membranes.

Although it is generally accepted that for a healthy human adult the plasma concentration is between 35 and 45 g dm^{-3} this may alter by 10-20% with posture or during exercise. Many diseases also lower the serum albumin levels such as nephrosis, hepatitis, cirrhosis, cancers, gastrointestinal diseases, hypergammaglobulinaemia, malnutrition, heart disease and hypothyroidism. Levels will also be lower during infancy, pregnancy, senility and in individuals suffering from the effects of stress, injury, alcohol, and acclimatisation to heat.

Higher levels of endogenous substances which competitively bind with albumin such as fatty acids or bilirubin will also decrease the level of albumin drug binding. The albumin molecule consists of a single polypeptide chain with an isoelectric point at pH 5.0. At pH 7.4

Fig. 1.1. Amino acid sequence of human serum albumin. Residues assigned by analogy with bovine serum albumin are shown in lower case letters.

Parent amino acid	Structure	Number of residues/ molecule
Aspartic acid (Asp)	$HO_2CCH_2CH(NH_2)CO_2H$	39
Asparagine (Asn)	$CONH_2CH_2CH(NH_2)CO_2H$	15
Threonine (Thr)	$CH_3CH(OH)CH(NH_2)CO_2H$	30
Serine (ser)	$HOCH_2CH(NH_2)CO_2H$	22
Glutamic acid (Glu)	$HO_2C(CH_2)CH(NH_2)CO_2H$	60
Glutamine (Gln)	$CONH_2CH_2CH_2CH(NH_2)CO_2H$	23
Proline (Pro)		25
Glycine (Gly)	$NH_2CH_2CO_2H$	12
Alanine (Ala)	$CH_3CH(NH_2)CO_2H$	63
Cystine/2 (Cys)	$SCH_2CH(NH_2)CO_2H$	34
Arginine (Arg)		25

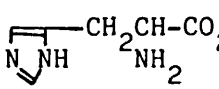
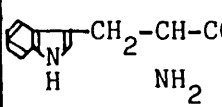
Parent amino acid	Structure	Number of residues/ molecule
Cysteine (CySH)	$HSCH_2CH(NH_2)CO_2H$	1
Valine (Val)	$(CH_3)_2CHCH(NH_2)CO_2H$	39
Methionine (Met)	$CH_3S(CH_2)_2CH(NH_2)CO_2H$	6
Isoleucine (Ile)	$CH_3CH_2CH(CH_3)CH(NH_2)CO_2H$	8
Leucine (leu)	$CH_3CHCH_2CH(NH_2)CO_2H$	61
Tyrosine (Tyr)	$p\text{-}HOC_6H_4CH_2CH(NH_2)CO_2H$	18
Phenylalanine(phe)	$C_6H_5CH_2CH(NH_2)CO_2H$	30
Histidine (His)		16
Lysine (Lys)	$NH_2(CH_2)_4CH(NH_2)CO_2H$	58
Tryptophan (Trp)		1

Table 1.1. Amino acid residual data for the human serum albumin molecule

(physiological pH) it, therefore, carries a net negative charge (arising from C terminal, aspartic, glutamic and tyrosine residues). It also carries a smaller number of positive charges (arising from histidine, N-terminal, Lysine and arginine residues).[2]

The albumin molecule appears to exist partly as an α-helix and partly as a random coil. At pH 4.0 albumin undergoes a conformational transition which results in an increased viscosity and greater electrophoretic mobility.

Further conformational changes occur at lower pH values. Thus it is important that drug binding studies should be performed at known, buffered, pH.

The amino-acid residue sequence of human serum albumin proposed by Brown[3] is shown in Fig.1.1. Further information is continued in Table 1.1. The structure is based on tryptic and chymotryptic peptides. The disulphide bridges are based on analogy with bovine serum albumin[4]. The structure is in agreement with those obtained by other laboratories[5-15] for various fragments of the molecule. Geisow[16] has described this structure as consisting of six α-helices (three double loops) which form a roughly cylindrical domain, the total molecule being made up of an association of three such domains (Fig.1.2).

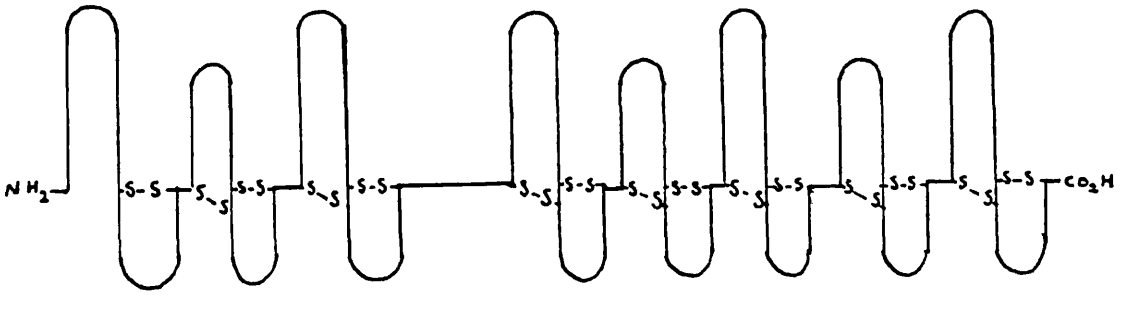


Fig.1.2. The double loop structure of serum albumin.[16]

1.2.2. Human serum albumin - preparation and purification

Commercially human serum albumin is still prepared by the method of Cohn[17] and his associates, this is usually obtainable as 'Cohn Fraction V'. Since the purity of Fraction V is only about 96%, re-crystallised, globulin free albumins are also commercially available, their preparation being generally based on the method described by Cohn, Hughes and Weare.[18]

Cohn Fraction V.[17]

Human blood plasma is cooled quickly to $0^{\circ}C$ without allowing ice formation. The pH of the solution is adjusted to 7.2 with an acetate buffer in an ethanol-water system so that the final ethanol concentration of the system is 8% by volume. The solution is cooled to between $-2^{\circ}C$ and $-3^{\circ}C$, a fraction ('Fraction I') precipitates out, consisting mainly of fibrinogen. The precipitate is removed by centrifugation. Adjustment of the solution to 25% ethanol at pH 6.9 and $-5^{\circ}C$ gives rise to a second precipitate consisting principally of β and γ-globulins ('Fraction II + III). The fraction contains nearly all of the immune globulins and the isoagglutinins. Nearly all the prothrombin is precipitated in this fraction. It also contains large amounts of cholesterol and other lipid substances.

After removal of this fraction the supernatant is adjusted to pH 5.2 and 18% ethanol at $-5^{\circ}C$. A fraction consisting mainly of α-globulin and lipids separates out (Fraction IV-1). The supernatant is next brought to a pH of $5.80 \pm .05$ and an ethanol concentration of 40%, the

ionic strength of the solution being 0.09 mol dm^{-3}. By centrifuging at $-5^{o}C$ a fraction (Fraction IV-4) is obtained consisting mainly of α-and β-globulins and some albumin.

At a pH of 4.8 and an ethanol concentration of 40% Fraction V is obtained. This fraction contains the bulk of the albumin present in human plasma.

Re-crystallisation

Human serum albumin may be re-crystallised from ethanol-water mixtures usually with the aid of some other organic reagent such as decanol, chloroform, or benzene. The most successful method used by Cohn et al[18] was by re-crystallising Fraction V at $-5^{o}C$ from a solution consisting of 25% ethanol, 0.2% decanol at a pH of 5.4, and an ionic strength of 0.15 mol dm^{-3}, attained by the addition of sodium acetate solution.

Laminar crystals are obtained in 90% yield after standing for several days.

Removal of fatty acids

Re-crystallised human serum albumin may still contain fatty acids bound to the protein. Chen[19,20] has described a method for their removal. To a 10% w/w solution of albumin in water is added charcoal (5% w/w) and the pH of the solution lowered to 3.0 by the addition of hydrochloric acid (0.2 mol dm^{-3}). The charcoal is removed by centrifugation, and the solution then brought to pH 7.0 by the addition of 0.2 mol dm^{-3} sodium hydroxide. The protein in this solution may be used directly, or re-precipitated at pH 4.8 as described previously.

1.3 Methods of studying protein-drug interactions

Many methods have been explored in the determination of albumin binding of small ions and molecules. The most important of these are outlined below. In this instance all species bound to the protein molecule will be referred to as 'ligands', even in the case of metals. This is a reversal of the usual convention in which the protein would be described as the ligand binding to the metal.

1.3.1. Dialysis[21,22].

This is by far the most widely used technique, and the major part of the data found in the literature concerning quantitative aspects of albumin-small molecule interactions has been determined by this means. At its simplest the technique takes the form of fig. 1.3.

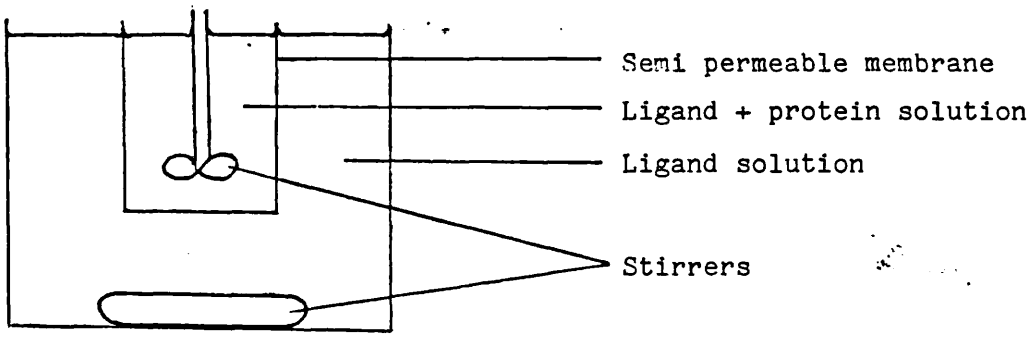


Fig.1.3. Schematic representation of a dialysis experiment.

The protein component is confined within a semi-permeable membrane through which unbound ligand molecules can freely diffuse. At equilibrium the unbound ligand activities on both sides of the membrane must be equal, and any increment in the protein compartment is presumed to represent bound ligand.

Being the most widely used technique for measurements of this kind comparison with other workers is easy.

However, the technique suffers from several drawbacks. Both protein and ligand may bind to the dialysis membrane. The Donnan effect must also be considered and appropriate corrections made in the case of ionic ligands, especially where the total ionic strength is low. Non-physiological conditions and prolonged equilibration times may cause bacterial contamination and protein denaturation.

1.3.2. Gel Filtration[23-25].

The method has certain similarities with equilibrium dialysis. Generally a sephadex column is equilibrated with a solution of ligand.

A small volume (relative to the total volume of the column) of protein, dissolved in the equilibrating solution is applied to the top of the column. The column is eluted with the ligand solution.

After passing through the column the solution in the vicinity of the protein will have essentially returned to the equilibrium concentration, having removed ligand from the gel. Consequently the solution eluted immediately behind the protein band shows a marked reduction in ligand concentration. The ligand concentration is usually followed by U.V/Visible spectrophotometry.

1.3.3. Ultracentrifugation[26,27]

If a solution containing protein and a small molecule is subjected to the influence of a centrifugal field there will be a redistribution of the molecules in accordance with the thermodynamic equations for sedimentation equilibrium. At relatively low centrifugal fields (about 10^4 x gravity) the redistribution of molecular mass about 10^5

is such that the concentration in the ultracentrifuge cell with a ligand column height of 3 mm varies about 30 fold when sedimentation equilibrium is achieved. Small molecules with molecular masses of about 10^2 under such field would be virtually uniformly distributed throughout the cell. At the same time the chemical equilibrium must be maintained at each level in the ultracentrifuge cell and, therefore, the total concentration of drug must vary with the concentration of albumin. Observation of the variation of protein concentration (e.g. by interferometry) and ligand concentration (e.g. by absorption spectrophotometry) can yield the information required to give dissociation constants and the number of binding sites per molecule in the system. The theory for the sedimentation equilibrium technique is rigorous and its application straightforward. Assumptions and approximations which must be made are minimal and subject to experimental test.

Sedimentation velocity methods have also been investigated, although they are theoretically not so satisfactory. Experimentally, however, they are more rapid, and often simpler to apply.

1.3.4. Electrophoresis[28-30]

Small molecules (especially ionic compounds) when bound to the albumin molecule should change the electrophoretic mobility of the molecule. However, since an H.S.A. molecule at pH 7.4 has about 100 positive charges the binding of two molecules, even if totally by an electrostatic interaction will only decrease this charge

by about 2%.

The method can be useful for obtaining qualitative information, and has the advantage that multiprotein mixtures can be used if necessary. It is usual for a Tiselius type apparatus to be used, whilst boundaries are observed by means of a Schlieren technique.

The success of the method depends on two characteristics of electrophoresis.

a) The velocity of an ion in a given potential gradient depends on its charge and, therefore, in part, on the number of small ions with which it is combined.

b) Although as the boundary moves the more highly charged ions move faster, there is a continuously instantaneously re-established equilibrium so that the boundary moves with a constant average velocity $\bar{U}p$, which is related in a linear fashion to the average molal ratio in the protein complex. The velocity is, of course, affected by the entire environment, (other electrolytes present, counterions etc.) so that the relation of $\bar{U}p$, and the extent of complex formation applies strictly only to conditions in which the environment undergoes no change other than small increments in ligand concentration. Even when this restriction is satisfied, it is necessary to make the assumption (which is known to be invalid for a number of linear polyelectrolytes) that the potential increment due to each anion bound is not altered as charges accumulate. i.e. the ratio of counterions accompanying the ion, to ions bound, does not depend on total or net charge. It is also necessary to assume that the frictional coefficient which depends on conformation does not depend on the extent of complex formation.

1.3.5. Nuclear Magnetic resonance.[31-35]

When a ligand is bound to a protein the relaxation characteristics of one or more of the protons of the ligand, or one or more of the protons of the protein may be altered.

This alteration may show up as a 'chemical shift', frequency change, or as the broadening of a line in the proton magnetic resonance spectrum.

Selective broadening of some of the protons resonances of the small molecule is always observed when it is bound to a protein, and has been interpreted as a sign of intimate contact between such protons, or the groups to which they belong, and the macromolecule. The chemical shifts are small and difficult to measure, they arise from rapid reversible changes of the state of the ligand from bound to unbound, and reflect not only the shifts in the nuclei which are caused by binding, but also the time average of the fraction of ligand bound.

1.3.6. Fluorescence Spectrophotometry[36-39]

Fluorescent molecules, when combining with protein, frequently show a change in the intensity or polarisation of the radiation emitted.

Daniel and Weber[40] for example, studied the interaction of 1-anilino-8-naphthalene sulphonate (A.N.S.) at various extents of combination with bovine serum albumin. The fluorescence spectra of the A.N.S.-B-S.A. complexes (fig.1.4) show that as the average number of molecules of ligand per molecule of protein ($\bar{n}$) increases, the A.N.S. fluorescence (λmax 469nm) increases, concurrently the protein fluorescence (λmax 343 nm) decreases.

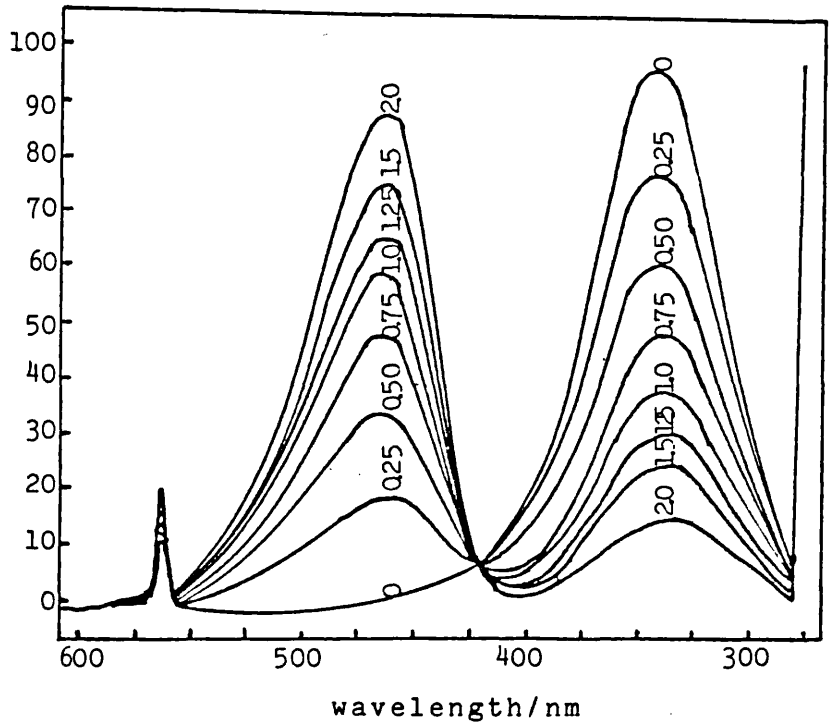


Fig.1.4. Fluorescence spectra of ANS-BSA at various values of $\bar{n}$. B-S.A. concentration 10g dm^{-3} in 0.1 mol dm^{-3} buffer pH7.0. Bandwidths of excitation and emission 3nm. An isoemissive point is observed at 416 nm.

They attributed this protein fluorescence to tyrosine and tryptophan residues. Inspection of the spectra indicated that the fluorescence of tyrosine was relatively less quenched than that of tryptophan.

They were able to treat the spectra mathematically, and to obtain values for the average number of ligand molecules bound to each albumin molecule under various conditions by making the following assumptions:-

(i) The quantum yield of free A.N.S. was negligably small compared with bound A.N.S.

(ii) The quantum yield of bound A.N.S. is at least to a good approximation independent of $\bar{n}$, or is a very slowly varying function of $\bar{n}$.

In the case of non-fluorescent molecules, fluorescent quenching of the tryptophan and tyrosine residues may be used for determining interactions between proteins and

small molecules[37]. Although it must be realised that such studies implicitly assume that binding occurs only at or very near to, the albumin tryptophan or tyrosine residues.

1.3.7. Ultra violet/visible spectrophotometry

The U.V/visible spectra of many compounds undergo changes in the presence of serum albumin. These are marked by a shift in the position of maximum absorbance, and a change in the overall shape of the absorption bands.

Klotz[41], appears to have been the first to investigate spectrophotometrically, interactions between organic anions and negatively charged proteins.

He chose for the organic anions three closely related compounds, Azosulphathiazole (fig.1.5), Orange I (fig.1.6), and Orange II (fig.1.7) using bovine serum albumin as the protein at pH 6.9

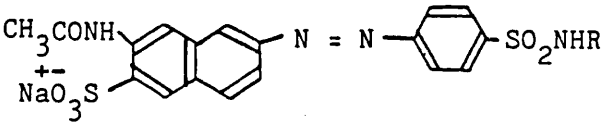


Fig.1.5 Azosulphathiazole

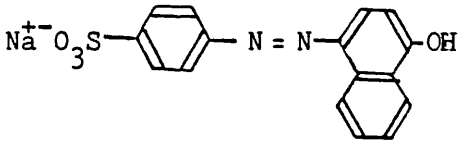


Fig.1.6 Orange I

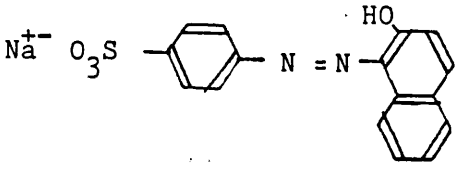


Fig.1.7 Orange II

Azosulphathiazole was shown by Klotz to obey Beer's law up to a concentration of approximately 10^{-3} mol dm^{-3}. From this he concluded that the dye existed in the monomeric state in aqueous solution at concentrations below 10^{-3} mol dm^{-3}. He also observed that the spectrum of the dye was independent of pH over the range 2 to 9.

On addition of a few hundredths of a per cent of bovine serum albumin the spectrum of Azosulphathiazole was significantly altered (fig.1.8).

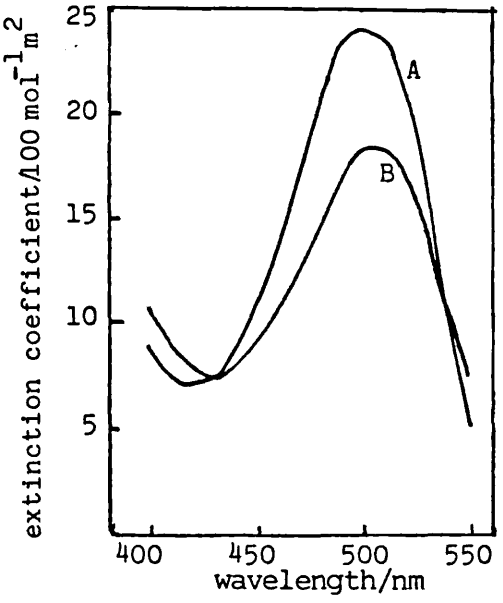


Fig.1.8 Absorption specta of Azosulphathiazole

A in buffer at pH 6.92

B in buffer containing B.S.A. (0.2%) at pH 6.90.

The alteration could not be attributed to the formation of dimers or polymers, as the dye had been firmly estabished as monomeric in this concentration range, nor were they attributable to a pH change. They must, therefore, have been due to combination of the protein with the dye anion to form an intermolecular complex.

Once an albumin concentration of 0.2% had been exceeded (for a dye concentration of approximately 1×10^{-5} mol dm^3) no further spectral change was observed, which Klotz concluded to be consistent with total binding of the dye by the protein.

Knowing, therefore, the spectral shapes for both free and bound dye it was possible to deduce, for any B.S.A.-dye solution, the concentration of bound drug from the equation,

$$A = (\varepsilon_f C_f + \varepsilon_b C_b)l$$

where A = the absorbance of the sample at a wavelength λ

l = the path length

ε_f = the extinction coefficient of the free ligand at the wavelength λ

C_f = the concentration of free ligand

ε_b = the extinction coefficient of the bound ligand at the wavelength λ

C_b = the concentration of bound ligand.

Similar changes were observed in the spectra of Orange I and Orange II, in the visible region, when these dyes interacted with bovine serum albumin, the extinction coefficients at the maxima being reduced, although no significant shift of the spectrum towards the red was observed. Methyl Orange also behaved similarly, but a shift in the spectrum towards the blue was observed on binding.

From these studies it seemed most likely that the sulphonate group common to all these substances was primarily responsible for the binding to the protein as in the case of albumin with detergents.

However, it appeared that the relative effect of the protein on the energies of the ground and excited states of the dyes was dependent on the nature of the entire molecule and not only the character of the binding sulphonate group.

Klotz also conducted some semi-quantitative competitive experiments with various simple organic acids containing one, or no aromatic ring.

On addition of the acid to a standard solution of Methyl Orange, or Azosulphathiazole, the dye was partially displaced from the protein, and thus the spectrum moved towards that of the free dye spectrum. Measurement of the extinction coefficient at the maxima allowed a comparison of the displacing ability of the acids.

Freedman and Johnson[42] used the spectrophotometric method of Klotz to determine binding constants for the dye Evans Blue, (p 12) with human, bovine, canine and rabbit serum albumins. They showed that for canine and bovine serum albumin the binding data fitted better to a two binding site model than to a single binding site model. In the case of human and rabbit serum albumin they obtained better values for a single site model. They also showed that for bovine serum albumin the binding data is dependent on the ionic strength of the buffer solution containing the dye-albumin system.

Glazer[43] extended the basic methods of Klotz in an investigation of the binding of Biebrich Scarlet (fig.1.9) to the enzyme α-chymotrypsin.

The visible spectrum of Biebrich Scarlet shows a red shift, and a decrease in the maximum extinction coefficient value in the presence of α-chymotrypsin (fig.1.10). Glazer ascribed this to protein binding of the dye. From the difference spectra produced as a result of the titration of Biebrich Scarlet with the enzyme, Glazer calculated a dissociation constant (without comment on the mathematical procedures involved) of 8.8×10^{-5} mol^{-1} dm^3, assuming a 1:1 complex. Using a gel filtration technique and a standard Langmuir isotherm treatment of the results he obtained a value for the dissociation constant of the complex as 8.9×10^{-5} mol^{-1} dm^3.

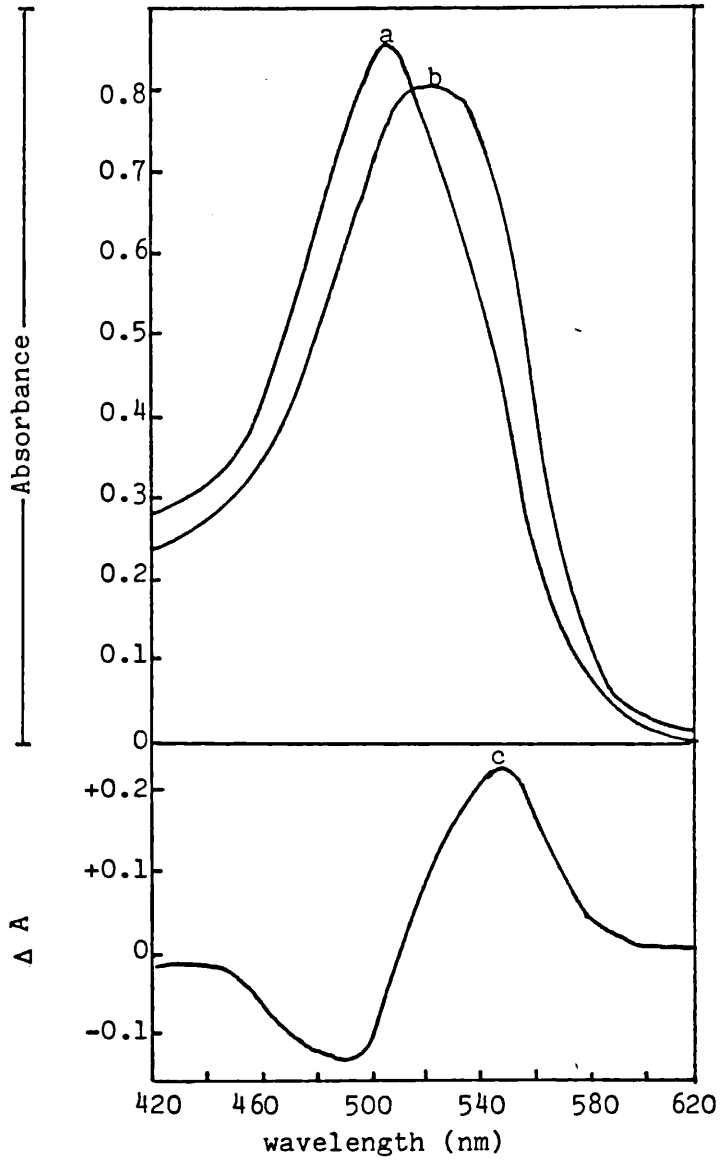


fig. 1.10 Effect of α-Chymotrypsin on the visible spectrum of Biebrich Scarlet. All spectra were obtained in 0.1 mol dm^{-3} phosphate buffer at 22°C at a Biebrich Scarlet concentration of 2.88 x 10^{-5} mol dm^{-3}. A 1 cm light path was used in all cases.

Curve (a) Biebrich Scarlet
Curve (b) Biebrich Scarlet in presence of α Chymotrypsin (1.8 x 10^{-4} mol dm^{-3})
Curve (c) Difference spectrum of Biebrich Scarlet and α-Chymotrypsin (1.8 x 10^{-4} mol dm^{-3}) vs Biebrich Scarlet.

Lang and Lasser[44] attempted a similar spectrophotometric study of the binding of Trypan Blue, fig.1.11, to bovine serum albumin.

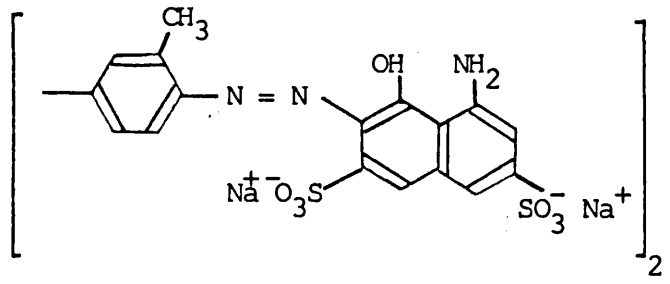


Fig.1.11 Trypan Blue

In this instance the shape of the difference spectrum was not independent of albumin concentration (see fig.1.12). This effect was explained as being due to a number of sets of sites on the albumin molecule giving rise to a number of complexes with differing molar extinction coefficients at any particular wavelength.

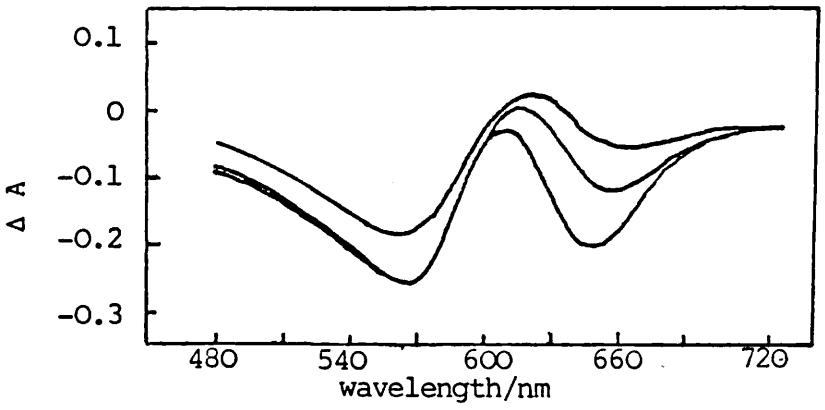


Fig.1.12. Difference spectra at pH 7.4 for the Trypan Blue to B.S.A. ratios indicated. Trypan Blue concentration = 2.56×10^{-5} mol dm^{-3}.

Shams-Eldeen, et al[45] used difference spectrophotometry to study the interaction of Sulindac with human serum albumin. They published a set of difference spectra without any indication of the absortion spectrum of

Sulindac either in the absence, or the presence of, H.S.A. They did not attempt to interpret the difference spectra quantitatively. They did, however, publish some fluorescence quenching data which suggested that Sulindac had an association constant of about 1 x 10^5 mol^{-1} dm^3, although this disagreed with dialysis data from the same series of experiments which gave a value of 6 x 10^4 mol^{-1} dm^3.

1.3.8. Ultrafiltration

Ultrafiltration is basically an extension of the dialysis technique which goes some way towards rectifying the problems encountered as a result of the long time scale required for dialysis.

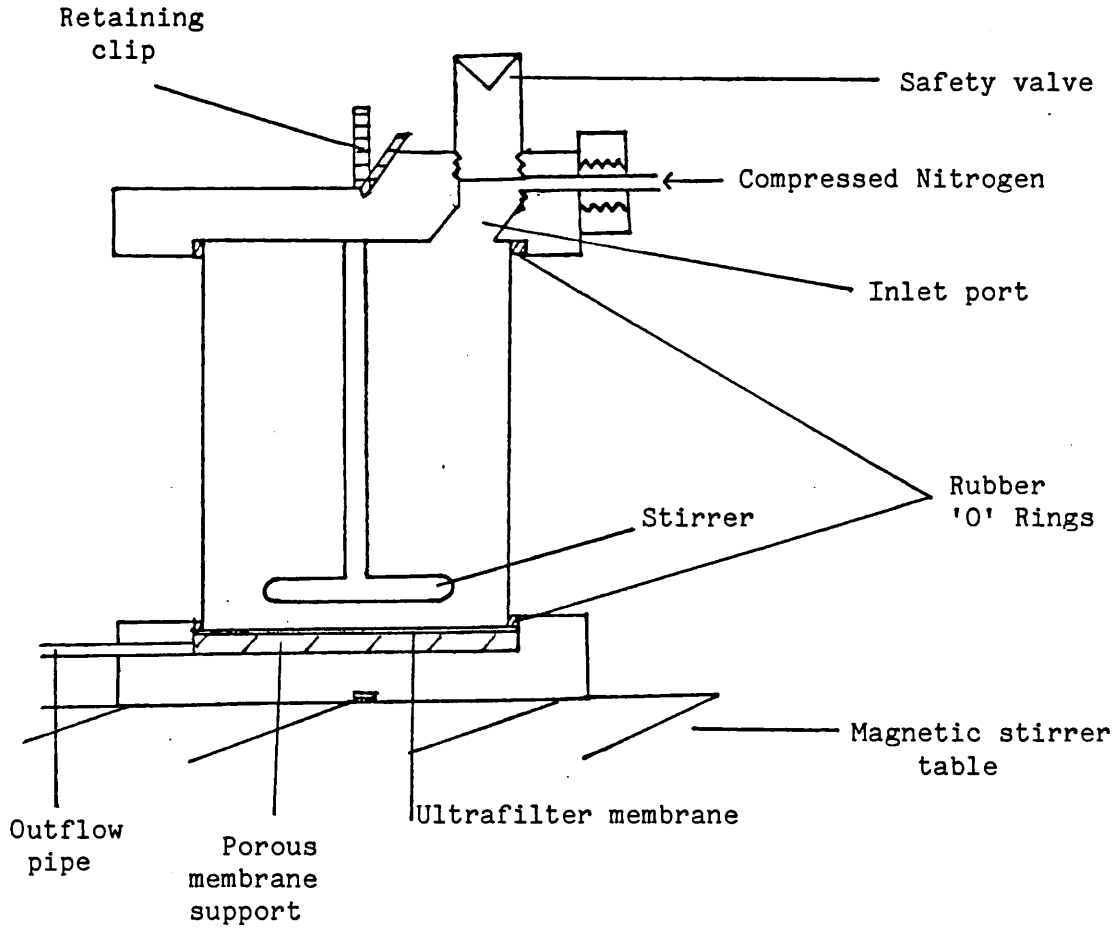


Fig.1.13 Diagramatic representation of the 'Amicon' ultrafiltration apparatus (Model 12)

A representation of a commercially available ultrafiltration apparatus is shown in fig.1.13.

A solution containing the protein and ligand to be observed is passed, under pressure, through an ultrafiltration membrane. This membrane will not allow macromolecules to pass whilst allowing small ligands to pass freely. The two major problems associated with the technique are those of binding to the filter and rejection by the filter membrane. Binding of both protein and ligand to membrane may occur, this is usually countered by equilibrating the membrane with the solution to be studied before filtration.

In many cases the ligand may not diffuse entirely freely across the ultrafiltration membrane, making the mathematical interpretation of data difficult.

The concentration of diffused ligand obtained at the outflow may be measured by any quantitative technique, although spectrophotometric measurement seems to be the most widely used.

Some workers[46] have modified the apparatus slightly to ensure that the protein concentration in the cell remains constant throughout the course of the experiment, in this instance the technique is known as 'diafiltration'.

These techniques have not been as widely used as dialysis.

Farese, Mager, and Blatt[47], have used ultrafiltration to determine diffusible calcium in serum. Blatt, Robinson and Bixler[48], made a limited study on the binding

of Methyl Orange to human serum albumin. They obtained binding data for the system although they gave no values for equilibrium constants. Their data was not in close agreement with the data of Klotz et al.[49], obtained by dialysis.

Crawford et al.[46] have studied the interaction of human serum albumin with Bromosulphthalein. Again they produced binding data but not binding constants or site numbers.

1.4. Interactions between albumin and small ligands

1.4.1. Cations

Albumin shows a much smaller capacity for binding cations than it does for anions. A number of interactions have been studied between metal ions and bovine serum albumin.

Hg^{2+} binds covalently at the sulphydryl group[50,51] whilst Cu^{2+} and Ni^{2+} bind at the square planar chelate ring formed by the α-amino nitrogen, the first two peptide nitrogens and the primary nitrogen of the imidazole ring of the third histidine residue[52-58]. Albumins such as canine or porcine albumin lacking the histidine residue in position 3 do not bind copper or nickel as tightly.

Mn^{2+} is thought to bind at the site formed between the $\alpha\text{-}NH_2$ group and the imidazole group[59], with $K = 2.8 \times 10^4$ mol^{-1} dm^3. Other metal cations appear to be less specific in their binding site and are bound less strongly, e.g. Co^{2+} ($K = 6.5 \times 10^3$ mol^{-1} dm^3),[60] Zn^{2+} ($K = 3 \times 10^2$ mol^{-1} dm^3)[61,62] and Cd^{2+} ($K = 1.7 \times$

10^2 mol^{-1} dm^3)[63-65]. The binding of calcium ion by serum albumin is important physiologically, but also shows only a weak affiliation with K = 1 x 10^2 mol^{-1} dm^3 [66-68].

1.4.2. Long chain anions

Anionic detergents are well known to bind strongly to human, bovine, and other serum albumins.

It is thought that one of the major functions of serum albumin is the transport of fatty acids and other long chain anions. Since long chain fatty acids are highly insoluble at physiological pH it is, therefore, necessary that they should be strongly bound by serum albumin.

Table 1.2 lists some binding data for organic anions binding with human, and with bovine, serum albumin.

Sites for binding fatty acids are probably hydrophobic clefts into which the aliphatic chains are inserted. In affinity chromatography, albumin attaches to the "tail" of palmitate[83] or other hydrocarbons[84] which have been immobilised on agarose.

It also appears that for a series of long chain anions possessing the same length of hydrophobic tail, their affinity for serum albumin increases as the polar end group goes from OH to CO_2H to SO_3^- to SO_4^-.

1.4.3. Anionic drugs

Binding studies of drugs to albumin have been extensively reviewed by Goldstein[85], and more recently by Meyer and Guttman[86] and Vallner[87].

Despite the size of the literature on the subject, a great part of this is qualitative or semi-quantitative.

Anion	Serum albumin	pH	K /mol^{-1} dm^{3}	n[a]	Refs
Oleate	Human	7.4	2.6×10^{8}	1	69
Oleate	Bovine	7.45	8.0×10^{7}	2	70
			8.0×10^{5}	5	
Palmitate	Human	7.4	6.2×10^{7}	1	69
Palmitate	Bovine	7.45	6.0×10^{7}	6-7	70
			3.0×10^{6}		
Linoleate	Bovine	7.4	1.3×10^{7}	2	70
			2.5×10^{6}	5	
Stearate	Bovine	7.45	1.1×10^{8}	2	70
			4.0×10^{6}	5	
Tetradecanoate	Bovine	7.45	4.0×10^{6}	7-8	70
			1.4×10^{6}		
Dodecanoate	Bovine	6.8	2.3×10^{5}	6-7	71
			1.6×10^{6}	2	70
			2.4×10^{5}	5	70
Decanoate	Bovine	6.8	6×10^{4}	6-7	71
Octanoate	Bovine	6.8	5×10^{4}	4-5	71
Bilirubin	Human	7.4	1×10^{8}	1	72
		7.4	7×10^{7}	1	73
Hematin	Human	7.5	5×10^{7}	1	74
L-thyroxine	Human	7.4	1.6×10^{6}	1	75
L-tryptophan	Human	7.4	1.6×10^{4}	2	76
Estradiol	Human	7.4	1.0×10^{5}	(1)	77
Progesterone	Human	7.4	3.7×10^{4}	(1)	77
Cortisol	Human	7.4	5.0×10^{3}	2	78
Corticosterone	Human	7.4	1.3×10^{4}	(1)	78
Aldosterone	Human	7.4	$<5 \times 10^{3}$	(1)	79
Testosterone	Human	7.4	4.2×10^{4}	(1)	80
Prostaglandin	Human	7.5	7.0×10^{4}	2	81
Urate	Human	7.4	3.0×10^{2}	(1)	82

[a] Parenthesis mean that n = 1 was assumed.

Table 1.2 Binding data for some organic anions.

It is common for the percentage of a drug bound to albumin to be quoted. This is misleading since the percentage bound is dependent on protein and drug concentrations. Even when binding constants have been determined for drug-albumin interactions, it is usual to assume a single binding site model, which appears from the work done here to be an erroneous assumption. In cases where the binding properties of the same substance have been studied by different workers, values of constants frequently vary widely.

Nevertheless it appears that albumin has a strong affinity for most anionic drugs, in some cases the affinity is so strong that little of the drug exists as the unbound species. Table 1.3 lists a few examples of drug-albumin interactions.

Compound	Primary affinity constant $mol^{-1}dm^3$	No. of Sites	Ref.
Sulphadiazene	3.6×10^3	1	88
Sulphisomidine	5.0×10^3	1.1	89
Sulphathiazole	2.96×10^3	2.0	90
Salicylate	4.0×10^5	1	91
	2.19×10^5	4	92
Warfarin	2.20×10^5	2	93
	1.4×10^6	1	94
	6.5×10^4	2.0	95
Phenylbutazone	2.7×10^5	2	98
	4.4×10^5	0.8	95
Oxacillin	4.7×10^3	1	96
Penicillin G	1.1×10^3	1	97
Ampicillin	5.0×10^2	1	96
Carbenicillin	2.0×10^3	1	96
Tolbutamide	4.1×10^4	1.4	99
Chlorpropamide	1.09×10^4	1.64	99

Table 1.3. Binding data for some anionic drugs with human serum albumin.

1.4.4. Anionic Dyes

Numerous anionic dyes have been studied for their interaction with blood proteins. Of these the azo-dyes are probably the most widely investigated group. In addition to those azo-dyes previously mentioned, Karush[100], has studied binding of Methyl Orange to bovine serum albumin by partition analysis and dialysis. He obtained Scatchard data (see p 45) for the dye protein system. Klotz and Luborsky[101] studied the effect of adding Glycine or β-Alanine to solutions containing Methyl Orange or p-aminoazobenzene, and bovine serum albumin. They found that addition of the amino-acids increased binding. They attributed this to both an increase in the dielectric constant of the solution and a specific interaction of the added amino acid.

Burkhard[102] conducted some competition experiments between Methyl Orange, anthranilic acids and bovine serum albumin, and between Methyl Orange, p-aminobenzoic acid and bovine serum albumin. He found that the anthranilate ion was more effective than the p-aminobenzoate ion in displacing Methyl Orange from protein surfaces.

Klotz et al.[103] carried out some spectrophotometric and dialysis experiments with a range of azo-dyes. These included Methyl Orange, Ethyl Orange, Propyl Orange, Butyl Orange, Methyl Red, ortho and para Methyl Red (fig.1.17) 4'-dimethylaminoazobenzene-3-phosphonate, 4'-dimethylaminoazobenzene-4-phosphonate and 4'dimethylaminoazobenzene-4-arsonate. From these studies it appeared that 'optical displacement is a measure of degree of binding' although this statement is not necessarily true in all

cases. (see Ch.8)

The same group of workers[104] measured the relative binding of Methyl Orange, Methyl Red, para Methyl and meta Methyl Red to bovine and human albumin. In all cases they observed greater binding to human albumin than to bovine albumin.

Karusch[105] has also studied, by dialysis, the binding of p-(2-hydroxy-5-methylphenylazo)-benzoic acid for which he proposed at 25°C, a two binding site model. He found the primary affinity constant to be 6.16×10^4 mol^{-1} dm^3 and the number of primary binding sites per molecule to be 4.66. The secondary sites (17.34 per molecule) had a binding constant of 1.90×10^3 mol^{-1} dm^3. He also showed[106] that the same dye competes with dodecyl sulphate in its reaction with bovine serum albumin.

Burkhard et al.[107] showed that binding of 4'-amino benzene-4-sulphonate increased if the amino nitrogen was alkylated, or the amino group removed.

French and Pritchard[108] showed that the spectral shape of dimethyl-p-aminoazobenzene depends on the buffer used, and also observed no spectral change on addition of bovine fibrinogen fraction I or bovine plasma albumin.

Uzman[109] showed that Methyl Orange binds more strongly to bovine serum albumin denatured with urea and guanidine hydrochloride, than to native protein.

A polarographic method for studing the binding of Evans Blue with rabbit plasma has been used[110], and dye binding curves have been produced.

1.5 Data Plotting

Many ways of presenting binding data have been suggested. The Langmuir isotherm is one such approach.[111]

Consider the reaction between a drug (D) and a single class of binding site on the protein (S).

$$D + S = DS$$

The association constant K is given by

$$K = \frac{[DS]}{[D][S]} \qquad (1.1)$$

Let the total concentration of sites be $[S_{tot}]$ and the fraction of sites bound be θ

Therefore $[DS] = [S_{tot}]\theta$

$$[S] = (1 - \theta)[S_{tot}]$$

$$K = \frac{[S_{tot}]\theta}{[D](1-\theta)[S_{tot}]}$$

$$K[D] = \theta(1 + K[D])$$

hence $$\theta = \frac{K[D]}{1 + K[D]}$$

The Langmuir isotherm is, therefore, a curve which approaches a straight line, gradient K as the free drug concentration approaches zero.

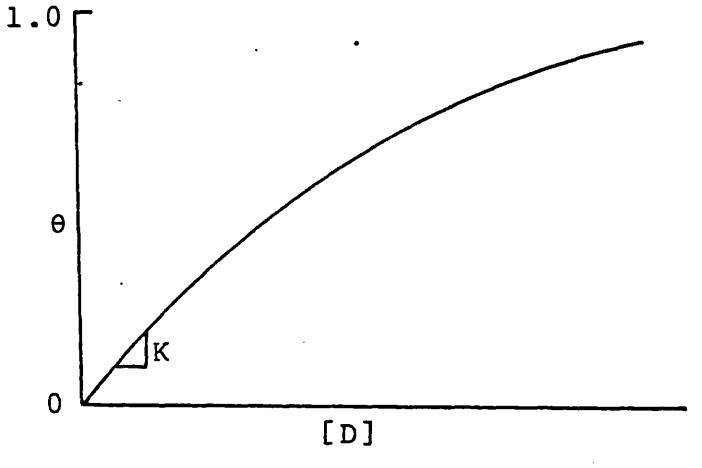


Fig. 1.14 Typical Langmuir isotherm

Much better approaches to the problem are provided by reciprocal, Klotz or Scatchard plots.

Reciprocal plots

From equation (1.1) and the relationship that

$$[S] = [S_{tot}] - [DS]$$

$$K = \frac{[DS]}{[D]([S_{tot}]-[DS])}$$

$$\frac{1}{K} = \frac{[D]([S_{tot}]-[DS])}{[DS]} \qquad (1.2)$$

$$\frac{1}{K} = [D][S_{tot}]\left(\frac{1}{[Ds]} - \frac{1}{[S_{tot}]}\right)$$

$$\frac{1}{K} \cdot \frac{1}{[D]}[S_{tot}] + \frac{1}{[S_{tot}]} = \frac{1}{[DS]} \qquad (1.3)$$

Since $[S_{tot}]$ is not known usually it is assumed that $[S_{tot}]$ is proportional to the total albumin concentration [P]

thus $$[S_{tot}] = n\,[P] \qquad (1.4)$$

n is, therefore, the number of sites per albumin molecule if $[S_{tot}]$ and [P] are in the same units.

Substituting 1.4 into 1.3.

$$\frac{1}{nk} \cdot \frac{1}{[D][P]} + \frac{1}{n[P]} = \frac{1}{[DS]} \qquad (1.5)$$

Thus at constant albumin concentration a plot of 1/[DS] versus 1/[D] gives a straight line, gradient 1/nK[P] and intercept 1/n[P].

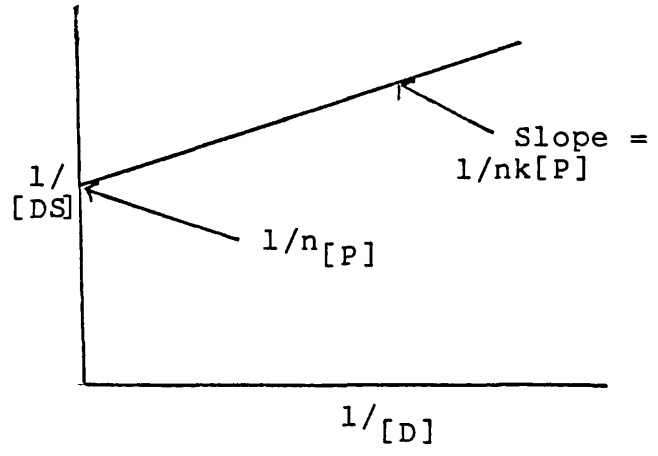


Fig. 1:15
Typical reciprocal plot

Alternatively with non constant albumin concentrations, a plot of [P]/[DS] versus 1/[D] gives a line of gradient 1/nk intercept 1/n. This form of plot is known, usually as a Klotz plot[41].

Scatchard Plots[112]

From equation (1.5)

$$\frac{1}{nk} \cdot \frac{1}{[D][P]} + \frac{1}{n[P]} = \frac{1}{[DS]}$$

$$\frac{1}{nk} \cdot \frac{[DS]}{[D][P]} + \frac{[DS]}{n[P]} = 1$$

$$\frac{[DS]}{[D][P]} = nk - \frac{[DS]}{[P]} \cdot K$$

Thus a plot of [DS]/[P] (often called r), versus r/[D], gives a straight line, intercept nk and gradient -k.

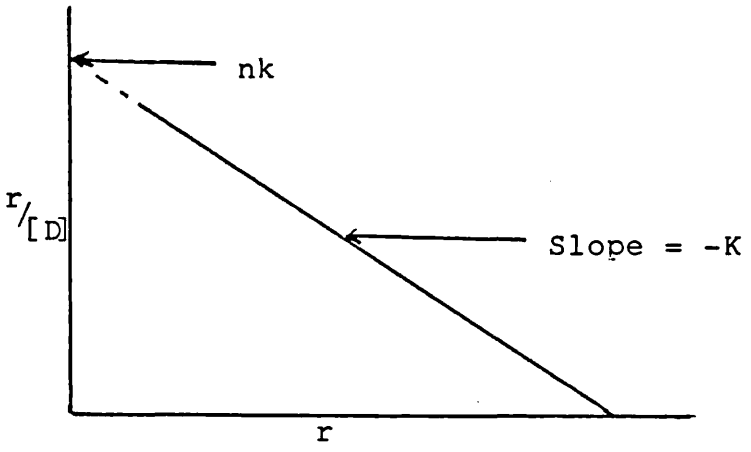


fig.1.16 Typical Scatchard plot

1.6 Objectives of this investigation

The objectives of this investigation were:

a) To develop a reliable, single phase technique to study the interactions between human serum albumin and organic anions.

b) To use the method to study a series of related compounds, and to correlate structural features of

the compounds with their binding characteristics.

c) To confirm these binding characteristics by means of a conventional multiphase method for measuring the binding of anions to proteins (ultrafiltration).

d) To develop a single phase method to study competitive interactions between anions and human serum albumin.

e) To correlate competitive studies with the single ligand binding studies.

CHAPTER 2

EXPERIMENTAL TECHNIQUES.

2.1. Materials

2.1.1. Buffer solutions

AnalaR potassium dihydrogen orthophosphate, and disodium hydrogen orthophosphate were purchased from B.D.H. Chemicals Ltd. All solutions used in this study were made up in phosphate buffer, pH 7.4, consisting of 0.05334 mol dm^{-3} $Na_2HPO_4.12H_2O$ and 0.01334 mol dm^{-3} KH_2PO_4.[113] The buffer solutions were always filtered prior to use, stored in the refrigerator, and never kept for more than a week.

2.1.2. Proteins

Human serum albumin, Cohn fraction V, human albumin, crystallised lyophilised and essentially globulin free, (H.S.A.C.L.E.G.F.), and bovine serum albumin, Cohn fraction V, were all purchased from the Sigma Chemical Company. Protein solutions were made in the following way:-
To avoid frothing, the buffer solution was added to a previously weighed portion of the crystalline material, slowly and down the side of the weighing vessel. The solution was allowed to stand for approximately twenty minutes to allow dissolution of the protein. The solution was then gently agitated to ensure mixing. If further dilution was necessary the contents of the vessel were poured slowly down the side into a volumetric flask, and made up to volume with buffer solution, always adding further buffer in a way so as to prevent frothing. Mixing was achieved by gentle agitation of the flask. Protein solutions were never used after two days.

2.1.3. Dyes and pharmaceuticals

Methyl Orange 1, Mordant Yellow 12, Mordant Yellow 7 and Mordant Yellow 10, were purchased from the Aldrich

Chemical Company Inc. 5-phenylazosalicylic acid and 4-hydroxyazobenzene-4'-sulphonic acid were supplied by the Alfred Bader Library of Rare Chemicals, a division of the Aldrich Chemical Company Inc. Bromophenol Blue and azobenzene were obtained from B.D.H. Chemicals Ltd., and Evans Blue from Hopkin and Williams Ltd. Warfarin was purchased from the Sigma London Chemical Company.

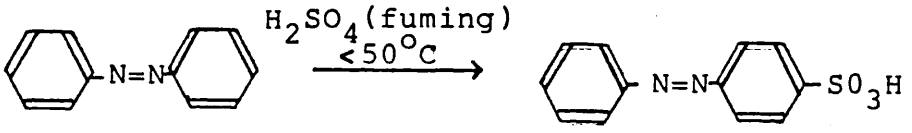
Sulindac was a gift from Merck, Sharpe and Dohme. M.T.T. was donated by the Wellcome Research Foundation.

None of these compounds were further purified.

In addition two further dyes, Alizarin Yellow GG and 4-azobenzenesulphonic acid were synthesised according to the procedures described below.

All the dyes and pharmaceuticals were found to obey the Beer-Lambert law over the concentrations used.

2.1.4. Synthesis of 4-azobenzenesulphonic acid trihydrate.[114]



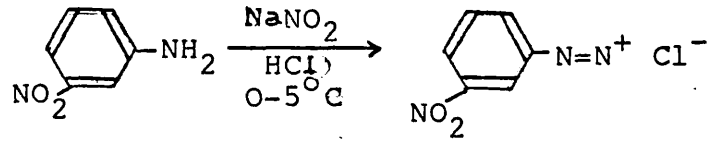
Azobenzene (10g) was pulverised by grinding in a mortar. $30cm^3$ of fuming sulphuric acid was placed in a $100cm^{-3}$ round bottomed flask equipped with an efficient stirrer. The powdered azobenzene was added slowly with stirring, and each portion was allowed to dissolve before the next was added. The temperature of the reaction mixture was maintained below 50^oC. The addition of the azobenzene required about thirty minutes. The mixture was then heated on a steam bath at 75^o-80^oC for 5 minutes. The mixture was cooled to 50^oC and poured slowly with vigorous stirring onto $150cm^3$ of ice. $30cm^3$ of concentrated HCl was added to the hot solution and

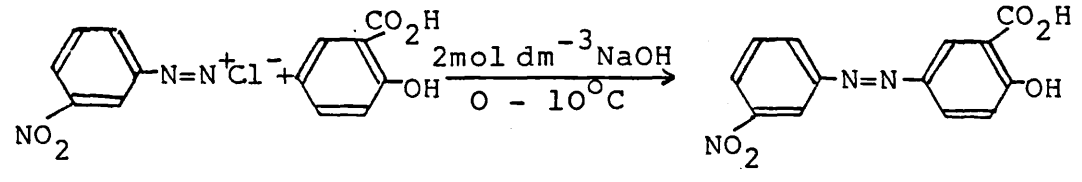
the mixture cooled and refrigerated overnight. The orange crystals of 4-azobenzenesulphonic acid were filtered on a Buchner funnel. The filtration was very slow. The precipitate was sucked as dry as possible, and whilst still moist was dissolved in 30 cm^3 of hot water. The solution was filtered, and 30cm^3 of 95% ethanol and 30 cm^3 of concentrated HCl were added to the filtrate. The mixture was cooled overnight in the refrigerator. The precipitate was filtered and washed with a cold mixture of 10 cm^3 each of water, alcohol and concentrated HCl, and then air dried.

The dry acid was dissolved in the minimum quantity of hot ethyl acetate and the solution filtered and cooled in the refrigerator overnight. The precipitate was filtered and re-crystallised from the minimum quantity of a mixture containing 30% ethanol, 30% water and 40% concentrated HCl.

The product was obtained in a yield of 53.2% and its purity checked by its melting point of 127-129°C (literature value = 127°C[115]), and an elemental analysis for nitrogen, carbon and hydrogen. (N = 9.0%, C = 45.4%, H = 5.0%, the theoretical composition is:- N = 8.85%, C = 45.5% and H = 5.06%).

2.1.5. Synthesis of Alizarin Yellow GG





3-nitroaniline was suspended and heated with a mixture of concentrated HCl ($25cm^3$) and water ($25cm^3$) to form a fine suspension of the hydrochloride. This was then cooled quickly in an ice-salt mixture and a solution of sodium nitrite (7.0g) in water ($15cm^3$) was added dropwise over 15 minutes, keeping the temperature at 0-5°C. Stirring was continued for a further 30 minutes, keeping the temperature at less than 10°C. The filtered, cold, solution was then added to a solution of salicylic acid (14g) in 2 mol dm^{-3} sodium hydroxide ($100cm^3$) containing crushed ice (150g). The dye was liberated by the addition of concentrated HCl. The yellow crystals were filtered on a Buchner funnel and washed with concentrated HCl. The product was then recrystallised by dissolving in the minimum quantity of hot ethyl acetate, filtering and then cooling. The recrystallisation was repeated twice. The product was obtained in a yield of 27.8%.

The infrared spectra of the acid and sodium salt was compared with Aldrich standard spectra, and the product analysed for nitrogen, carbon, and hydrogen (N = 14.9%, C = 54.1%, H = 3.1%, the theoretical composition is:- N = 14.64%, C = 54.37%, H = 2.79%).

2.2. Experimental methods

2.2.1. Method 1. Spectrophotometric titrations

All absorption spectra were measured using a Perkin Elmer 555 U.V/visible spectrophotometer, using a slit width of 1.0 nm. The cell compartments were electrically thermostated at 21.0°C (±0.1°C) using a Perkin Elmer C550-0555 Peltier type thermostat.

