THE DEVELOPMENT OF ENERGY TRANSFER IMMUNOASSAY METHODS

by

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A Doctoral Thesis
submitted in partial fulfilment of the requirements for
the award of
DOCTOR OF PHILOSOPHY
of the Loughborough University of Technology

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September 1980

ACKNOWLEDGEMENTS

I wish to express my grateful thanks to Dr. J.N.Miller and Professor J.W.Bridges for their help, encouragement and guidance during the period of research.

Thanks are also due to the following members of the technical staff who have provided expert technical assistance and constructive advice: Messrs. A.F.Bower, M.R.Coupe, M.K.Patel, A.G.Stevens, J.J.Swithenbank and Miss Jill Kristina Thorley.

The friendly co-operation given by the other research workers in the laboratory is acknowledged. In particular, I am most appreciative of the kind understanding and co-operation of Mr. H.N.Sturley who has been gracious enough to share his glassware and other laboratory equipment.

The Medical Research Council provided the financial support for this research which was carried out using the facilities of the Chemistry Department, Loughborough University of Technology.

Finally, I am most grateful to my mother for her encouragement and prayerful blessings for my welfare and success.

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SYNOPSIS

The use of fluorescein and rhodamine as donor and acceptor fluorescent labels in the development of energy transfer immunoassay (ETIA) methods was evaluated by developing an assay for human serum albumin. The sensitivity of the assay was found to depend on (i) the degrees of fluorophore labelling of antibody and antigen, (ii) concentrations of labelled antibody and antigen, (iii) the fluorimeter spectral bandwidth, and (iv) whether the donor (fluorescein) was conjugated to the antigen or the antibody. These results, including those relating to the stability of the labelled immune reactants on storage, lead to the conclusion that fluorescein and rhodamine are far from ideal as donor and acceptor. Nevertheless, the application of the assay to the analysis of test serum samples gave results that compared favourably with those obtained by electroimmunoassay.

Other potential donor and acceptor fluorescent labels were also investigated, viz. (a) fluorescamine and fluorescein, (b) MDPF and fluorescein, (c) dansyl chloride and rhodamine, (d) quinacrine and fluorescein, and (e) quinacrine and rhodamine. Of these, only (a) and (b) were found to be suitable donor-acceptor pairs, and they were applied to the development of a number of assays for both low and high molecular-weight analytes. Comparative studies of fluorescamine and MDPF as donor fluorescent labels together with fluorescein as the acceptor label were performed by developing immunoassay methods for the determination of human serum transferrin in four serum samples including a blood sample from the victim of a road traffic accident. Results

obtained were generally in good agreement with those found by the radial immunodiffusion method. An ETIA developed for nortriptyline and related tricyclic antidepressants was capable of detecting nanomolar concentrations of the drugs in pure solution and in spiked sera. Other ETIA's developed include a sandwich assay for the quantitation of human immunoglobulin A, and a direct assay for the determination of human immunoglobulin G.

The Fluram as well as the MDPF enhancement phenomena were also studied in detail and applied successfully to the development of fluorescence enhancement immunoassays for nortriptyline, human serum transferrin and immunoglobulin G.

Finally, the automation of an energy transfer immunoassay was successfully performed by using the principles of stopped-flow injection analysis with merging zones.

CHAPTER 1 INTRODUCTION

1.1 Energy Transfer

1.1.1 Competitive processes affecting an electronically excited molecule.

A molecule excited from the ground state S_0 to an upper singlet energy level S_2 may be deactivated by several competitive processes as outlined in the energy-level diagram of Fig.1.1

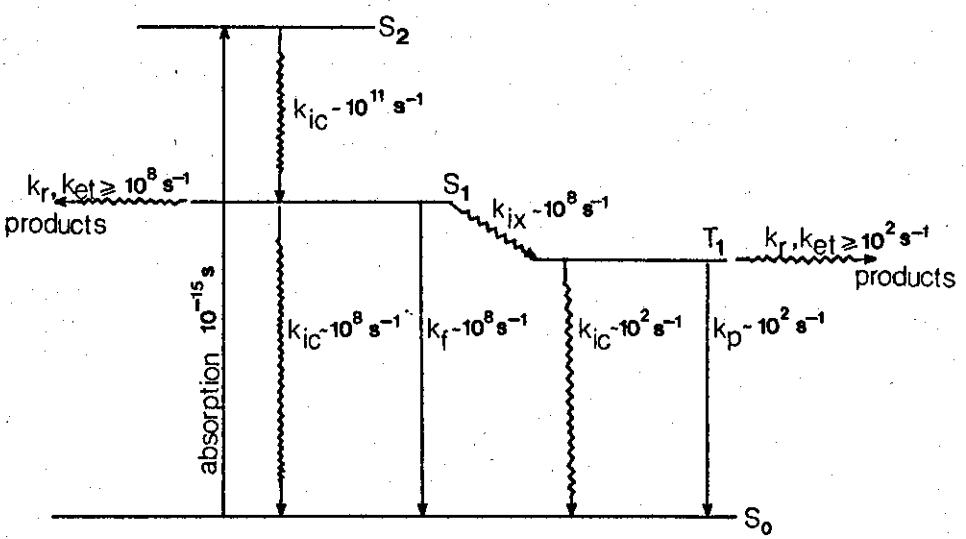


Fig 1.1 Competitive processes affecting a molecule in an excited state S_2 . Straight and wavy lines denote radiative and nonradiative processes, respectively.

S_0 = ground state ; S_1 = lowest excited singlet state ;
S_2 = initial excited state ; T_1 = lowest triplet state
k_r = rate constant for chemical reaction
k_{et} = rate constant for energy transfer
k_{ic} = rate constant for internal conversion
k_{ix} = rate constant for intersystem crossing
k_f = rate constant for fluorescence
k_p = rate constant for phosphorescence

From the initial excited state S_2, the molecule can (i) return rapidly (in ca. 10^{-13} to 10^{-11} s) to the lowest singlet

state S_1 by a nonradiative process called internal conversion, (ii) undergo a chemical reaction to yield products, or (iii) donate its excitation energy to a neighbouring molecule (acceptor), a process called energy transfer.

For a competitive process to compete effectively with the spontaneous decay (internal conversion) of the excited state, it must have a rate constant k_{cp} greater than the decay rate of the excited state from which it occurs, ie

$$k_{cp} \geq \frac{1}{\tau_s} \qquad (1.1)$$

where τ_s is the lifetime of the state involved. Thus, in this case, the competing processes - chemical reaction and energy transfer - are not probable from the excited state S_2 because their occurrence would imply that these competing processes could not be diffusion-controlled (with rate constant $\sim 10^9$ litre $mol^{-1} s^{-1}$), but must occur with a first order rate constant of at least 10^{11} litre $mol^{-1} s^{-1}$ (Hercules 1966).

The molecule from the lowest excited singlet state S_1 may return to the ground state S_0 by fluorescence or internal conversion; it may also go to the triplet state T_1 by a non-radiative spin-inversion process called intersystem crossing. Since the lifetime of the molecule in S_1 is ca. 10^{-8}s (k_f , k_{ic} and k_{ix} for $S_1 \longrightarrow S_0$ approximately $10^8 s^{-1}$), the competing processes of energy transfer and chemical reaction can also occur from S_1 .

From the lowest triplet state T_1 , the molecule can return to the ground state S_0 by internal conversion or phosphorescence. Furthermore, with T_1 having a comparatively long lifetime (10^{-4} to

10 s) the competitive processes of energy transfer and chemical reaction can also take place from the triplet state. Thus, energy transfer from both the lowest excited singlet S_1 and triplet T_1 states of a molecule (donor) to another molecule (acceptor) can occur, and the following possible types of energy transfer between donor and acceptor molecules will be discussed in the appropriate sections below:

singlet-singlet energy transfer
triplet-singlet energy transfer
singlet-triplet energy transfer
triplet-triplet energy transfer

1.1.2 Collisional deactivation of an electronically excited molecule

This is a diffusion-controlled process that involves a collisional contact between the excited donor D^* and the ground state acceptor A. The steps involved in a collisional deactivation of an excited donor may be represented as follows.

$$D + h\nu \longrightarrow D^* \qquad \text{(excitation)} \qquad (1.2)$$

$$D^* + A \longrightarrow D + A + kT \qquad \text{(deactivation without energy transfer to A)} \qquad (1.3)$$

$$D^* + A \longrightarrow D + A^* \qquad \text{(energy transfer)} \qquad (1.4)$$

The donor absorbs a photon hv and becomes an excited state molecule D^* which can then be deactivated to its ground state by emitting a photon hv' (fluorescence: $D^* \longrightarrow D + h\nu'$) or dissipating its excitation energy through interaction with a colliding acceptor molecule. The latter process is governed by the Stern-Volmer law (Stern and Volmer 1919),

$$\frac{F_0}{F} = 1 + k[A]\tau_d \qquad (1.5)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the acceptor respectively; τ_d is the lifetime of the excited state of the donor and k a proportionality constant. It can be deduced from the Stern-Volmer that the lifetime τ_d decreases with an increase in $[A]$. More collisions will occur the longer the excited state exists and therefore donors with long lifetimes are more susceptible to collisional deactivation.

Although Equations (1.2) to (1.4) above provide an adequate mathematical description of collisional energy loss, the physical process necessitates an energy dissipation of ca. 210 - 420 kJ mol^{-1}, which is much greater than what would have been expended in a simple bimolecular collision (Hercules 1966). Deactivation of the excited donor by the collisional process can be effected by the following mechanisms which <u>may</u> or <u>may not</u> involve an energy transfer to the acceptor.

1.1.2.1 <u>Collisional deactivation mechanisms NOT involving energy transfer</u>

(a) Enhanced intersystem crossing (spin-orbit coupling ; the external heavy atom effect).

$$^1D^* + A \longrightarrow {}^3D^* + A$$ } intersystem crossing enhanced in the presence of A

$^3D^* \longrightarrow D + h\nu$ phosphorescence

$^3D^* \longrightarrow D + kT$ internal conversion to ground state

(b) Enhanced internal conversion. The interaction with H-bonding acceptor involving vibrational coupling between D^* and A will lead to enhanced internal conversion of D^* to the ground state.

$$D^* + A \longrightarrow D + A + kT$$

(c) Exciplex formation followed by internal conversion to the ground state or diminished and red-shifted fluorescence.

$$D^* + A \longrightarrow (D\text{-}A)^* \longrightarrow D + A + h\nu'$$

exciplex

diminished fluorescence and red-shifted compared to monomeric donor fluorescence

$$D + A + kT$$

internal conversion to ground state

(d) Charge-transfer complex formation (by the reversible electron transfer mechanism)

<u>In nonpolar solvents</u>

$$D^* + A \rightleftharpoons D^*\!\!-\!\!A \longrightarrow (D^+\!\!-\!\!A^-)^* \longrightarrow D + A + h\nu'$$

encounter complex

charge-transfer complex

diminished, red-shifted fluorescence

$$D + A + kT$$

internal conversion to ground state

<u>In polar solvents</u>

$$D^* + A \rightleftharpoons D^*\!\!-\!\!A \longrightarrow D_s^+ + A_s^-$$

encounter complex

solvated ions

Triplets may also be produced via enhanced intersystem crossing in encounter complex:

$$^1D^*\!\!-\!\!A \longrightarrow {}^3D^* + A$$

or by ion-recombination reactions:

$$D^+ + A^- \longrightarrow {}^3D^* + A$$

1.1.2.2 Collisional deactivation mechanisms involving energy transfer

(a) Exchange mechanism (Dexter 1953)

When the donor and acceptor are in close collisional contact, the electron clouds of the colliding molecules will coalesce resulting in the indistinguishability of the highest energy electrons of the donor and acceptor. Exchange of electrons between donor and acceptor can take place so that, for example, an electron in a donor triplet state may become part of the electronic structure of the acceptor originally in the S_0 state. While the donor is deactivated to the ground state S_0 by acquiring an electron from the acceptor, simultaneously, the acceptor will be raised to the S_1 state by the acquisition of an electron from the donor in the triplet state (triplet - singlet energy transfer). The exchange mechanism of energy transfer is most efficient when the equilibrated excited state of the donor lies slightly higher than the Frank-Condon excited state of the acceptor. Singlet-singlet and triplet-singlet energy transfer between donor and acceptor are permitted by the exchange mechanism. Furthermore, the exchange mechanism can also effect an energy transfer which may involve the spin-forbidden transition of the acceptor, viz., singlet-triplet (occasionally) and triplet-triplet (quite commonly), provided that the donor singlet or triplet state is in a higher energy state than the acceptor triplet state (Schulman 1977). This is in marked contrast to the resonance energy transfer mechanism which precludes a spin-forbidden transition in the acceptor. (See Section 1.1.4 for further discussion). Since collisional

contact between donor and acceptor is a prerequisite for energy transfer by the exchange mechanism, the process is diffusion-controlled and is dependent on the viscosity of the medium.

(b) Delayed fluorescence

In rigid and viscous media, the phenomenon of delayed fluorescence is occasionally observed in addition to phosphorescence. It occurs at the wavelength of "normal" fluorescence (provided the molecule is also fluorescent at the temperature of measurement) but has a lifetime comparable to that of phosphorescence. Delayed fluorescence is a result of the excitation of the molecule in the triplet state back to the lowest excited singlet state (ie reverse intersystem crossing), and can be effected by two mechanisms. Delayed fluorescence brought about by thermal excitation (ie by warming) is exhibited only by certain molecules which have a small energy difference between the S_1 and T_1 states. This was first observed in eosin and hence the phenomenon has been classified as E-type delayed fluorescence. In the other mechanism for delayed fluorescence, two triplet molecules would collide resulting in the activation of one to the S_1 state and the deactivation of the other by internal conversion to the S_0 state (triplet-triplet annihilation). Since the phenomenon was first observed in pyrene it has been called P-type delayed fluorescence. The mechanism requires the simultaneous annihilation of two triplet molecules and so P-type delayed fluorescence is a biphotonic process which requires a sufficiently high concentration of the absorbing molecules. The intensity of P-type delayed fluorescence is proportional to the square of the intensity of the exciting light.

All the above mechanisms for the collisional deactivation of the excited donor would result in the fluorescence quenching of the donor (if it is fluorescent) with or without a concomitant energy transfer to the acceptor. As only the excited state of the donor is involved, this type of fluorescence quenching is diffusion-controlled and is called dynamic quenching. Donor fluorescence intensity can also be quenched if the number of potential excited state donor is decreased eg. by the complexation of ground state donor and acceptor ($D + A \longrightarrow D—A$) or the formation of a ground state dimer ($D + D \rightarrow D—D$). The dimer or donor-acceptor complex when excited has a zero or negligible quantum yield, and even though it may dissociate in the excited state to produce the luminescing species, the dissociation process is usually less efficient than the nonradiative conversion of the excited complex to its ground state. This type of quenching is not diffusion dependent and is referred to as static quenching.

1.1.3 Noncollisional energy transfer

Energy transfer that occurs over distances larger than the contact distances of molecular collision is called a noncollisional energy transfer (Forster 1947,1959;Perrin 1932). In resonance transfer, the energy of the excited donor is lost via transfer to the acceptor without any emission of radiation or any collisional contact between the donor and acceptor. Various other equivalent terms have also been used in the literature to describe this class of energy transfer process: sensitized fluorescence (if acceptor is also fluorescent), singlet-singlet nonradiative energy transfer, dipole-dipole

resonance transfer, long-range radiationless energy transfer, Forster-type energy transfer, etc. Resonance energy transfer is the term used in this thesis to describe this type of non-radiative energy transfer which should be distinguished from the "trivial" process of reabsorption of the donor emission by the acceptor. The efficiency of the trivial reabsorption process depends on (i) the spectral overlap between the donor emission spectrum and the acceptor absorption spectrum, and (ii) the Beer-Lambert law which states

$$I_a = I_o[1 - 10^{-abc}] \qquad (1.6)$$

where I_o and I_a are the intensities of the incident and absorbed radiation, a is the (molar) absorption coefficient, b the optical pathlength, and c the concentration. The qualifying word "trivial" used to describe the process of radiative energy transfer is a misnomer: life on earth depends upon the radiant energy from the sun. Livingstone (1957) called the process "trivial not because it is unimportant but because it is easy to explain".

1.1.4 Resonance energy transfer

1.1.4.1 Historical background

Historically, the first recorded observation of resonance energy transfer was made in 1923 by Cario and Frank who found that a gaseous mixture of mercury and thallium showed the emission spectra of both atoms even though the excitation radiation was set at the 254 nm mercury resonance wavelength. The experimental set-up in this case excluded the possibility of indirect excitation of thallium by reabsorption

of the mercury emission. Subsequent work by Bowen and Brocklehurst in 1955 on energy transfer between 1-chloro-anthracene and perylene showed very clearly that the non-collisional energy transfer was not brought about by the process of trivial reabsorption. That the resonance energy transfer process was also not a consequence of a collisional mechanism had already been established in 1949 by Forster (1949a,b) who found that the fluorescence quenching of trypaflavin by rhodamine B occurred over a distance of 7 nm, which was much greater than the collisional molecular dimensions. The efficiency of the resonance energy transfer process had been demonstrated earlier in 1947 by Bucher and Kaspers in their investigations on the transfer efficiency between the chromophoric groups in myoglobin. Their results were later confirmed by Weber and Teale (1959) who found an almost 100% transfer efficiency in this particular system. Even though the first work on the quantitative aspects of resonance energy transfer was done by J. and F. Perrin (1925,1927 and 1932), it was Forster who developed in 1948 a complete theory underlying the mechanism of the resonance transfer process.

1.1.4.2 Transfer mechanism

In the classical sense, an electron in a molecule which has been excited to a singlet or triplet state behaves like a charged oscillator with a characteristic frequency superimposed on its electrical field (which extends beyond the molecular dimension). Transfer of its excitation energy to a second molecule (acceptor) within this electrical field can take place if the electronic transition of the acceptor

corresponds to the frequency imposed on the electrical field by the excited oscillator (donor). This would require that the transitional energy difference from the excited state to the ground state in the donor is the same as the reverse process in the acceptor. However, an electronic transition in a molecular oscillator (cf. free atoms) with various vibrational transitions superimposed on it does not correspond to a fixed frequency. Instead, it is characterised by a whole distribution of frequencies. Consequently, a continuous spectrum of frequencies is also imposed on the electrical field in any one type of molecular electronic transition, and a certain frequency interval within this spectrum can cause the excitation of the acceptor. Maximum spectral overlap between donor emission and acceptor absorption is therefore an important prerequisite for resonance energy transfer to occur. Furthermore, there must also be an optimum relative orientation to permit strong interaction between the oscillators. As will be shown in Equation (1.10) below, the rate of resonance energy transfer varies inversely as the sixth power of the distance between the donor and acceptor, and there must be a certain minimum distance (the critical distance r_o, See Equation(1.10)) for efficient energy transfer (Stryer 1960). The mean intermolecular distance decreases with higher concentrations of both donor and acceptor, so resonance energy transfer is a concentration dependent phenomenon. Other parameters which determine the rate of resonance energy transfer include the lifetimes and therefore the spin multiplicities of the excited states of the donor, as well as the probabilities (ie molar absorptivities) of the electronic transitions involved in the

acceptor. The lowest excited singlet state of the donor has a shorter lifetime (10^{-8} s) than the triplet state (10^{-4} to 10 s). However, excitation to the lowest excited singlet state of an acceptor has a higher probability than transition from the ground to the triplet state (spin-forbidden transition). Thus, the acceptor can be excited to the excited singlet state via the resonance mechanism by a donor in the lowest excited singlet state (singlet-singlet energy transfer) or triplet state (triplet-singlet energy transfer). However, because of the spin-forbidden transition from the ground to the triplet state of an acceptor, singlet-triplet and triplet-triplet resonance energy transfer resulting, respectively, in singlet- and triplet- sensitized phosphorescence have very low probabilities and such phenomena are not generally observed.

The conditions for resonance energy transfer are very similar to those for trivial reabsorption (Refer Section 1.1.3) and special precautions must be taken to minimize the contributory effect of one on the observation of the other. In most studies of resonance energy transfer, trivial reabsorption can be obviated by keeping the absorbance (optical density) of the solution low (< 0.005 for 1 cm optical pathlength) or altering the optical layout for sample illumination (eg. front-surfance fluorescence measurements; smaller optical pathlength cuvette). Some of the characteristic properties associated with resonance transfer and trivial reabsorption had been identified by Forster (1959) and are summarized in Table 1.1 which can be used for distinguishing between the two mechanisms.

Table 1.1

Some characteristic properties of resonance energy transfer and trivial reabsorption (Forster 1959).

Properties	Resonance transfer	Reabsorption
Dependence on sample volume	None	Increase
Dependence on sample conc.	Increase	Increase
Dependence on viscosity	None	None
Donor lifetime	Decreased	Unchanged
Donor fluorescence spectrum	Unchanged	Changed
Absorption spectrum	Unchanged	Unchanged

1.1.4.3 Some quantitative aspects (Forster 1948,1951;Pesce 1971)

The field strength E at a distance r from an excited electron oscillating at a point D is given by

$$E = \frac{kqa}{\eta^2 r^3} \qquad (1.7)$$

where q is the electronic charge, k the orientation factor, a the amplitude of oscillation at D, η the refractive index, and r the distance between the acceptor A and the donor at D.

After time t of exact resonance, oscillator A has acquired the energy

$$W_A = \frac{q^2 E^2 t^2}{8m} \qquad (1.8)$$

where m is the mass of the electron. Thus, the interaction energy between the oscillators is proportional to the inverse sixth power of the distance (Equation (1.7) and (1.8)) and the rate of energy transfer between donor and acceptor is

$$\frac{-dW_D}{dt} = \frac{dW_A}{dt} = N_{D\rightarrow A}\, h\nu \qquad (1.9)$$

where $N_{D\rightarrow A}$ is the number of energy quanta ($h\nu$) transferred per unit time.

For a given donor-acceptor system, a critical distance r_o has been defined:

$$N_{D\rightarrow A} = \frac{1}{\tau_0}\left(\frac{r_o}{r}\right)^6 \qquad (1.10)$$

where τ_0 is the lifetime of the excited state of the donor in the absence of transfer. ($\frac{1}{\tau_0}$ is the rate of its deactivation in the absence of acceptor). Consequently from Equation (1.10), r_o is the distance at which there is an equal probability for resonance energy transfer and for intramolecular deactivation of the excited state of the donor (by radiative or nonradiative processes). An expression for r_o had been derived by Forster (1951), viz.,

$$r_o^6 = \frac{9 \times 10^6 (\ln 10)^2 k^2 c \tau_0 J_{\bar{\nu}}}{16 \pi^4 \eta^2 L^2 \bar{\nu}_o^2} \qquad (1.11)$$

where c = velocity of light, L = Avogadro's constant, $\bar{\nu}_o$ = arithmetic mean of the wave-number of maximum absorption and emission of donor, and $J_{\bar{\nu}}$ = overlap integral between emission spectrum of donor and absorption spectrum of acceptor.

The theoretical r_o values of some donor-acceptor pairs had been calculated (from Equation (1.11)) by Weber and Teale (1959) and Weber (1960) and these were generally in good

agreement with the experimental values - See Table 1.2 (Pesce et al 1971). (An experimental method for the determination of r_o by fluorescence depolarization had been described by Weber (1954,1960)).

Table 1.2 r_o values

Donor-acceptor pair	r_o/nm Theoretical from Eqn.(1.11)	Experimental
DNS - heme (ferrous)	6.3	4.3 - 6.7
DNS - heme (ferric)	5.8	3.7 - 6.9
Phenol-phenol	1.1	1.7
Indole-indole	2.3	1.6
Phenol-indole	1.5	2.0
Tryptophan-DNS	2.6	2.4

DNS = 1-Dimethylaminonaphthalene-5-sulphonate

1.2 Ligand Assay

1.2.1 Classical and labelled immunochemical methods

Immunochemical techniques which are widely used in the biomedical sciences exploit two outstanding properties of the immune response: sensitivity which refers to the ability of immunological reagents - antibodies or cellular receptors, or the secondary receptors such as complement and histamine - to respond to extremely minute quantities of the antigen; and the specificity of the immunological reaction which allows the detection of specific antigens in a matrix of potential interferences. The immunochemical methods differ, however, according to the mechanisms by which the antigen-antibody interaction is manifested. Classical methods such as precipitation, immunodiffusion, agglutination, complement fixation, anaphylaxis, etc are based on the gross or secondary and tertiary effects produced by the antigen-antibody interactions which are usually visible to the naked eye. Other methods utilize the increased sensitivity of the immunological interaction when the antibody or the antigen is bound to a distinctive label eg. isotope, enzyme. The use of labelled immune reactants converts to observable or detectable forms the primary antigen-antibody interactions which by themselves produce no visible effects at least in vitro. Labelled immunochemical methods generally trace their origins to the studies performed by Coons et al (1941,1942 and 1950) who used fluorescein-labelled antibody as a topographical marker for the localization of antigen in tissue cells. Other applications have included the specific identification and localization

of precipitation lines in immunodiffusion and immunoelectrophoresis (eg. radio-IEP), and the distribution of labelled lymphoid cells in the body. Table 1.3 lists the different types of labels and the corresponding immunochemical methods which had been developed on the same principles as Coon's immunofluorescence method. All these methods are essentially qualitative techniques which utilize the enhanced sensitivity associated with the use of labelled immune reactants for the detection and identification of antigens and antibodies in biological systems.

Table 1.3

Labelled immunochemical methods

Method/Technique	Label	Reference
Immunofluorescence	Fluorophore	Coons et al 1941, 1942 and 1950
Passive hemagglutination	Tanned red blood cell;erythrocyte	Boyden 1951; Borduas 1953
Immunoautoradiography	Radionuclide	Fitzgerald 1953
Immunoelectron-microscopy	Electron-dense compounds eg. ferritin	Singer 1951,1961
Immunoenzymatic method	Enzyme	Nakane 1966,1967; Avrameas 1966

1.2.2 Ligand assay

1.2.2.1 Introduction

The most significant quantitative application of labelled immune reactants was based on the use of radio-

isotopically labelled ligands in a technique called radio-immunoassay which was introduced in 1959 by the late Solomon Berson and his collaborator, Rosalyn Yalow. The radioimmunoassay methodology offers a technique to assay materials otherwise unmeasurable or detectable only with difficulty. The subsequent developments and exhaustive exploitation of the original principles of the method and its widespread and diversified application in the biomedical field stand as a testimony to the creative genius and industry of Berson and Yalow whose achievements were "accomplished by a spectacular combination of immunology, isotope research, mathematics and physics" (Nobel citation 1977).

Radioimmunoassay (RIA) was the first of what are now known collectively as ligand assays (Marks 1978), even though the technique has been variously termed in the literature depending on which aspect of the test principle is being emphasized in the definition. Thus radioimmunoassay has also been termed:

saturation analysis	antigen excess technique
displacement analysis	limited reagent technique
equilibrium analysis	isotope dilution technique
competitive binding assay	radioassay
competitive inhibition assay	radioligand assay
	radiosorbent assay

Notwithstanding the semantics, the general analytical concepts of radioimmunoassay are best expressed in the two terms competitive binding and saturation analysis (Zettner 1973), though the developments since the late 1960's have produced variants of the technique which are based on the principle of noncompetitive binding ie stoichiometric assays.See Section 1.2.2.3

1.2.2.2 Competitive binding assay (CBA)

The underlying theory of competitive binding assay is as follows:

L = Ligand (material to be assayed; the analyte)

L^* = Ligand tagged with a distinctive label eg. isotope

B = Binding agent; the binder eg. antibody Ab raised specifically against the ligand to be assayed; plasma binding proteins, etc.

On combining both L and L^* with B, the reaction of complex formation, which follows the law of mass action, can be represented as

$$\left[L + L^*\right] + \left[B\right] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \left[BL + BL^*\right] \qquad (1.12)$$

where $\left[L + L^*\right]$ = the combined molar concentration of the mixture of labelled and unlabelled ligand; $\left[B\right]$ = the molar concentration of the specific binding agent; $\left[BL + BL^*\right]$ = the combined molar concentration of the complexes between the binder and the unlabelled and labelled ligands; k_1 = the association constant; k_2 = the dissociation constant; and $k_a = k_1/k_2$ is the affinity constant of the binder (Parker 1976).

The complexes BL and BL^* can be looked upon as the bound fractions of L and L^* respectively, in contrast to the free or unbound ligand L and L^*. If a fixed quantity of B and L^* is used and the amount of B is adjusted so that it can bind (say) 50% of the total ligands, then at equilibrium the reaction system will contain both free unlabelled and labelled ligands (L and L^*) and both bound labelled and unlabelled ligands (BL and BL^*). It is implicit in this type of assay that the

binding properties of the binder do not distinguish between the unlabelled and labelled ligands ie. the binding sites of B possess identical affinity for L and L^*. (This assumption is not completely valid, but the problems arising therefrom are usually inconsequential in most assays).

Reproducibility of results would require that the system be made to attain equilibrium which is usually accomplished by allowing the reagents to react for a sufficiently long time (the incubation time) under fixed conditions. The time required to achieve this equilibrium may vary from several hours to days depending on the assay system (Zettner 1973). However, so long as sufficient time is allowed for establishing the equilibrium conditions, the order of addition of the reagents is not critical in "equilibrium" competitive binding assay. (Cf. Non-equilibrium competitive binding assay which also comes under various terms: sequential saturation, delayed addition, two-step analysis, etc (Zettner 1974)).

From Equation (1.12), it can be seen that since the amount of L^* and B are fixed, then the proportion of L^* bound to the binding agent is inversely proportional to the amount of unlabelled L. Conversely, the proportion of free L^* will be proportional to the amount of unlabelled L . Therefore, if various known amounts of L are added and BL^* or L^* is measured by the appropriate instrumentation, the relationship between the signal of the label and the concentration of L can be established. From this a standard curve can be obtained and used to quantitate the amount of L in an unknown sample.

In a heterogeneous assay, the quantitation of L by detecting the signal of the label in BL^* must be preceded by a separation step to separate BL^* from the reagent mixture containing the free L^*. If, however, the physicochemical properties of the label in the free and bound L^* are sufficiently different, then the change in the signal detection implicit in the label may be used to quantitate L without a need to separate BL^* from L^*. This type of assay is called homogeneous.

1.2.2.3 Noncompetitive binding assay

(a) Stoichiometric assay (immunometric; reagent-excess; labelled antibody)

In contrast to the competitive binding assay as exemplified by the radioimmunoassay, stoichiometric assay involves the use of labelled binding agent eg. labelled antibody Ab^*. In this connection, it must be emphasized that labelled binding agents are by no means precluded from use in some variants of competitive binding assay, just as it must not be inferred that labelled ligands cannot be used in non-competitive binding assay. See Sections 1.2.3.3 and 1.2.4 . The principal purpose of the stoichiometric assay, which was first suggested by Miles and Hales in 1968, is to improve the sensitivity which can be achieved with a given binding agent. In the reaction of the conventional RIA method (refer Equation (1.12)), there must be a limited number of binding sites available such that only a proportion of the ligands, labelled as well as unlabelled, is bound. In the stoichiometric assay, as exemplified by the immunoradiometric assay IRMA , a large

excess of the labelled binding agent is used to increase the forward (association) reaction, and a greater sensitivity potential might be expected. The assay method involves the addition of excess labelled binder B^* to standards (and unknown samples) of the ligand. After incubation, an excess of insolubilized ligand is added to bind the excess B^* which is removed by centrifugation. The amount of B^* in the final supernatant is thus directly proportional to the ligand initially present in the solution. Since the assay involves a separation of excess unbound B^* from B^*L, it is heterogeneous.

(b) Sandwich (two-site) assay

The sandwich assay (Addison and Hales 1971) is a variant of the stoichiometric assay in which a solid-phase antibody (the binding agent) is first reacted with the antigen (the ligand) and an excess of labelled antibody then added. The amount of labelled antibody associated with the solid-phase component is proportional to the original antigen concentration. The assay is also heterogeneous.

$$\vdash\!\text{Ab} \quad + \quad \text{Ag} \longrightarrow \vdash\!\text{AbAg} \xrightarrow{\text{Ab}^*} \text{AbAgAb}^*$$

1st reaction 2nd reaction

$\vdash$ denotes solid-phase

(c) Double sandwich assay (Beck and Hales 1975)

The double sandwich assay resembles the preceding method but uses an unlabelled antibody from a second species for the second reaction,

$$\vdash\!\text{Ab}_1\text{Ag} \xrightarrow{\text{Ab}_2} \vdash\!\text{Ab}_1\text{AgAb}_2$$

1st reaction 2nd reaction

A labelled heterologous antibody to the γ-globulin of this species is then used to produce the signal:

$$\vdash Ab_1AgAb_2Ab^*$$

3rd reaction

(Ab_1 and Ab_2 represent 1st and 2nd antibody, respectively)

An attractive feature of this assay system is that a single labelled preparation may serve for any number of different assays, provided only that they all employ the same species antibody at the second stage.

(d) Indirect sandwich assay for antibody detection

The most notable example of this approach is the radioallergosorbent test (RAST) of Wide et al (1967). Its greatest use is in identifying the concentrations of antibodies of defined immunoglobulin types.

It is unlikely that any of the stoichiometric assays will be applicable to the analyses of haptens. Hapten-antibody complex dissociates in the presence of solid-phase antigen, and sandwiches cannot be made with haptens because their small size would preclude the display of two independent binding sites.

1.2.2.4 Classification of ligand assay methods: Principles

Ligand assay methods are usually classified on the basis of one or more of the following principles.

(a) Separation methods (the original basis for classification)

eg. chromatoelectrophoresis
alcohol precipitation
charcoal and talc

second antibody
solid-phase Ab or Ag

Refer Ratcliffe (1974) for a review of methods for bound/free separation .

(b) Binding agents

eg. antibody

specific binding proteins (thyroid binding globulins with affinities for various circulating hormones; transcortin is a binding protein for cortisol).

cell membrane or tissue receptors (the uterine receptor for measuring estradiol)

(c) Nature of label

See Table 1.6 for a full list of the different types of labels that have been used in the development of ligand assays.

(d) Labelled materials

eg. labelled ligand (antigen)

labelled antibody (in stoichiometric and sandwich assay)

labelled 2nd Ab to 1st Ab (in indirect sandwich assay)

(e) Analytical principles

eg. competitive binding or saturation analysis

stoichiometric assay

sandwich (two-site) assay

indirect sandwich assay

homogeneous and heterogeneous assay

1.2.3 Isotopic and nonisotopic immunoassay methods

1.2.3.1 Developments in ligand assay techniques

The principal developments in ligand assay techniques after the introduction of the radioimmunoassay method in 1959 can be summarized as follows:

(a) the substitution of other binding agents for antibodies eg. plasma binding proteins, cell membranes and tissue receptors;

(b) the use of other radionuclides in addition to ^{131}I such as ^{125}I , ^{3}H , ^{14}C , and ^{75}Se ;

(c) the modification of the separation techniques (Ratcliffe 1974) which include the use of second antibody, solid-phase immune reactants (immunoadsorbents) on polystyrene tubes, beads and magnetizable particles;

(d) the use of labelled antibodies in assay techniques which are based on stoichiometric principles eg. immunoradiometric assay (IRMA) and sandwich assay;

(e) the use of nonisotopic labels as the tracer eg. enzymes, fluorophores, etc. See Table 1.6 ;

(f) the introduction of homogeneous assays for haptens eg.EMIT (Rubenstein 1972) where the difference in the physico-chemical properties of the label between the free and bound labelled ligands is utilized as a "substitution" for the separation step used in heterogeneous assays;

(g) the extension of the homogeneous assay concept to the measurement of macromolecules in addition to haptens eg. an EMIT assay for specific proteins (Gibbons et al 1979);

(h) the automation of heterogeneous ligand assays and the adaptation of the homogeneous assay technology to existing instrumentation; and

(i) the development of ultra-sensitive immunoassays in which two types of labels are used simultaneously to improve sensitivity eg. USERIA (Harris et al 1979) .

Many reviews have been published on the developments in the techniques as well as the underlying principles of the different types of ligand immunoassay . See Table 1.4 .

Table 1.4

Published reviews on ligand immunoassay methods

Subject	Reference
Radioimmunoassay	Parker(1972); Skelley et al(1979)
Enzymeimmuoassay	Scharpe et al(1976); Wisdom(1976); Schuurs et al(1977)
Fluorescence immunoassay	O'Donnell and Suffin(1979); Soini and Hemmila(1979)
Chemi- and Bio-luminescence immunoassay	Whitehead et al(1979)
Principles and Theory of Ligand Assay	Zettner(1973,1974); Walker(1977); Odell and Daughaday(1971)

1.2.3.2 Isotopic immunoassay methods: Advantages and disadvantages

The widespread and diversified applications of radioimmunological methods in the biomedical field may be related to the following advantages.

(a) High sensitivity (10^{-12} - 10^{-15} M) and specificity which are attainable with the use of radiolabelled materials.

(b) Wide applicability eg. analyses of hormonal and non-hormonal peptides, drugs, enzymes, parasitic, microbial and viral agents, etc. It was estimated that some 50 - 60 million radioimmunoassays were carried out worldwide in 1976 alone (New Scientist, 20 October 1977, p 144)

(c) Small sample size, usually from 10 - 100 µl .

(d) Speed of analysis and batch sampling capabilities, especially with the advent of multihead scintillation spectrometers for the analyses of several samples simultaneously.

The technique suffers, however, from certain drawbacks.

(1) Radiation hazards which impose several restrictions such as regulation of production, transport, handling and disposal of radioactive materials.

(2) The destructive process of radioactive decay limits the useful lifetime of assay kits and prevents long-term assay standardization.

(3) Radiation damage to immune reactants.

(4) Relatively expensive instrumentation and reagents, and a need for a special laboratory.

(5) The method being heterogeneous, the assay procedures are usually less amenable to automation.

1.2.3.3 Nonisotopic immunoassay methods

To overcome some of the limitations of radioimmunoassay methods, alternative immunological techniques are being developed which do not require the use of radiolabelled materials. These nonisotopic immunoassay methods (Table 1.6) take full advantage of the specificity and sensitivity that result from the application of antibodies but avoid the use of radionuclides. Other advantages are listed in Table 1.5 . A number of these nonisotopic immunoassays are already commercially available eg. EMIT (Syva Corporation), IMMUNO-FLUOR (BIO-RAD Laboratories), and FIAX/STiQ (International Diagnostic Technology). However, the success of these new immunochemical approaches will depend ultimately on the attainment of the sensitivity and specificity equivalent to that already achieved by the use of radiolabelled materials.

Some of the nonisotopic immunoassays listed in Table 1.6 are still in the developmental stage eg. voltammetric immunoassay, while others already developed have been found to unsatisfactory for routine application for a number of reasons. Thus, the use of free radicals in spin-immunoassay requires expensive electron spin resonance spectrometers for measurement. The utility of phage immunoassay is limited by the long periods

Table 1.5

Some advantages of nonisotopic immunoassay methods

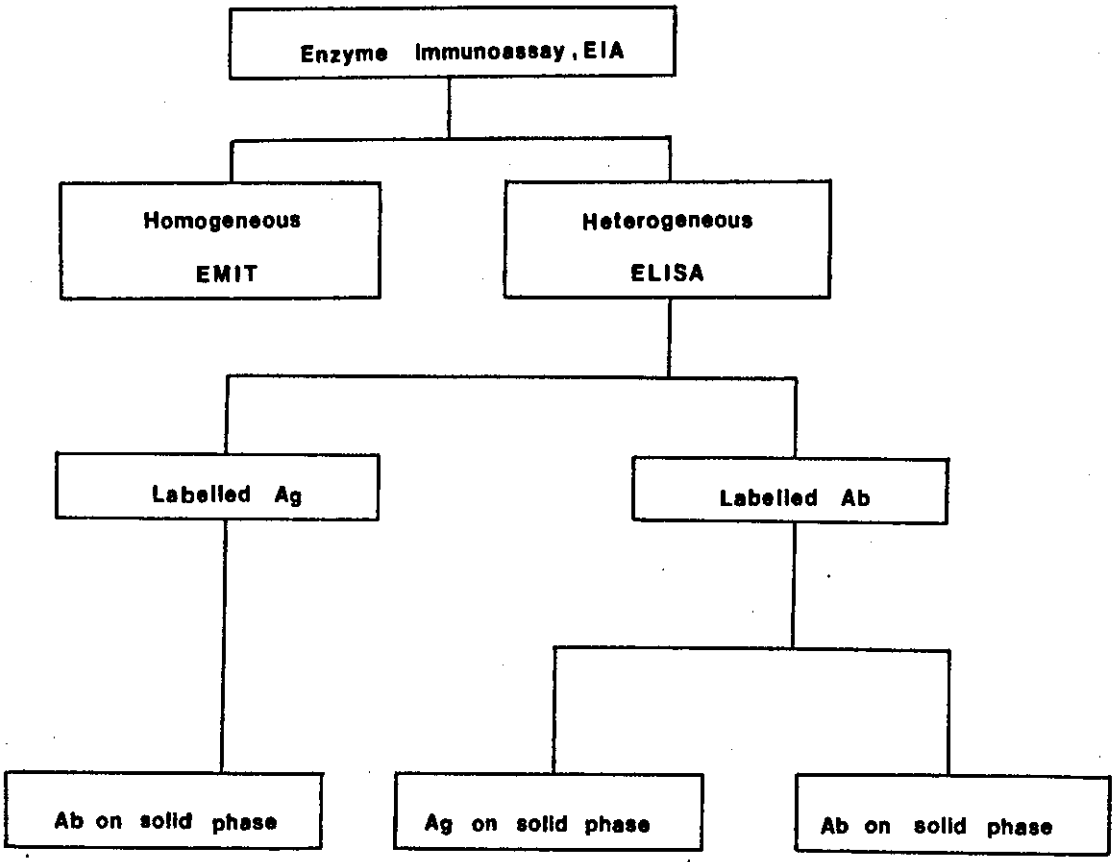
Equipment	:	Widely available and relatively inexpensive eg. uv/visible apectrophotometers, filter fluorimeters
Reagents	:	Comparatively cheap; long shelf-life eg. enzyme- and fluorescence-labelled materials
Label	:	Wide range (See Table 1.6). Choice of labels may allow multiple, simultaneous assays to be performed eg. in enzyme immunoassays. Physico-chemical properties of labels may also be exploited in addition to their use as signal tracers.
Assay procedure	:	Simple. Labelling procedure easily performed. Separation step may be obviated and thus there is a potential for automation.
Hazards	:	No special hazards.

of time required to culture the bacteria for the assay of phage activity. At the moment, it would appear that only enzyme and fluorescence immunoassays are capable of competing with, and possibly supplanting, RIA in a number of areas even though there are inherent limitations in each of these two nonisotopic immunoassays. Table 1.7a & b summarize the various types of enzyme immunoassay methods already developed. Fluorescence immunoassay methods will be discussed in detail in Section 1.2.4 .