Clean, dry Helma 'Tandem' spectrophotometric cells (path length of each compartment = 0.4375 cm) were initially balanced in the spectrophotometer by performing a background correction over the wavelength range to be measured.

1 cm^3 of dye solution was placed in one of the sample compartments whilst the remaining sample compartment, and those of the reference cell, were filled with 1 cm^3 of buffer solution. (The contents of the buffer filled compartment of the sample cell and the corresponding compartment of the reference cell were left unaltered throughout the course of the experiment.)

The U.V/visible absorption spectrum was then measured over the required wavelength range. Absorption measurements were recorded on a printer sequencer (Perkin Elmer PRS-10) at 10nm intervals over the described wavelength range.

0.01 cm^3 of a stock albumin solution and 0.01cm^3 of a dye solution of twice the sample strength were added to the sample cell by means of a micropipette. 0.01cm^3 of the albumin solution and 0.01 cm^3 of buffer solution were added to the reference cell. The contents of each cell was stirred by means of a separate Pasteur pipette. The same pipette was kept for each cell throughout the course of the titration, thus minimising losses from the cell.

A number of similar increments were performed. By this method the total dye concentration during each titration was maintained at a constant value.

The following spectrophotometric titrations were performed by Method 1:

Method 1.1:- Bromophenol Blue with H.S.A. fraction V

Dye concentration = 4.174×10^{-5} mol dm^{-3}

Stock protein concentration = 2.899×10^{-4} mol dm^{-3}

Number of protein increments = 12

Method 1.2:- Methyl Orange with H.S.A. fraction V

Dye concentration = 1.515×10^{-4} mol dm^{-3}

Stock protein concentration = 1.449×10^{-3} mol dm^{-3}

Number of protein increments = 17

Method 1.3:- Methyl Orange with B.S.A. fraction V

Dye concentration = 1.515×10^{-4} mol dm^{-3}

Stock protein concentration = 1.449×10^{-3} mol dm^{-3}

Number of protein increments = 7.

In addition, two further increments were made but using a $0.1cm^3$ micropipette.

Method 1.4:- Warfarin with H.S.A. fraction V

Dye concentration = 1.911×10^{-4} mol dm^{-3}

Stock protein concentration = 1.449×10^{-3} mol dm^{-3}

Number of protein increments = 9

Method 1.5:- Evans Blue with H.S.A. fraction V

Dye concentration = 7.183×10^{-5} mol dm^{-3}

Stock protein concentration = 7.247×10^{-4} mol dm^{-3}

Number of protein increments = 10

Method 1.6:- Evans Blue with H.S.A. fraction V

Dye concentration = 7.183×10^{-5} mol dm^{-3}

Stock protein concentration = 2.899×10^{-5} mol dm^{-3}

Number of protein increments = 10

Method 1.7:- Evans Blue with H.S.A. (C.L.E.G.F.)

Dye concentration = 7.748×10^{-5} mol dm^{-3}

Stock protein concentration = 8.015×10^{-5} mol dm^{-3}

Number of protein increments = 14.

Method 1.8 Mordant Orange 1 with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.8a	0.7792	3.672	14
1.8b	0.7792	3.672	14
1.8c	1.558	7.354	14
1.8d	1.558	7.334	14
1.8e	2.460	7.319	14
1.8f	2.445	7.280	14

Method 1.9 Mordant Yellow 12 with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.9a	2.397	7.944	14
1.9b	2.394	8.116	14
1.9c	3.586	7.749	14
1.9d	3.586	7.725	14
1.9e	5.405	7.341	14
1.9f	5.382	7.464	14

Method 1.10 Alizarin Yellow GG with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.10a	1.508	7.833	10
1.10b	1.679	3.819	10
1.10c	1.673	3.819	10
1.10d	1.401	3.819	10
1.10e	1.410	3.819	10
1.10f	1.059	3.819	10
1.10g	1.055	3.819	10

Method 1.11 Mordant Yellow 7 with H.S.A. (C.L.E.G.F.)

Method	Dye Concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.11a	1.578	7.465	5
1.11b	1.584	7.536	5
1.11c	2.292	7.552	5
1.11d	2.295	7.536	5
1.11e	2.840	7.377	5
1.11f	2.854	7.377	5

Method 1.12 5-phenylazosalicylic acid with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.12a	1.096	7.265	5
1.12b	1.102	7.246	5
1.12c	1.494	7.326	5
1.12d	1.500	7.341	5
1.12e	1.806	7.333	5
1.12f	1.809	7.345	5

Method 1.13 4-azobenzenesulphonic acid with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments.
1.13a	0.8808	7.536	6
1.13b	0.8838	7.536	6
1.13c	1.164	7.493	6
1.13d	1.165	7.493	6
1.13e	1.499	7.420	6
1.13f	1.503	7.420	6

Method 1.14 Mordant Yellow 10 with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.14a	1.285	7.312	5
1.14b	1.289	7.310	5
1.14c	1.664	7.449	5
1.14d	1.664	7.438	5
1.14e	2.112	7.328	5
1.14f	2.128	7.294	5

Method 1.15 4-hydroxyazobenzene-4'-sulphonic acid with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.15a	0.8881	7.694	5
1.15b	0.8964	7.710	5
1.15c	1.206	7.609	5
1.15d	1.213	7.632	5
1.15e	1.489	7.536	5
1.15f	1.502	7.536	5

Method 1.16 Bromophenol Blue with H.S.A. (C.L.E.G.F.)

Method	Dye concentration /10^{-5} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.16a	3.548	3.949	10
1.16b	3.565	3.949	10
1.16c	4.417	3.949	10
1.16d	4.415	3.949	10
1.16e	5.284	3.949	10
1.16f	5.297	3.949	10

Method 1.17 M.T.T. with H.S.A. fraction V

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.17a	6.130	2.899	5
1.17b	3.065	2.899	5

Method 1.18 Sulindac with H.S.A. fraction V

Method	Dye concentration /10^{-4} mol dm^{-3}	Stock protein concentration /10^{-4} mol dm^{-3}	Number of increments
1.18a	1.998	2.898	7
1.18b	0.9989	2.898	7

2.2.2 Method 2 Spectrophotometric titrations (competition reactions)

The method used was essentially the same as that used in a normal spectrophotometric titration, (Method 1), except that 1cm^3 of a mixture of Bromophenol Blue($\sim$4.400 x 10^{-5} mol dm^{-3}) and the competing dye were placed in the sample beam. As well as the 0.01 cm^3 of stock albumin solution, 0.01 cm^3 of a solution containing double the concentration of Bromophenol Blue, and double the concentration of competing dye were added to the sample cell. Thus the concentrations of both Bromophenol Blue and the competitor dye were maintained at a constant level throughout the course of the titration.

In each case the Bromophenol Blue spectrum was measured between 550 and 620 nm, and absorbances measured at 10 nm intervals. Ten increments of H.S.A. (C.L.E.G.F.) were made.

Method 2.1. Bromophenol Blue - Mordant Yellow 10, competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.1a	4.473	3.623	4.425
2.1b	4.442	3.623	4.425
2.1c	4.481	3.658	8.848
2.1d	4.487	3.658	8.848
2.1e	4.418	3.658	17.70
2.1f	4.429	3.658	17.70

Method 2.2. Bromophenol Blue - Mordant Yellow 12 competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. Stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.2a	4.459	3.888	4.459
2.2b	4.465	3.888	4.465
2.2c	4.437	3.888	8.852
2.2d	4.428	3.888	8.852
2.2e	4.432	3.859	17.70
2.2f	4.448	3.859	17.70

Method 2.3. Bromophenol Blue - 5-phenylazosalicylic acid competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. Stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.3a	4.426	3.713	4.418
2.3b	4.412	3.713	4.418
2.3c	4.440	3.713	8.836
2.3d	4.396	3.713	8.836
2.3e	4.404	3.903	2.002
2.3f	4.382	3.903	2.002

Method 2.4. Bromophenol Blue - 4-azobenzenesulphonic acid competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. Stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.4a	4.454	3.733	5.005
2.4b	4.396	3.733	5.005
2.4c	4.385	3.733	10.01
2.4d	4.385	3.733	10.01
2.4e	4.415	3.903	20.02
2.4f	4.421	3.903	20.02

Method 2.5. Bromophenol Blue - Mordant Yellow 7 competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. Stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.5a	4.465	3.835	4.433
2.5b	4.489	3.835	4.433
2.5c	4.437	3.835	8.867
2.5d	4.443	3.835	8.867
2.5e	4.440	3.835	17.73
2.5f	4.434	3.835	17.73

Method 2.6. Bromophenol Blue - Mordant Orange 1 competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. Stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.6a	4.445	3.675	4.435
2.6b	4.448	3.675	4.435
2.6c	4.468	3.675	8.871
2.6d	4.448	3.675	8.871
2.6e	4.476	3.661	17.74
2.6f	4.465	3.661	17.74

Method 2.7. Bromophenol Blue - Alizarin Yellow GG competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. Stock concentration /10^{-4} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.7a	4.415	3.849	4.263
2.7b	4.423	3.849	4.263
2.7c	4.390	3.788	8.525
2.7d	4.437	3.788	8.525
2.7e	4.398	3.788	17.05
2.7f	4.432	3.788	17.05

Method 2.8. Bromophenol Blue with 4-hydroxyazobenzene-4'-sulphonic acid competition

Method	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	H.S.A. stock concentration /10^{-5} mol dm^{-3}	Competing dye concentration /10^{-5} mol dm^{-3}
2.8a	4.438	3.848	4.418
2.8b	4.415	3.848	4.418
2.8c	4.434	3.743	8.835
2.8d	4.434	3.743	8.835
2.8e	4.418	3.743	17.67
2.8f	4.426	3.743	17.67

2.2.3. Method 3. Bromophenol Blue - Sulindac competition

As for Method 2, but using H.S.A. fraction V and only seven increments of the protein.

Bromophenol Blue concentration = 4.180×10^{-5} mol dm^{-3}

Stock albumin concentration = 2.899×10^{-4} mol dm^{-3}

Sulindac concentration = 4.050×10^{-3} mol dm^{-3}

2.2.4. Method 4. Interaction of M.T.T. with H.S.A. fraction V

Using the same instruments and conditions as described for Method 1, $1cm^3$ of pH 7.4 buffer solution were placed in the sample beam and reference beam. A baseline correction was performed over the wavelength range 250-400nm. To the sample cell was added $0.01cm^3$ of a solution containing 1.799×10^{-3} mol dm^{-3} of the drug and $0.01cm^3$ of buffer solution. The spectrum was measured over the described wavelength range. Seven further increments of the drug were made. The experiment was then repeated using solutions containing H.S.A. fraction V at concentrations of 5.798,

2.899 and 1.449 x 10^{-5} mol dm^{-3} in the sample and reference cells. 0.01cm^3 of double concentration albumin was added to the sample beam on addition of the drug to maintain the albumin concentration.

2.2.5. Method 5. Ultrafiltration rejection curve for Evans Blue

An Amicon UM10 filter was soaked in distilled water for one hour as recommended by the manufacturer. This was then placed in an Amicon ultrafiltration cell, model 12, maintained at 21°C in a thermostated room. 10 cm^3 of Evans Blue (1.192 x 10^{-5} mol dm^{-3}) were placed in the cell and about 5 cm^3 run off under a pressure of 5 p.s.i. of Nitrogen ('white spot' supplied by the British Oxygen Company). This was repeated twice to ensure equilibration. A further 10 cm^3 of dye were added to the cell and approximately 0.75 cm^3 fractions collected as they passed through the filter. Simultaneously 0.5 cm^3 fractions were collected from the interior of the cell. These fractions were diluted, their absorbance measured at 602nm, and the concentration of the solutions calculated ($\varepsilon 602$ = 4398 mol^{-1} m^2). The experiment was repeated, and then repeated again , using 2.384, 3.576, 4.768 and 5.960 x 10^{-5} mol dm^{-3} Evans Blue solutions. A rejection curve was thus obtained (curve (a)(p 164).

A solution of H.S.A. (C.L.E.G.F.) (1.449 x 10^{-5} mol dm^{-3}) was then passed through the filter. This process was repeated, and then Evans Blue solutions were ultrafiltered as described above. In this case one further Evans Blue solution of concentration 8.344 x 10^{-5} mol dm^{-3} was used.

2.2.6. Method 6. Ultrafiltration of Azo-dyes with H.S.A.

An Amicon YM 10 filter was soaked in distilled water and placed in the ultrafiltration cell. $10cm^3$ of the azo-dye/protein solution being studied were placed in the cell and approximately $1cm^3$ run off under pressure as described in Method 5. Collection of this fraction required about 10 minutes. This fraction was discarded. The contents of the cell were removed and a further $10cm^3$ of dye/protein solution added to the cell and 1 cm^3 ultrafiltered as before. The collected fraction was diluted to give a suitable absorbance at the wavelength of maximum absorbance and its absorbance measured. This process was repeated twice and then the whole experiment repeated with further azo-dye/protein mixtures. In this way the concentrations given in Chapter 6 were calculated (p 169-181).

2.3. Computation

All data was processed using programmes written in Fortran IV and run either on the University of London Computer Centre C.D.C. 7600, or on a VAX 11 Computer.

2.3.1. Matrix rank analysis

The matrix rank analysis programme, based on the method of Wallace and Katz[116] is reproduced in appendix 1.

2.3.2. U.V./visible spectrophotometric data fitting

The programme employed two 'packaged' sub-routines. The first of these was a routine for obtaining roots of polynominals by Newton's method (Nottingham Algorithms Group C02AEF) based on publications by Adams, Grant and Hitchins[117,118].

The second was a data 'best fit' package (C.E.R.N. Computer Centre 'Minuits'). The package used three data fitting techniques. The first of these used a Monte-Carlo search for the best minimum. The second ('Migrad') performed a local function minimisation using Fletchers "Switching" variation of the Davidson-Fletcher-Powell algorithm.[119]

The third ('Simplex') performed a simplex minimisation by the method of Nelder and Mead.[120]

The programme is reproduced in appendix 2.

2.3.3. Ultrafiltration data fitting

Binding parameters were obtained from ultrafiltration data using a simplex fitting routine.[120] Cubic equations were solved using the subroutine 'cubic'. The ultrafiltration data fitting routine is reproduced in appendix 3.

CHAPTER 3

THE APPLICATION OF MATRIX RANK ANALYSIS TO THE SPECTRA OF ANIONIC LIGAND - SERUM ALBUMIN INTERACTIONS.

3.1. Theoretical

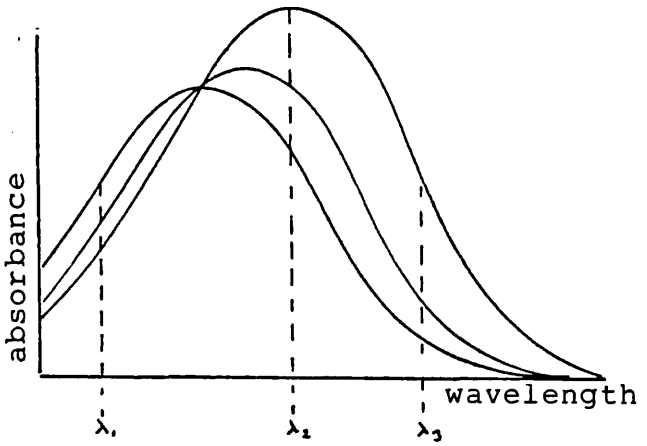


Fig.3.1. Sketch of spectral changes in a dye spectrum accompanying dye-protein interactions.

Consider a series of solutions containing two light absorbing species at a wavelength λ_i. The absorbance of the jth solution, per unit pathlength is given by

$$A_{ij} = \varepsilon_{i1} C_{ij} + \varepsilon_{i2} C_{2j}$$

where A_{ij} = the absorbance of the jth solution at the ith wavelength

ε_{i1} = the extinction coefficient at the ith wavelength of the 1st component

ε_{i2} = the extinction coefficient at the ith wavelength of the 2nd component

C_{1j} = the concentration of the 1st component in the jth solution

C_{2j} = the concentration of the 2nd component in the jth solution.

Consider now the absorbance of two solutions at two wavelengths. These can be written:-

$$A_{11} = \varepsilon_{11} C_{11} + \varepsilon_{12} C_{21}$$

$$A_{21} = \varepsilon_{21} C_{11} + \varepsilon_{22} C_{21}$$

$$A_{12} = \varepsilon_{11} C_{12} + \varepsilon_{12} C_{22}$$

$$A_{22} = \varepsilon_{21} C_{12} + \varepsilon_{22} C_{22}$$

It is a known property of simultaneous linear equations of this type that the determinant of a matrix of the absorbances is equal to the product of the determinants of the corresponding extinction coefficient and concentration matrices.

Thus:-

$$\begin{vmatrix} A_{11} & A_{12} \\ A_{21} & A_{22} \end{vmatrix} = \begin{vmatrix} \varepsilon_{11} & \varepsilon_{12} \\ \varepsilon_{21} & \varepsilon_{22} \end{vmatrix} \times \begin{vmatrix} C_{11} & C_{12} \\ C_{21} & C_{22} \end{vmatrix}$$

since all ε_{ij} and C_{nj} have finite values, the determinant of the absorbance matrix will be greater than zero, and given by

$$(A_{11}\ A_{22} - A_{21}\ A_{12}) = (\varepsilon_{11}\ \varepsilon_{22} - \varepsilon_{21}\ \varepsilon_{12})(C_{11}\ C_{22} - C_{21}\ C_{12})$$

Now consider the solutions to contain three species, but that the concentration of the third species is zero. The absorbance of any solution will be given by:-

$$A_{ij} = \varepsilon_{i1}\ C_{1j} + \varepsilon_{i2}\ C_{2j} + \varepsilon_{i3}\ C_{3j}$$

$$= \varepsilon_{i1}\ C_{1j} + \varepsilon_{i2}\ C_{2j} + \varepsilon_{i3}.0$$

for a 3 x 3 matrix, then, the corresponding determinants will be given by:-

$$\begin{vmatrix} A_{11} & A_{12} & A_{13} \\ A_{21} & A_{22} & A_{23} \\ A_{31} & A_{32} & A_{33} \end{vmatrix} = \begin{vmatrix} \varepsilon_{11} & \varepsilon_{12} & \varepsilon_{13} \\ \varepsilon_{21} & \varepsilon_{22} & \varepsilon_{23} \\ \varepsilon_{31} & \varepsilon_{32} & \varepsilon_{33} \end{vmatrix} \times \begin{vmatrix} C_{11} & C_{12} & C_{13} \\ C_{21} & C_{22} & C_{23} \\ 0 & 0 & 0 \end{vmatrix}$$

the concentration matrix determinant is given by:-

$$C_{11}\ (C_{22}.0 - C_{23}.0) - C_{12}\ (C_{21}.0 - C_{23}.0) + C_{13}$$

$$(C_{21}.0 - C_{22}.0)$$

and is thus equal to zero.

In this way it may be seen that the determinant of a 3 x 3 absorbance matrix for a system containing two species is zero. It

may also be seen that the determinants of matrices of all higher orders will also be zero.

In the general case, therefore, for solutions containing n species, the determinant of the absorbance matrix of order n + 1 should reduce to zero, since the non-existent (n + 1)th species always introduces a row of zeros into the concentration matrix.

Using similar reasoning it can also be seen that solutions containing a non-absorbing species will give an absorbance matrix with rank equivalent to the number of absorbing species.

In reality, however, there is a limit(s) on the accuracy to which absorbances can be measured, so that

$$A = A_{observed} \pm S$$

This error must be considered when considering whether a determinant is really zero.

Wallace and Katz[116] have described a method for matrix rank analysis of absorbance spectra.

The method consists of setting up in addition to the absorbance matrix A, a companion matrix S, whose elements S_{ij} are the estimated errors of A_{ij}. A is then reduced by a series of elementary operations to an equivalant matrix whose elements below the principal diagonal are all zero. S is also continually transformed during the reduction of A by computing new values of S_{ij} based on the propagation of errors in transforming A. The rank of A is then found from the number of non zero rows in the reduced matrix. An element in the A matrix is considered to be zero when it falls to a value lower than the equivalent value in the S matrix.

The A matrix is pivoted by an interchange of rows and

columns to place the element whose absolute value is the largest in position 1.1, S is transformed by the same row - column interchanges used in A. The resulting A matrix is transformed to A' by the operation

$$A'_{ij} = A_{ij} - \frac{A_{i1} \cdot A_{1j}}{A_{11}} \qquad (3.1)$$

performed on all except the first row.

This operation makes all elements in the first column identically zero except for the first one. S is transformed to S' by the equation for the propagation of errors in 3.1.[121]

$$S'_{ij} = \left[S_{ij}^2 + S_{1j}^2 \left(\frac{A_{i1}}{A_{11}}\right)^2 + S_{i1}^2 \left(\frac{A_{ij}}{A_{11}}\right)^2 + S_{11}^2 \left(\frac{A_{i1}A_{1j}}{A_{11}^2}\right)^2 \right]^{\frac{1}{2}} \qquad (3.2)$$

The submatrices formed by deleting the first row and column of A' and S' are then treated similarly to give A'' and S'' and so on until all the elements of the transformed A matrix below the principal diagonal are identically zero. It is then only necessary to compare elements on the principal diagonal of the transformed A and S matrices to determine how many rows are statistically non-zero, and, therefore, to find the experimental rank.

The main reason for pivoting the matrix to place the largest elements on the diagonal is to minimise the rate of propagation of errors. With this arrangement the coefficients of S_{ij}^2, S_{i1}^2 and S_{11}^2 in equation 3.2 can never exceed 1, and the value of an element in S' after an operation, therefore, cannot exceed twice the value of the largest of the elements in S that goes into its calculation. If the matrix A contains n rows and all the elements in the original S matrix are identical and equal to S_o, the largest possible value of S in the reduced matrix will be $2^{n-1}S_o$, but it will

usually be much smaller.

3.2. Matrix rank treatment of Bromophenol Blue - H.S.A. Spectra

Tables 3.1 and 3.2 show a typical analysis for Bromophenol Blue with various concentrations of H.S.A. fraction V (experimental method 1.1).(fig.3.2)

Every element of the original S matrix was taken as 0.003 and so is not reproduced here.

An element on the principal diagonal of the A matrix was taken to be definitely non-zero if it exceeded four times the corresponding S matrix element.

If an A matrix element on the diagonal exceeded, but was less than four times the corresponding matrix element, then this was taken to indicate the possible presence of a species.

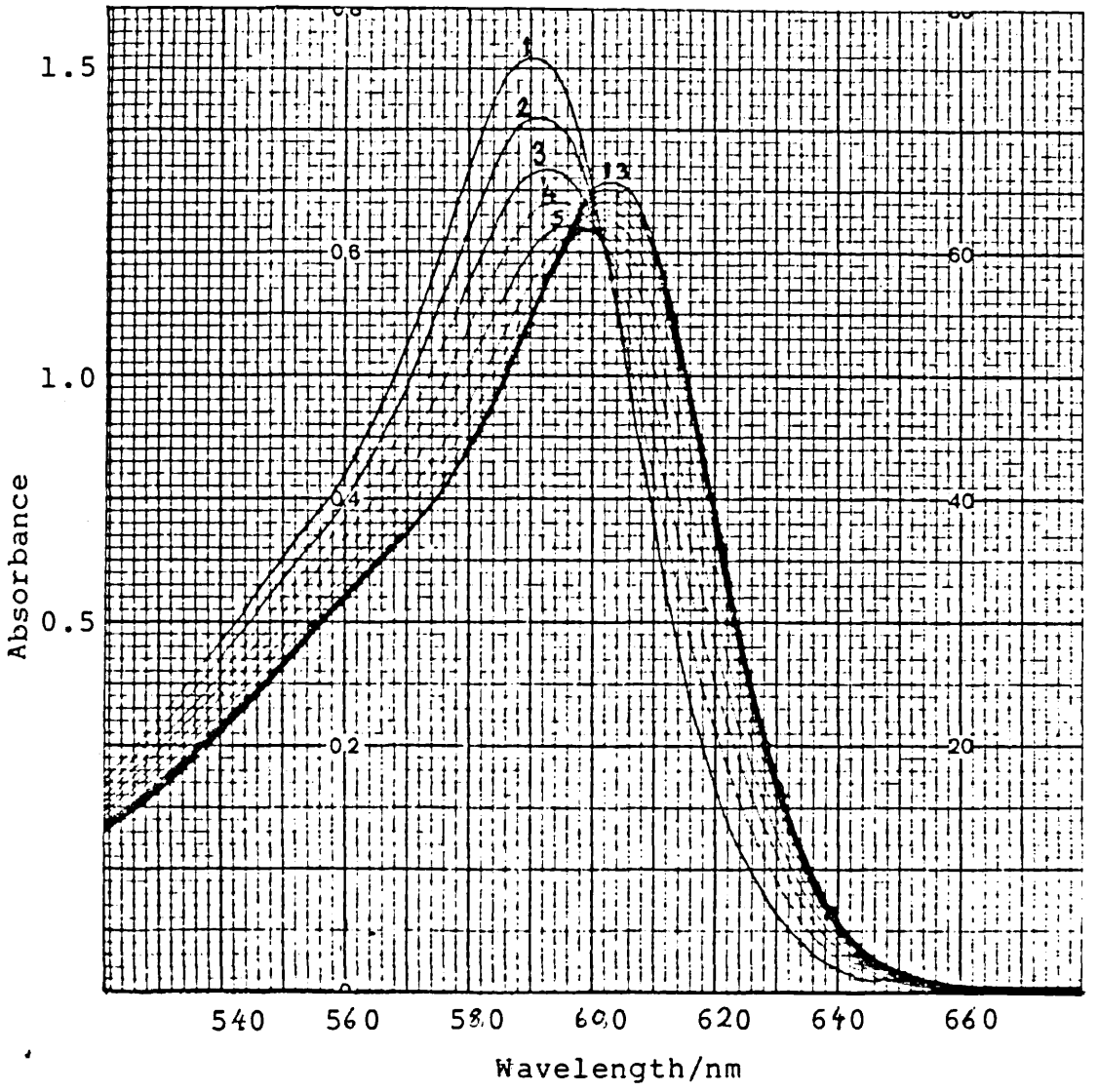
Any A matrix element which did not exceed the corresponding S matrix element was taken to have a value of zero.

The third element on the principal diagonal of the A matrix, for this example, has the value 0.047 whilst the corresponding S matrix element has the value 0.005. This firmly indicates the presence of 3 spectrophotometrically distinguishable species in solution. However, the fourth element has the value 0.007 which, being less than the S matrix elements value of 0.01 firmly excludes the possibility of a fourth species.

The simplest model available to explain the presence of three species in solution would be one in which there are two classes of binding site on the albumin molecule. Thus the three species consist of the free dye and two bound species of dye. The binding of the dye to the albumin sites

Fig.3.2 Absorbance spectra for the Titration of Bromophenol Blue (4.174×10^{-5} mol dm^{-3}) with H.S.A. fraction V

The albumin concentrations are as follows:-
/10^{-5} mol dm^{-3} (1) 0, (2) .2842, (3) .5573, (4) .8203, (5) 1.073, (6) 1.318, (7) 1.553, (8) 1.856, (9) 1.999, (10) 2.211, (11) 2.415, (12) 2.613, (13) 2.807.


1.5
1.0
0.5
Absorbance
0.8
0.4
0.2
0
60
40
20
1
2
3
13
540
560
580
600
620
640
660
Wavelength/nm

Albumin concentration /10^{-5} mol dm^{-3}	wavelength/nm												
	630	620	610	600	590	580	570	560	550	540	530	520	510
.0	.165	.396	.842	1.376	1.508	1.277	.995	.807	.674	.543	.416	.317	.243
.2842	.225	.490	.906	1.331	1.398	1.180	.935	.765	.640	.516	.398	.305	.234
.5573	.275	.573	.973	1.300	1.305	1.098	.884	.731	.611	.493	.382	.294	.226
.8203	.312	.635	1.022	1.271	1.227	1.030	.841	.702	.588	.474	.368	.284	.219
1.073	.343	.693	1.072	1.252	1.162	.971	.803	.677	.566	.457	.356	.276	.213
1.318	.368	.741	1.118	1.240	1.111	.923	.772	.656	.549	.442	.344	.267	.206
1.553	.380	.774	1.153	1.235	1.075	.889	.750	.641	.538	.433	.336	.260	.201
1.856	.395	.802	1.186	1.237	1.054	.866	.734	.629	.525	.421	.327	.254	.195
1.999	.403	.821	1.209	1.245	1.046	.855	.725	.622	.518	.414	.322	.249	.192
2.211	.408	.815	1.222	1.258	1.069	.864	.730	.624	.522	.415	.325	.252	.194
2.415	.380	.794	1.202	1.266	1.068	.867	.725	.626	.521	.412	.320	.249	.191
2.613	.394	.821	1.229	1.271	1.055	.852	.716	.612	.507	.406	.313	.242	.188
2.807	.386	.828	1.260	1.279	1.045	.843	.722	.619	.513	.401	.317	.247	.183

Absorbance

Table 3.1. Original A matrix for Bromophenol Blue (4.174×10^{-5} mol dm^{-3}) interactions with H.S.A. fraction V

FINAL A MATRIX

1.508	.396	.842	1.376	.165	1.277	.995	.807	.674	.543	.416	.317	.243
0	.677	.554	.325	.272	-.042	.032	.060	.046	.025	.029	.027	.015
0	0	.047	-.013	.042	.013	.015	.017	.019	.024	.016	.013	.016
0	0	0	.007	.001	-.008	-.018	-.017	-.017	-.008	-.013	-.012	-.003
0	0	0	0	.009	.001	.000	.008	-.001	.002	.004	.005	.002
0	0	0	0	0	.005	.001	.000	-.001	-.001	.002	.001	-.001
0	0	0	0	0	0	.004	-.002	.003	.001	-.000	-.002	.001
0	0	0	0	0	0	0	.002	-.001	-.001	.000	.000	-.000
0	0	0	0	0	0	0	0	.002	.000	.001	.002	.001
0	0	0	0	0	0	0	0	0	.006	-.008	-.011	-.000
0	0	0	0	0	0	0	0	0	0	.004	.004	.001
0	0	0	0	0	0	0	0	0	0	0	.000	-.001
0	0	0	0	0	0	0	0	0	0	0	0	-.001

FINAL S MATRIX

.003	.003	.003	.003	.003	.003	.003	.003	.003	.003	.003	.003	.003
0	.005	.004	.006	.004	.006	.005	.005	.004	.004	.004	.004	.004
0	0	.005	.008	.007	.008	.007	.006	.006	.005	.005	.005	.005
0	0	0	.010	.009	.009	.008	.007	.007	.007	.006	.006	.006
0	0	0	0	.026	.016	.028	.027	.012	.015	.020	.019	.009
0	0	0	0	0	.033	.018	.042	.011	.018	.027	.027	.011
0	0	0	0	0	0	.019	.042	.013	.020	.029	.028	.012
0	0	0	0	0	0	0	.020	.044	.020	.029	.029	.013
0	0	0	0	0	0	0	0	.039	.029	.038	.059	.017
0	0	0	0	0	0	0	0	0	.353	.238	.527	.193
0	0	0	0	0	0	0	0	0	0	.259	.157	.058
0	0	0	0	0	0	0	0	0	0	0	.178	.044
0	0	0	0	0	0	0	0	0	0	0	0	.258

Table 3.2 Final A and S matrices for Bromophenol Blue (4.174×10^{-5} mol dm^{-3}) interactions with H.S.A. fraction V

may be represented in the following way:-

$$D + S_1 \rightleftharpoons DS_1 \qquad (3.3)$$

$$D + S_2 \rightleftharpoons DS_2 \qquad (3.4)$$

where S_1 = the free primary binding site

S_2 = the free secondary binding site

D = free dye

DS_1 = the primary dye site complex

DS_2 = the secondary dye site complex

It is also assumed that the total concentration of the primary binding site (S_1^{tot}) is related to the concentration of albumin (P_{tot}) by the relationship

$$S_1^{tot} = n_1 \times P_{tot}$$

where n_1 is the number of sites per albumin molecule.

Similarly: $S_2^{tot} = n_2 \times P_{tot}$

If the concentration of D is large in comparison with both S_1^{tot} and S_2^{tot} equations 3.3 and 3.4 will lie almost entirely to the right hand side.

Therefore $[DS_1] = n_1 \cdot P_{tot}$ and $[DS_2] = n_2 \cdot P_{tot}$

assuming all three species to obey Beer's law, the absorbance per unit path length of a solution will be given by:-

$$A = \varepsilon_f[D] + \varepsilon_1[DS_1] + \varepsilon_2[DS_2] \qquad (3.5)$$

where ε_f, ε_1 and ε_2 are the corresponding molar extinction coefficients for the three species, free dye, primary site bound dye, and secondary site bound dye.

Under the conditions of a large relative free dye concentration, i.e. at the beginning of the titration, equation 3.5 can be written:-

$$A = \varepsilon_f[D] + \varepsilon_1 n_1[P_{tot}] + \varepsilon_2 n_2[P_{tot}]$$

$$A = \varepsilon_f[D] + (\varepsilon_1 n_1 + \varepsilon_2 n_2)[P_{tot}]$$

Since $[DS_1] = n_1[P_{tot}]$ and $[DS_2] = n_2[P_{tot}]$

If $[D_{bound}]$ is the total concentration of the bound dye then

$$[D_{bound}] = [DS_1]+[DS_2] = n_1 P_{tot} + n_2 P_{tot}$$

$$[P_{tot}] = \frac{[D_{bound}]}{(n_1+n_2)}$$

and hence

$$A = \epsilon_f[D] + \frac{(\epsilon_1 n_1 + \epsilon_2 n_2)}{(n_1+n_2)}[D_{bound}]$$

let $$\frac{(\epsilon_1 n_1 + \epsilon_2 n_2)}{n_1+n_2} = \epsilon_b$$

then $$A = \epsilon_f[D] + \epsilon_b[D_{bound}]$$

If at some point $\epsilon_f = \epsilon_b \equiv \epsilon_I$

$$A = \epsilon_I([D]+[D_{bound}]) \qquad (3.6)$$

Since $[D] + [D_{bound}]$ is constant for this titration at low albumin concentrations all spectra should pass through a single (isosbestic) point. This is observed at 602 nm. Furthermore, at low albumin concentrations the spectra will appear only to be those of a two species system.

Table 3.3 shows the effect of considering only the first six solutions in the titration in the matrix rank analysis. The first two elements on the principal diagonal of the final A matrix are clearly non-zero. The third element has the value 0.010 which is only very slightly larger than the corresponding S matrix value of 0.009.

Consider now the situation as the total site concentration becomes very large. Most of the dye will be bound in one of the complexes DS_1 or DS_2. Further changes in spectra will arise as a result of transfer of dye from one site to the other. The absorbance per unit path length will be given by

$$A = \epsilon_1[DS_1] + \epsilon_2[DS_2]$$

ORIGINAL A MATRIX

Albumin concentration / 10^{-5} mol dm^{-3}	wavelength/nm 620	600	580	560	540	520
0	.396	1.376	1.277	.807	.543	.317
.2842	.490	1.331	1.180	.765	.516	.305
.5573	.573	1.300	1.098	.731	.493	.294
.8203	.635	1.271	1.030	.702	.474	.284
1.073	.693	1.252	.971	.677	.457	.276
1.318	.741	1.240	.923	.656	.442	.267

absorbance

FINAL A MATRIX					
1.376	.396	1.277	.807	.543	.317
0	.384	-.228	-.071	-.047	-.019
0	0	.010	.007	.006	.004
0	0	0	.001	.000	.000
0	0	0	0	-.001	-.001
0	0	0	0	0	.001
FINAL S MATRIX					
.003	.003	.003	.003	.003	.003
0	.004	.007	.005	.005	.004
0	0	.009	.007	.006	.005
0	0	0	.008	.009	.010
0	0	0	0	.009	.010
0	0	0	0	0	.023

Table 3.3 Original and final matrices for the first six solutions of the Bromophenol Blue (4.174×10^{-5} mol dm^{-3}) H.S.A. fraction V titration.

Each element of the original S matrix was taken as 0.003

Again assuming overlap of the spectra, at the point of overlap an isosbestic point will occur.

In this example (Table 3.4) the final five solutions in the titration have very similar absorbances below 592 nm.

In this instance matrix rank analysis shows the definite existence of two species and the possible existence of a third since the third element on the principal diagonal of the A matrix has the value 0.014 whilst the third element of the error matrix has the value 0.008.

3.3 Matrix rank treatment of Methyl Orange - serum albumin spectra

The absorbance spectra obtained as a result of the interaction of a number of different compounds with albumin have been measured.

Klotz has reported that Methyl Orange shows rather different spectral changes with bovine (experimental method 1.3) as compared with human (experimental method 1.2) serum albumin. Figs. 3.3 and 3.4 confirm this observation.

Matrix rank analysis of the human serum albumin, Methyl Orange system indicates the existence of at least three species and possibly four. Analysis of the last ten spectra shows only the existence of two species, giving rise to the isosbestic point at 442 nm.

Rank analysis of the bovine serum albumin/Methyl Orange spectra shows the existence of at least two species and the probable existence of a third.

Tables 3.6 and 3.7 summarise the results of the matrix rank analysis of various ligand protein interactions.

ORIGINAL A MATRIX

Albumin concentration /10^{-5} mol dm^{-3}	wavelength/nm 620	600	580	560	540
1.999	0.821	1.245	0.855	0.622	0.414
2.211	0.815	1.258	0.864	0.624	0.415
2.415	0.794	1.266	0.867	0.626	0.412
2.613	0.821	1.271	0.852	0.612	0.406
2.807	0.828	1.279	0.843	0.619	0.401

Absorbance

FINAL A MATRIX				
1.279	0.828	0.843	0.619	0.401
0.000	0.035	0.001	0.015	0.021
0.000	0.000	0.014	0.004	0.003
0.000	0.000	0.000	0.007	0.002
0.000	0.000	0.000	0.000	0.002
FINAL S MATRIX				
0.003	0.003	0.003	0.003	0.003
0.000	0.006	0.006	0.005	0.005
0.000	0.000	0.008	0.007	0.007
0.000	0.000	0.000	0.016	0.015
0.000	0.000	0.000	0.000	0.019

Table 3.4 Original and final matrices for the final five solutions of the Bromophenol Blue (4.174 x 10^{-5} mol dm^{-3}) H.S.A. fraction V titration

Each element of the original S matrix was taken as 0.003

Fig. 3.3 Absorbance spectra for the titration of Methyl Orange (1.515×10^{-4} mol dm^{-3}) with H.S.A. fraction V

The protein concentrations /10^{-5} mol dm^{-3} were as follows:-
(1) 0, (2) 1.421, (3) 2.787, (4) 4.101, (5) 5.368, (6) 6.587, (7) 7.764, (8) 8.899, (9) 9.996, (10) 11.05, (11) 12.08, (12) 13.07, (13) 14.02, (14) 14.95, (15) 16.46, (16) 17.57, (17) 18.39, (18) 19.18

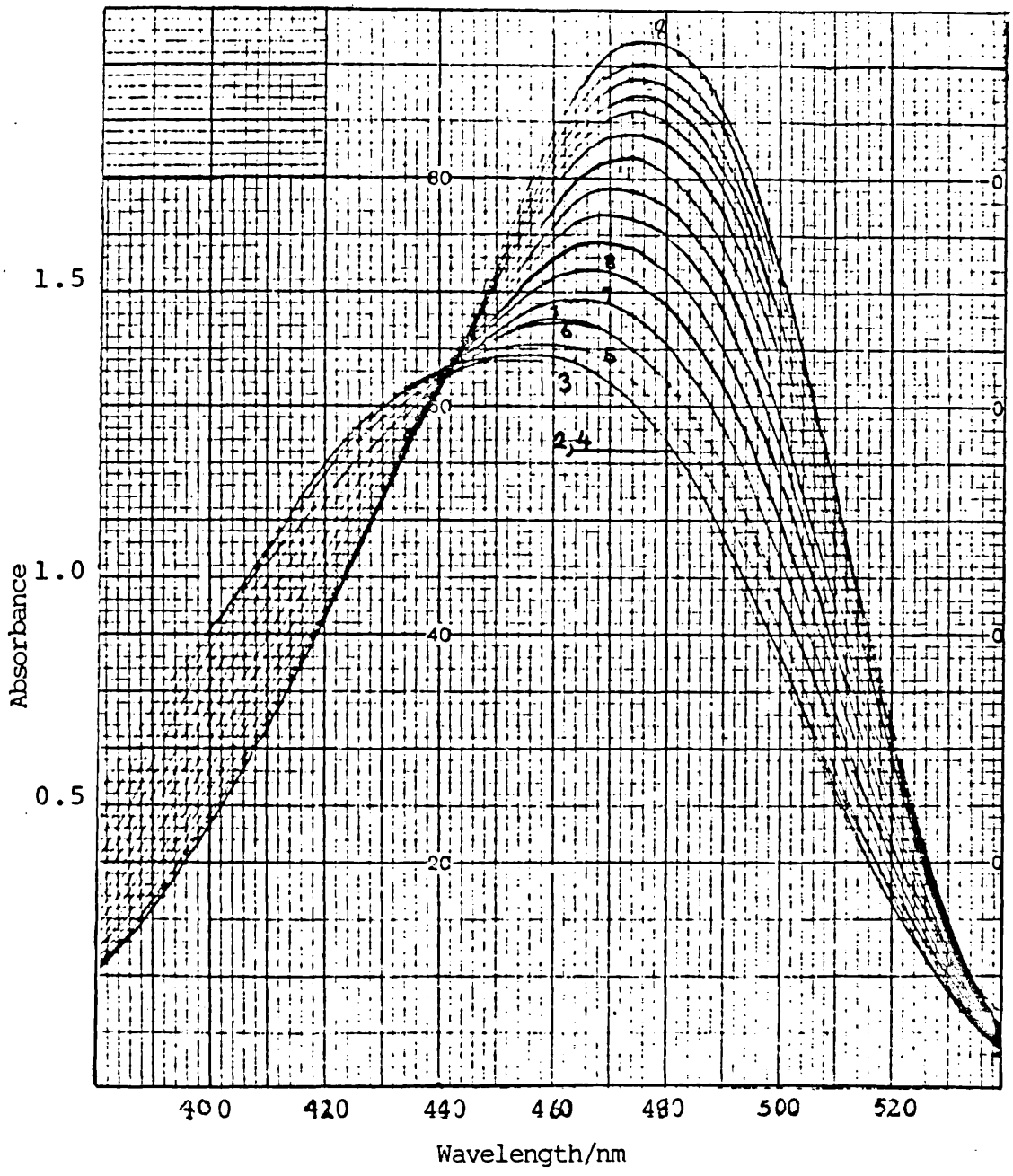
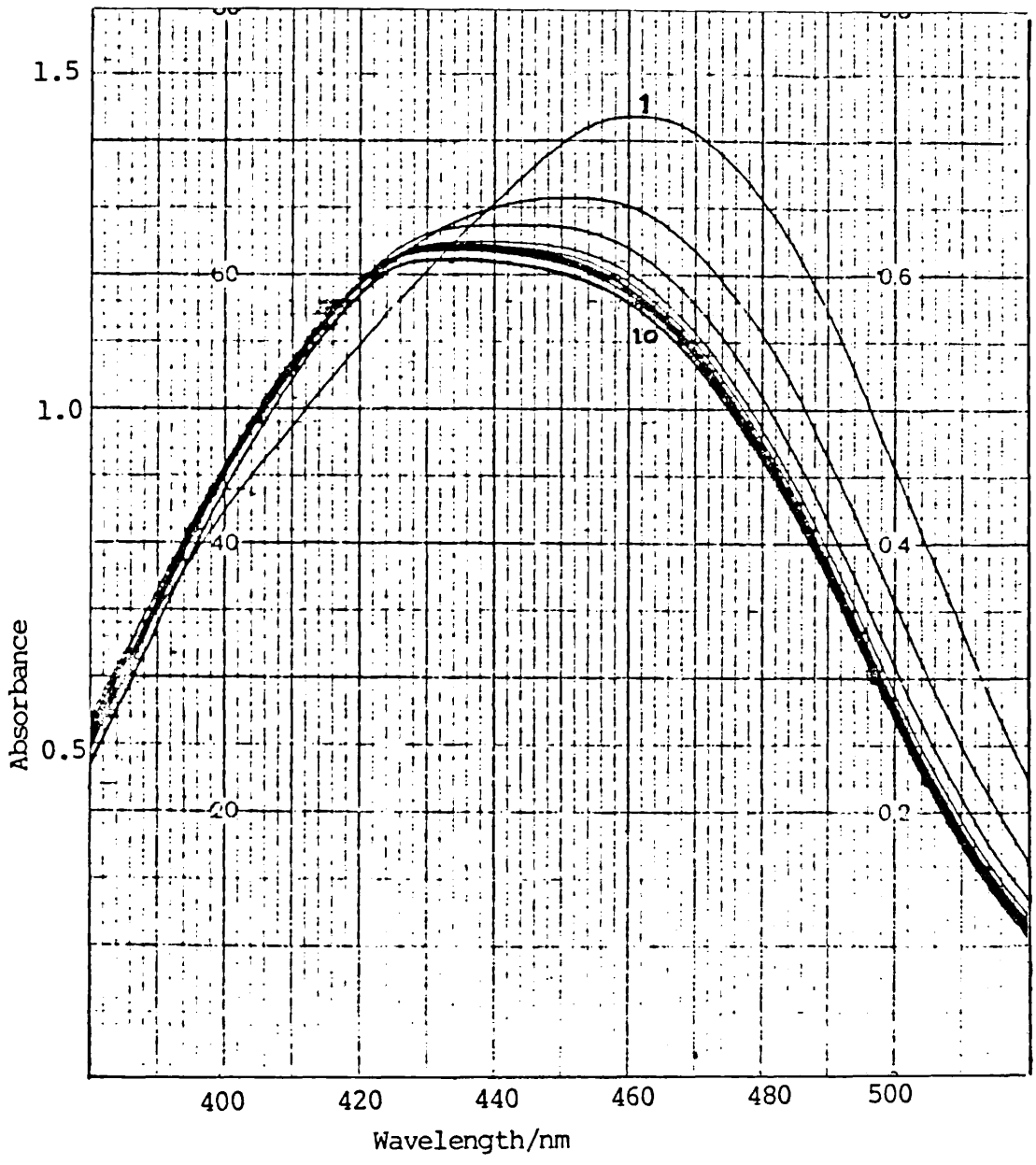

Absorbance
1.5
1.0
0.5
80
60
40
20
8
7
6
5
3
2,4
400
420
440
460
480
500
520
Wavelength/nm

Fig. 3.4 Absorbance spectra for the titration of Methyl Orange (1.515×10^{-4} mol dm^{-3}) with bovine serum albumin

The protein concentrations $/10^{-5}$ mol dm^{-3} are as follows:-

(1) 0, (2) 1.421, (3) 2.787, (4) 4.101 (5) 5.368, (6) 6.587, (7) 7.764, (8) 8.899, (9) 18.39, (10) 25.41.


1.5
1.0
0.5
Absorbance
1
0.6
0.4
0.2
400
420
440
460
480
500
Wavelength/nm

3.4 SUMMARY OF MATRIX RANK ANALYSIS OF THE SPECTRA OF VARIOUS ANIONS WITH SERUM ALBUMIN

Table 3.6 Matrix rank analysis data for various compounds with albumin

Compound	Protein	Albumin Concentration range /10^{-5} mol dm^{-3}	Ligand concentration /10^{-5} mol dm^{-3}	wavelength range /nm	Probable rank (m)	Value of m'th element on principal diagonal of A matrix	Value of M'th element on principal diagonal of S matrix	Possible rank (m')	Value of m'th element on principal diagonal of A matrix	Value of M'th element of principal diagonal of S matrix
Bromophenol Blue	H.S.A. fract. V	0 - 2.807	4.174	510 - 630	3	.047	.005	-	-	-
		0 - 1.318	4.174	520 - 620	2	.384	.004	3	.010	.009
		1.999 - 2.807	4.174	540 - 620	2	.035	.006	4	.014	.008
Methyl Orange	H.S.A. fract. V	0 - 19.18	15.15	320 - 560	3	.122	.007	4	.011	.008
		8.899- 19.18	15.15	340 - 540	2	.369	.005	-	-	-
	B.S.A. frac.V	0 - 25.41	15.15	340 - 520	2	.266	.006	3	.017	.006
Sulindac	H.S.A. frac.V	0 - 1.780	19.70	310 - 350	2	.162	.005	-	-	-
Warfarin	H.S.A. frac.V	0 - 11.05	19.11	260 - 340	2	.292	.004	3	.012	.007
Evans Blue	H.S.A. fract. V	0 - 6.039	7.183	500 - 700	3	.084	.008	4	.030	.009
		0 - 22416	7.183	460 - 740	2	.048	.007	5	.013	.011
	H.S.A. C.L.E. G.F.	0 - .8766	7.748	480 - 720	2	.031	.010	3	.031	.010
		.5527 - .8766	7.748	560 - 660	1	1.438	.003	2	.025	.007

Table 3.7 Matrix rank data for various azo dyes with humane serum albumin (crystallised, lyophilised and essentially globulin free)

Compound	Albumin concentration range /10^{-5} mol dm^{-3}	Ligand concentration /10^{-4} mol dm^{-3}	wavelength range /nm	Probable rank (m)	Value of m'th element on principal diagonal of A matrix	Value of m'th element on principal diagonal of S matrix	Possible rank (m')	Value of m'th element on principal diagonal of A matrix	Value of m'th element on principal diagonal of S matrix
Mordant Orange 1	0 - 4.015	0.7792	310 - 450	2	.221	.004	.3	.007	.006
	0 - 8.017	1.558	310 - 450	2	.505	.004	3	.017	.007
Mordant Yellow 12	0 - 8.029	5.405	310 - 450	3	.139	.013	4	.019	.017
Alizarin Yellow GG	0 - 6.528	1.508	300 - 400	3	.029	.007	5	.012	.009
Mordant Yellow 7	0 - 3.433	2.292	310 - 450	2	.291	.005	4	.009	.008
5-phenylazo salicylic acid	0 - 3.337	1.506	320 - 370	2	.198	.006	-	-	-
4-azobenzene sulphonic acid	0 - 3.426	1.501	300 - 350	2	.128	.007	-	-	-
Mordant Yellow 10	0 - 3.386	2.127	330 - 380	3	.252	.008	4	.059	.013
4-hydroxyazo benzene-4-sulphonic acid	0 - 3.426	1.497	320 - 420	2	.076	.006	-	-	-

3.5. Matrix rank analysis of Bromophenol Blue - H.S.A. spectra - in competition reactions.

In the presence of Sulindac, Bromophenol Blue shows no change in absorbance spectrum at wavelengths longer than 450 nm, even at relatively high Sulindac concentrations ([Sulindac] : [Bromophenol Blue]~100:1). However, the changes which occur in the absorbance spectrum of Bromophenol Blue on addition of human serum albumin are not the same as those which occur in the spectrum in the absence of Sulindac. Thus comparing fig 3.2 and 3.5, it can be seen that for essentially the same Bromophenol Blue and albumin concentrations, different sets of spectra are obtained in the presence and absence of Sulindac.

These differences between the sets of data in the presence and absence of Sulindac may be thought to arise as a result of competition between Sulindac and Bromophenol Blue for binding sites on the albumin molecule.

Sulindac appears to bind to a single site on the albumin molecule, since matrix rank analysis of Sulindac-albumin absorbance spectra shows a rank of 2, indicating 2 species present, a free and a bound species.

Bromophenol Blue appears to bind to two sites on the molecule since a matrix of absorbance spectra for this system has a rank of 3.

The reactions may be represented thus:-

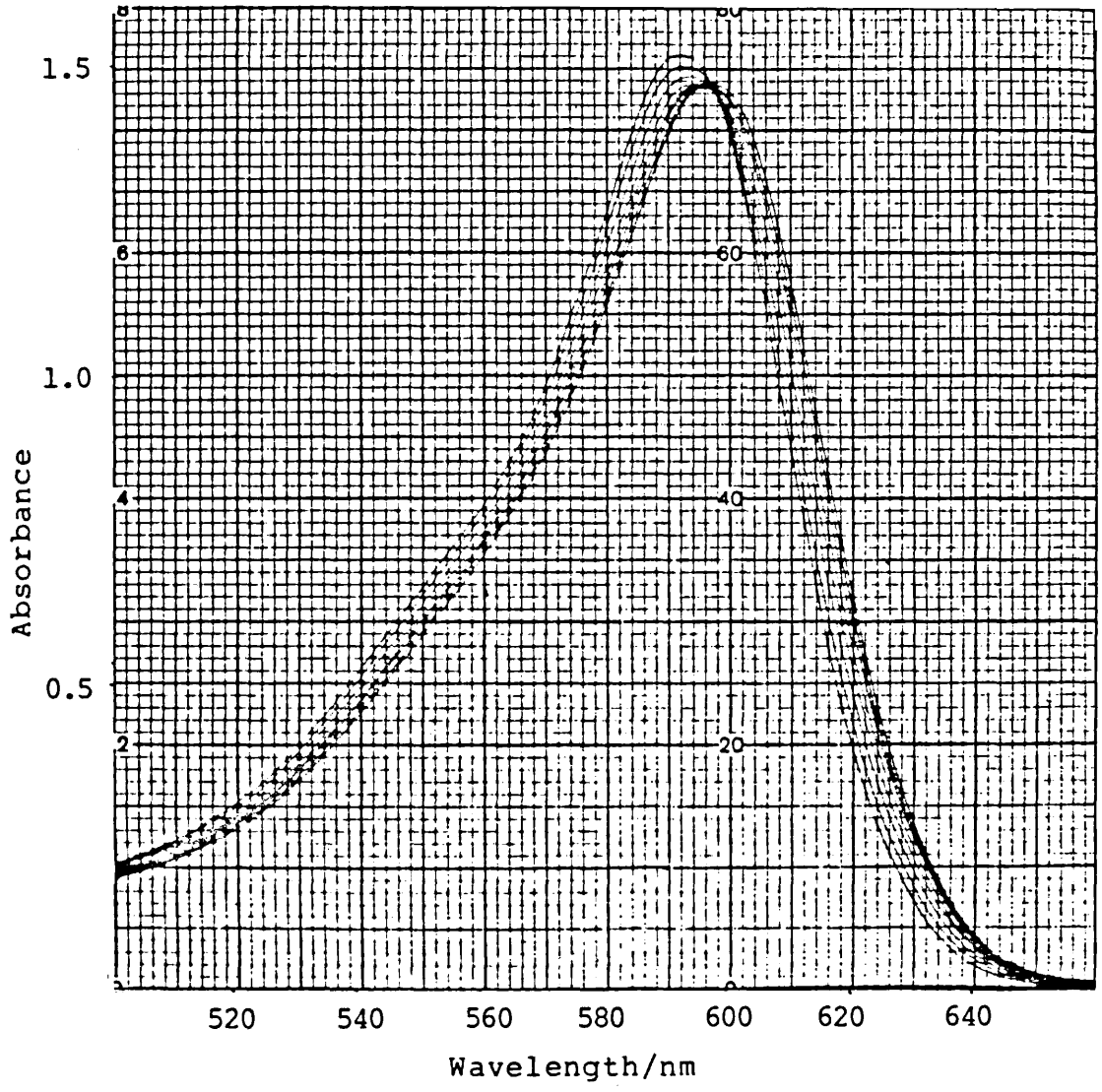
$$D + S_1 \rightleftharpoons DS_1 \qquad (3.7)$$

$$B + S_1 \rightleftharpoons BS_1 \qquad (3.8)$$

$$B + S_2 \rightleftharpoons BS_2 \qquad (3.9)$$

Fig. 3.5. Absorbance spectra for the titration of Bromophenol Blue (4.180×10^{-5} mol dm^{-3}) with H.S.A. (fraction V) in the presence of Sulindac (4.050×10^{-3} mol dm^{-3})

Albumin concentration /10^{-5} mol dm^{-3} were as follows:-
(1) 0, (2) .2842, (3) .5573, (4) .8203, (5) 1.073, (6) 1.318, (7) 1.553, (8) 1.856


1.5
1.0
0.5
Absorbance
520
540
560
580
600
620
640
Wavelength/nm

where D = Sulindac

B = Bromophenol Blue

DS_1 = the Sulindac-site complex

BS_1 = the primary Bromophenol Blue complex

BS_2 = the secondary Bromophenol Blue complex

S_1 = the unbound primary site

S_2 = the unbound secondary site

In the presence of a large excess of Sulindac, equation 3.7 will lie almost entirely to the right, the concentration of S_1 will, therefore, be small, and the principal Bromophenol Blue reaction will be that given by equation 3.9. Under these conditions a matrix rank analysis of the Bromophenol Blue spectra at wavelengths greater than 450 nm, on addition of human serum albumin, might be expected to behave as a single site system, and thus give a matrix of rank 2.

Table 3.8 shows that although the system tends towards a two site system there is still some secondary site present which suggests interaction of Sulindac with both sites.

Matrix rank analysis data for competition reactions between various azo-dyes and Bromophenol Blue with human serum albumin are shown in Table 3.9.

ORIGINAL A MATRIX

Albumin concentration (10^{-5} mol dm^{-3})	600	580	560	540	520	500	480	460
0	1.400	1.233	.769	.508	.301	.199	.208	.375
.2842	1.423	1.192	.757	.497	.292	.197	.207	.377
.5573	1.415	1.176	.740	.484	.281	.191	.205	.373
.8203	1.435	1.161	.726	.468	.273	.186	.204	.381
1.073	1.443	1.132	.717	.464	.272	.186	.203	.374
1.318	1.465	1.113	.707	.456	.267	.183	.205	.374
1.553	1.458	1.075	.688	.442	.261	.178	.200	.374
1.856	1.478	.062	.676	.429	.250	.172	.199	.380

FINAL A MATRIX							
1.478	1.062	.676	.429	.250	.172	.199	.380
0	.227	.129	.102	.064	.036	.020	.015
0	0	.010	.008	.003	.004	.001	-.000
0	0	0	.005	.002	.001	.001	-.003
0	0	0	0	.002	.001	.001	-.006
0	0	0	0	0	.003	-.002	.001
0	0	0	0	0	0	.002	-.001
0	0	0	0	0	0	0	.001
FINAL S MATRIX							
.003	.003	.003	.003	.003	.003	.003	.003
0	.006	.005	.005	.004	.004	.004	.004
0	0	.008	.006	.006	.005	.005	.006
0	0	0	.006	.008	.006	.005	.006
0	0	0	0	.005	.006	.009	.006
0	0	0	0	0	.024	.014	.011
0	0	0	0	0	0	.013	.017
0	0	0	0	0	0	0	.020

Table 3.8. Original and final matrices for Bromophenol Blue (4.180×10^{-5} mol dm^{-3}) with H.S.A.(fraction V) in the presence of Sulindac (4.050×10^{-3} mol dm^{-3})

Table 3.9 Matrix rank data for competition reactions between Bromophenol Blue and various azo dyes with human serum albumin (crystallised, lyophilised and essentially globulin free).

Compound	Albumin concentration range /10^{-5} mol dm^{-3}	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Competing ligand concentration /10^{-4} mol dm^{-3}	wavelength range /nm	Probable rank (m)	Value of m'th element on principal diagonal of A matrix	Value of m'th element on principal diagonal of S matrix	Possible rank (m')	Value of m'th element on principal diagonal of A matrix	Value of m'th element on principal diagonal of S Matrix
Mordant Orange 1	0 - 3.051	4.476	1.774	550 - 620	2	.658	.005	3	.017	.007
Mordant Yellow 12	0 - 3.240	4.437	1.770	550 - 640	3	.024	.005	4	.012	.010
Alizarin Yellow GG	0 - 3.157	4.398	1.705	550 - 620	2	.564	.005	-	-	-
Mordant Yellow 7	0 - 3.196	4.440	1.773	550 - 620	2	.464	.005	3	.012	.006
5-phenylazo salicylic acid	0 - 3.094	4.404	1.767	550 - 620	2	.531	.005	-	-	-
4-phenyl-azobenzene sulphonic acid	0 - 3.252	4.415	2.002	550 - 620	2	.641	.005	-	-	-
Mordant Yellow 10	0 - 3.048	4.418	1.770	550 - 650	2	.582	.005	4	.0010	.009
4-hydroxyazo benzene-4 sulphonic acid	0 - 3.120	4.418	1.767	550 - 620	2	.631	.005	3	.016	.007

CHAPTER 4.

DEVELOPMENT OF A SPECTROPHOTOMETRIC TECHNIQUE FOR THE MEASUREMENT OF THE BINDING OF DYES TO BLOOD PROTEINS

4.1. Interaction of M.T.T. with H.S.A.

The changes which occur in the spectra of coloured compounds when they bind to proteins appeared to be a potentially useful means of studying these interactions, since such methods are free from apparatus binding problems associated with dialysis, the most frequently used technique.

M.T.T. gives a weakly coloured, yellow, aqueous solution, with an absorbance maximum at 275nm with a shoulder at 295nm (fig.4.2). On addition of human serum albumin fraction V (experimental method 4) the absorbance at 295nm increases, giving a well defined peak rather than a shoulder (figs. 4.3-4-5). Spectra in which the drug concentrations remain constant (experimental method 1.17) were also recorded (fig. 4.6, 4.7).

It was initially thought that the new peak (λmax 295) was due to a very large movement of the main peak (λmax 274) on binding of the drug to human serum albumin. Fig. 4.6 and 4.7 clearly show, however, that on adding excess of albumin to the drug the peak at 274nm still remains, although moved slightly to 276nm.

On saturation the peak at 295nm assumes a steady absorbance value. Using the Klotz[41] interpretation of these spectra it is assumed that this represents the spectrum of the fully bound species of drug. Thus at any wavelength, assuming Beer's law to apply, the absorbance A is given by:-

$$A = \varepsilon_B' [DS]l \qquad (4.1)$$

Where ε_B' is the molar extinction coefficient of the bound drug, at a particular wavelength, [DS] is the concentration of drug bound to the protein, which in the presence of excess

Fig. 4.2 - 4.5. Absorbance spectra for solution containing 0, 5.798×10^{-5}, $2,899 \times 10^{-5}$ and 1.449×10^{-5} mol dm^{-3} human serum albumin fraction V respectively.

In all cases the drug concentrations are $/10^{-5}$ mol dm^{-3}

(1) 1.76, (2) 3.46, (3) 5.09,
(4) 6.66, (5) 8.18, (6) 9.64,
(7) 11.04, (8) 12.40.

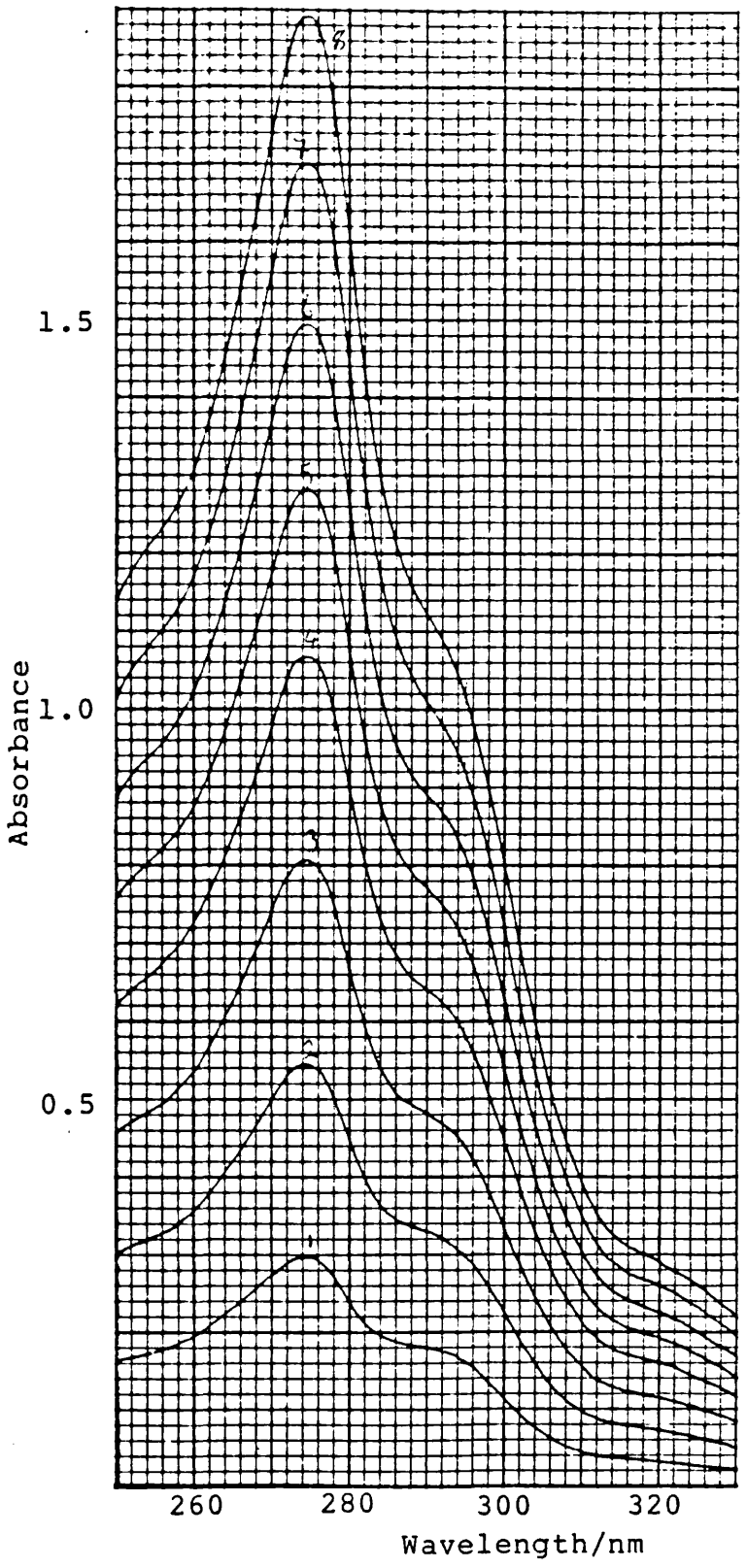


Fig.4.2.

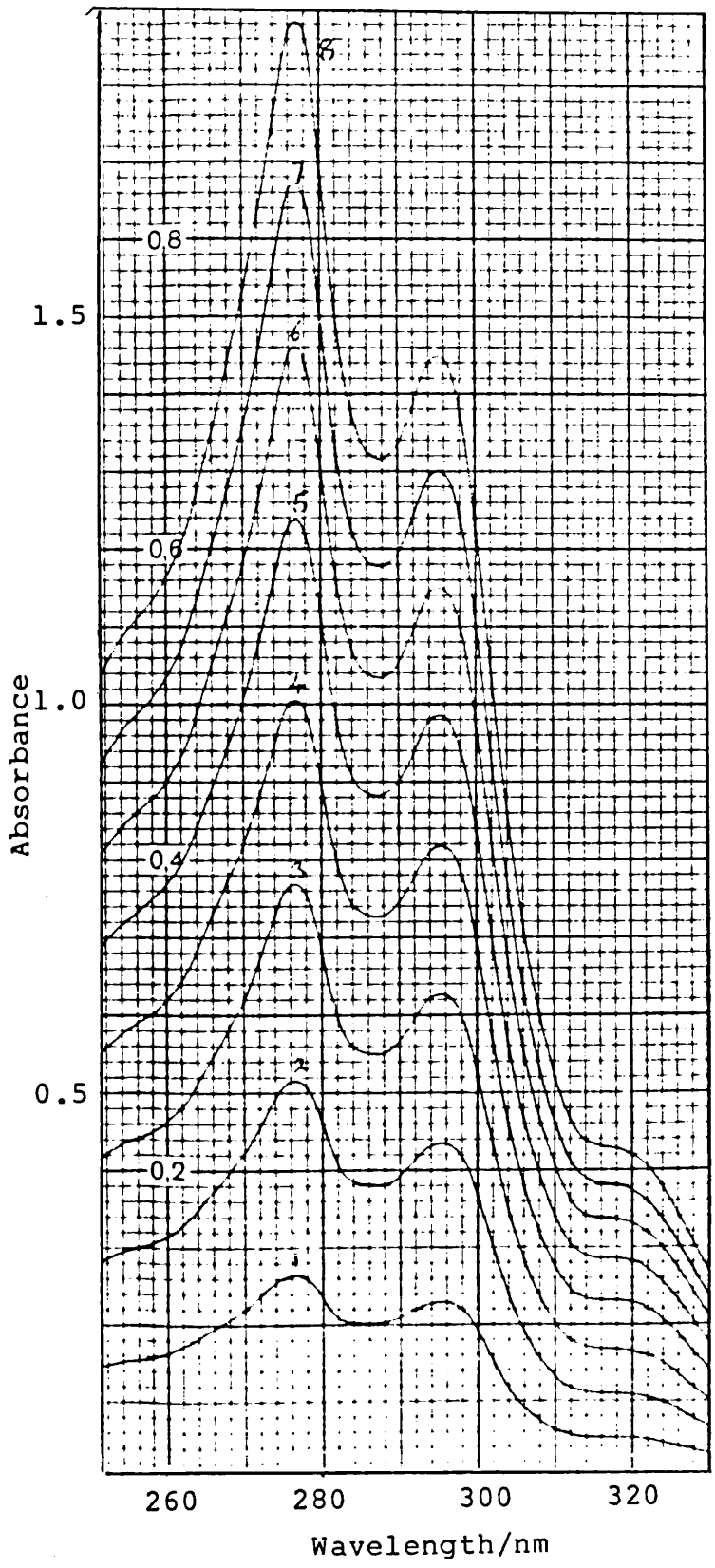

Absorbance
1.5
1.0
0.5
0.8
0.6
0.4
0.2
260
280
300
320
Wavelength/nm
1
2
3
4
5
6
7
8

Fig.4.3.

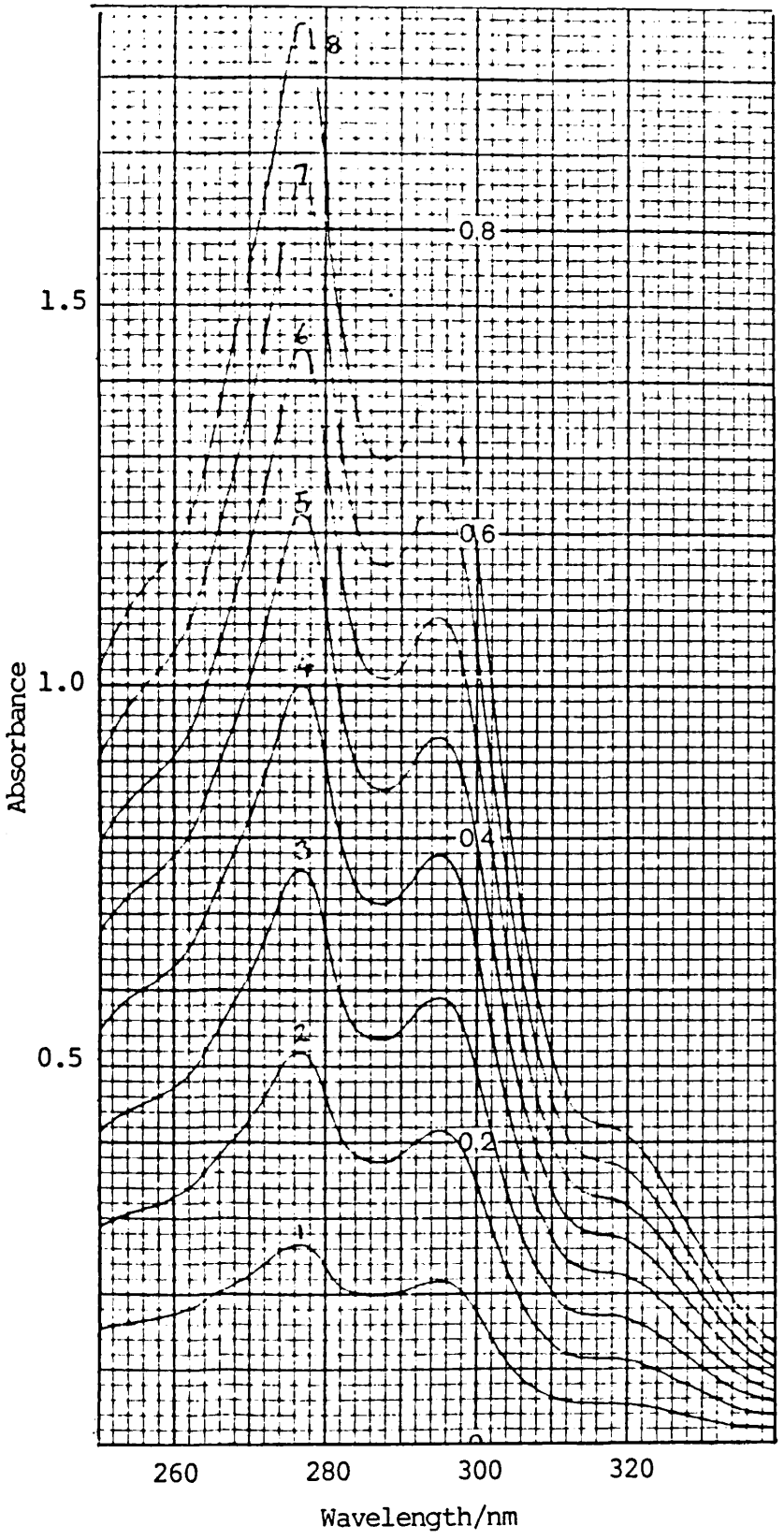

Absorbance
1.5
1.0
0.5
0.8
0.6
0.4
0.2
0
260
280
300
320
Wavelength/nm
1
2
3
4
5
6
7
8

Fig.4.4.

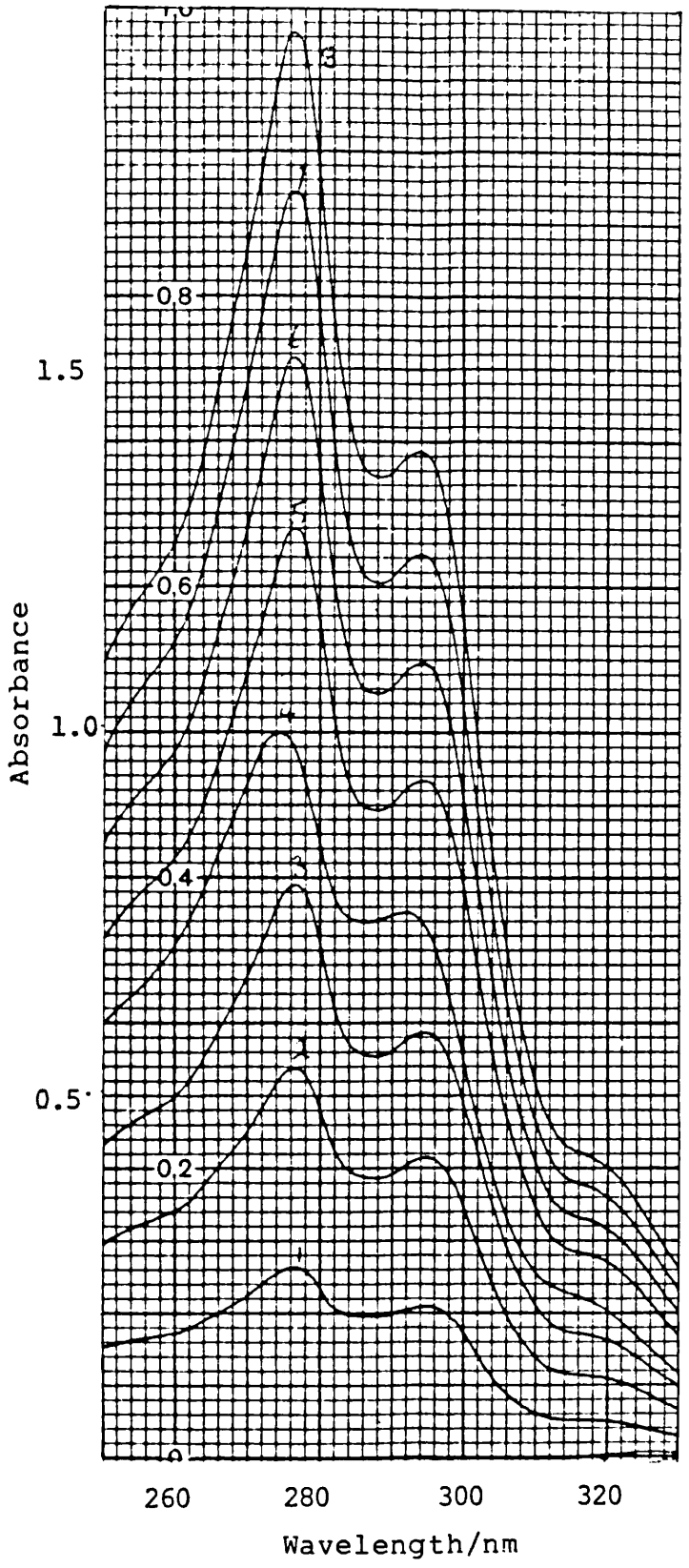


Fig.4.5

Fig 4.6 and 4.7

Interaction of M.T.T. with H.S.A. fraction V (experimental method 1.17) drug concentrations are 6.130×10^{-4} mol dm^{-3} and 3.065×10^{-4} mol dm^{-3} respectively

Albumin concentrations/10^{-5} mol dm^{-3}

(1) 0, (2) 2.841, (3) 5.580, (4) 8.203, (5) 10.72, (6) 13.17

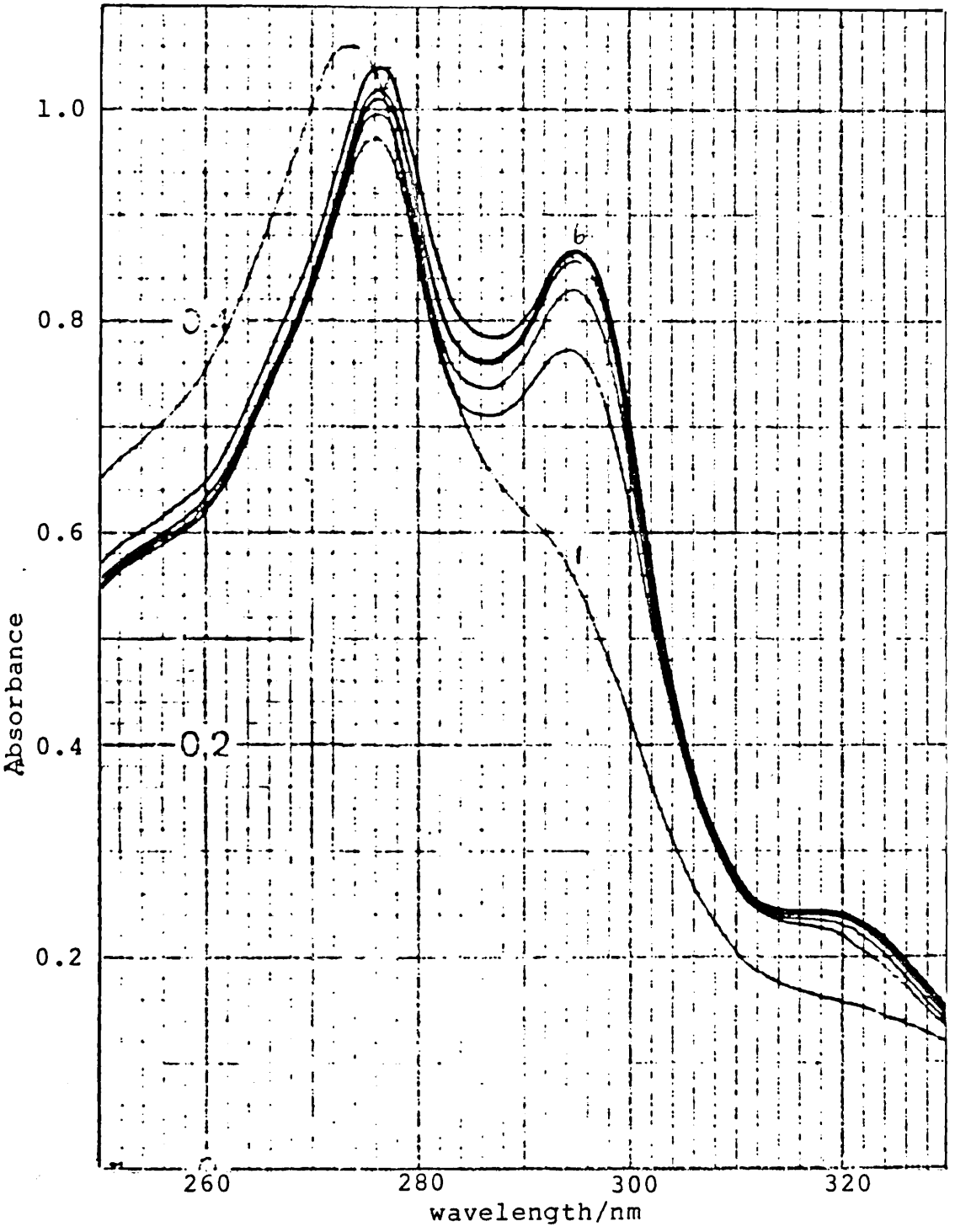


Fig.4.6.

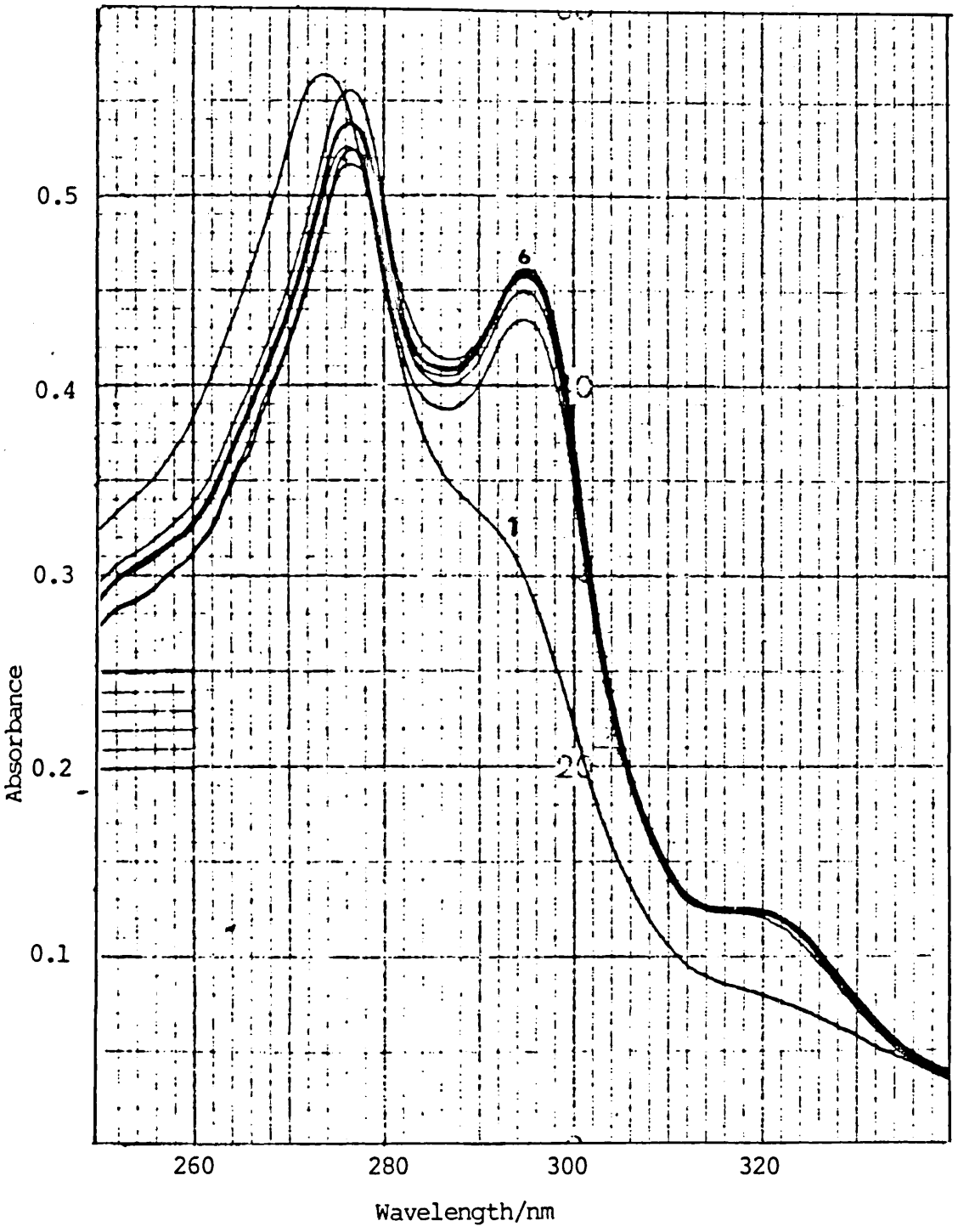


Fig.4.7.

protein is equivalent to the total drug concentration, and 1 is the pathlength.

Similarly, in the absence of protein, the absorbance is given by:-

$$A = \varepsilon_f [D]1 \qquad (4.2)$$

Where ε_f is the molar extinction coefficient at a particular wavelength of the free dye and [D] is its concentration. Assuming the equilibrium to apply:

$$D + S = DS \qquad K = \frac{[DS]}{[D][S]} \qquad (4.3)$$

Where [S] is the concentration of free site, and K is the association constant for the reaction, at any intermediate concentration of protein the absorbance at any wavelength will be a composite of free and bound species and also the absorbance due to the protein.

In the sample beam the absorbance (A_{Sa}) is given by

$$A_{Sa} = A_{DF} + A_{DB} + A_n + A_{FP} + A_{BP} + A_{SYS} \qquad (4.4)$$

where A_{DF} = the absorbance due to free drug

A_{DB} = the absorbance due to bound drug

A_n = the absorbance due to chromophores on the albumin which do not participate in the reaction

A_{FP} = the absorbance due to unbound binding sites on the albumin molecule

A_{BP} = the absorbance due to bound binding sites on the albumin molecule

A_{SYS} = absorbance due to buffer, cells, etc.

The free site concentration is given by

$$[S] = [S_{tot}] - [DS]$$

where $[S_{tot}]$ = the total site concentration.

Substituting corresponding extinction coefficients and concentrations

$$A_{Sa} = \varepsilon_F[D] + \varepsilon'_B[DS] + A_n + \varepsilon_{FP}([S_{tot}]-[DS]) + \varepsilon_{BP}[DS] + A_{sys} \quad (4.5)$$

Similarly in the reference beam

$$A_{ref} = A_n + \varepsilon_{FP}[S_{tot}] + A_{sys} \quad (4.6)$$

thus the observed absorbance is given by

$$\frac{A}{l} = \varepsilon_F[D] + (\varepsilon'_B + \varepsilon_{BP} - \varepsilon_{FP})[DS] \quad (4.7)$$

let $$\varepsilon'_B + \varepsilon_{BP} - \varepsilon_{FP} = \varepsilon_B \quad (4.8)$$

and since $[D] = [D_{tot}] - [DS]$ where $[D_{tot}]$ is the total drug concentration

$$\frac{A}{l} = \varepsilon_F[D_{tot}] + (\varepsilon_B - \varepsilon_F)[DS] \quad (4.9)$$

When [D] is very small, i.e., in the presence of a large excess of albumin ε_B may be determined.

Thus values for [DS] may be calculated from any absorbance (Table 4.1).

From figs.4.6 and 4.7 at 295nm $\varepsilon_B = 3314 \text{ mol}^{-1}\text{m}^2$ $\varepsilon_f = 1935 \text{ mol}^{-1}\text{m}^2$ $\varepsilon_B - \varepsilon_f = 1379 \text{ mol}^{-1}\text{m}^2$

(A) Absorbance at 295nm	A/l /m^{-1}	$[D_{tot}]$ /mol m^{-3}	$f[D_{tot}]$ /m^{-1}	[DS] mol m^{-3}	[D] /mol m^{-3}	$1/[DS]$ /$mol^{-1}m^3$	$1/[D]$ /$mol^{-1}m^3$
H.S.A. concentration = 5.798×10^{-5} mol dm^{-3}							
.225	51.4	.0176	34.1	.0125	.0051	80.0	196
.435	99.4	.0346	67.0	.0235	.0111	42.6	90.1
.625	142	.0509	98.5	.0321	.0188	31.2	53.2
.818	187	.0666	129	.0421	.0245	23.8	40.8
.983	226	.0818	158	.0481	.0336	20.8	29.8
1.150	263	.0964	187	.0555	.0409	18.0	24.4
1.300	297	.1103	214	.0606	.0498	16.5	20.1
1.445	330	.1240	239	.0656	.0584	15.2	17.1
H.S.A. concentration = 2.899×10^{-5} mol dm^{-3}							
.218	49.8	.0176	34.1	.0114	.0062	87.7	161
.415	94.9	.0346	67.0	.0202	.0144	49.5	69.4
.590	135	.0509	98.5	.0263	.0246	38.0	40.6
.778	178	.0666	129	.0355	.0311	28.2	32.2
.932	213	.0818	158	.0398	.0420	25.1	23.8
1.085	248	.0964	187	.0447	.0517	22.4	19.3
1.241	284	.1104	214	.0509	.0595	19.6	16.8
1.390	318	.1240	239	.0565	.0675	17.7	14.8
H.S.A. concentration = 1.449×10^{-5} mol dm^{-3}							
.205	46.9	.0176	34.1	.0928	.0083	107	120.5
.415	94.9	.0346	67.0	.0202	.0144	49.5	69.4
.582	133	.0509	98.5	.0250	.0259	40.0	38.6
.752	172	.0666	129	.0311	.0355	32.2	28.2
.935	214	.0818	158	.0403	.0415	24.8	24.1
1.095	250	.0964	187	.0463	.0501	21.6	20.0
1.241	284	.1104	214	.0509	.0595	19.6	16.8
1.382	316	.1240	239	.0552	.0688	18.1	14.5

Table 4.1. Binding data for M.T.T.

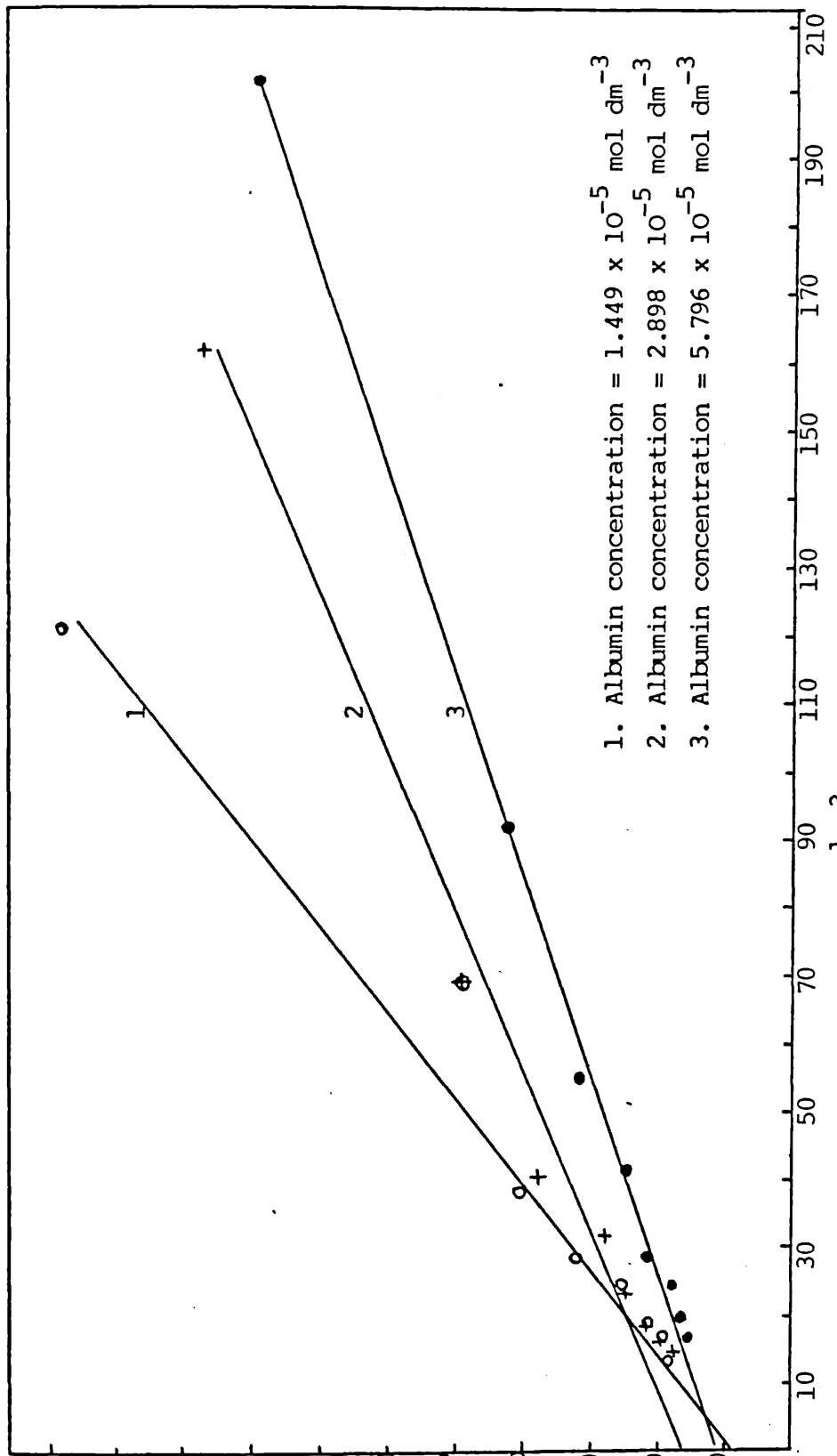


Fig. 4.8. Reciprocal plot for M.T.T.

Calculating slopes and intercepts by linear regression, the values shown in Table 4.2 are obtained.

Table 4.2.

Albumin concentration /10^{-5} mol dm^{-3}	Intercept /mol^{-1} m^3	Gradient	n[P] /mol m^{-3}	n	K /10^4 mol^{-1} dm^3
1.449	10	0.35	0.10	6.9	2.9
2.899	15	0.44	0.067	2.31	3.4
5.798	8	0.81	0.13	2.27	9.9

In fact it is unlikely that the absorbance of albumin can be thought to change in a regular way, since binding may be accompanied by a conformational change. In order to overcome the problems encountered as a result of changes taking place in the albumin spectrum, compounds for further study were chosen, which absorbed at wavelengths greater than 300nm where the absorbance of albumin is small, and thus changes in the absorbance spectrum of albumin at wavelengths greater than 300nm would be negligable.

4.2 Sulindac - H.S.A. interactions

Sulindac[45] has an absorbance maximum at 322nm. The spectrum shows that, on addition of human serum albumin, there is a red shift, and the maximum absorbance diminishes (fig. 4.9 and 4.10).

One clear consequence of the equation

$$\frac{A}{l} = \varepsilon_f [D] + \varepsilon_b [DS]$$

is that if the spectra of free and bound species overlap, i.e. at some point $\varepsilon_f = \varepsilon_b$, and if the total drug concentration remains constant for a series of solutions, then all the spectra of the series will pass through that point. (The isosbestic point)

The Sulindac, human serum albumin system, shows a clearly defined isosbestic point at 338nm. Data for this system is shown in Tables 4.3 and 4.4.

Standard deviation for the free drug concentrations were calculated from the equation

$$S = \frac{(\Sigma[D_i^2]) - (\Sigma[D_i]^2)}{(n-1)} \qquad (4.10)$$

the coefficient of variation is given by

$$C = \frac{S}{[\bar{D}]} \times 100\% \qquad (4.11)$$

$[\bar{D}]$ = the mean free drug concentration.

Figs. 4.9 and 4.10 Interaction of Sulindac with H.S.A. fraction V (experimental method 1.18) Drug concentrations are 1.998×10^{-4} and 9.989×10^{-5} mol dm^{-3} respectively

Albumin concentrations/10^{-6} mol dm^{-3}
(1) 0, (2)2.841 (3) 5.580, (4) 8.203,
(5) 10.72, (6) 13.17, (7) 15.52,
(8) 17.80.

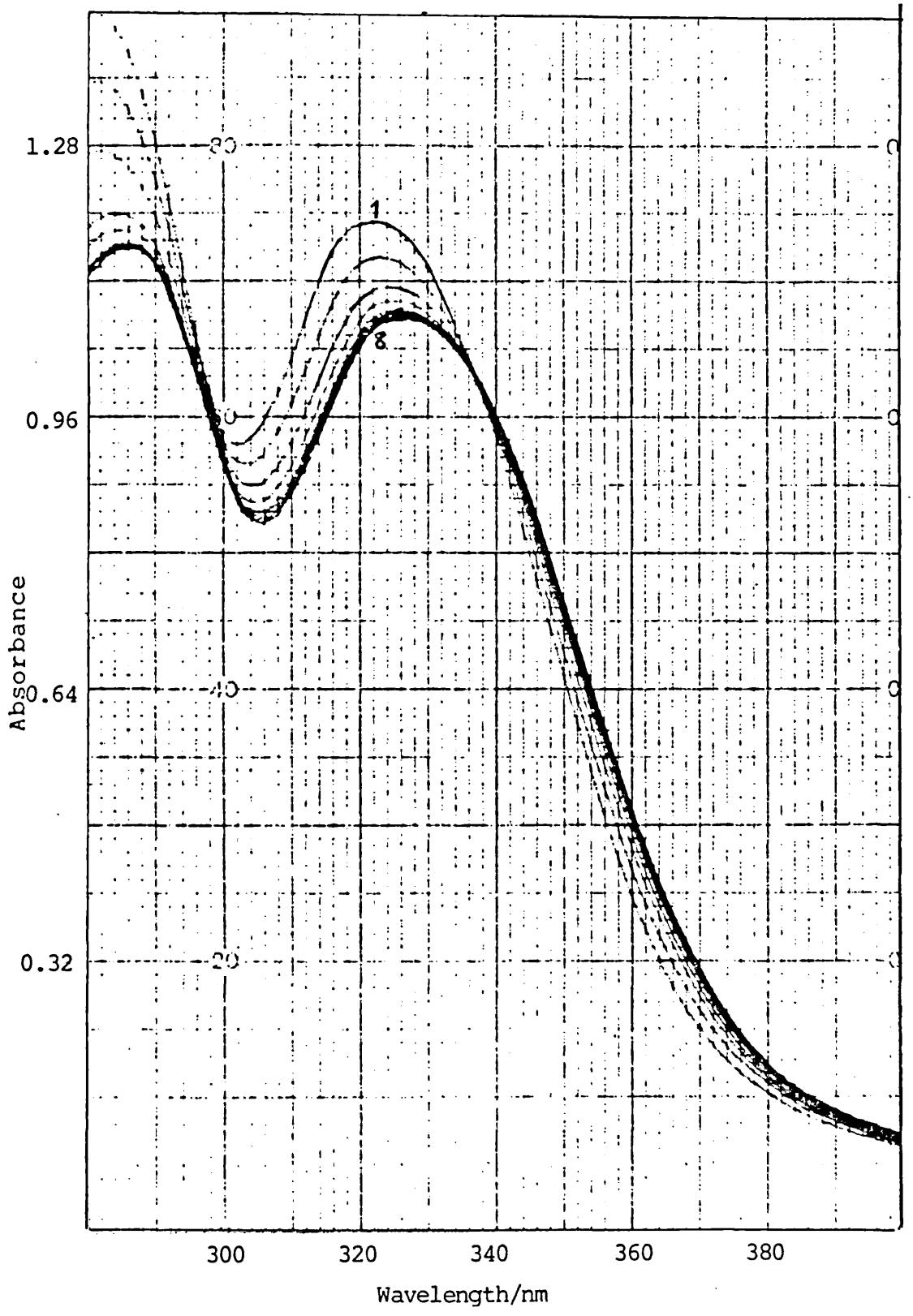

1.28
0.96
0.64
0.32
Absorbance
1
8
300
320
340
360
380
Wavelength/nm

Fig. 4.9.

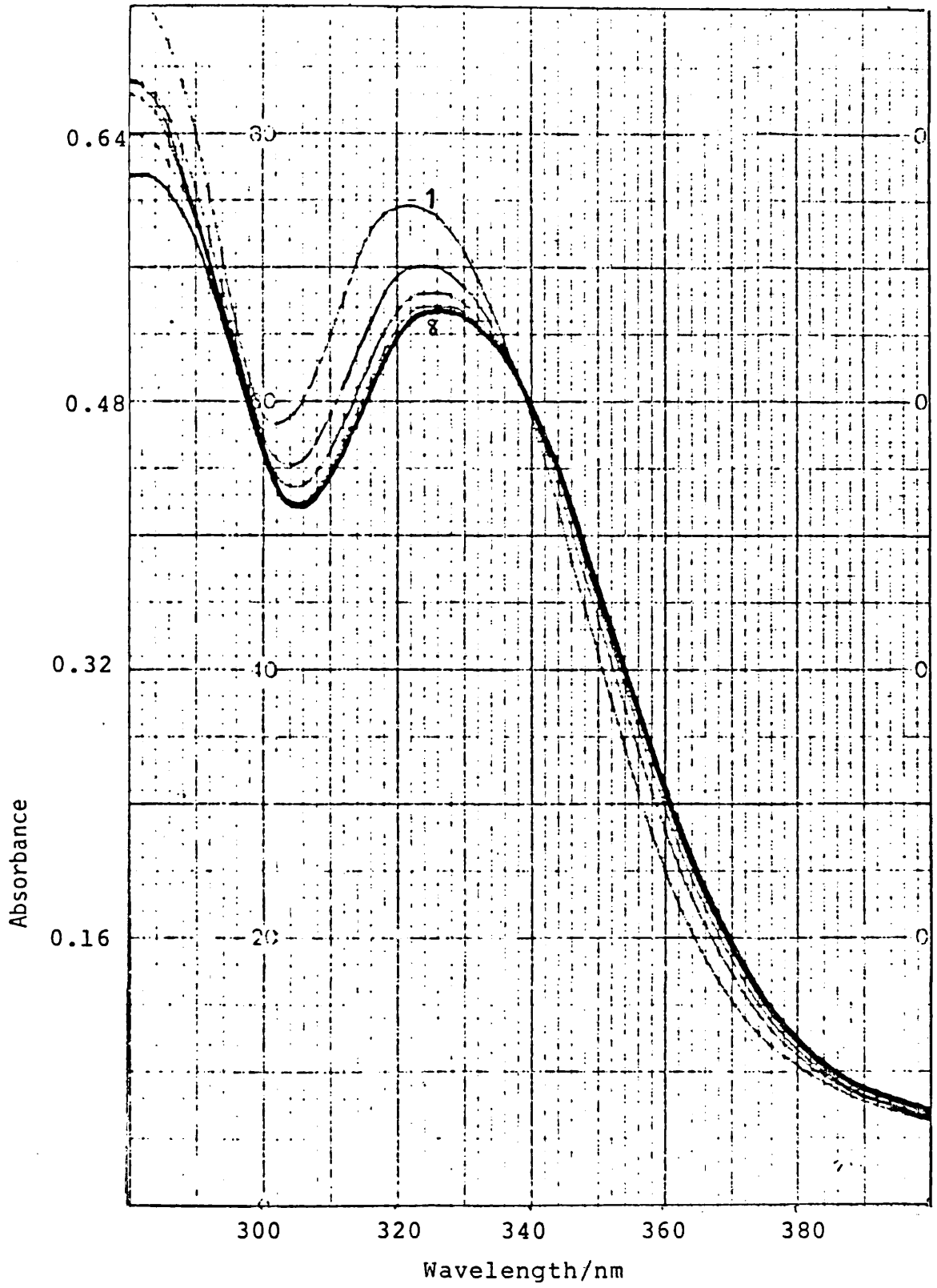

0.64
0.48
0.32
0.16
Absorbance
300
320
340
360
380
Wavelength/nm
1
8

Fig.4.10.

Total Sulindac concentration = 1.998×10^{-4} mol dm^{-3}

Albumin concentration /10^{-6} mol dm^{-3}	Wavelength/nm 330	328	326	324	322	320	318	316	314	312	310	Calculated [D] /10^{-5} mol dm^{-3}
2.841	13.66	13.55	13.57	13.64	13.52	13.55	13.36	13.50	13.52	13.75	13.68	
5.580	8.36	8.17	8.08	8.10	8.04	8.01	8.04	7.68	7.99	8.17	8.15	
8.203	4.69	4.69	4.73	4.73	4.51	4.52	4.45	4.50	4.47	4.41	4.30	
10.72	2.24	2.26	2.44	2.43	2.44	2.37	2.39	2.42	2.35	2.47	2.31	
13.17	1.63	1.56	1.53	1.62	1.46	1.47	1.52	1.54	1.53	1.69	1.54	
Total Sulindac concentration = 9.848×10^{-5} mol dm^{-3}												
2.841	5.17	5.07	4.86	4.80	4.77	4.73	4.68	4.58	4.59	4.61	4.46	
5.580	2.50	2.38	2.36	2.15	2.16	2.04	1.98	2.02	1.84	1.66	1.69	
8.203	0.89	0.79	0.83	.76	.79	.75	.73	.64	.46	.38	.31	

Table 4.3. Data for Sulindac - H.S.A. interactions

Total drug concentration /10^{-5} mol dm^{-3}	Total H.S.A. concentration /10^{-6} mol dm^{-3}	Mean free drug concentration /10^{-5} mol dm^{-3}	Mean bound drug concentration /10^{-5} mol dm^{-3}	Standard deviation in free drug values /10^{-5} mol dm^{-3}	Free drug concentration coefficient of variation (%)	$\frac{[DS]}{[P][D]}$ 10^{5} $mol^{-1} dm^{3}$	$\frac{[DS]}{[P]}$
19.98	2.841	13.57	6.41	0.106	0.8	1.66	22.6
19.98	5.580	8.07	11.91	0.166	2.1	2.64	21.3
19.98	8.203	4.55	15.43	0.144	3.2	4.13	18.8
19.98	10.72	2.37	17.61	0.077	3.2	6.93	16.4
19.98	13.17	1.55	18.43	0.069	4.4	9.03	14.0
9.85	2.841	4.76	5.09	0.208	4.4	3.77	17.9
9.95	5.580	2.07	7.78	0.275	13.3	6.73	13.9
9.85	8.203	0.67	9.18	0.195	29.2	16.7	11.2

Table 4.4. Mean data and Scatchard data for Sulindac - H.S.A. interactions.

$K = 1.109 \times 10^{5}$ mol^{-1} dm^{3}

$n = 22.8$

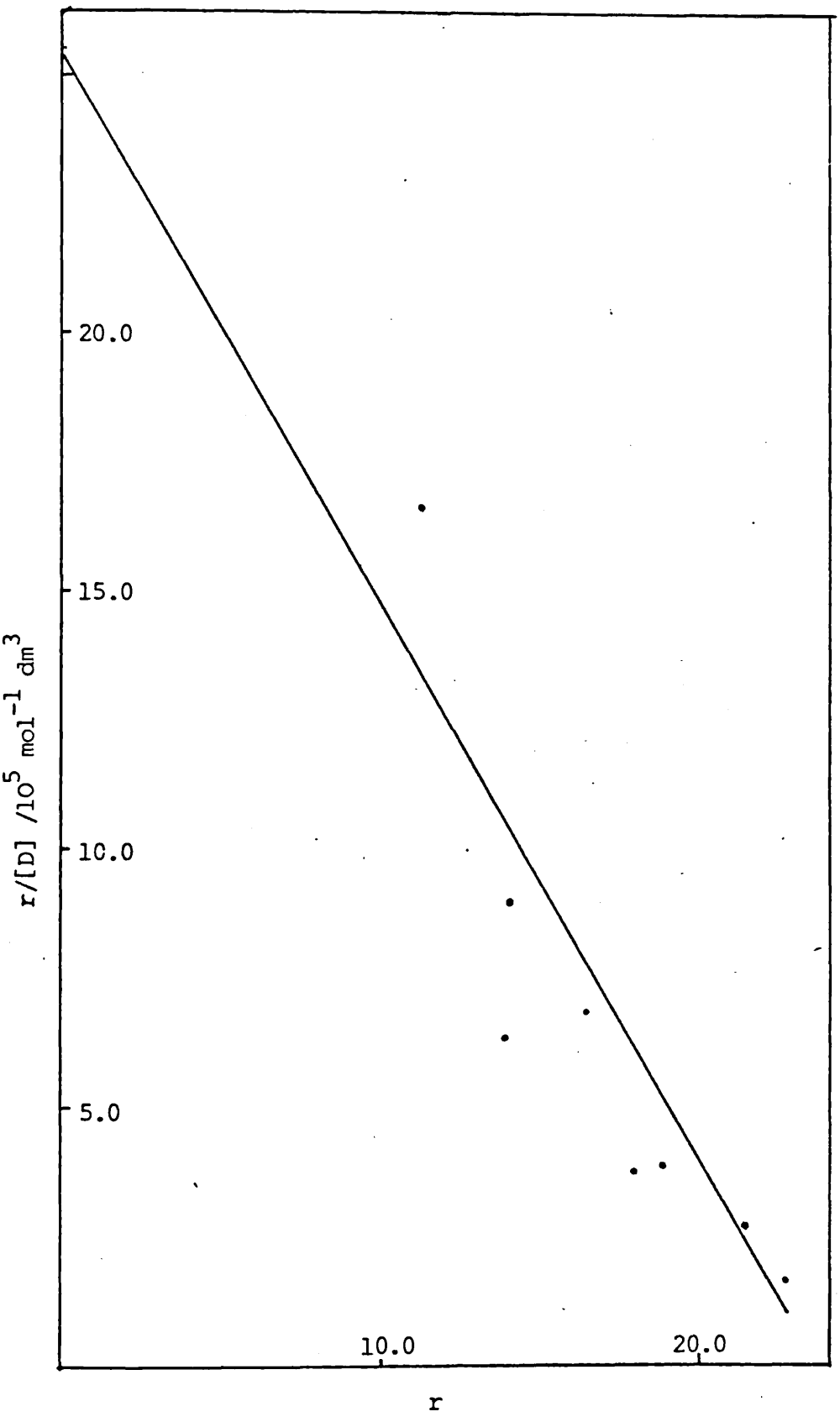


Fig.4.11. Scatchard plot for Sulindac-H.S.A. interactions.

4.3 Mordant Orange-1 - H.S.A. interactions

The spectrum of the azo dye Mordant Orange 1, when complexed with human serum albumin (experimental method 1.8), shows no isosbestic point (fig.4.12). This, together with matrix rank analysis (see ch.3), indicates that the simple single site model is not sufficient to explain the behaviour of this system. The next simplest model is one in which the drug may bind independently to two classes of sites on the albumin molecule in the following way:-

Consider the two equilibria

$$\begin{array}{ccc} D + S_1 & \overset{K_1}{\rightleftharpoons} & DS_1 \\ D_f\ (B-x) & & x \end{array} \qquad K_1 = \frac{[DS_1]}{[S_1][D]} = \frac{x}{(B-x)D_f} \qquad (4.12)$$

$$\begin{array}{ccc} D + S_2 & \overset{K_2}{\rightleftharpoons} & DS_2 \\ D_f\ (C-y) & & y \end{array} \qquad K_2 = \frac{[DS_2]}{[S_2][D]} = \frac{y}{(C-y)D_f} \qquad (4.13)$$

Where x = the concentration of the first bound species DS_1

y = the concentration of the second bound species DS_2

D_f = the concentration of free drug

B = the total concentration of the primary site S_1

C = the total concentration of the secondary site S_2

D_{tot} = the total dye concentration

n_1 = the number of primary binding sites per albumin molecule

n_2 = the number of secondary binding sites per albumin molecule

P = the total albumin concentration

thus $B = n_1 P$ and $C = n_2 P$

also $$x = \frac{K_1 B D_f}{(K_1 D_f + 1)} \qquad (4.14)$$

$$y = \frac{K_2 C D_f}{(K_2 D_f + 1)} \qquad (4.15)$$

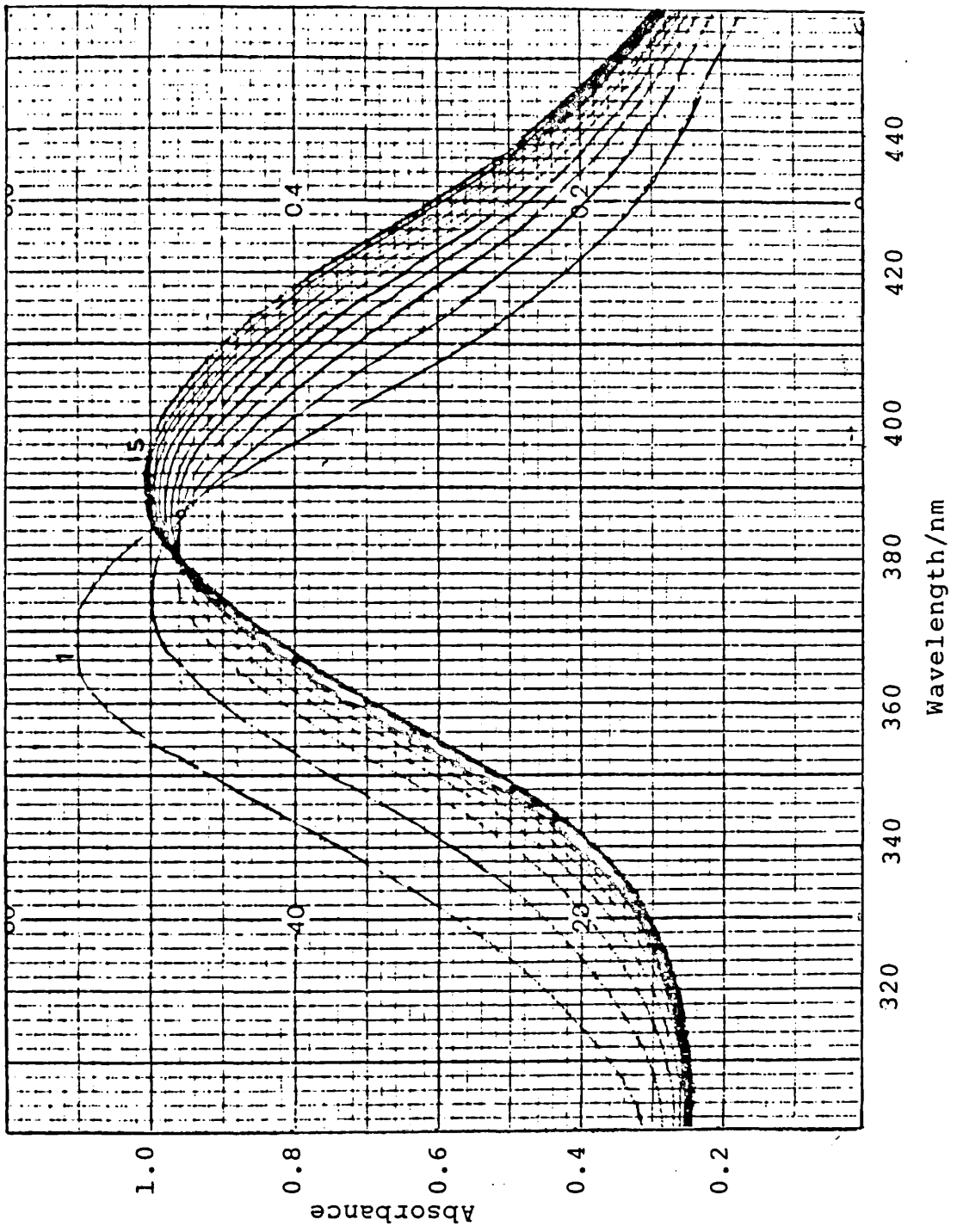
Therefore since $D_{tot} = D_f + x + y$

$$D_f = A - \frac{K_1 B D_f}{K_1 D_f + 1} - \frac{K_2 C D_f}{K_2 D_f + 1} \qquad (4.16)$$

Fig. 4.12 Interaction of Mordant Orange 1 with H.S.A.