Table 1.6 Ligand Immunoassay Technique

Immunoassay technique	Label	Reference
Isotopic		
Radioimmunoassay RIA	Radionuclide	Yalow & Berson 1959a,b and 1960
Nonisotopic		
Viroimmunoassay; Phage immunoassay	Bacteriophage	Haimovich 1966; Makela 1966
Hemagglutination inhibition technique HIT	Erythrocyte (cell)	Adler & Liu 1971
Enzyme immunoassay EIA	Enzyme	Engvall 1971a,b; Van Weeman 1971
Free radical assay technique FRAT; Spin immunoassay	Free radical	Leute et al 1972a,b
Fluorescence immunoassay FIA	Fluorophore	Aalberse 1973
Bioluminescence immunoassay BLIA	eg.ATP-labelled ligand with firefly luciferase detection	Carrico et al 1976
Chemiluminescence immunoassay CLIA	eg. Luminol with H_2O_2/haem detection	Pratt et al 1978
Metalloimmunoassay MIA	Metal ion complexes	Cais et al 1977
Voltammetric immunoassay VIA	Electroactive group	Weber & Purdy 1979; Heineman et al 1979
Isotopic & Nonisotopic		
Ultra-sensitive enzymatic radioimmunoassay USERIA	Enzyme and radionuclide	Harris et al 1979

Table 1.7a Classification of EIA methods



Ia: Competitive assay for Ag detection. Cf. classical RIA

Ib: Noncompetitive assay for Ab detection: Ag^{*} + Ab. Then excess ⊢Ab added to react with remaining Ag^{*}

IIa: Noncompetitive assay for Ag detection. Also called immunoenzymometric assay. Cf. IRMA

IIb: Competitive assay for Ab detection: ⊢Ag + Ab + Ab^{*} ⇌ ⊢AgAb + ⊢$AgAb^{*}$

IIIa: Sandwich assay for Ag detection

IIIb: Indirect sandwich assay [using labelled 2nd Ab] for Ab detection. Cf. RAST

Table 1.7b

Enzyme immunoassay methods

Method/Technique*	Reference
Heterogeneous (ELISA)	
Ia	Engvall and Perlmann 1971a,b; Van Weeman and Schuurs 1971
Ib	Schuurs and Van Weeman 1972
IIa	Maiolini et al 1975a
IIb	Schuurs and Van Weeman 1972
IIIa	Maiolini et al 1975b
IIIb	Engvall and Perlmann 1972
Homogeneous (EMIT)	Rubenstein et al 1972 Rowley et al 1975

* Refer Table 1.7a for a classification of EIA methods

1.2.4 Fluorescence immunoassay

Quantitative analyses of ligands using fluorescence immunoassay methods may be accomplished in one of two ways

(*) The fluorophores are used as labels or probes in the same way that radioactive isotopes are used in radioimmunoassays. Like RIA, these assays are heterogeneous, and similar methods to those used in RIA can be implemented as separation procedures eg. precipitation with ammonium sulphate, polyethylene glycol and various other organic solvents, double-

antibody precipitation and solid-phase separation. To date, solid-phase fluorescence immunoassay has received the most attention, and many variations of the technique have been developed and reported (See Table 1.8a and b)

In the solid-phase system, once the separation has been achieved then the fluorescent label can either be stripped off the solid-phase using a denaturant such as dilute alkali and measured in a standard fluorimeter, or the fluorescence can be recorded using a front surface attachment in a fluorimeter. With the latter method, the separation procedure is relatively straightforward and there is the added advantage of one less analysis step when reading the label attached to the solid-phase. Furthermore, endogenous interferences from the sample can be removed or minimized after the separation step. Of course, the sensitivity of the assay in this case will be limited by the optical properties of the solid-phase material as well as light scattering interferences. These drawbacks can be overcome by using solid-phase materials which are not strongly absorbent at the excitation wavelength, or fluorescent at the emission region. Light scattering interferences can be removed by using cut-off filters, polarizers or specially designed instrumentation for front surface fluorescence measurements. Another major problem of the solid-phase system relates to the difficulty of producing reproducible reagents: the attachment of antibody on solid-phase by physical adsorption is not very reproducible over long periods of time and subject to antibody loss on storage, and covalent coupling often results in the reduction of antibody binding capacity. The latter problem can be overcome by

using secondary antibody immobilized on the solid-phase which binds the primary antibody. However, the need to use considerable amounts of the secondary antibody will be a constraining factor as far as assay cost is concerned.

(2) When a fluorescent-labelled ligand L^* binds to the antibody (the binder B), a change in the physicochemical properties of the fluorophore can occur as a result of the differences in the solvation effects, hydrophobic binding, hydrogen binding and ionic interactions within the micro-environment of the antibody binding site. In particular, if the fluorescence properties of the bound labelled ligand (BL^*) are sufficiently different from those of the unbound labelled ligand L^*, then this change in the fluorescence properties may be used to quantitate the ligand (ie the analyte) in an immunoassay without a separation of BL^* and L^*. This type of assay is called homogeneous fluorescence immunoassay and the fluorescence properties which have been studied to date include:

Fluorescence polarization
Fluorescence quenching and enhancement
Fluorescence energy transfer
Fluorescence lifetime (time-resolved fluorimetry)

In addition, conjugates have also been used as labels which depend on additional enzymatic reactions to yield fluorescent products (Burd 1977).

(a) Fluorescence polarization

Polarization of fluorescence is based on the relative orientation of the excitation and emission oscillators.

The degree of polarization p can be calculated from readily measurable quantities:

$$p = \frac{I_V - GI_H}{I_V + GI_H} \qquad (1.13)$$

where I_V = the vertical component (ie parallel to the excitation polarization)

I_H = the horizontal component (ie perpendicular to the excitation polarization)

G = the grating factor

p is a function of the molecular size and shape, the viscosity of the medium η, the decay time τ of the excited state, and the temperature T. The Perrin equation for a spherical molecule as derived by Weber (1952a) is:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_o} - \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V}\right) \qquad (1.14)$$

where p_o is the polarization observed in the absence of rotary Brownian motion, V the molar volume assuming a spherical molecule, and R is the gas constant.

When a small fluorescent-labelled hapten or antigen L^* reacts with the antibody, amongst other effects, the rotary motion of L^* is retarded due to the increase in effective size. There will thus be a corresponding increase in the degree of polarization, which will be reversed by the addition of unlabelled ligand. The fluorescence polarization immunoassay requires a fluorophore with a relatively long decay time (>20 ns), and though it is a relatively simple technique its application in routine analysis is limited by the expense for

instrumentation and endogenous background.

(b) Fluorescence quenching and enhancement

Following the formation of the ligand-antibody complex, the changed molecular environment within the immune complex will alter the electronic energy distribution of both the ligand and the fluorophore that is attached to it. In most cases, this will result in the label fluorescence being quenched (Shaw 1977), while in others an enhancement phenomenon may be observed. Smith (1977) had reported that the fluorescence quantum yield of fluorescein-labelled thyroxine (T_4) was increased approximately four-fold upon binding to antibody. The increase in fluorescence was explained in terms of the quenching effect of iodine (the heavy atom effect), which was partly dispersed as the thyroxine binds to the antibody. Similar enhancement immunoassays using the more convenient fluorescamine (Fluram , Roche Diagnostics Ltd) and its closely related analogue, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) , have also been developed. However, the mechanism underlying these fluorescamine-based assays (Miller 1979) is not the same as that described above for fluorescein-labelled T_4 . The "Fluram" enhancement phenomenon appears to be a general effect with all the fluorescamine-labelled antigens studied so far, and this has been applied to the development of fluorescence enhancement immunoassays for nortriptyline (Chapter 5) , human serum transferrin (Chapter 6) and immunoglobulin G (Chapter 7).

In a general immunochemical method described by Zuk et al (1979), the formation of an immune complex of a

fluorescent-labelled ligand sterically protects the fluorophore from binding by antibodies to it. The addition of unlabelled ligand prevents the formation of the fluorescent-labelled ligand immune complex, and allows the anti-fluorophore antibodies to quench the fluorescence by binding to the fluorophore. This homogeneous assay technique has been variously termed in the literature, viz., fluorescence protection immunoassay (Zuk et al 1979), indirect quenching fluoroimmunoassay (Nargessi et al 1978), and double-receptor fluorescence immunoassay (Ullman 1973).

(c) Fluorescence energy transfer

This is another general immunochemical method for the assay of haptens and proteins first described by Ullman et al (1976). In this method, ligand labelled with the donor fluorescent molecule is reacted with its specific antibody labelled with the acceptor molecule. Since resonance energy transfer is based on a short-range (< 5 nm) nonradiative mechanism (Refer Section 1.1.4), it is only within the molecular dimension of the immune complex formed that transfer of energy from the donor group to the acceptor group can take place. The energy transfer effects are manifested by the quenching of the donor fluorescence intensity and (possibly) the enhancement of the acceptor fluorescence intensity. Competitive binding by added unlabelled ligand will inhibit the interaction between labelled ligand and antibody, and the consequent reversal of the quenching and enhancement effects may be used to quantitate the ligand ie the sample.

Since the assay involves a change in the fluorescence properties of one or both of the labels, the assay does not require a separation step ie it is homogeneous. Furthermore, the simultaneous observation of the quenching and enhancement effects may be used to distinguish specific ligand-antibody combination from non-specific environmental effects on the fluorescence intensity. The assay requires, however, the use of pure ligand and monospecific antibody for the preparation of labelled immune reactants because the presence of non-specific labelled materials in the assay system will increase the background fluorescence and thus limit sensitivity. So, an alternative sandwich type of assay may be used with a poly-valent ligand in which separate portions of the antibody are labelled with the donor and acceptor groups. Unlabelled ligand causes the aggregation of the separately labelled antibody components and the effects of energy transfer are again observed. With this approach, the use of pure or enriched ligand is not necessary.

The development of energy transfer immunoassay methods for the analyses of low and high molecular-weight ligands will be described in the appropriate chapters below.

(d) Time-resolved fluorimetry

In time-resolved fluorescence measurements, a fast light pulse (<1ns) is used to excite the fluorescent label whose excited state has a lifetime τ between 100 ns - 1 μs. Measurement of fluorescence is started after a certain time

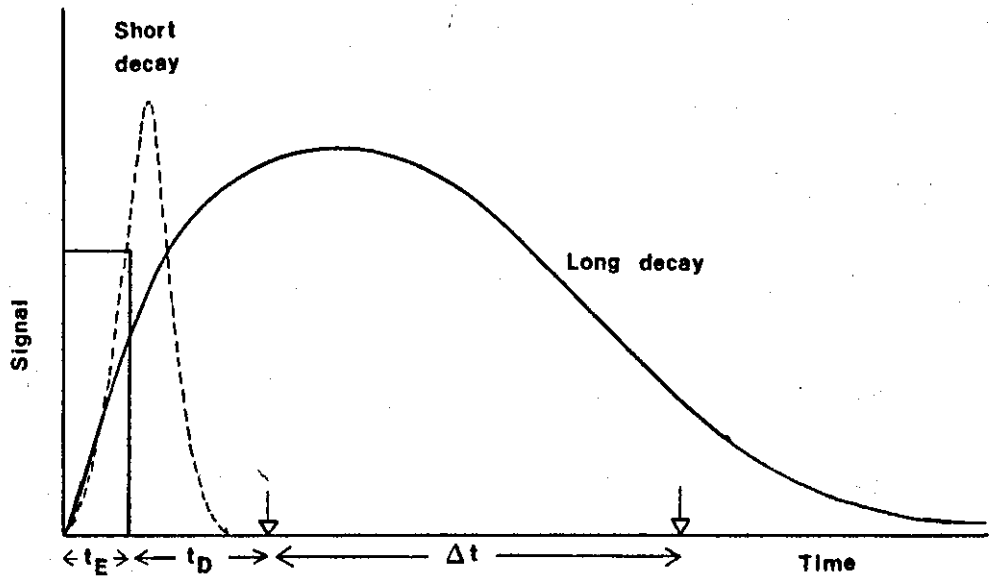


Fig 1.2 Diagram of time resolved fluorescence measurements

t_E = Excitation pulse time (<1 ns)

t_D = Delay time

Δt = Counting time interval

(the delay time t_D) has elapsed when the background fluorescence with a decay time of less than 10 ns would have been reduced almost to zero (See Fig 1.2). The fluorescence of the label is measured at certain time intervals (Δt, the counting time), starting from time t_D . Thus time resolved fluorimetry provides an elegant way of removing non-specific background interferences with a consequent improvement in sensitivity.

Two types of time resolved systems have been used to measure the decay times of fluorescent labels:(i) for short decay times up to 200 ns, and (ii) for long decay times up to 1 μs . A time resolved fluorimeter for short decay time measurements requires a very fast PMT detector together with

either a nanosecond spark discharge lamp or a dye laser controlled by a nitrogen laser for excitation. These instruments are expensive and are commonly used for such fluorophores as the pyrene derivatives, N-(3-pyrene)-maleimide and pyrene butyrate (with τ ca. 100 ns), and some other organic labels with decay times less than 20 ns. Measurement of long decay times (1 μs - 1 ms) can be performed with an inexpensive and commercially available xenon-discharge lamp for excitation and an ordinary PMT detector equipped with an electronic switch (switching time $\leq$1 μs). Such an instrument is suitable for work with the rare earth metal chelates eg. europium and terbium complexed with various β-diketones and salicylates exhibit very long decay times from 50 μs to 1 ms.

To date, there has been no published report of a time resolved fluorescence immunoassay. However, if suitable chelates are available and if these can be conjugated to the immune reactants without producing adverse immunological effects, then it can be envisaged that time-resolved fluorescence immunoassays may attain even greater sensitivity than RIA (Wieder 1978).

(e) Enzymatic reaction to yield fluorescent products.

$$L^* \xrightarrow{\text{enzyme}} \text{fluorescent products} \qquad (1.15a)$$

$$B + L^* \rightleftharpoons BL^* \xrightarrow{\text{enzyme}} \text{no reaction} \qquad (1.15b)$$

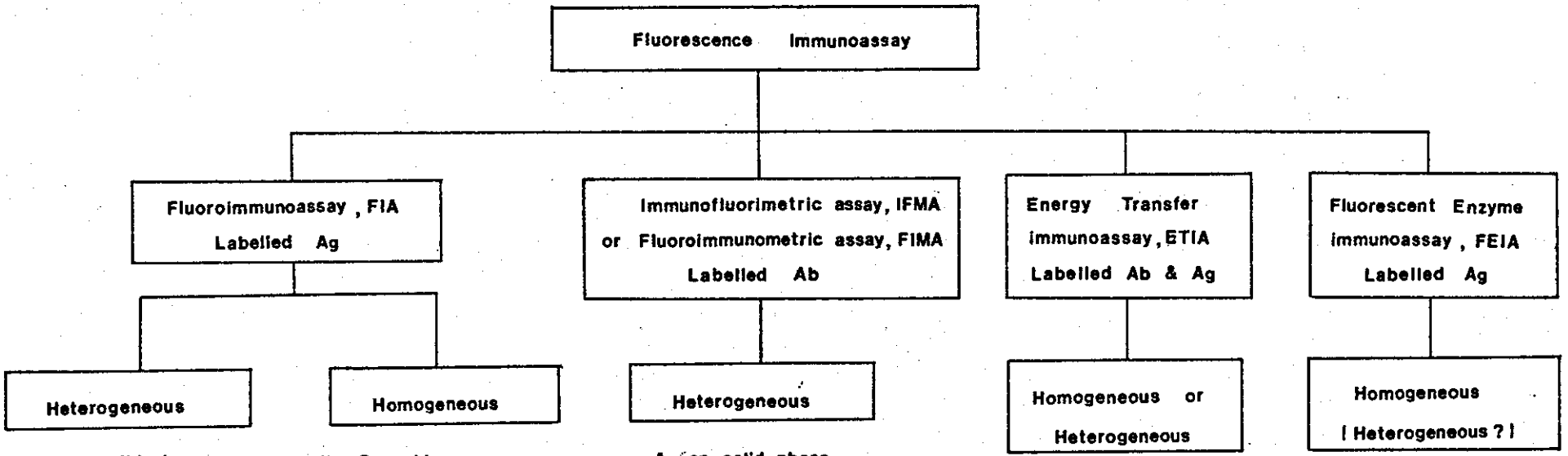
$$BL^* + L \xrightarrow[\text{displacement of } L^* \text{ by L in competitive binding reactions}]{} BL + L^* \longrightarrow \text{fluorescent products} \qquad (1.15c)$$

The assay is based on the enzymatic reaction with the substrate-labelled ligand to yield fluorescent products (Equation (1.15a)). The reaction principles as given above show that the substrate-labelled ligand conjugate as an enzymatic substrate is inactivated when it is bound to the antibody (the binder B) - Equation(1.15b) This inactivation is relieved by the presence of unlabelled ligand in competitive binding reactions (Equation (1.15c)). Hence, the rate of production of fluorescence will be related to the ligand (sample) concentration. Since the assay utilizes a change in the physicochemical properties of the substrate-labelled ligand upon binding to antibody, no separation step is required in the procedure. This fluorescence enzyme immunoassay (FEIA) has also been termed as homogeneous reactant-labelled fluorescence immunoassay (Burd 1977). Applications of this immunoassay method include the analyses of gentamicin (Burd 1977) and phenytoin in serum (Wong 1979).

A common drawback of all the homogeneous fluorescence assays described above is the difficulty of removing the endogenous background of the test sample. Though the assay sensitivities as low as 0.1 nmol have been reported in the literature, interferences may reduce this limit by several orders of magnitude. Nevertheless, the assays being homogeneous, the experimental procedures are more amenable to automation

A classification of the various types of fluorescence immunoassay together with the references are given in Table 1.8a and 1.8b .

Table 1.8a Classification of FIA methods



Ab on solid-phase

Ia: Competitive assay for Ag detection. Cf. classical RIA

Ib: Noncompetitive assay for Ab detection [?]

IIa: Quenching

IIb: Enhancement

IIc: Protection; [indirect quenching; double-receptor assay]

IId: Polarization

IIe: Lifetime; [Time-resolved fluorimetry]

IIf: Others ?

Ag on solid-phase

IIIa: Noncompetitive assay for Ag detection. Cf. IRMA

IIIb: Competitive assay for Ab detection ?

Ab on solid-phase

IIIc: Sandwich assay for Ag detection.

IIId: Indirect sandwich [using labelled 2nd Ab] for Ab detection ? Cf. RAST

IVa: Competitive assay. Ag and Ab labelled with donor and acceptor, respectively.

IVb: Sandwich assay: Separate portions of Ab labelled with donor and acceptor.

IVc: Solid-phase ETIA

Va: Substrate-labelled

Vb: Enzyme-labelled

Vc: Cofactor-labelled

Table 1.8b Fluorescence immunoassay methods

Method/Technique*	Reference/Remarks
Fluoroimmunoassay	
Heterogeneous	
Ia	Nargessi 1978a ; Kamel 1978
Ib	? Analogous to type Ib of EIA;(see Table 1.7a & b)
Homogeneous	
IIa	Shaw et al 1977
IIb	Smith 1977
IIc	Ullman 1973 : double-receptor Zuk 1978 : fluorescence protection Nargessi 1978b : indirect quenching
IId	Watson et al 1976
IIe,f	? Time-resolved immuoassay may have the potential for even more sensitivity than RIA (Wieder 1978)
Immunofluorimetric assay	
IIIa	eg. FIAX/STiQ system; Blanchard 1978
IIIb	? Analogous to type IIb of EIA; (see Table 1.7a,b)
IIIc	eg. IMMUNO-FLUOR system; Aalberse 1973; Burgett 1977
IIId	? Cf. RAST. Analogous to type IIIb of EIA (see Table 1.7a,b)
Energy Transfer Immunoassay	
IVa,b	Ullman et al 1976
IVc	? See Chapter 7
Fluorescent Enzyme Immunoassay	
Va	Burd et al 1977
Vb	These assays generally make use of chemi- and bioluminescence as the detection system. See Whitehead 1979
Vc	

*Refer Table 1.8a

1.3 Fluorophores as Labels in Fluorescence Immunoassay

1.3.1 General requirements for fluorescence labels

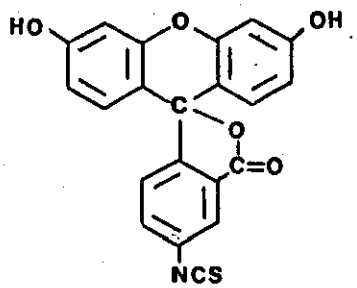
Most labels utilized in fluorescence immunoassays were developed for other purposes (eg. as a topographical marker in immunofluorescence microscopy and as a probe in the investigation of the structural changes in proteins, etc.), and there are deficiencies in these labels. The most significant drawback relates to the broad absorption and emission bands because this reduces the specificity of the label. In resonance energy transfer studies, a principal criterion in the choice of suitable donor and acceptor fluorescent labels is the absence of spectral overlap between the excitation spectra of both labels in addition to the prerequisite that there must be optimum spectral overlap between the donor emission and acceptor excitation. This important factor is well illustrated by the dependence on the fluorimeter spectral bandwidth of the energy transfer efficiency between fluorescein and rhodamine when these are used as labels in energy transfer immunoassays (See Chapter 3 ; also refer Schiller 1975). In the search for new fluorescent labels designed for specific types of assays, in particular for labels for time-resolved fluorimetry, attention has focussed on the fluorescing metal chelates which exhibit large Stoke's shifts and long decay times (Soini and Hemmila 1979). These emit well-defined, spectrally distinct peaks that would permit simultaneous measurements

Table 1.9 Some properties of an ideal fluorescence label

Property		Desirable Characteristics
Molar absorbance	:	As high as possible, $> 10^4$
Quantum yield	:	As high as possible especially in buffered aqueous solutions at pH 7 - 8.
Excitation and emission wavelengths	:	The excitation and emission wavelengths must be as far removed as possible from those background regions where interferences are significant. In most immunoassays, these interferences arise from the serum samples and the antiserum which have excitation and emission maxima at ca. 340 nm and 470 nm, respectively (Soini 1979). High protein concentration also causes scattering interferences (Rayleigh, Raman and 2nd order scattering). So, wavelength requirements are (a) λ_f between 500-700 nm, (b) a Stoke's shift > 50 nm, and (c) λ_{ex} in the visible region to avoid uv irradiation of the sample.
Stability	:	Should be stable both under measurement conditions and on storage so that long-term standardization of assay procedures can be performed.
Conjugation to ligand	:	Conjugation procedures must be simple and capable of producing reproducibly pure reagents. Labels must possess flexibility in functional group accessibility and form stable covalent bonds to the compounds under investigation without producing any adverse immunological effects.
Solubility	:	High solubility in aqueous solutions so that even the use of heavily-labelled antibodies or antigens will not decrease the solubility of the conjugates.

without interference. However, the widespread application of the existing and new fluorescent labels in immunoassays is limited by the deficiencies in our knowledge of the electronic structural details of the label and the intricate changes in the electronic energy distribution when the labelled immune complex is formed. The desirable characteristics of an ideal fluorescence label are summarized in Table 1.9

1.3.2 Fluorophores and the chemistry of their conjugation to functional groups in ligands eg. proteins



Fluorescein isothiocyanate FITC

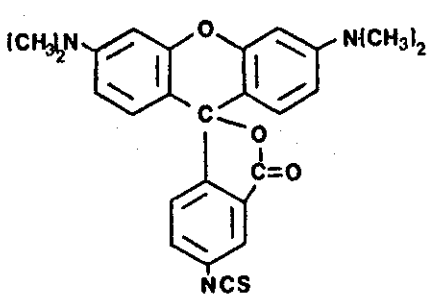
$C_{21}H_{11}NO_5S = 389.38$

$$R{-}N{=}C{=}S + \begin{cases} H_2N{-}Protein \rightarrow R{-}NH{-}CS{-}NH{-}Protein & (1.16a) \\ HS{-}Protein \rightarrow R{-}NH{-}CS{-}S{-}Protein & (1.16b) \end{cases}$$

Fluorescein isothiocyanate is by far the most commonly used label in fluorescence immunoassays because of its high quantum yield (0.85) and molar absorptivity (7.2×10^4 $M^{-1}cm^{-1}$). Its disadvantages include a small Stoke's shift (20-30 nm), the presence of two isomers in commercial preparation which causes heterogeneity in conjugation (Brandtzaeg 1973; Goding 1976), and its limited solubility in water though dimethylsulphoxide

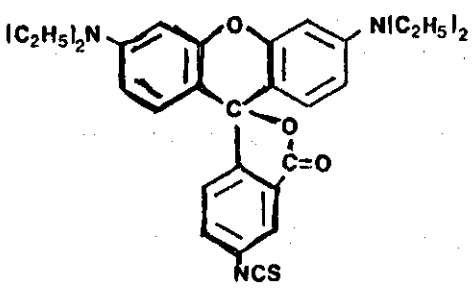
(DMSO) may be used as the solvent (up to 30% DMSO in carbonate buffer) without causing any deleterious effects on gamma-globulins (Bergquist and Nilsson 1974; Goding 1976). Blakeslee and Baines (1976) had reported that the replacement of the isothiocyanate functional group with the dichlorotriazinyl group would produce substantially improved stability, homogeneity and simplicity of conjugation.

The next important group of fluorescent labels are the various rhodamines: tetramethylrhodamine isothiocyanate (TRITC), rhodamine-B-isothiocyanate (RBITC), and lissamine rhodamine-B200 sulphonyl chloride (RB200SC). Compared to FITC, the rhodamine emission wavelengths fall in the 585-595 nm region but their relative fluorescence intensities are less (Brandtzaeg 1975; McKinney 1975). Advantages of the rhodamine labels include their stability and homogeneity (Brandtzaeg 1973; Chadwick 1958)



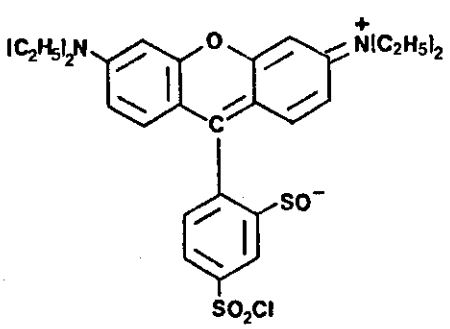
TRITC

$C_{25}H_{21}N_3O_3S = 443.51$



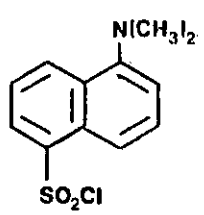
RBITC

$C_{29}H_{29}N_3O_3S = 499.63$



RB200SC

$C_{27}H_{29}N_2O_4S_2Cl = 545.11$



Dansyl Chloride

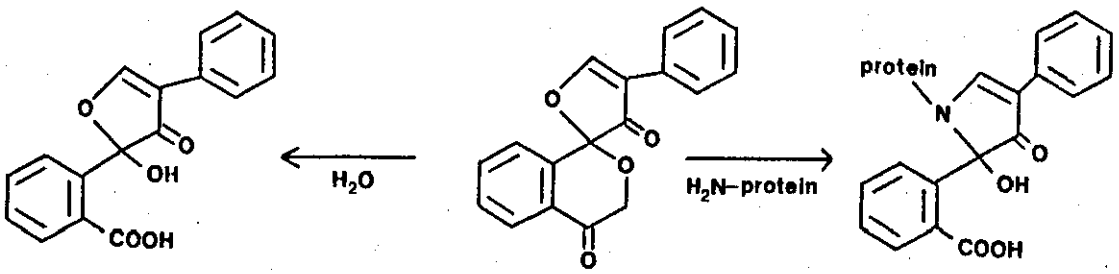
$C_{12}H_{12}NO_2SCl = 269.75$

$$R\text{-}SO_2Cl + \begin{cases} H_2N\text{—Protein} \longrightarrow R\text{-}SO_2\text{—NH-Protein} & (1.17a) \\ HO\text{-}C_6H_4\text{-Prot} \longrightarrow R\text{-}SO_2\text{—O-}C_6H_4\text{-Prot} & (1.17b) \end{cases}$$

Dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride; DNS-Cl) has a comparatively low quantum yield (0.3) and its excitation and emission wavelengths overlap those of serum (Soini 1979). Its sensitivity to polarity changes has been utilized to investigate the formation of antigen-antibody complexes (Parker 1970).

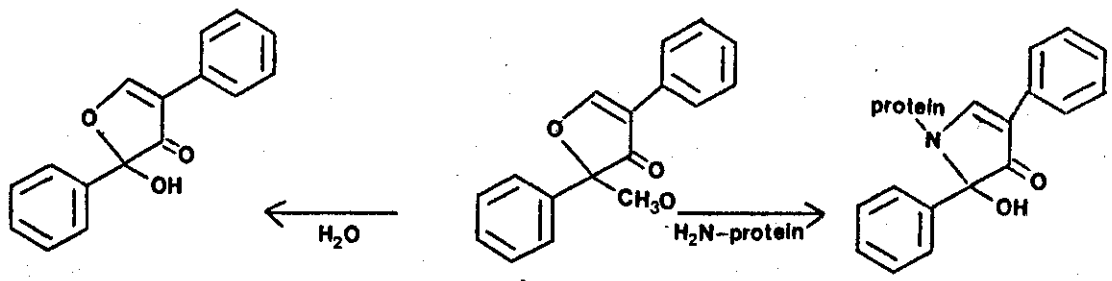
4-phenylspiro[furan-2(3),1'-phthalan-3,2-dione] (Fluorescamine;Fluram) and its closely related analogue, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF), react rapidly with primary amino groups to yield fluorescent products (Weigele 1972, 1973). The use of fluorescamine in immunoassays has been reported (Katsch et al 1974; Handley 1979), and although the fluorescent conjugate has a relatively small quantum yield (0.1), its sensitivity to polarity changes may be exploited (Miller 1979). It had been claimed (Handschin and Ritschard 1976) that the fluorescence of MDPF was more stable than that of fluorescamine, but comparative studies performed by Cukor(1976) and Egwu(1977) had shown that MDPF-based assays did not compare favourably with the radioallergosorbent tests or with assays using fluorescein-labelled IgG. Both fluorescamine and MDPF are readily hydrolysed to non-fluorescent products so that separation to remove unreacted hydrolysed products from the labelled conjugates is optional (Handschin 1976).

Hydrolysate (non-fluorescent) | Fluorogenic reagent (non-fluorescent) | Conjugate (Fluorescent)



Fluorescamine FL

$C_{17}H_{10}O_4$ = 278.27

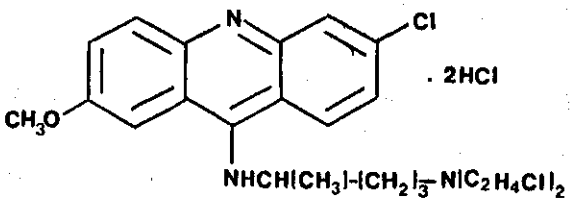


MDPF

$C_{17}H_{14}O_3$ = 266.3

Quinacrine [atebrine; 9-(4-diethylamino-1-methyl-butylamino)-6-chloro-2-methoxy acridine], a known fluorescent drug with antimalarial properties, has been used in cytological studies for investigating cellular components eg. chromosomes and submitochondrial particles. Quinacrine mustard (QM) differs from the parent compound in that the N-methyl groups are replaced by chloroethyl moieties that are capable of reacting with primary amino groups under alkaline conditions (Price et al 1968). The potential application of quinacrine and its mustard as fluorescent labels has been evaluated by Chen(1976). The

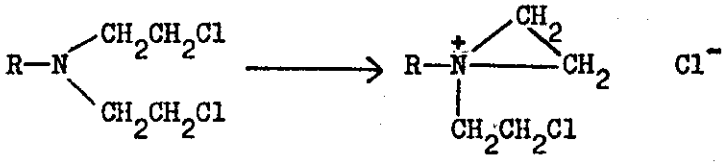
advantages appear to be that QM labels proteins readily, has good light stability, is water soluble, and the mustard conjugates are useful for polarization studies. However, the quantum yields of mustard conjugates are generally low, varying from 0.1 to 0.3 depending on the protein. Absorption bands at 250, 350 and 450 nm offer a choice of excitation wavelengths but the broad emission spectrum (see Fig.4.1a&b) may limit the applicability of the label.



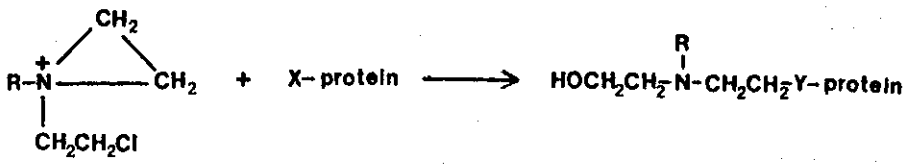
Quinacrine mustard dihydrochloride

$C_{23}H_{28}Cl_3N_3O.2HCl = 541.76$

The reaction of mustards with proteins occurs via a cyclic intermediate (Dandliker and Portman 1971)

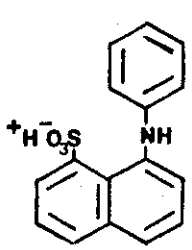


The intermediate is readily hydrolysed in aqueous media, but it reacts even more readily with a variety of protein groups, the second reactive group on the open chain being hydrolysed and not leading to cross-linking (Herriot 1946; Fraenkel-Conrat 1959; Webb 1966)



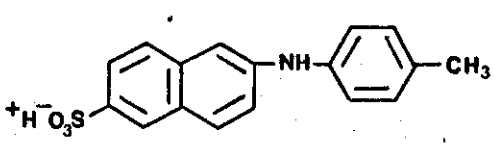
X	Y
HOOC–	–O–CO–
HS–	–S–
HO–	–O–
H_2N–	–NH–

1-Anilino-8-naphthalenesulphonic acid (ANS) and toluidino-naphthalene sulphonic acid (TNS) are used extensively as fluorescent probes for the polarity of their environment because the quantum yield increases and the emission maximum shifts towards the shorter wavelengths as the environmental polarity decreases (Stryer 1968; Brand 1972; Kanaoka 1977). Being not covalently bound to proteins, these hydrophobic probes are not suitable for use as fluorescent labels in immunoassays.



ANS

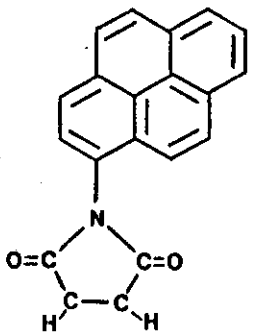
$C_{16}H_{13}NO_3S$ = 299.34



TNS

$C_{17}H_{15}NO_3S$ = 313.37

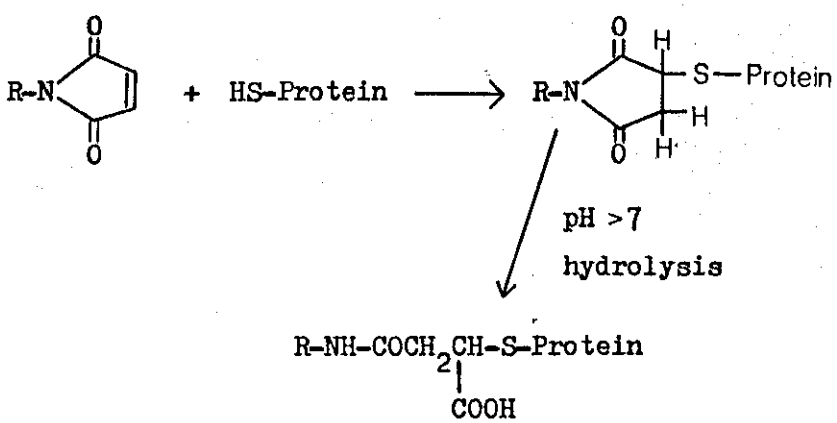
The pyrene derivatives such as N-(3-pyrene) maleimide (NPM), which combines the selective reactivity of maleimide towards



N-(3-pyrene) maleimide, NPM

$C_{20}H_{11}NO_2$ = 281.31

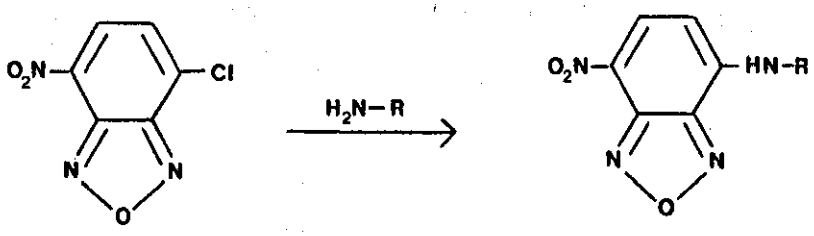
thiols with the spectral properties of pyrene, are generally not suitable for conventional immunoassay (Weltman 1973). However, the long decay time of the fluorescence may have applications in time-resolved fluorimetry. The reaction of N-substituted maleimide with the thiol functional group may be represented as follows.



At pH 7 the reaction is specific for thiol groups; below pH 6 the rate is slow, and above pH 7 the reagent begins to hydrolyse and groups other than thiol may also be attacked.

Other less commonly used fluorophores include the following:

(a) 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (Ghosh 1968)



NBD-Cl

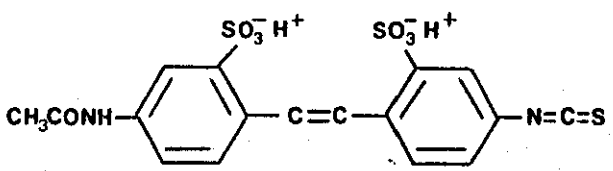
$C_6H_2N_3O_3Cl$ = 199.55

non-fluorescent

NBD-conjugate

highly fluorescent

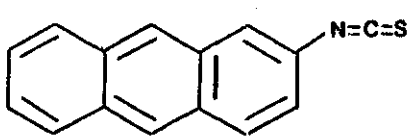
(b) 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) $C_{17}H_{14}N_2O_7S_3$ = 454.49



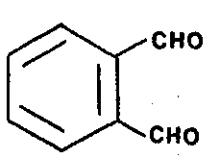
The utility of SITS as a fluorescent label is limited by the decrease in fluorescence intensity and the changes in absorption spectrum under prolonged excitation (Chen 1969)

(c) Anthracene-2-isothiocyanate (Chen 1969)

$C_{15}H_9NS$ = 235.30



(d) Ortho-phthaldialdehyde (OPT) ; $C_8H_6O_2$ = 134.13



1.3.3 Luminescence properties; molar absorption coefficients of fluorophores conjugated to proteins.

Some luminescence properties of the commonly used fluorescent labels are listed in Table 1.10. Data on λ_{ex} and λ_f represent the most probable values as these may vary depending on (i) whether the label is bound or free, (ii) the species protein to which the label is bound, and (iii) the degree of labelling (F:P ratio) of the conjugate. The corrected excitation and emission spectra of the fluorescent conjugates used in the energy transfer immunoassay investigations can be found in the appropriate chapters. Concentrations of the bound fluorescent labels are assayed spectrophotometrically using the molar absorption coefficients given in Table 1.10 . Further comments on these generally accepted values published in the literature are given below.

Comments on the molar absorption coefficients ϵ listed in Table 1.10

(a) FITC : The value of 7.2×10^4 $M^{-1}cm^{-1}$ at 493 nm for the bound fluorescein thiocarbamide group in conjugated proteins was calculated by Chen (1969) from the data of McKinney et al (1964) .

(b) RBITC : The assumed value of 1.23×10^4 $M^{-1}cm^{-1}$ at 557 nm for RBITC conjugates is based on the finding that the free isothiocyanate in 50% ethanol has that extinction at 555 nm (Chen 1969)

(c) TRITC : The determination of TRITC in conjugates remains uncertain. While crystalline TRITC conjugates show one

stable absorbance peak at 555 nm, the amorphous conjugates exhibit two absorbance maxima in the regions 515-520 nm and 550-555 nm with the relative intensities of these two maxima varying according to the conjugation ratio (Bergquist and Nilsson 1974). Thus, of the TRITC conjugates, only those labelled with crystalline TRITC can be assessed in terms of F:P ratio. Cebra and Goldstein (1965) had suggested that the ratio of absorbance measurements at 280/515 nm be taken as a measure of the degree of labelling of TRITC conjugates, and this had been accepted by Amante (1972) and Brandtzaeg (1973):

$$\text{TRITC-conjugated IgG (mg/ml)} = \frac{A_{280} - 0.56\, A_{515}}{1.4}$$

(with the protein concentration of conjugated IgG based on $E^{1\%}_{280} = 14.0$). Bergquist and Nilsson (1974) had suggested using a 280/550 nm ratio instead, because they considered it undesirable to use a wavelength (515 nm) which represented low fluorescence for calculations. There is as yet no accepted method available for the evaluation of amorphous TRITC conjugates.