Mordant Orange 1 concentration = 1.558×10^{-4} mol dm^{-3}.

Albumin concentrations/10^{-5} mol dm^{-3}
(1) 0, (2) .7214, (3) 1,415, (4) 2.083
(5) 2.726, (6) 3.344, (7) 3.942,
(8) 4.519, (9) 5.075, (10) 5.613,
(11) 6.133, (12) 6.636, (13) 7.122,
(14) 7.593, (15) 8.049.


Absorbance
1.0
0.8
0.6
0.4
0.2
320
340
360
380
400
420
440
Wavelength/nm
1
15
2
4
8
23
40

The solution to this equation is:-

$$K_1 K_2 D_f^3 + (K_1 + K_2 - D_{tot} K_1 K_2 + B K_1 K_2 + C K_1 K_2) D_f^2 + (1 - D_{tot} K_1 - D_{tot} K_2 + K_1 B + K_2 C) D_f - D_{tot} = 0 \qquad (4.17)$$

At any wavelength, assuming that all three species obey Beer's law, the absorbance A is given by:-

$$\frac{A}{l} = \varepsilon_f D_f + \varepsilon_1 X + \varepsilon_2 Y \qquad (4.18)$$

Where ε_f, ε_1 and ε_2 are the respective molar extinction coefficients of the three species D_f, DS_1 and DS_2.

If all seven constants are known(K_1, K_2, n_1, n_2, ε_f, ε_1, ε_2) the absorbance of any solution of the albumin-dye system, at a given wavelength, can be readily calculated.

In reality only ε_f is known. However, the other constants may be obtained by guessing values for them, substituting into 4.17 and 4.18, and comparing calculated absorbances with those measured experimentally. The guesses are then refined until calculated and measured absorbances match as closely as possible.

The cubic equation 4.17 is solved by Newton's method and has three real roots, only one of these, however, permits both x and y to be positive. The accuracy of the guesses is obtained from the relationship

$$S = (A_{obs} - A_{calc})^2$$

Where A_{obs} is the observed absorbance, and A_{calc} is the calculated absorbance.

Results for the H.S.A. - Mordant Orange system are shown in Table 4.5.

It may be seen that although the results for the two

experiments agree closely, good correlation between observed and calculated absorbances may be obtained by using quite different values for the unknown constants. This serves to illustrate the point that for fitting routines of this nature there are frequently a number of equally mathematically acceptable sets of parameters.

Matters may be improved by minimising S for absorbances of the solutions at five different wavelengths. There is also an isosbestic point between the two bound species at 380nm which may be observed when the albumin concentration is high and hence the free dye concentration approaches zero. The method has the advantage that spectra for the three species may be plotted. Any data sets which produce sharp discontinuities in the spectra may be eliminated.

In this instance absorbances were obtained at 10nm intervals for each spectrum. The five wavelengths chosen other than the isosbestic point wavelength were those at which the largest changes in absorbance were observed, in this case 350, 360, 370, 410 and 420 nm. The remaining extinction coefficients at 380 and 390 nm were obtained by maintaining K_1, K_2 n_1 and n_2 at the best values found and adjusting the four unknown extinction coefficients to their best values.

Table 4.6 and 4.7 show data for the best fits found at a dye concentration of 1.558×10^{-4} mol dm^{-3}. Tables 4.8 and 4.9 summarise data obtained for the same system at different dye and albumin concentrations.

Table 4.5 Typical absorbance and fitting data for the Mordant Orange - H.S.A. system at 360nm

Experimental method 1.8d
(Dye concentration = 1.558×10^{-6} mol dm^{-3})

Total albumin concentration /10^{-5} mol dm^{-3}	Observed absorbance/cm at 360nm	Calculated absorbance/cm at 360nm
.7187	2.023	2.020
1.410	1.819	1.823
2.075	1.714	1.716
2.715	1.653	1.652
3.332	1.609	1.610
3.928	1.582	1.581
4.501	1.568	1.560
5.055	1.543	1.544
5.591	1.531	1.531
1.109	1.518	1.520
6.609	1.513	1.512
7.094	1.506	1.505
7.564	1.495	1.499
8.017	1.493	1.494

Constants

$K_1 = 6.60 \times 10^3$ mol^{-1} dm^3
$K_2 = 8.35 \times 10^3$ mol^{-1} dm^3
$n_1 = 13.71$
$n_2 = 6.39$
$\epsilon_1 = 980.8$ mol^{-1} m^2
$\epsilon_2 = 771.1$ mol^{-1} m^2
$\epsilon_f = 1525$ mol^{-1} m^2

Experimental method 1.8c
(Dye concentration = 1.558×10^{-4} mol dm^{-3})

Total albumin concentration /10^{-5} mol dm^{-3}	Observed absorbance/cm at 360nm	Calculated absorbance/cm at 360nm
.7214	1.993	2.002
1.415	1.817	1.808
2.083	1.721	1.704
2.726	1.634	1.642
3.344	1.589	1.602
3.942	1.566	1.573
4.519	1.550	1.552
5.075	1.536	1.536
5.613	1.525	1.524
6.133	1.518	1.514
6.636	1.506	1.505
7.122	1.497	1.498
7.593	1.495	1.492
8.049	1.493	1.487

Constants

$K_1 = 1.58 \times 10^3$ mol^{-1} dm^3
$K_2 = 6.80 \times 10^3$ mol^{-1} dm^3
$n_1 = 10.66$
$n_2 = 18.00$
$\epsilon_1 = 896.4$ mol^{-1} m^2
$\epsilon_2 = 899.0$ mol^{-1} m^2
$\epsilon_f = 1525$ mol^{-1} m^2

Table 4.6 Absorbance and fitting data for the Mordant Orange-1-H.S.A. system at multiple wavelengths

(Experimental method 1.8d)

albumin conc^{n} /10^{-5} mol dm^{-3}		wavelength/nm 350	360	370	380	390	400	410	420
		Absorbance/cm							
.7187	OBS	1.653	2.023	2.258	2.320	2.181	1.895	1.568	1.250
	CALC	1.645	2.019	2.254	2.321	2.181	1.896	1.568	1.241
1.410	OBS	1.442	1.819	2.103	2.242	2.199	1.991	1.705	1.367
	CALC	1.442	1.821	2.103	2.246	2.197	1.987	1.699	1.376
2.075	OBS	1.335	1.714	2.021	2.201	2.213	2.048	1.785	1.474
	CALC	1.335	1.716	2.024	2.210	2.218	2.056	1.794	1.476
2.715	OBS	1.269	1.653	1.975	2.187	2.238	2.112	1.870	1.559
	CALC	1.272	1.653	1.977	2.190	2.238	2.111	1.869	1.555
2.332	OBS	1.227	1.609	1.945	2.178	2.256	2.155	1.927	1.621
	CALC	1.231	1.612	1.946	2.179	2.256	2.155	1.929	1.619
3.928	OBS	1.198	1.582	1.922	2.171	2.272	2.192	1.977	1.673
	CALC	1.201	1.583	1.924	2.172	2.272	2.192	1.977	1.672
4.501	OBS	1.184	1.568	1.915	2.174	2.293	2.231	2.023	1.719
	CALC	1.180	1.561	1.908	2.167	2.285	2.221	2.017	1.715
5.055	OBS	1.163	1.543	1.897	2.165	2.297	2.247	2.050	1.749
	CALC	1.163	1.544	1.895	2.163	2.296	2.246	2.049	1.750
5.591	OBS	1.150	1.531	1.888	2.162	2.306	2.267	2.075	1.778
	CALC	1.150	1.531	1.886	2.160	2.305	2.266	2.076	1.779
6.109	OBS	1.138	1.518	1.877	2.158	2.311	2.281	2.096	1.799
	CALC	1.139	1.520	1.878	2.158	2.313	2.283	2.099	1.803
6.609	OBS	1.134	1.513	1.872	2.158	2.322	2.297	2.119	1.824
	CALC	1.131	1.511	1.871	2.157	2.320	2.297	2.118	1.824
7.094	OBS	1.127	1.506	1.865	2.155	2.325	2.306	2.128	1.833
	CALC	1.124	1.504	1.866	2.156	2.325	2.310	2.134	1.841
7.564	OBS	1.115	1.495	1.858	2.153	2.325	2.315	2.146	1.856
	CALC	1.118	1.498	1.862	2.155	2.330	2.320	2.147	1.856
8.014	OBS	1.113	1.493	1.858	2.158	2.334	2.331	2.165	1.877
	CALC	1.113	1.493	1.858	2.154	2.334	2.329	2.159	1.869

	wavelength/nm 350	360	370	380	390	400	410	420
ϵ_1/mol^{-1}m^2	630.8	870.5	1129	1376	1599	1696	1650	1488
ϵ_2/mol^{-1}m^2	798.3	1049	1256	1376	1352	1213	1018	796.8
ϵ_f/mol^{-1}m^2	1302	1534	1630	1585	1397	1136	886.4	677.5

$K_1 = 2.54 \times 10^5$ mol^{-1} dm^3 $\quad n_2 = 16.9$

$K_2 = 1.02 \times 10^4$ mol^{-1} dm^3 $\quad$ Dye conc^{n} = 1.558×10^{-4} mol dm^{-3}

$n_1 = 2.14$

Table 4.7 Absorbance and fitting data for the Mordant Orange-1-H.S.A. System at multiple wavelengths
(Experimental method 1.8c)

wavelength/nm

Albumin concentration /10^{-5} mol dm^{-3}		350	360	370	380	390	400	410	420
.7214	OBS	1.625	1.993	2.233	2.299	2.169	1.888	1.573	1.250
	CALC	1.620	2.003	2.234	2.302	2.170	1.885	1.569	1.245
1.415	OBS	1.415	1.817	2.078	2.222	2.183	1.984	1.703	1.390
	CALC	1.414	1.806	2.082	2.226	2.186	1.977	1.704	1.385
2.083	OBS	1.312	1.721	2.021	2.192	2.208	2.027	1.799	1.486
	CALC	1.312	1.703	2.005	2.192	2.208	2.048	1.801	1.487
2.726	OBS	1.250	1.634	1.957	2.171	2.229	2.107	1.877	1.563
	CALC	1.253	1.643	1.960	2.174	2.228	2.104	1.875	1.565
3.344	OBS	1.209	1.589	1.922	2.160	2.245	2.151	1.931	1.623
	CALC	1.216	1.602	1.931	2.164	2.246	2.148	1.933	1.626
3.942	OBS	1.186	1.566	1.909	2.158	2.263	2.187	1.977	1.669
	CALC	1.190	1.574	1.911	2.157	2.260	2.183	1.978	1.674
4.519	OBS	1.173	1.550	1.895	2.155	2.277	2.213	2.011	1.707
	CALC	1.171	1.553	1.896	2.153	2.272	2.212	2.014	1.712
5.075	OBS	1.163	1.536	1.888	2.153	2.286	2.242	2.048	1.749
	CALC	1.157	1.536	1.885	2.150	2.282	2.234	2.043	1.743
5.613	OBS	1.147	1.525	1.877	2.151	2.295	2.258	2.071	1.771
	CALC	1.146	1.524	1.876	2.148	2.290	2.253	2.067	1.768
6.133	OBS	1.141	1.518	1.872	2.149	2.299	2.272	2.089	1.792
	CALC	1.138	1.513	1.869	2.146	2.297	2.268	2.086	1.789
6.636	OBS	1.131	1.506	1.861	2.144	2.297	2.274	2.094	1.797
	CALC	1.131	1.505	1.863	2.145	2.303	2.281	2.103	1.806
7.122	OBS	1.122	1.497	1.856	2.142	2.302	2.286	2.112	1.817
	CALC	1.125	1.498	1.859	2.144	2.308	2.292	2.116	1.820
7.593	OBS	1.120	1.495	1.856	2.144	2.313	2.299	2.130	1.838
	CALC	1.120	1.492	1.855	2.143	2.312	2.301	2.128	1.833
8.049	OBS	1.118	1.493	1.854	2.142	2.313	2.309	2.139	1.849
	CALC	1.116	1.487	1.851	2.142	2.315	2.309	2.138	1.844

wavelength/nm

	350	360	370	380	390	400	410	420
ϵ_1/mol^{-1}m^2	634.9	846.5	1118	1369	1591	1705	1649	1479
ϵ_2/mol^{-1}m^2	795.7	1074	1261	1369	1340	1180	1001	786.6
ϵ_f/mol^{-1}m^2	1298	1531	1626	1578	1391	1131	883.5	672.5

$k_1 = 2.16 \times 10^5$ mol^{-1} dm^3

$k_2 = 1.15 \times 10^4$ mol^{-1} dm^3

$n_1 = 2.37$

$n_2 = 16.9$

Dye conc^n $= 1.558 \times 10^{-4}$ mol dm^{-3}

	Mean extinction coefficient values for dye concentration 7.792×10^{-5} mol dm^{-3} / 10^3 mol^{-1} m^2			Mean extinction coefficient values for dye concentration 1.558×10^{-4} mol dm^{-3} / 10^3 mol^{-1} m^2			Mean extinction coefficient values for dye concentration 2.453×10^{-4} mol dm^{-3} / 10^3 mol^{-1} m^2			Mean extinction coefficient value for all concentrations / 10^3 mol^{-1} m^2		
wavelength /nm	ε_1	ε_2	ε_f	ε_1	ε_2	ε_f	ε_1	ε_2	ε_f	ε_1	ε_2	ε_f
350	.728	.678	1.30	.637	.797	1.30	.686	.760	1.30	.684	.745	1.30
360	.942	.955	1.53	.859	1.06	1.53	.925	1.01	1.53	.909	1.01	1.53
370	1.16	1.18	1.62	1.13	1.26	1.63	1.17	1.22	1.62	1.15	1.22	1.62
380	1.35	1.35	1.58	1.38	1.38	1.58	1.37	1.37	1.58	1.37	1.37	1.58
390	1.48	1.36	1.39	1.60	1.35	1.40	1.60	1.36	1.39	1.56	1.36	1.39
400	1.51	1.22	1.13	1.71	1.20	1.14	1.67	1.24	1.12	1.63	1.22	1.13
410	1.45	1.02	.874	1.65	1.01	.886	1.62	1.06	.874	1.57	1.03	.878
420	1.27	.814	.666	1.49	.798	.677	1.42	.854	.668	1.39	.822	.670

Table 4.8 The extinction coefficients calculated for the Mordant Orange 1 - H.S.A. system

	Dye concentration/10^{-4} mol dm^{-3}			
	0.7792	1.558	2.453	Mean
$10^{-5} \times K_1/mol^{-1}\ dm^3$	2.20	2.35	3.01	2.52
$10^{-4} \times K_2/mol^{-1}\ dm^3$	1.47	1.07	0.98	1.17
n_1	2.82	2.26	1.76	2.28
n_2	11.4	16.9	15.6	14.6

Table 4.9 Binding constants and proportionality constants obtained for the Mordant Orange 1 - H.S.A. system

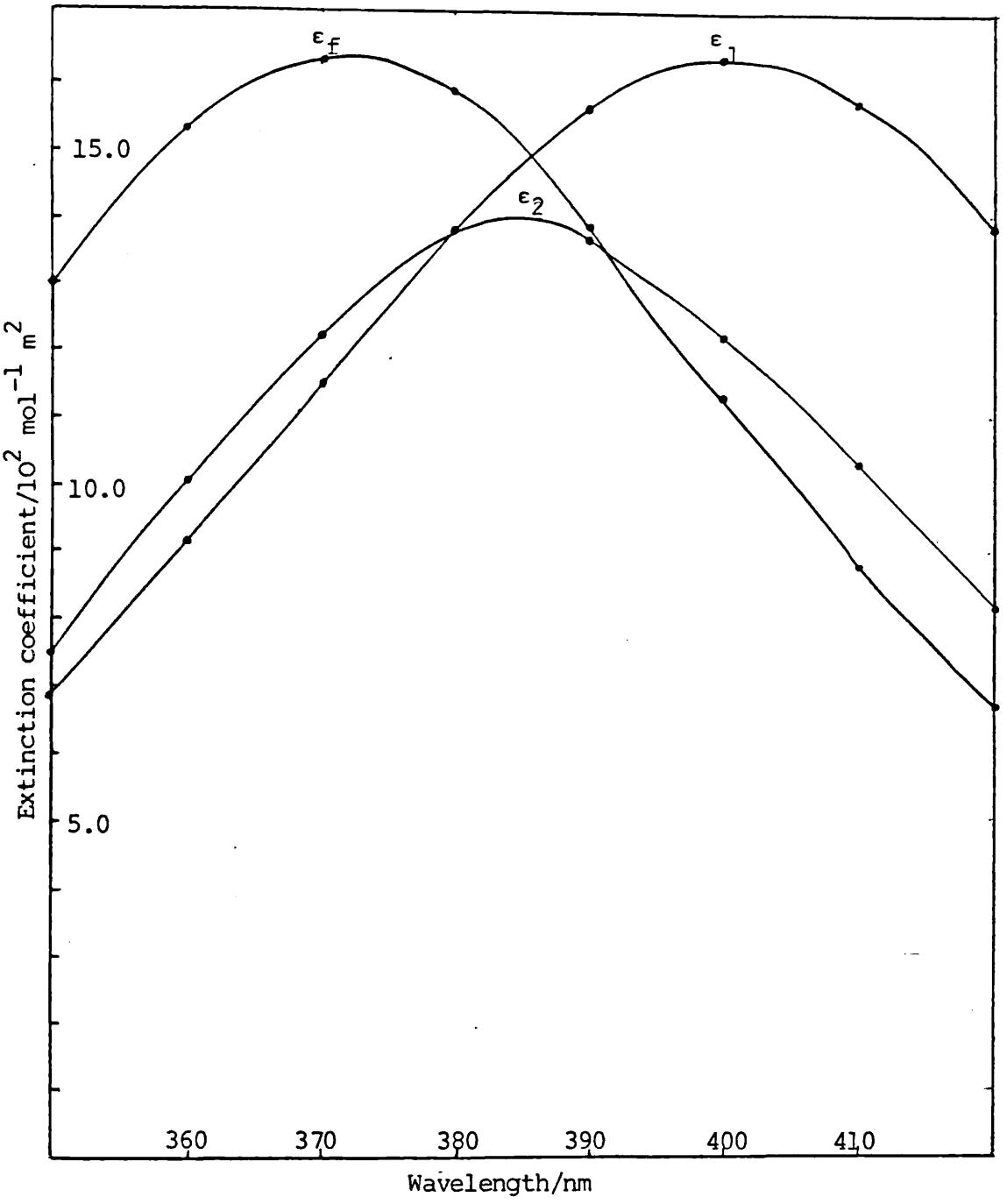


Fig.4.13 Resolved extinction coefficient for the three dye species present in the Mordant Orange 1 - H.S.A. system.

CHAPTER 5

THE SPECTROPHOTOMETRIC MEASUREMENT OF THE INTERACTIONS BETWEEN SOME ORGANIC ANIONS AND HUMAN SERUM ALBUMIN

5.1. Mordant Yellow - H.S.A. interactions

The method described in Chapter 4 to obtain binding parameters for Mordant Orange 1, was applied to the Mordant Yellow 12/human serum albumin system (Experimental method 1.9). A typical series of spectra is shown in fig.5.1. The overall change in the dye spectrum on binding with albumin is not as pronounced as that with Mordant Orange 1. Nevertheless a matrix rank analysis of the spectra indicates the existence of three species (p87) in solution, of which one is the free dye. Since there is a virtual isosbestic point at 387nm which hardly moves throughout the course of the titration it is not possible to use this point in the same way as for Mordant Orange 1, because absorbance changes at this point are so small. Therefore, five wavelengths were used for the 'best fit' determination, 350, 360, 370, 440 and 450 nm. It was found that the most consistent values for the binding parameters were obtained by 'pooling' the results from three separate titrations. Several different permutations of the sets of data were tried, and found to give essentially the same binding parameters (Table 5.6).

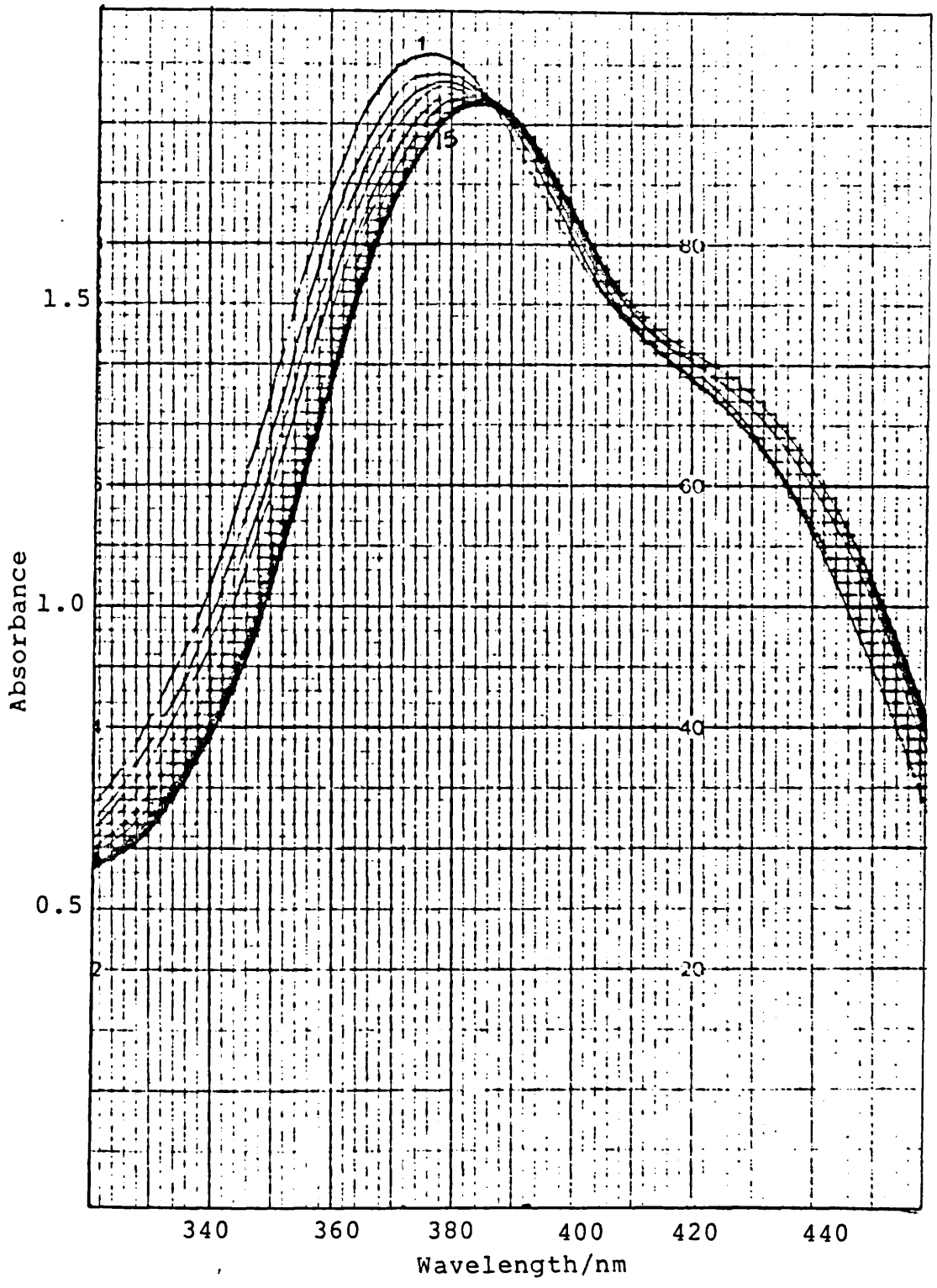
Table 5.1 - 5.3 give absorbance and fitting data for the Mordant Yellow 12 - H.S.A. system, as a result of pooling data from 3 titrations. The binding parameters obtained are given in Table 5.4.

Extinction coefficient values at the remaining wavelengths were obtained by fixing K_1, K_2, n_1 and n_2 at the best values previously found and adjusting the unknown extinction coefficients to their best values.

Fig. 5.1. Interaction of Mordant Yellow 12 with H.S.A. (Experimental Method 1.9e)

Mordant Yellow 12 concentration = 5.405×10^{-4} mol dm^{-3}

Albumin concentrations were as follows/10^{-5} mol dm^{-3}
(1) 0, (2) 0.7197, (3) 1.412, (4) 2.078, (5) 2.719, (6) 3.337, (7) 3.932, (8) 4.507, (9) 5.062, (10) 5.599, (11) 6.117, (12) 6.619, (13) 7.104, (14) 7.574, (15) 8.029.


1
15
80
60
40
20
1.5
1.0
0.5
Absorbance
340
360
380
400
420
440
Wavelength/nm

Albumin concentration /10^{-5} mol dm^{-3}		Wavelength/nm 350	360	370	440	450
			Absorbance/cm			
.7788	OBS	1.198	1.536	1.794	1.211	1.013
	CALC	1.193	1.536	1.797	1.203	1.004
1.528	OBS	1.118	1.454	1.733	1.243	1.056
	CALC	1.112	1.450	1.732	1.229	1.041
2.248	OBS	1.063	1.394	1.685	1.266	1.083
	CALC	1.062	1.396	1.690	1.260	1.077
2.942	OBS	1.038	1.360	1.659	1.289	1.111
	CALC	1.029	1.359	1.660	1.286	1.107
3.611	OBS	1.006	1.330	1.630	1.305	1.131
	CALC	1.006	1.334	1.639	1.307	1.130
4.256	OBS	.994	1.314	1.618	1.321	1.150
	CALC	.989	1.315	1.623	1.324	1.147
4.878	OBS	.978	1.301	1.607	1.335	1.161
	CALC	.976	1.300	1.611	1.337	1.161
5.478	OBS	.960	1.280	1.591	1.344	1.173
	CALC	.966	1.289	1.602	1.347	1.172
6.059	OBS	.983	1.303	1.589	1.353	1.182
	CALC	.958	1.280	1.594	1.355	1.180
6.620	OBS	.951	1.275	1.582	1.376	1.202
	CALC	.952	1.273	1.588	1.362	1.188
7.162	OBS	.946	1.262	1.573	1.371	1.195
	CALC	.947	1.267	1.584	1.367	1.193
7.687	OBS	.928	1.243	1.559	1.374	1.200
	CALC	.942	1.263	1.579	1.372	1.198
8.196	OBS	.951	1.271	1.577	1.385	1.209
	CALC	.939	1.259	1.576	1.375	1.202
8.688	OBS	.935	1.257	1.570	1.385	1.214
	CALC	.936	1.255	1.573	1.379	1.206

Table 5.1. Absorbance and fitting data for the Mordant Yellow 12/H.S.A. system

Experimental method 1.9a. Dye concentration $= 2.397 \times 10^{-4}$ mol dm^{-3}

Albumin concentration /10^{-5} mol dm^{-3}		Wavelength/nm 350	360	370	440	450
		absorbance/cm				
.7597	OBS	1.819	2.334	2.713	1.790	1.486
	CALC	1.835	2.352	.2.728	1.787	1.482
1.490	OBS	1.721	2.226	2.635	1.815	1.525
	CALC	1.731	2.242	2.648	1.802	1.513
2.193	OBS	1.655	2.169	2.576	1.838	1.559
	CALC	1.658	2.164	2.589	1.829	1.550
2.870	OBS	1.595	2.103	2.535	1.870	1.595
	CALC	1.607	2.109	2.545	1.860	1.586
3.522	OBS	1.554	2.053	2.501	1.899	1.630
	CALC	1.569	2.067	2.512	1.889	1.619
4.151	OBS	1.527	2.027	2.475	1.920	1.650
	CALC	1.540	2.035	2.486	1.914	1.647
4.758	OBS	1.513	2.009	2.469	1.943	1.675
	CALC	1.517	2.009	2.464	1.936	1.670
5.344	OBS	1.490	1.982	2.441	1.943	1.689
	CALC	1.498	1.989	2.447	1.936	1.690
5.910	OBS	1.479	1.968	2.434	1.954	1.705
	CALC	1.483	1.971	2.433	1.955	1.707
6.458	OBS	1.481	1.961	2.427	1.970	1.723
	CALC	1.470	1.957	2.421	1.970	1.721
6.987	OBS	1.458	1.952	2.418	1.989	1.730
	CALC	1.460	1.945	2.411	1.984	1.733
7.499	OBS	1.458	1.945	2.418	1.998	1.746
	CALC	1.450	1.935	2.402	1.995	1.743
7.995	OBS	1.449	1.931	2.402	2.011	1.744
	CALC	1.443	1.926	2.395	2.005	1.752
8.476	OBS	1.447	1.934	2.411	2.009	1.762
	CALC	1.436	1.918	2.388	2.013	1.760

Table 5.2 Absorbance and fitting data for the Mordant Yellow 12/H.S.A. system.

Experimental method 1.9c. Dye concentration = 3.586×10^{-4} mol dm^{-3}

Albumin concentration /10^{-5} mol dm^{-3}		Wavelength/nm 350	360	370	440	450
			Absorbance/cm			
.7197	OBS	2.855	3.616	4.153	2.672	2.201
	CALC	2.831	3.613	4.161	2.687	2.217
1.412	OBS	2.699	3.483	4.071	2.686	2.233
	CALC	2.713	3.489	4.073	2.690	2.240
2.078	OBS	2.635	3.390	3.998	2.706	2.270
	CALC	2.614	3.385	3.997	2.702	2.268
2.719	OBS	2.553	3.303	3.936	2.718	2.293
	CALC	2.536	3.303	3.936	2.723	2.300
3.337	OBS	2.478	3.280	3.902	2.759	2.341
	CALC	2.478	3.240	3.888	2.750	2.334
3.932	OBS	2.446	3.207	3.861	2.779	2.370
	CALC	2.432	3.191	3.849	2.779	2.367
4.507	OBS	2.386	3.141	3.824	2.807	2.395
	CALC	2.396	3.151	3.816	2.806	2.398
5.062	OBS	2.363	3.131	3.790	2.821	2.432
	CALC	2.365	3.117	3.789	2.833	2.427
5.599	OBS	2.318	3.065	3.753	2.850	2.446
	CALC	2.339	3.088	3.765	2.857	2.453
6.117	OBS	2.331	3.063	3.749	2.873	2.473
	CALC	2.316	3.062	3.744	2.878	2.476
6.619	OBS	2.279	3.026	3.719	2.894	2.491
	CALC	2.297	3.041	3.726	2.898	2.497
7.104	OBS	2.313	3.035	3.710	2.905	2.507
	CALC	2.279	3.021	3.710	2.915	2.516
7.574	OBS	2.249	2.987	3.701	2.923	2.521
	CALC	2.264	3.004	3.695	2.931	2.533
8.029	OBS	2.231	2.978	3.689	2.923	2.533
	CALC	2.251	2.989	3.683	2.946	2.548

Table 5.3 Absorbance and fitting data for the Mordant Yellow 12/H.S.A. system.

Experimental method 1.9e. Dye concentration = 5.405×10^{-5} mol dm^{-3}

	wavelength/nm				
	350	360	370	440	450
ε_1/mol^{-1} m^2	319.4	443.4	587.7	659.8	591.1
ε_2/mol^{-1} m^2	488.8	635.4	752.6	452.1	375.6
ε_f/mol^{-1} m^2	548.5	694.1	788.0	497.5	406.4

$K_1 = 1.28 \times 10^5$ mol^{-1} dm^3
$K_2 = 1.27 \times 10^4$ mol^{-1} dm^3
$n_1 = 4.18$
$n_2 = 18.3$

Table 5.4. Binding parameters and extinction coefficients used for the absorbance fitting data in tables 5.1 - 5.3.

Titration	Dye concentration /10^{-4}mol dm^{-3}	Albumin conc^n /10^{-5} mol dm^{-3}
1A	2.397	.7788 - 8.688
2A	3.586	.7597 - 8.476
3A	5.405	.7197 - 8.029
1B	2.394	.7957 - 8.877
2B	3.566	.7573 - 8.449
3B	5.382	.7317 - 8.163

Table 5.5. Concentration ranges for the six Mordant Yellow 12/H.S.A. titrations.

Data sets used	n_1	n_2	K_1 /10^5 mol^{-1} dm^3	K_2 /10^4 mol^{-1} dm^3
1A, 2A, 3A	4.18	18.3	1.28	1.27
1B, 2B, 3B	4.44	17.2	1.92	1.78
1B, 2A, 3A	4.26	15.7	1.43	1.52
1A, 2B, 3B	4.33	20.5	1.72	1.30
1A, 2B, 3A	4.19	16.4	1.30	1.26
1B, 2A, 3B	4.42	19.4	1.82	1.52
1A, 2A, 3B	4.24	21.7	1.64	1.30
1B, 2B, 3A	4.26	15.0	1.43	1.49

Table 5.6. Binding parameters obtained with various permutations of the Mordant Yellow 12 - H.S.A. absorbance data sets.

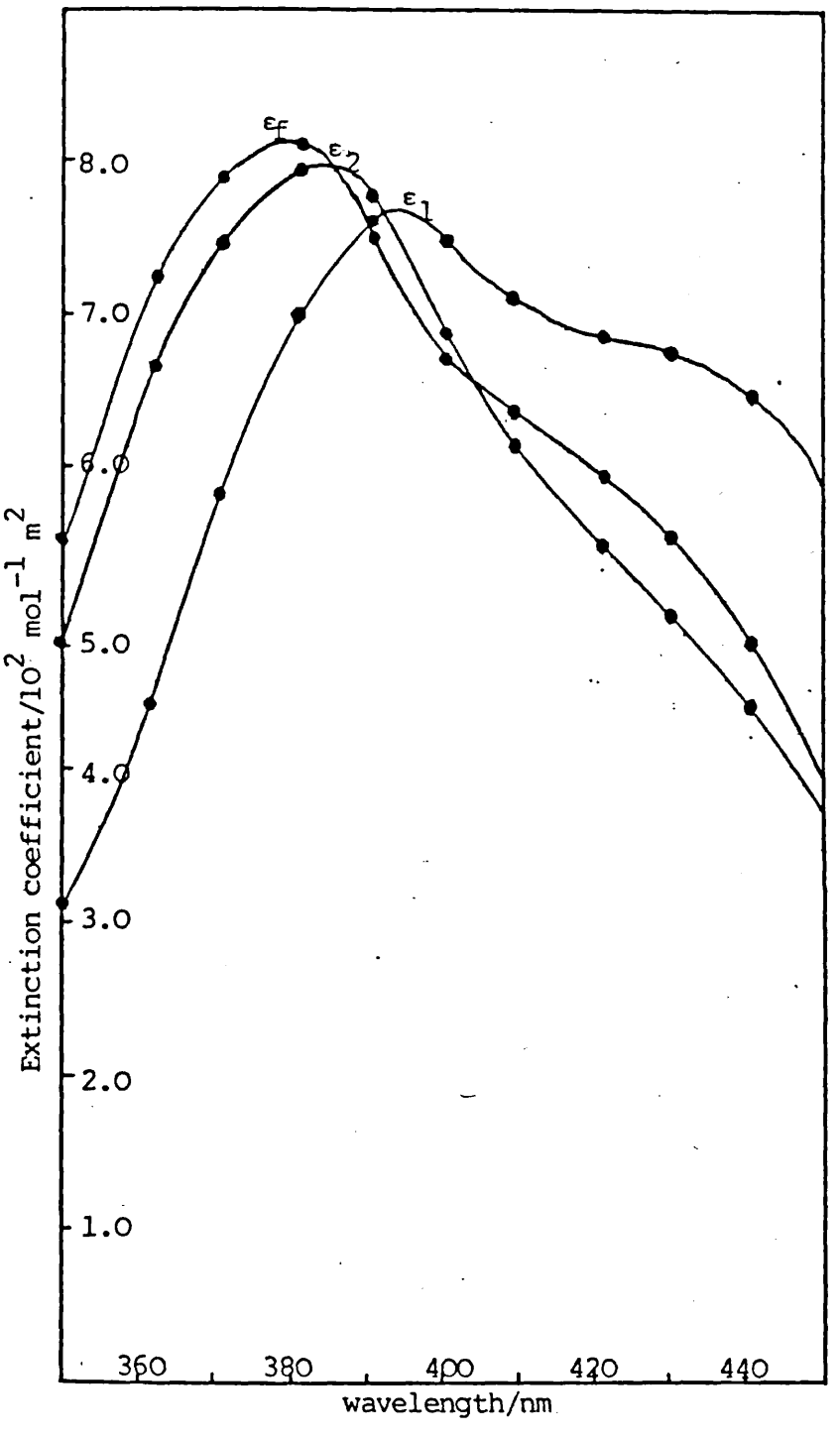


Fig 5.2 Resolved extinction coefficients for the three dye species present in the Mordant Yellow 12 - H.S.A. system (experimental methods 1.9a, 1.9c, 1.9e).

As a means of comparing these results with others obtained in this work, and with other workers, it is useful to plot data in the most widely accepted form; either as a Scatchard plot (Fig.5.3) or as a Klotz plot (Fig.5.4) as described in Chapter 1 (p 45). It is also useful to plot the theoretical free drug concentration against total dye concentration for a constant albumin concentration (in this case 1.000×10^{-5} mol dm^{-3}), which gives an easily visualised concept of the extent to which the dye is bound by the protein (Fig.5.5) and also gives a direct comparison with ultrafiltration methods (see Chapter 6). A computer programme was, therefore, written which calculated and drew theoretical Scatchard, Klotz and binding extent plots when given the binding parameters K_1, K_2, n_1 and n_2 for the dye. In this instance two sets of parameters and their mean parameters were used, these are given in Table 5.7.

Data set	n_1	n_2	K_1 /10^5 mol^{-1} dm^3	K_2 /10^4 mol^{-1} dm^3
A	4.18	18.3	1.28	1.27
B	4.44	17.2	1.92	1.78
M(Mean)	4.31	17.7	1.60	1.53

Albumin Concentration = 1.000×10^{-5} mol dm^{-3}

Mordant Yellow 12 concentration = 5.000×10^{-5} to 5.000×10^{-4} mol dm^{-3}

Table 5.7 Parameters used for Klotz, Scatchard and extent of binding plots.

In this instance the total bound drug concentration = $[DS_1] + [DS_2]$

The quantity r is defined as $\frac{[DS_1]+[DS_2]}{[P]}$

where [P] = the protein concentration.

The interaction of a number of other dyes have also been investigated with H.S.A. in an exactly analagous way to that described for Mordant Yellow 12. Mean values for the four binding parameters K_1, K_2, n_1 and n_2 are presented in Table 5.17, together with other experimental information. The resolved spectra for the three species of each dye are shown in Fig.5.6 to 5.12.

The theoretical Scatchard and Klotz plots for all the dyes are shown in Fig. 5.13 and 5.14. The theoretical binding extent plot is shown in Fig.8.1.

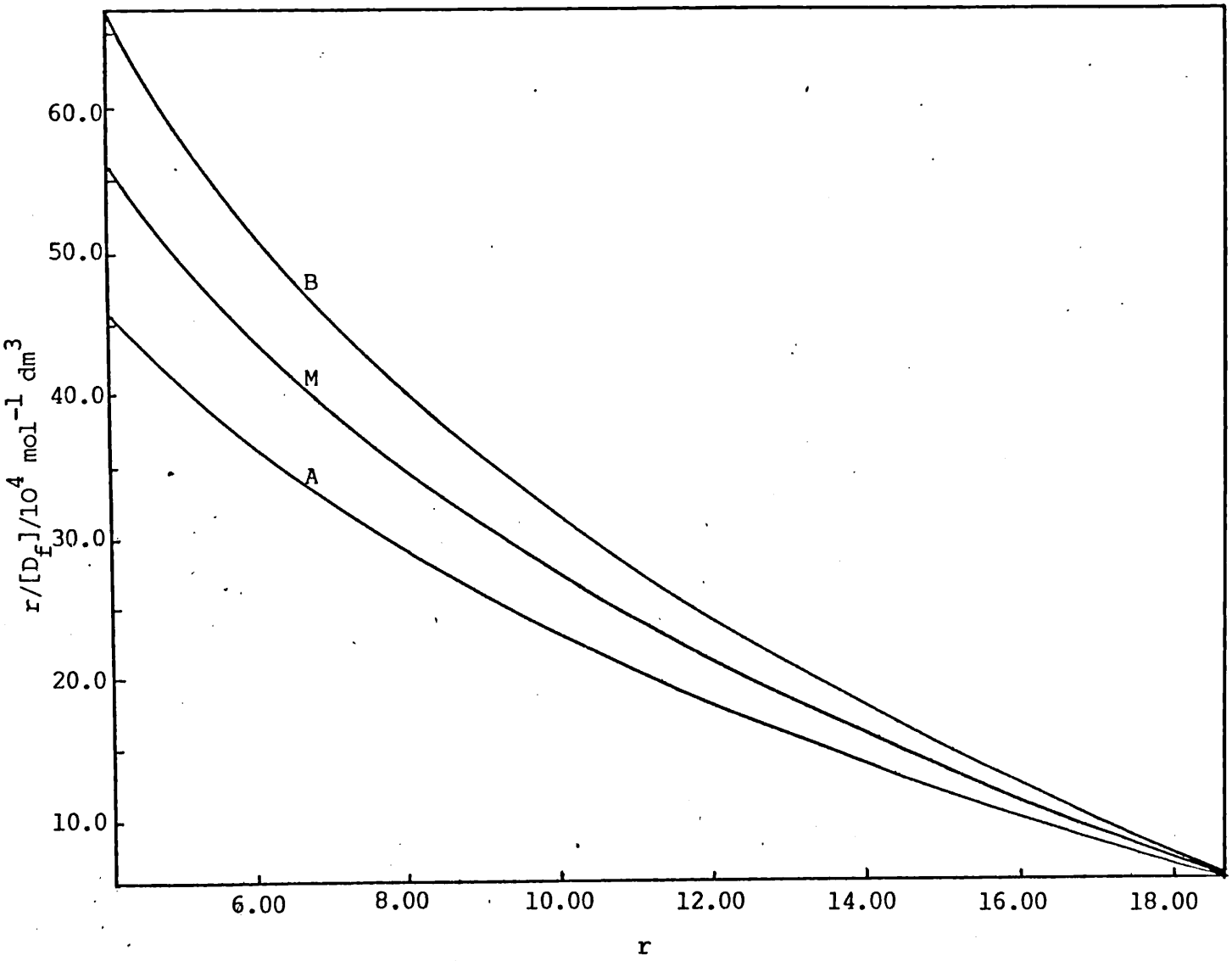
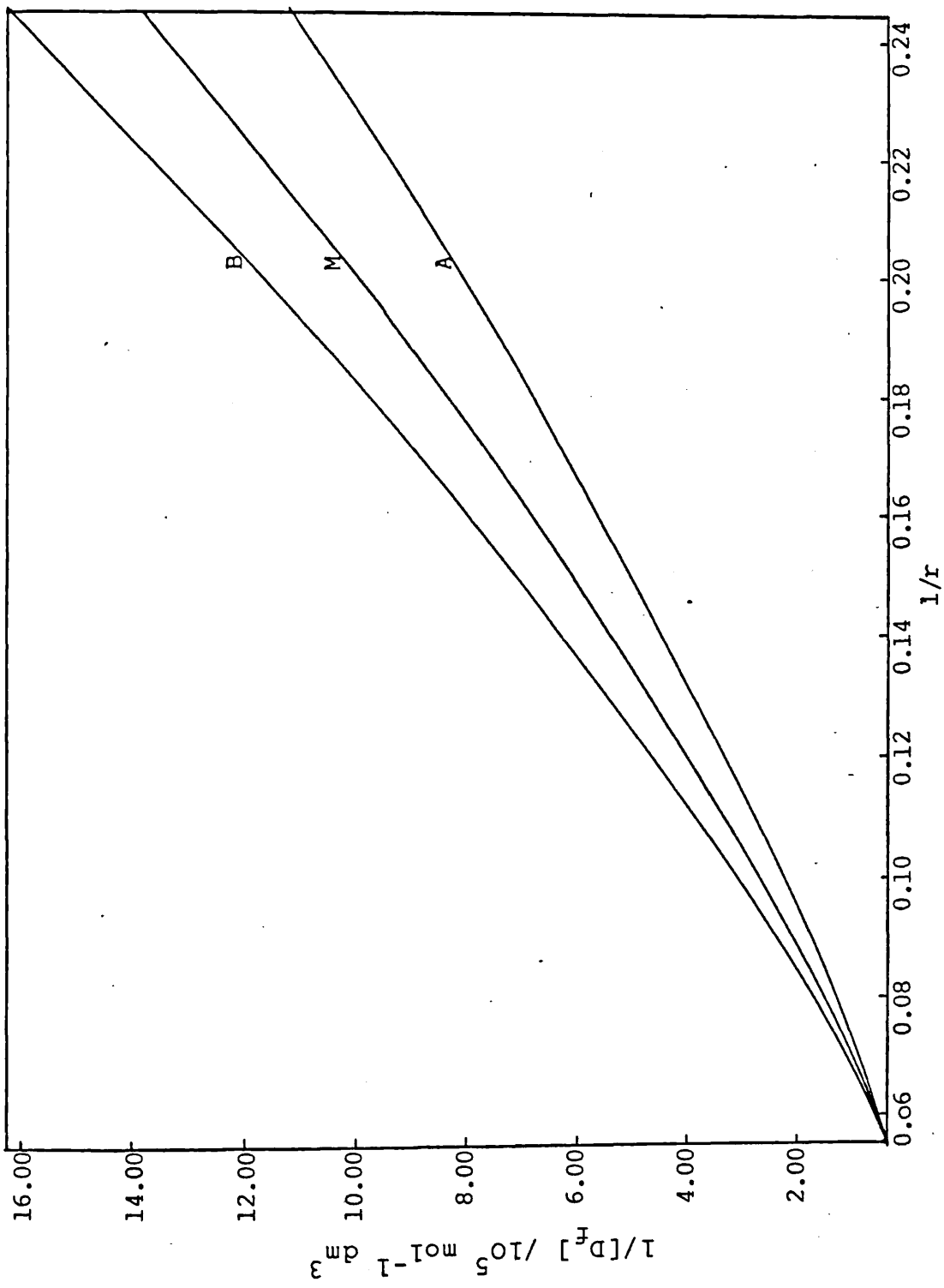


Fig.5.3
Theoretical Scatchard plot for the interaction of Mordant Yellow 12 with H.S.A.

See Table 5.7 for meaning of B, M and A.

Fig.5.4

Theoretical Klotz plot for the interaction of Mordant Yellow 12 with H.S.A.



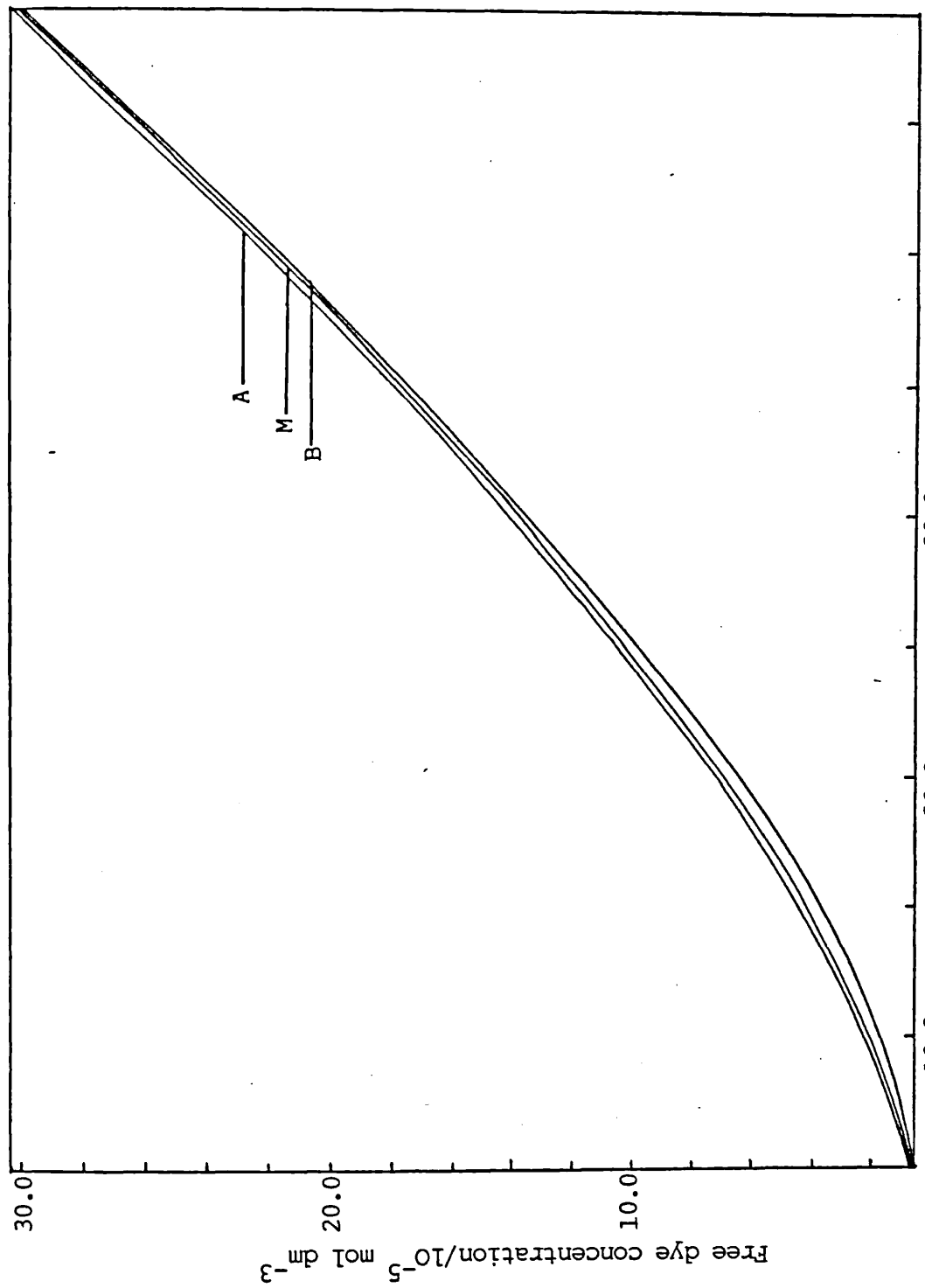


Fig.5.5
Theoretical binding extent plot for Mordant Yellow 12 with H.S.A. (1_3^{000} x 10^{-5} mol dm^{-3})

5.2 Resolved spectra for various dye- H.S.A. interactions

Tables 5.8 - 5.16 Resolved spectra data for various dyes with H.S.A.

Table 5.8 Mordant Yellow 12

wavelength /nm	ε_1 (mean) /100 mol^{-1} m^2	ε_2 (mean) /100 mol^{-1} m^2	ε_f /100 mol^{-1} m^2
350	3.28	4.89	5.48
360	4.55	6.34	6.94
370	5.98	7.52	7.88
380	7.12	8.00	8.15
390	7.77	7.83	7.73
400	7.60	6.95	6.93
410	7.10	6.09	6.39
420	6.90	5.59	6.06
430	6.92	5.13	5.64
440	6.56	4.52	4.97
450	5.87	3.74	4.06

Table 5.9 Mordant Yellow 7

wavelength /nm	ε_1 (mean) /1000mol^{-1} m^2	ε_2 (mean) /1000mol^{-1} m^2	ε_f /1000mol^{-1} m^2
330	0.535	0.798	0.948
340	0.704	0.994	1.17
350	0.945	1.22	1.39
360	1.21	1.33	1.48
370	1.38	1.27	1.39
380	1.41	1.08	1.16
390	1.26	0.817	0.852
400	0.932	0.606	0.598

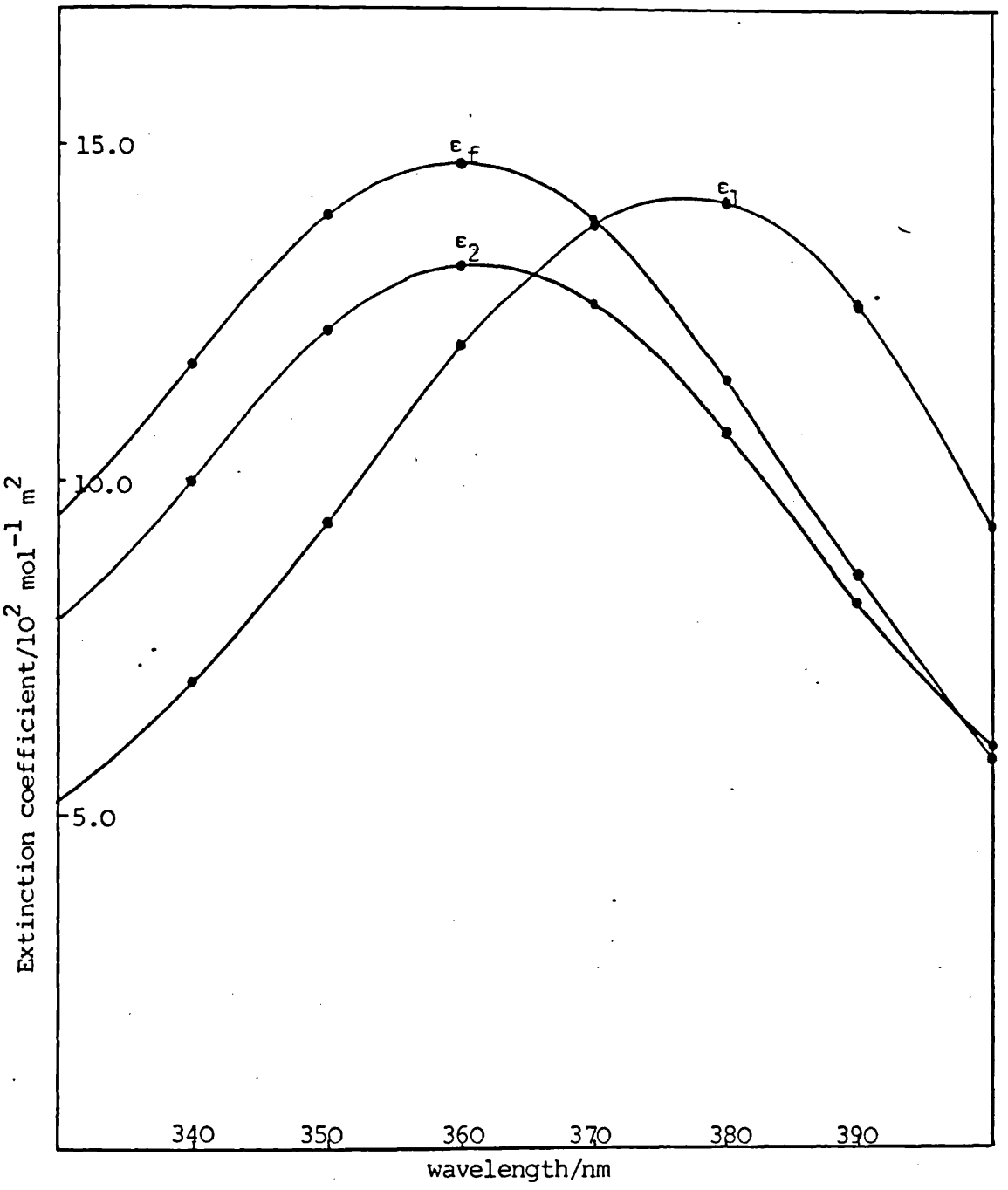


Fig. 5.6 Resolved extinction coefficients for the three dye species present in the Mordant Yellow 7 - H.S.A. system.

Table 5.10 Alizarin Yellow GG

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2 (mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
320	0.946	1.33	1.46
330	1.22	1.65	1.81
340	1.54	1.96	2.13
350	1.91	2.21	2.35
360	2.17	2.20	2.27
370	2.16	1.93	1.92
380	1.95	1.47	1.43
390	1.49	1.00	0.930

Table 5.11 4-azobenzenesulphonic acid

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2 (mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
300	1.32	1.23	1.69
310	1.82	1.63	2.11
320	2.11	1.97	2.33
330	2.20	1.97	2.20
340	1.98	1.71	1.77
350	1.48	1.17	1.15

Table 5.12 5-Phenylazosalicylic acid

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2 (mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
320	0.866	1.38	1.48
330	1.25	1.61	1.78
340	1.68	1.82	2.02
350	2.33	1.91	2.09
360	2.70	1.71	1.83
370	2.72	1.33	1.39

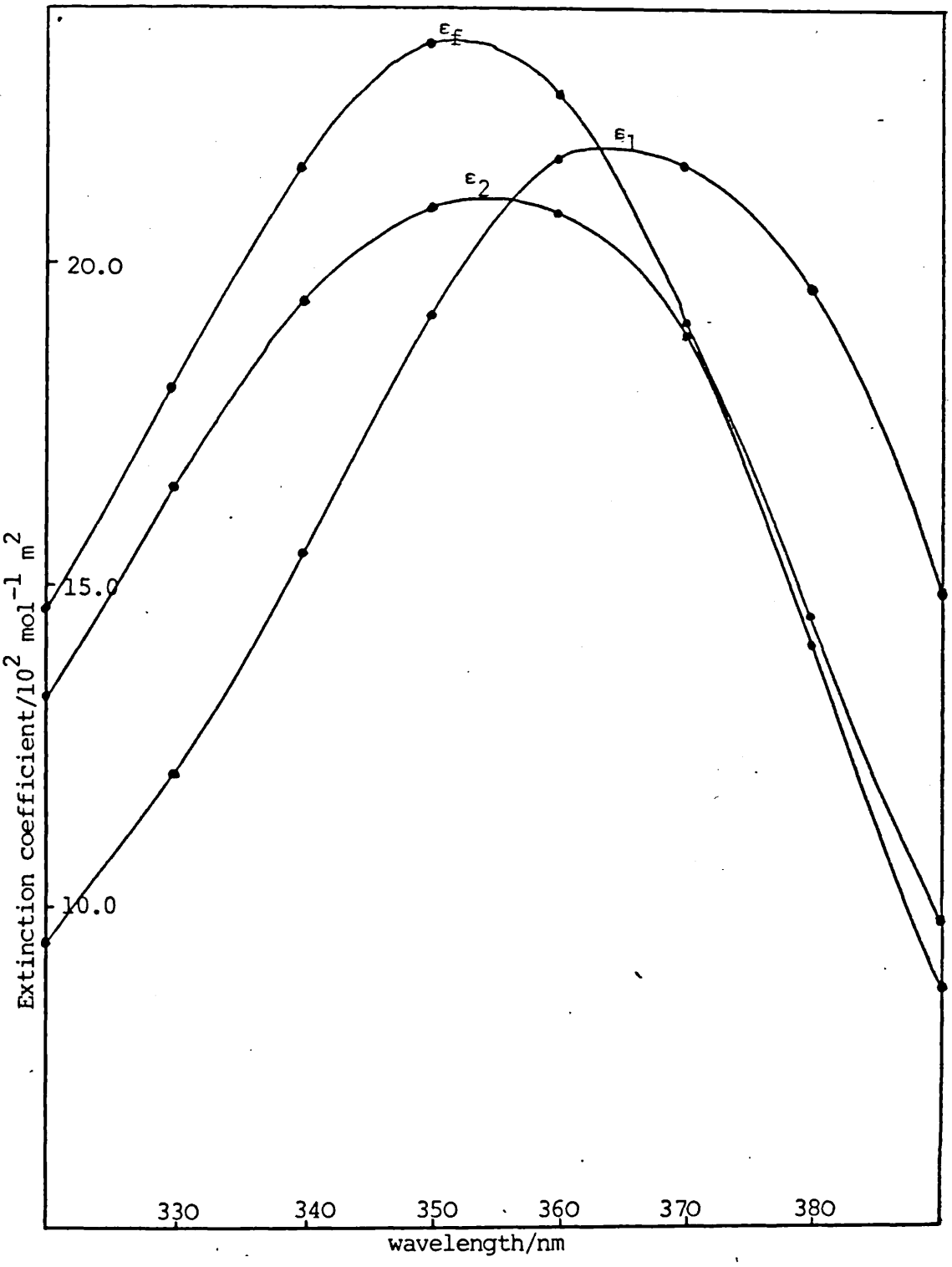


Fig.5. 7. Resolved extinction coefficients for the three dye species present in the Alizarin Yellow GG - H.S.A. system.

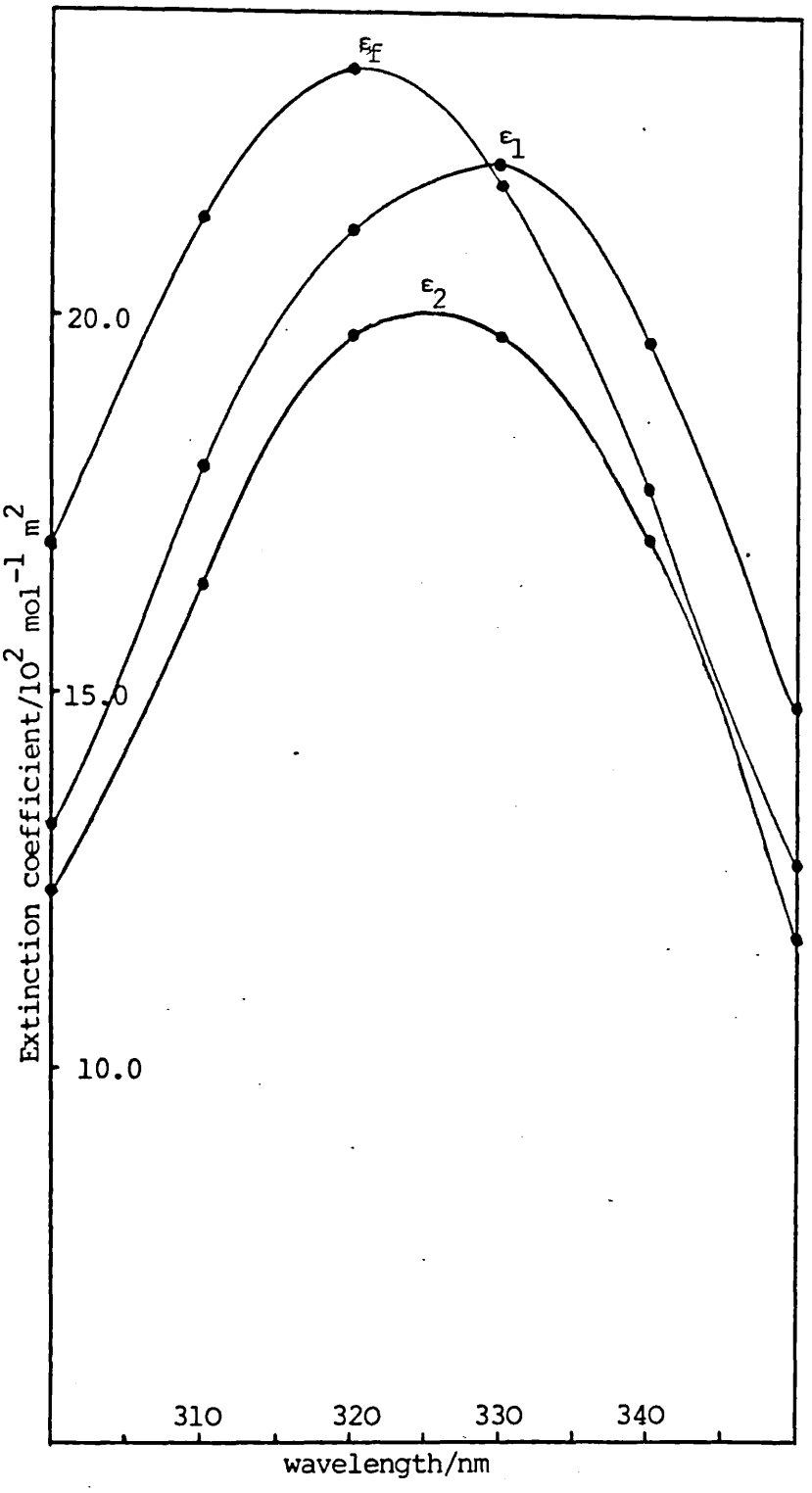


Fig. 5.8 Resolved extinction coefficients for the three dye species present in the 4-azobenzene sulphonic acid - H.S.A. system.

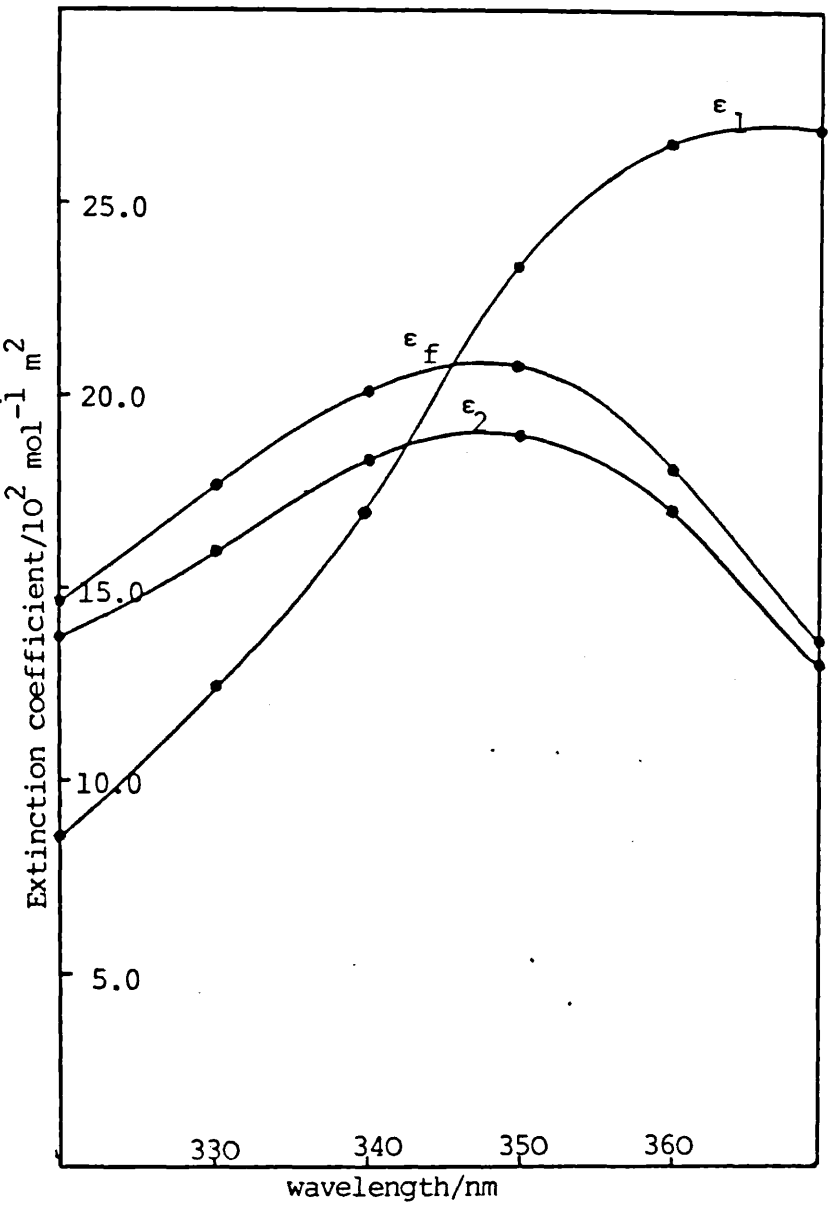


Fig. 5.9 Resolved extinction coefficients for the three dye species present in the 5-phenylazosalicylic acid - H.S.A. system.

Table 5.13 4-hydroxyazobenzene-4'-sulphonic acid

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2 (mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
320	1.15	0.825	1.25
330	1.59	1.14	1.69
340	2.01	1.48	2.06
350	2.29	1.75	2.28
360	2.30	1.88	2.24
370	2.19	1.71	1.98
380	1.89	1.46	1.60

Table 5.14 Mordant Yellow 10

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2(mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
330	.991	1.19	1.45
340	1.26	1.47	1.74
350	1.57	1.71	1.94
360	1.83	1.67	1.89
370	1.84	1.50	1.61
380	1.63	1.24	1.21
390	1.25	0.853	0.801

Table 5.15 Mordant Orange 1

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2 (mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
350	0.684	0.745	1.30
360	0.909	1.01	1.53
370	1.15	1.22	1.62
380	1.37	1.37	1.58
390	1.56	1.36	1.39
400	1.63	1.22	1.13
410	1.57	1.03	0.878
420	1.39	0.820	0.670

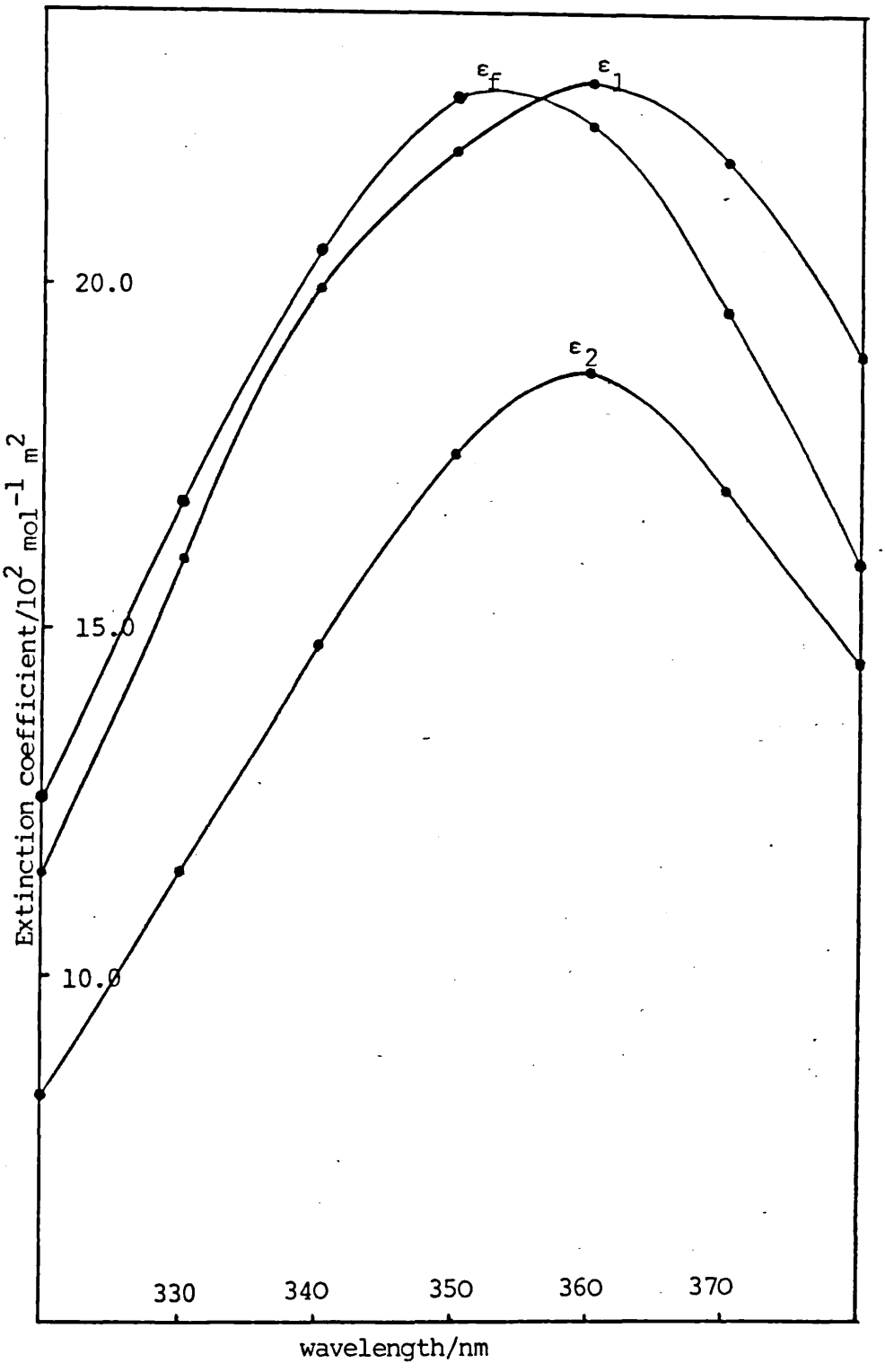


Fig.5.10 Resolved extinction coefficients for the three dye species present in the 4-hydroxyazobenzene-4'-sulphonic acid - H.S.A. system.

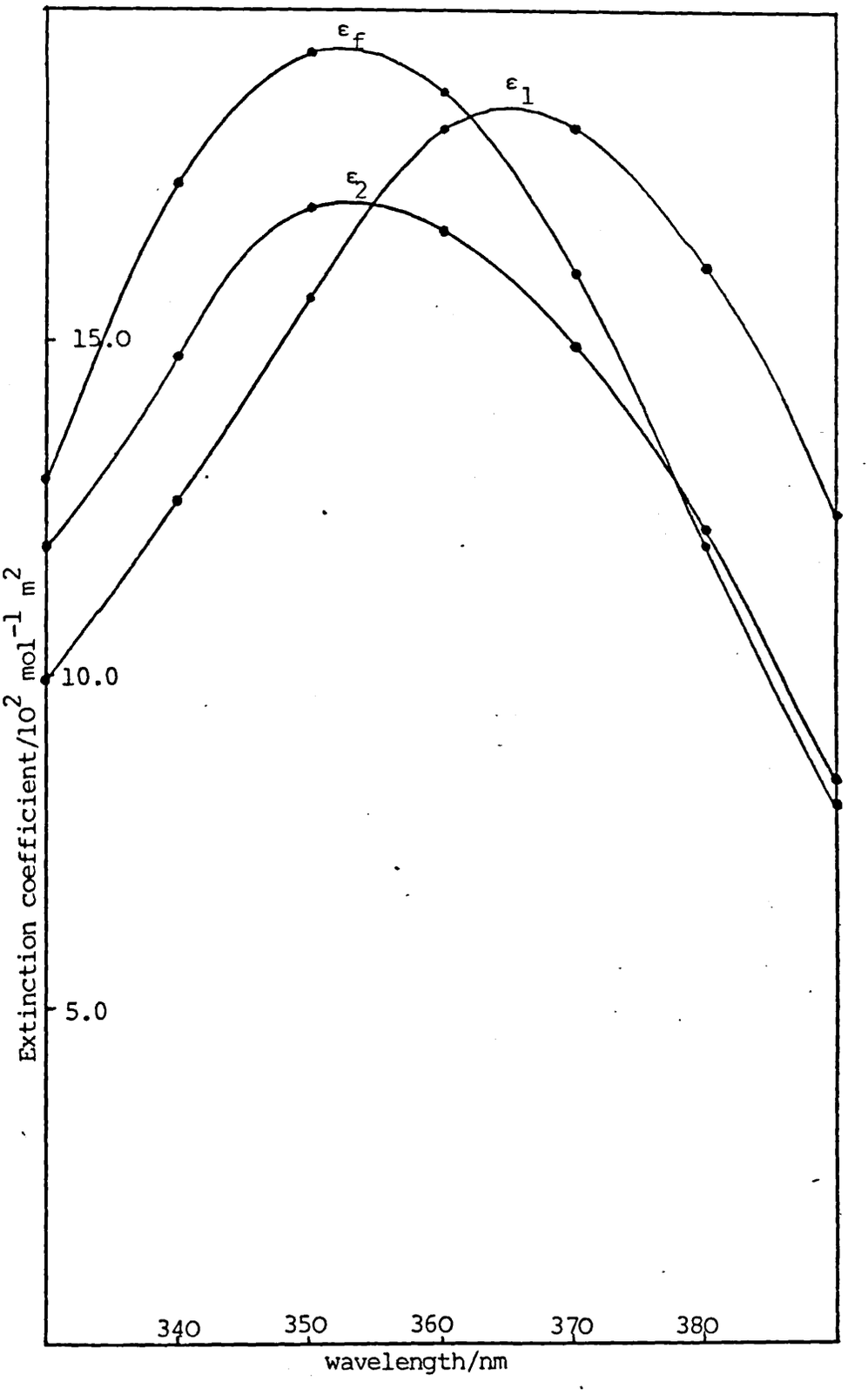


Fig. 5.11 Resolved extinction coefficients for the three dye species present in the Mordant Yellow 10 - H.S.A. system.

Table 5.16 Bromophenol Blue

wavelength /nm	ε_1 (mean) /1000 mol^{-1} m^2	ε_2 (mean) /1000 mol^{-1} m^2	ε_f /1000 mol^{-1} m^2
570	3.66	4.06	5.44
580	4.54	4.47	6.98
590	6.04	4.87	8.26
600	7.87	4.98	7.30
610	7.84	4.57	4.24
620	5.09	3.69	1.83

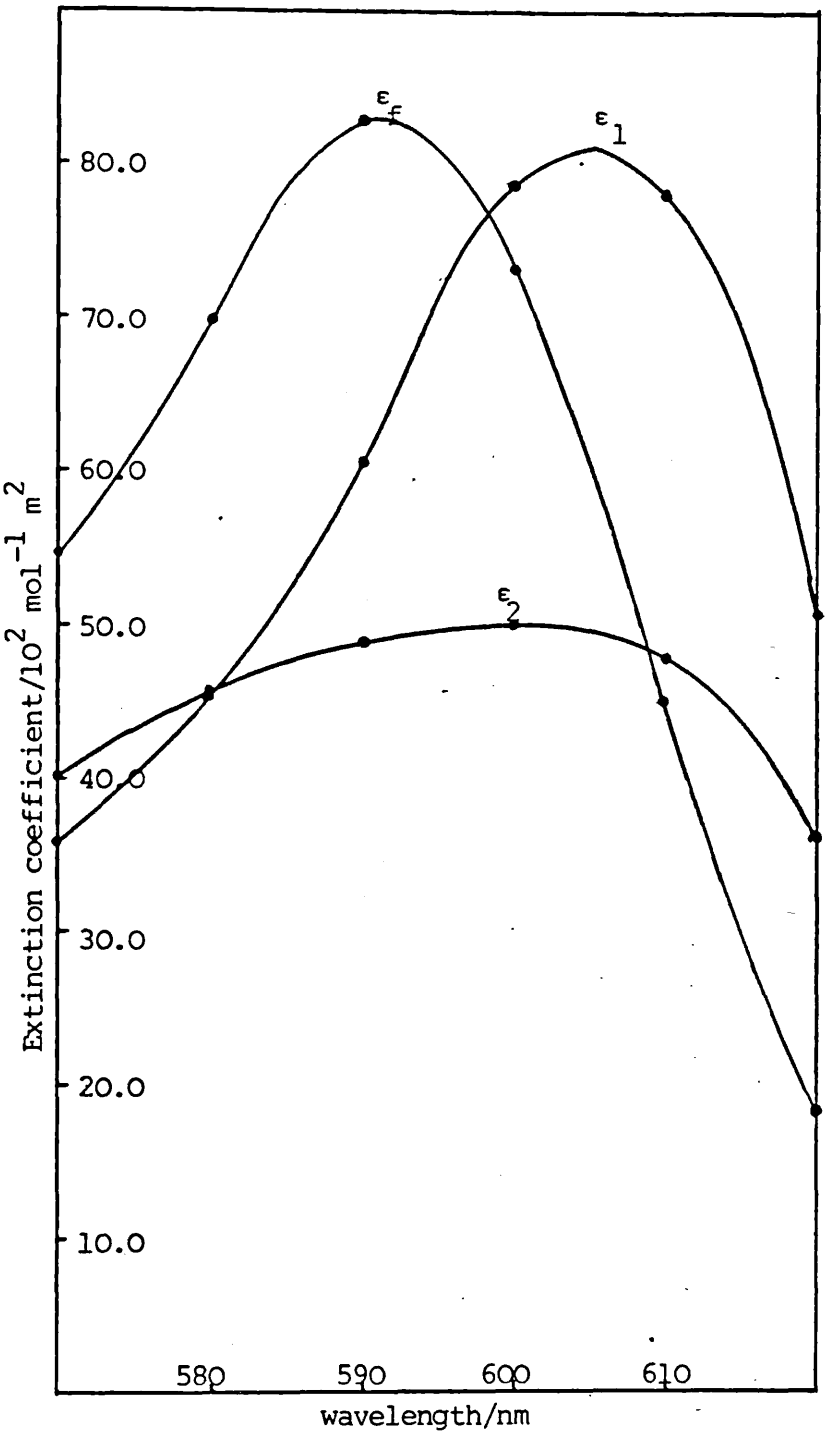


Fig. 5.12 Resolved extinction coefficients for the three dye species present in the Bromophenol Blue - H.S.A. system.

5.3 Binding Data for dye - H.S.A. interactions

Compound	Mean K_1 /10^5 mol^{-1} dm^3	Mean K_2 /10^4_3 mol^{-1} dm^3	n_1	n_2	Experimental method (see Ch. 2)
Mordant Yellow 12	1.60	1.53	4.31	17.7	1.9
Mordant Yellow 7	2.00	1.06	3.41	6.87	1.11
Alizarin Yellow GG	3.71	2.18	5.46	10.9	1.10
4-azobenzene sulphonic acid	3.65	0.929	1.14	4.61	1.13
5-phenylazo-salicylic acid	3.81	4.08	1.31	9.39	1.12
4-hydroxy-azobenzene-4'-sulphonic acid	2.33	1.65	2.17	4.18	1.15
Mordant Yellow 10	7.82	3.90	2.93	2.68	1.14
Mordant Orange 1	2.52	1.15	2.28	14.62	1.8
Bromophenol Blue	11.5	3.67	1.78	3.46	1.16

Table 5.17 Summary of binding parameters for H.S.A. dye interactions.

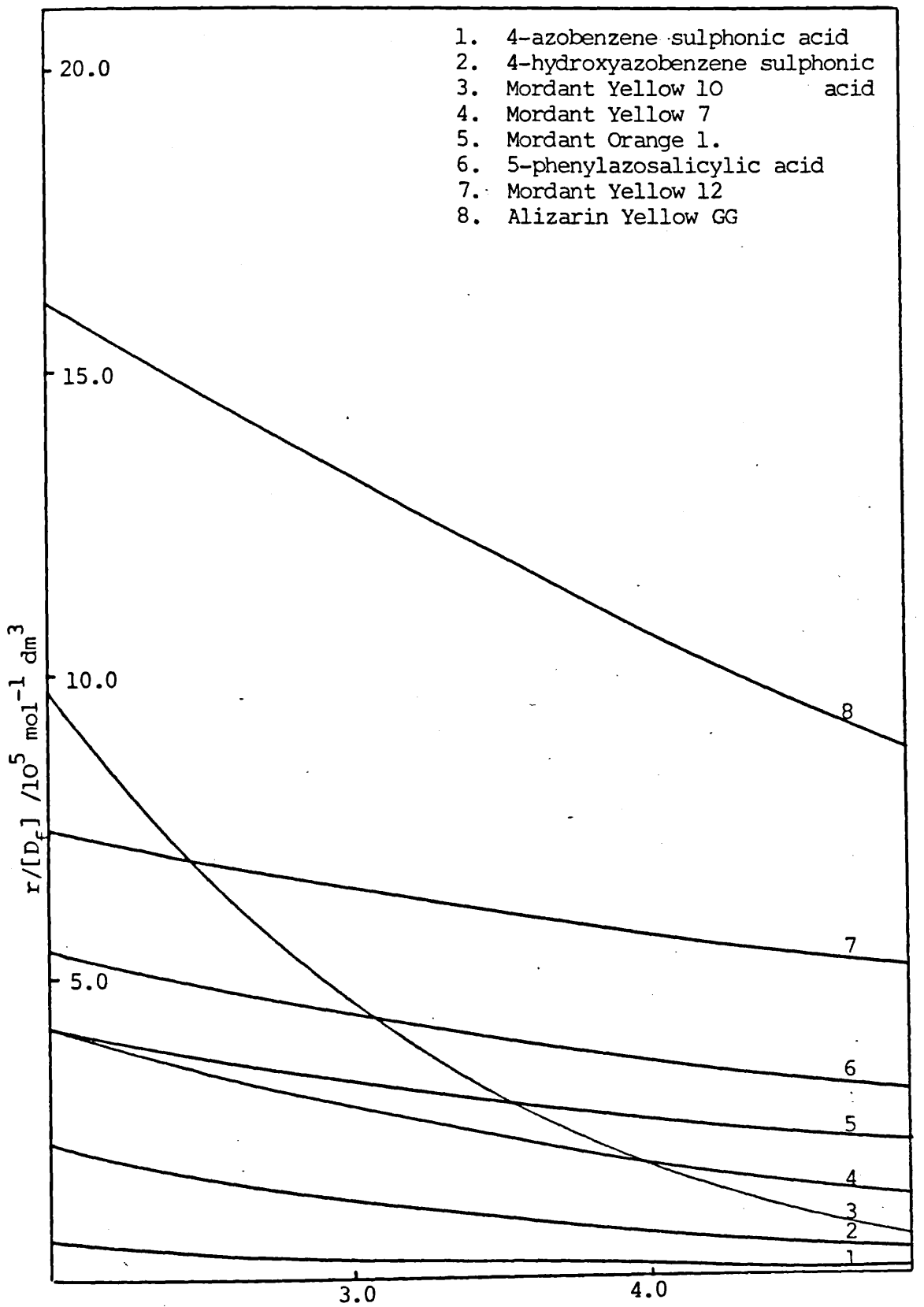


Fig. 5.13 Theoretical Scatchard plots for the azo dyes with H.S.A. (U.V/visible data fitting method).

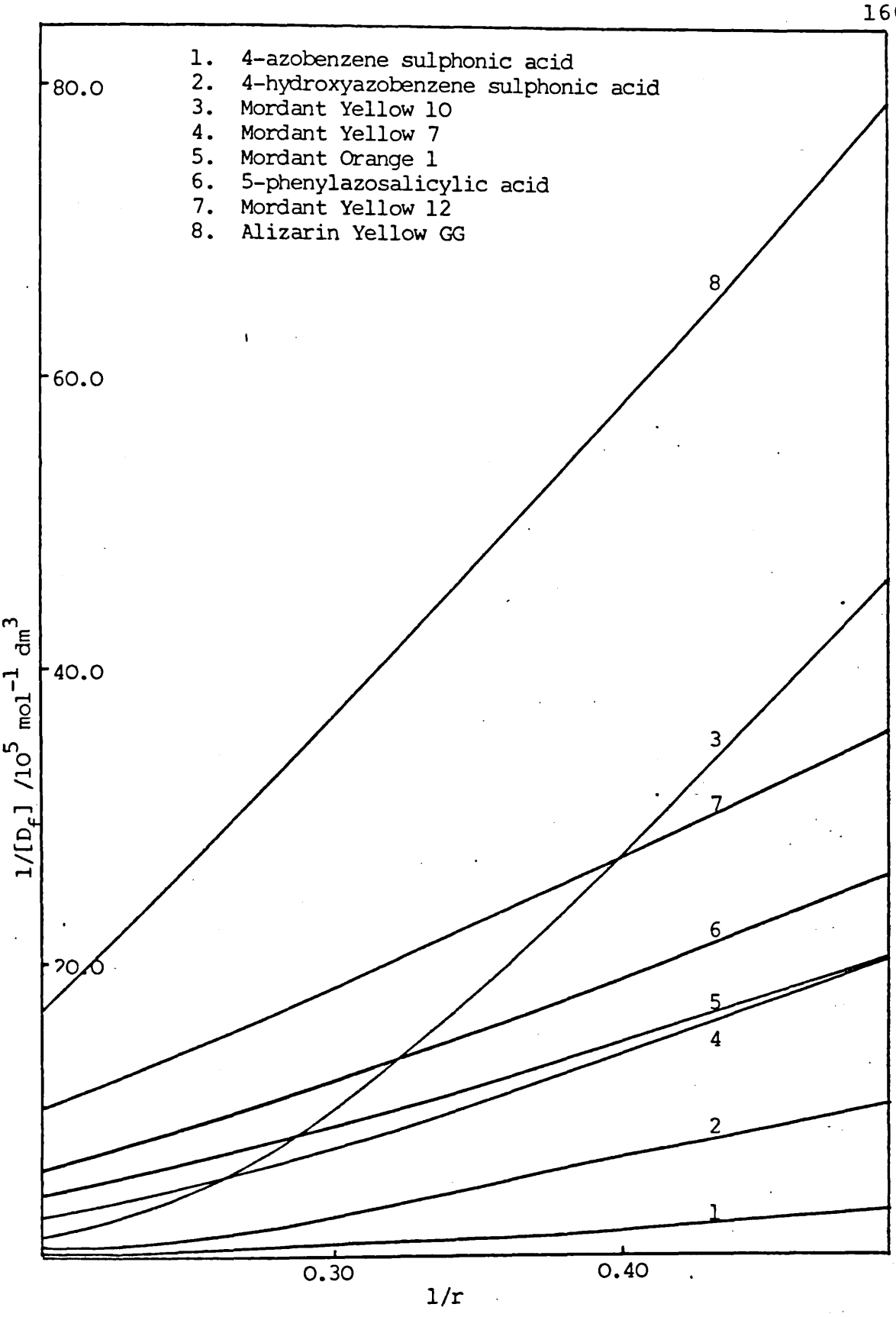


Fig. 5.14 Theoretical Klotz plots for the azo dyes with H.S.A. (U.V./visible data fitting method)

CHAPTER 6

THE APPLICATION OF ULTRAFILTRATION TO THE STUDY OF THE INTERACTIONS OF HUMAN SERUM ALBUMIN WITH AZOBENZENE DERIVATIVES.

The principal advantage of ultrafiltration over the spectroscopic techniques is that values for free ligand concentrations are determined experimentally rather than via an intermediate data fitting step. Practically, however, the technique suffers from rather more inherent errors.

6.1. The changing volume system

It may be shown that in the ideal system, equilibrium data can be obtained directly by measurement of the effluent ligand concentration in the following way:-

Consider the cell initially to contain a volume of solution V. Assuming firstly a single, independent site system the following equilibrium applies

$$D + S_1 \rightleftharpoons DS_1 \qquad K = \frac{[DS_1]}{[D][S_1]}$$

Initially the following equations apply

$$[D] = \frac{M_D}{V} \qquad [S] = \frac{M_s}{V} \qquad [DS] = \frac{M_{DS}}{V}$$

Where M_D, M_S and M_{DS} are the masses of free ligand, free site and ligand site complex within the cell.

Therefore $$K = \frac{M_{DS}}{V} \Big/ \frac{M_S}{V}.[D] = \frac{M_{DS}}{M_S[D]}$$

Let the volume of solution in the cell decrease by an amount δV. Therefore

$$K = \frac{M_{DS}}{(V-\delta V)} \Big/ \frac{M_S}{(V-\delta V)}.[D] = \frac{M_{DS}}{M_S[D]}$$

In the ideal case free ligand diffuses perfectly through the filter, whilst the protein and ligand-protein complex are completely rejected. The concentration of free ligand [D], therefore remains unaltered throughout the course of

the experiment.

By similar reasoning it may be shown that in the case of a two independent site system the following equations apply:-

$$K_1 = \frac{M_{DS1}}{M_{S1}[D]} \qquad K_2 = \frac{M_{DS2}}{M_{S2}[D]}$$

where the subscripts 1 and 2 refers to the two classes of site.

In the case of cooperativity between sites (see discussion p216)the technique may still be applied.

$$D + P \overset{K_1}{\rightleftharpoons} DP \qquad K_1 = \frac{DP}{[P][D]} \text{ or } K_1 = \frac{M_{DP}}{M_P}[D]$$

$$DP + D \overset{K_2}{\rightleftharpoons} D_2P \qquad K_2 = \frac{D_2P}{[DP][D]} \qquad K_2 = \frac{M_{D2}P.[D]}{M_{DP}}$$

$$D_nP + D \overset{K_{n+1}}{\rightleftharpoons} D_{(n+1)}\cdot P \qquad K_{n+1} = \frac{D_{(n+1)}P}{[D_nP][D]} \qquad K_{n+1} = \frac{M_{D(n+1)}}{[M_{Dn}P][D]}$$

where [P] = free protein concentration

$[D_iP]$ = Concentration of the ith ligand-protein complex

[D] = The free ligand concentration

M_P = The mass of free protein in the cell

$M_{Di}P$ = The mass of the ith ligand-protein complex in the cell.

This theory is not applicable, however, if:

1) An increase in protein concentration lead to an alteration in the number of sites or their equilibrium constants. This may arise as a result of protein polymerisation or conformational change.

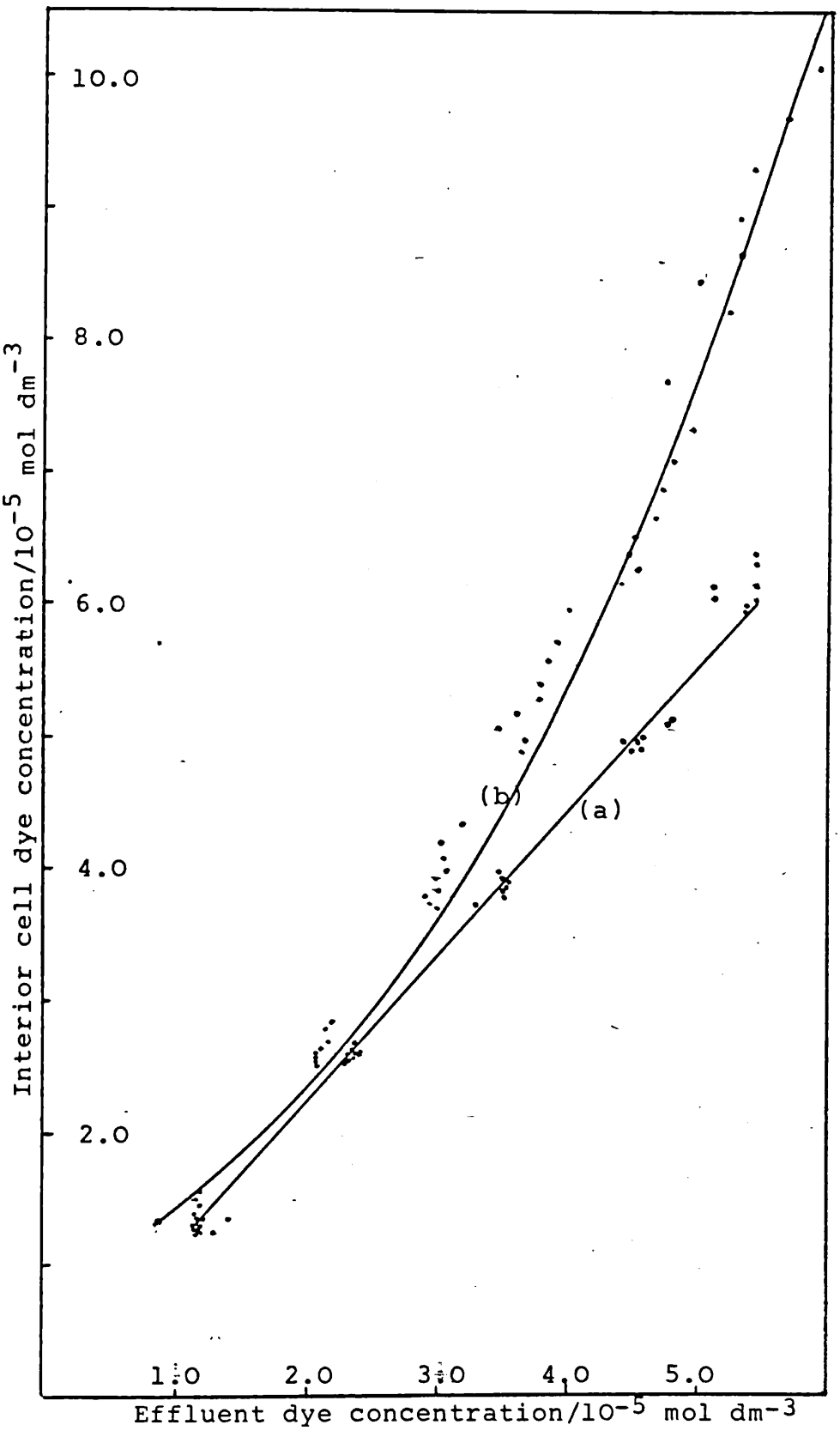


Fig. 6.1 Rejection of Evans Blue by UM10 ultrafilter (experimental method 5)

2) At higher concentrations a larger proportion of protein or dye is bound to the membrane or cell walls.

3) The free dye is significantly rejected by, or some of the protein passes through, the ultrafilter.

6.2. Membrane and cell wall binding

In order to counter this effect the apparatus is initially equilibrated with the solution. Since the extent of dye or protein binding may depend on their concentration an equivalent volume to that in the test solution is run off.

6.3. Membrane rejection

This is a commonly occuring phenomenon and depends on both the ligand and type of ultrafilter used.

Evans Blue is seriously rejected by Amicon UM10 filters. Fig. 6.1, curve (a), shows the effect of running Evans Blue through a UM10 filter (experimental method 5) and measuring internal and external dye concentrations. Rejection is even more pronounced if the cell is firstly equilibrated with a dye-albumin solution. Curve (a) is the regression 'best straight line', whilst curve (b) is fitted to a polynomial of the type:-

$$y = a + bx + Cx^2$$

minimising the function $\sum_{o}^{i}(y-ye)_i^2$

Where ye is the experimental interior cell dye concentration, and y is the calculated value. Rejection to this extent makes interpretation of binding data very difficult.

None of the phenylazobenzoic or phenylazosalicylic acid derivatives used in this study were seriously rejected by Amicon YM10 filters.

The rejection problems encountered with the UM10 filter may have been partially due to using a buffer solution of concentration 0.0666 mol dm^{-3} in phosphate, which exceeds the manufacturer's recommendation of 0.05 mol dm^{-3} maximum. A UM2 filter was also tried, but filtration was unacceptably slow.

Both YM10 and UM10 filters have a nominal cut-off molecular weight of 10000. The UM2 filter has a nominal cut off of 1000.

The effects of rejection may be minimised by allowing only a small volume of the test solution to pass through the filter ($\sim 1 cm^3$) before measuring the concentration.

6.4. Ultrafiltration data fitting

Assuming the two independent classes of site system described in Chapter 4 (p117), the concentration of free dye may be calculated from given equilibrium constants and binding site numbers by the equation

$$K_1K_2D_f^3 + (K_1 + K_2 - D_{tot}K_1K_2 + BK_1K_2 + CK_1K_2)D_f^2 + (1 - D_{tot}K_1 - D_{tot}K_2 + K_1B + K_2C)D_f - D_{tot} = 0 \qquad (6.1)$$

where $B = n_1P$ and $C = n_2P$

Best values for K_1, K_2, n_1 and n_2 for ultrafiltration data obtained with the azo dyes and human serum albumin described in experimental method 6 , were calculated in the following way:-

Theoretical values of D_f were initially calculated for a series of solutions from guessed values of K_1, K_2, n_1 and n_2. These were compared with the measured D_f values. The binding parameters were then adjusted by means of a 'simplex' routine so as to minimise the function

$$\sum_{o}^{i} ([D_f exp]_i - [D_f calc]_i)^2$$

where $[D_f exp]_i$ = The experimentally measured concentration of the ith dye solution.

$[D_f calc]_i$ = The calculated concentration of the ith dye solution.

The roots of the cubic equation were determined directly, rather than by using Newton's method, using the following method:-

If $a x^3 + bx^2 + cx + d = 0$

the equation may be transformed to

$$y^3 + 3py + 2q = 0 \qquad (6.2)$$

where $2q = \frac{2b^2}{27a}3 - \frac{bc}{3a}2 + \frac{d}{a}$

$$3p = \frac{3ac - b^2}{3a^2}$$

and $y = x + b/3a$.

It can be shown that where $D = q^2 + p^3$

If D>O then the equation 6.2 has 1 solution (one real and two imaginary roots)

If D<O then the equation has 3 solution (three different roots).

If D = O then the equation has one solution for p = q = O (three coincident zero roots) and 2 solutions for $p^3 = -q^2 \neq O$ (two of three real roots coincide).

In the case of D>O the real root may be found using Cardan's formula

$$y = u + v$$

where $u = \sqrt[3]{-q+\sqrt{q^2 + p^3}}$

$$v = \sqrt[3]{-q + \sqrt{q^2 + p^3}}$$

In the case of $D \leq 0$ the three real roots are obtained from the formulae:-

$$y_1 = -2r \cos \tfrac{1}{3} \phi$$

$$y_2 = +2r \cos\left(\frac{\cos^{-1}(-1)}{3} - \frac{\phi}{3} \right)$$

$$y_3 = +2r \cos\left(\frac{\cos^{-1}(-1)}{3} + \frac{\phi}{3} \right)$$

where $\phi = \cos^{-1} \frac{q}{r}$ and where all trigonometric functions are in radian measure.

As before, in the real situation there are usually three real roots to the equation 6.1, of which only one allows x and y to be positive. Although a single root may be found if the simplex adjusts any parameter to an unreal value.

6.5. Results

Table 6.1 gives data for the ultrafiltration of Mordant Yellow 7 with human serum albumin (experimental method 6) at the best parameter fit. Tables 6.2 to 6.8 give experimental ultrafiltration data for seven other azo dyes with human serum albumin. Figures 6.2 to 6.9 show the result of the ultrafiltration of the eight dyes. In each case curve (a) is the 'best fit' curve, using the best values found for K_1, K_2, n_1 and n_2. Curve (b) is the theoretical ultrafiltration curve from the binding parameters obtained by the U.V/visible data fitting method in chapter 5.

Total dye concentration /10^{-4} mol dm^{-3}	Theoretical concentration of 1^{o}ry site bound dye/10^{-5} mol dm^{-3}	Theoretical concentration of 2^{o}ry site bound dye/10^{-5} mol dm^{-3}	Theoretical free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	3.289	4.317	4.239	4.129
5.000	3.289	4.317	4.239	4.157
5.000	3.289	4.317	4.239	4.131
4.500	3.284	4.241	3.747	3.692
4.500	3.284	4.241	3.747	3.711
4.500	3.284	4.241	3.747	3.728
4.000	3.277	4.147	3.258	3.263
4.000	3.277	4.147	3.258	3.189
4.000	3.277	4.147	3.258	3.262
3.500	3.268	4.026	2.771	2.857
3.500	3.268	4.026	2.771	2.809
3.500	3.268	4.026	2.771	2.613
3.000	3.255	3.867	2.288	2.265
3.000	3.255	3.867	2.288	2.319
3.000	3.255	3.867	2.288	2.248
2.500	3.236	3.649	1.811	1.880
2.500	3.236	3.649	1.811	1.856
2.500	3.236	3.649	1.811	1.851
2.000	3.205	3.337	1.346	1.414
2.000	3.205	3.337	1.346	1.406
2.000	3.205	3.337	1.346	1.383
1.500	3.146	2.864	0.8989	0.8970
1.500	3.146	2.864	0.8989	0.9280
1.000	3.146	2.864	0.8989	0.9430
1.000	3.007	2.108	0.4885	0.5160
1.000	3.007	2.108	0.4885	0.4310
1.000	3.07	2.108	0.4885	0.4940
0.500	2.493	0.9448	0.1562	0.1280
0.500	2.493	0.9448	0.1562	0.1330
0.500	2.493	0.9448	0.1562	0.1080

$K_1 = 1.91 \times 10^5$ $mol^{-1}dm^3$ $K_2 = 1.49 \times 10^4$ $mol^{-1}dm^3$ $n_1 = 3.28$, $n_2 = 4.52$

Table 6.1. Ultrafiltration fitted data for Mordant Yellow 7 with human serum albumin (1.000×10^{-5} mol dm^{-3})

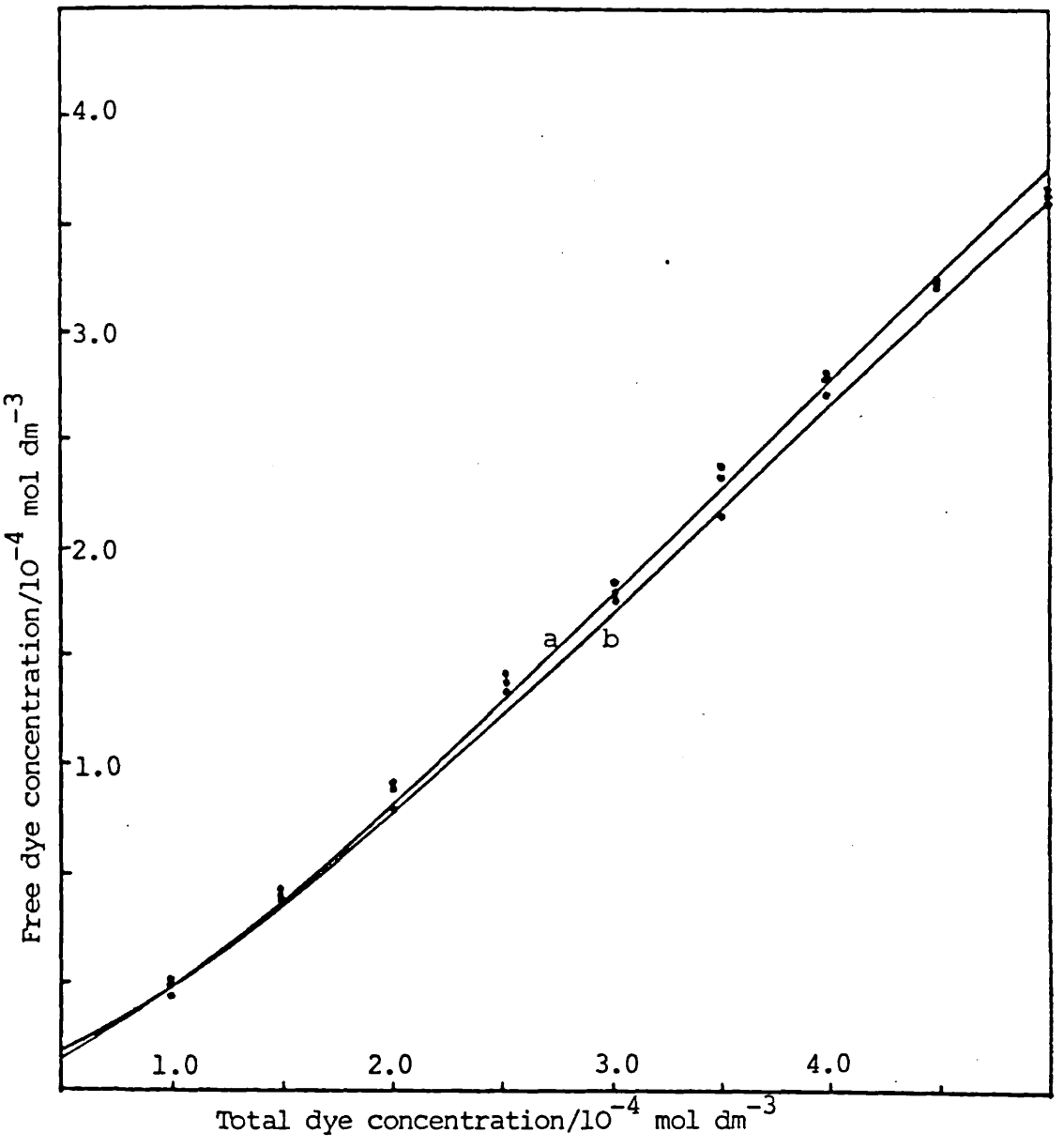


Fig. 6.2 Ultrafiltration curve for Mordant Yellow 7 with H.S.A. (1.000×10^{-5} mol dm^{-3})

Binding parameters for fitted line (a)

$K_1 = 1.91 \times 10^5$ mol^{-1} dm^3 $K_2 = 1.49 \times 10^4$ mol^{-1} dm^3

$n_1 = 3.28$ $n_2 = 4.52$

Binding parameters for line (b) (from Ch. 5 p158)

$K_1 = 2.00 \times 10^5$ mol^{-1} dm^3 $K_2 = 1.06 \times 10^4$ mol^{-1} dm^3

$n_1 = 3.41$ $n_2 = 6.87$

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	4.288	4.200	4.226
4.500	3.821	3.979	3.825
4.000	3.375	3.344	3.330
3.500	2.837	2.967	2.856
3.000	2.356	2.440	2.363
2.500	1.976	1.886	1.969
2.000	1.499	1.545	1.489
1.500	1.068	1.089	1.059
1.000	0.632	0.633	0.645
0.500	0.244	0.218	0.232

Table 6.2. Ultrafiltration data for Mordant Yellow 10 with human serum albumin (1.000 x 10^{-5} mol dm^{-3})

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	2.962	3.012	2.961
4.500	2.722	2.545	2.666
4.000	2.296	2.176	2.289
3.500	1.932	1.884	1.915
3.000	1.473	1.392	1.489
2.500	1.063	1.127	1.034
2.000	0.771	0.760	0.727
1.500	0.441	01463	0.415
1.000	0.226	0.243	0.210
0.500	0.097	0.080	0.078

Table 6.3. Ultrafiltration data for Mordant Orange 1 with human serum albumin (1.000 x 10^{-5} mol dm^{-3})

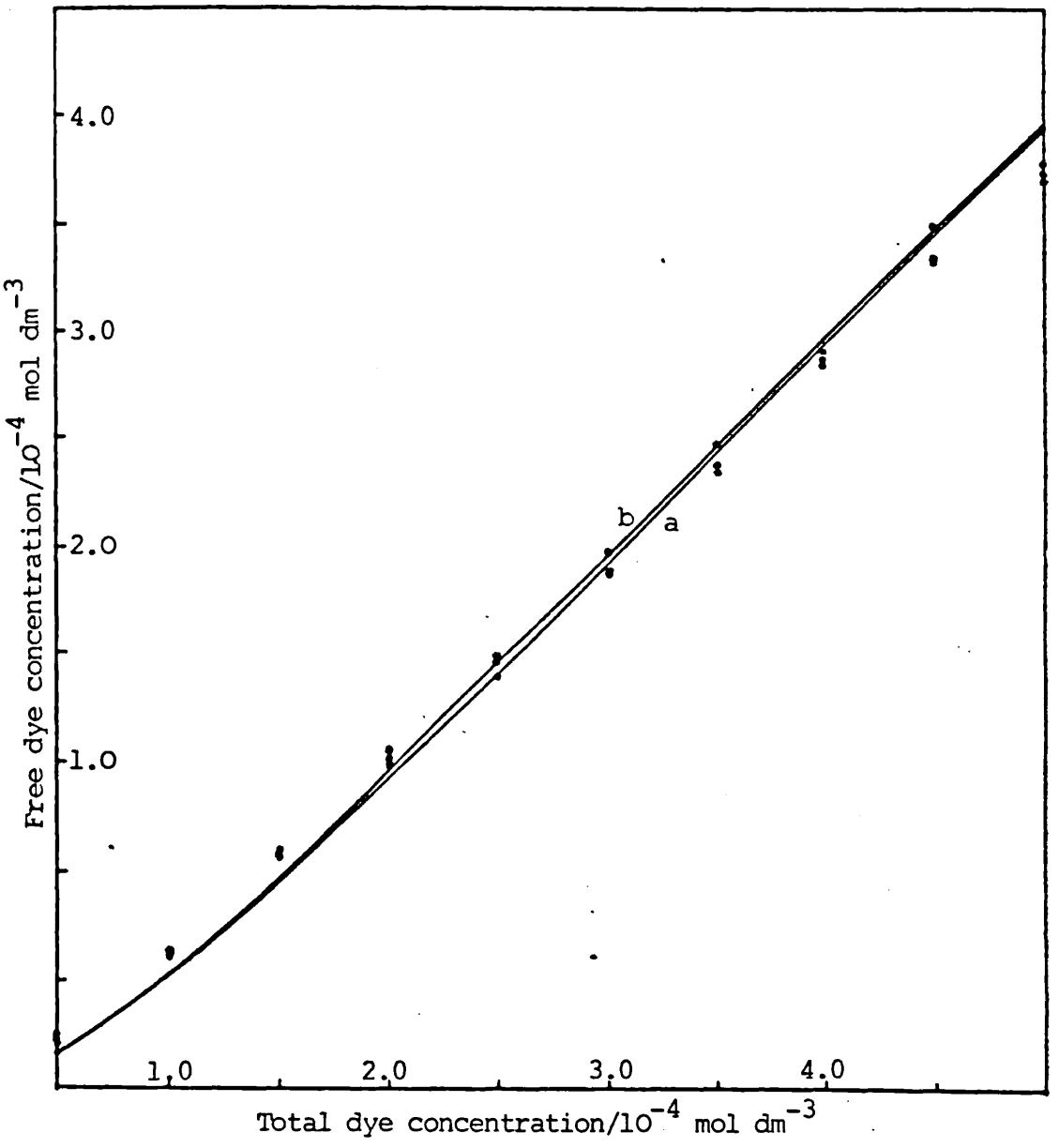


Fig. 6.3 Ultrafiltration curve for Mordant Yellow 10 with H.S.A.