(d) RB200SC : It is not possible to obtain a molar absorption coefficient for lissamine rhodamine-B conjugates since the absorption spectrum varies with the extent of labelling (Chen 1969; Brandtzaeg 1973; Chadwick 1958)

(e) DNS-Cl : Results obtained by Chen (1968) showed that for the most accurate work, no single absorption coefficient could be assumed for the dansyl group in conjugates of all proteins. Data from Hartley and Massey (1956) and Chen (1968) would suggest that a value of $3.4 \times 10^3\ M^{-1}\ cm^{-1}$

at 340 nm should be a close approximation to the actual absorption coefficient in many dansyl conjugates. Chen (1968) also found that the value of 4.3×10^3 $M^{-1}cm^{-1}$ (Weber 1952b) generally employed to calculate the degree of labelling might result in large errors in the determination of the dansyl groups.

(f) Fluram and MDPF : Chen (1974) found that the molar absorption coefficient of a number of fluorescamine-conjugated amino acids at 388 nm was 6450 100 $M^{-1}cm^{-1}$: this value was then used to calculate the dye:protein (F:P) ratios of fluorescamine-conjugated proteins even though Chen recognised that there might be some difference in the absorption of the fluorescamine conjugated to amino acids and proteins.

Handschin and Ritschard (1976) investigated the individual molar absorption coefficients of MDPF and FL covalently bound to goat and rabbit gamma-globulins and found that they were almost identical ($\epsilon^{385}_{\text{MDPF-}\gamma\text{-globulin}}$ = 6400 ± 60 $M^{-1}cm^{-1}$; $\epsilon^{385}_{\text{FL-}\gamma\text{-globulin}}$ = 6300 ± 100 $M^{-1}cm^{-1}$). Furthermore, these values are not significantly different from those provisionally found by Weigele et al (1973) for FL-conjugated amino acids. The assumption was thus made that the average molar absorption coefficients of bound MDPF and FL are the same for a great number of proteins.

(g) QM : The degree of labelling of quinacrine conjugate is determined by measurement of absorbances at 425 nm and 280 nm. At pH 7.4, the quinacrine mustard is found to have $\epsilon_{425} = 7.8 \times 10^3$ $M^{-1}cm^{-1}$ and $\epsilon_{280} = 4.56 \times 10^4$ $M^{-1}cm^{-1}$ (Chen 1976). If the absorbance of the conjugate is measured

at 425 nm, the amount contributed by the label to the total absorbance at 280 nm can be calculated if it is assumed that the optical characteristics of quinacrine mustard in the free and bound states are equivalent. The remainder of the absorbance at 280 nm is thus from the protein itself. Protein concentrations are than calculated using the extinction coefficients at 280 nm.

(h) SITS : The molar absorption coefficient of SITS conjugates is assumed to be the same as that of SITS dissolved in water, which was found by Chen (1969) to be $3.7 \times 10^4\ M^{-1}cm^{-1}$ at 345 nm. SITS reacts with γ-globulins to give conjugates with an absorption at 323 nm and a peak emission at 430 nm.

Table 1.10 Luminescence properties of some commonly used fluorophores

Fluorophore	τ/ns	ϕ_f	ϕ_b	λ_e/nm	λ_f/nm	ϵ/$M^{-1}cm^{-1}$	λ_a/nm	Reference
FITC	4·5		0·85	493	~520	$7{\cdot}2 \times 10^4$	493	McKinney et al, 1964; Chen, 1969
RBITC	3		0·7	550	585	$1{\cdot}23 \times 10^4$	557	Chen, 1969; Brandtzaeg, 1973, 1975; McKinney, 1975
TRITC								See Note c
RB200SC	<1		0·04	530 565	595 710			See Note d
DNS-Cl	14	0·2	0·3	340	480–520	$3{\cdot}4 \times 10^3$	340	Chen, 1968; Hartley et al, 1956
FL	7	0	0·1	~395	~485	6300 ± 100	385	Handschin et al, 1976;
						6450 ± 100	388	Chen, 1974
MDPF		0	0·1	~395	~485	6400 ± 60	385	Handschin et al, 1976
QM	4–13		0·1–0·3	250, 350, 450	~520 broad			See Note g
ANS	16	0	0·9	385	471			Kanaoka, 1977
NPM	100	0		340	375, 392			Weltman et al, 1973
NBD-Cl				468	530	$1{\cdot}29 \times 10^4$	468	Ghosh, 1968; Huang, 1975
SITS	1·0–1·3			323	430	$3{\cdot}7 \times 10^4$	345	Chen, 1969
Anthracene-ITC	29		0·6	357	460	$3{\cdot}04 \times 10^3$	357	Ibid; Harrington et al, 1956

Abbreviations used are defined in Section 1.3.2

ϕ_f, ϕ_b : quantum yield of free and bound fluorophore, respectively.

τ : lifetime

ϵ : molar extinction coefficient of bound fluorophore at the absorption wavelength λ_a

1.4 The Development of Energy Transfer Immunoassay Methods. Proposed Investigations.

The principal intention of this project is to develop a number of energy transfer immunoassay (ETIA) procedures, and to compare their sensitivity and ease of application with those of existing immunoassays and other analytical techniques.

In the first part of this work detailed studies will be performed to identify the experimental variables that affect the degree of energy transfer (and hence the sensitivity of the analysis) in a given assay system. For this purpose fluorescein and rhodamine as donor-acceptor fluorescent labels will be used to develop an energy transfer assay for human serum albumin. The suitability of this label pair for other applications of the ETIA method will be evaluated, and the sensitivity of the energy transfer assay in the analysis of serum samples compared with the electroimmunoassay.

Other potential donor-acceptor pairs will also be studied. These include (a) quinacrine and fluorescein; (b) quinacrine and rhodamine; (c) dansyl chloride and rhodamine; (d) fluorescamine and fluorescein; and (e) MDPF and fluorescein. The development of a single pair of labels which might be of value in the analysis of the widest possible range of materials would be very desirable, since it would permit the use of an extremely simple filter fluorimeter with filters providing fixed exciting and emitting wavelengths. In practice, however, it seems more likely that a number of different systems

would be required to facilitate the labelling of antigens containing different functional groups.

Upon the successful completion of the above investigations, a number of immunoassays for both low and high molecular-weight antigens will be developed, and comparisons made with other established immunochemical methods. In order to increase the versatility of the ETIA method, different variants of the technique will also be developed, including homogeneous direct and indirect (or sandwich) ETIA, and heterogeneous solid-phase ETIA. A principal advantage of the homogeneous ETIA method is its ease of automation because, in contrast to the heterogeneous assay, the troublesome separation step is not required. Flow injection analysis principles will be applied to the automation of an energy transfer immunoassay.

Experiments will also be carried out to determine the changes in the emission inetnsities when antigens labelled with various types of fluorophores are reacted with specific unlabelled antibodies. In particular, the fluram (and the analogous MDPF) enhancement effects will be studied in detail and applied to the development of FIA methods for the determination of drugs and biological macromolecules.

CHAPTER 2 MATERIALS, INSTRUMENTATION, AND GENERAL EXPERIMENTAL PROCEDURES

2.1 Materials (including purification and pretreatment steps)

Materials	Source/Description
Antibodies	
Purified immunoglobulin fractions of monospecific rabbit antisera to the following human proteins:	DAKO-immunoglobulins Ltd., Mercia Brocades Ltd., Weybridge, Surrey.
Albumin;	
Colostrum immunoglobulin A, specific for α-chains and secretory piece;	
Immunoglobulin G, specific for γ-chains;	
α-2-Macroglobulin;	
Transferrin;	
Sheep anti-desmethylnortriptyline antiserum.	Guildhay Antisera, Surrrey Batch No.A HP/S/1 (Gift from Prof.Bridges). Purification to yield IgG fraction of the antiserum is described below.
Human proteins	
Albumin, lyophilised with electrophoretic purity 100 %	Hoechst (UK) Limited, Hounslow, Middlesex.
Transferrin, lyophilised with min. purity 99 % and max. Fe concentration = 20 μg/g	
Standard human serum	

Materials	Source/Description
Human proteins	
Immunoglobulin A, lyophilised	Calbiochem Ltd., Bishops, Stortford. Prepared from human colostrum from the method of Got (1965)
Immunoglobulin G, lyophilised	Miles Laboratories Ltd., Stoke Poges, Slough.
Normal human serum samples	Donated by healthy laboratory workers.
Fatal road traffic accident blood sample	Home Office; Ref. CRE 2/79 Sample was centrifuged to remove lysed cells, sand particles, etc. Sodium azide was added as preservative to the serum. Stored frozen before analysis.
Animal proteins	
Bovine albumin, Fn V (powder). Purity 96-99 % albumin. Assayed N = 15.4 %	Sigma (London) Chemical Co.Ltd., Poole, Dorset.
Bovine γ-globulin, Cohn Fn II. Electrophorectic purity 99 % with <1 % NaCl. Assayed N = 15.0 %	Sigma (London)
Tricyclic Antidepressants	
Desmethylnortriptyline	Gift from Prof. Bridges
Protriptyline ; Desipramine Nortriptyline ; Imipramine Amitriptyline ; Chlomipramine Butriptyline ;	Gift from Dr. L.A.Gifford

Materials	Source/Description
Chemicals, Reagents, etc.	
1-Dimethylaminonaphthalene-5-sulphonyl chloride (DNS-Cl)	BDH Chemicals Ltd., Poole, Dorset.
Rhodamine-B isothiocyanate, RBITC	
Fluorescein isothiocyanate isomer I (10 % on Celite)	Calbiochem Ltd.,
Fluram (Fluorescamine) MDPF	Roche Diagnostics Ltd., Welwyn Garden City
Quinacrine mustard diHCl	Sigma(London)
8-Anilino-1-naphthalene-sulphonic acid, ANS (the ammonium salt)	Sigma(London)
DEAE-Sephacel; DEAE-Sephadex A50; Sephadex G-25 (medium grade); Column PD-10 (G-25M)	Pharmacia Ltd., Hounslow, Middlesex.
Immunobead Reagent Coupling Kit R containing immunobead matrix, control reagent, and EDAC.	BIO-RAD Laboratories, Watford, Hertfordshire.
M-Partigen Immunodiffusion Plates for transferrin determination	Hoechst (UK) Ltd.
"Cellogel" Cellulose acetate	Whatman Labsales Ltd., Maidstone, Kent.

All other buffer salts and reagents were of Analar or equivalent grade.

Separation of sheep immunoglobulins on DEAE-Sephacel

Buffer 1 : 0.0175M phosphate buffer pH 6.8

Buffer 2 : 0.08M phosphote buffer pH 6.6

Buffer 3 : 0.3M phosphate buffer pH 6.5

Procedure :

The methodology of Mollison (1972) was employed using DEAE-Sephacel instead of DEAE-cellulose. 2 ml of the sheep anti-nortriptyline antiserum were equilibrated on a Pharmacia PD-10 Sephadex G-25M column with buffer 1 . The dilution factor was ca. 1.5 . The equilibrated sample was applied to a short column (1.5 x 10.5 cm) of DEAE-Sephacel and then eluted with buffer 1 to obtain the first peak (see Fig 2.1). Subsequent stepwise elution with buffer 2 and 3 gave the second and third peak respectively. The flowrate of the elution was 0.28 ml min^{-1} and each collected fraction contained 1.3 ml.

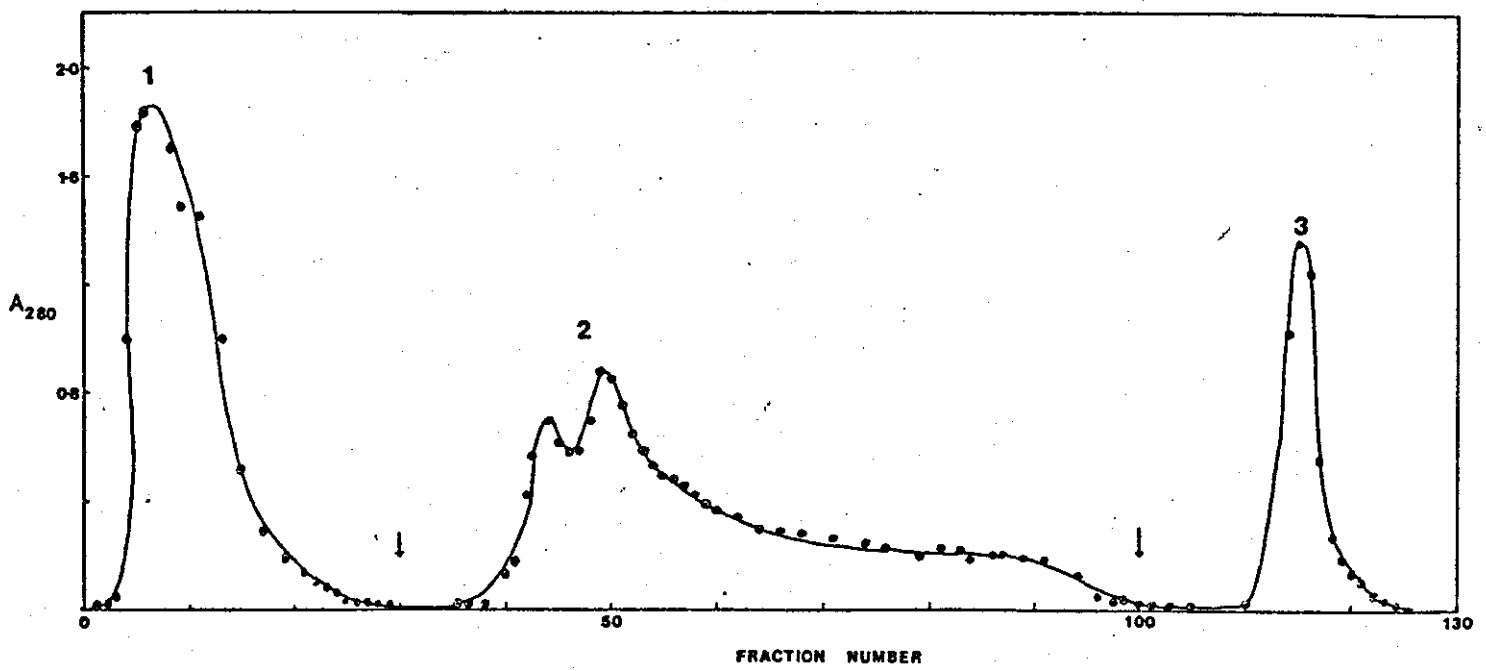


Figure 2.1 Fractionation of sheep serum immunoglobulins on DEAE-Sephacel. Peak 1 contained mainly IgG; peak 2 contained IgA with traces of IgG; and peak 3 contained mainly IgM. Arrows indicate the change of buffers used in the stepwise elution.

2.2 Instrumentation

The MPF-44B Fluorescence Spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks) fitted with a DCSU-2 corrected spectrum unit was used to record all corrected excitation and emission spectra. It was also used in addition to the Fluoricord Spectrofluorimeter (Baird-Atomic Ltd., Braintree, Essex) to record other uncorrected spectra. Quantitative fluorimetry was measured using both the MPF-44B and the Fluoripoint Spectrofluorimeter (Baird-Atomic Ltd.) fitted with an R928 side-on photomultiplier (Hakuto International, Leigh-on-Sea, Essex) for improved long-wavelength sensitivity. Thermostatted 10 mm path length silica cuvettes were used in all fluorimetric measurements. In each case, the spectrofluorimeter used will be specified. For flow-injection analysis work, the detector used was a Perkin-Elmer Model 1000M Filter Fluorimeter fitted with a flow-cell specially adapted with silica tubing of 1 mm id. See chapter 8

Spectrophotometric absorption measurements were recorded using the Pye-Unicam SP-600 UV Spectrophotometer.

Electroimmunodiffusion experiments were performed in water-cooled electrophoresis chambers powered by a Shandon Southern (Model Vokam SAE 2761) Power Supply unit.

All other equipment used will be described in the appropriate chapters.

2.3 General Experimental Procedures

2.3.1 Standard methods for the preparation of fluorescent protein conjugates

The use of a purified homogeneous fraction of the protein to be labelled is a prerequisite for a standardized conjugation procedure, regardless of the type of fluorophore. This is particularly true of antiserum which, if labelled without a prior purification step to remove non-specific constituents, would yield fluorescent conjugates with both specific and non-specific binding properties (Brandtzaeg 1973). A purified immunoglobulin fraction of the antiserum should therefore be used in the preparation of labelled antibody conjugates. Wood et al (1965) had reported that the relative homogeneity in the isoelectric point of isolated IgG molecules was the basis for the characterization of labelled IgG conjugates by anionic exchange chromatography. However, the IgG molecule which accounts for 70% or more of a large number of mammal serum immunoglobulins is among the most difficult to label (Brandtzaeg 1973), and this had been demonstrated earlier by the work of McKinney et al(1964) and subsequently confirmed by White (1970). In order that labelled immune reagents may be reproducibly prepared, labelling procedures and conditions must be optimized and standardized with respect to the following experimental parameters.

(a) Mode of addition of label

A number of approaches may be used:

(1) The label in dry, finely divided form is added gradually and with efficient stirring to the buffered protein solution. The method was first proposed by Marshal et al (1958) for

the preparation of fluorescein-labelled antibody. Reisberg (1970) had reported that the reactivity of fluorescein was reduced after being dissolved, and so there is an advantage in some cases of adding the label as a dry powder directly to the protein solution. The method is simple and economical on label consumption but requires a good microbalance. Protein damage is minimal .

(2) The label is first dissolved in a small volume of organic solvent eg. acetone, dioxane and DMSO, and then added gradually to the protein solution with continuous stirring. With this method, protein denaturation attributable to the organic solvents is inevitable.

(3) The label is adsorbed on filter paper (Goldman 1957) or cellulose powder (Celite) (Rinderknecht 1962) from which it is gradually released into the protein solution. The method facilitates the weighing of small amounts of the label.

(4) The protein solution is dialysed against a solution of the label (Clark and Shepard 1963)

(b) Buffer concentration and pH effect

For all commonly used labels, a medium alkaline buffer eg. carbonate or phosphate, is used with the protein concentration generally kept between 10 - 60 mg ml^{-1} (Dandliker and Portman 1971). Buffer concentration used is dependent on the label functional group involved in the conjugation reaction. For example, while a 0.05M buffer may be adequate for isocyanate and isothiocyanate, a buffer concentration of 0.5M may be required for the sulphonyl chloride group.

The effect of pH on conjugation reaction efficiency had been thoroughly investigated by McKinney et al (1964) in the case of labelling with fluorescein isothiocyanate. It was found that the speed of fluorescein labelling of serum proteins was increased with increasing pH within the range pH 6 to 10, with the optimal pH at 9.5. Thus, pH control (maintained by buffers or continuous adjustment with added Na_2CO_3 or NaOH) is an absolute prerequisite for the attainment of standardized conjugation since it had been found (Goldstein and Morthland 1967) that even a pH drop from 9.5 to 9.1 could reduce the efficiency of fluorescein-labelling by about 30% .

(c) Effect of temperature and light

The former practice was to keep the reaction temperature at 0°C because of reduced protein denaturation. However, results on the fluorescein-labelling of serum proteins reported by Klugerman (1965) and McKinney et al (1964) showed that labelling at room temperature was many times faster without any disadvantages being apparent so long as organic solvents were absent. All labelling experiments described below (Section 2.3.1.1) for the preparation of fluorescent protein conjugates were carried out at room temperature, except where otherwise mentioned. Protection against light is a precaution necessary only insofar as the label and/or the conjugate are easily photodegradable.

Isolation of labelled conjugates from unreacted label

Before gel filtration became available, the methods

used to remove unreacted label and other low molecular weight substances from the labelled conjugate are time-consuming and often result in considerable protein loss and damage. These methods include charcoal and tissue powder adsorption, dialysis and solvent extraction. Gel filtration (on a column of Sephadex, for example) is now the preferred method because it is (i) efficient in removing low molecular weight label and organic solvent from the protein conjugate, (ii) gentle on proteins, (iii) rapid, and the protein recovery is high (Killander 1961; George 1961).

Fractionation of conjugates with respect to the degree of labelling.

With few exceptions, a labelling procedure usually produces a heterogeneous population of labelled conjugates because of the differing reactivity of the macromolecular functional groups. The labelled conjugates, after having been separated from unreacted label by gel filtration, must therefore be further fractionated with respect to the degree of labelling by ion-exchange chromatography or electrophorectic methods. Even after this fractionation step, it is very likely that the resulting purified product still consists of a large number of isomers corresponding to the different conjugation points to which the label has been chemically bound. It has remarked (O'Donnell and Suffin 1979) that " the purification of labelled ligands is more art than science at present" even though recent developments in such analytical separation techniques as HPLC may help to reduce the tedium and labour involved in producing reproducibly pure labelled immune reactants.

Methods for the determination of protein and label concentrations in conjugates

Label concentration can easily be assayed spectrophotometrically using absorption or fluorescence measurements, though these methods are subject to a number of uncertainties. Thus, the original assumption (Creech and Jones 1940), that the extinction coefficients of the bound and free label were equivalent, was found to be not valid by the work of Dandliker et al (1964) and Tengerdy and Chang (1966) who respectively found greater absorption by the free label compared to the protein conjugate by factors of 1.12 and 1.18 . Changes in the extinction coefficient and shifts in the absorption maximum can occur when a label is chemically bound to a protein. Likewise, the fluorescence spectrum is also very dependent on the experimental conditions and can undergo even greater changes than the absorption spectrum. An alternative and more accurate approach to the quantitation of the bound label is to incorporate a radioactive tracer into the label. However, the work involved in radioactive labelling of the fluorescent group may be laborious.

Protein determination by absorption measurements is also subject to the same difficulties outlined above, but the effects on protein absorption are usually less significant than those on the label. Consequently, the determination of the protein content of the conjugate can be obtained from the known (molar or specific) extinction coefficients of the proteins (Table 2.1), by nitrogen analysis (eg.micro-Kjeldahl) , or some colorimetric method such as the Lowry or biuret method. Spectrophotometric determination of proteins based on absorbance measurements must be corrected for absorption by the dye at the wavelength of

measurement (usually 280 nm) as well as the type of protein used. The Lowry method (Lowry et al 1951) for protein determination is based on the reaction between proteins and Folin-Ciocalteau reagent mediated by copper salts. The resulting colour reaction is recorded at 700 nm, which makes it a method of choice for fluorescent conjugates labelled with FITC, the rhodamines, DNS-Cl etc because these do not absorb near the 700 nm region. Methods used to characterize labelled conjugates with respect to protein concentration and fluorophore:protein (F:P) ratio include the following:

(1) The protein content is determined by the Lowry method and the label is assayed spectrophotometrically using the molar absorption coefficients of the bound labels given in Table 1.10 on page 58

(2) Use of nomograph and formula.

For fluorescein-labelled immunoglobulins, protein concentration and molar F:P ratio can be found from the nomograph of Well et al (1966). A similar nomograph given by The and Feltkamp (1970) or, alternatively, Brighton's formula (Brighton 1970) may also be used. [Refer also to Brusman(1971) for some theoretical and practical considerations in the spectrophotometric determination of fluorescein:protein ratio.]

(3) Other specific methods will be described where appropriate in Section 2.3.1.1

Table 2.1 Molecular weights and extinction coefficients of some human and animal proteins.

Protein	MW	Ref.	$\epsilon^{1\%}_{1\,cm}$ (280)	Ref.
Human				
Albumin	66241	Putman(1975)	5.94[a]	Berrens(1965)
IgA	360000-720000	White et al (1978)	13.4	Grey(1972)
IgG	150000	White et al (1978)	13.8	Grey(1972)
α-2-Macro-globulin	725000	Putman(1975)		
Transferrin	76500	Putman(1975)	11.4[b]	Feeney and Komatsu(1966)
			14.3[c]	Perkins(1966)
Animal				
Bovine albumin	69000	Longworth (1971)	6.14[a]	Berrens(1965)
Bovine γ-globulin	160000	Longworth (1971)	14.1	Longworth (1971)
Rabbit IgG	140000	Mamet-Bratle (1970)	13.5	Stevenson (1970)
Sheep IgG	145000		15.0	

a: pH 7.0 in 0.01M phosphate buffer

b: apotransferrin

c: Fe^{3+}.Transferrin

2.3.1.1 Preparation of fluorescent protein conjugates used in energy transfer immunoassay studies.

Fluorescein-labelled conjugates.

Proteins labelled:
lyophilised human serum albumin (100% pure);
monospecific rabbit immunoglobulins to human serum albumin, transferrin and IgG, and to human colostrum IgA;
the IgG fraction of sheep anti-nortriptyline antiserum.

Procedure: The method of Rinderknecht (1962) utilising fluorescein isothiocyanate isomer I adsorbed on Celite (10 %) was used to label the protein. Celite was separated from the labelled solution mixture by centrifugation, and the unreacted dye was removed using a short column of Sephadex G-25 (medium grade) equilibrated with 0.01M phosphate buffer pH 7.2 . The labelled protein was fractionated on a column of DEAE-Sephadex A-50 using a 0.075M - 1.5M NaCl gradient in 0.01M phosphate buffer pH 7.2 . (In the case of FITC-labelled sheep IgG, the salt gradient used was 0.025M - 1.5M NaCl).

The protein content and molar F:P of fluorescein-labelled antibodies were found from the nomograph of Well et al (1966). For fluorescein-labelled albumin the F:P ratio was determined using the Lowry method to determine the protein concentration, and a value of 7.2×10^4 $M^{-1}cm^{-1}$ at 493 nm for the molar absorption coefficient of the fluorescein thiocarbamide group (Chen 1969).

Rhodamine-labelled conjugates

Proteins labelled:
lyophilised human serum albumin (100 % pure); monospecific rabbit immunoglobulins to human serum albumin and IgG .

Procedure: Rhodamine conjugates were prepared by reacting the protein with an acetone solution of rhodamine-B isothiocyanate. The reagent solution was added, slowly and with stirring, to a solution of the protein maintained at pH 9.5 with solid sodium carbonate. After stirring for a further hour, excess reagent was removed and the conjugate fractionated by ion-exchange chromatography as described above for FITC-labelled conjugates. In determining the F:P ratios, the rhodamine group was assumed to have a molar absorption coefficient of 1.23×10^4 $M^{-1}cm^{-1}$ at 557 nm (Chen 1969)

Fluorescamine- and MDPF-labelled conjugates

Proteins labelled:
lyophilised human serum albumin , transferrin, IgG;
monospecific rabbit immunoglobulins to human colostrum IgA.

Procedure: The preparation of these conjugates was based on the methodology of Weigele et al (1973) with some modifications. The weighed protein (25 - 35 mg) was dissolved in 2 ml of 0.1M sodium phosphate buffer pH 9.5 . Depending on the molar F:P ratio required, 50 μl portions of the fluorescamine or MDPF solution in acetone (3.0 -4.0 mg ml^{-1}) were added (using a Hamilton microlitre syringe) to the protein solution with stirring for one hour. Separation of the labelled conjugate from the hydrolysed products and acetone was performed on a column (1.5 x 15 cm) of Sephadex G-25 (medium grade) equilibrated with 0.01M sodium phosphate buffer pH 7.2 containing 0.145M NaCl. The Lowry method was applied to the determination of the protein concentration of the conjugate. The bound label was assayed using a value of 6400 $\pm$ 60 $M^{-1}cm^{-1}$ and 6300 $\pm$ 100 $M^{-1}cm^{-1}$ at 385 nm for the molar absorption coefficients of the conjugated MDPF and FL groups, respectively (Handschin and Ritschard 1976).

Molar fluorophore:protein (F:P) ratio

Trans-FL			Trans-M		
Added	Recovered	%	Added	Recovered	%
2.0	1.85	92.5	2.7	2.6	96.3
4.2	3.9	92.8	4.2	4.1	97.6
7.2	7.0	97.2	6.5	6.3	96.9
10.0	9.79	97.9	9.0	8.8	97.8
12.8	12.6	98.4	13.0	12.8	98.5
		av. 95.8			av.97.4

IgG-FL			IgG-M		
Added	Recovered	%	Added	Recovered	%
8.5	6.6	77.6	11.2	6.8	60.7
14.9	9.3	62.4	16.3	9.87	60.6
22.8	16.7	73.2	25.0	16.0	64.0
		av. 71.1			av. 61.8

Alb-FL		
Added	Recovered	%
6.74	6.2	92.0
10.7	10.0	93.5
16.1	14.7	91.3
		av. 92.3

Quinacrine-labelled human immunoglobulin G

Procedure: Quinacrine mustard conjugates of human IgG were prepared by adding various amounts of the mustard dissolved in tri-distilled water to 1.0 -1.5 % w/v protein solutions in 0.1M $NaHCO_3$. The mixture was vigorously agitated on a flask-shaker for one hour. Labelled protein was separated from

unreacted mustard on Sephadex G-25 (medium grade) equilibrated with 0.01M phosphate buffer pH 7.2 containing 0.145M NaCl. The protein concentration and molar F:P of the conjugate was found using the method described by Chen (1976). See page 56

Dansyl-labelled albumin

Procedure: DNS-Cl dissolved in acetone (3 - 5 mg ml^{-1}) were added, slowly and with stirring, to the protein solution (16 - 20 mg dissolved in 2 ml 0.1M $NaHCO_3$) kept at ice-bath temperature. After mixing for 1½ hours, the undissolved materials were removed by centrifugation and the solution allowed to come to room temperature. Organic solvent and unbound dye were removed by gel filtration on Sephadex G-25 (medium grade) equilibrated with 0.01M phosphate buffer pH 7.2 . (Conjugate and unbound dye were easily visualized on the column). The protein content of the conjugate was measured by the Lowry method, and the bound DNS assayed spectrophotometrically assuming an ϵ value of 3.4×10^3 $M^{-1}cm^{-1}$ at 340 nm (Chen 1968).

2.3.1.2 Preparation of fluorescamine- and MDPF-labelled desmethylnortriptyline (DNT)

Optimization of derivative formation procedure

(a) Fluorescamine-labelled DNT (DNT-FL)

Procedure 1 (pH effect on the reaction): 0.5 ml of 8.27×10^{-4}M fluorescamine in acetone was rapidly added to 2.5 ml of 9.64×10^{-6}M DNT in 0.05M phosphate buffer of pH (7.5, 8.0, 8.5,..., 12.0). The mixture was vigorously mixed on a vortex mixer and then equilibrated in the waterbath at 25°C for 5 minutes before fluorescence intensities $I_f(485)$ were recorded in 10 mm path

length silica cuvette using the Baird-Atomic Fluoripoint Spectrofluorimeter with λ_{ex} = 390 nm and spectral bandwidth = 8 nm. In each case, final labelled product (DNT-FL) concentration was 8.0×10^{-6}M and the molar excess of added FL = 17.2 .

Procedure 2 (Molar excess of FL for quantitative reaction):

2.1 ml of 1.15×10^{-5}M DNT solution in 0.05M phosphate buffer pH 9.0 were rapidly and thoroughly mixed on vortex with various amounts of 1.37×10^{-3}M FL in acetone. In each case, the appropriate amount of pure acetone was added to the reaction mixture to give the final total volume of 3 ml. This was to ensure that any changes in fluorescence intensity recorded were not the result of co-solvent polarity effects. The solutions were equilibrated at 25°C for 5 minutes before fluorescence readings $I_f(485)$ were recorded using the same experimental conditions as above. Final concentration of DNT-FL = 8.0×10^{-6}M .

Note: In all subsequent experiments, solutions of DNT-FL were prepared with a 25-fold molar excess of the label in 0.01M sodium phosphate buffer at pH 9.0 .

(b) MDPF-labelled DNT (DNT-M)

Procedure: The optimization experiments were performed as described above except that the molar excess of MDPF in the study of the pH effect was 20.3, and the solutions were buffered at pH 10.0 in the determination of the molar excess of MDPF required for quantitative reaction with DNT.

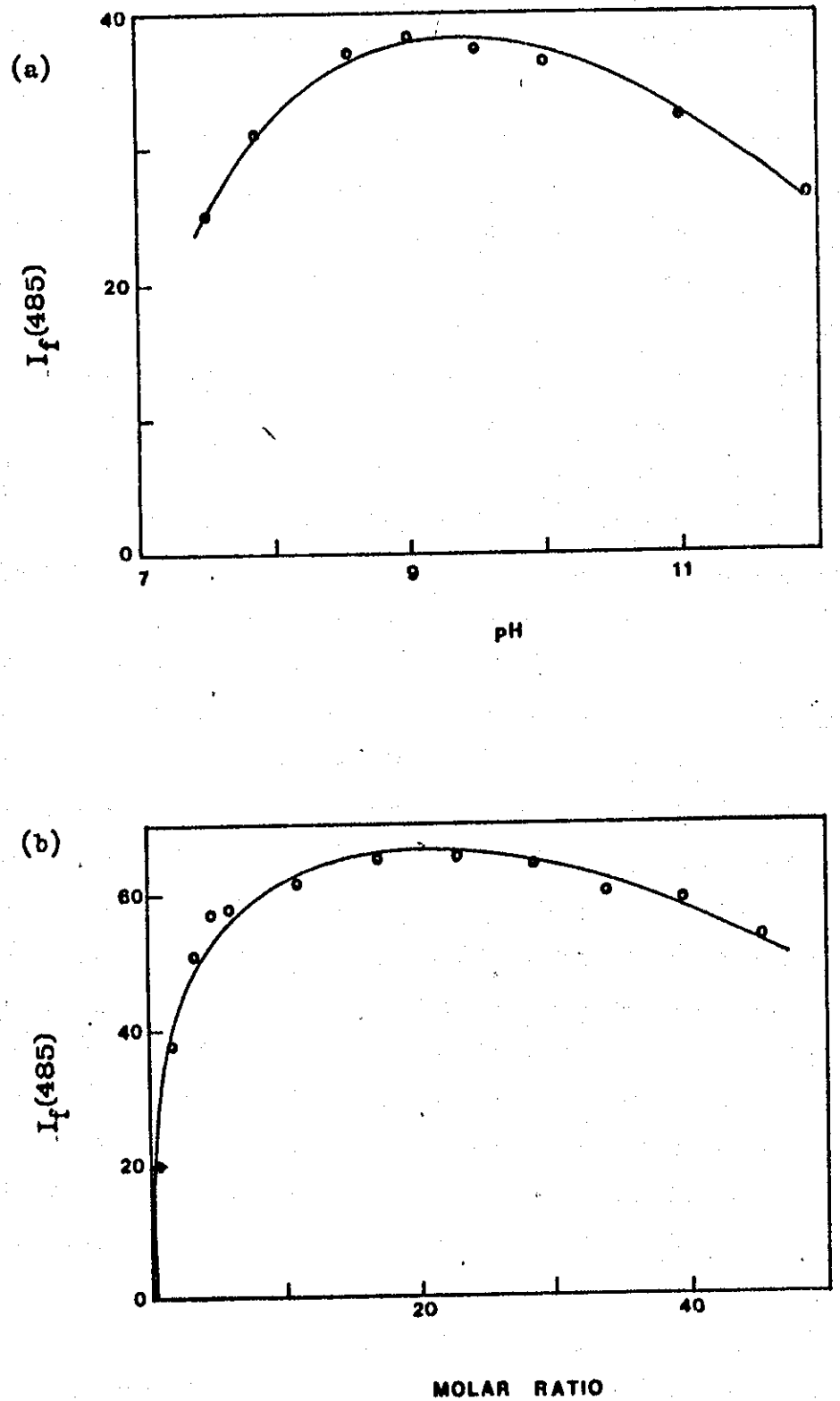


Figure 2.2

Conditions of (a) pH and (b) molar excess of fluorescamine required for quantitative reaction between desmethylnortriptyline and fluorescamine. Molar ratio = fluorophore : drug

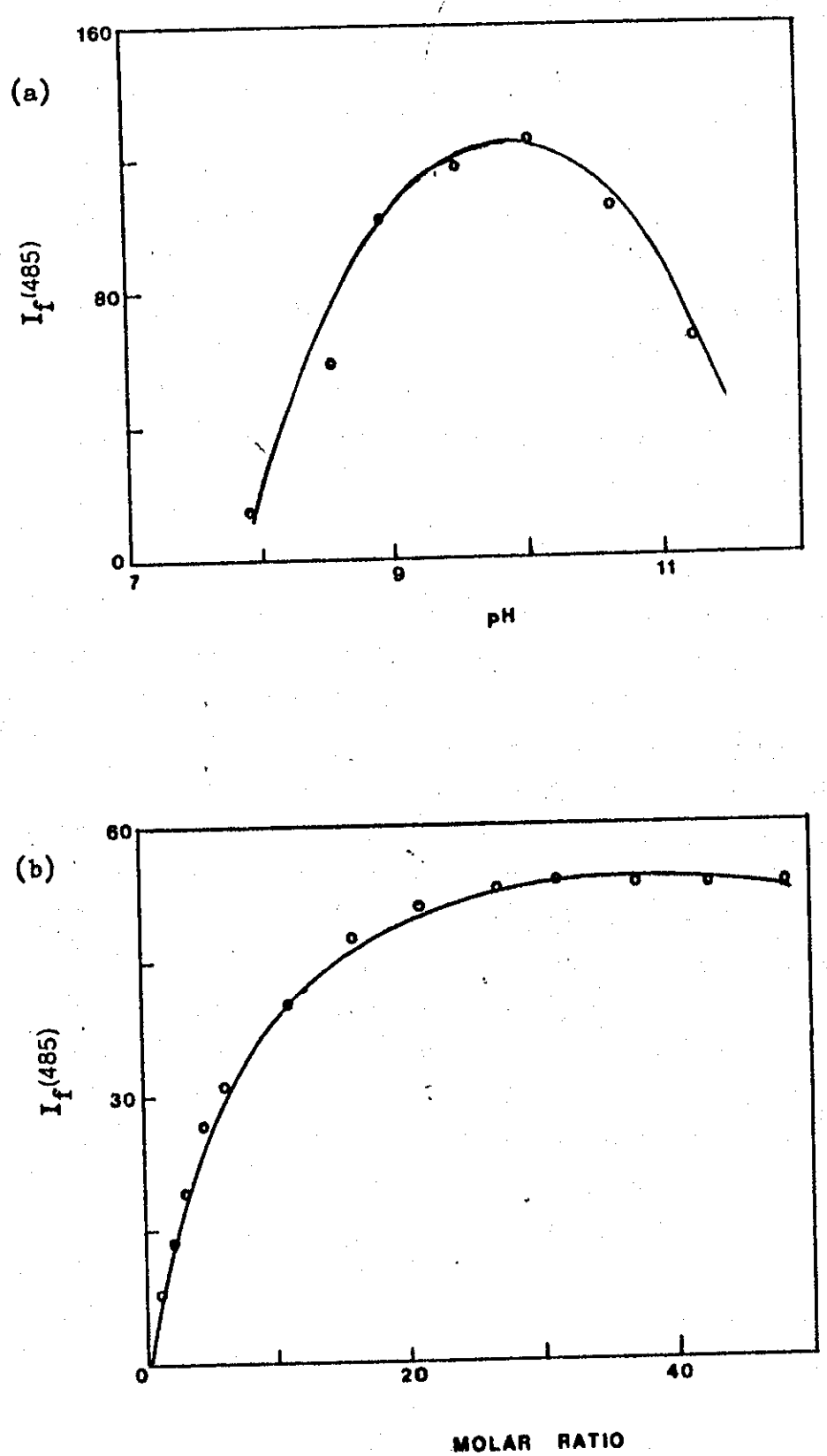


Figure 2.3

Conditions of (a) pH and (b) molar excess of MDPF required for quantitative reaction between desmethylnortriptyline and MDPF. Molar ratio = MDPF : drug .

Table 2.2 Abbreviations used to denote fluorescent conjugates

Protein/Ligand	Label	Conjugate
Antibody (Ab)[a]	FITC	Ab-F
	Fluram	Ab-FL
	RBITC	Ab-R
Albumin	DNS-Cl	Alb-D
	FITC	Alb-F
	Fluram	Alb-FL
	MDPF	Alb-M
	RBITC	Alb-R
Desmethylnortriptyline	Fluram	DNT-FL
DNT	MDPF	DNT-M
Immunoglobulin G	Fluram	IgG-FL
IgG	MDPF	IgG-M
	Quinacrine	IgG-Q
Transferrin	Fluram	Trans-FL
	MDPF	Trans-M

a : The antigen for which the antibody is specific should be obvious from the context; where ambiguity may arise, subscript will be used to denote the specific antigen. Thus, Ab_X means antibody specific for antigen X.

2.3.2 Electroimmunodiffusion and Radial immunodiffusion

Determination of albumin concentration by electro-immunodiffusion was performed on Cellogel cellulose acetate strips containing anti-albumin antibodies (Dakopatts). 1 µl samples of diluted serum standards (Hoechst) and appropriately diluted serum samples were applied on the Cellogel strip using 1 µl microcap pipette. A barbiturate buffer pH 8.6, I = 0.02 (barbitone 0.736 g l^{-1} , sodium barbitone 4.12 g l^{-1}) was used in the electrophoresis which was carried out in a water-cooled electrophoresis chamber at a constant voltage of 120 V for 2 hours. After the staining (Coomassie Blue RL, 0.25 % in methanol:acetic acid:water, 5:1:5 by volume) and destaining procedures to locate the rocket-shaped precipitin peaks, albumin concentrations of the test samples were determined from the plot of rocket heights against concentrations of standards.