Binding parameters for fitted line (a)

$K_1 = 6.76 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 2.08 \times 10^4$ mol^{-1} dm^3

$n_1 = 3.25$ $\quad n_2 = 3.29$

Binding parameters for line (b) (from Ch.5 p 158)

$K_1 = 7.82 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 3.90 \times 10^4$ mol^{-1} dm^3

$n_1 = 2.93$ $\quad n_2 = 2.68$

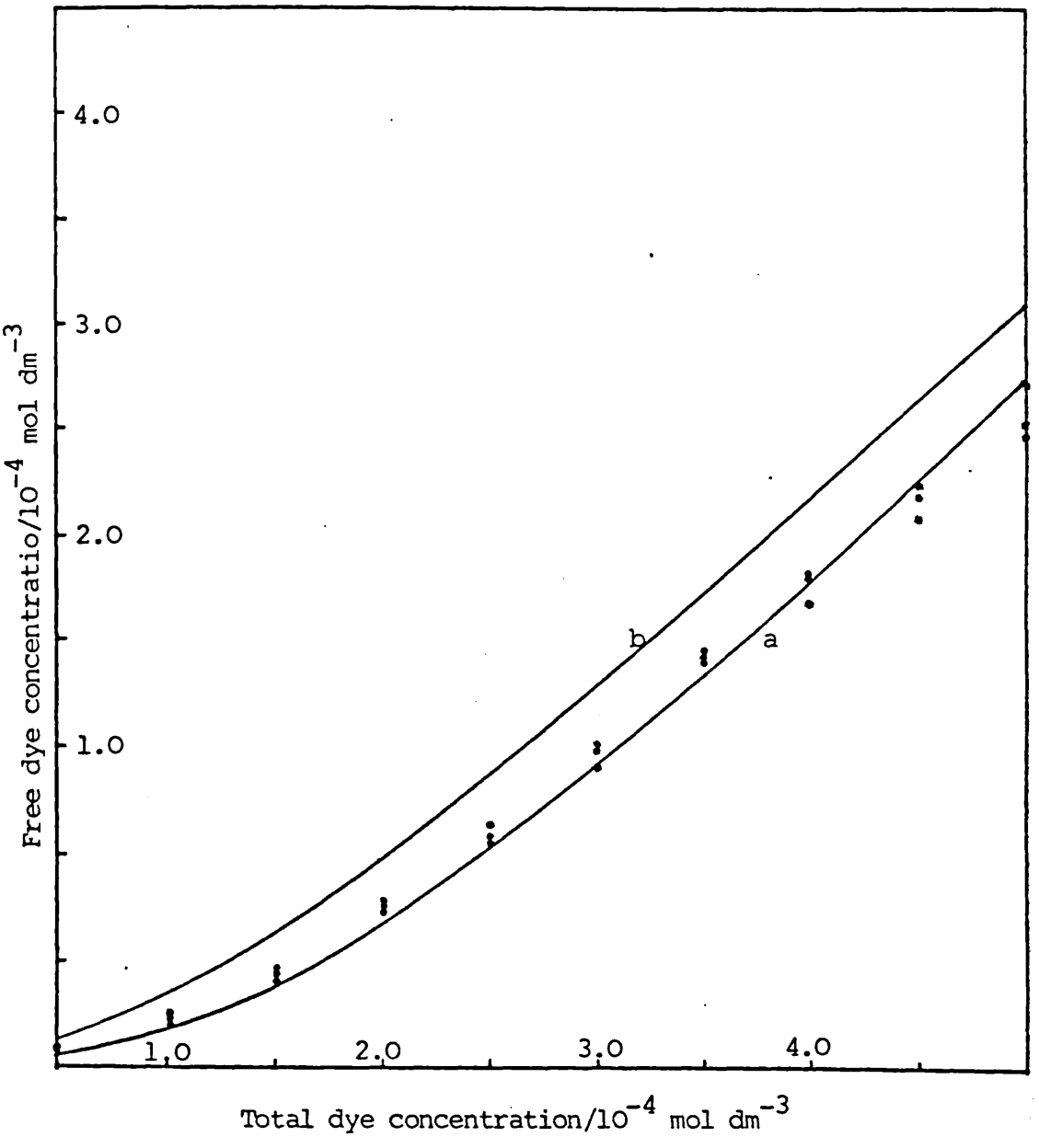


Fig. 6.4 Ultrafiltration curve for Mordant Orange 1 with H.S.A. (1.000×10^{-5} mol dm^{-3})

Binding parameters for fitted line (a)

$K_1 = 1.14 \times 10^{-5}$ mol^{-1} dm^3 $\quad K_2 = 2.31 \times 10^{4}$ mol^{-1} dm^3

$n_1 = 3.08$ $\quad n_2 = 16.67$

Binding parameters for line (b) (from Ch. 5 p158)

$K_1 = 2.52 \times 10^{5}$ mol^{-1} dm^3 $\quad K_2 = 1.15 \times 10^{4}$ mol^{-1} dm^3

$n_1 = 2.28$ $\quad n_2 = 14.62$

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	3.352	3.168	3.265
4.500	2.899	3.148	2.969
4.000	2.793	2.707	2.771
3.500	2.249	2.352	2.242
3.000	1.860	1.944	1.896
2.500	1.285	1.441	1.344
2.000	1.162	1.039	1.109
1.500	0.724	0.765	0.709
1.000	0.486	0.433	0.467
0.500	0.229	0.198	0.212

Table 6.4 Ultrafiltration data for Mordant Yellow 12 with human serum albumin (1.000 x 10^{-5} mol dm^{-3})

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	3.867	3.887	3.710
4.500	3.412	3.480	3.321
4.000	2.834	2.967	2.862
3.500	2.426	2.542	2.420
3.000	2.085	1.978	2.064
2.500	1.648	1.566	1.614
2.000	1.190	1.169	1.162
1.500	0.780	0.749	0.716
1.000	0.400	0.382	0.372
0.500	0.126	0.130	0.130

Table 6.5 Ultrafiltration data for 5-phenylazosalicylic acid with human serum albumin (1.000 x 10^{-5} mol dm^{-3})

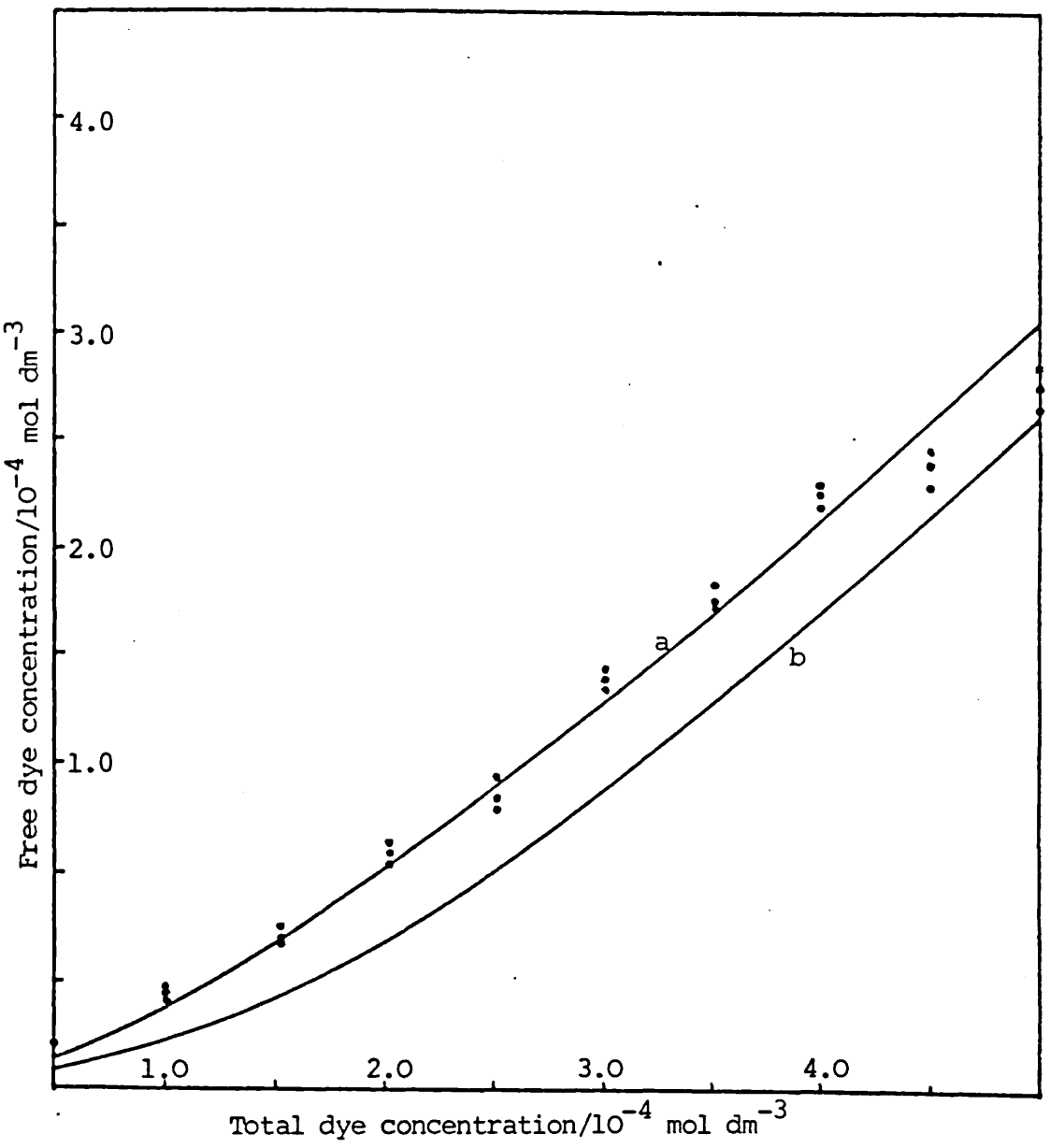


Fig. 6.5 Ultrafiltration curve for Mordant Yellow 12 with H.S.A. (1.000×10^{-5} mol dm^{-3})

Binding parameters for fitted line (a)

$K_1 = 3.78 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 1.04 \times 10^4$ mol^{-1} dm^3

$n_1 = 4.47$ $\quad n_2 = 13.39$

Binding parameters for fitted line (b) (from Ch.5 p158)

$K_1 = 1.60 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 1.53 \times 10^4$ mol^{-1} dm^3

$n_1 = 4.31$ $\quad n_2 = 17.70$

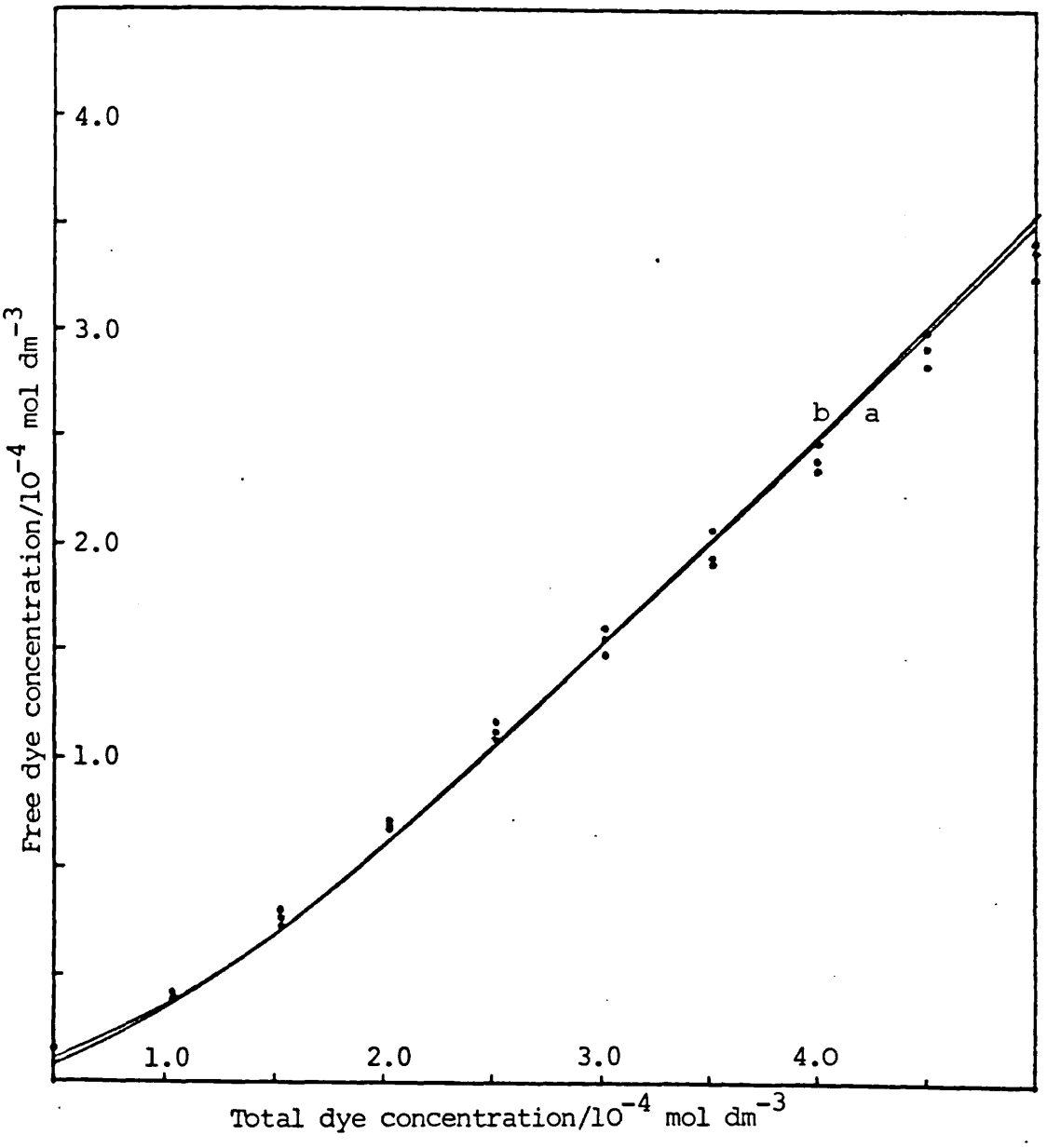


Fig. 6.6 Ultrafiltration curve for 5-phenylazosalicylic acid with H.S.A. (1.000×10^{-5} mol dm^{-3})

Binding parameters for fitted line (a)

$K_1 = 4.57 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 3.24 \times 10^4$ mol^{-1} dm^3

$n_1 = 1.75$ $\quad n_2 = 11.66$

Binding parameters for line (b) (from Ch.5 p158)

$K_1 = 3.81 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 4.08 \times 10^4$ mol^{-1} dm^3

$n_1 = 1.31$ $\quad n_2 = 9.39$

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	4.382	4.174	4.382
4.500	3.903	4.080	3.984
4.000	3.522	3.479	3.483
3.500	2.921	3.017	2.970
3.000	2.537	2.646	2.559
2.500	2.175	2.068	2.054
2.000	1.662	1.645	1.669
1.500	1.222	1.225	1.202
1.000	0.702	0.724	0.726
0.500	0.352	0.338	0.335

Table 6.6 Ultrafiltration data for 4-hydroxyazobenzene-4'-sulphonic acid with human serum albumin (1.000×10^{-5} mol dm^{-3})

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	4.076	4.040	4.217
4.500	3.669	3.404	3.474
4.000	3.102	2.842	2.925
3.500	2.393	2.326	2.526
3.000	2.205	2.270	2.255
2.500	1.837	1.839	1.852
2.000	1.425	1.396	1.451
1.500	1.030	1.007	0.962
1.000	0.578	0.581	0.572
0.500	0.215	0.191	0.190

Table 6.7 Ultrafiltration data for azobenzene-4-sulphonic acid with human serum albumin (1.000×10^{-5} mol dm^{-3})

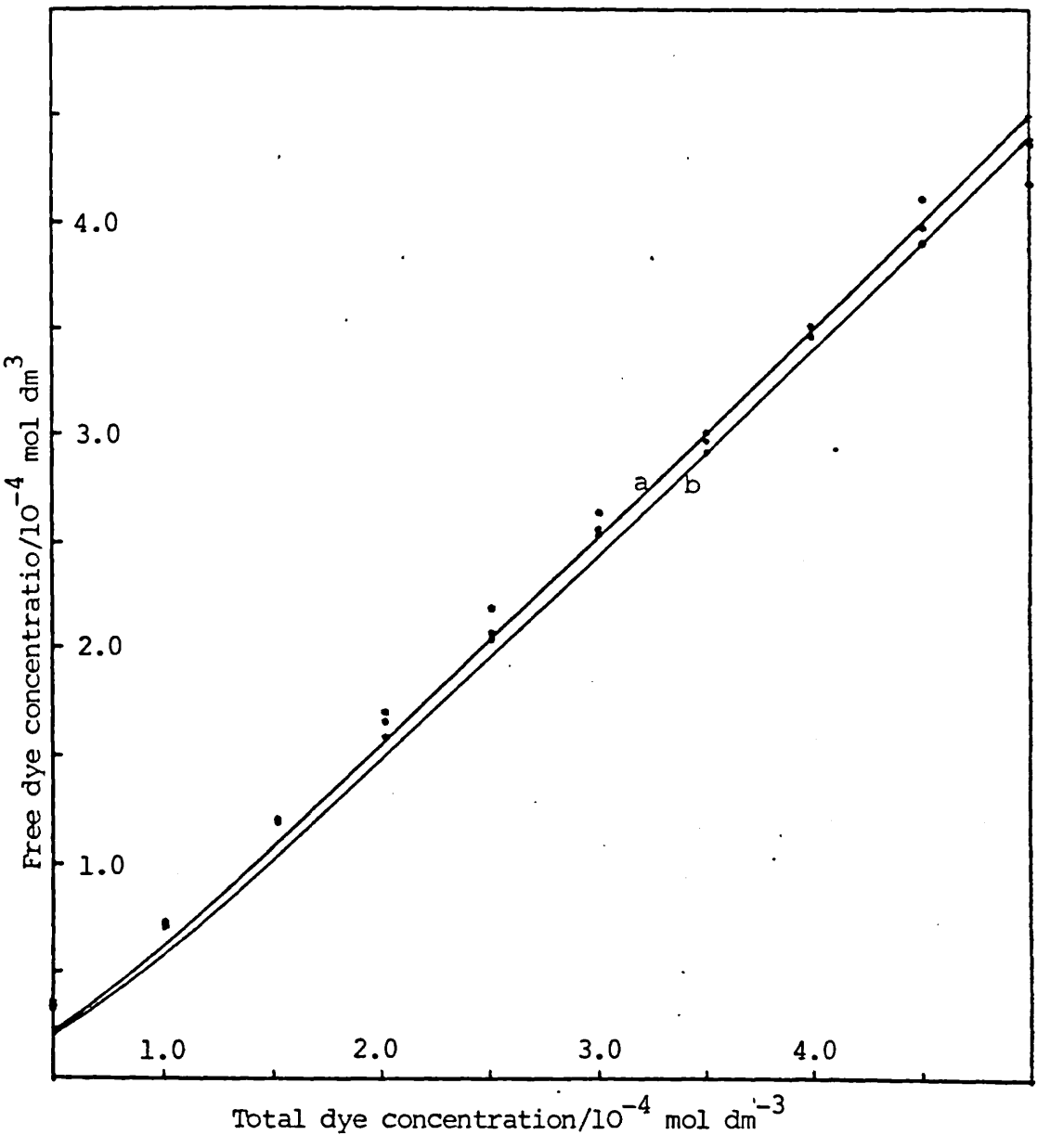


Fig. 6.7 Ultrafiltration curve for 4-hydroxyazobenzene-4'-sulphonic acid with H.S.A. (1.000 x 10^{-3} mol dm^{-3})

Binding parameters for fitted line (a)

$K_1 = 2.96 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 7.58 \times 10^3$ mol^{-1} dm^3

$n_1 = 2.37$ $\quad n_2 = 4.43$

Binding parameters for line (b) (from Ch.5 p158)

$K_1 = 2.33 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 1.65 \times 10^4$ mol^{-1} dm^3

$n_1 = 2.17$ $\quad n_2 = 4.18$

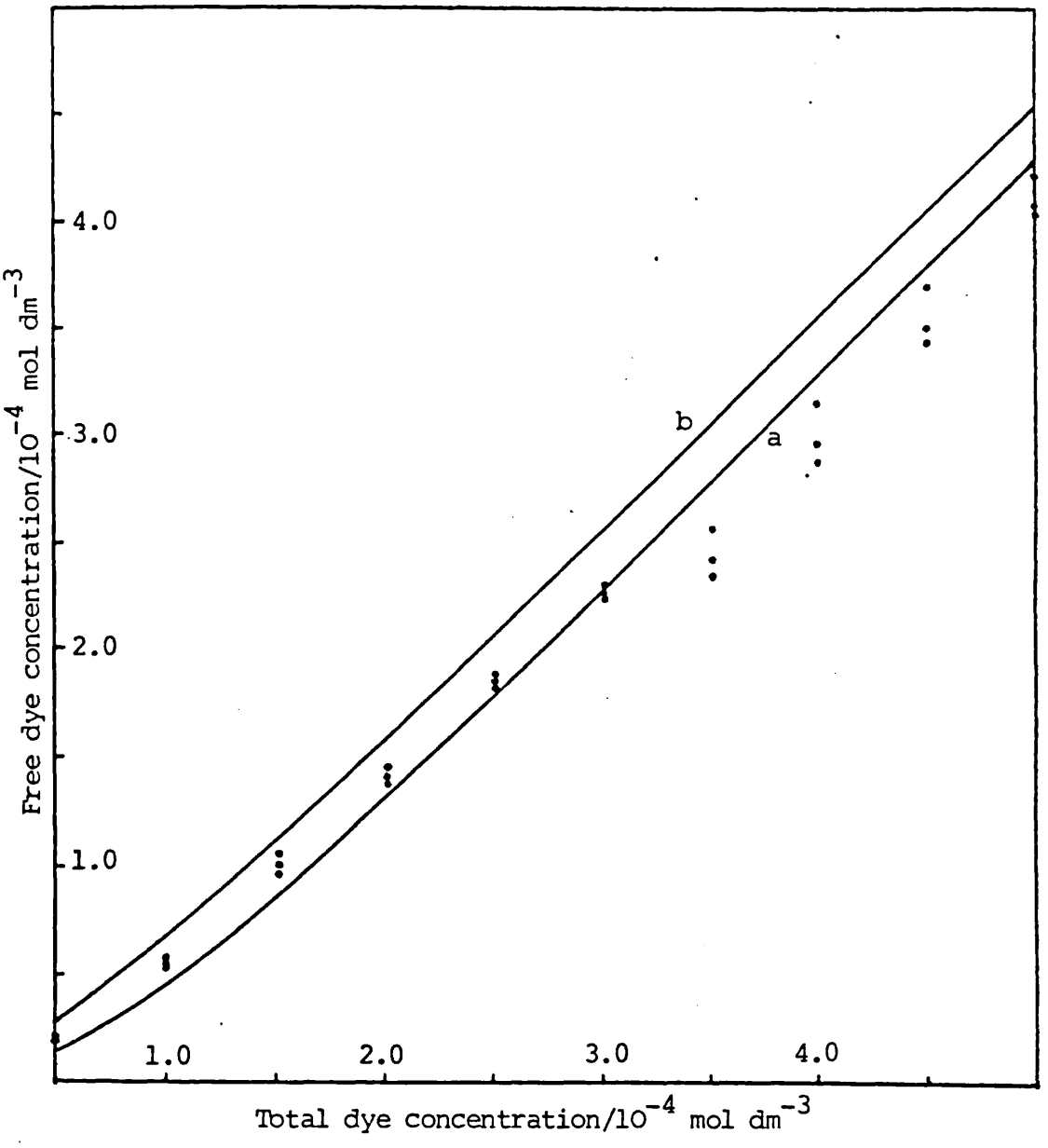


Fig. 6.8 Ultrafiltration curve for 4-azobenzene-sulphonic acid.

Binding parameters for fitted line (a)

$K_1 = 4.79 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 2.37 \times 10^4$ mol^{-1} dm^3

$n_1 = 1.73$ $\quad n_2 = 6.48$

Binding parameters for line (b) (from Ch.5 p158)

$K_1 = 3.65 \times 10^5$ mol^{-1} dm^3 $\quad K_2 = 9.29 \times 10^3$ mol^{-1} dm^3

$n_1 = 1.14$ $\quad n_2 = 4.61$

Total dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}	Experimental free dye concentration /10^{-4} mol dm^{-3}
5.000	3.358	3.512	3.600
4.500	3.172	3.278	3.245
4.000	2.780	2.802	2.799
3.500	2.366	2.348	2.329
3.000	1.920	1.908	1.834
2.500	1.315	1.376	1.456
2.000	1.144	1.098	1.077
1.500	0.762	0.684	0.690
1.000	0.399	0.381	0.381
0.500	0.169	0.127	0.118

Table 6.8 Ultrafiltration data for Alizarin Yellow GG with human serum albumin (1.000 x 10^{-5} mol dm^{-3})

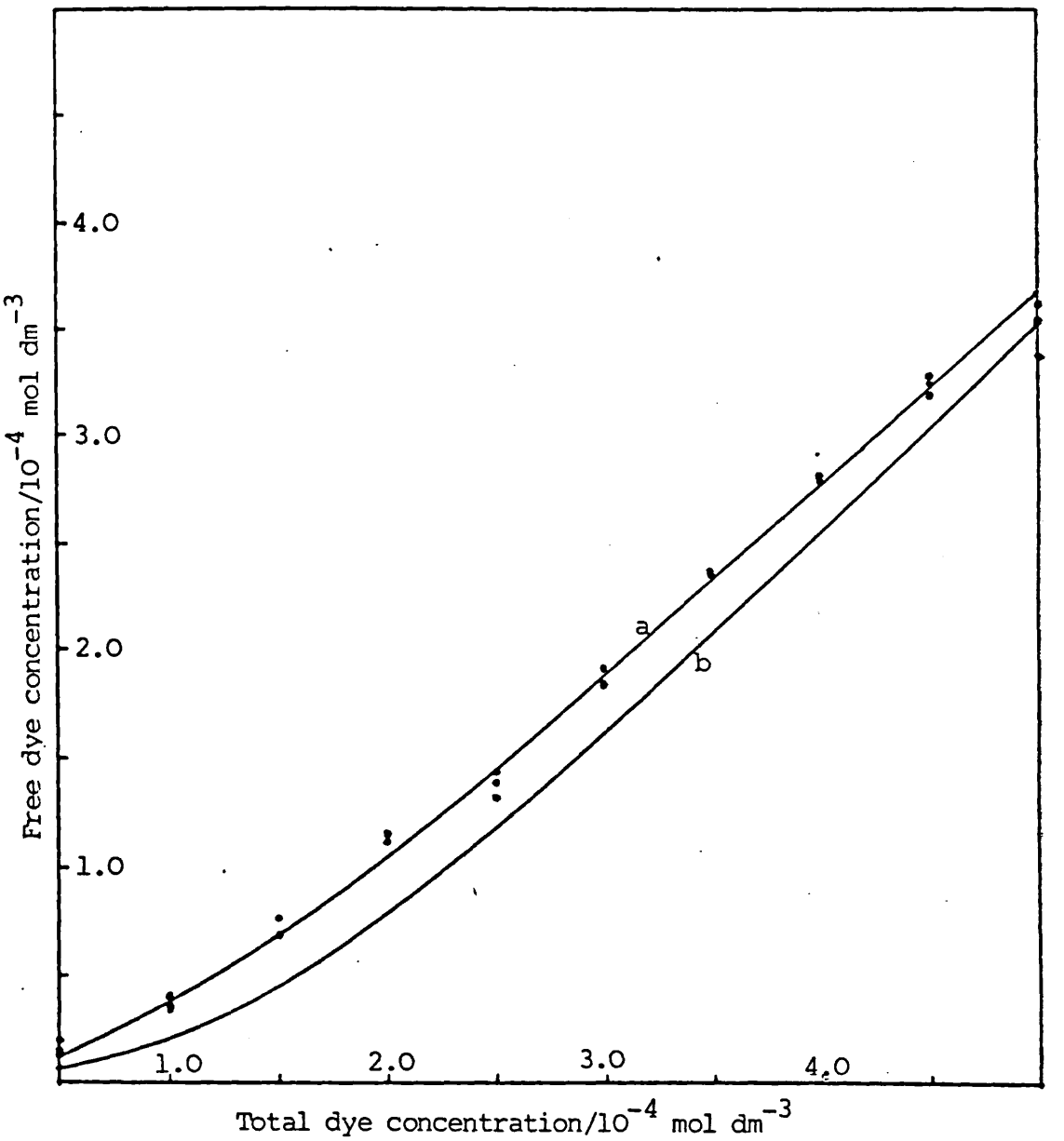


Fig. 6.9 Ultrafiltration curve for Alizarin Yellow GG with H.S.A. (1.000×10^{-5} mol dm^{-3})

Binding parameters for fitted line (a)

$K_1 = 5.98 \times 10^5$ mol^{-1} dm^3 $K_2 = 7.66 \times 10^3$ mol^{-1} dm^3

$n_1 = 4.18$ $n_2 = 14.89$

Binding parameters for fitted line (b) (from Ch.5 p158)

$K_1 = 3.71 \times 10^5$ mol^{-1} dm^3 $K_2 = 2.18 \times 10^4$ mol^{-1}

$n_1 = 5.46$ $n_2 = 10.9$

CHAPTER 7

SPECTROPHOTOMETRIC STUDIES OF COMPETITION REACTIONS BETWEEN SOME AZOBENZENE DERIVATIVES AND BROMOPHENOL BLUE, WITH HUMAN SERUM ALBUMIN.

7.1. The two independent site model for competitive reactions

The shapes of a series of absorption spectra, produced as a result of the titration of a solution of Bromophenol Blue with human serum albumin, can be altered by the introduction of another dye. This effect occurs even though the second dye has no absorption of its own over the wavelength range measured. The effect is not due to a direct interaction between the two dyes. For all the compounds described in this Chapter, over the wavelength range measured, the spectrum of Bromophenol Blue (with no albumin present) is unaffected by the introduction of the second dye.

The effect is clearly shown in Fig. 7.1 - 7.3, which show the absorption spectra for the titration of Bromophenol Blue with human serum albumin in the presence of successively larger Mordant Yellow 10 concentrations.

As described in Chapter 4, the spectrum of Bromophenol Blue changes as H.S.A. is added, but, in general, the changes in shape, are smaller if a second dye is present. Furthermore the change in shape is even smaller when the dye concentration is increased.

This seems to indicate that less Bromophenol Blue is bound by the albumin when the other dye is present, and shows competition between the two dyes for the albumin molecule.

If the two site model for the interaction of Bromophenol Blue with human serum albumin is assumed, as proposed in Chapter 4, then the concentration of the free dye, and the two bound species in any solution can be calculated from

Fig 7.1. Interaction of Bromophenol Blue with H.S.A. (experimental method 1.16c)

Bromophenol Blue concentration = 4.417×10^{-5} mol dm^{-3}.

Albumin concentrations/10^{-5} mol dm^{-3} were as follows:-

(1) 0, (2) .3872, (3) .7595, (4) 1.118, (5) 1.463, (6) 1.795, (7) 2.116, (8) 2.425, (9) 2.724, (10) 3.012, (11) 3.291.

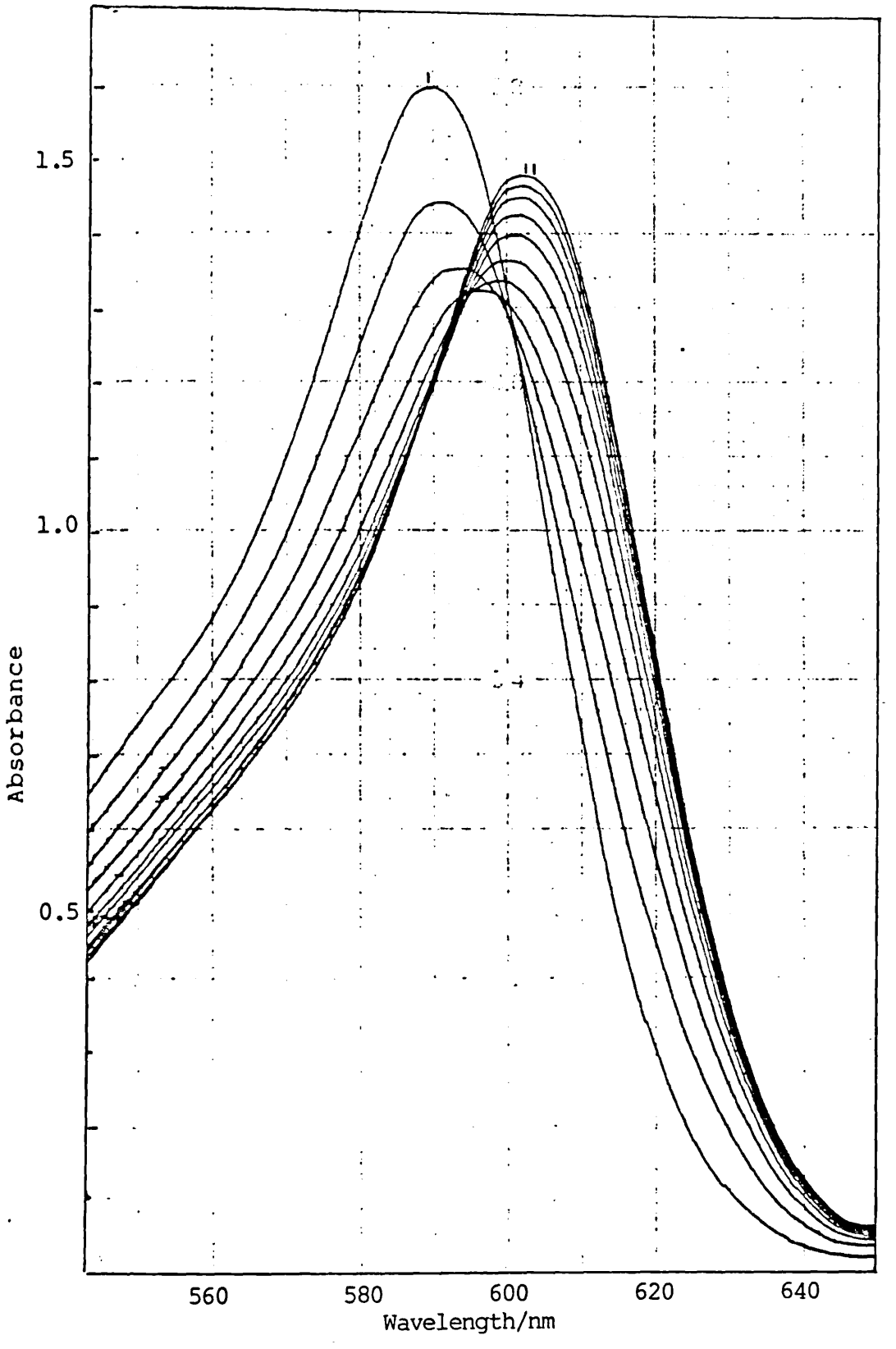

1.5
1.0
0.5
Absorbance
560
580
600
620
640
Wavelength/nm
I
II

Fig. 7.2 Interaction of Bromophenol Blue (4.442×10^{-5} mol dm^{-3}) with H.S.A. (experimental method 2.1b), in the presence of Mordant Yellow 10 (4.425×10^{-5} mol dm^{-3})

Albumin concentrations/10^{-5} mol dm^{-3} were as follows:-

(1) 0, (20 0.3552, (3) 0.6968, (4) 1.025, (5) 1.342, (6) 1.647, (7) 1.941, (8) 2.225, (9) 2.499, (10) 2.763.

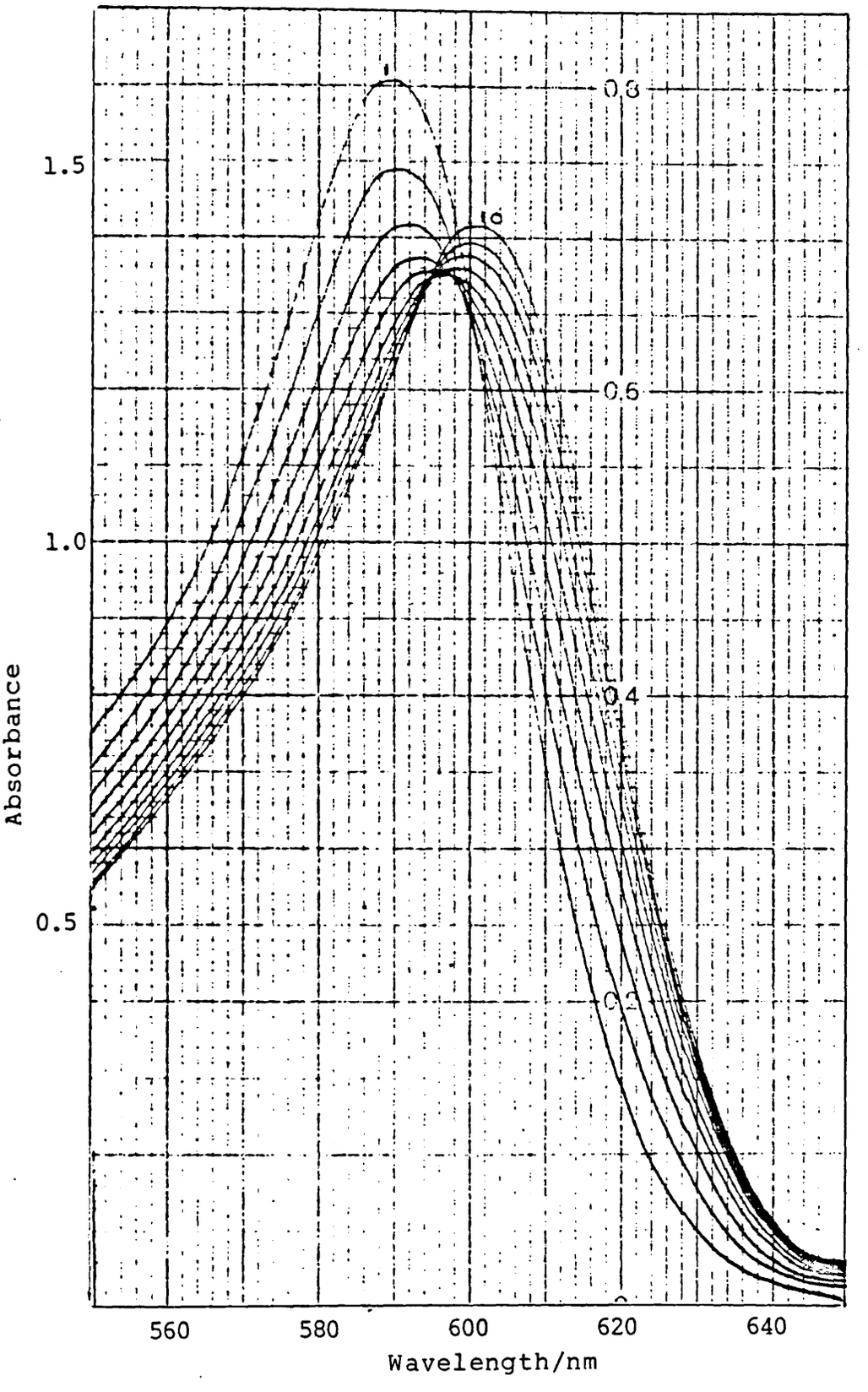
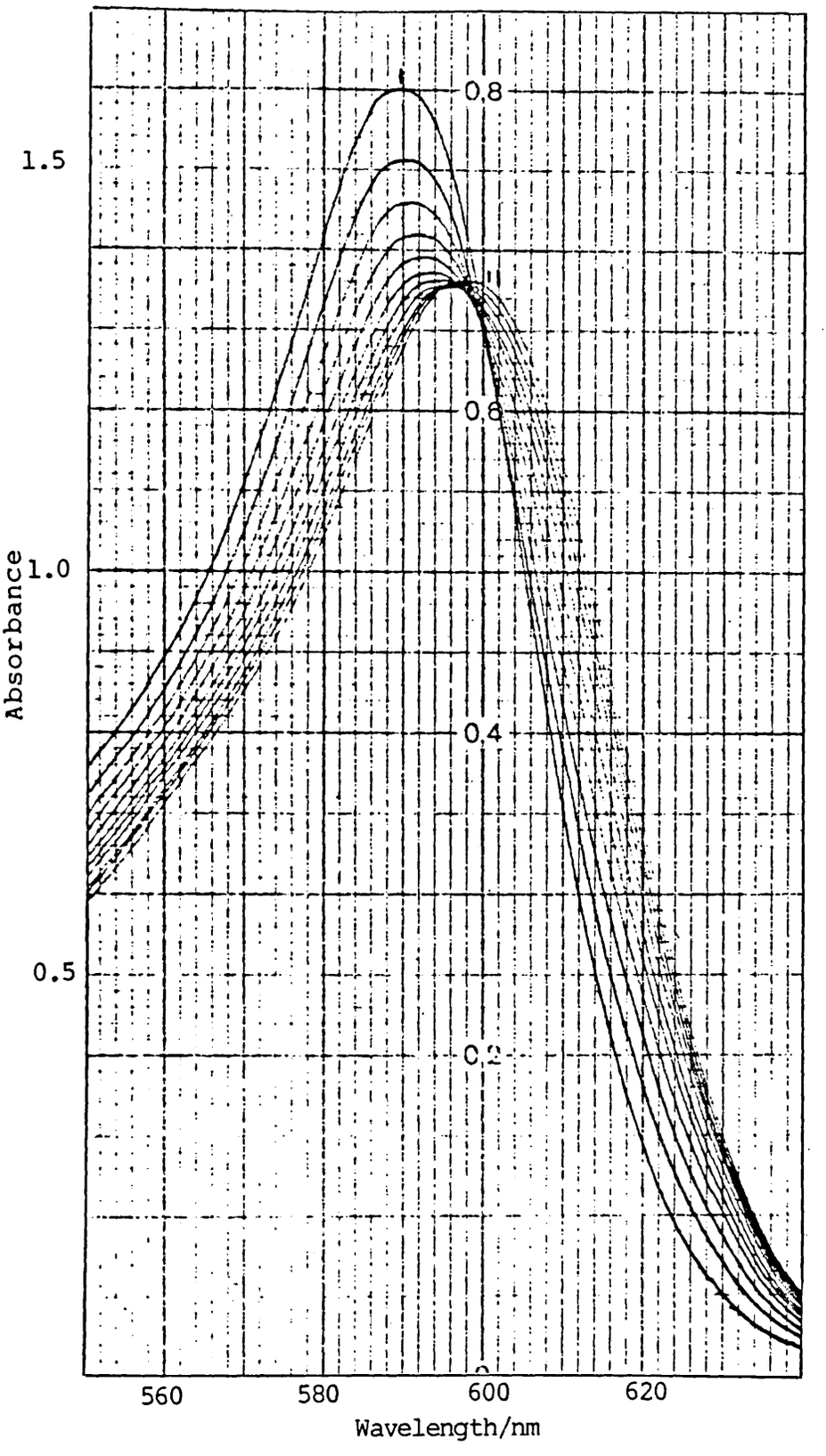

1.5
1.0
0.5
Absorbance
0.8
0.6
0.4
560
580
600
620
640
Wavelength/nm

Fig. 7.3 Interaction of Bromophenol Blue (4.429 x 10^{-5} mol dm^{-3}) with H.S.A. (experimental method 2.1f) in the presence of Mordant Yellow 10 (1.770 x 10^{-4} mol dm^{-3})

Albumin concentrations/10^{-5} mol dm^{-3} were as follows:-

(1) 0, (2) 0.3586, (3) 0.7034, (4) 1.035, (5) 1.355, (6) 1.663, (7) 1.960, (8) 2.246, (9) 2.523, (10) 2.790, (11) 3.048.


0.8
0.6
0.4
0.2
1.5
1.0
0.5
Absorbance
560
580
600
620
Wavelength/nm

the extinction coefficients calculated in Chapter 5, by the following method:-

If $[B_f]$, $[BS_1]$ and $[BS_2]$ are the concentrations of free Bromophenol Blue and the two albumin bound species respectively, the absorbance (A_1) of the solution at a wavelength λ_1, per unit pathlength is given by:-

$$A_1 = \varepsilon_{1f} [B_f] + \varepsilon_{11} [BS_1] + \varepsilon_{12} [BS_2] \qquad (7.1)$$

where ε_{1f}, ε_{11} and ε_{12} are the molar extinction coefficients for the three species B_f, BS_1 and BS_2 at the wavelength λ_1. Similarly for the same solution at two further wavelengths λ_2 and λ_3. The competiting dye does not absorb at λ_1, λ_2 or λ_3.

$$A_2 = \varepsilon_{2f} [B_f] + \varepsilon_{21} [BS_1] + \varepsilon_{22} [BS_2] \qquad (7.2)$$

$$A_3 = \varepsilon_{3f} [B_f] + \varepsilon_{31} [BS_1] + \varepsilon_{32} [BS_2] \qquad (7.3)$$

Therefore, by re-arranging 7.1, 7.2 and 7.3 to eliminate $[BS_2]$ from the equations, the following may be written:-

$$\varepsilon_{22} (\varepsilon_{11} [BS_1] + \varepsilon_{1f} [B_f] - A_1) = \varepsilon_{12}(\varepsilon_{21} [BS_1] + \varepsilon_{2f} [B_f] - A_2) \qquad (7.4)$$

$$\varepsilon_{32} (\varepsilon_{11} [BS_1] + \varepsilon_{1f} [B_f] - A_1) = \varepsilon_{12} (\varepsilon_{31} [BS_1] + \varepsilon_{3f} [B_f] - A_3) \qquad (7.5)$$

Substituting the equalities

$$A = (\varepsilon_{1f}\ \varepsilon_{22} - \varepsilon_{2f}\ \varepsilon_{12}) \qquad A' = (\varepsilon_{1f}\ \varepsilon_{32} - \varepsilon_{31}\ \varepsilon_{12})$$

$$B = (\varepsilon_{11}\ \varepsilon_{22} - \varepsilon_{21}\ \varepsilon_{22}) \qquad B' = (\varepsilon_{11}\ \varepsilon_{32} - \varepsilon_{31}\ \varepsilon_{12})$$

$$C = (A_2\ \varepsilon_{12} - A_1\ \varepsilon_{22}) \qquad C' = (A_3\ \varepsilon_{12} - A_1\ \varepsilon_{32})$$

7.4 and 7.5 become on re-arrangement

$$A\ [B_f] + B\ [BS_1] + C = 0 \tag{7.6}$$

$$A'\ [B_f] + B\ [BS_1] + C' = 0 \tag{7.7}$$

equating for $[B_f]$ and $[BS_1]$

$$[B_f] = \frac{BC' - B'C}{B'A - BA'} \tag{7.8}$$

$$(BS_1] = \frac{CA' - C'A}{AB' - A'B} \tag{7.9}$$

$$(BS_2] = -\left[\frac{\varepsilon_{1f}(BC' - B'C) + \varepsilon_{11}\ (CA' - C'A) - A_1(B'A - BA')}{\varepsilon_{12}\ (B'A - BA')} \right] \tag{7.10}$$

The simplest competitive model for a two site system is one in which there are n_1 mutual primary sites and n_2 mutual secondary sites on the albumin molecule. Thus for Bromophenol Blue

$$B_f + S_1 \overset{K_{B1}}{\rightleftharpoons} BS_1 \qquad K_{B1} = \frac{[BS_1]}{[B_f][S_1]} \tag{7.11}$$

$$B_f + S_2 \overset{K_{B2}}{\rightleftharpoons} BS_2 \qquad K_{B2} = \frac{BS_2}{[B_f][S_2]} \tag{7.12}$$

and for the competing dye

$$D + S_1 \overset{K_1}{\rightleftharpoons} DS_1 \qquad K_1 = \frac{[DS_1]}{[D][S_1]} \tag{7.13}$$

$$D + S_2 \overset{K_2}{\rightleftharpoons} DS_2 \qquad K_2 = \frac{[DS_2]}{[D][S_2]} \tag{7.14}$$

Therefore from 7.11 and 7.12

$$[S_1] = \frac{[BS_1]}{[B_f].K_{B1}}$$

$$[S_2] = \frac{[BS_2]}{[B_f].K_{B2}}$$

If the total primary binding site $[S_{1tot}]$ is given by

$$S_{1\,tot} = [DS_1] + [BS_1] + S_1$$

then $DS_1 = S_{1\,tot} - [BS_1] - [S_1]$

Similarly $DS_2 = S_{2\,tot} - [BS_2] - [S_2]$

If the total competing dye concentration is $[D_{tot}]$ then

$$[D] = [D_{tot}] - [DS_1] - [DS_2]$$

Hence K_1 and K_2 can be calculated from 7.13 and 7.14.

Competition experiments between Bromophenol Blue and eight azo dyes were performed as described in Ch.2 (experimental method 2). Absorbances for the solutions at the five wavelengths at which the largest changes in absorbance occurred were used in the calculation of the concentrations of the three Bromophenol Blue species present, using the calculated ε values for the three Bromophenol Blue species (see p 156).

Equations 7.8, 7.9 and 7.10 require only three absorbances for each solution. A Fortran computer program was written which obtained values for the concentrations of the three Bromophenol Blue species for every permutation of the five absorbance values. Thus ten values for the concentration of the three Bromophenol Blue species were calculated for each solution.

Tables 7.1 - 7.8 give examples of one titration for each of the eight azo compounds. Table 7.9 summarises the equilibrium constant values obtained for each compound.

In calculating the values for K_1 and K_2 there are a small number of data sets which give negative values for the constants. These drastically affect the mean K_1 and K_2 values. Therefore, as well as the mean values of K_1 and K_2, a mean value has also been calculated in which any data set which gives a negative value for K_1 or K_2 is disregarded. The number of data sets giving positive values is given.

It may be more meaningful to plot the free Bromophenol Blue concentration against albumin concentration in the presence of a competitor. Fig. 7.4 and 7.5 show the data plotted in this way.

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue mean concentration /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 1^{o}ry site /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 2^{o}ry site /10^{-6} mol dm^{-3}	Mean K_1 /10^{4} mol^{-1} dm^{3}	Mean K_2 /10^{4} mol^{-1} dm^{3}
0.3784	0.994	1.255	1.489	0.917	0.471	3.400	0.5164	5.571	9.13	0.509
0.7422	0.933	1.161	1.391	1.015	0.574	2.511	1.127	8.360	3.75	0.726
1.092	0.886	1.090	1.321	1.094	0.652	1.860	1.645	9.629	2.93	0.881
1.429	0.847	1.034	1.265	1.162	0.712	1.369	2.086	9.951	2.59	1.07
1.754	0.822	1.004	1.233	1.207	0.752	1.100	2.362	9.739	3.28	1.28
2.068	0.802	0.976	1.212	1.240	0.780	0.8886	2.633	8.956	3.53	1.67
2.370	0.790	0.958	1.192	1.276	0.790	0.7662	2.831	7.953	4.12	2.31
2.662	0.776	0.942	1.185	1.298	0.820	0.6195	3.038	7.400	4.19	4.91
2.944	0.772	0.934	1.187	1.317	0.832	0.5547	3.204	6.463	5.34	11.1

Table 7.1. Competition data for Bromophenol Blue (4.432×10^{-5} mol dm^{-3} and human serum albumin in the presence of Mordant Yellow 12 (1.770×10^{-4} mol dm^{-3})

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570 nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue mean concentration /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 1^{o}ry site /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 2^{o}ry site /10^{-5} mol dm^{-3}	Mean K_1 /10^{5} mol^{-1} dm^{3}	Mean K_2 /10^{4} mol^{-1} dm^{3}
.3586	1.009	1.278	1.508	0.873	0.431	3.661	0.2535	0.5315	5.21	0.596
.7033	0.973	1.221	1.447	0.931	0.499	3.085	0.5950	0.7854	3.27	1.05
1.035	0.946	1.173	1.398	0.977	0.549	2.611	0.8757	0.9946	2.97	1.25
1.355	0.920	1.147	1.360	1.020	0.597	2.323	1.069	1.087	3.43	1.73
1.663	0.892	1.102	1.324	1.063	0.641	1.944	1.421	1.105	2.45	1.86
1.959	0.876	1.080	1.303	1.091	0.671	1.731	1.605	1.137	2.58	2.18
2.246	0.858	1.057	1.280	1.124	0.698	1.536	1.818	1.106	2.58	2.69
2.523	0.844	1.035	1.262	1.158	0.728	1.318	2.040	1.113	2.38	2.80
2.790	0.834	1.022	1.251	1.184	0.753	1.171	2.198	1.114	2.44	3.08
3.048	0.824	1.006	1.238	1.210	0.774	1.016	2.366	1.109	2.37	3.20

Table 7.2. Competition data for Bromophenol Blue (4.429×10^{-5} mol dm^{-3}) and human serum albumin in the presence of Mordant Yellow 10 (1.770×10^{-4} mol dm^{-3})

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue mean concentration /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 1^{o}ry site /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 2^{o}ry site /10^{-6} mol dm^{-3}	Mean K_1 /10^{5} mol^{-1} dm^{3}	Mean K_2 /10^{4} mol^{-1} dm^{3}
.3759	1.016	1.291	1.522	0.862	0.416	3.801	.1926	4.428	10.19	1.28
.7372	0.981	1.233	1.459	0.913	0.472	3.255	0.5007	6.813	5.33	1.87
1.086	0.951	1.191	1.416	0.959	0.526	2.848	0.7966	7.960	4.35	2.39
1.421	0.925	1.153	1.378	1.002	0.568	2.503	1.078	8.522	3.93	3.07
1.743	0.902	1.119	1.346	1.042	0.610	2.180	1.345	9.120	3.62	3.44
2.054	0.882	1.092	1.320	1.080	0.641	1.948	1.593	8.840	3.57	4.51
2.354	0.863	1.066	1.297	1.110	0.674	1.713	1.812	8.974	3.53	4.99
2.644	0.848	1.045	1.279	1.138	0.701	1.522	2.009	8.921	3.62	5.79
2.925	0.833	1.026	1.259	1.172	0.729	1.335	2.203	8.840	3.66	6.53
3.196	0.818	1.007	1.246	1.203	0.749	1.205	2.443	7.551	3.88	9.86

Table 7.3. Competition data for Bromophenol Blue (4.434×10^{-5} mol dm^{-3}) and human serum albumin in the presence of Mordant Yellow 7 (1.773×10^{-4} mol dm^{-3})

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue mean concentration /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 1°ry site /10^{-5} mol dm^{-3}	Bromophenol Blue mean concentration at 2°ry site /10^{-6} mol dm^{-3}	Mean K_1 /10^5 mol^{-1} dm^3	Mean K_2 /10^5 mol^{-1} dm^3
.3589	1.016	1.291	1.531	0.895	0.431	3.772	0.3939	2.946	2.23	0.310
.7039	0.968	1.218	1.452	0.962	0.499	3.129	0.8207	4.849	1.65	0.479
1.036	0.915	1.138	1.366	1.019	0.559	2.489	1.234	6.520	1.29	0.775
1.356	0.881	1.086	1.311	1.081	0.618	1.991	1.601	7.861	1.09	1.18
1.664	0.849	1.038	1.265	1.136	0.669	1.565	1.971	8.323	0.871	-13.3
1.961	0.826	1.003	1.229	1.175	0.708	1.236	2.215	9.178	0.814	2.03
2.248	0.805	0.973	1.203	1.209	0.733	1.017	2.477	8.481	0.771	-1.65
2.525	0.795	0.958	1.189	1.235	0.759	0.8425	2.625	8.908	0.790	1.21
2.792	0.785	0.946	1.181	1.254	0.775	0.7500	2.775	8.259	0.894	9.44
3.051	0.779	0.938	1.176	1.273	0.789	0.6710	2.899	7.851	1.037	-1.39

Table 7.4. Competition data for Bromophenol Blue (4.465×10^{-5} mol dm^{-3}) and human serum albumin in the presence of Mordant Orange 1 (1.774×10^{-4} mol dm^{-3})

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 1^{o}ry site /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 2^{o}ry site /10^{-6} mol dm^{-3}	K_1 /10^{4} mol^{-1} dm^{3}	K_2 /10^{4} mol^{-1} dm^{3}
0.3826	1.003	1.278	1.511	0.878	0.424	3.752	0.3380	3.090	36.7	2.57
0.7506	0.954	1.205	1.439	0.963	0.504	3.090	0.8916	4.066	13.5	4.35
1.104	0.911	1.141	1.380	1.042	0.573	2.530	1.429	4.160	8.67	19.6
1.446	0.874	1.089	1.330	1.109	0.631	2.077	1.872	4.079	7.48	-3.72
1.774	0.844	1.043	1.289	1.167	0.681	1.669	2.269	4.135	6.68	-17.3
2.091	0.818	1.007	1.259	1.216	0.721	1.374	2.624	3.370	6.36	-2.61
2.397	0.799	0.982	1.237	1.251	0.751	1.164	2.869	2.908	6.91	-0.916
2.692	0.785	0.967	1.226	1.278	0.770	1.063	3.073	1.684	8.40	5.27
2.977	0.776	0.951	1.212	1.299	0.787	0.9123	3.210	1.872	9.64	16.0
3.252	0.767	0.940	1.205	1.313	0.798	0.8425	3.336	1.165	12.4	-0.411

Table 7.5. Competition data for Bromophenol Blue (4.421×10^{-5} mol dm^{-3}) and human serum albumin in the presence of 4-azobenzenesulphonic acid (2.002×10^{-4} mol dm^{-3})

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 1^{o}ry site /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 2^{o}ry site /10^{-6} mol dm^{-3}	K_1 /10^{5} mol^{-1} dm^{3}	K_2 /10^{5} mol^{-1} dm^{3}
0.3714	1.027	1.314	1.556	0.883	0.416	3.983	.3181	1.597	4.90	1.01
0.7283	0.996	1.268	1.513	0.948	0.474	3.543	.7297.	1.937	3.00	1.69
1.072	0.963	1.220	1.469	1.017	0.531	3.109	1.178	1.751	2.28	-1.71
1.403	0.935	1.178	1.430	1.083	0.589	2.686	1.579	2.140	2.01	-0.983
1.722	0.906	1.137	1.393	1.140	0.642	2.304	1.958	2.193	1.84	-1.46
2.029	0.882	1.101	1.361	1.198	0.686	1.972	2.324	1.866	1.72	0.145
2.326	0.867	1.076	1.340	1.254	0.732	1.667	2.635	2.205	1.67	0.0844
2.613	0.841	1.043	1.311	1.282	0.752	1.479	2.901	0.8414	1.89	2.93
2.889	0.823	1.022	1.298	1.316	0.776	1.342	3.180	-0.8369	2.27	-2.91
3.157	0.818	1.014	1.285	1.338	0.799	1.191	3.245	0.3319	2.94	2.60

Table 7.6 Competition data for Bromophenol Blue (4.432×10^{-5} mol dm^{-3} and human serum albumin in the presence of Alizarin Yellow GG (1.705×10^{-4} mol dm^{-3}

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 1^{o}ry site /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 2^{o}ry site /10^{-5} mol dm^{-3}	K_1 /10^{4} mol^{-1} dm^{3}	K_2 /10^{3} mol^{-1} dm^{3}
0.3670	0.990	1.253	1.481	0.900	0.464	3.420	0.4231	0.6020	18.0	3.54
0.7200	0.927	1.161	1.385	0.999	0.571	2.550	1.046	0.8461	5.25	6.72
1.059	0.878	1.088	1.313	1.073	0.645	1.912	1.556	0.9533	3.55	7.98
1.387	0.842	1.034	1.258	1.129	0.701	1.427	1.923	1.059	3.61	7.27
1.702	0.816	0.996	1.222	1.171	0.740	1.102	2.217	1.076	3.74	7.51
2.006	0.797	0.967	1.195	1.197	0.764	0.8727	2.418	1.086	4.05	7.68
2.299	0.787	0.954	1.18	1.220	0.783	0.7459	2.554	1.078	4.70	8.58
2.582	0.776	0.940	1.171	1.238	0.795	0.6560	2.700	1.003	5.31	11.9
2.855	0.774	0.935	1.166	1.252	0.807	0.5729	2.761	1.044	5.92	12.1
3.119	0.764	0.925	1.161	1.266	0.820	0.5152	2.899	0.9481	6.50	14.8

Table 7.7. Competition data for Bromophenol Blue /4.426×10^{-5} mol dm^{-3}, and human serum albumin in the presence of 4-hydroxyazobenzene-4'-sulphonic acid /1.763×10^{-4} mol dm^{-3}

Albumin concentration /10^{-5} mol dm^{-3}	Absorbance at 570nm	Absorbance at 580nm	Absorbance at 590nm	Absorbance at 610nm	Absorbance at 620nm	Free Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 1°ry site /10^{-5} mol dm^{-3}	Bromophenol Blue concentration at 2°ry site /10^{-6} mol dm^{-3}	K_1 /10^5 mol^{-1} dm^3	K_2 /10^5 mol^{-1} dm^3
0.3640	1.023	1.310	1.553	0.866	0.404	4.026	0.2799	1.146	5.28	1.50
0.7139	0.987	1.262	1.510	0.932	0.461	3.619	0.7381	0.4437	2.73	1.90
1.051	0.958	1.221	1.477	0.998	0.514	3.251	1.187	- 0.3847	2.13	-1.31
1.375	0.929	1.181	1.442	1.057	0.564	2.896	1.585	- 0.8833	1.98	-0.998
1.688	0.902	1.143	1.409	1.115	0.611	2.558	1.971	- 1.418	1.88	-10.01
1.989	0.877	1.108	1.379	1.168	0.653	2.254	2.330	-2.053	1.86	-6.95
2.280	0.855	1.078	1.353	1.210	0.690	1.992	2.662	-2.425	1.98	-1.39
2.561	0.836	1.051	1.330	1.251	0.723	1.753	2.899	-2.838	2.15	-3.19
2.832	0.820	1.030	1.312	1.284	0.746	1.589	3.127	-3.634	2.58	2.78
3.094	0.806	1.012	1.298	1.318	0.774	1.420	3.353	-4.179	3.14	-1.12

Table 7.8 Competition data for Bromophenol Blue (4.404×10^{-5} mol dm^{-3}) and human serum albumin in the presence of 5-phenylazosalicylic acid (1.767×10^{-4} mol dm^{-3})

Competitor dye	Competitor dye Concentration /10^{-5} mol dm^{-3}	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Mean K_1 /10^5 mol^{-1} dm^3	Mean K_2 /10^5 mol^{-1} dm^3	Mean K_1 using only + ve values /10^5 mol^{-1} dm^3	Mean K_2 using only + ve values /10^5 mol^{-1} dm^3	Total number of calculated values	Total number of positive values
5-phenylazo-salicylic acid	4.418	4.412	6.67	4.80	15.90	9.26	100	52
	4.418	4.426	38.4	18.59	76.3	38.0	100	53
	8.836	4.396	-0.294	-2.09	6.27	4.61	100	63
	8.836	4.440	-0.152	-3.94	7.53	4.41	100	63
	17.67	4.404	2.57	-1.88	3.28	15.1	100	34
	17.67	4.382	2.58	-0.683	2.91	19.7	100	39
Mordant Yellow 7	4.433	4.489	4.23	-0.324	11.53	1.45	90	50
	4.433	4.465	37.0	1.06	70.3	20.4	90	51
	8.867	4.437	11.6	2.99	17.3	5.01	100	87
	8.867	4.444	0.620	-0.126	9.37	1.67	100	87
	17.73	4.434	4.57	0.437	4.57	4.37	100	100
	17.73	4.440	4.16	0.462	4.16	0.462	100	100
Mordant Yellow 10	4.424	4.473	5.87	0.437	9.93	1.14	100	71
	4.424	4.442	7.46	1.28	9.82	1.78	100	84
	8.848	4.481	5.24	0.805	5.24	0.805	100	100
	8.848	4.487	3.51	0.454	3.51	0.454	100	100
	17.70	4.418	3.63	0.207	3.42	0.213	100	97
	17.70	4.429	2.97	0.204	2.97	0.204	100	100
4-azobenzene sulphonic acid	5.005	4.396	18.3	24.6	33.35	32.3	100	59
	5.005	4.454	2.68	-0.444	8.05	3.34	100	59
	10.01	4.385	2.17	-0.212	3.01	2.40	100	83
	10.01	4.385	2.87	-0.139	2.61	8.45	100	82
	20.02	4.421	1.17	0.229	1.22	1.17	100	79
	20.02	4.415	1.09	0.523	1.12	1.41	100	81
4-hydroxyazobenzene -4'-sulphonic acid	4.418	4.415	0.705	-0.178	2.15	0.477	100	62
	4.418	4.434	1.018	-0.135	2.47	0.398	100	63
	8.835	4.434	2.625	0.726	2.50	0.766	100	95
	8.835	4.434	2.273	0.597	2.22	0.637	100	94
	17.67	4.418	0.611	0.0795	0.555	0.0832	100	96
	17.67	4.426	0.606	0.088	0.592	0.0889	100	99

Table 7.9 Binding data for the competition of azo dyes and Bromophenol Blue with H.S.A.