M-Partigen Immunodiffusion plates (Hoechst) were used for the quantitative determination of serum proteins. The procedure followed for the radial immunodiffusion experiments was as directed in the enclosed instructions.

CHAPTER 3 ENERGY TRANSFER IMMUNOASSAY : A STUDY OF THE EXPERIMENTAL PARAMETERS IN AN ASSAY FOR HUMAN SERUM ALBUMIN.

3.1 Introduction

In the first description of energy transfer immunoassay (ETIA) by Ullman et al (1976), fluorescein (donor) and rhodamine (acceptor) were used in the development of assay methods for measuring both low molecular-weight (morphine, codeine) and high molecular-weight (human IgG) species with detectability at levels above 100 pM. The same donor-acceptor fluorescent labels were also used in other applications of the method reported in subsequent papers from the same laboratory. These include assays for the quantitation of human IgA in samples containing from 80 - 450 mg dl^{-1} of the immunoglobulin (Rodgers et al,1978), and IgM in the range 60 - 260 mg dl^{-1} (Eimstad et al, 1978). All the above assays have made use of the competitive binding principle in the direct labelling method, or the sandwich assay principle in the indirect labelling method. In the former, purified or enriched antigen and antibody are respectively labelled with the donor and acceptor fluorescent groups. The average distance between donor and acceptor within the antigen-antibody complex formed should be sufficient to permit resonance energy transfer. This will lead to the quenching of the donor fluorescence and (possibly) the enhancement of the acceptor fluorescence. These effects will be reversed by the inclusion of unlabelled antigens (present in standards and samples) in competitive reactions for the limited antibody binding sites. In the alternative sandwich assay, separate portions of the antibody are labelled with the donor and acceptor molecules. Unlabelled antigen causes the aggregation of the separately

labelled antibodies, and the effects of energy transfer are again observed. The sandwich assay does not require the use of purified antigens but a high degree of specificity is required of the antibodies so as to avoid the excessive fluorescence background due to inactive fluorescein-labelled proteins. Sandwiches cannot be made with hapten-antibody combinations, so the sandwich assay method is applicable to only multivalent ie. macro-molecular antigens.

Since energy transfer from donor to acceptor involves a change in the fluorescence properties of one or both of the labels, the assay is homogeneous. That is, the separation of antibody-bound and free fractions of the antigen is not necessary, and hence this type of assay is easier to automate compared to the heterogeneous system. Furthermore, the simultaneous measurement of the quenching and enhancement effects gives a more accurate assessment of the specific antigen-antibody combinations, and so offers a simple method for distinguishing genuine energy transfer effects from spurious environmental effects on the fluorescence intensity.

In this chapter, the development of an energy transfer assay for low concentrations of human albumin is described, and the suitability of fluorescein and rhodamine as fluorescent donor and acceptor labels in ETIA evaluated. The effects on the assay system of several experimental variables were investigated, including (i) the fluorophore:protein (F:P) ratio, (ii) concentrations of labelled antigen and antibody, (iii) the fluorimeter spectral bandwidth, and (iv) whether, for greater resonance transfer efficiency, the donor (fluorescein) should be conjugated to the antigen or the antibody. The assay method was applied to the

analyses of test serum samples and the results compared with those found by electroimmunoassay. Studies on the specificity of the assay system as well as the stability of labelled immune reactants on storage will also be described.

Human blood contains at least 100 individual proteins, and albumin which constitutes 60 per cent by weight is the most abundant. Its relatively small molecular size (molecular weight = 66241; Putman,1975) confers upon it the important role of maintaining through its osmotic pressure effect the circulating fluid within the vascular system. It binds with many physiological substances (eg. bilirubin, steroid hormones), drugs, antibiotics etc., and is therefore an important transport protein for many substances in blood. Albumin is synthesized in the liver and is a precursor for tissue proteins.

In clinical laboratories, a simple, rapid and precise quantitative assay for albumin is essential for the diagnosis of vascular, cirrhotic and nephrotic defects, nutritional deficiencies of other proteins, and many other disease states. A summary of the different methods which had been developed for the analysis of albumin in human blood plasma was given by Peters (1977).

3.2 Experimental

Quantitative fluorimetry was performed in thermostatted 10 mm path length silica cuvettes using the Baird-Atomic Fluoripoint Spectrofluorimeter. All determinations were made at 37 C after samples had been incubated at this temperature for 20 minutes before measurement. Corrected excitation and emission

spectra were recorded at 25 C.

All fluorimetric measurements were corrected for the fluorescence background due to the buffer (saline(0.145M)-phosphate buffer, pH 7.2, 0.01M) and added proteins. Contributions to the background from rhodamine-labelled antibodies were found to be negligible.

For stability studies, labelled protein solutions were stored in the dark at 4 C and examined for their fluorescent and immunological characteristics over periods of several months. If a labelled protein solution had turned turbid with precipitation, it was first centrifuged to remove the precipitates and the molar F:P of the recovered conjugates was then re-analysed as described in Section 2.3.1.1 . After re-standardization, such reagents were used in the usual way.

3.3 Results and Discussion

Energy transfer between fluorescein and rhodamine within the antigen-antibody complex was demonstrated by addition of rhodamine-labelled anti-albumin antibodies (Ab-R) to a fixed concentration of albumin-fluorescein (Alb-F). The degree of energy transfer was assessed by measuring the quenching of fluorescein emission at 520 nm and the enhancement of rhodamine emission at 580 nm (an excitation wavelength of 470 nm was used throughout). The resonance transfer efficiency of the fluorescein-rhodamine system was expected to be high because there was significant overlap between the fluorescein emission spectrum and the rhodamine excitation spectrum - see Fig.3.1 . However, the

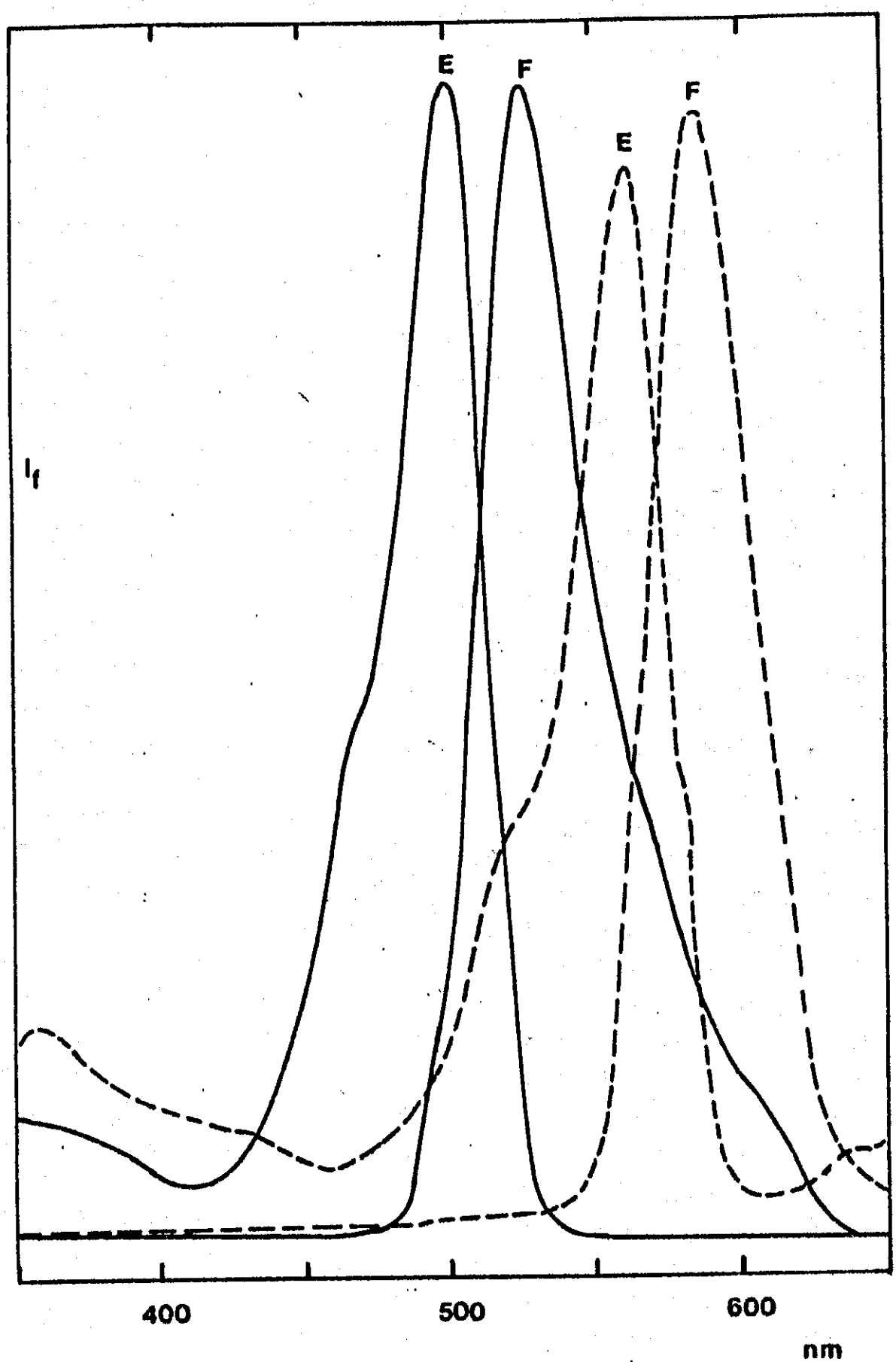


Figure 3.1 Corrected excitation (E) and fluorescence (F) spectra of albumin-fluorescein conjugate, label : protein ratio = 1.3 :1 (————), and of rhodamine-antibody conjugate, label : protein ratio = 7.5 : 1, (- - - -).

magnitude of the energy transfer effects (and hence the sensitivity) of this particular assay system was found to depend on several other factors, including the molar ratio of labelled antigen and labelled antibody; the fluorophore:protein ratios of the labelled molecules; and whether the antigen was labelled with fluorescein and the antibody with rhodamine or vice-versa. These factors will be considered in turn.

Fig. 3.2 shows that addition of increasing amounts of Ab-R to a fixed concentration of a lightly-labelled Alb-F (molar F:P = 1.3) caused fluorescein quenching and rhodamine enhancement to increase approximately linearly up to at least a 12-fold molar excess of antibody. At very low (<0.5) antibody:antigen ratios, however, significant changes in fluorescence intensity were not observed. For any given mixture of Alb-F and Ab-R, the rhodamine enhancement was rather greater than fluorescein quenching. This result may be related to the small non-specific enhancement effect that occurred when unlabelled anti-albumin antibodies were added to fluorescein-labelled albumin (Fig. 3.2), and which partially offsets the quenching due to energy-transfer. Ullman et al (1976) had also noted a similar slight increase in fluorescence intensity when unlabelled anti-morphine antibody was reacted with morphine-fluorescein molecules. Possibly, in both the cases, the enhancement phenomenon is due to the change in the polarity of the medium within the environment of the bound antigen-fluorescein molecules. It is also clear from Fig. 3.2 that observations of rhodamine enhancement, as well as of fluorescein quenching, may be feasible in this type of fluorescence immunoassay, although in the present work the absolute intensity of the rhodamine fluorescence was found to be generally low. However, the background fluorescence

of blood serum and other biological fluids has been found (Soini et al, 1979) to be generally greater at shorter wavelengths, so interferences from this cause will be reduced if, instead of measurement of fluorescein quenching at 520 nm, the rhodamine enhancement at 580 nm is measured. All previous studies on energy transfer (Ullman et al, 1976; Rodgers et al 1978; Eimstad et al, 1978) have made use of only fluorescein quenching measurements

The figure also shows that the magnitude of the quenching and enhancement effects is strongly dependent on the number of rhodamine molecules bound to the antibody molecules, with heavily-labelled conjugates producing the largest effects especially at high antibody : antigen ratios. The effect of the degree of rhodamine labelling on the quenching of albumin-fluorescein fluorescence by excess labelled antibodies can be related to the greater probability of one or more rhodamine groups being attached close to the antigen-binding site : energy transfer is thus more efficient because of the lower mean distance between the fluorescein and rhodamine groups.

Legends for Figures

Fig. 3.2 Enhancement of rhodamine fluorescence (580 nm) and quenching of fluorescein fluorescence (520 nm) when increasing amounts of rhodamine-labelled antibody are added to an albumin-fluorescein conjugate (label:protein ratio = 1.3:1). The degree of labelling of the antibody is indicated by the n values: n = moles of rhodamine per mole of antibody. The dotted line shows the slight enhancement of fluorescein fluorescence (520 nm) when unlabelled antibody is added to the same albumin-fluorescein conjugate. Pure solutions of fluorescent conjugates have arbitrary fluorescence intensities of 100. Excitation wavelength : 470 nm.

Fig. 3.3 Effects of adding rhodamine-labelled antibody to an albumin-fluorescein conjugate (label:protein ratio = 4:1). Other details as in Fig. 3.2.

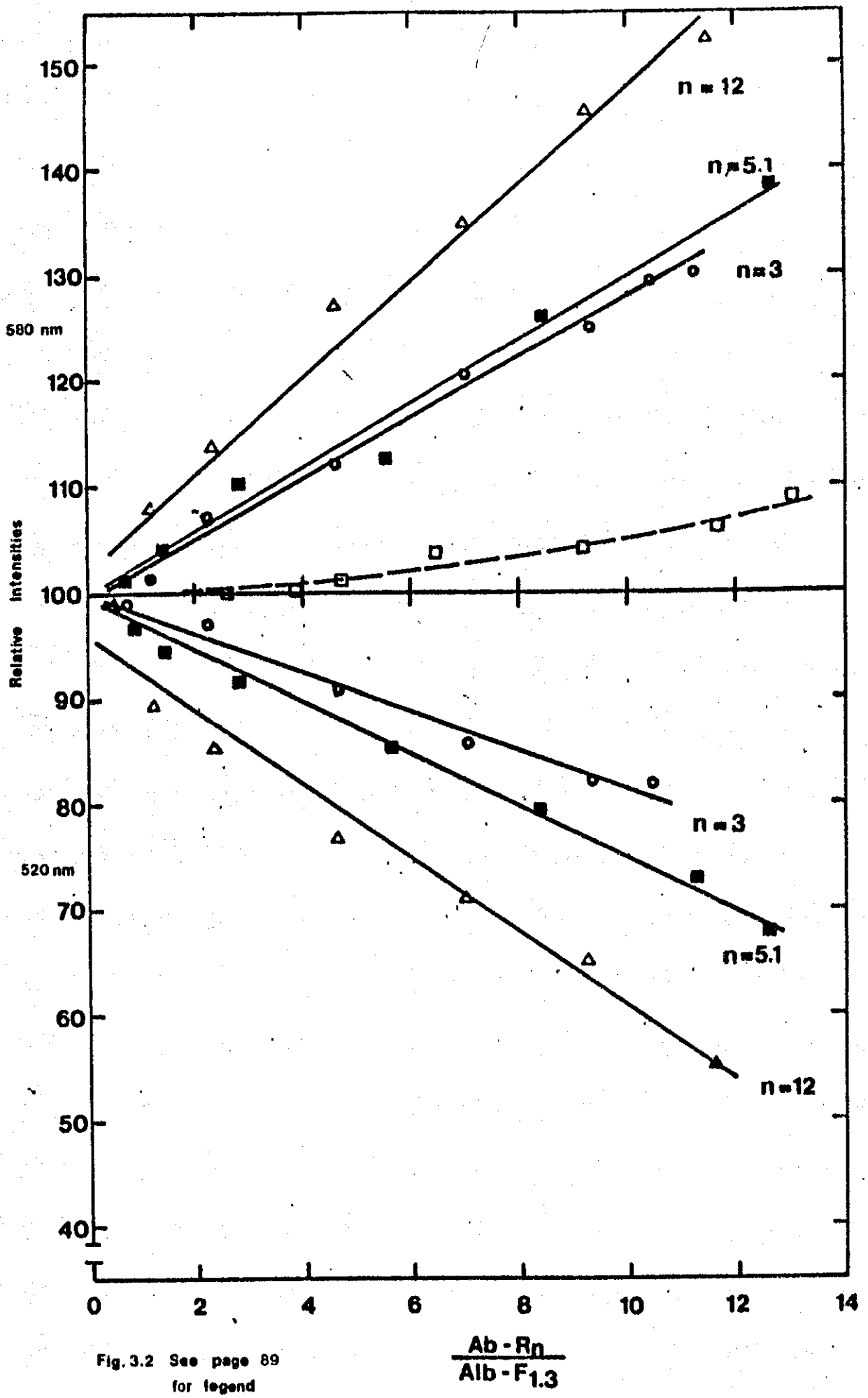


Fig. 3.2 See page 89 for legend

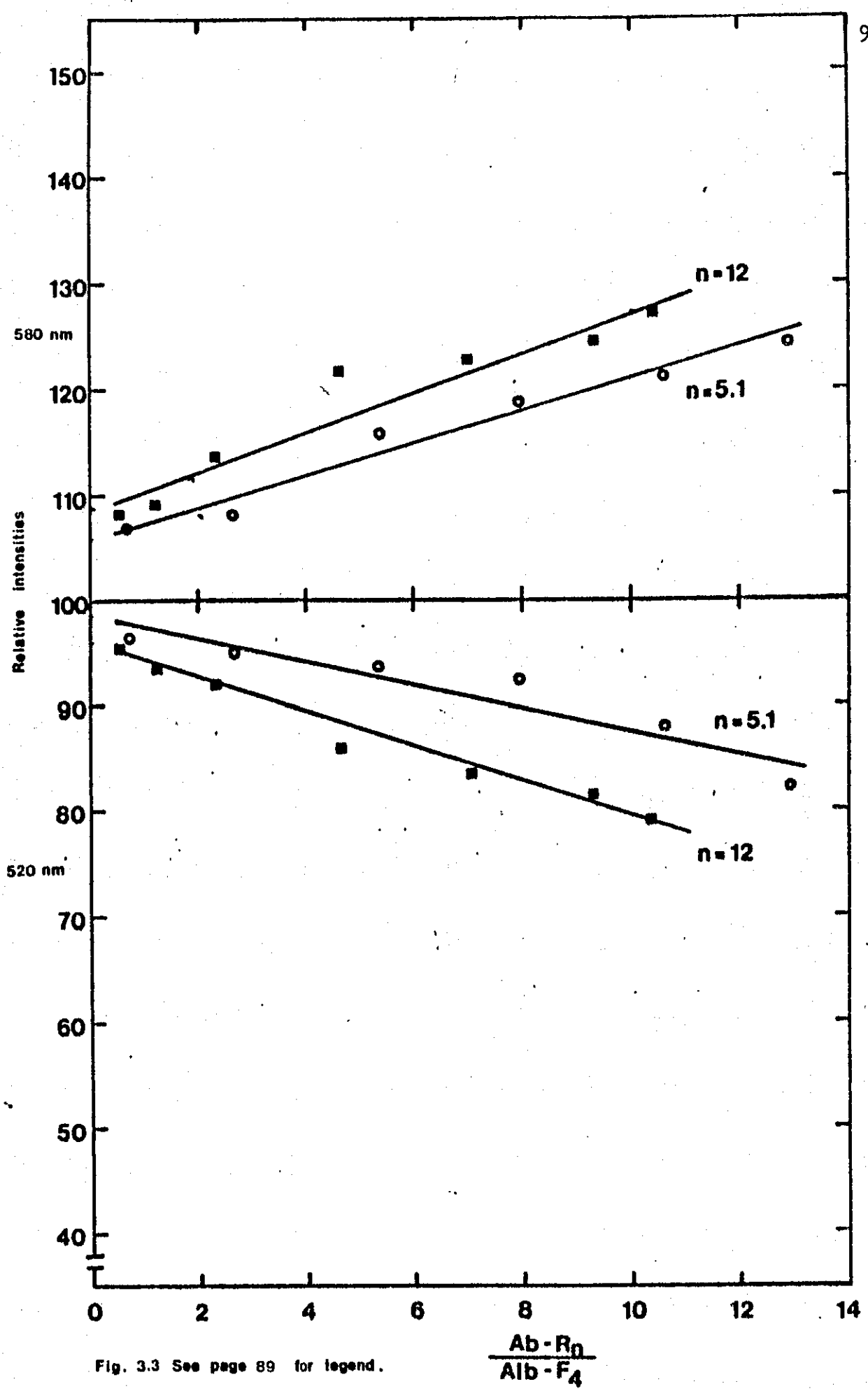


Fig. 3.3 See page 89 for legend.

Comparison of Figures 3.2 and 3.3 shows that the degree of labelling of the albumin-fluorescein molecule is also an important factor. For a given rhodamine-labelled solution, and a given antibody:antigen ratio, the efficiency of resonance transfer from fluorescein to rhodamine is less for an albumin-fluorescein with a molar F:P of 4.0 than for a conjugate in which the molar F:P is only 1.3. That is, the more heavily-labelled albumin conjugate is less susceptible to quenching by the energy transfer mechanism than the lightly-labelled one. The principal reason for this effect is likely to be that, in the heavily-labelled conjugates, fluorescein-fluorescein energy transfer occurs in competition with fluorescein-rhodamine energy transfer. The corrected excitation and emission spectra of an albumin-fluorescein conjugate overlap to a considerable degree, (Fig. 3.1). The fluorescein-fluorescein energy transfer is thus likely to be quite efficient. These results show that the optimum conditions for the observation of large proportional changes in the fluorescence intensities of fluorescein and rhodamine labels include the use of lightly-labelled fluorescein (donor) conjugates, and heavily-labelled rhodamine (acceptor) conjugates. A few experiments were also performed in which the labels were reversed ie. an antibody solution lightly labelled with fluorescein (label: protein ratio = 1.4:1) was the donor of energy, and a heavily-labelled albumin-rhodamine conjugate (label:protein ratio = 8.0:1) was the acceptor. Results of all these experiments show that the measured quenching and enhancement effects were substantially less than those found using albumin-fluorescein and antibody-rhodamine conjugates. It is naturally possible that the opposite result will be obtained with a different antigen.

The sensitivity of the assay system was found to be dependent on the total concentration of the labelled reactants. Fig. 3.4 shows that in an assay system where the concentration of albumin-fluorescein was 4.8×10^{-9} and the molar labelled antibody : labelled antigen 14.6, albumin concentrations of 10^{-9}M and below could be detected. (This is well below the concentration of albumin in normal urine - ca. 10^{-7}M, so the energy transfer assay might be suitable for this application - Cf. Woo et al,1979) When both labelled albumin and labelled antibody were present at 10-fold higher concentration, however, the sensitivity of the assay is much reduced. In the latter conditions, not only was a higher concentration of sample albumin necessary to dissociate labelled albumin-labelled antibody complexes, but inner-filter effects would also reduce the observed fluorescence intensity changes. If the labelled immune reactants were present in very low concentrations, however, the maximum instrument gain setting (producing worsening signal to noise ratios) had to be used to measure the fluorescence signals : in this work, the limit of detection of albumin in pure solution was found to be ca. 4.5×10^{-10}M . The sensitivities of different fluorimeters differ substantially in practice, so an instrument specially designed to give optimum results with fluorescein-labelled speceis might be able to improve on this detection limit considerably.

A further important limitation on the sensitivity of a fluorimeter in this assay is the spectral bandwidth. Fig. 3.5 shows that the largest energy transfer effects, measured by determining the quenching of fluorescence of labelled albumin, were observed when both the excitation and emission monochromators on the instrument were used with narrow bandwidths. In the illustrated experiment, the quenching observed with excitation

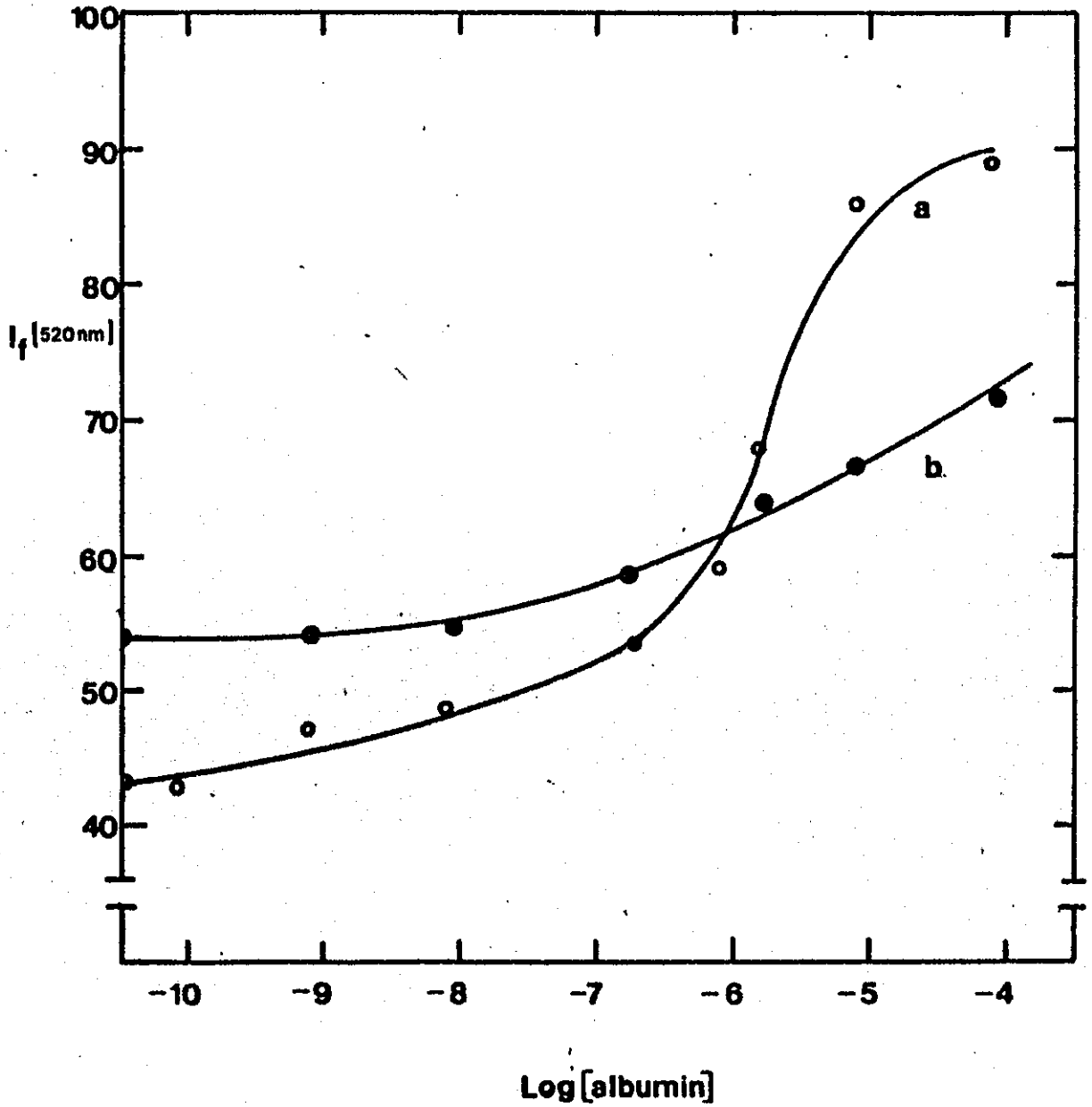


Figure 3.4

Changes in fluorescence intensity at 520 nm when unlabelled albumin is added to mixtures containing (a) 4.8×10^{-8}M albumin-fluorescein conjugate (label : protein ratio 1.3 : 1) and 7×10^{-7}M antibody-rhodamine conjugate (label : protein ratio 12 : 1), and (b) the same conjugates each at one-tenth the concentrations in (a).

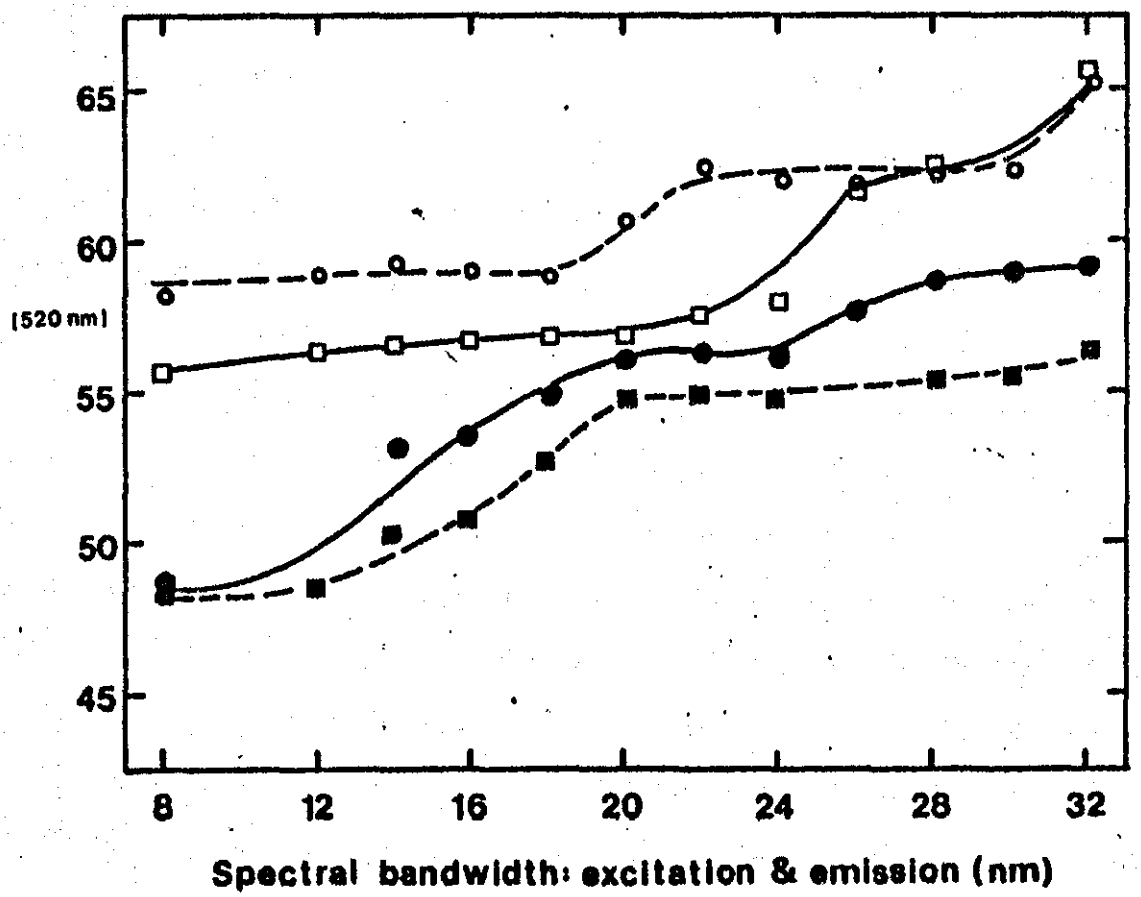


Figure 3.5

Effects of spectral bandwidths on the efficiency of energy transfer (measured at 520 nm) between an albumin-fluorescein conjugate (9.6×10^{-9}M; label:protein ratio, 1.3:1) and an antibody-rhodamine conjugate (1.6×10^{-7}M; label:protein ratio, 16.5:1). The albumin-fluorescein conjugate alone has a fluorescence intensity of 100.

—●—●— = emission bandwidth 12 nm, excitation bandwidth varied;

—□—□— = emission bandwidth 32 nm, excitation bandwidth varied;

—■—■— = excitation bandwidth 12 nm, emission bandwidth varied;

—o--o— = excitation bandwidth 32 nm, emission bandwidth varied.

Excitation wavelength, 470 nm.

and emission bandwidths of 8 and 12 nm respectively was 51.5 %; when both monochromators were used with bandwidths of 32 nm, the observed quenching was only 35 %. This effect occurs because the excitation spectrum of the rhodamine label partially overlaps that of the fluorescein label (Fig. 3.1). Thus, when the fluorimeter is used with large bandwidths some direct excitation of the rhodamine occurs at a nominal exciting wavelength of 470 nm, in addition to and in competition with the energy transfer excitation. This problem has been previously noted during other studies of energy transfer phenomena (Schiller, 1975) : one practical effect in the present application is to reduce the value of filter fluorimeters in fluorescein-rhodamine energy transfer assays.

The excellent specificity of the assay is exemplified in Fig. 3.6. Addition of human IgG to a mixture of fluorescein-labelled albumin and rhodamine-labelled anti-albumin produced no significant changes in fluorescence intensity. Furthermore, excellent agreement between the energy-transfer method (using fluorescein quenching measurements) and the electroimmunoassay method was obtained when four diluted serum samples were studied (Table 3.1). The energy-transfer assay also showed excellent precision, particularly when the serum samples were diluted 500 times to minimise fluorescent background interference.

The development of a useful fluorescence immunoassay requires that the fluorescent labelled reagents are reasonably stable both under measurement conditions and on storage. A long shelf-life for such reagents will facilitate long-term assay standardization and is indeed one of the major potential advantages of fluorescence immunoassay methods compared to isotopic assays. In this work, fluorescein-labelled proteins

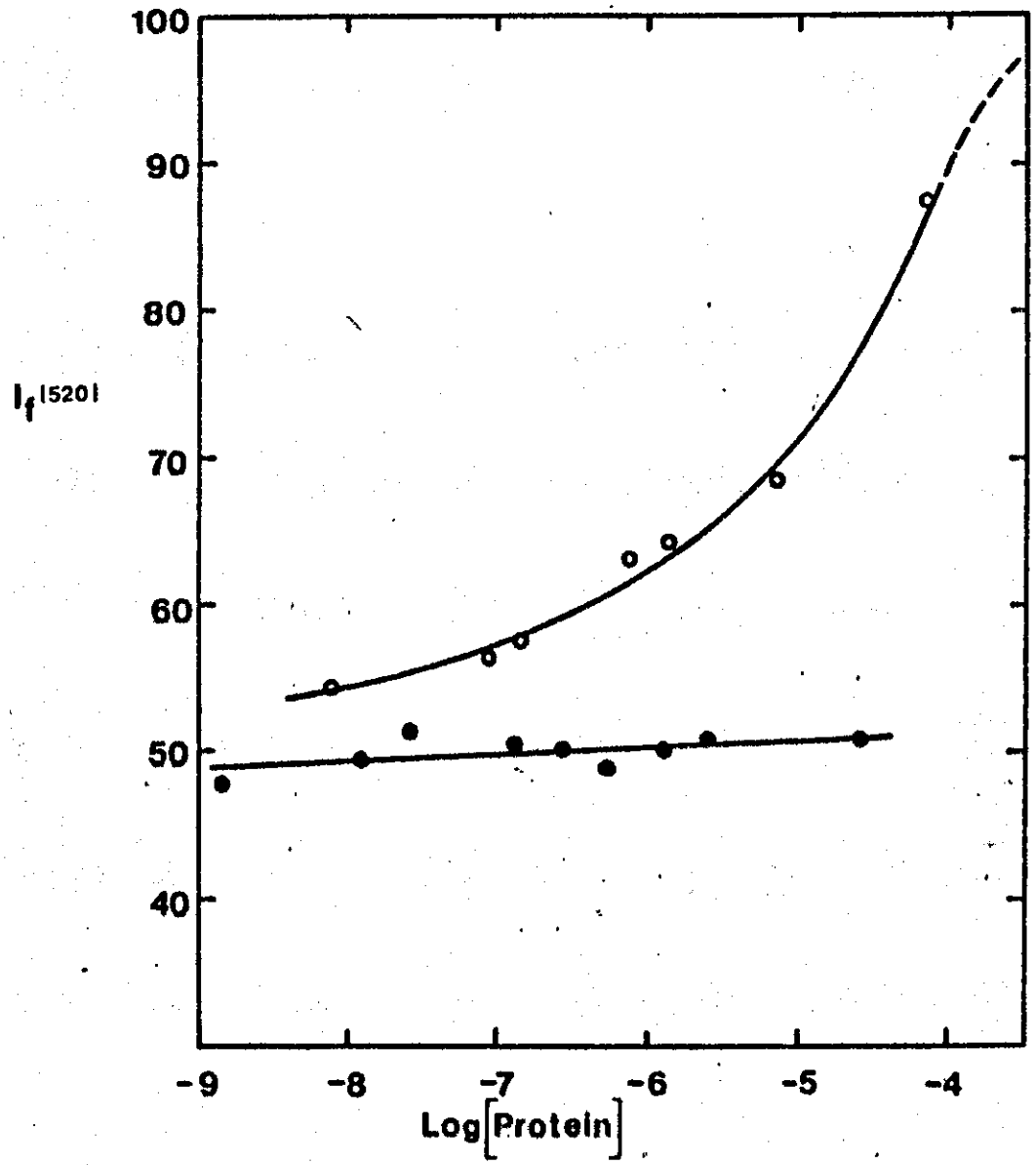


Figure 3.6

Effects of adding albumin (—o——o—) and human immunoglobulin G (—●——●—) to a solution containing an albumin-fluorescein conjugate (4.8×10^{-9}M ; label:protein ratio, 1.3:1) and an anti-albumin-rhodamine conjugate (5×10^{-8}M ; label:protein ratio, 8.1:1). The albumin-fluorescein conjugate alone has arbitrary fluorescence intensity of 100. Excitation wavelength, 470 nm; bandwidths, 12 nm.

Table 3.1

Determination of albumin in diluted human serum samples:
comparison of energy-transfer and electroimmunoassay methods

		Energy-Transfer (E-T) Assay				Electroimmunoassay	
Sample	Dilution for E-T assay	Same day Concentration mg. dl^{-1}	(n=10) cv %	Different days Concentration mg. dl^{-1}	(n=10) cv %	Same day Concentration mg. dl^{-1}	(n=10) cv %
1	x50	3370±130	3.8	3180±220	6.9	3300±160	5.0
2	x50	3400±110	3.1	3570±230	6.4	3700±260	7.0
3	x500	3970±120	3.0	4070±160	4.0	4100±180	4.4
4	x500	3510±80	2.3	3620±180	4.9	3700±120	3.3

stored in the dark at 4 C were found to be stable (in respect of fluorescent and immunological characteristics) over periods of several months. Rhodamine-labelled antibody solutions, on the other hand, exhibited turbidity and precipitation after only a few weeks in the same storage conditions. Heavily-labelled fractions (F:P 10) were particularly vulnerable to such losses. Thus, a preparation of rhodamine-labelled anti-albumin, with an initial rhodamine:protein ratio of 13.0 was found, after storage at 4 C for 12 weeks, removal of precipitate by centrifugation, and analysis, to have a rhodamine:protein ratio of only 9.3. After re-standardization, such rhodamine-labelled samples were still usable but the sensitivity of the assay was thus reduced, so such reagents are clearly not ideal.

From these results it is apparent that, while energy-transfer immunoassays using fluorescein and rhodamine as the donor and acceptor labels are perfectly practicable, these fluorophores are by no means ideal for use in such assays. Particular disadvantages include the poor stability of rhodamine-labelled antibodies, and the overlap of the fluorescein and rhodamine absorption spectra. In addition, the Stokes shift for fluorescein is relatively small, so interference from scattered light may limit the sensitivity of the assay in biological samples; and in the present work the intensity of the rhodamine emission at 580 nm was too feeble to permit its routine use in a sensitive assay. Results of the investigations performed to identify pairs of labels that overcome most of these disadvantages are discussed in the next chapter.

CHAPTER 4 EVALUATION OF SOME POTENTIAL DONOR-ACCEPTOR FLUORESCENT LABELS APPLICABLE IN ENERGY TRANSFER IMMUNOASSAY

4.1 Introduction

All the applications of ETIA so far reported (Ullman et al 1976; Rodgers et al 1978; Eimstad et al 1978) have made use of fluorescein as the donor label because of its high absorption coefficient and fluorescence quantum yield, and rhodamine as the acceptor label because of its good spectral overlap with the fluorescein emission maximum. An evaluation of these two labels described in Chapter 3 has shown that they are not an ideal pair of labels for use in such assays. The principal deficiencies of the fluorescein-rhodamine energy transfer assay system can be attributed to :

(i) the small Stokes shift of the fluorescein molecule (ca 30 nm) which makes fluorimetric measurements at the emission maximum wavelength (520 nm) very liable to interferences from Rayleigh (and possibly Raman) scatter especially in assays of biological samples;

(ii) the partial overlap of the absorption spectra of fluorescein and rhodamine which reduces the assay sensitivity when large spectral bandwidths for fluorescein excitation at 490 nm are used; and

(iii) the poor stability of the rhodamine-labelled protein solutions on storage, which prevents long-term assay standardization.

To date, most fluorescence immunoassays (FIA) have utilized fluorescent molecules which were originally developed

for other applications. For example, fluorescein which is perhaps the most commonly used fluorophore in FIA (eg.Aalberse 1973; Watson et al 1976; Smith 1977; Shaw et al 1977; McGregor et al 1978; etc) was initially employed by Coons et al (1942) in immunofluorescence microscopy as a topographical marker for the localization of antigens or antibodies in tissue cells. Not surprisingly, a large number of the commonly (and commercially) available fluorescent labels have been found to be unsuitable for specific applications in FIA. The special requirements of a fluorescent label for use in FIA severely limit the choice to only a few fluorophores. A further limitation is imposed by the specific requirements of the energy transfer assay method which requires, amongst other conditions, the optimum spectral overlap of the donor emission spectrum with the acceptor excitation spectrum.

This chapter describes the preliminary investigations which were performed to identify suitable donor-acceptor pairs amongst the many commonly available fluorescent labels. The development of a single pair of labels applicable to the widest possible range of materials would be very desirable, although in practice it is more likely that a number of different donor-acceptor systems would be required to facilitate the labelling of a range of ligands with different chemically active groups. The following donor-acceptor pairs were studied:

(a) quinacrine and fluorescein

(b) quinacrine and rhodamine

(c) dansyl chloride and rhodamine

(d) fluorescamine and fluorescein

(e) MDPF and fluorescein (to be described in Chapters 6 and 7)

Of the above, only (d) and (e) were found to be suitable donor-acceptor pairs. Fluorescamine (donor) and fluorescein (acceptor) were used to develop an energy transfer assay for human albumin in an evaluation of their applicability to the development of similar assays for other analytes. The general methodology used in the albumin assay was later extended to the development of assays for nortriptyline and related tricyclic antidepressants (Chapter 5), human serum transferrin (Chapter 6), and human immunoglobulin G and A (Chapter 7)

Experiments were also performed to investigate the changes in the fluorescence intensities when unlabelled specific antibodies were reacted with antigens labelled with different types of fluorophores (quinacrine, DNS, fluram and MDPF). In these studies, albumin-fluorescamine conjugates in particular were found to exhibit large increases in the fluorescence intensity when excess unlabelled anti-albumin antibodies were added. Further investigations (to be described in subsequent chapters) confirm that the fluram enhancement phenomenon is a general effect observed with all the fluorescamine-labelled antigens studied in this work.

4.2 Experimental

The donor-acceptor pairs listed above were studied using the following labelled proteins (see Table 2.2 for abbreviations used).

(a) IgG-Q and Ab-F
(b) IgG-Q and Ab-R
(c) Alb-D and Ab-R
(d) Alb-FL and Ab-F

Quantitative fluorimetric studies of fluorescamine-fluorescein energy-transfer were performed with a Baird-Atomic Fluoripoint Spectrofluorimeter. The MPF-44B Fluorescence Spectrophotometer was used in experiments to evaluate other donor-acceptor pairs. Thermostatted 10 mm path length silica cuvettes were used in all fluorescence measurements at 35 C after preincubation of the reagents at the same temperature for 60 minutes (for experiments on (a) and (b)) or 45 minutes (for experiments on (c) and (d)). Corrections were made for the background fluorescence contributed by the buffer, added proteins and labelled antibodies. The buffer used in all the experiments was 0.01M phosphate buffer, pH 7.2, containing 0.145M NaCl.

The spectra shown in Figure 4.10 were recorded with a Baird-Atomic Fluoricord Spectrofluorimeter connected to a Model 27000 chart recorder; other spectra were recorded in the corrected mode using the MPF-44B together with the Model 56 recorder.

4.3 Results and Discussion

The condition of maximum spectral overlap (the $J_{\bar{\nu}}$ factor in Equation (1.11)) is an important prerequisite for efficient energy transfer by the resonance mechanism. In a given donor-acceptor system where this condition amongst others is fulfilled, the transfer efficiency can be expected to be high; at the same time, the observation of the energy transfer effects can be complicated by other factors related to the spectral characteristics of both the donor and acceptor labels. This is exemplified in Figure 4.1(b) which shows the good spectral

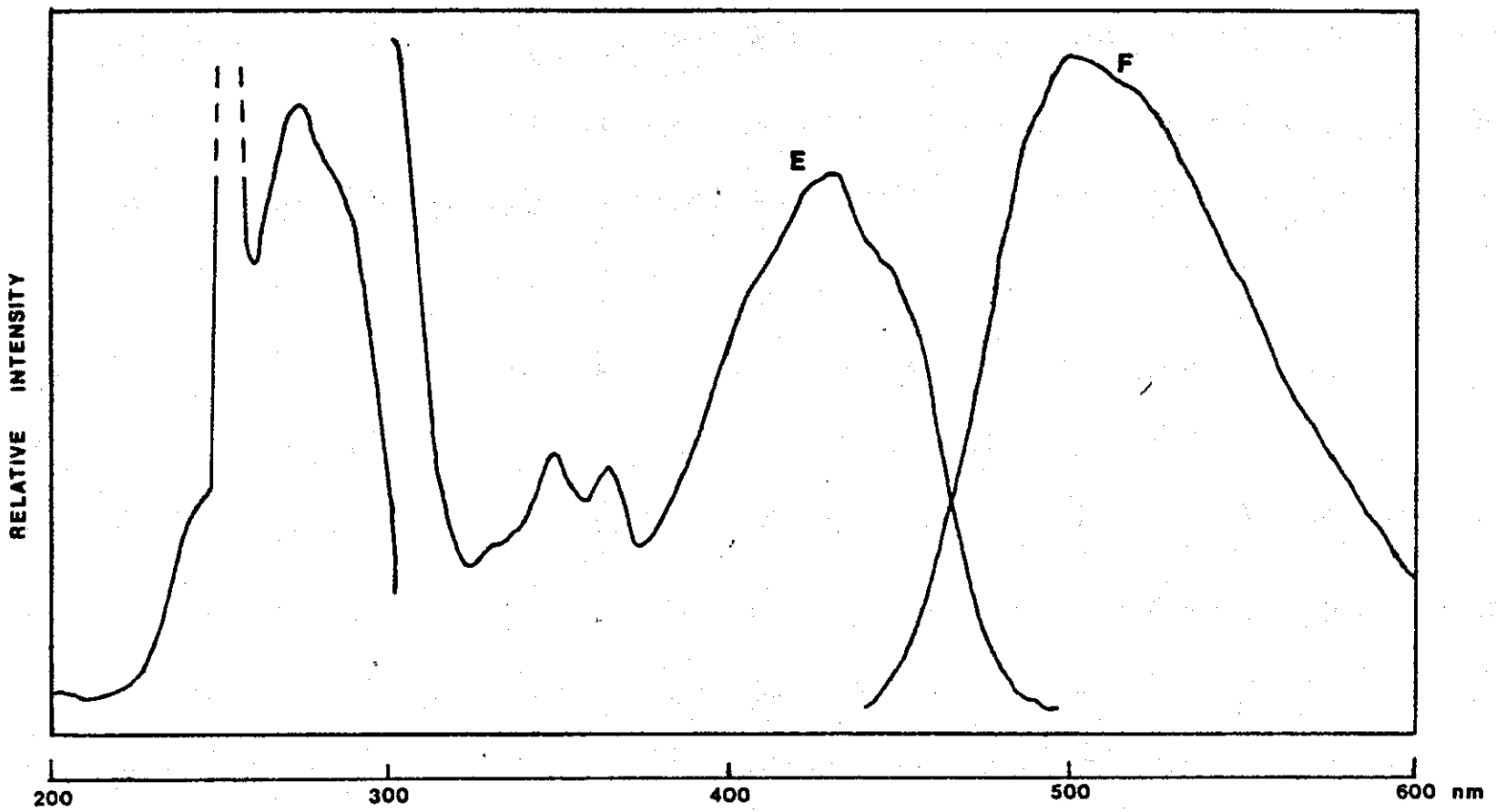


Figure 4.1(a) Corrected excitation (E) and fluorescence (F) spectra of an IgG-quinacrine conjugate (label : protein ratio, 1.9:1). Instrument gain setting was reduced 3.3 times when recording from 200 to 300 nm.

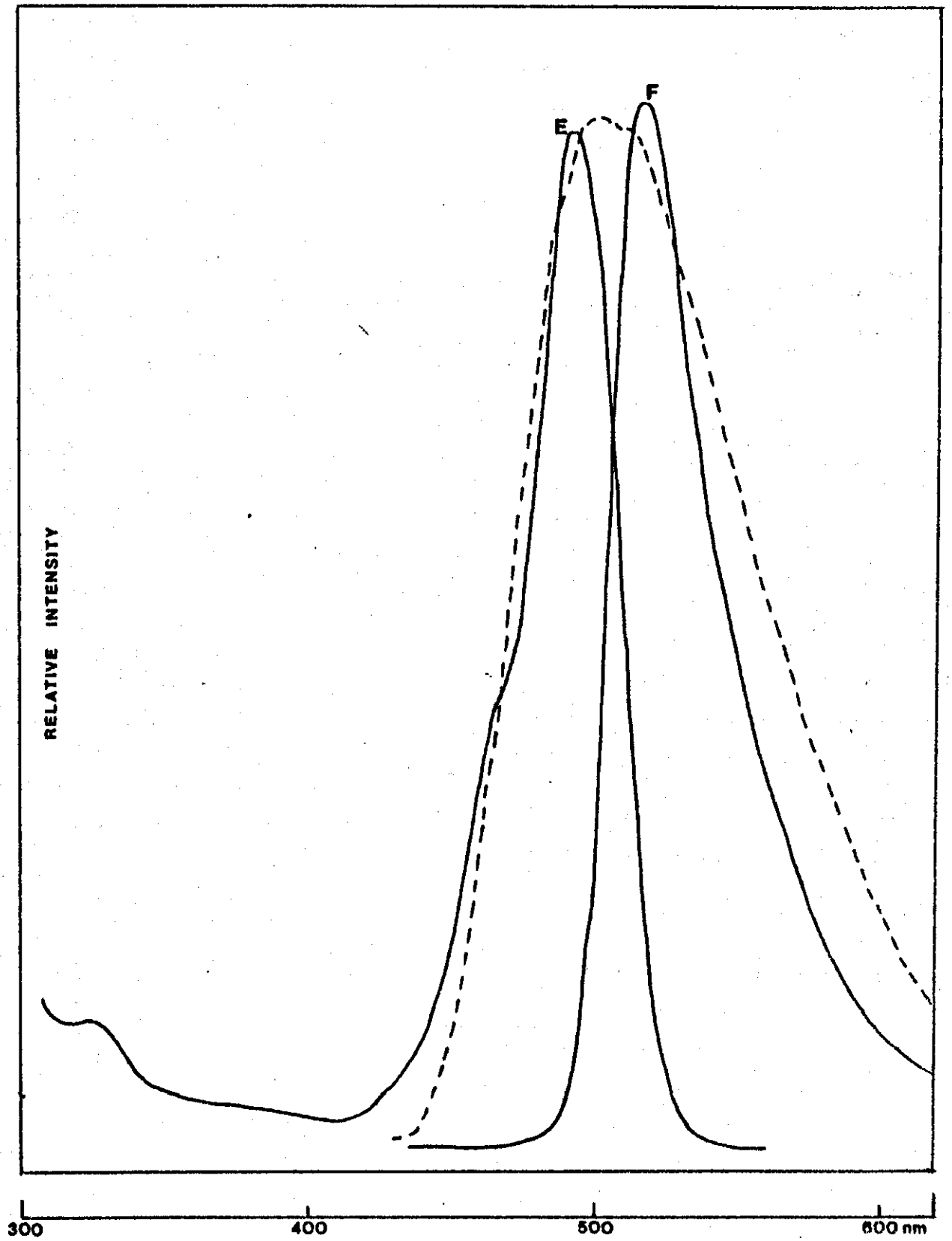


Figure 4.1(b) Corrected excitation (E) and fluorescence (F) spectra of a fluorescein-labelled anti-IgG conjugate (label:protein ratio, 1.5:1) (———). Fluorescence spectrum of an IgG-quinacrine conjugate (label:protein ratio, 1.9:1) is shown by dotted lines.

overlap of fluorescein with the quinacrine emission spectrum. This desirable condition for the observation of energy transfer between quinacrine and fluorescein is offset, however, by the broad spectral bandwidth of the quinacrine fluorescence spectrum, which also overlaps the fluorescein emission spectrum to a considerable degree. One practical limitation of this result in the study of quinacrine-fluorescein energy transfer will be the difficulty of measuring accurately the enhancement of fluorescein and the quenching of quinacrine emission intensities. Clearly, these two labels are not suitable for use as donor-acceptor labels in ETIA.

In order to overcome the shortcomings of fluorescein as the acceptor label in the above system, rhodamine as an acceptor of energy was investigated as a potential replacement for fluorescein. Even though the spectral overlap between quinacrine emission and rhodamine excitation spectra is less than optimum (Fig. 4.2), rhodamine was nevertheless employed in this investigation because, if efficient energy transfer from quinacrine to rhodamine could occur, then the measurement of the rhodamine enhancement intensity at 585 nm would be more convenient compared to the measurement of the fluorescein emission intensity at 520 nm (for reasons already discussed in Chapter 3). The efficiency of quinacrine-rhodamine energy transfer was investigated by addition of rhodamine-labelled anti-IgG conjugates to a fixed concentration of IgG-quinacrine. The resultant energy transfer effects were assessed by measuring the quenching of quinacrine emission at 500 nm and the rhodamine enhancement at 585 nm (an excitation wavelength of 425 nm was used). Figures 4.3 and 4.4 show that in the reaction between a lightly labelled

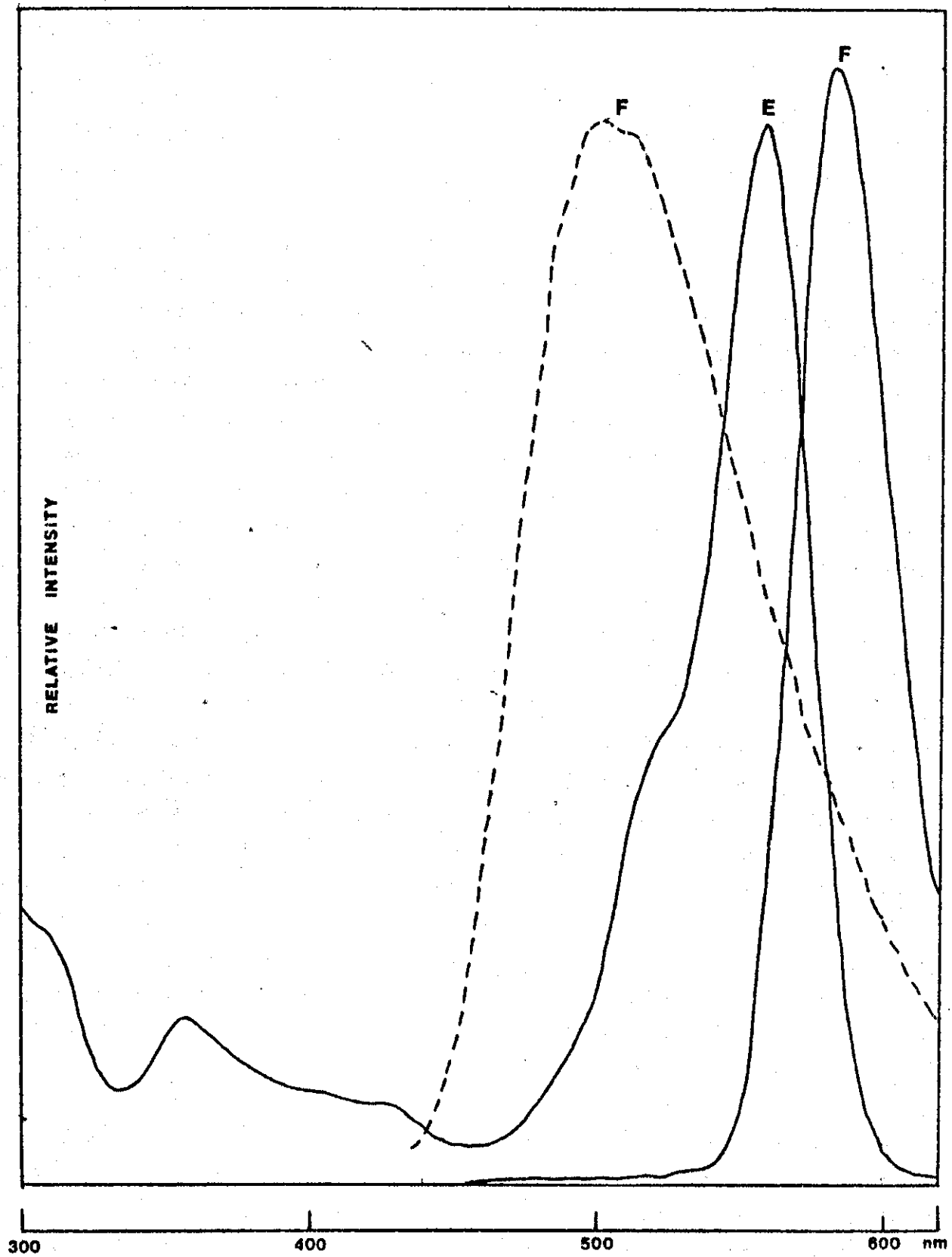


Figure 4.2 Corrected excitation (E) and fluorescence (F) spectra of a rhodamine-labelled anti-IgG conjugate (label:protein ratio, 8.3:1) (———). Fluorescence spectrum of an IgG-quinacrine conjugate (- - -) reproduced from Fig. 4.1(b).

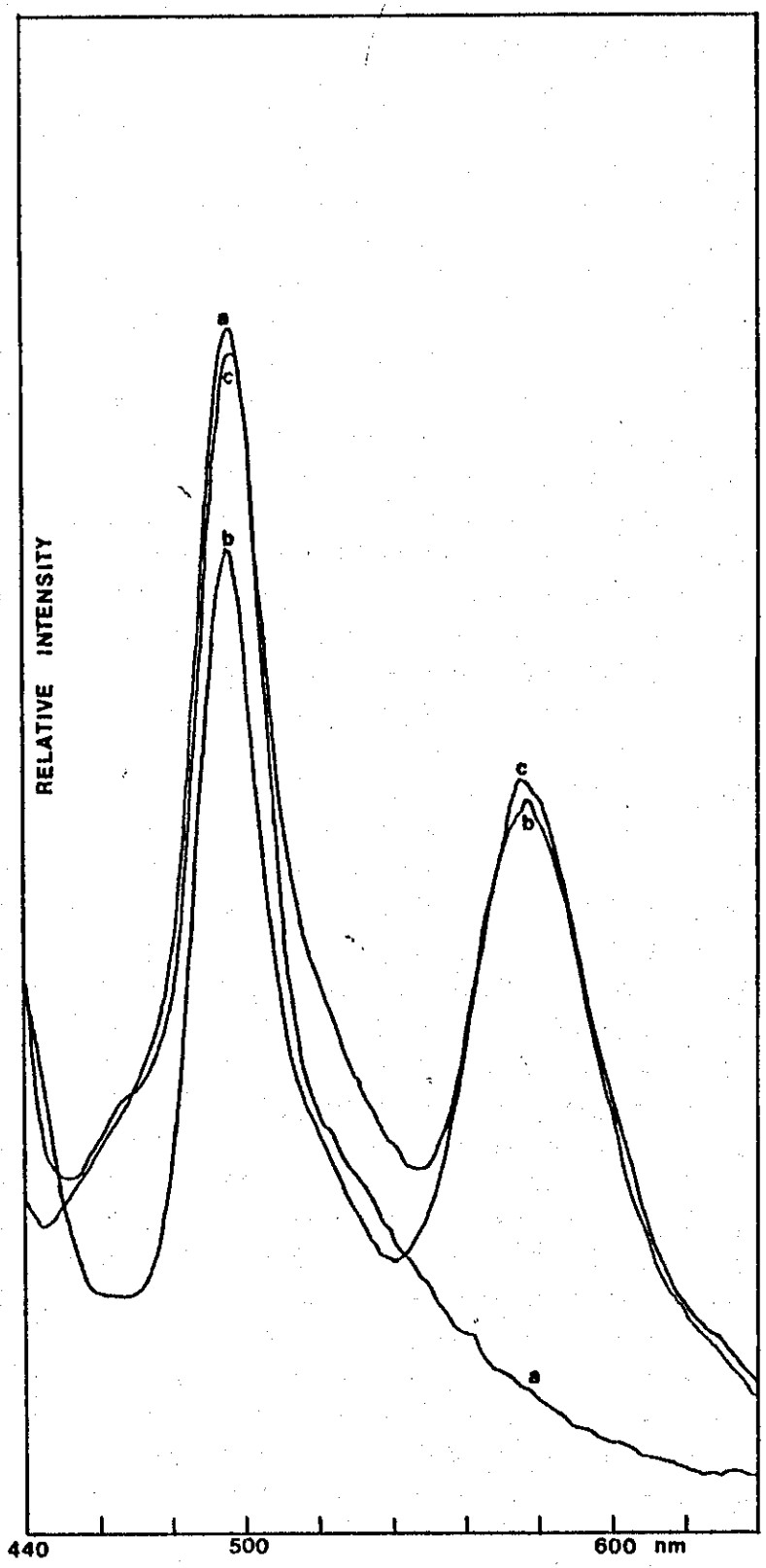


Figure 4.3 (a) and (b) are respectively the emission spectra of IgG-quinacrine conjugate (8.95 nM; label:protein ratio,1.9:1) and rhodamine-labelled anti-IgG conjugate (22.9 nM; label:protein ratio, 8.3:1). (c) is the emission spectrum of a mixture containing both the conjugates at the concentrations stated.

Excitation wavelength, 425 nm ; bandwidths, 10 nm .

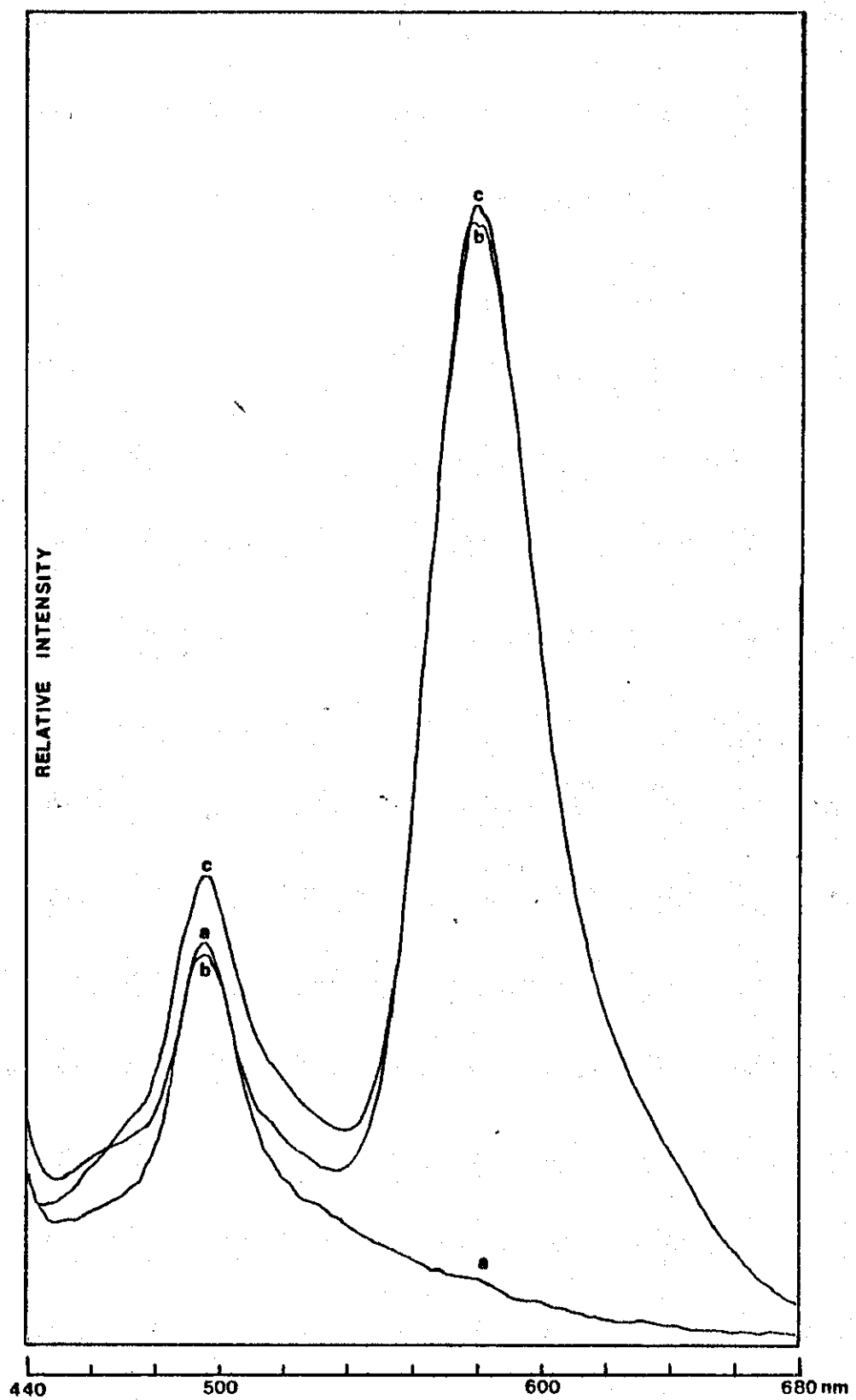


Figure 4.4 As in Fig. 4.3 , except that the rhodamine-labelled anti-IgG conjugate concentration is 1.07×10^{-7}M.

IgG-quinacrine conjugate (label:protein ratio, 1.9:1) and its specific antibody heavily labelled with rhodamine (label:protein ratio, 8.3:1), no significant changes in the fluorescence intensities of quinacrine and rhodamine were observed either at low (2.56) or high (12.8) molar labelled antibody:labelled antigen ratios. The negligible transfer effects observed in this case was at first thought to be due to the low degree of labelling in the IgG-quinacrine molecules, but similar negligible transfer effects were again observed when more heavily labelled IgG-quinacrine conjugates (label:protein ratios, 4.2:1 and 8.6:1) were used with the same preparation of rhodamine-labelled anti-IgG conjugates. Furthermore, the accurate determination of quinacrine quenching effect at 500 nm was made more difficult by the overlapping Raman scatter peak (Fig. 4.5); a small increase (ca 20 %) in the IgG-quinacrine emission intensity was also noted when excess unlabelled anti-IgG antibodies were added (Fig. 4.5). The latter effect would have more than nullified whatever small decreases in quinacrine emission caused by energy transfer. These results demonstrate clearly that quinacrine is not a suitable donor label for use in ETIA studies.

Legend for Figure 4.5

Emission spectra showing the changes in the fluorescence intensity of mixtures containing a fixed concentration of IgG-quinacrine conjugate (1.79×10^{-8} M ; label:protein ratio, 1.9:1) and increasing amounts of unlabelled anti-IgG antibodies.

B = emission spectrum of buffer alone; the Raman scatter peak occurs at 500 nm.

① = emission spectrum of IgG-quinacrine conjugate alone.

1 = ① - B. All other spectra were corrected for the added Ab. 2,(Ab:Ag ratio, 3.8:1) ; 3,(7.6:1) ; 4,(11.3:1) ; 5,(15.0:1) ; and 6,(18.9:1). Excitation wavelength, 425 nm; bandwidths, 10 nm.

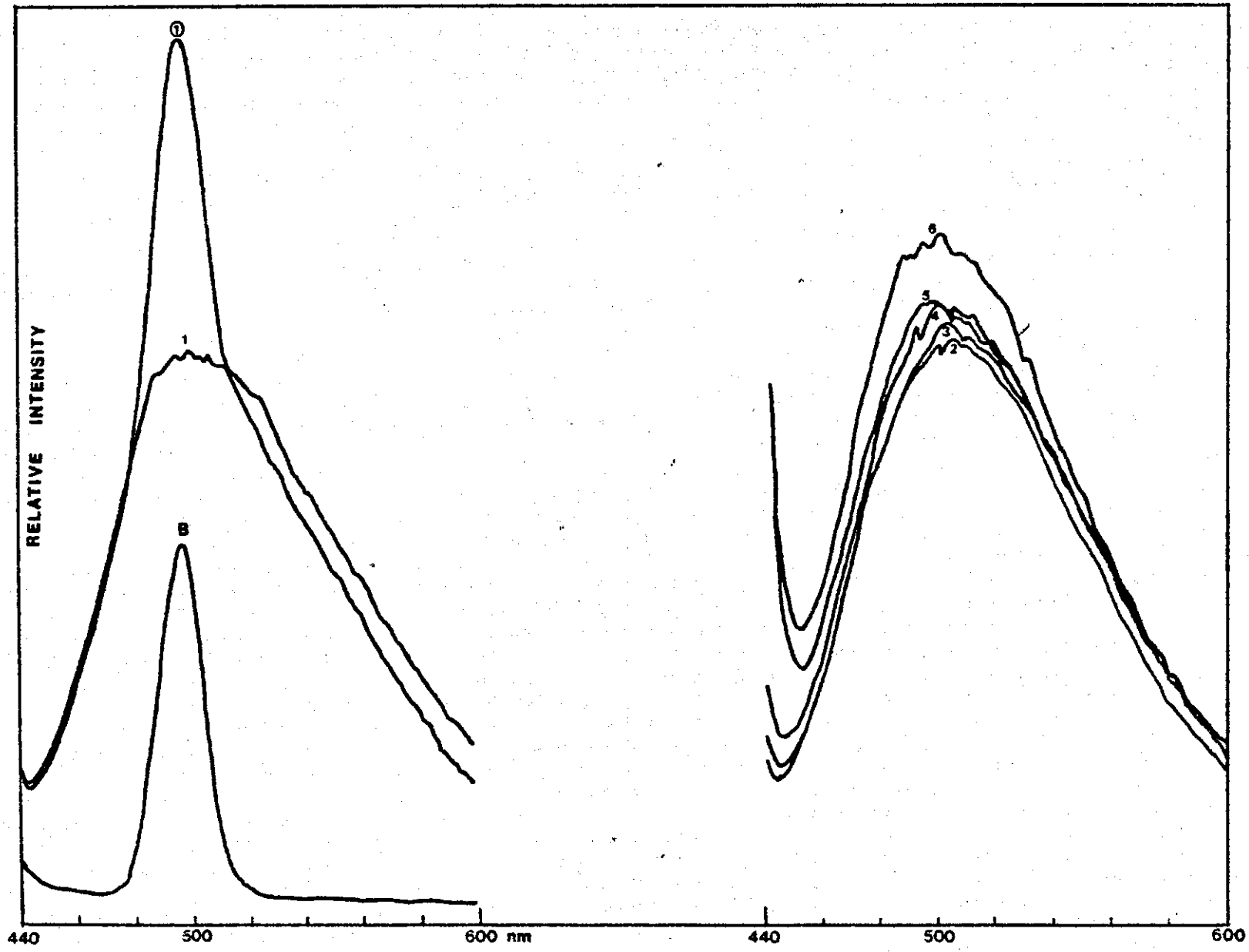


Figure 4.5

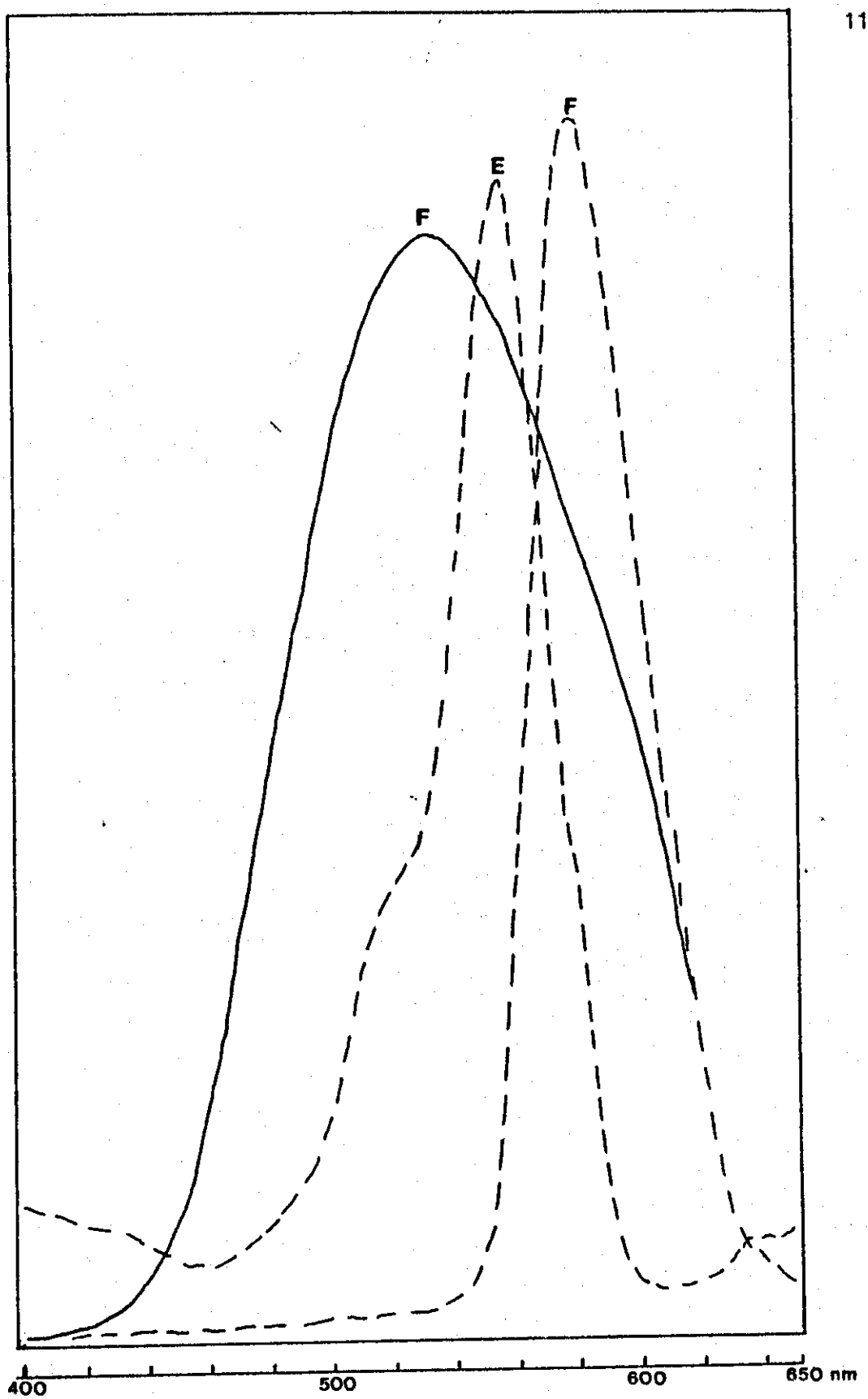


Figure 4.6 Corrected excitation (E) and fluorescence (F) spectra of a rhodamine-labelled anti-albumin conjugate (- - -) (label: protein ratio, 7.5:1), and of an albumin-dansyl conjugate (———) (label:protein ratio, 16:1)

The overlap of dansyl emission and rhodamine excitation spectra is shown in Figure 4.6. The efficiency of energy transfer in the dansyl-rhodamine system was assessed by adding rhodamine-labelled anti-albumin conjugates to a fixed concentration of dansylated albumin (dansyl:protein ratio, 16:1), and measuring the decrease in the dansyl emission intensity at 530 nm and the relatively small increase in the rhodamine emission intensity at 585 nm (excitation wavelength, 340 nm). See Figure 4.7. At more than a 12-fold molar excess of labelled antibody conjugates, the quenching of the dansyl emission intensity amounted to no more than 30 per cent in the two cases where the degree of rhodamine labelling was 7.1 (Fig. 4.7a) and 11.6 (Fig. 4.7b).(A small increase in the dansyl emission intensity was also observed when excess unlabelled anti-albumin was added to a same concentration of the albumin-dansyl conjugate - Fig.4.8; this effect may partially offset the quenching of dansyl emission intensity by rhodamine-labelled antibodies). Interestingly, in all these studies, there did not seem to be a proportional increase in the rhodamine enhancement intensity at 585 nm comparable to the observed decrease in the dansyl emission intensity at 530 nm. A possible explanation for this observation is likely to be that, in this particular system, the nonradiative deactivation processes are highly efficient in competitively diminishing the sensitized rhodamine fluorescence intensity. A few experiments were also performed using lightly labelled albumin-dansyl conjugates (dansyl:albumin ratios, 4.8:1 and 6.0:1). Results relating to the energy transfer effects observed in these studies were not at all satisfactory because the feeble fluorescence signals from low concentrations of these lightly labelled conjugates required the use of maximum gain setting

in the fluorimeter : this inevitably produced worsening signal to noise ratios. It is apparent from the above results that while energy transfer from the dansyl to the rhodamine group can take place within the labelled antibody-labelled antigen complex, the magnitude of the transfer effects was not sufficiently large to permit its application to the development of a sensitive assay for albumin. It is naturally possible that a greater magnitude of the transfer effects may be obtained using a different antigen, although it is quite likely that even in such an assay system the rhodamine enhancement signal at 585 nm will be too feeble to be utilized for accurate fluorimetric measurements. Moreover, the excitation and emission spectra of dansyl chloride overlap those of serum and other biological fluids (Soini et al,1979), and so fluorimetric measurements of the dansyl emission intensity at 530 nm are very liable to fluorescence background interferences in assays of biological materials.

Legend for Figure 4.7

Emission spectra showing the changes in the fluorescence intensity of mixtures containing a fixed concentration of albumin-dansyl conjugate (5.85 nM ; dansyl:albumin ratio, 16:1) and increasing amounts of antibody-rhodamine conjugates (label: protein ratios, (a) 7.1:1 ; (b) 11.6:1).

R : emission spectrum of albumin-dansyl conjugate alone

Fig. 4.7(a) 1,(Ab:Ag ratio, 3.5:1) ; 2,(7.0:1) ; 3,(10.6:1) ; 4,(14.1:1) ; 5,(17.1:1).

Fig. 4.7(b) 1,(Ab:Ag ratio, 2.6:1) ; 2,(5.1:1) ; 3,(7.7:1) ; 4,(10.2:1) ; 5,(12.8:1).

Emission spectra of added antibody-rhodamine conjugates alone are indicated by numerals within circles. Excitation wavelength, 340 nm ; bandwidths, 6nm.