Competitor dye	Competitor dye concentration /10^{-5} mol dm^{-3}	Bromophenol Blue concentration /10^{-5} mol dm^{-3}	Mean K_1 /10^5 mol^{-1} dm^3	Mean K_2 /10^5 mol^{-1} dm^3	Mean K_1 using only + ve values /10^5 mol^{-1} dm^3	Mean K_2 using only + ve values /10^5 mol^{-1} dm^3	Total number of calculated values	Total number of positive values
Alizarin Yellow GG	4.423	4.263	-1.15	-9.25	11.9	3.47	100	53
	4.263	4.415	1.36	-1.56	11.3	2.67	100	49
	8.525	4.437	-1.09	-7.37	9.84	3.28	100	62
	8.525	4.390	1.42	-4.42	6.59	3.42	100	62
	17.05	4.398	2.29	2.96	2.31	5.50	100	73
	17.05	4.432	2.45	0.140	2.55	2.19	100	74
Mordant Orange 1.	4.435	4.448	2.212	0.669	5.75	3.44	100	42
	4.435	4.445	1.92	0.449	4.74	2.11	100	44
	8.871	4.468	-0.419	-4.73	2.53	1.67	100	62
	8.871	4.468	1.30	-1.07	2.93	2.40	100	66
	17.74	4.476	1.20	0.645	1.27	1.14	100	91
	17.74	4.465	1.14	-0.0845	1.19	2.02	100	90
Mordant Yellow 12.	4.426	4.459	0.805	-0.278	2.31	1.22	100	69
	4.426	4.465	-0.643	-0.642	1.53	0.737	100	66
	8.852	4.428	0.532	-0.118	0.990	1.38	100	92
	8.852	4.437	0.745	0.153	1.09	0.721	100	91
	17.70	4.448	0.460	0.282	0.469	0.289	90	88
	17.70	4.432	0.432	0.502	0.438	0.550	90	88

Table 7.9 (continued)

Fig. 7.4 and 7.5. Binding plots for Bromophenol Blue at approximately constant concentration (4.430×10^{-5} mol dm^{-3}) in the presence of competitor dyes

The competitor dye concentrations were as follows:-

1. 5-Phenylazosalicylic acid, 1.767×10^{-4} mol dm^{-3}
2. Mordant Yellow 7, 1.773×10^{-4} mol dm^{-3}
3. Mordant Yellow 10, 1.770×10^{-4} mol dm^{-3}
4. 4-azobenzenesulphonic acid 2.002×10^{-4} mol dm^{-3}
5. 4-hydroxyazobeneze-4'-sulphonic acid, 1.767×10^{-4} mol dm^{-3}
6. Alizarin Yellow GG, 1.705×10^{-4} mol dm^{-3}
7. Mordant Orange 1, 1.774×10^{-4} mol dm^{-3}
8. Mordant Yellow 12, 1.770×10^{-4} mol dm^{-3}

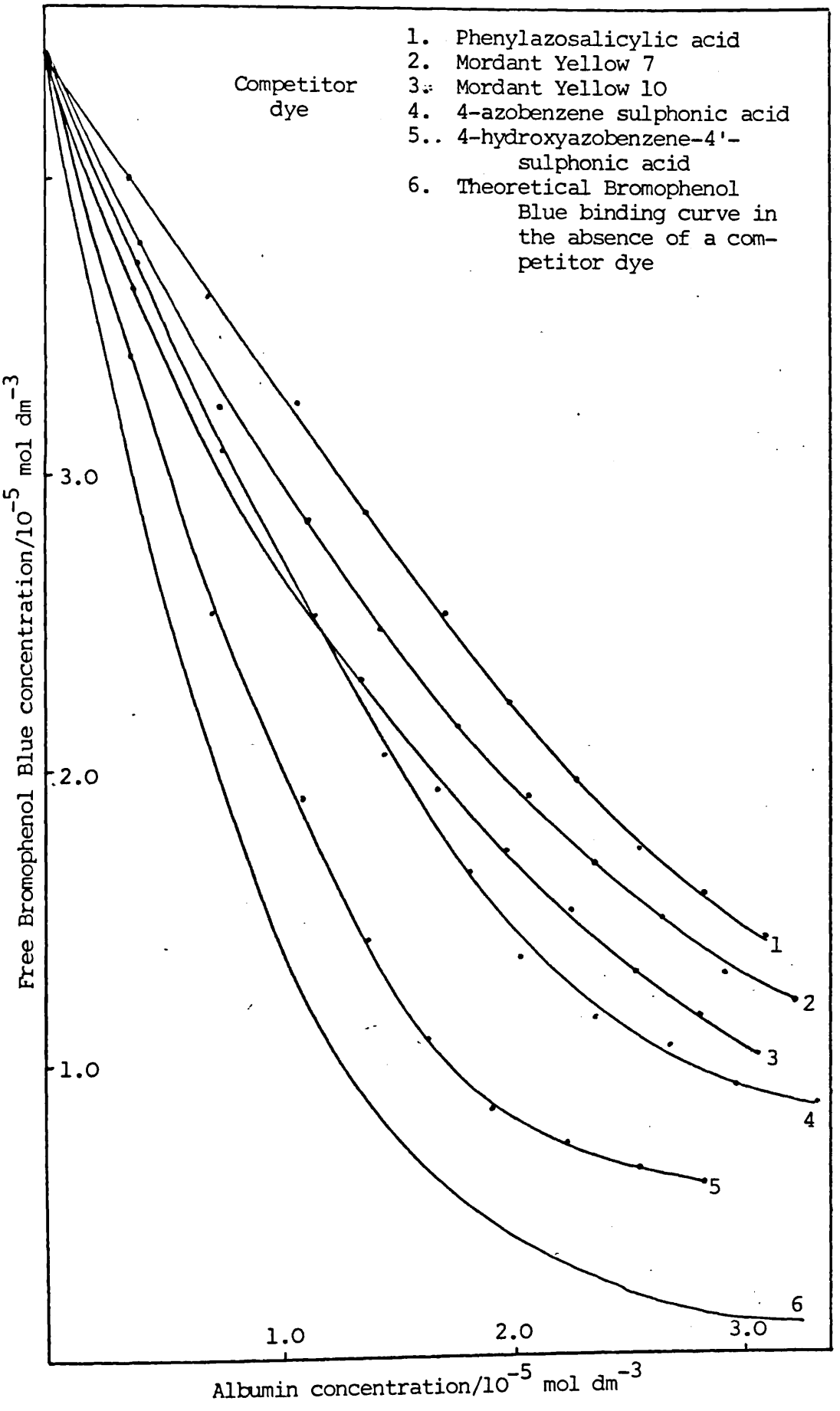


Fig. 7.4. The effect of Competitor dyes on the binding of Bromophenol Blue to H.S.A.

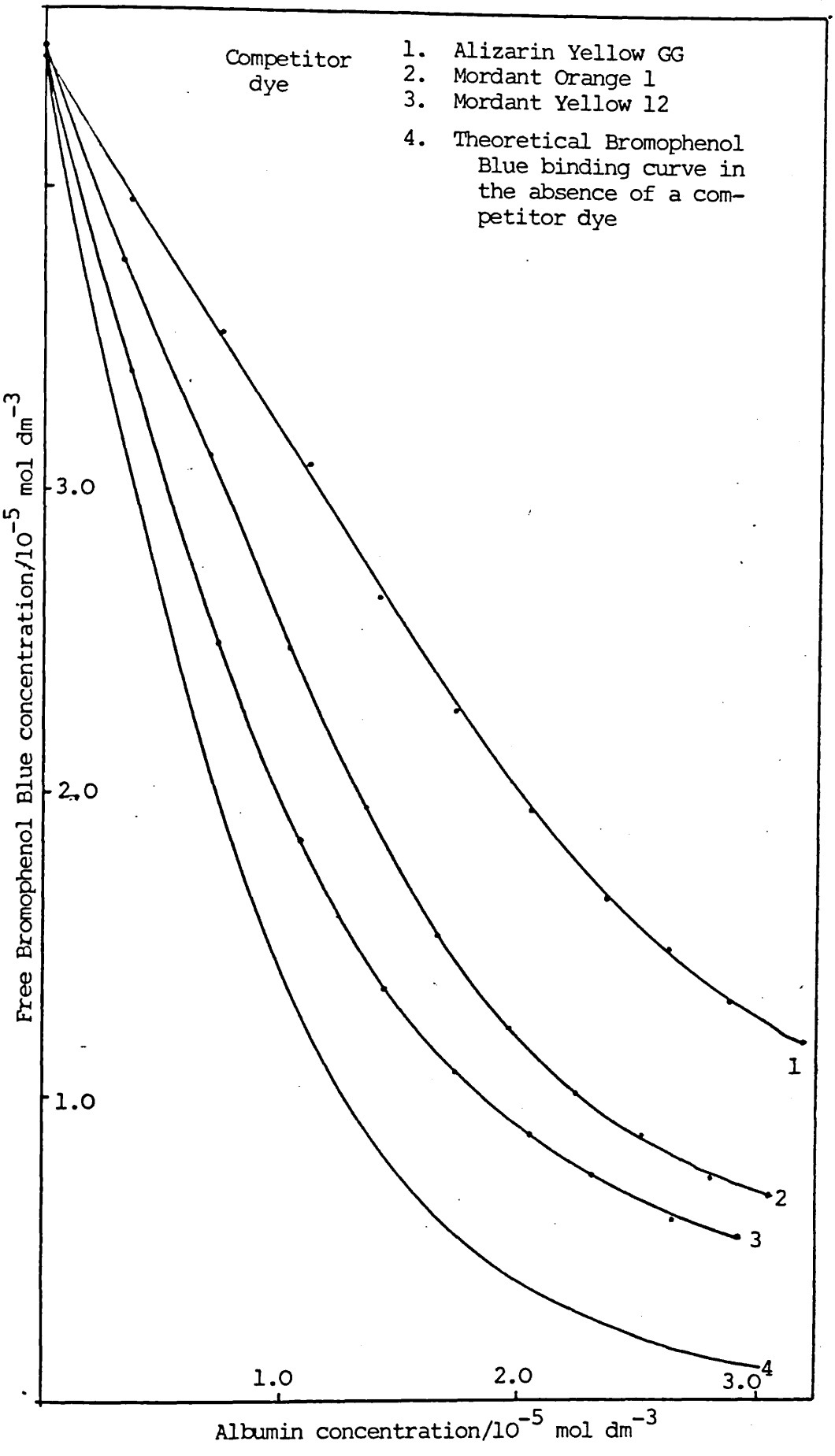


Fig. 7.5 The effect of Competitor dyes on the binding of Bromophenol Blue to H.S.A.

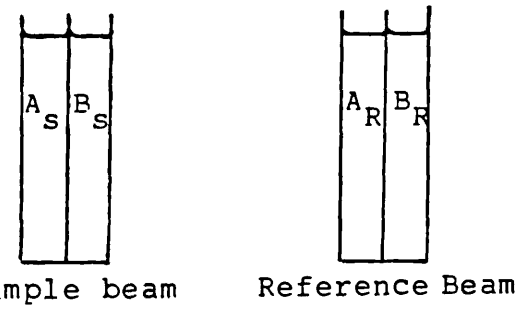
CHAPTER 8

DISCUSSION.

8. 1. Preliminary Spectrophotometric experiments

Spectrophotometry appeared to be a very suitable technique for studying ligand-protein interactions, provided that changes occur in the spectrum of either the ligand or the protein. Some preliminary experiments were, therefore, conducted, which eventually led up to the technique described in Chapter 4, for studying the interactions of M.T.T. with H.S.A. - fraction V.

Initially the protein used was plasma, separated by centrifugation from whole human blood. Various drug-plasma solutions were studied, the absorbance of the drug being measured at a single wavelength. Inconsistent results led to the substitution of H.S.A. fraction V for the plasma. In the most recent experiments this has been substituted with crystallised lyophilised H.S.A. It was also found that spectral changes at wavelengths, shorter than 300 nm were inconsistent. This is thought to be due to complex conformational effects taking place in the protein when it binds to ligands. Some early experiments were conducted, so that difference spectra were measured, using a tandem cell technique. This technique was performed in the following way:-



Tandem quartz cells.

After performing the usual baseline correction for the two cells a drug-protein solution was placed in both A_S and B_S compartments of the 'tandem' cells. A_R was filled with a

protein solution of twice the concentration of the protein in the sample compartments A_S and B_S and B_R was filled with a drug solution of twice the concentration of the drug in A_S and B_S. A difference spectrum over a wavelength range was then recorded. A titration was then performed in a similar way to that described in method 1.

The method has no inherent advantage over techniques measuring the whole U.V/visible spectrum of each of the drug-protein solutions. In fact, there must be a greater uncertainly in the reproducibility of the experiment since for any series of experiments, four titrations must be simultaneously performed rather than two in the conventional method. Furthermore, visualisation of the spectral changes involved is more difficult from difference spectra than from whole spectra. For this reason the technique was abandoned in favour of measurement of the whole absorption band.

Some first and second derivative spectra of the drug-protein solutions were also measured. The spectra should show more clearly than normal spectra the existence of new bands emerging as shoulders on the absorption bands of the free drug spectrum. However, for quantitative work amplitudes of first and second derivitive spectra at a particular wavelength must be measured from the chart paper rather than from a digital read out. This leads to larger inaccuracies, than in the normal mode, and so derivitive spectrophotometry was not used further.

8.2. M.T.T.-H.S.A. interactions

M.T.T. shows a very clear change in spectrum on addition of H.S.A. fraction V. A new band appearing with λmax at 295nm. Of all the compounds studied this is the only example in which a new side band appears, rather than a smooth progression of the spectrum towards a different shape. Since the treatment of the experimental data given in Chapter 4 failed to give consistent values for the equilibrium constants and number of binding sites, the data should possibly be treated as a two binding site case. In this case the band at 295nm is that of the primary bound species, and the secondary bound species has a spectrum similar in position and shape to that of the free drug. Even so, it is doubtful whether spectral changes at these wavelengths can be treated meaningfully when albumin spectral changes are largely contributing to the spectral alteration. In this case the absorption bands of the protein and of the ligand overlap.

8.3. Sulindac - H.S.A. interactions

U.V/visible spectra of Sulindac show a well defined isosbestic point when a solution of a constant concentration of the drug is titrated with H.S.A. This probably indicates the presence of one class of binding sites. The data may be treated to give the number of a single class of binding sites per protein molecule and an equilibrium constant. Even so, the fact that this treatment is successful does not exclude the possibility of there being more than one class of binding sites. In the two independent sites model, the spectrum of the secondary complex may be almost identical with that of the free drug. Although

Matrix rank analysis shows the existence of two drug species, this does not exclude the possibility of there being more species, since there may be two or more spectrophotometrically indistinguishable species present.

8.4. Matrix rank analysis

Upon the addition of H.S.A. the spectra of the majority of compounds studied changed in ways not consistent with there being a single class of binding site on the albumin molecule. Matrix rank analysis was introduced as a means of estimating the numbers of species in solution.

The technique should not be subject to many experimental errors due to the fact that the accuracy of the method depends only on the purity of the compounds used and the accuracy to which absorbances can be measured.

8.4.1. Warfarin

Matrix rank analysis of Warfarin-H.S.A. interactions seems to show the definite existence of a single class of binding sites and the possible existence of a second.

The literature contains many references to Warfarin-H.S.A. interactions. Much of the more recent literature,[94, 95, 122, 123] suggests that Warfarin binds to several different sets of sites, although there is a strong implication[95,124], that this may be via a cooperative rather than an independent site mechanism (see section 8.5.4.).

8.4.2. Sulindac

As already pointed out, Sulindac spectra indicate that there are only two spectrophotometrically distinguishable species in solution. However, even at very high Sulindac concentrations competition studies between Sulindac

and Bromophenol Blue still indicate three Bromophenol Blue species in solution.

If Sulindac has only one class of binding sites, which is also one of the classes of Bromophenol Blue binding sites, then at high concentration of Sulindac, this class of sites should be effectively unavailable to Bromophenol Blue. The Bromophenol Blue-H.S.A. system should, under such conditions, appear to be a one class of binding sites system. Since it appears that under such conditions binding of Bromophenol Blue to both classes of site is impared, there may also be more than one class of Sulindac binding site.

8.4.3. Methyl Orange

Matrix rank analysis of the spectra of Methyl Orange-H.S.A. spectra, in common with most of the following azobenzene derivatives shows the probable existence of two types of binding site. Analysis of the spectra at high albumin concentrations, at which the free dye concentration is likely to be very low, show the existence of only two species, which is consistent with this model. It is highly significant that the spectral changes on addition of bovine serum albumin differs from those with H.S.A., and suggests that the primary B.S.A. site is rather different from that in H.S.A.

8.4.4. Azobenzene derivatives

The spectra of 5-phenylazosalicylic acid, 4-azobenzene sulphonic acid, and 4-hydroxyazobenzene-4'-sulphonic acid, only indicate the existence of two distinguishable species. There may only be one bound species, although it seems more likely that two or more species have rather similar

spectra as shown in Chap. 5. Matrix rank analysis of the other azo dyes are consistent with there being two binding sites on the albumin molecule.

8.5. The spectrophotometric data fitting technique

It is important to realise that there are a number of equally valid models which may be applicable to a set of data. The following section describes some of the options available.

8.5.1. Non-specific binding

An option which is usually ignored in discussions of this nature is that in which the ligand does not bind to any specific site. Alternatively, there may be a large number of sites, but they are each only available when the albumin molecule is in a particular conformation. Since the albumin molecule exists partly as an αHelix and partly as a random coil, the very large number of conformations available would make binding of this nature effectively non-specific.

8.5.2. Specific independent site binding

Single class of binding sites:-

A frequently used model[125-127] for obtaining binding constants and binding site numbers is that of the protein having a single class of binding sites. From this study it would appear that single class independent site systems are rare; the only case here in which this model could be applied being that of Sulindac with H.S.A. Although matrix rank analysis of the spectra of 5-phenylazosalicylic acid, 4-azobenzenesulphonic acid and 4-hydroxyazobenzene-4'-sulphonic acid with H.S.A. can only differentiate two species in solution which may indicate single class site binding.

Since many studies in which the single class site model is used interpet data via the Scatchard plot (see p 4 5) it should be observed that Scatchard plots for a two inde-

pendent class site system can give apparently linear plots, e.g. the theoretical plots given in Fig. 5.13 (p159) for all the dyes except for Mordant Yellow 10, Mordant Yellow 7 and 4-hydroxyazobenzene-4'-sulphonic acid could easily be interpreted as straight lines, especially if they were plotted through data showing the degree of scatter common to multi-phase methods for the measurement of drug-blood protein binding.

8.5.3. Specific multipule independent site binding

In these cases there are two or more classes of independent sites. If there are j classes of site, then the equilibrium constant for each class of site is given by

$$K_j = \frac{[S_jD]}{[S_j][D]} \qquad (8.1)$$

for the reaction $S_j + D \rightleftharpoons S_jD$

where $[S_j]$ is the concentration of the unoccupied jth class of sites, $[D]$ is the concentration of the ligand and $[S_jD]$ is the concentration of the ligand-site complex.

The mole ratio of bound ligand per mole of total protein (r) is given by:-

$$r = \sum_{1}^{n} r_j = \sum_{1}^{n} \frac{K_j[D]}{1 + K_j[D]} \qquad (8.2)$$

where n is the number of classes of binding sites on each protein molecule. In some instances it is more convenient to consider the system from the macromolecular point of view.[128] In this case the sequential addition of ligand to the protein (P) is considered. The equilibrim constants for the sequential binding are K_i^s.

Thus $P + D \rightleftharpoons PD_1$ $\qquad K_1^S = \dfrac{[PD_1]}{[P][D]}$

$PD_1 + D \rightleftharpoons PD_2$ $\qquad K_2^S = \dfrac{[PD_2]}{[PD_1][D]}$

$PD_{(i-1)} + D \rightleftharpoons PD_i$ $\qquad K_i^S = \dfrac{[PD_i]}{[PD_{(i-1)}][D]}$

in which case

$$r = \frac{K_1^S[D] + K_1^S K_2^S [D]^2 \ .....}{1 + K_1^S[D] + K_1^S K_2^S [D]^2 \ .....}$$

and $K_1^S = K_1 + K_2 + \ldots K_2 = \sum_{J_1=1}^{n} K_{j1}$

$$K_1^S K_2^S = K_1 K_2 + K_1 K_3 + ... + K_1 K_n + K_2 K_3 + K_2 K_4 + ... + K_2 K_2$$

$$= \sum_{J_1=1}^{n-1} \sum_{J_2=j_1+1}^{n} K_{j1} K_{j2}$$

$$K_1^S K_2^S ..... K_I^S = \sum_{J_1=1}^{n-i+1} \sum_{J_2=J_1+1}^{n-i+2} ..... \cdot \sum_{J_1=J_{i-1}+1}^{n} K_{j1} K_{j2} .....K_{ji}$$

8.5.4. Cooperative site binding.

Several authors,[40,129,130] have suggested that ligand macromolecular interactions may take place cooperatively. Parsons and Vallner[131] have given a thorough treatment of cooperativity.

Cooperative binding of a ligand refers to the situation where occupation of a binding site on a macromolecule by a ligand affects in some way the subsequent binding of the ligand to other sites on the macromolecule. The cooperativity is positive when subsequent binding of the ligand by the macromolecule is facilitated, and negative when it is impaired.

Positive and negative cooperativity are often thought to arise as a result of a conformational change in the macromolecule when bound to the ligand. This mechanism is referred to as allosterism.

Cooperativity may arise as a result of very small displacements of the peptide chains, or perhaps merely from strains in the structure of the macromolecule.

Both positive and negative cooperativity may also be caused by electrostatic interactions between bound ligand molecules.

Positive cooperativity may result from the creation of new binding sites on the macromolecule when the ligand binds to a pre-existing site. Conversely, negative cooperativity may arise from the destruction of some pre-existing sites caused by the binding of ligand to another site. The sites could be destroyed as a result of steric interference between bound ligand molecules or as a result of a conformational change in the macromolecule, or again by electrostatic effects.

Cooperative binding involving conformational changes is likely to introduce a large entropy term to the equation for the free energy of the reaction, and there must, therefore, be a correspondingly large, negative, enthalpy term.

In their three related papers, Parsons and Vallner [131] consider the cases of one site creators of binding sites, two site creators of sites, and destruction of pre-existing binding sites, and positive and negative site site cooperativity. In every case they show how Scatchard plots can be created with convex, concave, or linear shapes depending

on the relative magnitudes of the equilibrium constants at the various sites.

8.5.5. Choice of model used

Throughout this work all data, with the exception of Sulindac-H.S.A. interactions, have been interpreted assuming a two independent site model. The n and K values obtained give concave theoretical Scatchard plots. These plots, however, could also be consistent with a model using three or more independent sites, or with a cooperative model.

The single largest error factor in the titration of the azo-dyes with albumin was in the accuracy of the micro-pipette with which albumin could be added to the cell, introducing an error factor of $\pm$ 0.5%.

The average error between the measured and calculated values of J absorbance values for a parameter fit is given by

$$f = 100 \times \sum_{1}^{N} \frac{(A_J\text{measured} - A_J\text{calculated})^2}{A_J\ \text{measured}} \Big/ N$$

If f is greater than 1% any change in the binding parameters K_1, K_2 n_1, n_2 and the extinction coefficients ε_1 and ε_2 which produce a decrease in the value of f, represents a real improvement in the parameter fit. If f falls below 1% any change in the parameters producing a decrease in f does not represent a meaningful improvement, i.e. all parameter sets producing f values of less than 1% are equally applicable to the system.

The values quoted for the binding parameters in this work are the best values, i.e. s (and hence f) having the minimum values. In no case did f exceed 1% and in many cases

did not exceed 0.5%. This means that no meaningful improvement could be made to improve the fit of measured to calculated data because the fit is better than the reproducibility of the experimental data. It also means that there are a number of parameter sets which are equally as good as those quoted.

Although using a three site model would introduce a further three parameters into the equations to be fitted, which would undoubtedly enable a mathematical improvement in the 'goodness of fit', this would not represent a real physical improvement.

It is also not really justifiable to apply a three site model when in most cases matrix rank analysis of the spectra indicate only three spectrophotometrically distinguishable species (one being the unbound species).

Equally a cooperative model could probably be used to produce as good a fit as the independent site model, but it could not be decided solely from the spectrophotometric experiments described in Chap. 5, or the ultrafiltration experiments which is the better model.

It would appear that the model used gives good agreement between projected dye binding curves from the best fit parameters obtained from the spectrophotometric method described in Chap. 5 (fig.8.1), and those obtained experimentally by ultrafiltration (fig.8.2). It is apparent that different sets of binding parameters can yield almost identical binding curves. For example, in fig. 6.4. curves a and b are almost identical, yet the values of K_1, K_2, n_1 and n_2 are significantly different. It appears, therefore,

that the most significant thing is not the individual numbers, but the values that they give when combined together in an equation such as (4.17).

$$K_1K_2D_f^3 + (K_1+K_2 - D_{tot}\,K_1K_2 + BK_1K_2 + C\,K_1K_2)D_f^2 + (1 - D_{tot}\,K_1 - D_{tot}\,K_2 + K_1\,B + K_2\,C)D_f - D_{tot} = 0 \qquad (4.17)$$

The binding curves produced by equation (4.17) for the eight azo-dyes are summarised in Figs. 8.1 and compared with corresponding ultrafiltration curves in Fig.8.2, the binding parameters used being in Table 8.1.

Comparison of binding constants obtained from the spectrophotometric and ultrafiltration techniques.

Compound	Spectrophotometric				Ultrafiltration			
	K_1 /10^5 dm^3 mol^{-1}	K_2 /10^4 dm^3 mol^{-1}	n_1	n_2	K_1 /10^5 dm^3 mol^{-1}	K_2 /10^4 dm^3 mol^{-1}	n_1	n_2
4-azobenzenesulphonic acid	3.65	0.929	1.14	4.61	4.79	2.37	1.73	6.48
Mordant Yellow 10	7.82	3.90	2.93	2.68	6.76	2.08	3.25	3.29
4-hydroxyazobenzene 4'sulphonic acid	2.33	1.65	2.17	4.18	2.96	7.58	2.37	4.43
Mordant Yellow 7	2.00	1.06	3.41	6.87	1.91	1.49	3.28	4.52
5-phenylazosalicylic acid	3.81	4.08	1.31	9.39	4.57	3.24	1.75	11.66
Mordant Orange 1	2.52	1.15	2.28	14.62	1.14	2.31	3.08	16.67
Alizarin Yellow GG	3.71	2.18	5.46	10.90	5.98	0.766	4.18	14.89
Mordant Yellow 12	1.60	1.53	4.31	17.70	3.78	1.04	4.47	13.39

Table 8.1. Table of binding parameters for the series of azobenzene derivatives

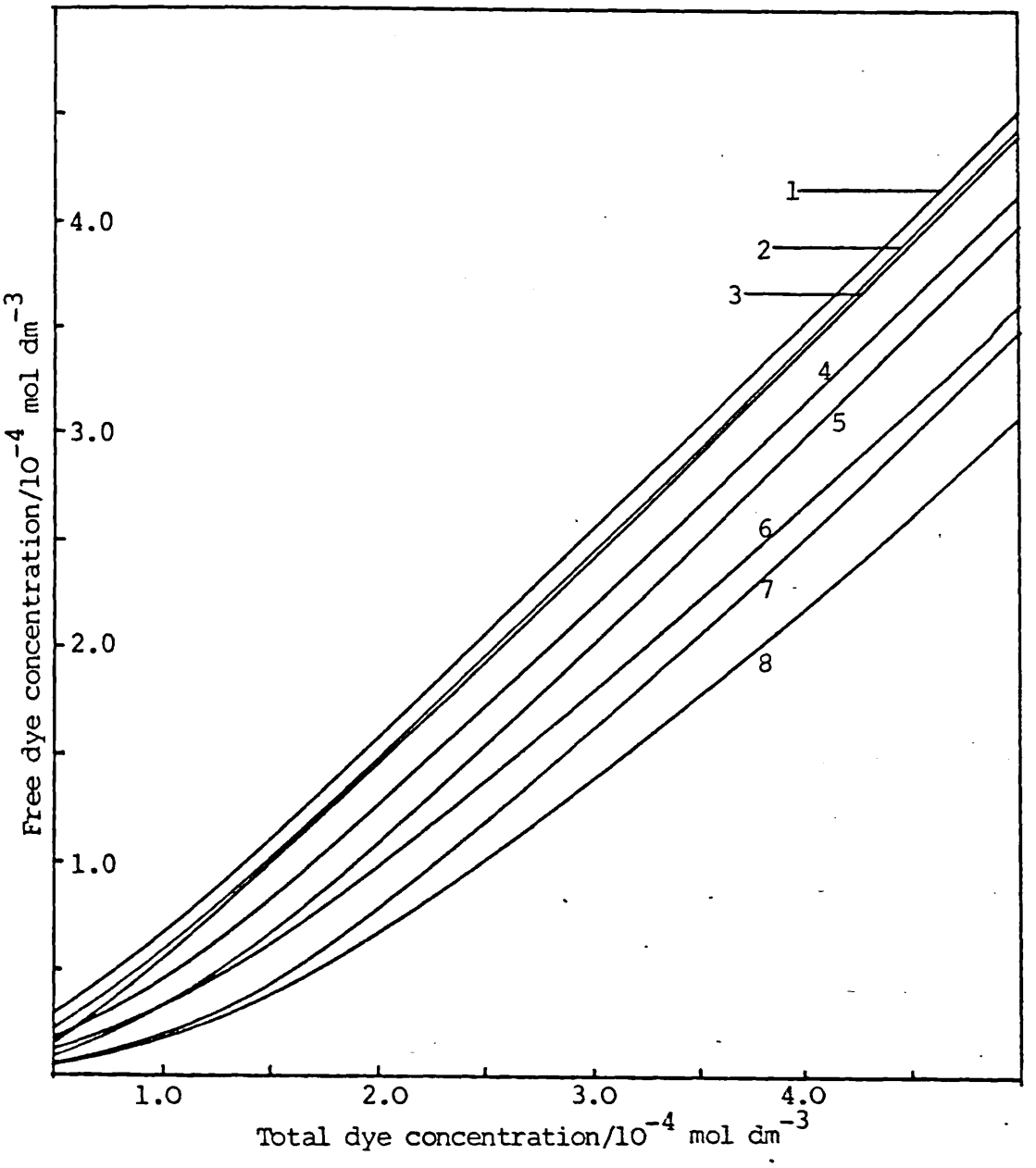


Fig. 8.1. Theoretical binding extent plots for azo dyes with human serum albumin (1.000 x 10^{-5} mol dm^{-3}) (U.V./visible data fitting method)

1. 4-azobenzene sulphonic acid
2. Mordant Yellow 10
3. 4-hydroxyazobenze-4'-sulphonic acid
4. Mordant Yellow 7.
5. 5-phenylazosalicylic acid
6. Mordant Orange 1
7. Alizarin Yellow GG
8. Mordant Yellow 12.

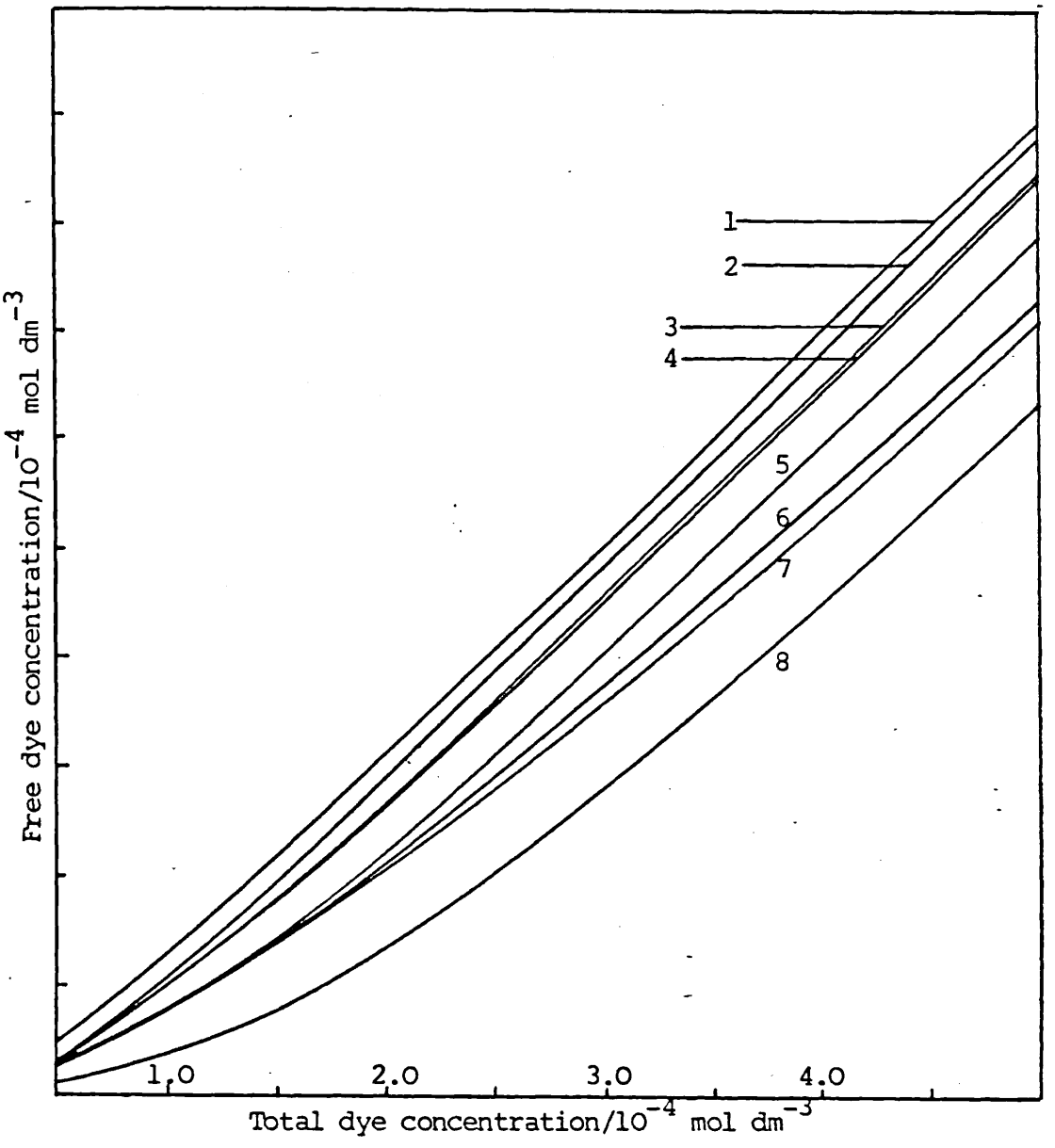


Fig. 8.2. Theoretical binding extent plots for azo dyes with human serum albumin (1.000×10^{-5} mol dm^{-3}) (Ultrafiltration method)

1. 4-hydroxyazobenzene-4'-sulphonic acid
2. Mordant Yellow 10
3. 4-azobenzene sulphonic acid
4. Mordant Yellow 7
5. 5-phenylazosalicylic acid
6. Alizarin Yellow GG
7. Mordant Yellow 12
8. Mordant Orange 1.

8.6. Correlation between structure and binding characteristics

In order to establish any correlation between structural features of molecules and the degree to which they bind to H.S.A. it is useful to rank the various binding parameters for the compounds used. (Table 8.2)

In terms of the total binding of the compounds to H.S.A. (fig. 8.1 and 8.2), the two methods correlate well. The three weakest binders, 4-azobenezenesulphonic acid, Mordant Yellow 10 and 4-hydroxyazobenzensulphonic acid are all acids containing a sulphonate group. As such they are likely to be less hydrophobic than members of the group containing only a carboxylate group as the base function. In both series of experiments Mordant Yellow 7 is next in terms of total binding. The introduction of the methyl group to the salicylate ring is likely to increase hydrophobicity although the strength of binding may be offset to some extent by steric hindrance. The trend of increasing binding with increasing hydrophobicity continues with 5-phenylazosalicylic acid. Introduction of a nitro or amino-group to the phenyl ring increases the extent of binding further, and must be attributable to a specific interaction between these groups and a receptor site on the albumin molecule. It is not certain whether the individual values K_1 K_2 n_1 and n_2 have any physical significance. The degree to which the azo compounds are bound seems to be dictated largely by the value of n_2, and there is good correlation between the ranking orders of total binding and the value of n_2 for each series of experiments. (Table 8.2) There is also good agreement between the ranking orders of n_2 for the two series of experiments.

The ranked sequences of n_1, for the two series of experiments, agree even more closely, although it is hard to correlate the sequence with structural features of the molecules. There is poor agreement between the two series of experiments for K_1 and K_2 values and it must be concluded that although these values have similar orders of magnitude they are probably only of mathematical significance.

	Total Binding	K_1	K_2	n_1	n_2
Spectrophotometric method increasing values ↓	4-ABSA	MY12	4-ABSA	4-ABSA	MY10
	MY10	MY7	MY7	5-PASA	4-OHABSA
	4-OHABSA	4-OHABSA	MO1	4-OHABSA	4-ABSA
	MY7	MO1	MY12	MO1	MY7
	5-PASA	4-ABSA	4-OHABSA	MY10	5-PASA
	MO1	AYGG	AYGG	MY7	AYGG
	AYGG	5-PASA	MY10	MY12	MO1
	MY12	MY10	5-PASA	AYGG	MY12
Ultrafiltration method increasing values ↓	4-OHABSA	MO1	AYGG	4-ABSA	MY10
	MY10	MY7	MY12	5-PASA	4-OHABSA
	4-ABSA	4-OHABSA	MY7	4-OHABSA	MY7
	MY7	MY12	MY10	MO1	4-ABSA
	5-PASA	5-PASA	MO1	MY10	5-PASA
	AYGG	4-ABSA	4-ABSA	MY7	MY12
	MY12	AYGG	5-PASA	AYGG	AYGG
	MO1	MY10	4-OHABSA	MY12	MO1

Table 8.2. Ranked binding data for the two series of experiments

Abbreviations:-

4-ABSA	= 4 azobenzenesulphonic acid
4-OHABSA	= 4-hydroxyazobenzenesulphonic acid
5-PASA	= 5-phenylazosalicylic acid
AYGG	= Alizarin Yellow GG
MY10	= Mordant Yellow 10
MY7	= Mordant Yellow 7
MO1	= Mordant Orange 1
My12	= Mordant Yellow 12

8.7. Comparison of Spectrophotometric and Ultrafiltration methods for studying dye-protein interactions

8.7.1. Spectrophotometry

The most striking advantage of this technique is the reproducibility which can be obtained. For any particular dye-albumin mixture, titrations of dye with albumin give values of absorbance which agree to within 1%. The technique has the advantage of having only one phase, thereby eliminating the problems of dye membrane association encountered with multiphase methods such as dialysis and ultrafiltration. The method requires only small quantities of reagents, and, since determination is rapid, problems of reagent deterioration are removed.

The method is applicable to ligands with appreciable absorbances at wavelengths longer than 300nm. (At shorter wavelengths albumin absorbs radiation strongly, changes in the protein spectra are more complex than those of the dye, possibly as a result of configurational changes).

Assuming that the two independent site model is a reasonable approximation to the real system, the technique has the advantage that changes in the concentration of all three dye components are being observed at the same time. With dialysis or ultrafiltration the concentration of only one component is measured. Under certain conditions the ratio of the two bound species (which depends on the relative binding constants of the two species) can vary by large amounts whilst the free dye concentration remains fairly constant. It is essential that the spectrophotometer used for the determinations has good precision and reproducibility in both absorbance and wavelength readings.

(The Perkin Elmer 555 used in this study specifies a precision of ± 0.2nm in the wavelength reading and ± 0.002A at 1A in the ordinate reading).

There is also a certain amount of qualitative information which can be drawn from the spectral changes both in terms of amplitude and direction of shift in wavelength of the spectra.

The technique does suffer from the drawback that only a limited range of dye-albumin concentrations can be studied. It also has the necessary prerequisite that the ligand studied must show changes in their U.V/visible spectra when added to albumin or some other protein in question.

8.7.2. Ultrafiltration

The main advantage of the technique is that free dye concentrations are measured directly, and not via an intermediate theoretical step.

The technique is fairly rapid and compares favourably in this respect with the spectrophotometric method. Theoretically the method is simple, provided that no account has to be made of dye rejection. The technique is applicable to any dye-protein system provided that there are no serious membrane binding or rejection problems. Any dye-protein ratio can be studied provided that the effluent dye concentration is large enough to be measured. This is a considerable advantage over the spectrophotometric method in which the concentrations which can be used are rather limited.

As well as the problems of rejection and membrane

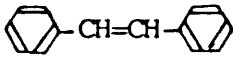
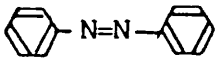
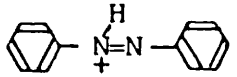
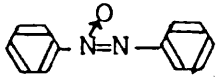
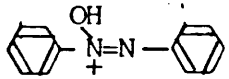
binding, the technique requires rather large quantities of reagents. It does not provide any qualitative information. Experimental data points exhibit a rather large degree of scatter ($\pm$ 3.5% at high dye concentrations and as high as $\pm$ 18% at low dye concentrations).

8.8. The spectrum of azobenzene

Isoelectronic molecules in many instances have similar electronic spectra. Azobenzene and stilbene are isoelectronic and they, as well as other azobenzene derivatives, exhibit very similar electronic spectra. They consist of a very intense band at relatively long wavelength, varying from about 300nm in stilbene to about 420nm in the conjugate acid of azobenezene. A second band occurs with lower intensity at about 230nm in all compounds. In addition, some of the compounds have an additional low-intensity band at longer wavelength. Jaffé and Orchin[132] assign the high intensity long wavelength band to the $^1B - {}^1A$ transition, since this transition should occur at the longest wavelength and its great intensity identifies it as a V - N transition. The shorter wavelength transition is insensitive to the nature of the bridge bonding the phenyl groups, provided that the conjugation of the system is not significantly altered. This indicates that either both upper and lower energy levels are equally displaced, or that neither is displaced at all by modification of the bridge. Making reasonable assumptions for Coulomb and resonance integrals for the various bridge atoms it can be shown[133] that the energy levels of the two occupied symmetric orbitals (ν orbitals) and the corresponding unoccupied ones (ω orbitals) are unaffected by the bridging group. Accordingly Jaffe and Orchin[134] assign the short wavelength band to a $^1H - {}^1A$ transition. The spectral characteristics of some analogues of azobenzene are reproduced in Table 8.3.

Table 8.3. The spectra of azobenzene and analogues.

The wavelength of absorption (λ) and the extinction coefficient (ε) of azobenzene and its analogues

Transition										
	λ /nm	ε /1000 mol^{-1}m^2	λ /nm	ε /1000 mol^{-1}m^2	λ /nm	ε /1000 mol^{-1}m^2	λ /nm	ε /1000 mol^{-1}m^2	λ /nm	ε /1000 mol^{-1}m^2
$^1W \leftarrow {}^1A$			420	0.076	300	0.30				
$^1B \leftarrow {}^1A$	297	2.70	314	1.92	418	2.69	322	1.54	383	1.45
$^1G \leftarrow {}^1A$	~275		281	0.55	382	1.48	282	0.50	377	0.20
$^1G \leftarrow {}^1A$	~260		258	0.24	352	0.48	260	0.78	298	0.46
$^1H \leftarrow {}^1A$	226	1.50	236	0.74	236	0.80	238	0.82	~232	0.29
$^1H \leftarrow {}^1A$ or $^1C \leftarrow {}^1A$			223	0.98	~220	~0.5	231	0.94		

8.8.1. Solvent effects

The strongest U.V. band in azo compounds undergoes a shift towards the blue upon passing from a polar to a non-polar solvent. Thus azobenzene[135] has a peak maximum at 342nm in ethanol, but at 338.5nm in hexane. Correspondingly there is a small increase in the value of the extinction coefficient at the wavelength of maximum absorbance on passing from ethanol to hexane.

8.9. Spectral changes on binding of Azo-dyes to H.S.A.

When the series of azobenzene derivatives in this study bind to H.S.A., the long wavelength band corresponding to the V - N transition undergoes changes which have a number of features in common which may be summarised as follows:-

All the spectra exhibit a shift to longer wavelength on addition of H.S.A.

From the calculated spectra which used the two independent site model, two further observations can be made.

(a) In almost all cases the spectrum of the more strongly bound species moves towards the red by about 10-30nm. The value of the extinction coefficient at λmax for the more strongly bound species being roughly the same as for the free species.

(b) In most cases the spectrum of the more weakly bound species moves towards the red by only a small amount (less than 10nm). The value of the extinction coefficient at λmax being smaller than that of either the free dye or the strongly bound species.

Even if the two independent site model is incorrect any equilibrium model used to explain the spectral changes

will show that the more weakly bound dye species shows a decrease in the extinction coefficient at λmax and a small red shift, whilst the more strongly bound species have larger extinction coefficients at λmax and show a larger red shift.

The question that immediately arises is whether, as Klotz suggested (Ch.1 p 41), 'optical displacement is a measure of degree of binding'. The red shift in the spectra upon saturation of the dyes with H.S.A. are summarised in Table 8.4.

Compound	Red shift/nm
4-ABSA	6
4-OHABSA	6
5-PASA	8
MY7	9
MY10	11
AYGG	12
MY12	14
MO1	22

Table 8.4. Summary of red shifts in the azo-dye spectra.

Although these red shifts are only approximate, it is apparent that the three strongly binding dyes also show relatively large red shifts. In terms of the resolved spectra from the two site model, there is hardly any correlation between the degree of binding and red shifts of the spectra as shown in Table 8.5.

Compound	Red shift in ε_1 /nm	Red shift in ε_2 /nm
4-ABSA	10	5
4-OHABSA	10	5
5-PSSA	15	<5
MY7	20	<5
MY10	15	<5
AYGG	15	<5
MY12	15	5
MO1	30	15

Table 8.5.

It would appear, therefore, that the largest displacements do occur in the strongly bound dyes, it is not possible to predict the order of binding strength solely by measuring the red shift in the observed spectra.

8.10. The nature of dye-H.S.A. interactions

It has been suggested by some workers[136] that H.S.A. preferentially binds anionic drugs by some form of electrostatic interaction. From the work described here it seems most unlikely that ionic interactions are the chief mechanism by which azo dyes are bound to H.S.A. If this were the case then the diacids such as Mordant Yellow 10 and Mordant Yellow 7 (for which both carbonyl and sulphonate groups are totally dissociated at pH 7.4) would be expected to be the most strongly bound dye, whereas, in fact, Mordant Yellow 10 is one of the most weakly bound dyes. Furthermore, in terms of electrostatic attraction, both of these dyes should bind equally, if there were any difference in binding, Mordant Yellow 7 would be expected to be the weaker due to steric hindrance by the methyl group in the salicylate ring. In fact, Mordant Yellow 7 is more strongly bound than Mordant Yellow 10.

Hydrophobicity appears to be an important factor in the degree to which dyes are bound by H.S.A. It might be suggested that since all the dyes used are hydrophobic, binding of the dyes to the protein might be comparable to the solvent distribution that would occur if an organic solvent were introduced to an aqueous solution of the dye, i.e. the dye will distribute between the organic environment of the protein and the aqueous solution in much the same way as it would distribute between aqueous and organic phases.

Hydrophobic effects have been suggested by many workers to be the major contributing factor in the interaction of proteins with most neutral drugs. The term "hydrophobic bonding" may, in fact, be a misleading term.[137, 138] It seems likely that bonding of this type is entropically driven, largely as a result of the disruption of the quazi-crystalline water structure around the non-polar groups in aqueous solution.[139]

Hansch et al.[140] have found excellent correlation between binding of various compounds to bovine serum albumin and the partition coefficients of the compounds between octanol and water.

Scholtan[141] has shown that for some sulphonamides, antibiotics, cardenolides and steroid hormones, series of these compounds show increased binding with the increasing hydrophobic character of their members. Similarly series of penicillins[142], p-hydroxybenzoic acid esters[143], and homologous series of fatty acids[144], sulphates, sulphonates and alcohols[145, 146], hydrocarbons[147], aromatic compounds

and alkanes[148], have all been shown to bind more strongly as the hydrophobicity of their members is increased.

However, binding of the azo-dyes is not entirely analagous with partitioning of the dyes between water and an organic solvent. On binding, the dye spectra undergo a red shift, rather than the expected blue shift associated with a change to a less polar environment. There is also a decrease, rather than an increase, in the value of their extinction coefficient at λ max. Furthermore, spectral shifts associated with solvent changes are small (rarely more than 5nm), which suggests that binding between the dye molecules and H.S.A. involves forces other than those of the solvent-partitioning type of effect. The spectral changes of the azobenzene analogues in Table 8.3 may yield a clue to the binding forces involved.

Azobenzenes conjugate acid shows a strong red shift of 104nm for its $^1B \leftarrow {}^1A$ U.V. band, and a corresponding increase in the extinction coefficient at λmax. Azoxybenzene and its conjugate acid also exhibit red shifts in comparison with azobenzene although in these cases there is a decrease in their extinction coefficients at λmax. It would appear, therefore, that the spectral changes in this work are consistent with the dye azo-group donating electrons to an electrophilic group on the albumin molecule, and that any potential binding site should contain an electrophilic group.

Residues such as tyrosine, phenylalanine and tryptophan may all potentially form complexes by delocalisation of electrons into their π-systems.

It seems likely, therefore, in view of the apparent correlation between binding and hydrophobicity, and the probability of electron donation by the azo group, that the strongest binding sites will be those in highly apolar regions, with an electron accepting group. One such possibility is in the tryptophan region of the H.S.A. molecule.

211
Lys. Ala. Trp. Ala. Val. Ala Arg.

This region has been suggested by Reynolds et al.[146] as a likely binding site for organic molecules.

Introduction of an a amino group to the phenyl ring in Mordant Yellow 12 increases the extent of binding. This must be due to binding with further acidic sites on the albumin molecule. Mordant Orange 1 and Alizarin Yellow GG, on the other hand, containing nitro groups in their phenyl ring are likely to bind to basic regions containing groups such as arginine or lysine.

The question of the location of drug binding sites on H.S.A. has been discussed by a number of authors. Fehske et al.[149] have reviewed much of the work and concluded that H.S.A. has at least five identifiable binding sites.