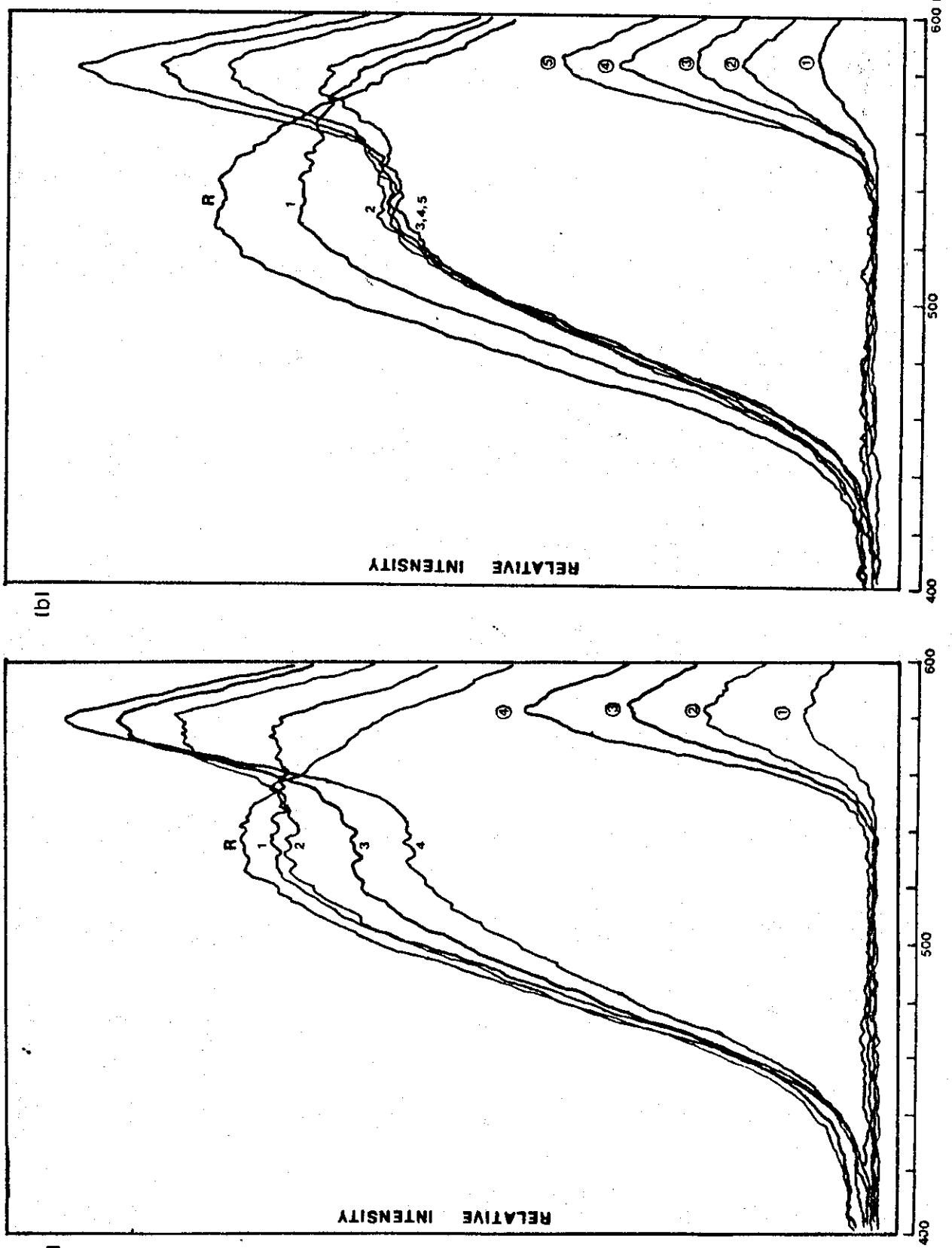

(b)
RELATIVE INTENSITY
R
1
2
3,4,5
①
②
③
④
⑤
400
500
600 nm
(a)
RELATIVE INTENSITY
R
1
2
3
4
①
②
③
④
430
500
600

Fig. 4.7

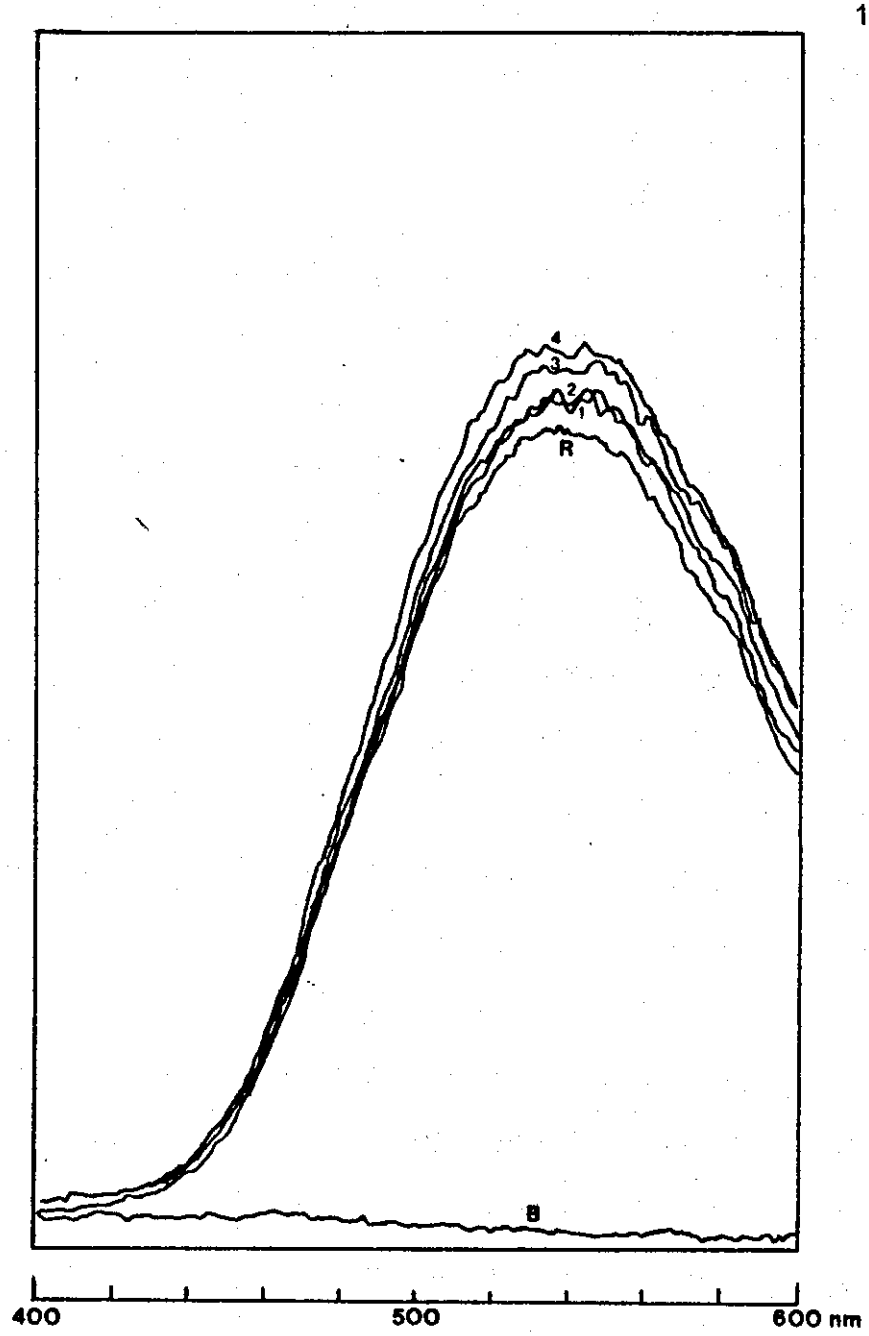


Figure 4.8

Emission spectra showing the changes in the fluorescence intensity of mixtures containing a fixed concentration of albumin-dansyl conjugate (5.85 nM; dansyl:albumin ratio, 16:1) and increasing amounts of unlabelled anti-albumin antibodies.

B : emission spectrum of phosphate buffer alone.

R : emission spectrum of albumin-dansyl conjugate alone.

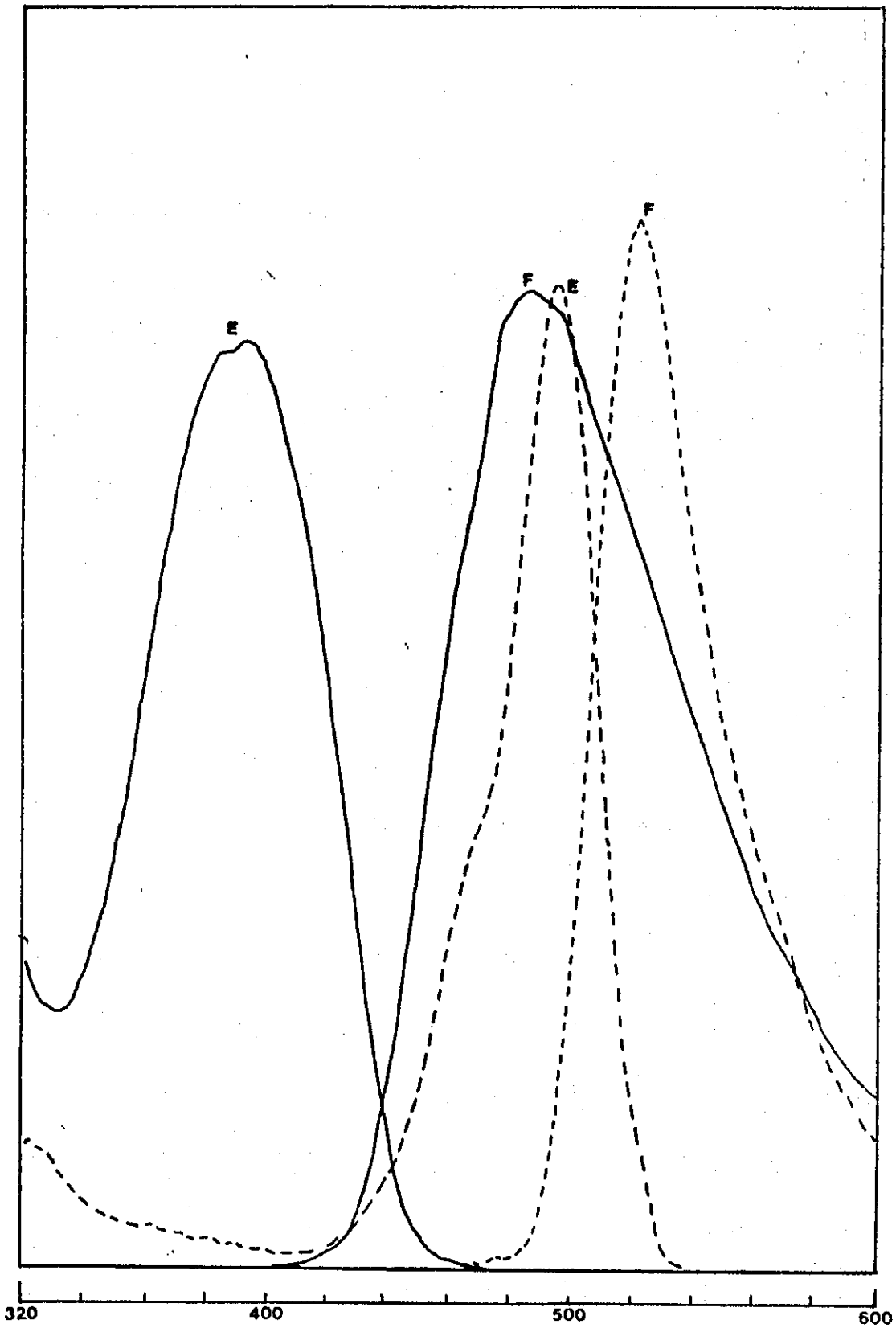
1,(Ab:Ag ratio, 3.7:1) ; 2,(7.5:1) ; 3,(11.2:1) ; and 4,(14.9:1). Excitation wavelength, 340 nm; bandwidths, 6 nm.

Figure 4.9 shows that the maximum of the fluorescamine emission spectrum overlaps strongly the maximum of the fluorescein excitation spectrum. Transfer of energy from fluorescamine to fluorescein can therefore be expected to be efficient, and this is exemplified in Figure 4.10 by the quenching of fluorescamine and the enhancement of fluorescein emission intensities. Quantitative fluorimetric studies of fluorescamine- fluorescein energy transfer were performed by adding increasing amounts of fluorescein-labelled anti-albumin antibodies to a fixed concentration of albumin-fluorescamine conjugate (label:protein ratio, 10.0 : 1), and measuring the decreases in fluorescamine emission intensity at 485 nm and the simultaneous increases in fluorescein emission intensity at 535 nm (an excitation wavelength of 390 nm was used). Figure 4.11 shows the changes in intensities of fluorescamine and fluorescein emission at various molar labelled antibody : labelled antigen ratios. In the two cases where the degrees of fluorescein labelling were 1.4:1 (Fig. 4.11a) and 3.2:1 (Fig. 4.11b), the decrease in fluorescamine emission intensity varied approximately linearly with the added antibody conjugates. At a 6-fold molar excess of labelled antibody conjugates, the overall quenching effect in both the cases amounted to no more than 20 per cent. In the same conditions, the observed proportional enhancement of the fluorescein emission intensity was considerably greater, amounting to more than 110 per cent increase. The most likely cause for this apparent disparity between the quenching and enhancement effects can be related to the exceptionally large increases in the albumin-fluorescamine conjugate emission intensity when excess unlabelled anti-albumin was added (Figure 4.12). The figure also shows that the enhancement phenomenon, termed the "fluram enhancement effect" in this

thesis, was strongly dependent on the number of fluorescamine molecules bound to the albumin, with heavily-labelled conjugates producing the largest effects. Neither the addition of non-specific bovine gammaglobulins to a solution of albumin-fluorescamine conjugate, nor the addition of unlabelled anti-albumin antibodies to a solution of albumin-fluorescein conjugate produced any significant increases in the fluorescence intensities of the conjugates (Fig. 4.12). Experiments performed using other antigens labelled with fluorescamine also gave results similar to those obtained with albumin-fluorescamine conjugate, thus confirming that the fluram enhancement phenomenon is indeed a general effect (see Chapters 5,6 and 7). A probable mechanism for this effect has been described by Miller (1979).

An ETIA for albumin based on the fluorescamine-fluorescein label pair would have to make use of only the fluorescein enhancement measurements because the quenching of the fluorescamine emission intensity by energy transfer is counteracted simultaneously by the fluram enhancement effect. In the assay for albumin, the optimum conditions required include the use of heavily labelled albumin-fluorescamine conjugate (label:protein ratio, 10:1) and the antibody lightly labelled with fluorescein (label:protein ratio,1.5:1). The molar excess of labelled antibody to labelled antigen in the assay should preferably be not too large ($<$ a 6-fold molar excess of labelled Ab) because at high concentrations of the antibody-fluorescein conjugates, the magnitude of the energy transfer effects observed will be diminished by inner filter effect. The standard curves shown in Figure 4.13 permit low concentrations of albumin (sub-nanomolar levels) in pure solution and in serum samples to be detected. Subsequent chapters will describe the applications of the fluorescamine-fluorescein label pair to the development of ETIA's for other analytes.

Figure 4.9 Corrected excitation (E) and fluorescence (F) spectra of an albumin-fluorescamine conjugate (label:protein ratio, 5.8:1) (———), and of an antibody-fluorescein conjugate (label:protein ratio, 3.2:1) (- - -).



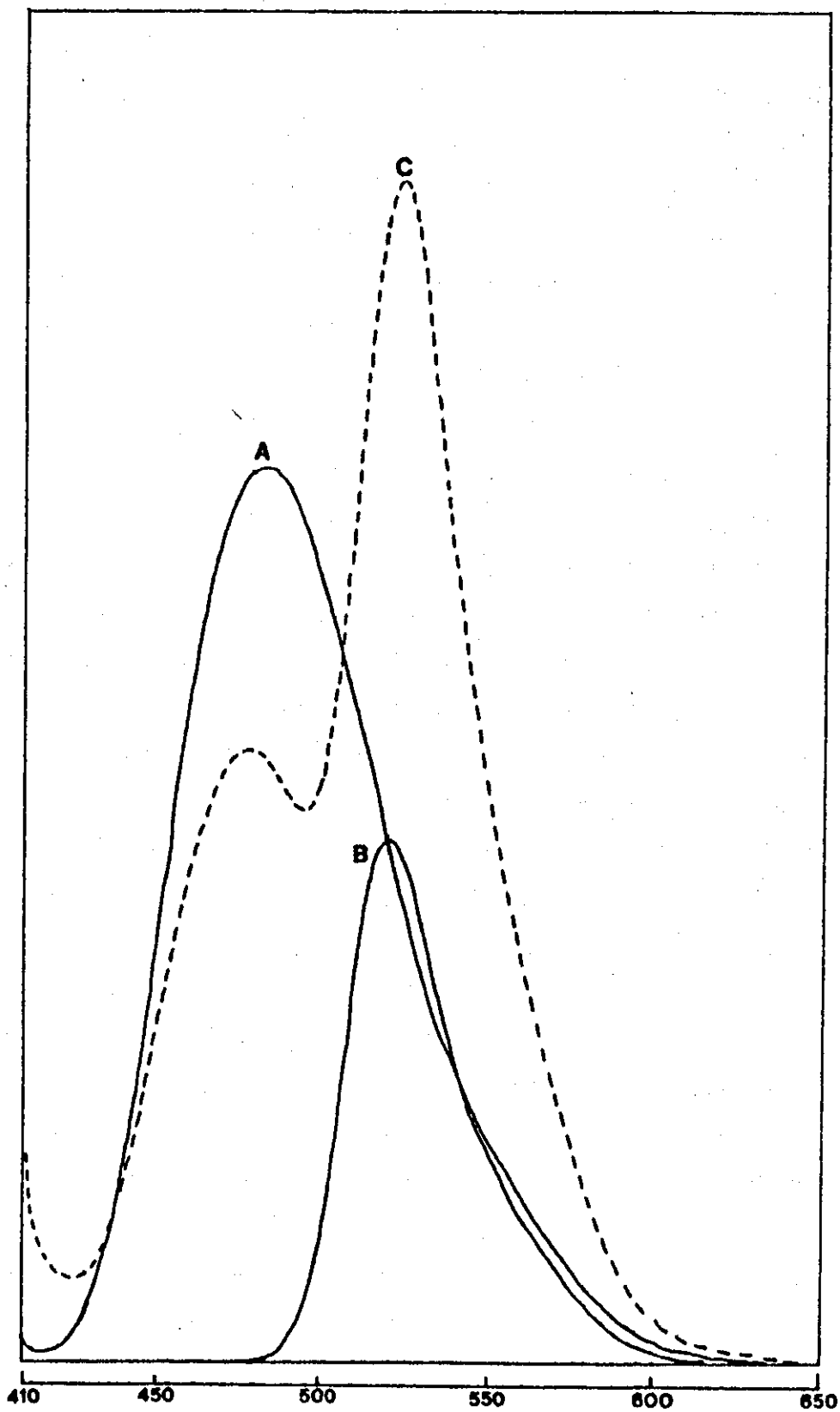


Figure 4.10

Emission spectra of (a) an albumin-fluorescamine conjugate (1.5×10^{-7}M ; label:protein ratio, 6.2:1) ; (b) an antibody-fluorescein conjugate (4.67×10^{-7}M ; label:protein ratio, 1.4:1) ; and (c) a mixture containing both the conjugates at the concentrations stated. λ_{ex} = 390 nm ; bandwidths, 12 nm

Legends for Figures

Figure 4.11

Quenching of fluorescamine emission intensity $I_f(485)$ and the enhancement of fluorescein emission intensity $I_f(535)$ as increasing amounts of antibody-fluorescein conjugates (label: protein ratio, (a) 1.4:1 ; (b) 3.2:1) are added to a fixed concentration of albumin-fluorescamine conjugate (5.7×10^{-8}M ; label:protein ratio, 10:1). Corrections were made for contributions to the background fluorescence by added labelled antibodies. Excitation wavelength, 390 nm ; bandwidths, 10 nm.

Figure 4.12

The Fluram Enhancement Effect.

Increases in fluorescamine emission intensities $I_f(485)$ of albumin-fluorescamine conjugates when unlabelled anti-albumin antibodies are added:

6.2×10^{-8}M Alb-FL ; label:protein ratio, 6.2:1 (—●—●—●—)

7.4×10^{-8}M Alb-FL ; label:protein ratio, 10:1 (—×—×—×—)

7.0×10^{-8}M Alb-FL ; label:protein ratio, 14.7:1 (—□—□—□—)

Effect on the fluorescamine emission intensity at 485 nm when non-specific bovine gammaglobulins are added to an albumin-fluorescamine conjugate (7.4×10^{-8}M ; label:protein ratio, 10:1) is shown by (▲ ▲ ▲), Excitation wavelength used for all the above measurements, 390 nm; bandwidths, 10 nm.

Changes in the fluorescein emission intensity at 530 nm when unlabelled anti-albumin antibodies are added to an albumin-fluorescein conjugate (4.8×10^{-8}M ; label:protein ratio,1.3:1) are indicated by (o o o). Excitation wavelength, 470 nm ; bandwidths, 8 nm. All fluorescent conjugates alone have arbitrary intensity of 100.

Figure 4.13

Changes in the fluorescence intensity at 535 nm when unlabelled albumin in pure solutions (—x—x—) and in standard serum (—I—I—) is added to mixtures containing 2.3×10^{-8}M Alb-FL (label:protein ratio,10:1) and 1.4×10^{-7}M Ab-F (label:protein ratio, 1.4:1). λ_{ex} = 390 nm ; bandwidths, 10 nm . Error bars are standard deviations for 4 measurements.

Fig. 4.11 a

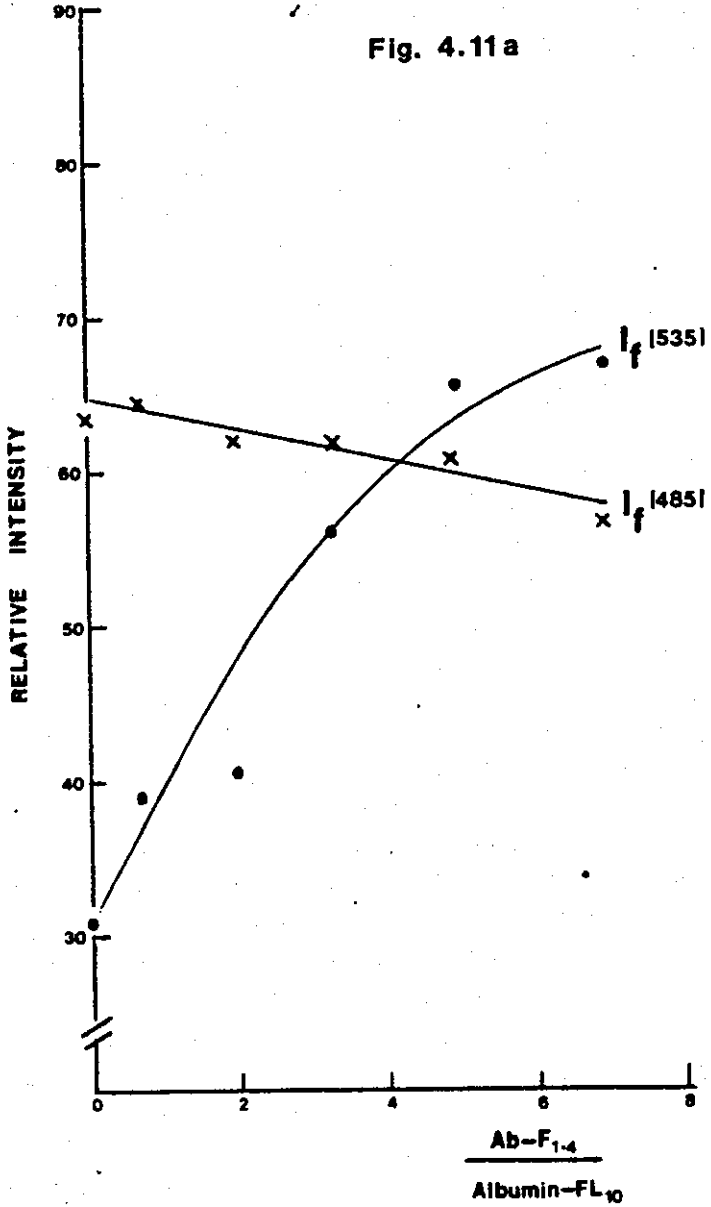
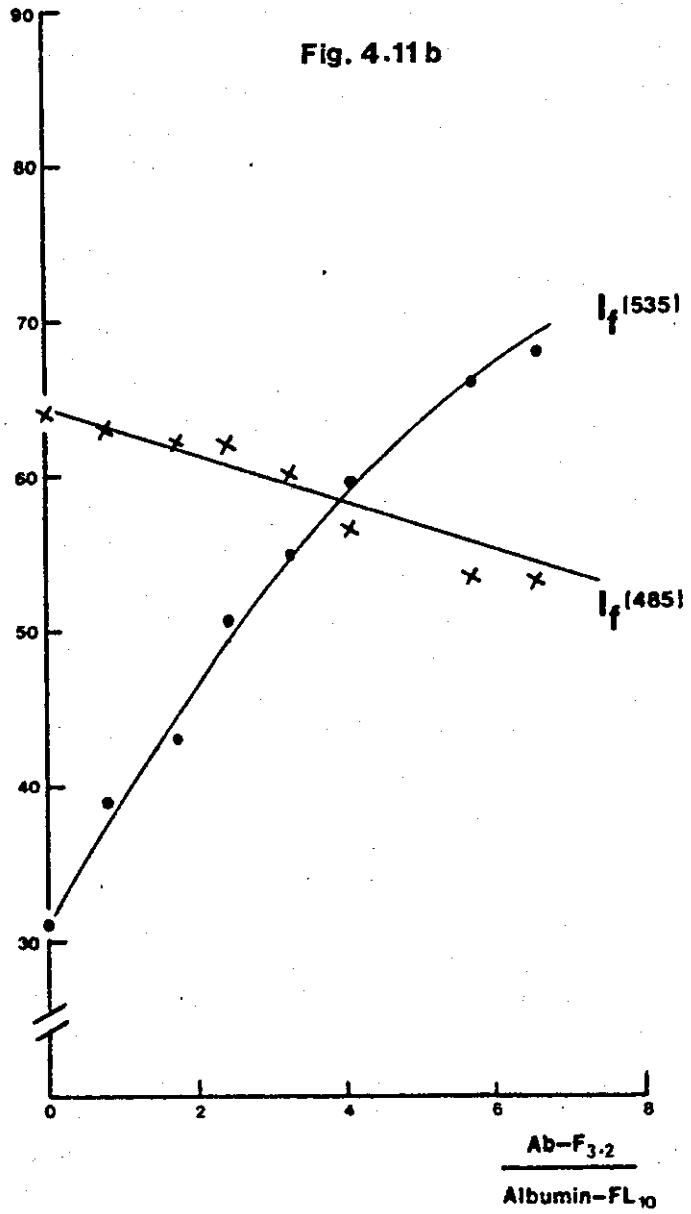


Fig. 4.11 b



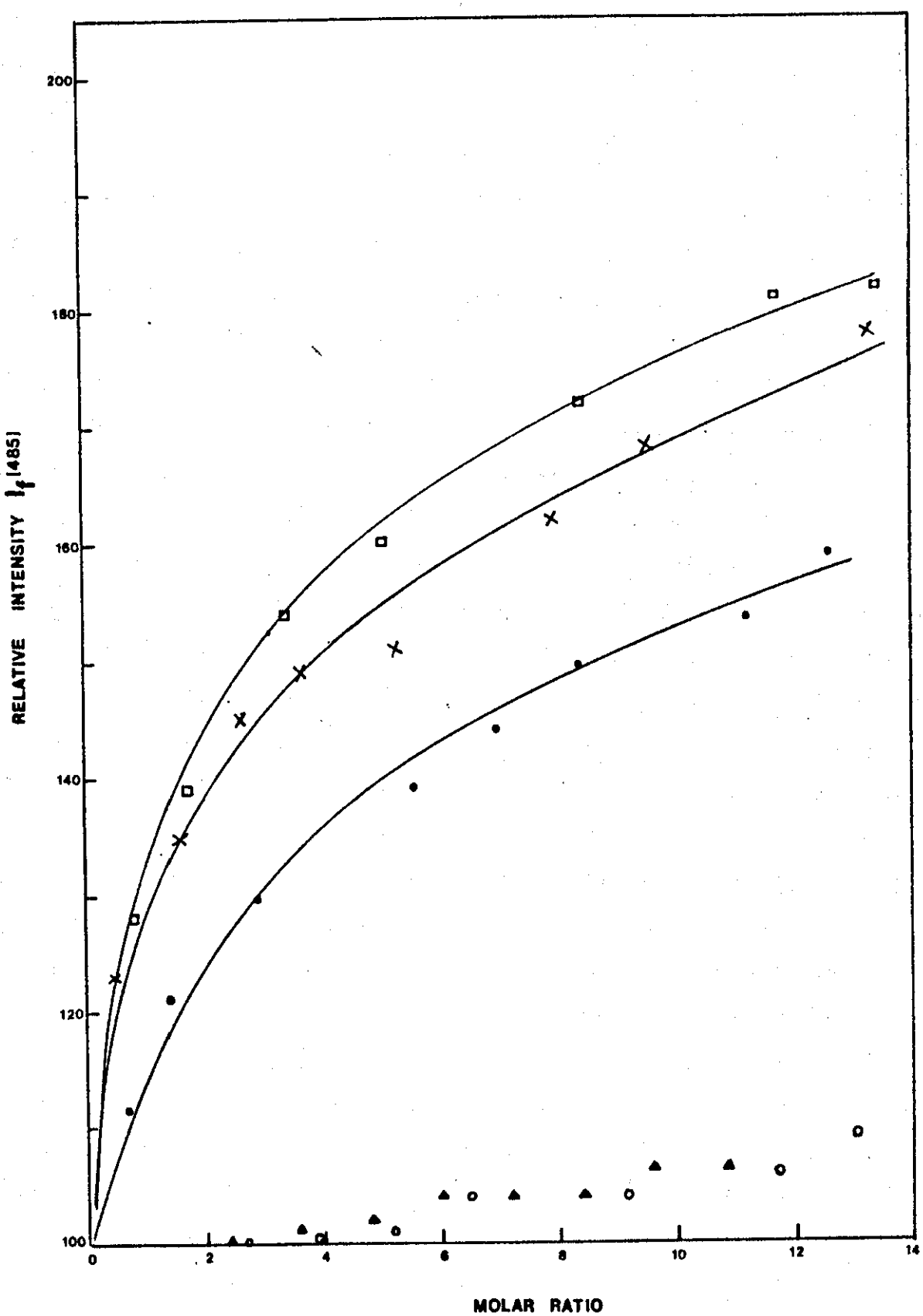

RELATIVE INTENSITY I_f (485)
200
180
160
140
120
100
0
2
4
6
8
10
12
14
MOLAR RATIO

Figure 4.12

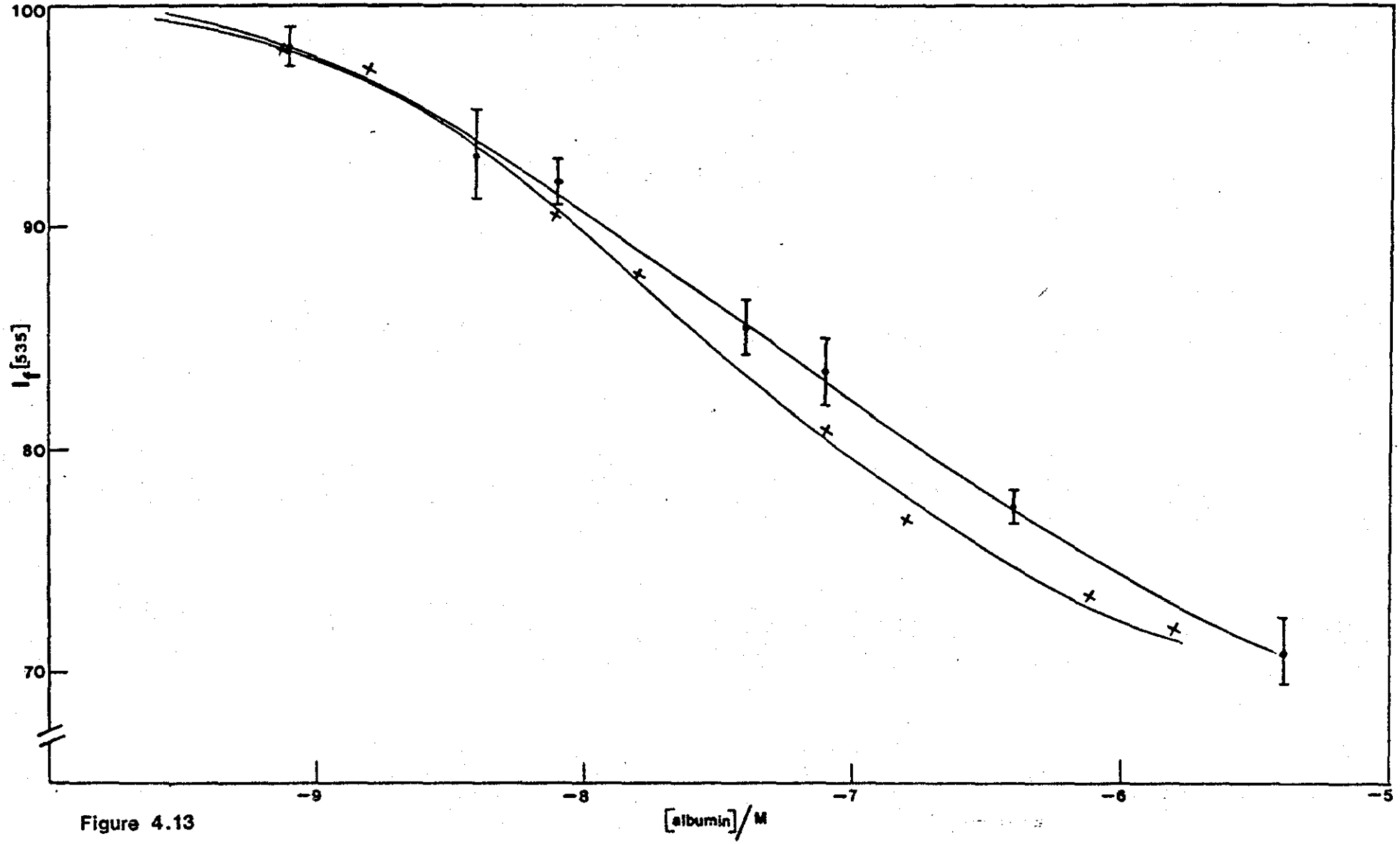


Figure 4.13

CHAPTER 5 DEVELOPMENT OF FLUORESCENCE IMMUNOASSAY METHODS FOR NORTRIPTYLINE AND RELATED TRICYCLIC ANTIDEPRESSANTS

5.1 Introduction

In view of the widespread use of tricyclic antidepressants, TAD, (see Table 5.1) for the alleviation of depression, there exists in the clinical laboratories a need for a rapid, sensitive and precise method for the quantitative measurement of these exogenous compounds in human blood serum and urine. Reported analytical techniques for measuring tricyclic antidepressants in biological fluids have been based on spectrophotometric (Wallace et al,1967), isotopic (Hammer et al,1967), chromatographic (Borga et al,1972 ; Braithwaite et al,1971 ; Faber et al,1974), and immunochemical procedures including a radioimmunoassay (Aherne et al,1976 ; Maguire et al,1978) and a double-antibody enzyme immunoassay (Al-Bassam et al,1978) for nortriptyline.

This chapter describes the development of two fluorescence immunoassay methods for the determination of nortriptyline in human blood serum : an energy transfer assay in which fluorescamine is used as the energy donor and fluorescein as the acceptor, and a fluorescence enhancement immunoassay in which the fluram enhancement effect is utilized. An evaluation of fluorescamine and fluorescein as donor-acceptor labels applicable in ETIA was described in the last chapter. It was found that the fluorescamine-fluorescein energy transfer within the albumin-antibody complex was efficient. A large increase in the fluorescein emission intensity at 535 nm and a relatively small decrease (offset by the fluram effect : see Section 4.3) in the

fluorescamine emission intensity at 485 nm were observed. For the foregoing reason, the energy transfer assay that was developed for albumin made use of only the fluorescein enhancement measurements.

In several respects, fluorescamine and fluorescein are superior to fluorescein and rhodamine as donor-acceptor fluorescent labels in ETIA. Compared to fluorescein as the donor label, fluorescamine has a large Stokes shift of ca. 100 nm, and so with the excitation wavelength of fluorescamine set at 390 nm, the fluorimetric measurement of the fluorescein enhancement emission at 535 nm will be less susceptible to scatter interferences. Further, large fluorimeter slit widths for greater assay sensitivity may be used because, in contrast to the fluorescein-rhodamine system, the effect on the fluorescamine-fluorescein energy transfer of the fluorimeter spectral bandwidth is negligible. Fluorescamine derivatives of proteins and other analytes containing the primary amine group can be easily prepared without the need to separate the labelled conjugates from excess unreacted fluorescamine molecules which are rapidly hydrolysed to non-fluorescent products (Udenfriend et al,1972). And compared to rhodamine-labelled protein conjugates, fluorescein-antibody conjugates are more stable, being usable and free from turbidity or precipitation after storage for several months in the dark at 4 C

5.2 Experimental

Quantitative fluorimetric studies were performed in 10 mm path length silica cuvettes using a Fluoripoint fluorimeter fitted with a temperature-controlled cell compartment. All reagents were were incubated at 30 C for 30 minutes before measurements were

recorded at the same temperature. Desmethylnortriptyline and the immunoglobulin G fraction of sheep anti-nortriptyline antiserum were labelled with fluorescamine and fluorescein respectively as described in Section 2.3.1. For studies on serum samples, the sera were diluted 1:1 v/v with saline (0.145 M)-phosphate buffer, pH 9.0, 0.01M, containing known concentrations of drugs. The spiked sera were then further diluted 50-fold prior to analysis.

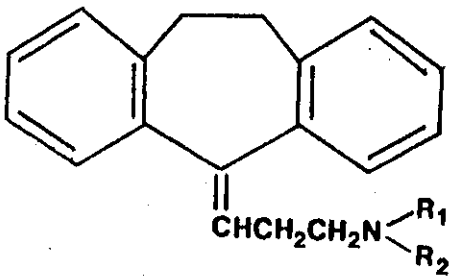
Studies on the fluram effect of nortriptyline-fluorescamine conjugate made use of the IgG and IgM fractions of the sheep antiserum (Figure 2.1). A value of $E^{1\%}_{280} = 15$ was used to calculate the protein concentrations of the sheep immunoglobulins. Assumed molecular weights for sheep IgG and IgM molecules are 145000 and 900000 respectively.

In all fluorimetric measurements, corrections were made for the background fluorescence from added proteins, labelled antibodies, serum and buffer. Tricyclic antidepressants were found not to contribute to the background. The following non-standard abbreviations are used to denote the drugs studied in this work.

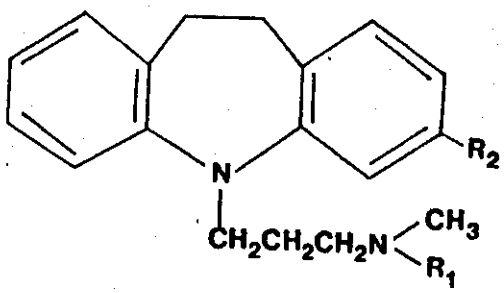
AT	: Amitriptyline	DP	: Desipramine
BT	: Butriptyline	IP	: Imipramine
CP	: Chlomipramine	NT	: Nortriptyline
DNT	: Desmethylnortriptyline	PT	: Protriptyline

DNT-FL : Desmethylnortriptyline-fluorescamine conjugate

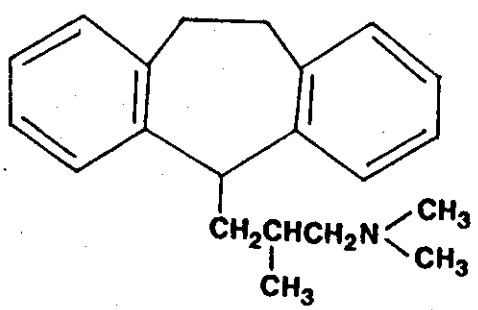
Table 5.1 **Some Tricyclic Antidepressants (TAD)**



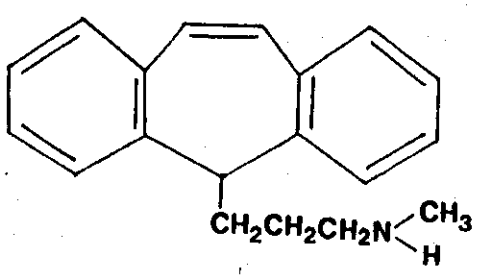
Amitriptyline	$R_1 = CH_3$	$R_2 = CH_3$
Nortriptyline	$R_1 = CH_3$	$R_2 = H$
Desmethylnortriptyline	$R_1 = H$	$R_2 = H$



Chlomipramine	$R_1 = CH_3$	$R_2 = Cl$
Desipramine	$R_1 = H$	$R_2 = H$
Imipramine	$R_1 = CH_3$	$R_2 = H$



Butriptyline



Protriptyline

5.3 Results and Discussion

The fluram enhancement effect of DNT-FL conjugate was first investigated by measuring the changes in the fluorescamine emission intensity at 485 nm as increasing amounts of unlabelled sheep anti-nortriptyline IgG molecules were added to a fixed concentration of DNT-FL conjugate (7.5×10^{-7}M); an excitation wavelength of 390 nm was used in this work. Figure 5.1 shows that in the presence of a 14.3-fold molar excess of unlabelled sheep IgG , the DNT-FL conjugate emission intensity increased by more than 170 per cent. The absence of non-specific enhancement effect is also illustrated in the figure which shows that when a comparable molar excess of non-specific human IgG, in lieu of the sheep IgG, was added to a solution of DNT-FL conjugate, the measured increase in the fluorescamine emission intensity of the conjugate was less than 2 per cent. This relatively small increase in the fluorescence intensity can be attributed to the viscosity change in the solution medium when protein was added. The specificity of antibody binding of DNT-FL conjugate was demonstrable by the observation that addition of excess unlabelled DNT caused the labelled DNT-antibody complex to dissociate, and consequently the fluram enhancement effect to be reversed. The effects of addition of DNT on the fluorescence intensity of a solution containing 1.5×10^{-8}M DNT-FL conjugate and 1.16×10^{-7}M sheep IgG molecules are shown in Figure 5.2. These standard graphs permit the detection of DNT at concentrations as low as 3.5 nM in aqueous solution and 6.5 nM in spiked sera.

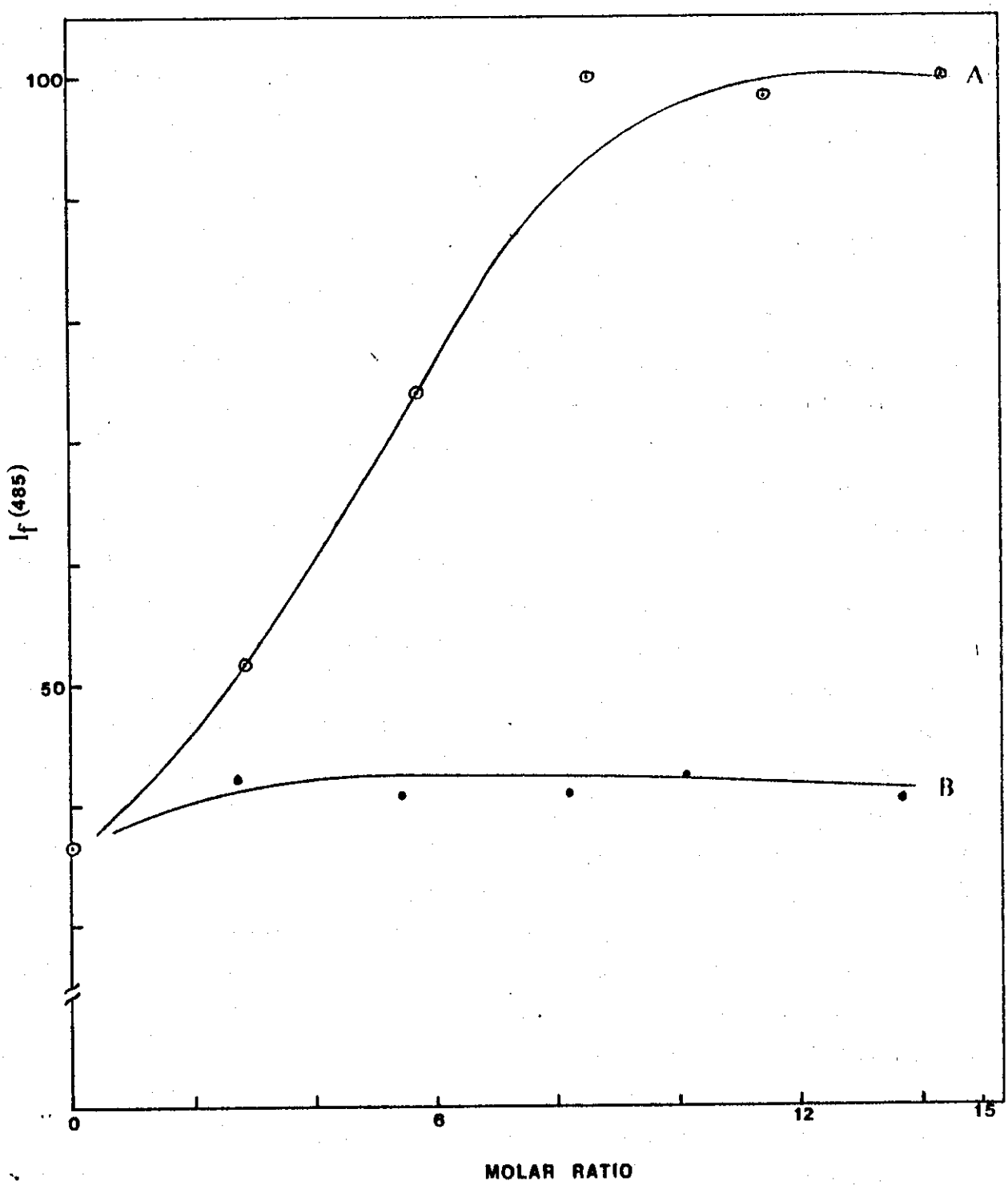


Figure 5.1

Changes in the fluorescence intensity at 485 nm of a solution of desmethylnortriptyline-fluorescamine conjugate (7.5×10^{-7}M) when sheep anti-nortriptyline IgG (graph A) and non-specific human IgG molecules (graph B) are added. Excitation wavelength, 390 nm; bandwidths, 20 nm.