1. Indole and benzodiazepine binding site

The site binds several indole derivatives and benzodiazepines, as well as L-tryptophan, diazepan, dansylsarcosine, iopanoic acid, and fluorbiprofen, it also binds many other drugs with very different structures. The site is thought to be formed, as a result of the tertiary structure of H.S.A., between several regions on the molecule. The major part of this binding site lies between residues 124-298, but parts of this site may be also located in the frag-

ment containing residues 299-585. Arginine 145, Histidine 146, and Lysine 194 are involved in this site, as also is Tyrosine 410.

2. Warfarin and Azapropazone binding site

It is thought[150, 151, 152] that this site is composed largely of Tryptophan 214, since modification of this residue reduces the binding of Warfarin and iodipamide to this site. However, modification of the residue does not affect the binding of Azapropazone, and it was concluded that, in fact, the region consists of two overlapping sites. It seems very likely that the Warfarin site binds the azo dyes studied here since this site is known to bind salicylic acid derivatives as well as Warfarin,[153] and a very large number of unrelated drugs.[123]

3. Digitoxin binding site

The site is specific for few drugs. The position and nature of the site is as yet unknown, although some tyrosine residues have been implicated.[149]

4. Bilirubin binding site

Bilirubin binds to at least two binding sites, evidence as to the exact nature and position of the site is contradictory, although it is known that bilirubin inhibits the binding of typical Warfarin site binders.

5. Fatty acid binding site

Again, the exact position of the site has not been identified, although the region around amino acid residue 422 has been suggested.[154, 155] The site is not thought to be identical with the Warfarin site, but may be one of the bilirubin sites.

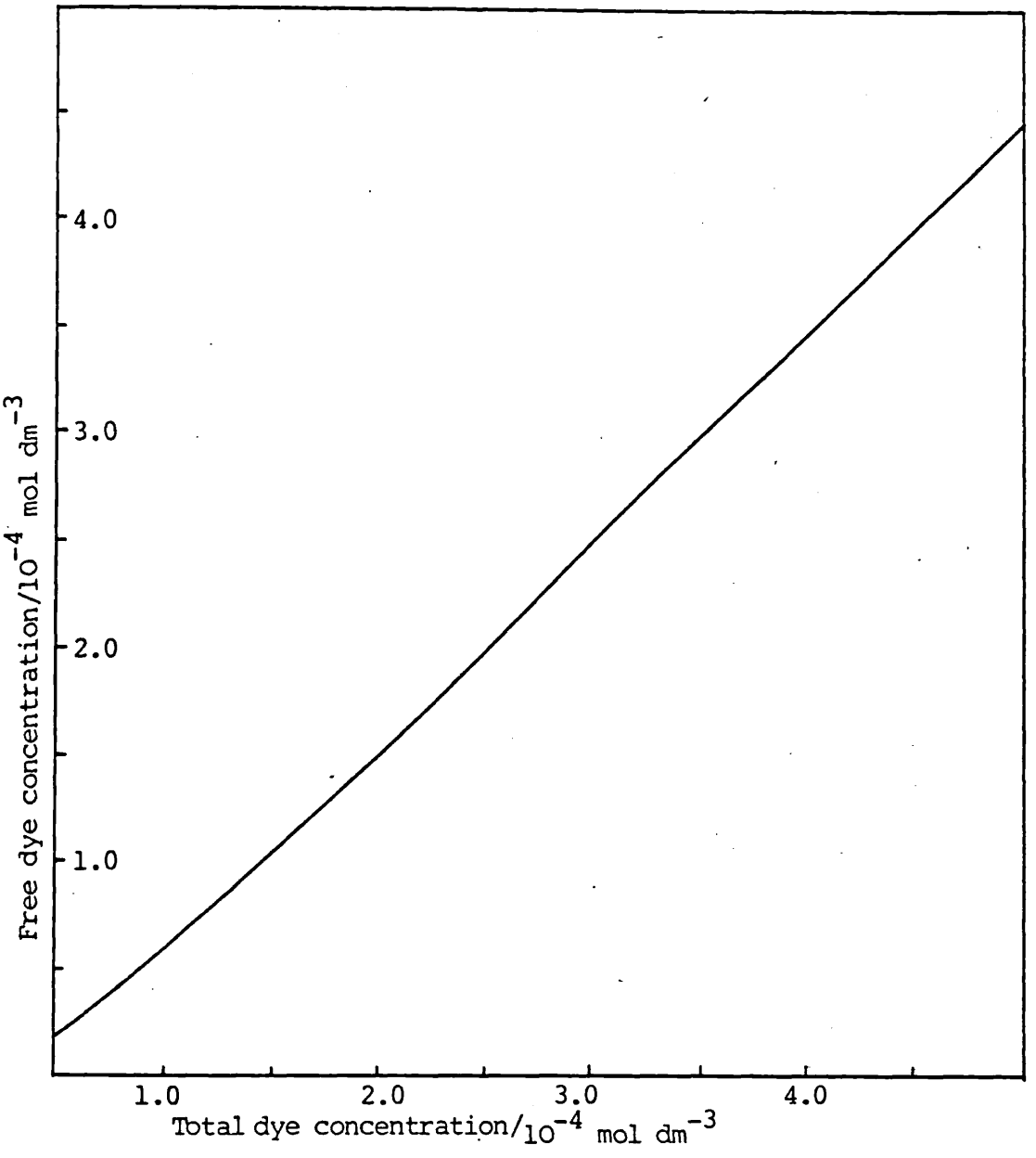


Fig.8.3. Theoretical binding extent plot for Bromophenol Blue with human serum albumin (1.000 x 10^{-5} mol dm^{-3}) (U.V/visible data fitting method).

8.11 Competition reactions

Competitive interactions between organic molecules and serum albumin have been well known for many years. Klotz[41] conducted some competition experiments between azosulphathiazole and various simple organic acids, and was able to rank them in order of strength of competition.

It has already been pointed out that binding sites may be classified according to the displacement of certain marker compounds from them.

Although semi-quantitative competition experiments are well known, the quantitative treatment of competitive systems has not been dealt with in such detail.

Meisner et al.[156] have described a study of the interaction of bovine serum albumin with the ligand-competitor pairs, (1) octanoate-chlorophenoxyisobutyrate (2) palmitate-stearate (3) stearate-chlorophenoxyisobutyrate and (4) 8-anilinonaphthalenesulphonate (A.N.S.)-chlorophenoxyisobutyrate. Using a simple two independent neutral site model such as will be described below, they were able to account for the competitive interactions of the pair (1) and (2), but not (3) and (4).

Kalbitzer and Stehlik[157] have described in some detail various mathematical models for competitive macromolecular interactions. They consider the case of a single binding class of independent sites. They also consider the case of competing ligands for cooperative as well as independent binding sites.

Although the mathematics for such systems can be developed relatively easily, except in the simplest case

of one independent site, there is usually no intrinsic solution for the binding parameters. These must, therefore, be obtained by some form of fitting procedure.

It has already been shown that even for single ligands it is usually difficult to differentiate between models, and the problem must become more acute when even more unknown binding parameters are introduced into the equations to be fitted.

8.11.1. Bromophenol Blue as a competitor

Bromophenol Blue was chosen as a competitor because it was found to undergo competition reactions with all the azobenzene derivatives studied. In addition, it has a spectrum with absorption bands at significantly higher wavelengths than the azo dyes. Therefore, the spectral changes monitored at these wavelengths are independent of the spectral changes in the competing azo dye. Furthermore these spectral changes of Bromophenol Blue, on addition of H.S.A., are large in comparison with those observed for many dyes.

Bromophenol Blue is seen to be a relatively weakly bound dye if Fig.8.3 is compared with Fig.8.1 and 8.2. As such it should be easily displaced from H.S.A. by the competing dyes.

In retrospect it might have been better to have chosen another azo dye, e.g. Evans Blue, as the standard displaced ligand, since this may have reduced the possibility of there being non-mutual binding sites on the albumin molecule.

8.11.2. Sequence of strength of competition

From Fig.7.4 and 7.5 the strength of competition at albumin concentrations of between 1.5 and 3.0 x 10^{-5} mol dm^{-3} for the dyes is in the order

MY12 4-OHABSA MO1 4-ABSA MY10 MY7 AYGG 5-PASA

(for abbreviations see above p226)

If the Mordant Yellow 12 and Mordant Orange 1 and Alizarin Yellow GG curves are disregarded, then the sequence is exactly that of the ultrafiltration experiments, except that the positions of Mordant Yellow 10 and 4-azobenzenesulphonic acid are reversed.

8.11.3. Improvements to the Model

The double independent mutual site model seems to be a very poor method for obtaining competitor dye binding constants. In no case are the values of K_1 and K_2, obtained from the competition experiments, consistent for a range of dye concentrations.

Probably the simplest improvement would be to consider that there are four classes of site present in the system. The first class is the Bromophenol Blue primary site (S_{B1}) to which the competitor dye may also bind with an equilibrium constant of K_1. The second class is a primary site (S_{D1}) to which the dye also binds, with the same affinity as to S_{B1}, but from which Bromophenol Blue is excluded. Similarly there are corresponding secondary sites. Thus the following equilibria can be written:-

$$B_f + S_{B1} \overset{K_{B1}}{\rightleftharpoons} BS_{B1} \qquad K_{B1} = \frac{[BS_{B1}]}{[B_f][S_{B1}]} \qquad (8.1)$$

$$D + S_{B1} \overset{K_1}{\rightleftharpoons} DS_{B1} \qquad K_1 = \frac{[DS_{B1}]}{[S_{B1}][D]} \qquad (8.2)$$

$$D + S_{D1} \overset{K_1}{\rightleftharpoons} DS_{D1} \qquad K_1 = \frac{[DS_{D1}]}{[S_{D1}][D]} \qquad (8.3)$$

$$B_f + S_{B2} \overset{K_{B2}}{\rightleftharpoons} BS_{B2} \qquad K_{B2} = \frac{[BS_{B2}]}{[S_{B2}][B_f]} \qquad (8.4)$$

$$D + S_{B2} \overset{K_2}{\rightleftharpoons} DS_{B2} \qquad K_2 = \frac{[DS_{B2}]}{[S_{B2}][D]} \qquad (8.5)$$

$$D + S_{D2} \overset{K_2}{\rightleftharpoons} DS_{D2} \qquad K_2 = \frac{[DS_{D2}]}{[S_{D2}][D]} \qquad (8.6)$$

The following relationships may also be written

$$[D_{tot}] = [D] + [DS_{B1}] + [DS_{B2}] + [DS_{D1}] + [DS_{D2}] \qquad (8.7)$$

$$[B_{tot}] = [B_f] + [BS_{B1}] + [BS_{B2}] \qquad (8.8)$$

$$[S_{D1tot}] = [DS_{D1}] + [S_{D1}] \qquad (8.9)$$

$$[S_{D2tot}] = [DS_{D2}] + [S_{D2}] \qquad (8.10)$$

$$[S_{B1tot}] = [S_{B1}] + [BS_{B1}] + [DS_{B1}] \qquad (8.11)$$

$$[S_{B2tot}] = [S_{B2}] + [BS_{B2}] + [DS_{B2}] \qquad (8.12)$$

where:-

$[BS_{B1}]$ = the concentration of Bromophenol Blue bound at the common primary site.

$[B_f]$ = the concentration of free Bromophenol Blue

$[S_{B1}]$ = the concentration of unbound common primary site

$[DS_{B1}]$ = the concentration of competitor bound at the common primary site

$[D]$ = the concentration of free competitor

$[BS_{B2}]$ = the concentration of Bromophenol Blue bound at the common secondary site

$[S_{B2}]$ = the concentration of unbound common secondary site

$[DS_{B2}]$ = the concentration of competitor bound at the common secondary site

$[S_{D1}]$ = the concentration of unbound, Bromophenol Blue excluded, primary site

$[S_{D2}]$ = the concentration of unbound, Bromophenol Blue excluded, secondary site

$[D_{tot}]$ = the total competitor concentration

$[B_{tot}]$ = the total Bromophenol Blue concentration

$[S_{D1tot}]$=the total Bromophenol Blue excluded, primary site concentration

$[S_{D2tot}]$=the total, Bromophenol Blue excluded, secondary site concentration

$[S_{B2tot}]$=the total common secondary site concentration

If n_{B1}, n_{B2}, n_{D1} and n_{D2} are numbers relating the correspondingly subscripted total dye concentration to the total protein concentration ([P]) then:-

$$[S_{D1tot}] = n_{D1}[P]$$

$$[S_{D2tot}] = n_{D2}[P]$$

$$[S_{B1tot}] = n_{B1}[P]$$

$$[S_{B2tot}] = n_{B2}[P]$$

If all K and n values were known then equations 8.1 to 8.12 should be soluble since there are twelve unknown and twelve equations. Undoubtedly the most satisfactory way of fitting this model to experimental data would be to guess K and n values, calculate $[BS_{B1}]$, $[BS_{B2}]$ and [B] and from the known extinction coefficients of these three species to calculate theoretical absorbances of a series of solutions. The K and n values could be refined to minimise the difference between measured and theoretical absorbances in much the same way as described in Chap. 4.

The equations can easily be reduced to two equations in two unknowns, but, unfortunately, both involve a cubic term. This means that on substitution there are nine possible solutions. Although, on re-substitution some values give unrealistic values for the other unknown concentrations, there are still a number of possible real solutions. It has not been possible to write a curve fitting routine which can effectively resolve this situation.

A further possibility would be to re-arrange equations (8.1), (8.2) and (8.3)

$$[S_{B1}] = \frac{[BS_{B1}]}{K_{B1}[B_f]} \tag{8.13}$$

$$[DS_{B1}] = K_1[S_{B1}][D] \tag{8.14}$$

$$[DS_{D1}] = K_1[S_{D1}][D] \tag{8.15}$$

Substituting these equations into (8.9) and (8.11)

$$[S_{D1tot}] = K_1[S_{D1}][D] + [S_{D1}] \tag{8.16}$$

$$S_{B1tot} = \frac{[BS_{B1}]}{K_{B1}[B_f]} + [BS_{B1}] + \frac{K_1[BS_{B1}][D]}{K_{B1}[B_f]} \tag{8.17}$$

and thus by substitution and re-arrangement

$$[S_{D1}] = \frac{BS_{B1}[S_{D1tot}]}{K_{B1}B_f([S_{B1tot}]-[BS_{B1}])} \tag{8.18}$$

A fitting routine could calculate values for BS_{B1}, BS_{B2} and B_f as previously, and, by guessing the K and n values, calculate $[S_{D1}]$. By re-substitution into any equation other than (8.7) all other unknown concentrations can be calculated, since there are now only four unknowns and six unused equations (excluding equation 8.7).

The 'goodness of fit' of the binding parameters is

then assessed using equation 8.7 to calculate D_{tot} values for the series of solutions, and comparing them with the actual D_{tot} values by the function:

$$S = \sum_{1}^{n} (D_{tot}^{calc} - D_{tot}^{exp})_i^2$$

A better model might consider the existence of six classes of site, which as well as the four classes mentioned above, would include primary and secondary classes of site to which Bromophenol Blue could bind, but from which the competitor was excluded.

If the simple model by which the results in Chap. 7 were calculated (Model 1) is compared with the first of the two models (Model 2) above, the results obtained can be largely explained. In Model 2 there are two effects which contribute to the degree to which competition occurs. The first of these is direct competition for the mutual binding sites between Bromophenol Blue and the competitor. The second is the binding of competitor to the non-mutual sites. If the competitor has a large number of non-mutual sites available, then much of the dye will be bound at these sites and the free dye concentration much reduced. The dye will thus effectively become a weak competitor, even though it has a larger affinity for the mutual sites than another competitor which has fewer non-mutual sites.

Since Model 1 ignores the existence of non-mutual sites the free dye concentration will be lower than it should be since the equilibrium constants are calculated from the equation:-

$$K_i = \frac{[DS_i]}{[D][S_i]}$$

K_i will be larger than it should be. Furthermore the effect will be worst at low competitor concentrations, and so K_i will decrease as the total concentration of competitor increases. This is seen in Tables 7.8 and 7.9 to be generally the case.

The largest errors in the values of K_i will also occur at low dye concentrations. The competitive effect, and thus the differences between the Bromophenol Blue spectrum in the presence and absence of competitor, will be smallest at these concentrations.

8.12. Conclusions

In conclusion this study has shown that spectrophotometric changes in the spectra of organic acids are compatible with binding to human serum albumin. Changes in binding characteristics brought about by changes in structure of the series of azobenzene derivatives agree with previous theories for the binding of organic acids to serum albumins, hydrophobicity being the most important factor. It seems likely that the lone tryptophan residue of the Warfarin binding site is at least partly involved in the binding of these compounds.

Although the ultrafiltration technique gives less reproducible results than the spectrophotometric method it gives a direct measure of binding of a ligand to a protein. It seems that the ligand binding curve is more meaningful than individual binding parameters. It is essential to realise that in most data fitting routines there are always a number of equally, mathematically correct, parameter sets. It is also important to measure spectral changes over as wide a range of concentration and wavelength as possible, single wavelength measurements are subject to large errors in fitting techniques. This should be borne in mind when comparing rate constants obtained from single wavelength measurements with the equilibrium constants.

Bromophenol Blue is displaced from human serum albumin by the azobenzene derivatives, and the spectra of Bromophenol Blue -albumin mixtures in the presence of these dyes are in agreement with this observation. Even so the spectral changes have only been partially interpreted and a

full description of these competitive reactions awaits explanation.

Further studies with a larger series of azo dyes should enable one to obtain quantitative structure activity relationships for binding of such dyes to albumin. A study of pH effect and comparisons of binding with other albumins, e.g. bovine, horse etc., may enable the sites of binding to be given with more certainty. Use of Warfarin as a competitor was not considered because of its position of absorption and because the reproducibility of data using Warfarin is rather poor. Almost each group of workers produces a new set of binding parameters.

APPENDIX 1

PROGRAM FOR MATRIX RANK ANALYSIS

```
      PROGRAM RANK
C     PROGRAM WRITTEN BY M.ELBOURNE AUGUST 1979 FROM A SIMILAR
C     PROGRAM BY DR GERRARD OF BP.
C     IT PERFORMS A MATRIX RANK ANALYSIS OF U.V/VISIBLE SPEC DATA.
C     DATA IS READ ON DATA CARDS THUS.....
C     1.IDENTIFICATION.NOT MORE THAN 80 CHARACTERS
C     2.NO OF SOLUTIONS,NO OF WAVELENGTHS(2I4)
C     3. THE WAVELENGTHS USED(IN ORDER)(I5 FORMAT)
C     4.SOLUTION MARKERS(20A4)
C     5.THE A MATRIX ONE ROW PER CARD(F6.3).(THE MATRIX MUST BE SQUARE)
C     6. THE S MATRIX,ONE CARD(F5.3)
C     ROWS= SOLN NUMBER,COLUMNS = WAVELENGTHS
C     FOR FURTHER INFORMATION CONSULT J.PHYS.CHEM.68,3891-.
C      ------------------------------------------------------------
      DIMENSION A(20,20),S(20,20),IDENT(20),NWAVE(20),NSOLN(20)
        CHARACTER IDENT*4,NSOLN*4
C
C     READ IN DATA
C
      DO 30 I=1,20
      DO1 J= 1,20
      A(I,J) = 0.0
      S(I,J) = 0.0
    1 CONTINUE
   30 CONTINUE
      READ(5,98)IDENT
   98 FORMAT(20A4)
      READ(5,99)NS,NW
   99 FORMAT(2I4)
      READ(5,50)(NWAVE(K),K=1,NW)
   50 FORMAT(16I5)
```

```
      READ(5,49)NSOLN
   49 FORMAT(20A4)
      DO 2 I=1,NS
    2 READ(5,100)(A(I,J),J=1,NW)
  100 FORMAT(13F6.3)
      READ(5,101)ER
  101 FORMAT(F5.3)
      DO 3 I= 1,NS
      DO 31 J= 1,NW
      S(I,J) = ER
   31 CONTINUE
    3 CONTINUE
      WRITE(6,1009)
 1009 FORMAT(/,1X,'$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$')
      WRITE(6,97)IDENT
   97 FORMAT(1H ,20A4)
      WRITE(6,1010)
 1010 FORMAT(1X,'SOLUTION NUMBERS IN ROWS-WAVELENGTHS IN COLUMNS')
      WRITE(6,1007)
 1007 FORMAT(/,1X,'ORIGINAL A MATRIX',/)
      WRITE(6,51)(NWAVE(K),K=1,NW)
   51 FORMAT(/,6X,20I6)
      WRITE(6,52)
   52 FORMAT(1X,'------------------------------------------------------
     1-----------------------------------------------')
      DO 22 I = 1,NS
   22 WRITE(6,1020)NSOLN(I),(A(I,J),J=1,NW)
 1020 FORMAT(1X,A4,' ',20F6.3)
      WRITE(6,1008)
 1008 FORMAT(/,1X,'ORIGINAL S MATRIX ',/)
      WRITE(6,51)(NWAVE(K),K=1,NW)
```

```
      WRITE(6,52)
      DO 23 I = 1,NS
   23 WRITE(6,1020)NSOLN(I),(S(I,J),J=1,NW)
C
C     SHIFT MATRICES TO LARGEST AT A(1,1) AND S(1,1)
C
      M=2
      K=1
      NSS=NS-1
      DO4 L= 1,NSS
      IROW =0
      AM=A(K,K)
      DO32 I=K,NS
      DO 10 J=K,NW
      IF(A(I,J).GT.AM) GO TO 11
      GO TO 10
   11 AM=A(I,J)
      IROW=I
      ICOL=J
   10 CONTINUE
   32 CONTINUE
      IF(IROW.EQ.0)GO TO 20
      DO12 J=K,NW
      COPY=A(IROW,J)
      A(IROW,J)=A(K,J)
      A(K,J) = COPY
      COPY=S(IROW,J)
      S(IROW,J)=S(K,J)
   12 S(K,J)=COPY
      DO 13 I=K,NS
      COPY=A(I,ICOL)
```

```
      A(I,ICOL)=A(I,K)
      A(I,K)=COPY
      COPY=S(I,ICOL)
      S(I,ICOL)=S(I,K)
      S(I,K)=COPY
   13 CONTINUE
   20 CONTINUE
C
C      MATRIX TRANSPOSE
C
      DO 21 I =M,NS
      DO 15 J=M,NW
      A(I,J)=A(I,J)-A(I,K)*A(K,J)/A(K,K)
      S1=S(I,J)*S(I,J)
      S2=S(K,J)* S(K,J)*((A(I,K)/A(K,K))**2)
      S3=S(I,K)*S(I,K)*((A(K,J)/A(K,K))**2)
      S4=S(K,K)*S(K,K)*((A(I,K)*A(K,J)/A(K,K)*A(K,K))**2)
   15 S(I,J)=SQRT(S1+S2+S3+S4)
      A(I,K)=0.0
      S(I,K)=0.0
   21 CONTINUE
      M=M+1
    4 K=K+1
C
C     RANK ANALYSIS
C
      IRC = 0
      IR= 0
      DO 5 I=1,NS
      AD=ABS(A(I,I))
      SD=ABS(S(I,I))
```

```
      IF(AD.GT.SD)IR=IR+1
      SD=SD*4
    5 IF(AD.GT.SD)    IRC=IRC+1
C
C       OUTPUT
C
      IF(IR.EQ.IRC)GO TO 6
      WRITE(6,1002)IRC,IR
 1002 FORMAT(/,1X,'THERE ARE PROBABLY',I2,',AND POSSIBLY',I2,
     1',SPECIES PRESENT')
      GO TO 7
    6 CONTINUE
      WRITE(6,1003)IR
 1003 FORMAT(/,1X,'THERE ARE',I2,' SPECIES PRESENT.')
    7 WRITE(6,1004)
 1004 FORMAT(/,1X,'FINAL A MATRIX',/)
      DO8 I=1,NS
    8 WRITE(6,1005)(A(I,J),J=1,NW)
 1005 FORMAT(1X,20F6.3)
      WRITE(6,1006)
 1006 FORMAT(/,1X,'FINAL S MATRIX',/)
      DO9 I= 1,NS
    9 WRITE(6,1005)(S(I,J),J=1,NW)
      WRITE(6,1009)
 2000 CONTINUE
      STOP
      END
```

APPENDIX 2

U.V/VISIBLE DATA MINIMISATION PROGRAM

```
JOB(UACA028DSC,J12,T1200,M6600)              M.P.ELBOURNE.....NO CARDS...
ATTACH(NAG,NAG7F,ID=PUBLIC)
ATTACH(PROG,MINUITS,ID=PUBLIC)
LIBRARY(NAG)
FTN(PMD)
MAP(PART)
LOAD(PROG)
LDSET(MAP=B/ZZZZMP,PRESET=NGINF)
LGO.
**EOR**
      PROGRAM BROMO(INPUT,OUTPUT,TAPE 1=INPUT,TAPE 2 = OUTPUT)
      CALL MINUITS
      STOP
      END
      SUBROUTINE FCN(N,V,S,Z,IFLG)
C*********************************************************************
C
C
C       FCN IS A SUBROUTINE WHICH CALCULATES THE GOODNESS OF FIT
C       OF A SET OF PARAMETERS.THIS IS RETURNED TO MINUITS AS
C       THE VARIABLE S.
C
C       PARAMETERS AND VARIABLES
C
C       Z     AN ARRAY CONTAINING THE PARAMETERS TO BE ADJUSTED
C             BY MINUITS. Z(1) AND Z(2) ARE THE PRIMARY AND
C             SECONDARY BINDING CONSTANTS,Z(3) AND Z(4) ARE THE
C             CORRESPONDING BINDING SITE NUMBERS(N1 AND N2).
C             Z(5)-Z(9) ARE THE PRIMARY COMPLEX EXTINCTION
C             COEFFICIENTS AND Z(10)-Z(14) ARE THE SECONDARY
C             COMPLEX EXTINCTION COEFFICIENTS AT THE FIVE
```

```
C              CHOSEN WAVELENGTHS
C
C       N      THE NUMBER OF PARAMETERS IN Z (IN THIS CASE 14)
C
C       V      A WORKSPACE ARRAY
C
C     IFLG     A MARKER TO INDICATE THE STAGE REACHED BY THE
C              FITTING ROUTINE GIVEN BY THE FOLLOWING
C              INTEGERS
C              IFLG=1       INITIALLY SET VALUE
C              IFLG=2       ROUTINE MOVING UNSATISFACTORILLY
C                           TOWARDS BEST MINIMUM
C              IFLG=3       BEST MINIMUM FOUND
C              IFLG=4       ROUTINE MOVING SATISFACTORILY
C                           TOWARDS BEST MINIMUM AND REQUIRES
C                           FURTHER PARAMETER ADJUSTMENT
C
C     NDATA    THE NUMBER OF EXPERIMENTS TO BE INCLUDED IN
C              THE FIT
C
C       M      THE NUMBER OF SOLUTIONS IN THE EXPERIMENT
C
C     AB(I,J)  AN ARRAY CONTAINING THE ABSORBANCE OF THE JTH
C              SOLUTION AT THE ITH WAVELENGTH
C
C     A(J)     THE CONCENTRATION OF DYE IN THE JTH SOLUTION
C              (MOL/L)
C
C     PSTOCK   AN ARRAY CONTAINING THE STOCK ALBUMIN
C              CONCENTRATION IN EACH EXPERIMENT(G/L)
C
```

```
C      EF       THE FREE DYE EXTINCTION COEFFICIENTS
C               AT THE CHOSEN WAVELENGTHS
C
C      IWAVEL   AN ARRAY CONTAINING THE FIVE CHOSEN
C               WAVELENGTHS
C
C      PATH     THE PATH LENGTH
C
C
C*********************************************************************

       DIMENSION Z(15),IWAVEL(5),V(15),PSTOCK(3),
      1 CFN(50),XN(50),YN(50),OBS(5,50),B(50),C(50),OBISOS(50)
       COMMON/MIKE/A(50),P(50),AB(5,50),EF(5),ABCALC(5,50),
      1ABISOS(50),CALISO(50),EFISOS
       GOTO(701,702,703,704)IFLG
  701  CONTINUE
       N=14
       MTOT =0
       READ(1,5)NDATA
     5 FORMAT(I3)
       DO 772 MNRUNS=1,NDATA
       READ(1,10)M
    10 FORMAT(I3)
 C
 C     SET SOLUTION MARKERS
 C
       MBEG =MTOT+1
       MTOT=MTOT+M
       DO 15 J=MBEG,MTOT
       READ(1,20)(AB(I,J),I=1,5)
```

```
   15 CONTINUE
   20 FORMAT(11F7.3)
      READ(1,21)A(MBEG)
   21 FORMAT(E10.3)
      MGO=MBEG+1
      DO 71 JNO=MGO,MTOT
   71 A(JNO)=A(MBEG)
      READ(1,22)PSTOCK(MNRUNS)
  22  FORMAT(F7.4)
      MCON=1
C
C     CALCULATE ALBUMIN CONCENTRATION IN EACH SOLUTION
C
      DO 73 JNO=MBEG,MTOT
      P(JNO)=FLOAT(MCON)*PSTOCK(MNRUNS)*10./(1000. +FLOAT(MCON)*20. )
      MCON=MCON+1
   73 CONTINUE
  772 CONTINUE
      READ(1,23)(EF(J),J=1,5)
   23 FORMAT(5F8.1)
      READ(1,26)(IWAVEL(J),J=1,5)
   26 FORMAT(5I5)
      READ(1,25)PATH
   25 FORMAT(F6.4)
      WRITE(2,99)
C
C     WRITE OUT INPUT DATA
C
   99 FORMAT(1H ,*STARTING POINT*)
      WRITE(2,100)(Z(I),I=1,4)
      WRITE(2,101)(IWAVEL(J),J=1,5)
```

```
      WRITE(2,102)(Z(I),I=5,9)
      WRITE(2,103)(Z(I),I=10,14)
      WRITE(2,104)(EF(I),I=1,5)
  101 FORMAT(1H0,*WAVELENGTH*,3X,5I9)
  102 FORMAT(1H ,*    E1    *,5X,5F9.1)
  103 FORMAT(1H ,*    E2    *,5X,5F9.1)
  104 FORMAT(1H ,*    EF    *,5X,5F9.1)
      WRITE(2,120)
      M=MTOT
      WRITE(2,555)M
  555 FORMAT(1H ,*M =*,I3)
      DO 28 K=1,5
      DO 27 J=1,M
C
C     CONVERT ABSORBANCES TO VALUES AT UNIT PATH LENGTH
C
      AB(K,J)=AB(K,J)/PATH
   27 CONTINUE
   28 CONTINUE
  704 CALL FUNCT(M,N,Z,S)
  702 RETURN
  703 CALL FUNCT(M,N,Z,S)
      CALL WRFRN2(Z(1),Z(2),Z(3),Z(4),CFN,XN,YN,M)
C
C     WRITE FINISHING POINT DATA
C
      WRITE(2,555)M
      WRITE(2,150)
  150 FORMAT(1H ,*FINISHING POINT.....................*)
      WRITE(2,100)(Z(I),I=1,4)
  100 FORMAT(1H ,*K1=*,E10.3/1H ,*K2=*,E10.3,/1H ,*N1=*,F5.2,/1H ,*N2=*
```

```
     1  ,F5.2)
        WRITE(2,200)S
  200 FORMAT(1H ,*VARIATION =*,E10.3)
        WRITE(2,202)
  202 FORMAT(1H ,*STOCK ALBUMIN CONCENTRATIONS*)
        WRITE(2,203)
  203 FORMAT(1H ,*----------------------------*)
        DO 205 NALB=1,NDATA
        WRITE(2,204)FSTOCK(NALB)
  204 FORMAT(1H ,8X,F6.2,*G/L*)
  205 CONTINUE
        WRITE(2,201)
  201 FORMAT(1H ,*TOTAL DRUG  TOTAL ALB  TOTAL SITE1  TOTAL SITE2  BOUND1
     1 DRUG  BOUND2 DRUG  FREE DRUG*)
        WRITE(2,120)
  120 FORMAT(1H *-------------------------------------------------------
     1----------------------------*)
        DO 126 K=1,5
        DO 125 I=1,M
C
C       CONVERT AB TO ORIGINALLY READ IN VALUES
C
        OBS(K,I)=AB(K,I)*PATH
  125 CONTINUE
  126 CONTINUE
         DO 127 I=1,M
        B(I) = Z(3)*P(I)/69000.
        C(I) = Z(4)*P(I)/69000.
        WRITE(2,130)A(I),P(I),B(I),C(I),XN(I),YN(I),CFN(I)
  130 FORMAT(1H ,E10.3,3X,F7.4,2X,E10.3,3X,E10.3,3X,E10.3,3X,E10.3,2X,
     1E10.3)
```

```
  127 CONTINUE
      WRITE(2,101)(IWAVEL(J),J=1,5)
      WRITE(2,102)(Z(I),I=5,9)
      WRITE(2,103)(Z(I),I=10,14)
      WRITE(2,104)(EF(I),I=1,5)
      WRITE(2,120)
      WRITE(2,105)(IWAVEL(J),J=1,5)
      WRITE(2,106)
      WRITE(2,107)
      DO 110 J=1,M
      WRITE(2,108)J,(OBS(K,J),AB(K,J),K=1,5)
  110 CONTINUE
      WRITE(2,120)
      WRITE(2,105)(IWAVEL(J),J=1,5)
      WRITE(2,106)
      WRITE(2,109)
      DO 111 J=1,M
      WRITE(2,108)J,(AB(K,J),ABCALC(K,J),K=1,5)
  111 CONTINUE
  105 FORMAT(1H ,*WAVELENGTH*,7X,I3,*NM*,4(13X,I3,*NM*))
  106 FORMAT(1H ,4X,5(13X,*-----*))
  107 FORMAT(1H ,*SOLUTION*,5X,5(*ABS OBS  ABS/CM*,3X))
  108 FORMAT(1H ,I6,4X,10F9.3)
  109 FORMAT(1H ,*SOLUTION*,5X,5(*ABS/CM   ABSCALC*,2X))
  143 RETURN
      END
      SUBROUTINE FUNCT(M,N,Z,S)
C     ************************************************************
C
C
C       FUNCT CALCULATES THE ERROR SQUARE TERM 'S'
```

```
C
C
C     ************************************************************
      DIMENSION Z(15),CFN(50),XN(50),YN(50),E(5,2)
      COMMON/MIKE/A(50),P(50),AB(5,50),EF(5),ABCALC(5,50),
     1ABISOS(50),CALISO(50),EFISOS
      CALL WRFRN2(Z(1),Z(2),Z(3),Z(4),CFN,XN,YN,M)
C     ************************************************************
C     THE ARRAYS CFN,XN,AND YN CONTAIN THE CONCENTRATIONS OF FREE
C     DYE,PRIMARY COMPLEX AND SECONDARY COMPLEX RESPECTIVELY
C     RETURNED FROM WRFRN2 FOR EACH SOLUTION
C     ************************************************************
      S=0.
      E(1,1)=Z(5)
      E(2,1)=Z(6)
      E(3,1)=Z(7)
      E(4,1)=Z(8)
      E(5,1)=Z(9)
      E(1,2)=Z(10)
      E(2,2)=Z(11)
      E(3,2)=Z(12)
      E(4,2)=Z(13)
      E(5,2)=Z(14)
      DO 2 K=1,5
      DO 1 I=1,M
      ABCALC(K,I)=EF(K)*CFN(I)+ABS(E(K,1))*XN(I)+ABS(E(K,2))*YN(I)
      F= AB(K,I)-ABCALC(K,I)
      S=S+F*F
    1 CONTINUE
    2 CONTINUE
      RETURN
```

```
      END

      SUBROUTINE WRFRN2(F1,F2,F3,F4,DFKEEP,XSTORE,YSTORE,M)
C     ************************************************************************
C
C
C     WRFRN2 CALCULATES THE CONCENTRATION OF THE THREE DYE SPECIES
C     PRESENT IN EACH SOLUTION
C
C     ************************************************************************
      REAL N1,N2,K1,K2
      DIMENSION X(50),Y(50),DF(50),
     1COEF(4),ROOT(3),XROOT(3),KNOTE(3),YSTORE(50),XSTORE(50),DFKEEP(50)
      COMMON/MIKE/A(50),P(50),AB(50),EF(5),ABCALC(5,50),ABISOS(50),
     1CALISO(50),EFISOS
      K1=ABS(F1)
      K2=ABS(F2)
      N1=ABS(F3)
      N2=ABS(F4)
      NPLUS = M+1
      I=0
      COEF1 = K1*K2
   35 I=I+1
      IF(I.EQ.NPLUS)GO TO 2000
C
C     CALCULATE THE TOTAL CONCENTRATION OF EACH SET OF SITES
C
      B = ABS(N1)*P(I)/69000.
      C = ABS(N2)*P(I)/69000.
C
C     CALCULATE THE COEFFICIENTS OF THE EQUATION
```

```
C
      COEF(1)= COEF1
      Q = K2*K1+(B+C-A(I))*K1*K2
      R = 1. - A(I)*K1-A(I)*K2 + B*K1 + C*K2
      S = -A(I)
      TO = 1.E-8
      COEF(2)=Q
      COEF(3)=R
      COEF(4)=S
      N=4
      IFAIL =0
      IT =0
      CALL C02AEF(COEF,N,ROOT,XROOT,TO,IFAIL)
C     ************************************************************
C     THE THREE ROOTS ARE NOW IN THE ARRAY 'ROOT'.
C     ANY UNREAL PARTS ARE IN THE ARRAY  'XROOT'.
C     N IS THE NUMBER OF COEFFICIENTS
C     TO IS A TOLERANCE VALUE
C     IFAIL IS AN INTEGER WHICH MAY BE USED TO DETERMINE
C     THE TYPE OF FAILURE SHOULD C02AEF FAIL
C
C     ************************************************************
      DO 900 K=1,3
      IF(ABS(XROOT(K)).GT.1.E-10)GO TO 900
      IF(ROOT(K) .GE. A(I))GOTO900
      IF(ROOT(K) .LE. 0.)GOTO900
      IT =IT+1
      KNOTE(IT)=K
  900 CONTINUE
      IF(IT.EQ.1)GO TO 1050
      WRITE(2,1003)IT,K1,K2,N1,N2,A(I),ROOT,XROOT
```

```
 1003 FORMAT(1H ,*THERE ARE NO PROPER ROOTS*,I4/' VARIABLES ',
     1 5(1PE10.3)/' ROOTS',6E10.3)
       STOP4
 1050 KN = KNOTE(1)
C
C      FIND THE GENUINE ROOT
C
       DFKEEP(I) = ROOT(KN)
       XSTORE(I) = K1*ROOT(KN) *B/(1. + K1*ROOT(KN))
       YSTORE(I) = A(I) - XSTORE(I) - ROOT(KN)
       GOTO35
 2000 RETURN
       END
**EOR**
```

APPENDIX 3

ULTRAFILTRATION DATA FITTING PROGRAM

```
      PROGRAM CURFIT
C     ***************************************************************
C
C
C     CURFIT IS A ROUTINE FOR FINDING THE BEST FIT OF THE
C     PARAMETERS N1, N2, K1 AND K2 TO ULTRAFILTRATION DATA
C
C     VARIABLES
C
C     IDENT    THE TITLE OF THE EXPERIMENT
C
C     FREEM    AN ARRAY CONTAINING CONTAINING THE
C              EXPERIMENTALLY MEASURED FREE DYE
C              CONCENTRATIONS
C
C     DTOT     AN ARRAY CONTAINING THE TOTAL DYE CONCENTRATIONS
C
C     FREE     AN ARRAY CONTAINING THE CORRESPONDING
C              THEORETICAL FREE DYE CONCENTRATIONS
C
C     XBOUND   AN ARRAY CONTAINING THE THEORETICAL
C              CONCENTRATION OF DYE BOUND AT THE
C              PRIMARY SITE
C
C     YBOUND   AN ARRAY CONTAINING THE THEORETICAL
C              CONCENTRATION OF DYE BOUND AT THE
C              SECONDARY SITE
C
C     PLASMA   THE ALBUMIN CONCENTRATION (G/L)
C
C      PY      THE CONCENTRATION OF PRIMARY SITE (MOL/L)
```

```
C
C        PX       THE CONCENTRATION OF SECONDARY SITE (MOL/L)
C
C       ITAP      AN INTEGER WHICH, WHEN SET TO 1, WILL NOT ALLOW
C                 THE BINDING PARAMETERS TO FALL TO UNREALISTIC
C                 VALUES. ALL OTHER VALUES OF ITAP ALLOW THE
C                 PARAMETERS TO FALL TO ANY VALUE
C
C        NSA      THE NUMBER OF SAMPLES
C
C       CONV3     CONVERSION FACTORS TO CONVERT K1 AND K2
C       AND       TO THE SAME ORDER OF MAGNITUDE AS N1 AND N2
C       CONV4
C
C        V        AN ARRAY CONTAINING THE PARAMETERS TO BE MINIMISED
C
C       FUN       THE FUNCTION TO BE MINIMISED
C
C       ************************************************************
      EXTERNAL FUN,MONIT
      DIMENSION V(4),W(100),IDENT(20)
      REAL N1,N2,K1,K2
      COMMON FREE(50),XBOUND(50),YBOUND(50),DTOT(50),PX,PY,NSA,
     1 CONV3,CONV4,FREEM(50),ITAP
      CHARACTER IDENT*4
      READ(5,666)ITAP
  666 FORMAT(I3)
      N=4
      STEP=2.0
      M=100
      READ(5,5)IDENT
```

```
  5   FORMAT(20A4)
      WRITE(6,6)IDENT
  6   FORMAT(1H ,20A4)
      IF(ITAP.EQ.1)WRITE(6,888)
888   FORMAT(1HO,'  NO CONSTRAINTS ')
      IF(ITAP.NE.1)WRITE(6,889)
889   FORMAT(1HO,' CONSTRAINTS ON')
      READ(5,10)PLASMA
 10   FORMAT(F6.4)
      PLASMA=PLASMA/69000.
      WRITE(6,11)PLASMA
 11   FORMAT(1H ,'PLASMA CONC=',E12.4,'MOL/L')
      READ(5,15)NSA
 15   FORMAT(I3)
      WRITE(6,12)NSA
 12   FORMAT(1H ,I3,'SOLUTIONS')
      READ(5,20)(DTOT(I),FREEM(I),I=1,NSA)
 20   FORMAT(2F10.3)
      DO 21 I=1,NSA
      DTOT(I)=DTOT(I)*1.0E-4
 21   FREEM(I)=FREEM(I)*1.0E-4
      READ(5,30)N1,N2,K1,K2
 30   FORMAT(2F10.4,2E12.4)
      CONV1=LOG10(K1)
      CONV2=LOG10(K2)
      ICONV1=INT(CONV1)
      ICONV2=INT(CONV2)
      CONV3=10**(ICONV1)
      CONV4=10**(ICONV2)
      V(1)=N1
      V(2)=N2
```

```
      V(3)=K1/CONV3
      V(4)=K2/CONV4
      PX=PLASMA*N1
      PY=PLASMA*N2
      WRITE(6,40)
 40   FORMAT(1H ,'STARTING POINT')
      WRITE(6,303)N1,N2,K1,K2
      WRITE(6,304)V(1),V(2),V(3),V(4)
304   FORMAT(1H ,'PARAMETERS',4E12.4)
      CALL WRFRN(N1,N2,K1,K2)
      WRITE(6,201)
      WRITE(6,202)
      WRITE(6,303)(DTOT(I),XBOUND(I),YBOUND(I),FREE(I),FREEM(I),
     1 I=1,NSA)
      WRITE(6,202)
      FFFF = FUN(V,N)
      WRITE(6,49)FFFF
 49   FORMAT(1H ,'FUNCTION=',E12.4)
      CALL SIMPLEX(V,N,FUN,STEP,MONIT,W,M)
      WRITE(6,50)
 50   FORMAT(1H ,'FINISHING POINT')
      N1=V(1)
      N2=V(2)
      K1=V(3)*CONV3
      K2=V(4)*CONV4
      FFFF=FUN(V,N)
      WRITE(6,49)FFFF
      CALL WRFRN(N1,N2,K1,K2)
      WRITE(6,201)
201   FORMAT(1H0,'  TOTAL DRUG   BOUND1 DRUG   BOUND2 DRUG'
     1 '   FREE DRUG   FREE (EXPTL)')
```

```
      WRITE(6,202)
  202 FORMAT(1H ,'----------------------------------------'
     1 '------------')
      WRITE(6,303)(DTOT(I),XBOUND(I),YBOUND(I),FREE(I),FREEM(I),
     1 I=1,NSA)
  303 FORMAT(1H ,E12.4,2E14.4,2E12.4)
      WRITE(6,301)N1,N2,K1,K2
  301 FORMAT(1HO,'N1=',F12.4,'N2='F12.4,/1HO,'K1=',E12.4,'K2='E12.4)
      STOP
      END
      FUNCTION FUN(V,N)
C     ******************************************************************
C
C
C     THE ERROR FUNCTION CALCULATING SUBPROGRAM
C
C     ******************************************************************
      COMMON FREE(50),XBOUND(50),YBOUND(50),DTOT(50),PX,PY,NSA,
     1 CONV3,CONV4,FREEM(50),ITAP
      REAL K1,K2, N1,N2
      DIMENSION V(4)
      K1=CONV3*V(3)
      K2=CONV4*V(4)
      N1=V(1)
      N2=V(2)
      IF(ITAP.EQ.1)GO TO 555
      IF(K1.LT.1.E+5)GO TO 777
      IF(K2.LT.7.0E+3)GO TO 777
      IF(N1.LT.0.8)GO TO 777
      IF(N2.LT.0.8)GO TO 777
  555 CONTINUE
```

```
      CALL WRFRN(N1,N2,K1,K2)
      FUN=0.
      DO 10 I=1,NSA
      FX=FREEM(I)-FREE(I)
      FUN=FUN+FX*FX
   10 CONTINUE
      FUN=FUN*100000000.
      GO TO 1000
  777 FUN=100000.
 1000 RETURN
      END
      SUBROUTINE WRFRN(N1,N2,K1,K2)
C     ************************************************************
C
C     WRFRN CALCULATES THE CONCENTRATIONS OF THE THREE DYE
C     SPECIES IN EACH SOLUTION
C
C     ************************************************************
      DIMENSION ROOT(3)
      REAL N1,N2,K1,K2
      COMMON FREE(50),XBOUND(50),YBOUND(50),DTOT(50),PX,PY,NSA,
     1 CONV3,COV4,FREEM(50),ITAP
C
C     CALCULATE THE COEFFICIENTS OF THE CUBIC EXPRESSION
C
      A=K1*K2
      DO 1000 I=1,NSA
      B=K2+K1+(PX+PY-DTOT(I))*K1*K2
      C=1. -DTOT(I)*K1-DTOT(I)*K2+PX*K1+PY*K2
      D=-DTOT(I)
      CALL CUBIC(A,B,C,D,ROOT,IFLG)
```

```
      IF(IFLG.EQ.2)GO TO 20
C
C     FIND THE ACTUAL ROOT
C
      DO 100 K=1,3
      IF(ROOT(K).GE.DTOT(I))GO TO 100
      IF(ROOT(K).LE.0.)GO TO 100
      ROOT1=ROOT(K)
      GO TO 21
  100 CONTINUE
   20 ROOT1=ROOT(1)
   21 FREE(I)=ROOT1
      XBOUND(I)=K1*FREE(I)*PX/(1.+K1*FREE(I))
      YBOUND(I)=DTOT(I)-XBOUND(I)-FREE(I)
 1000 CONTINUE
      RETURN
      END
```

```
      SUBROUTINE CUBIC(A,B,C,D,ROOT,IFLG)
      DIMENSION ROOT(3)
C     ******************************************************
C
C
C     SUBROUTINE CUBIC ******
C
C
C     THIS FINDS THE REAL ROOTS OF A CUBIC EQUATION
C     OF THE TYPE  AX**3 + BX**2 + CX + D
C
C     THE CALLING ROUTINE MUST SUPPLY THE COEFFICIENTS
C     A,B,C AND D. IF THERE ARE 3 REAL ROOTS (RETURNED
C     TO THE 3 DIMENSIONAL ARRAY 'ROOT')IFLG WILL BE SET
C     AT 1. IF THERE IS ONLY ONE REAL ROOT(RETURNED TO
C     ROOT(1)),IFLG WILL BE SET TO 2.
C
C
C     ******************************************************
C
C
C     TEST TO FIND THE NUMBER OF REAL ROOTS
C
      Q=((2.*(B**3))/(27.*(A**3))-(B*C)/(3.*(A**2))+D/A)/2.
      P=((3*A*C-B**2)/(3*(A**2)))/3.
      R=SQRT(ABS(P))
      IF((Q**2+P**3).LE.0.)GO TO 100
      IF((Q**2+P**3).GT.0)GO TO 200
C
C     THREE REAL ROOTS CALCULATED FROM CIRCULAR FORMULAE
C
```

```
  100   PHI=ACOS(Q/(R**3))
        ROOT(1)=-2.*R*COS(PHI/3.)-B/(3*A)
        ROOT(2)=2.*R*COS(2*ACOS(-1.)/6.-PHI/3.)-B/(3*A)
        ROOT(3)=2.*R*COS(2*ACOS(-1.)/6.+PHI/3.)-B/(3*A)
        IFLG=1
        RETURN
C
C       ONE REAL ROOT CALCULATED FROM CARDANS FORMULA
C
  200   IG=0
        IH=0
        G=-Q+SQRT(Q**2+P**3)
        H=-Q-SQRT(Q**2+P**3)
        IF(G.LT.0)IG=1
        IF(H.LT.0)IH=1
        G=ABS(G)
        H=ABS(H)
        U=EXP(LOG(G)/3.)
        V=EXP(LOG(H)/3.)
        IF(IG.EQ.1)U=-U
        IF(IH.EQ.1)V=-V
        ROOT(1)=U+V-B/(3*A)
        IFLG=2
        RETURN
        END
```

```
      SUBROUTINE SIMPLEX(B,N,F,STEP,MONIT,W,M)
*
*     MARKTOOLS ROUTINE --- SIMPLEX
*
*     NELDER-MEAD OPTIMIZATION OF A FUNCTION OF N-VARIABLES
*
*     SIMPLEX METHOD WITH AXIAL SEARCH
*
*     REF :   CNMC    J.NASH
*
*     PARAMETERS
*
*             B       ARRAY DIMENSIONED N CONTAINING THE
*                     THE STARTING VALUES OF THE N PARAMETERS
*
*             N       DIMENSION OF B
*
*             F       REAL FUNCTION TO BE MINIMIZED
*                     THIS SHOULD BE DECLARED AS EXTERNAL IN THE
*                     CALLING ROUTINE AND BE OF THE FORM
*
*                      REAL FUNCTION F(B,N)
*
*             STEP    STARTING INCREMENT FOR ALL PARAMETERS
*
*             MONIT   INTEGER FUNCTION WHICH WILL BE CALLED BY
*                     SIMPLEX WHEN DESCENDING THE N-DIMENSIONAL
*                     SIMPLEXES
*                     THIS SHOULD BE OF THE FORM
*
*                     INTEGER FUNCTION MONIT(IFCNT,FHI,FLOW)
```

```
*
*                       WHERE :
*                               FLOW    IS THE LOWEST VALUE OF THE
*                                       FUNCTION F IN THE CURRENT SIMPLEX
*
*                               FHI     IS THE HIGHEST VALUE OF THE
*                                       FUNCTION F IN THE CURRENT SIMPLEX
*
*                               IFCNT   IS THE NUMBER OF FUNCTION EVALUATIONS
*                                       THAT HAVE BEEN MADE SO FAR
*
*                       IF MONIT RETURNS A NON-ZERO VALUE TO SIMPLEX
*                       THEN CONTROL RETURNS FROM SIMPLEX TO THE CALLING
*                       ROUTINE
*                       ELSE (I.E. THE RETURNED VALUE IS ZERO)
*                           THE OPTIMIZATION CONTINUES
*
*               W       A WORKSPACE ARRAY WHICH SHOULD BE DIMENSIONED
*                       M >= (N+1)*(N+2) = N^2 + 3*N + 2
*
*               M       DIMENSION OF WORKSPACE ARRAY W (SEE ABOVE)
* ======================================================================
      DIMENSION B(N)
      DIMENSION W(M)
      INTEGER H,C
      PARAMETER(EPS   =       1.0E-6)
      EXTERNAL MONIT
      IND(I,J)        =       (N+1)*(J-1) + I
*
*
      C       =       N + 2
```

```
      ALPHA   =       1.0     ! REFLECTION FACTOR
      BETA    =       0.5     ! CONTRACTION FACTOR
      GAMMA   =       2.0     ! EXTENSION FACTOR
*
      CF      =       F(B,N)
      IFCOUNT =       1
      W(IND(N+1,1))   =       CF
      DO 10 I=1,N
              W(IND(I,1)) = B(I)
 10   END DO  ! I
*
      L       =       1
      SIZE    =       0.0
      DO 20 J=2,N+1
              DO 30 I=1,N
                      W(IND(I,J)) = B(I)
 30           END DO  ! I
              T       =       STEP
 1100         W(IND(J-1,J)) = B(J-1) + T
              IF(ABS(W(IND(J-1,J))-B(J-1)) .LT. EPS) THEN
                      T       =       10.0*T
                      GO TO 1100
              END IF
              SIZE    =       SIZE + ABS(T)
 20   END DO  ! J
*
*     CALCULATE FUNCTION VALUE THROUGHOUT CURRENT SIMPLEX
*
 111  SSIZE   =       SIZE
      DO 40 J=1,N+1
              IF(J.NE.L) THEN
```

```
                        DO 45 I=1,N
                                B(I) = W(IND(I,J))
 45                     END DO ! I
                        CF      =       F(B,N)
                        IFCOUNT =       IFCOUNT + 1
                        W(IND(N+1,J)) = CF
                END IF
 40     END DO ! J
*
 222    CONTINUE
*
*       ORDER FUNCTION VALUES
*
        L       =       1
        H       =       1
        N1      =       1
        VL      =       W(IND(N+1,1))
        VH      =       VL
        DO 50   J=2,N+1
                T = W(IND(N+1,J))
                IF (T .LT. VL) THEN
                        VL      =       T
                        L       =       J
                END IF
                IF(T .GE. VH) THEN
                        N1      =       H
                        H       =       J
                        VH      =       T
                END IF
 50     END DO  ! J
*
```

```
*       CALL MONITORING ROUTINE SUPPLIED BY USER
*
        IF(MONIT(IFCOUNT,VH,VL) .NE. 0) RETURN
*
        IF(ABS(VL - VH) .LE. EPS) GO TO 9000
*
        DO 60 I=1,N
                T = -W(IND(I,H))
                DO 65 J=1,N+1
                        T = T + W(IND(I,J))
 65             END DO ! J
                W(IND(I,C)) = T/N       ! SAVES CENTROID
 60     END DO  ! I
*
*       REFLECTION
*
        DO 70 I=1,N
                B(I) = (1.0 + ALPHA)*W(IND(I,C)) - ALPHA*W(IND(I,H))
 70     END DO  ! I
*
        CF      =       F(B,N)
        IFCOUNT =       IFCOUNT + 1
*
        IF(CF .LT. VL) GO TO 1000       ! GOING DOWN
*
        IF(CF .LT. W(IND(N+1,N1))) GO TO 2000
*
        IF(CF .LT. VH) THEN
                DO 80 I=1,N
                        W(IND(I,H)) = B(I)
 80             END DO  ! I
```

```
              W(IND(N+1,H)) = CF
         END IF
*
*        REDUCTION ALONG A LINE
*
         DO 90 I=1,N
              B(I) = (1.0 - BETA)*W(IND(I,H)) + BETA * W(IND(I,C))
 90      END DO  ! I
*
         CF      =       F(B,N)
         IFCOUNT =       IFCOUNT + 1
*
         IF(CF .LT. W(IND(N+1,H)) ) GO TO 2000 ! GOING DOWN
*
*        OTHERWISE CONTRACT
*
         SIZE    =       0.0
         DO 100 J=1,N+1
              IF(J.NE.L) THEN
                   DO 105 I=1,N
                   W(IND(I,J)) = BETA*(W(IND(I,J))-W(IND(I,L)))
     1                           + W(IND(I,L))
                   SIZE = SIZE + ABS(W(IND(I,J)) - W(IND(I,L)))
 105               END DO  ! I
              END IF
 100     END DO  ! J
         IF(SIZE .LT. SSIZE) GO TO 111 ! HAVE DECREASED SO KEEP GOING
         WRITE(*,*) 'FAILURE TO DECREASE'
         GO TO 9000
*
 1000    CONTINUE
```

```
*               EXTEND THE SIMPLEX ALONG A LINE
      DO  120 I=1,N
              T = GAMMA*B(I) + (1.0 - GAMMA)*W(IND(I,C))
              W(IND(I,C)) = B(I)
              B(I) = T
 120  END DO  ! I
      W(IND(N+1,C)) = CF
      CF = F(B,N)
      IFCOUNT =       IFCOUNT + 1
*
      IF(CF .GE. W(IND(N+1,C))) THEN
*             RESET SINCE NO PROGRESS IN LOWERING CF
              DO 130 I=1,N
                      B(I) = W(IND(I,C))
 130          END DO ! I
              CF      =       W(IND(N+1,C))
      END IF
*             CHANGE SIMPLEX
 2000 DO 140 I=1,N
              W(IND(I,H)) = B(I)
 140  END DO ! I
      W(IND(N+1,H)) = CF
      GO TO 222
 9000 CONTINUE
*             COULD CALL AXIAL SEARCH ROUTINE HERE TO TRY AND PUSH FURTHER
      RETURN
      END
```

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