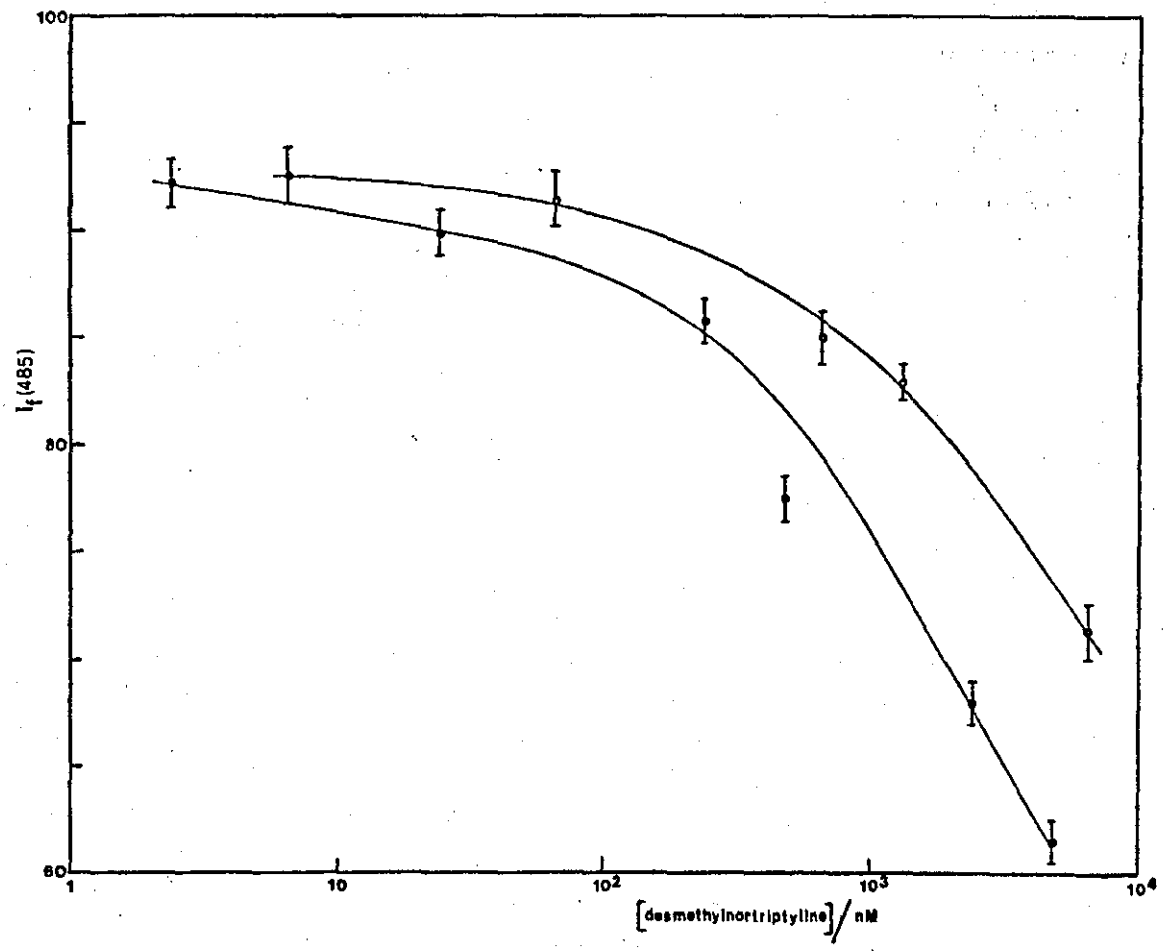


Figure 5.2

Fluorescence intensity changes at 485 nm as increasing amounts of unlabelled desmethylnortriptyline in pure solution (—●—●—) and in spiked sera (—○—○—) are added to a reagent mixture containing desmethylnortriptyline-fluorescamine conjugate (1.5×10^{-8}M) and unlabelled sheep immunoglobulin G molecules (1.16×10^{-7}M). Fluorescence intensity of the reagent mixture without unlabelled desmethylnortriptyline is assigned an arbitrary value of 100. Excitation wavelength, 390 nm ; bandwidths, 20 nm.

Experiments were also performed to investigate the changes in the fluorescence intensity when the IgM fraction of the same sheep antiserum was added to a solution of DNT-FL conjugate. Results of these investigations are given in Table 5.2. Comparison of the tabulated results with Figure 5.1 shows that a greater fluram enhancement effect was observed with the IgM molecules than with the IgG molecules at comparable immunoglobulin to DNT-FL conjugate molar ratios. It is naturally possible that a standard curve for DNT similar to that shown in Figure 5.2 could be obtained using a reagent mixture containing IgM molecules and fluorescamine-labelled DNT conjugate. Since a greater fluram effect was observed with the IgM molecules, an improvement on the detection limits by several orders of magnitude might be attainable if IgM-containing reagents were used in the assay. Due to a lack of IgM protein available, investigations into these possibilities were not performed.

Table 5.2

Fluram enhancement effect of DNT-FL conjugate when IgM molecules are added.

	$I_f(485)$	% Increase
7.5×10^{-7}M DNT-FL	40	-
IgM/DNT-FL (mol/mol)		
0.34	58	45
0.69	85	112
1.38	168	320
2.75	182	355
4.14	182	355

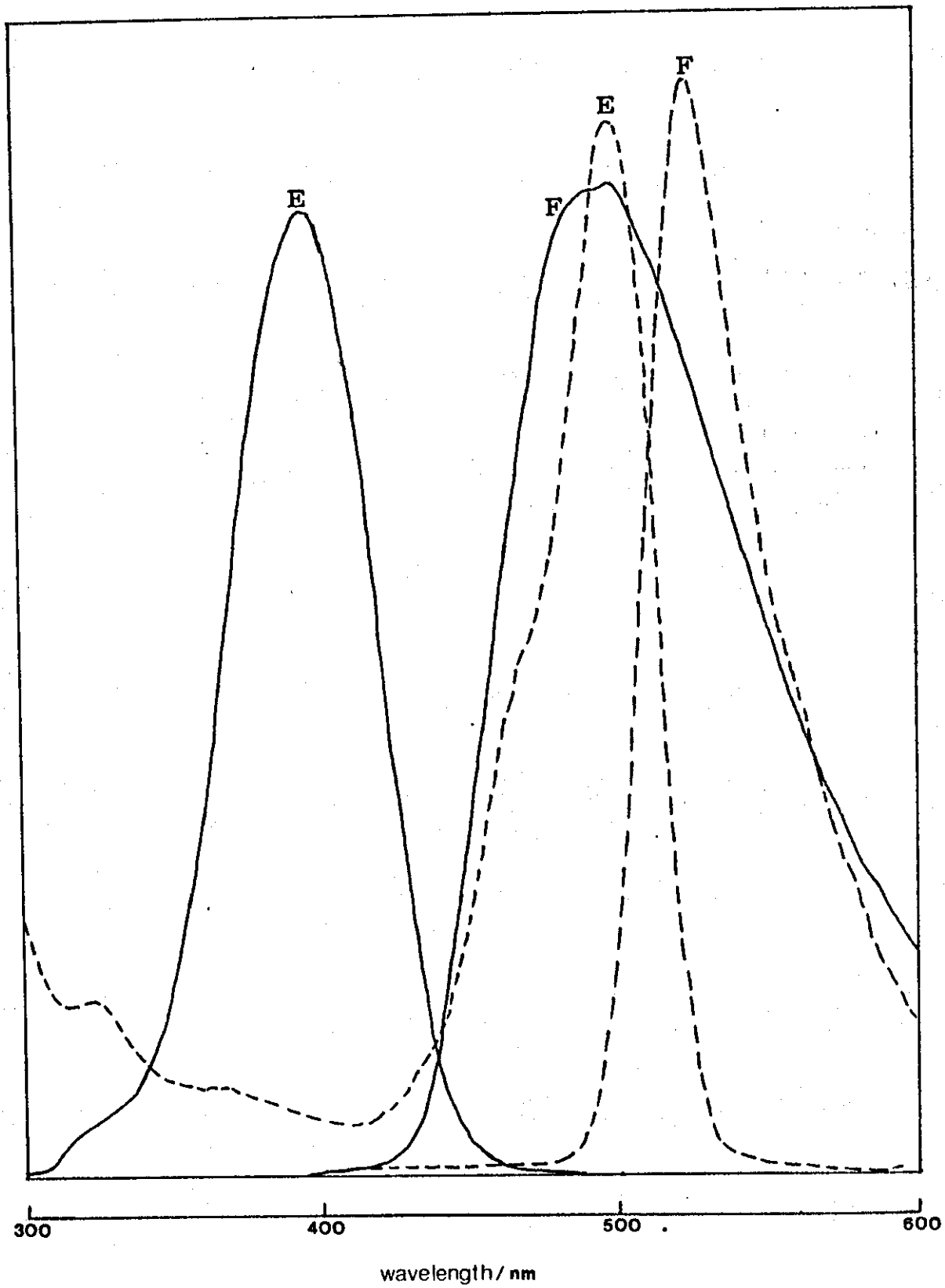


Figure 5.3

Corrected excitation (E) and fluorescence (F) spectra of a desmethyl-nortriptyline-fluorescamine conjugate (———), and of a sheep anti-nortriptyline IgG-fluorescein conjugate (label:protein ratio, 3.5:1) (- - - -)

The corrected spectra (Figure 5.3) of the fluorescamine derivative of desmethylnortriptyline showed excitation and fluorescence maxima at 390 nm and 490 nm, respectively. The excitation and fluorescence maxima of fluorescein-sheep anti-nortriptyline IgG conjugate were at 495 nm and 520 nm respectively. There is therefore the required excellent overlap between the fluorescence spectrum of the donor label (fluorescamine) and the excitation spectrum of the acceptor (fluorescein). Further, the overlap between the excitation spectra of the two labels is negligible : the efficiency of the observed energy transfer is thus undiminished even when large spectral bandwidths are used in the fluorimeter. Filter fluorimeters can therefore be conveniently used in this ETIA, in contrast to the case of fluorescein-rhodamine label pair (see Chapter 3).

Energy transfer was demonstrated by adding fluorescein-labelled antibodies (fluorescein to protein ratio, 7.5:1) to a 40 nM solution of fluorescamine-labelled desmethylnortriptyline. The degree of energy transfer was assessed from the quenching effect of the fluorescamine emission at 480 nm and the enhancement of the fluorescein emission at 530 nm. A 3.4-fold molar excess of labelled antibody reduced the intensity of the fluorescamine derivative emission by 60 per cent and produced a 4.5-fold enhancement of the fluorescein enhancement (Figures 5.4(a) & (b)). As noted in previous studies of energy transfer using antigens labelled with fluorescamine as the donor label, the observed proportional reduction of the fluorescamine emission intensity, being partially offset by the fluram enhancement effect, was very much less than the corresponding proportional increase of the

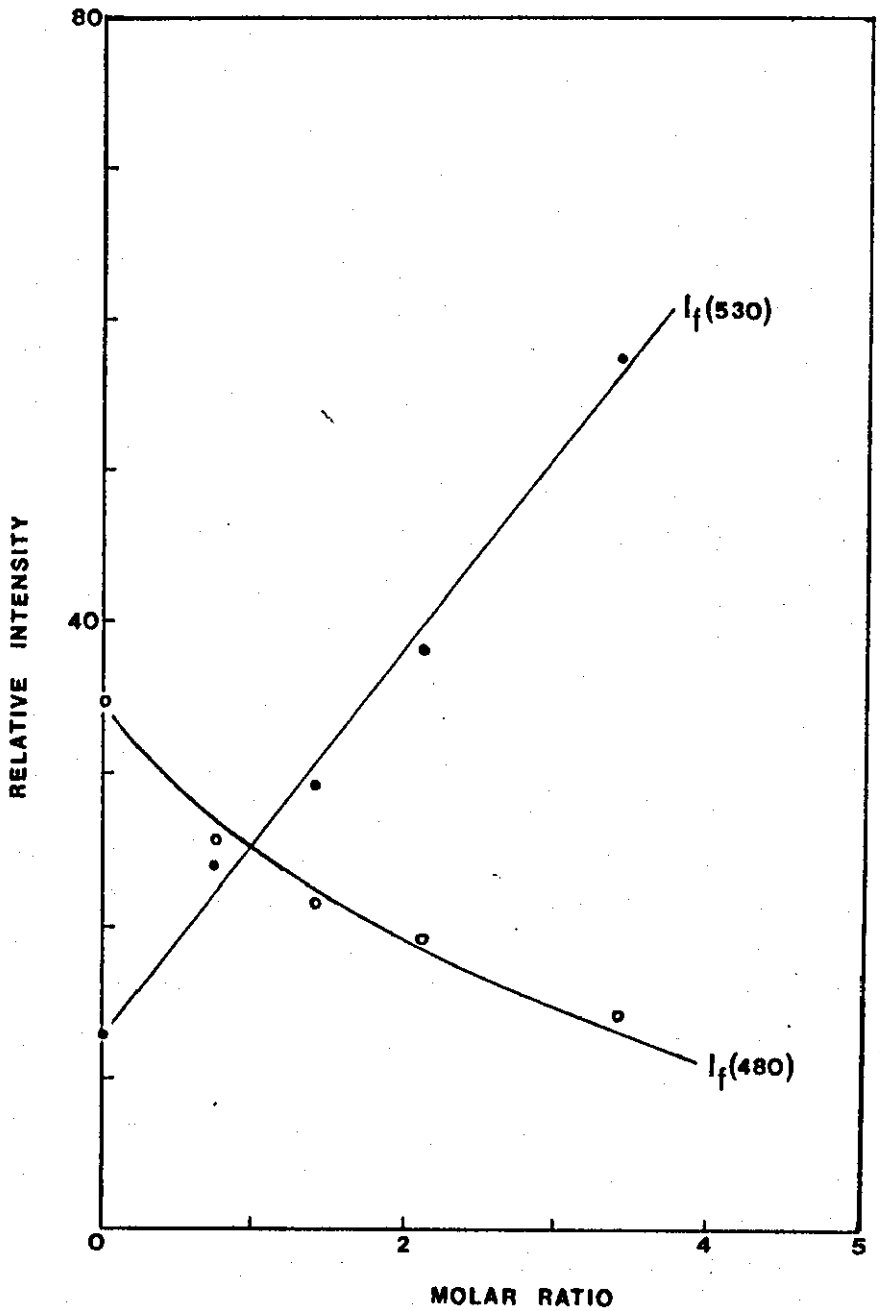


Figure 5.4(a)

Quenching of fluorescamine (at 480 nm) and enhancement of fluorescein (at 530 nm) emission intensities when fluorescein-labelled antibody conjugate (label:protein ratio, 7.5:1) are added to a fixed concentration of desmethylnortriptyline-fluorescamine conjugate (4.0×10^{-8}M). Excitation wavelength, 390 nm ; bandwidths, 20 nm. Molar ratio, labelled Ab:labelled Ag.

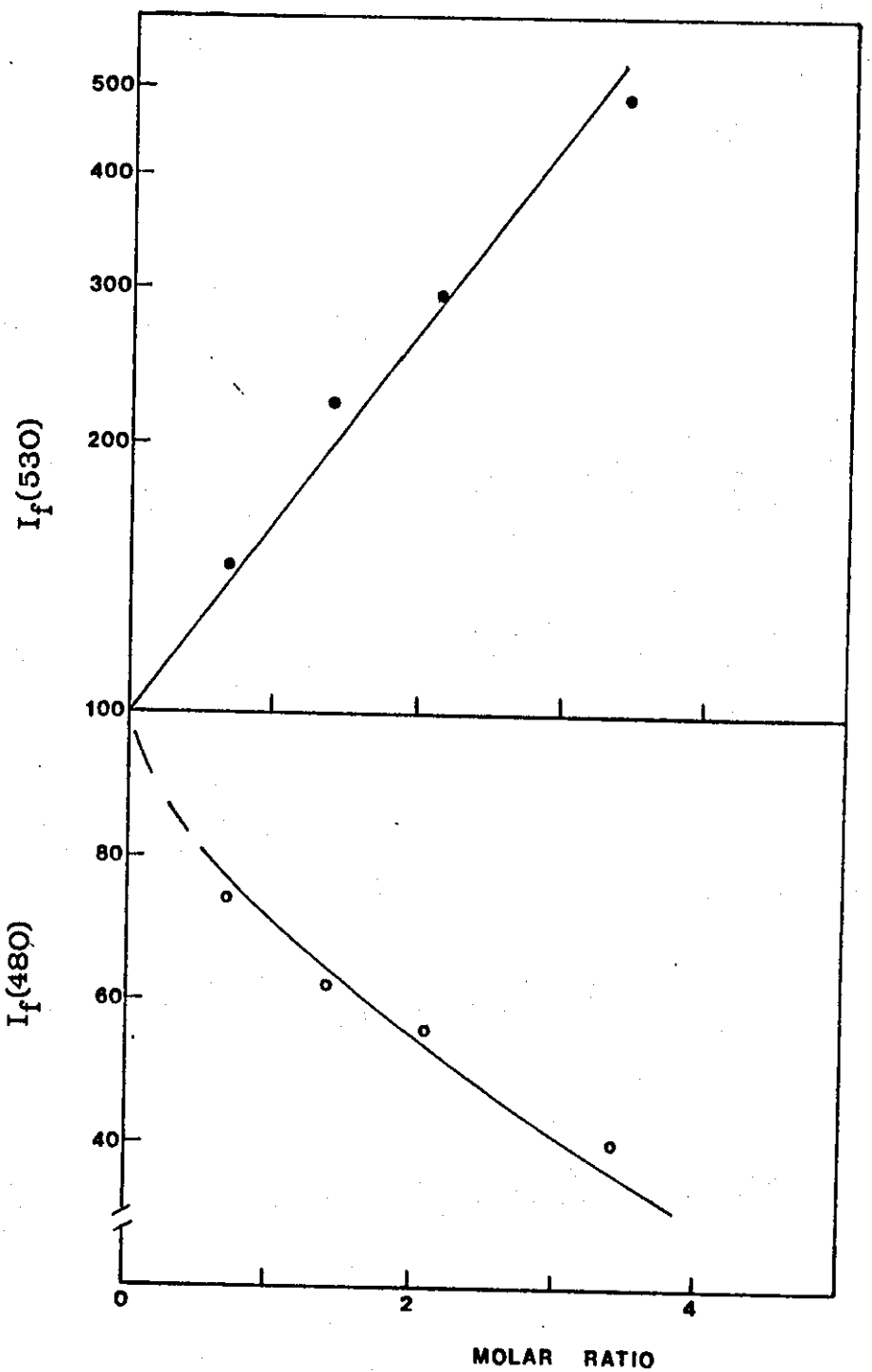


Figure 5.4(b)

Same as in Figure 4.5(a) except that a value of 100 has been arbitrarily assigned to the fluorescence intensity of the fluorescamine derivative alone.

fluorescein fluorescence. In practice, this would mean that fluorimetric determinations in this type of ETIA can make use of only the fluorescein enhancement measurements. Figures 5.4(a) and (b) also show that the fluorescence of both labels changed approximately linearly as the molar ratio of fluorescein-antibody to fluorescamine-nortriptyline increased from 0.7 to 3.4. Addition of unlabelled desmethylnortriptyline reversed these effects, producing, for example, a reduction in the fluorescein-antibody conjugate fluorescence at 530 nm. This reduction was used to set up standard graphs for the determination of tricyclic antidepressants in pure solutions and in spiked sera (Figure 5.5). In these assays the ratio of labelled antibody to labelled antigen was 2.3:1. The limit of detection of desmethylnortriptyline was about 2 nM (0.5 ng ml^{-1}) in pure solution and about 6 nM in spiked sera. The precision of the assay was high (mean coefficient of variation = 1.1 %)

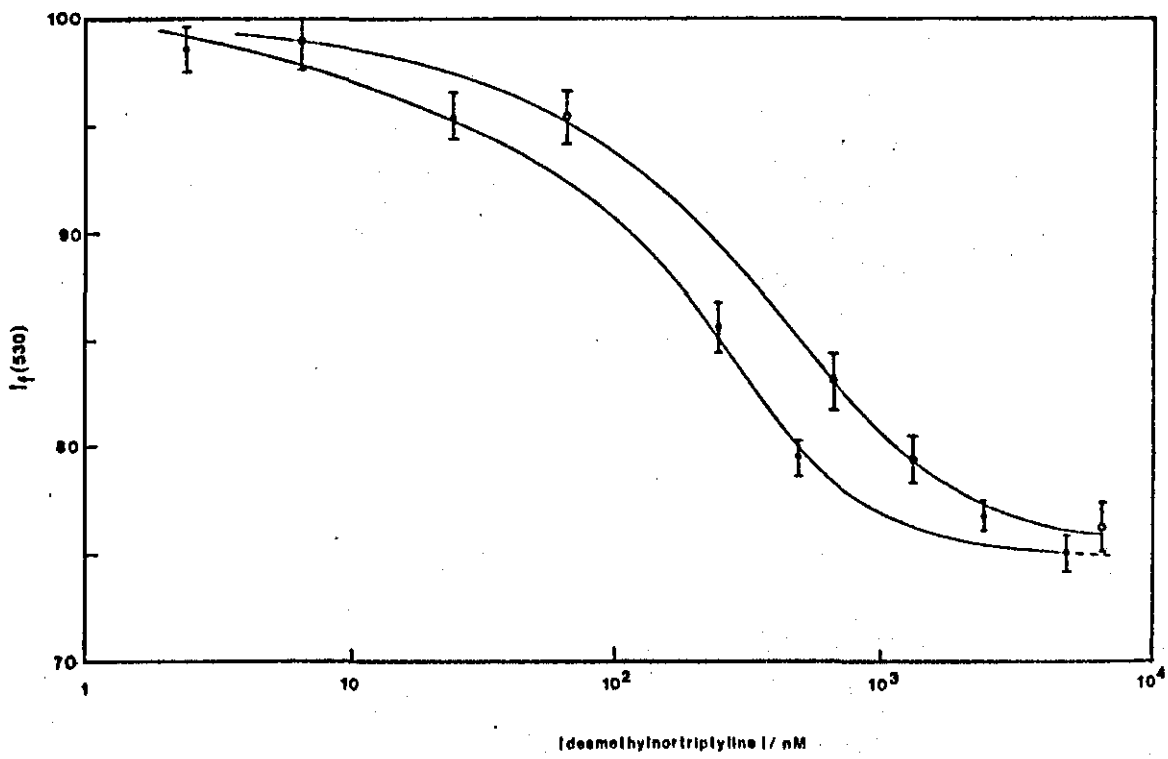


Figure 5.5 Changes in the fluorescence intensity upon combining DNT in pure solution (—●—) and in spiked sera (—○—) with 4.8×10^{-8} M DNT-FL conjugate and 1.1×10^{-7} M IgG-fluorescein conjugate (fluorescein:protein ratio, 7.5:1). Excitation wavelength, 390 nm; bandwidths, 10 nm.

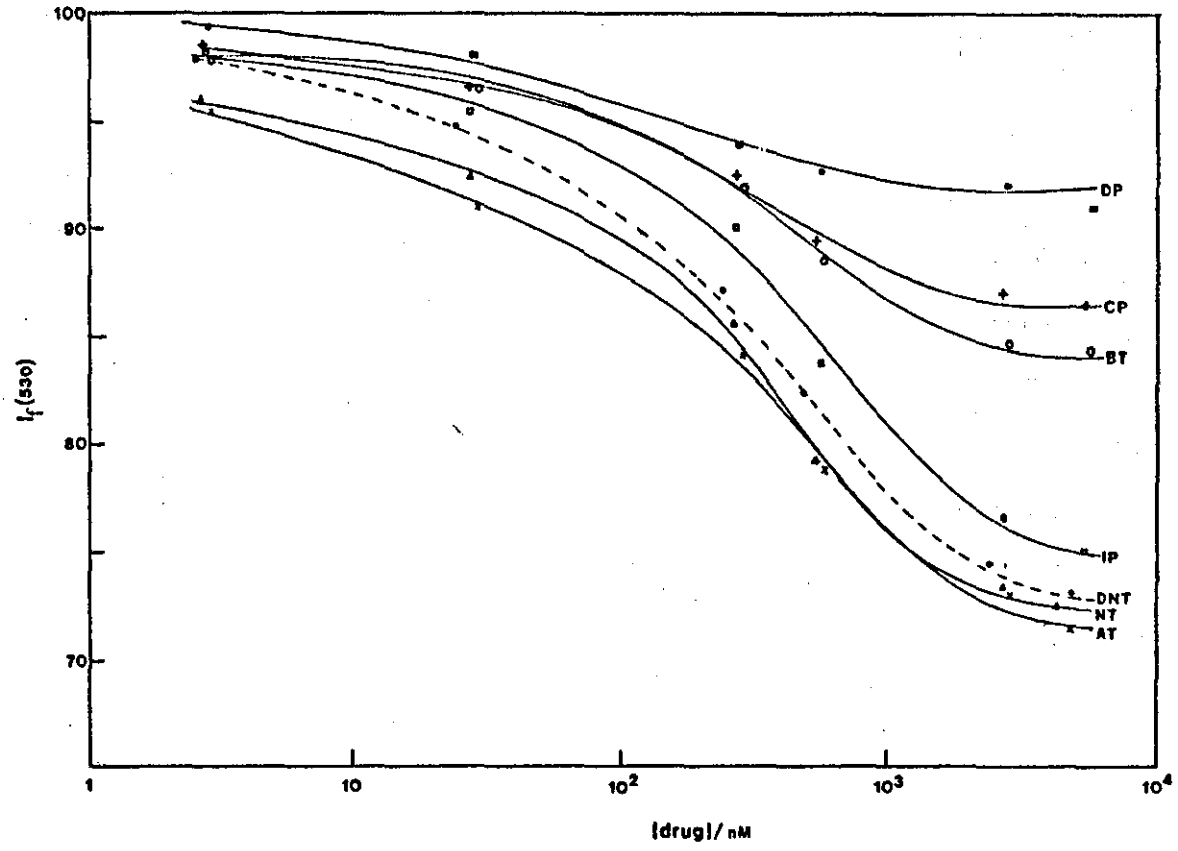


Figure 5.6 Changes in the fluorescence intensities upon combining DNT and other tricyclic antidepressants with mixtures containing 4.0×10^{-8}M DNT-FL conjugate and 1.1×10^{-7}M IgG-fluorescein conjugate. A value of 100 is arbitrarily assigned to the fluorescence intensity of the reagent mixture alone ie. without any unlabelled drug. Excitation wavelength, 390 nm; bandwidths, 12 nm.

Cross-reactivity studies (Figure 5.6) showed that nortriptyline, amitriptyline, imipramine and protriptyline generated fluorescence changes similar to those found for desmethylnortriptyline. Desipramine showed little reactivity in the assay, while chlomipramine and butriptyline showed partial reactivity. An enzyme immunoassay (Al-Bassam et al,1978) for tricyclic antidepressants, using antiserum from the same source

as this work, showed similar cross-reactivities; such effects do not preclude the use of the assay in practice as these drugs are normally administered singly. Other immunoassay studies of nortriptyline showed that the major metabolite, 10-hydroxy-nortriptyline, would not substantially interfere in the assay although present in greater amounts than nortriptyline itself (Maguire et al,1978; Ziegler et al,1976b).

The fluorescence immunoassay methods described provide several advantages over other heterogeneous assays for nortriptyline and tricyclic antidepressants (Maguire et al,1978; Al-Bassam et al,1978). The sensitivities of both fluorescence assays (from 0.5 - 1.5 ng ml^{-1}) are more than adequate for the routine measurement of nortriptyline and other TAD's in plasma samples. The normal plasma levels of nortriptyline lie in the range 50 - 250 ng ml^{-1} (Ziegler et al,1976a). Desmethylnortriptyline-fluorescamine conjugates required for use in both the assays can be prepared very easily and quickly immediately prior to the assay. The fluorescence assay methods are homogeneous, so the need for the troublesome separation step required in heterogeneous assays is eliminated. Finally, the prior extraction of plasma sample is not required, thus allowing for a more rapid assay.

CHAPTER 6 HOMOGENEOUS FLUORESCENCE IMMUNOASSAY METHODS FOR HUMAN SERUM TRANSFERRIN.

Comparative Studies of Fluorescamine and MDPF as Labels in Fluorescence Immunoassays.

6.1 Introduction

6.1.1 Properties and clinical significance of transferrin.

It was found by Holmberg and Laurell (1945;1947) that iron was principally transported in human blood plasma by the specific iron-binding β_1-globulin which they named transferrin (also called siderophilin). There are about 20 genetic variants of transferrin (Giblett, 1969), all apparently compatible with good health. At a normal blood pH, each transferrin molecule (molecular weight, 76500) can bind two atoms of Fe(III) to form a pink complex, although under normal circumstances the transferrin binding sites are only one third saturated. The concentration of transferrin in human blood plasma is normally in the range 200 - 400 mg dl^{-1} which may be decreased in cases of nephrosis (where there is a loss of transferrin in the urine), malignant neoplasia, or when there is a hereditary deficiency of the protein as in patients with a severe hypochromic anemia (Heilmeyer, 1964). Increased serum transferrin levels are observed in iron deficiency anemia and in the last months of pregnancy. Estrogenic substances such as those found in oral contraceptives can also cause an increase in the concentration of transferrin as well as other transport proteins in plasma (Laurell et al, 1968).

6.1.2 Determination of transferrin.

Transferrin levels may be measured directly by immunological techniques or indirectly by measuring the maximum amount of iron the serum can bind, ie. the total iron-binding capacity (TIBC). The indirect technique overestimates transferrin by 10 - 20 % because the metal binds to proteins other than transferrin when the latter is more than half saturated (van der Heul et al, 1972). Transferrin may be quantitated immunochemically by the radial immunodiffusion, electroimmunodiffusion or immuno-nephelometric methods. Data comparing the immunochemical, radio-isotopic and colorimetric procedures for the measurement of total iron binding capacity and transferrin appear in recent literature (Buffone et al, 1978, and references 1 to 4 cited therein; Daigneault et al, 1978). In this work, two fluorescence immunochemical methods for determining low concentrations of transferrin in serum samples have been developed. The first method is based on the enhancement in the fluorescence intensities of the fluorescamine and MDPF derivatives of transferrin on binding to anti-transferrin antibodies (fluorescence enhancement assays). The second method is an energy transfer assay in which either fluorescamine or MDPF as the energy donor is used in conjunction with fluorescein as the acceptor.

The excellent sensitivities attainable in the energy transfer assays for human albumin (Chapter 4) and nortriptyline (chapter 5) show that the fluorescamine-fluorescein label pair are suitable for use in other applications of this ETIA method. Likewise, the sensitive fluorescence assays that were developed also for albumin and nortriptyline demonstrate clearly that the fluram enhancement effect can be generally applied to the analysis of both

low and high molecular-weight antigens. MDPF resembles fluorescamine in many respects: molecular structure, chemical reactions and luminescence characteristics (page 49). However, some workers (Handschin et al, 1976; Weigele et al, 1973a) have reported that MDPF provides several advantages over fluorescamine, including the greater fluorescence stability of the MDPF conjugates over a wide pH-range. This stability has been attributed to the lack of a free carboxylic group, which in fluorescamine derivatives may give rise to an intramolecular nonfluorescent lactone (Weigele et al,1973a). The additional availability of MDPF to complement (if not to substitute for) fluorescamine in these FIA methods will certainly enhance the versatility of these fluorogenic reagents and enlarge the rather limited number of useful fluorophores for fluoroimmunological techniques.

This chapter describes the experiments carried out to examine the comparative merits of fluorescamine and MDPF as fluorophores for specific applications in FIA. The bases for comparison include (a) the efficiency of the fluorophores as donors of energy (together with fluorescein as the acceptor) in ETIA; (b) the magnitude of the fluram and MDPF enhancement effects; (c) the accuracy and precision of the assay methods in the analyses of serum samples; and (d) the stability of the fluorescent conjugates under storage conditions.

6.2 Experimental

The Baird-Atomic Fluoripoint fluorimeter was used to record all quantitative fluorimetric measurements. The reagents were incubated at 30 C for at least 30 minutes before measurements

were made in 10 mm path length silica cuvettes thermostatted at the same temperature. Corrections were made for fluorescence background contributed by the phosphate buffer (0.01M, pH 7.2, containig 0.145M NaCl), added proteins including serum samples, and labelled antibodies. Corrected spectra were recorded at 25 C using the Perkin-Elmer MPF-44B fitted with the DCSU-2 corrected spectra unit.

Radial immunodiffusion was performed using M-Partigen Transferrin Immunodiffusion plates according to the recommendations of the manufacturer (Behringwerke AG, Marburg/Lahn, West Germany- through Hoechst (UK) Ltd.). Zone diameters were measured after a 48-hour incubation at room temperature.

Serum samples were diluted 5- and 500-fold, respectively, prior to analysis by the immunodiffusion and energy transfer assay methods. Standard human serum (Behringwerke AG) was used as a standard.

All fluorescent protein solutions were kept in the dark at 4 C. After six weeks in storage conditions, MDPF- and fluorescamine-labelled transferrin conjugates were found to be usable. Furthermore, no significant difference in the fluorescence properties between the two transferrin conjugates was evident. A solution of antibody-fluorescein conjugate, after four months in the same storage conditions, was found to be usable although a very slight precipitation of the conjugate was also noted. This was easily removed by centrifugation and the recovered sample was found not to have suffered any significant loss in immunological or luminescence properties.

6.3 Results and Discussion

As noted above, the two fluorogenic reagents (MDPF and fluorescamine) have many properties in common, and so in analogy with the fluram enhancement effect of fluorescamine derivatives, it can reasonably be expected that a similar enhancement effect will also be observed with MDPF-labelled derivatives. This expectation is indeed borne out by experimental results. Figure 6.1 shows that the increases in the emission intensities at 485 nm of transferrin-fluorescamine (Trans-FL) and transferrin-MDPF (Trans-M) conjugates are strongly dependent on the molar excess of the added antibodies and the fluorophore:transferrin ratios of the conjugates. In all the transferrin conjugates examined, a greater magnitude of the enhancement effect was obtained with a more heavily-labelled conjugate than a lightly-labelled one. However, the results available are not sufficient to enable one to conclude which of the two transferrin conjugates (Trans-FL or Trans-M) exhibited the greater enhancement effect, even though it would appear from Figure 6.1 that the fluram enhancement effect was more pronounced compared to the MDPF enhancement effect. An accurate determination of the relative magnitude of these two effects would require the use of transferrin conjugates with the same degree of fluorescamine and MDPF labelling. The effect on the fluorescence spectrum of a transferrin-fluorescamine conjugate on binding to increasing amounts of unlabelled antibodies is shown in Figure 6.2. Except for a general increase in the fluorescence intensity of the transferrin conjugate with added antibodies, no spectral distortion is evident and there is no shift in the emission spectrum maximum.

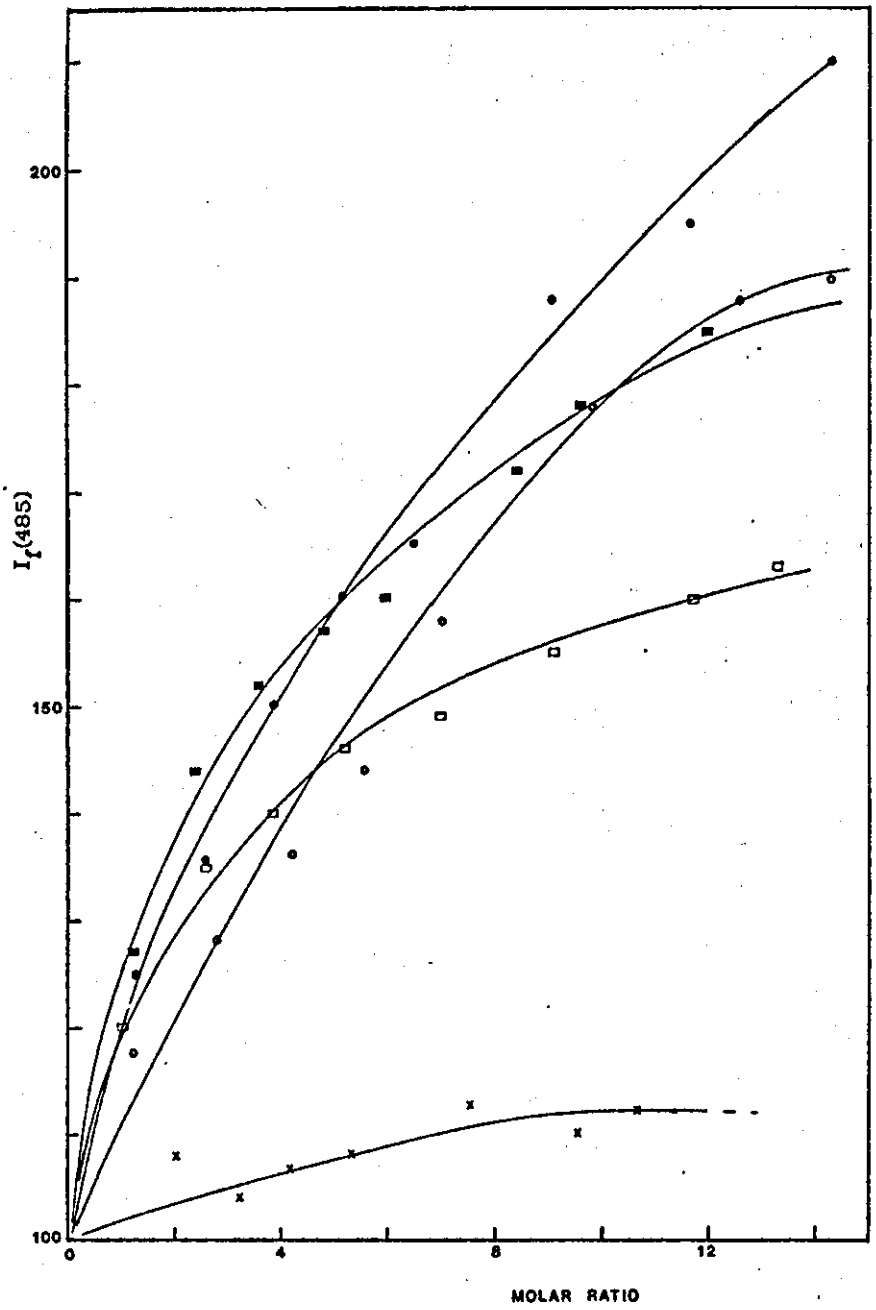


Figure 6.1

Changes in the fluorescence intensities at 485 nm of mixtures containing a fixed concentration of transferrin-fluorescamine or transferrin-MDPF conjugates and increasing amounts of unlabelled antibodies:

6×10^{-8}M Trans-M conjugate (label:protein ratio,2.8:1) (–□––□–);
6×10^{-8}M Trans-M conjugate (label:protein ratio,6.4:1) (–■––■–);
9×10^{-8}M Trans-FL conjugate(label:protein ratio,3.7:1) (–o––o–);
9×10^{-8}M Trans-FL conjugate(label:protein ratio,7.8:1) (–●––●–).

The changes in the fluorescence intensity of mixtures containing a fixed concentration of Trans-FL conjugate and increasing amounts of non-specific human IgG molecules are shown by (–x––x–). Pure solutions of fluorescent conjugates have arbitrary fluorescence intensity of 100. λ_{ex},390 nm; bandwidths, 8nm; molar ratio = Ab:Ag

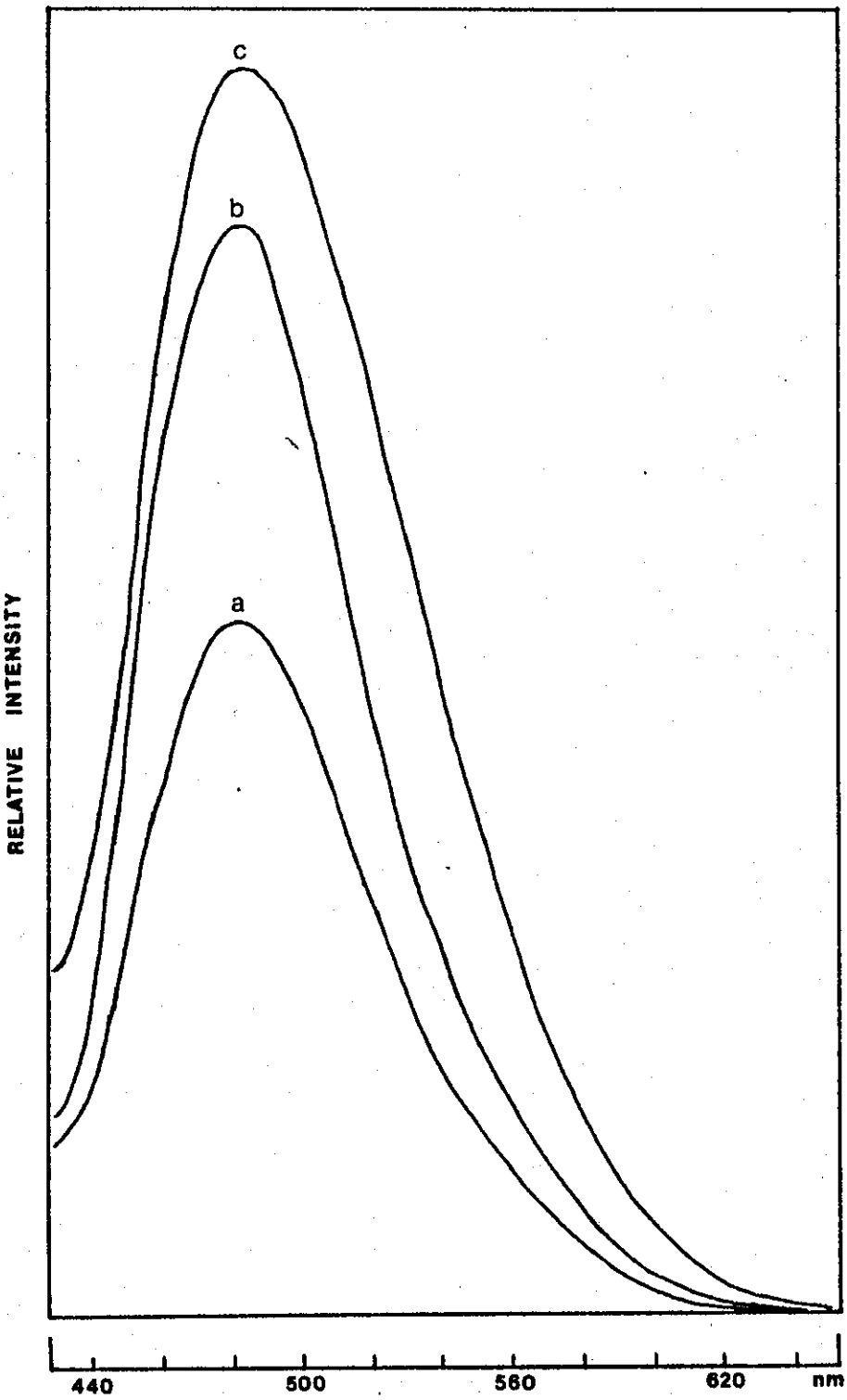


Figure 6.2

Spectra showing the changes in the fluorescence intensities of mixtures containing a fixed concentration of Trans-FL conjugate (7×10^{-8}M; label:protein ratio, 7.8:1) and increasing amounts of antibodies: (a) without antibody; (b) Ab:Ag molar ratio, 5.2:1); (c) Ab:Ag molar ratio, 9.1:1). Excitation wavelength, 390 nm; bandwidths, 8 nm.

Figure 6.1 also shows that the addition of a 10.6-fold molar excess of non-specific human IgG to a solution of transferrin-fluorescamine conjugate (label:protein ratio, 3.7:1) produced only a 12 % increase in the fluorescamine emission intensity at 485 nm, thus confirming that the fluram (and by analogy, the MDPF) enhancement phenomenon is a consequence of specific antigen-antibody combinations, and not of spurious environmental effects on the fluorescence intensity. The specificity of antibody binding of the fluorescent transferrin conjugates was further demonstrated by the reversal of the enhancement effects when unlabelled transferrin molecules (in serially diluted standard serum samples) were added to dissociate the fluorescent antigen-antibody complex. The resultant decreases in the fluorescence intensities of transferrin-fluorescamine and transferrin-MDPF conjugates were used to set up standard graphs for the determination of serum transferrin concentrations (Figure 6.3). In these assays, the molar excess of unlabelled antibodies were 12:1 in 3×10^{-8}M Trans-FL conjugate (label:protein ratio, 7.8:1), and 11.8:1 in 3.4×10^{-8}M Trans-M conjugate (label:protein ratio,7:1). The limits of detection in both assays are comparable (5 nM = 4 mg dl^{-1}).

Comparison of Figures 6.4 and 6.5 shows that the corrected excitation and fluorescence spectra of the fluorescamine and MDPF derivatives of transferrin are almost identical: the excitation and fluorescence maxima of both conjugates occur at 390 nm and 490 nm, respectively. The corresponding maxima for a fluorescein-labelled antibody conjugate occur at 495 nm and 525 nm, respectively. The condition of optimum spectral overlap between the donor (FL or MDPF) emission and the acceptor (fluorescein)

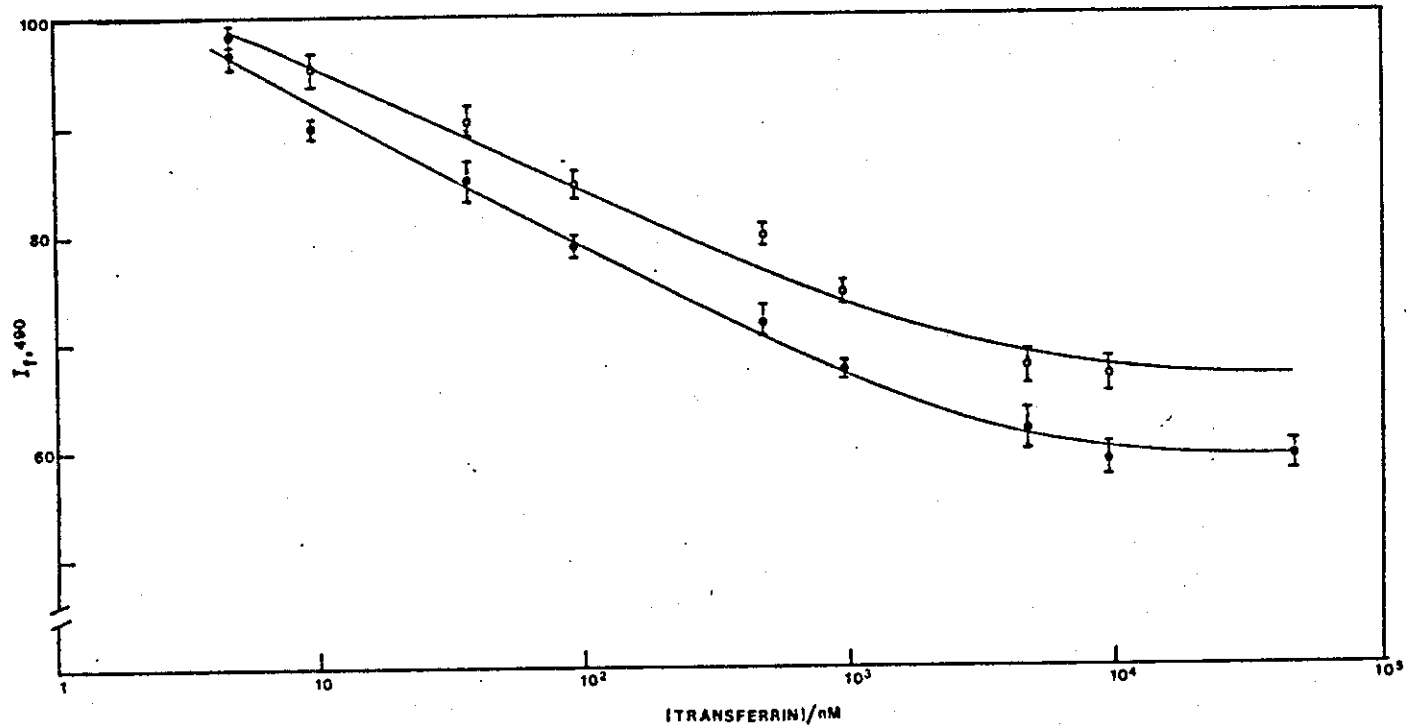


Figure 6.3 Fluorescence intensity changes at 485 nm as increasing amounts of unlabelled transferrin molecules (in serially diluted standard serum samples) are combined with mixtures containing (a) 3×10^{-8}M Trans-FL conjugate (label: protein ratio, 7.8:1) and 3.6×10^{-7}M antibody molecules (—●—● —); (b) 3.4×10^{-8}M Trans-M conjugate (label:protein ratio, 7:1) and 4×10^{-7}M antibody molecules (—○—○—). Reagent mixtures without added unlabelled antibodies have arbitrary fluorescence intensity of 100. Error bars represent standard deviations for 3 measurements. Excitation wavelength, 390 nm; bandwidths, 6 nm.

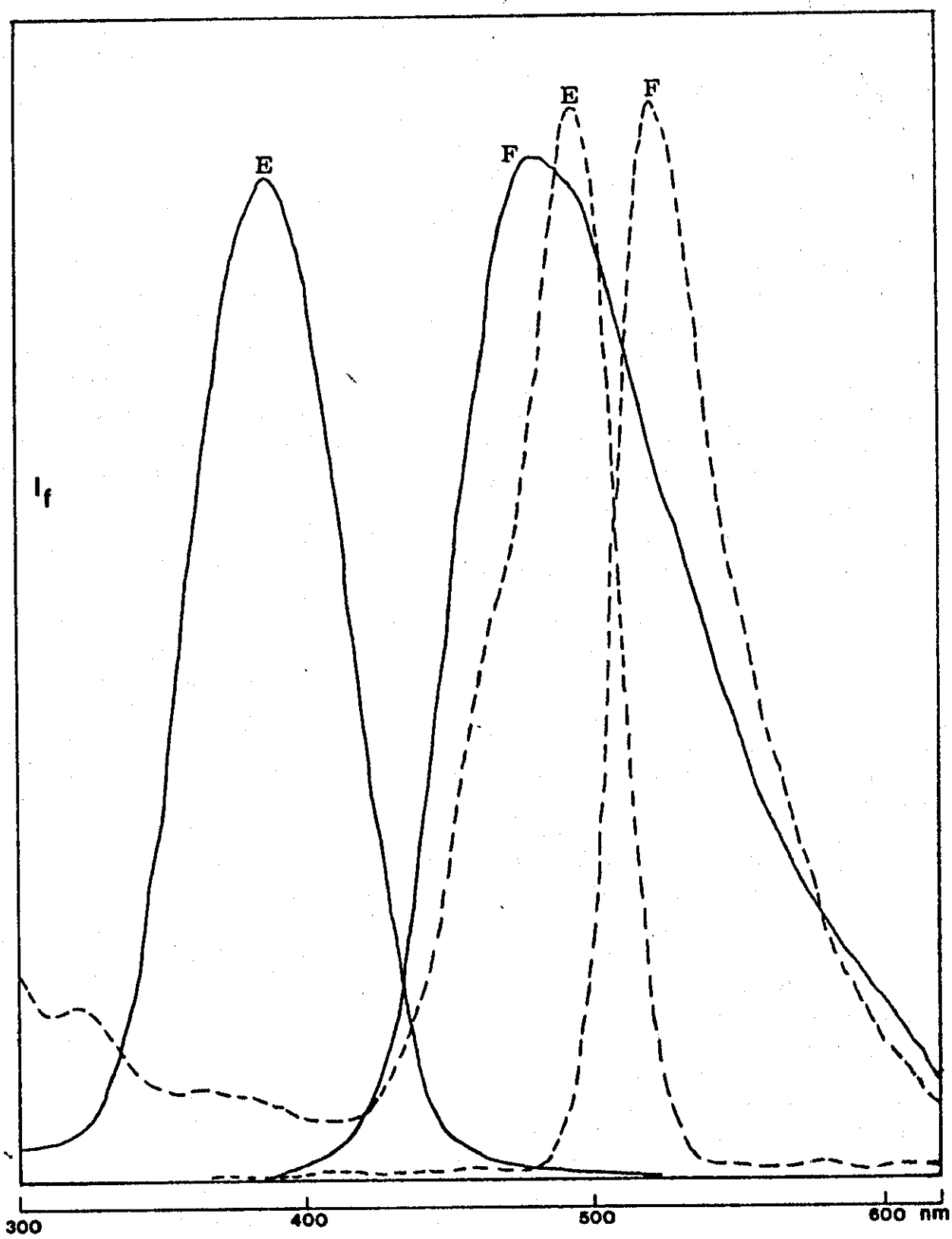


Figure 6.4 Corrected excitation (E) and fluorescence (F) spectra of a transferrin -fluorescamine conjugate (label:protein ratio, 3.9:1) (———), and of an antibody-fluorescein conjugate (label:protein ratio, 2.7:1) (- - - -).

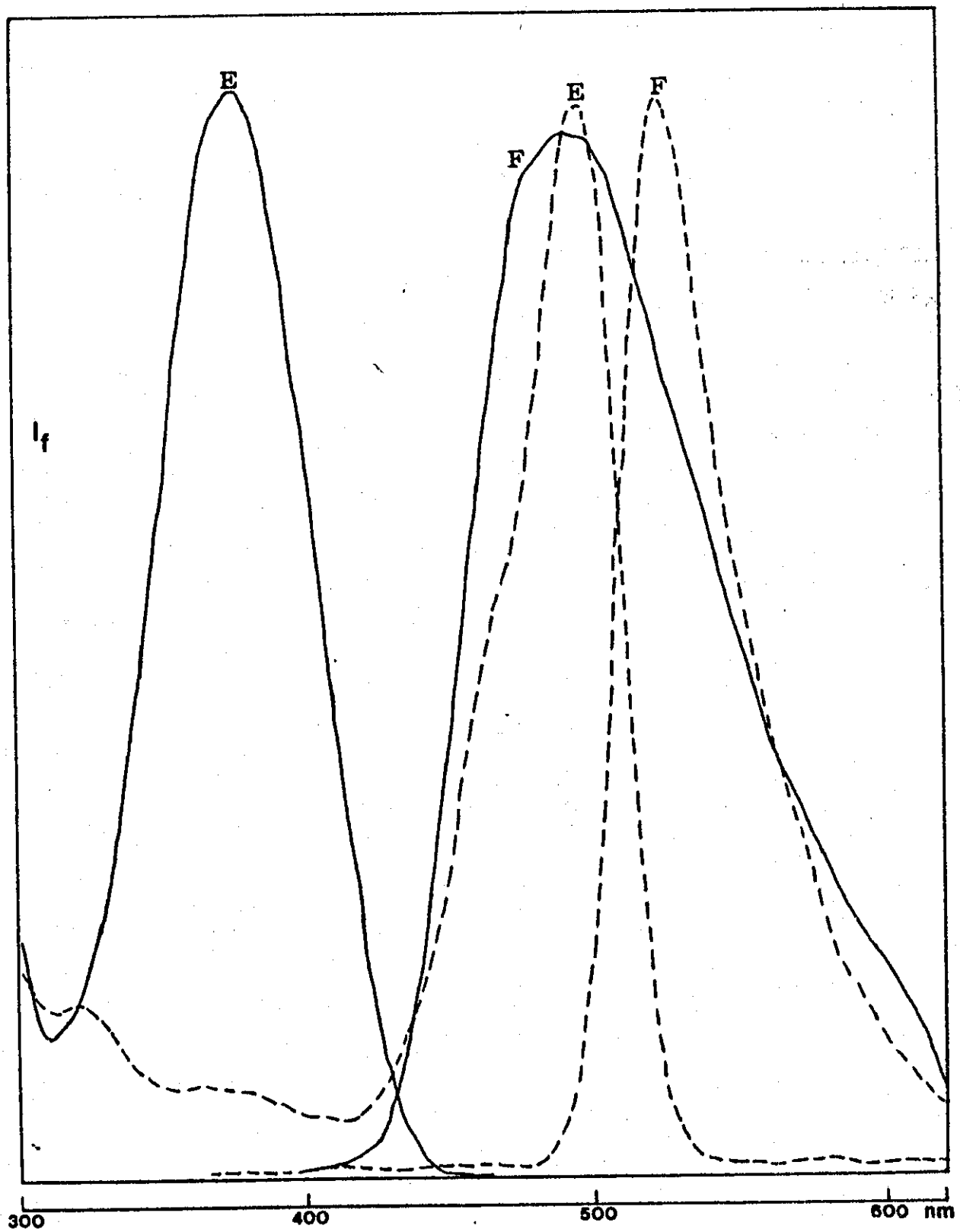


Figure 6.5 Corrected excitation (E) and fluorescence (F) spectra of a transferrin-MDPF conjugate (label:protein ratio, 3.2:1)(——). Spectra of an antibody-fluorescein conjugate (- - -) reproduced from Figure 6.4.

excitation spectra obtains, and therefore a high degree of energy transfer in the fluorescamine-fluorescein as well as in the MDPF-fluorescein systems can be expected.

Experiments to study the energy transfer effects of the two donor-acceptor pairs were performed by adding increasing amounts of antibody-fluorescein conjugates to solutions containing a fixed concentration of Trans-M conjugate (3×10^{-8}M; label: protein ratio,13.2:1), and also to solutions containing a fixed concentration of Trans-FL conjugate (7×10^{-8}M; label:protein ratio, 13.9:1). The degrees of energy transfer were assessed by measuring the decreases in the donor (FL or MDPF) emission intensities at 485 nm and the concomitant increases in the acceptor (fluorescein) emission intensity at 530 nm (excitation wavelength, 390 nm). Figures 6.6 and 6.7 show that addition of increasing amounts of the labelled antibody molecules to the transferrin conjugates caused the quenching and the enhancement effects in the two donor-acceptor systems to increase approximately linearly up to a 4-fold molar excess of labelled antibodies. Thereafter, while the fluorescein emission intensities continued to increase linearly up to an antibody:antigen molar ratio of 6:1, the fluorescamine as well as the MDPF emission intensities appeared to be less susceptible to the quenching effect. In addition, for a given antibody:antigen molar ratio, the proportional increase in the enhancement effect was rather greater than the proportional decrease in the quenching effect. As noted and explained previously in other studies of the fluorescamine-fluorescein energy transfer (Chapters 4 and 5), this apparent disproportionality between the quenching and enhancement effects can be related to the counter-acting fluram and MDPF enhancement

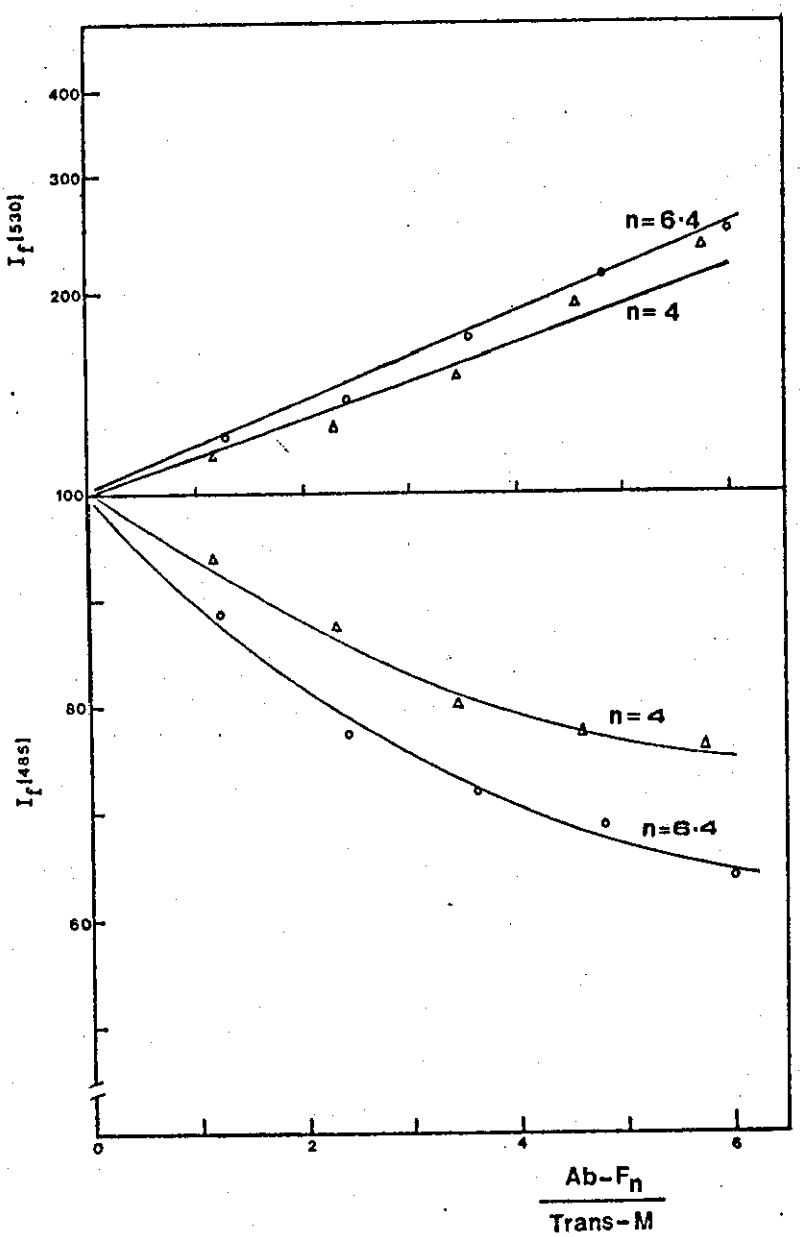


Figure 6.6 Enhancement of fluorescein fluorescence (530 nm) and quenching of MDPF fluorescence (485 nm)-when increasing amounts of fluorescein-labelled antibody molecules are added to solutions containing a fixed concentration of Trans-M conjugate (3×10^{-8}M; label:protein ratio, 13.2:1). The degree of labelling of antibody is indicated by the "n" values: n = moles of fluorescein per mole of antibody. An arbitrary value of 100 is assigned to the fluorescence intensity of a pure solution of transferrin-MDPF conjugate. Excitation wavelength, 390 nm; bandwidths, 8 nm.

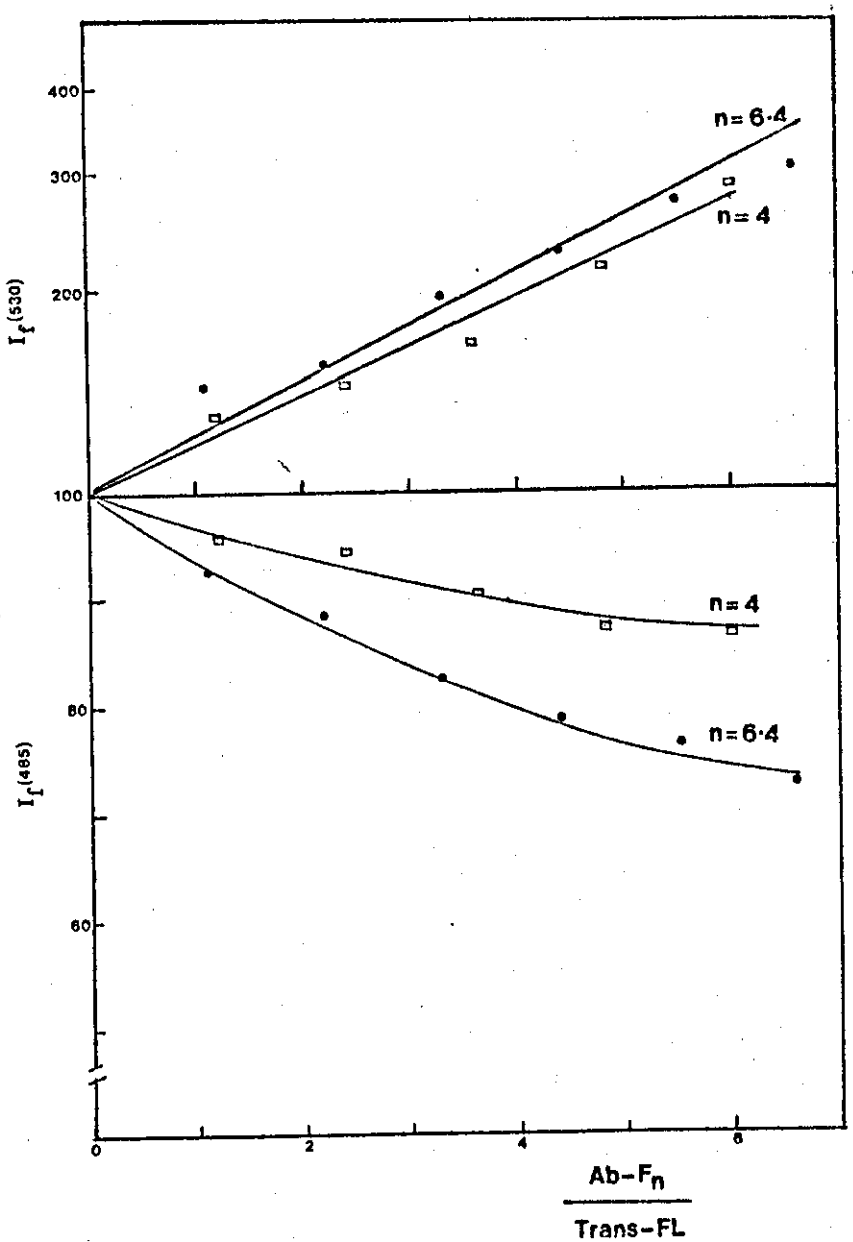


Figure 6.7
As in Figure 6.6 except that a transferrin-fluorescamine conjugate was used (7×10^{-8} M; label:protein ratio, 13.9:1)

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effects which partially offset the quenching of the fluorescamine and MDPF emission intensities caused by resonance energy transfer. As shown in the same figures, the magnitude of the energy transfer effects in the two donor-acceptor systems is also dependent on the degree of fluorescein labelling of the antibody conjugates. The use

of a more heavily-labelled antibody-fluorescein conjugate appeared to cause the quenching effect to be increased more than the enhancement effect. An explanation for this observation is likely to be that in the condition of a higher concentration of fluorescein molecules, the observed enhancement in the fluorescein emission intensity would be much diminished by inner filter effects due to the high background fluorescence. These results suggest that the optimum conditions required in the assays for transferrin should include the use of heavily-labelled fluorescamine- or MDPF-transferrin conjugates (label:protein ratio, ca. 12:1) (donor) and a lightly-labelled antibody-fluorescein conjugate (label:protein ratio, $\leq$ 4:1) (acceptor). The molar excess of labelled antibody in the assay should be kept low (labelled Ab:labelled Ag molar ratio, ca.4:1). These conditions are essentially similar to those required in the energy transfer assay for albumin described in Chapter 4. Typical standard graphs for the determination of transferrin in pure solution and in serum samples are shown in Figures 6.8 and 6.9.

The excellent specificity of the assays was demonstrated by the absence of cross-reactivities when two of the major constituents of serum proteins, albumin and IgG, were included in the assay medium (Figure 6.10). Furthermore, good agreement between the energy transfer assays and the immunodiffusion method was obtained when four serum samples were analysed for their transferrin contents. These results are summarized in Table 6.1. The precision of the two ETIA methods was comparable (mean CV = 6.6 %). Transferrin concentrations found in the three samples taken from healthy donors all lie within the normal range (200-400 mg dl^{-1}). On the other hand, the blood sample taken from the victim of a road traffic accident was found to have a very low transferrin

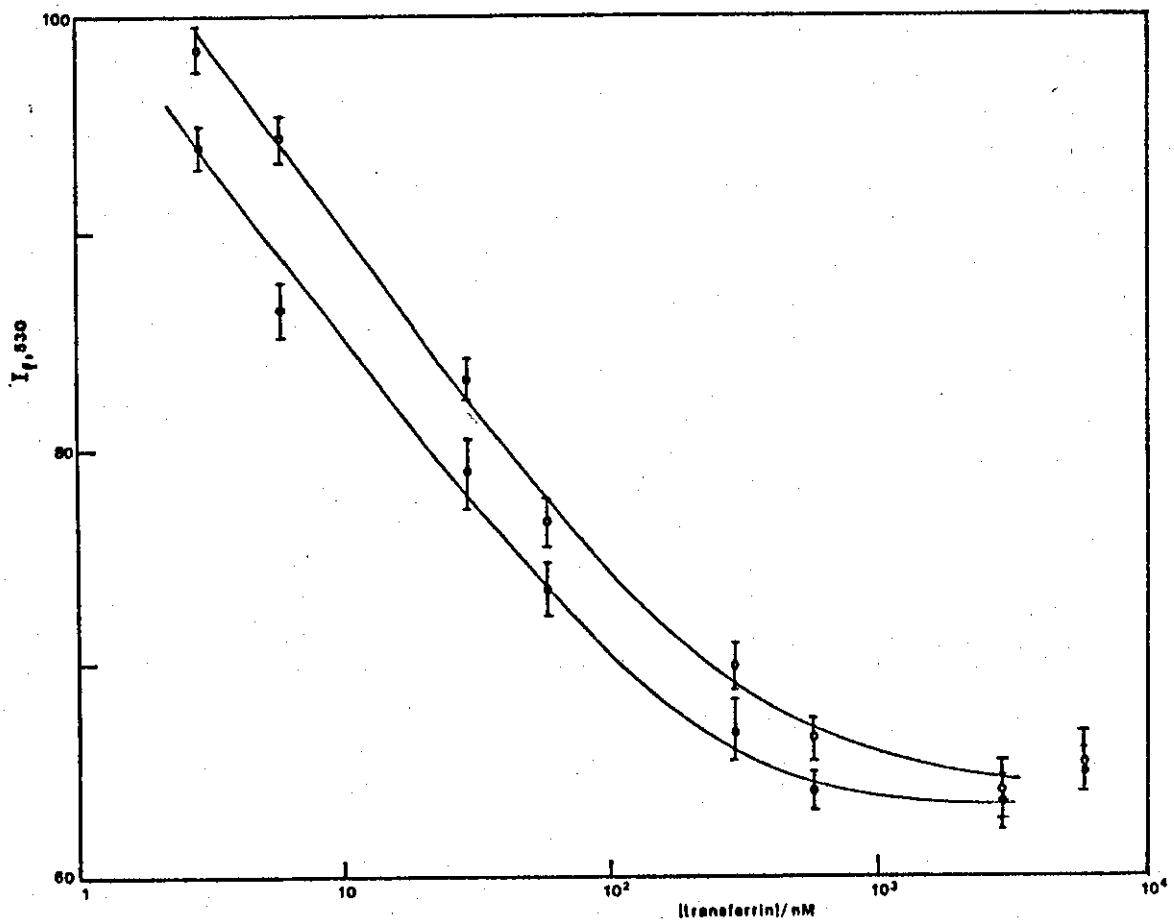

Figure 6.8

Standard graphs for transferrin determination.
Changes in the fluorescein fluorescence intensity at 530 nm when unlabelled transferrin molecules in aqueous solutions are added to reagent mixtures containing (a) 5×10^{-8}M Trans-M conjugate (label: protein ratio,13.2:1) and 1.8×10^{-7}M Ab-F conjugate (label:protein ratio,4:1) (—o—o—) ; (b) 4×10^{-8}M Trans-FL conjugate (label: protein ratio, 13.9:1) and 1.8×10^{-7}M Ab-F conjugate (label:protein ratio,4:1) (—•—•—).
Excitation wavelength, 390 nm; bandwidths, 12 nm. Error bars represent standard deviations for 4 measurements.

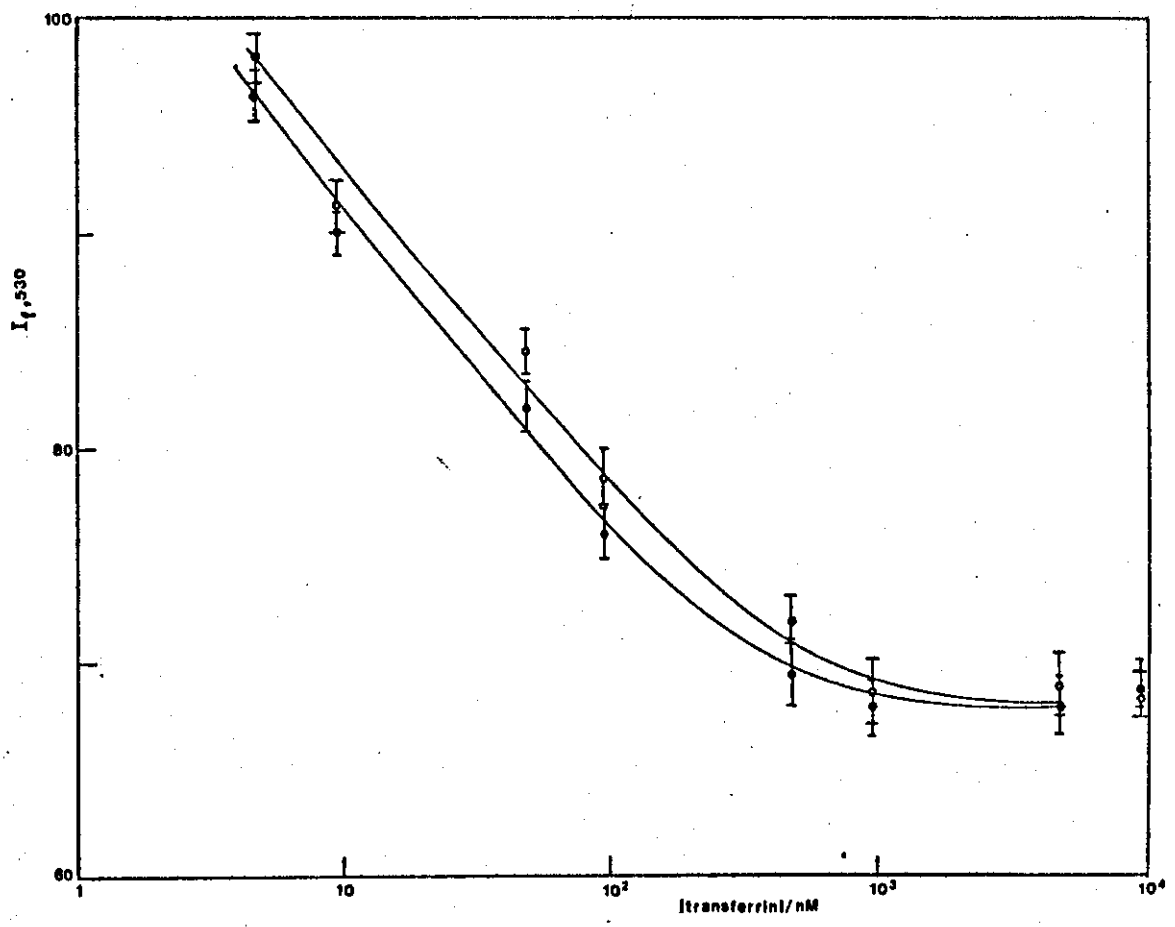

Figure 6.9 As in Figure 6.8 except that serially diluted standard serum solutions were used as standards.

concentration. Bacterial degradation, as evidenced by the putrid smell emitted from the sample, might have been the cause for this low level of transferrin.

It is apparent from these results that both fluorescamine-fluorescein and MDPF-fluorescein are suitable donor-acceptor labels for use in other applications of the ETIA method. There is no significant difference in properties between fluorescamine and MDPF when these are used as donors of energy together with fluorescein as the acceptor. In the assessment of the relative magnitude of the fluram and MDPF enhancement effects,

it was found that such effects are strongly dependent on the fluorophore:protein ratios of the conjugates as well as the molar excess of unlabelled antibodies added. So an accurate determination of the comparative magnitude of these two enhancement effects must make use of transferrin conjugates with the same degree of fluorophore labelling. However, in view of the close similarities between the two fluorogenic labels, it seems reasonable to assume that, all other conditions being equal, the fluram and MDPF enhancement effects are about comparable in magnitude. Thus, for applications in FIA, the choice between these two fluorophores would be dictated by the simple economic consideration of cost and availability.

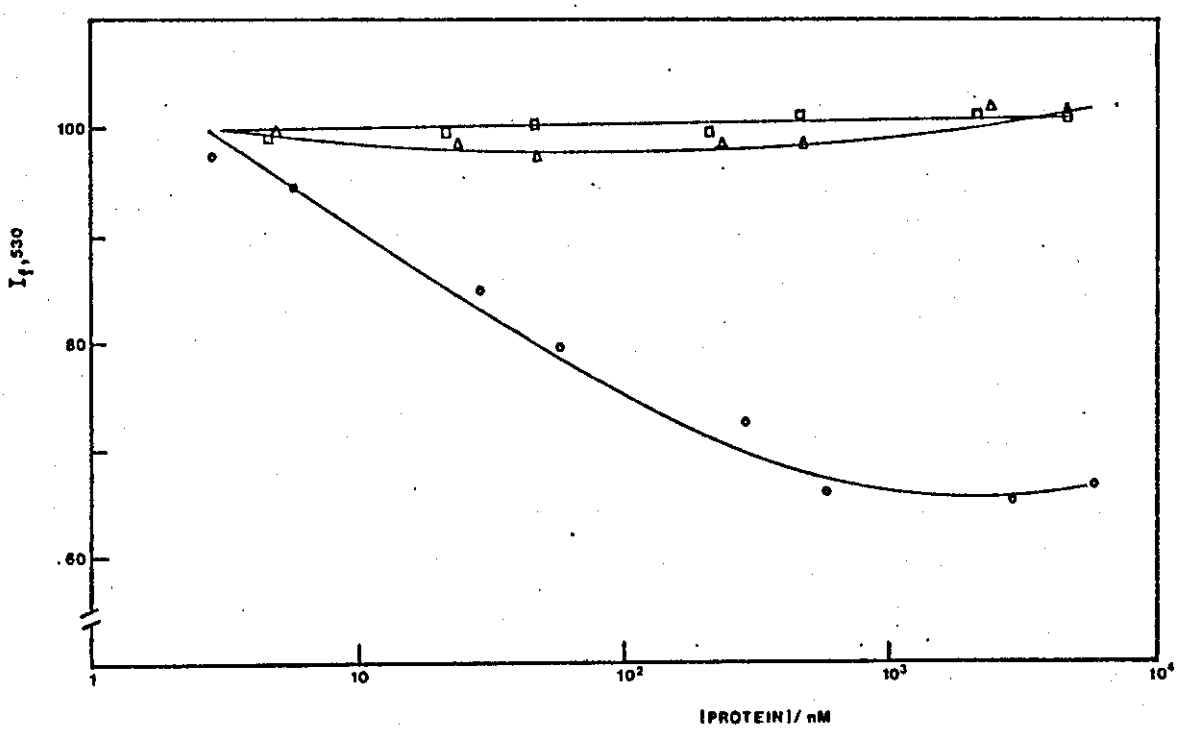

Figure 6.10 Effect of adding transferrin (—o—o—), albumin (—Δ—Δ—) and human IgG (—□—□—) to a solution mixture containing 4.0×10^{-8}M Trans-FL conjugate (label:protein ratio,13.9:1) and 1.8×10^{-7}M Ab-F conjugate (label:protein ratio,4:1). An arbitrary value of 100 is assigned to the fluorescence intensity of the solution mixture without any added proteins.
Excitation wavelength, 390 nm; bandwidths, 12 nm.

Table 6.1

Determination of Serum Transferrin

Sample	Radial immunodiffusion	Energy Transfer Immunoassay	
		FL as donor	MDPF as donor
	n = 5	n = 5	n = 5
1	220 ± 16.5	250 ± 18	240 ± 16
2	255 ± 13	260 ± 17.5	275 ± 18
3	260 ± 20	290 ± 16	285 ± 20
RTA sample	135 ± 6.8 (n = 4)	152 ± 9.4 (n = 4)	-

All concentrations in mg dl^{-1}. The serum samples were diluted 5- and 500-fold prior to analysis by the immunodiffusion and energy transfer assay methods, respectively.
RTA = Road Traffic Accident

CHAPTER 7 DEVELOPMENT OF FLUORESCENCE IMMUNOASSAY METHODS FOR HUMAN IMMUNOGLOBULIN G AND A

7.1 Introduction

7.1.1 The immunoglobulins G and A. Properties and clinical significance.

The immunoglobulins are a family of structurally and functionally related serum glycoproteins which exhibit antibody activity. The basic structure of the immunoglobulin molecule consists of two identical heavy polypeptide chains and two identical light polypeptide chains joined together by interchain disulphide bonds and noncovalent forces (Edelman 1973; Porter 1973). There are, however, variations which account for the class and subclass differences and their varying antigen-binding capacities. Based on differences in heavy chain composition and molecular weights, the immunoglobulins are classified into five classes, namely, immunoglobulin (Ig) G, IgA, IgM, IgD and IgE. Heterogeneity within classes allows for subclasses (Refer, for example, White et al (1978), p 930).

The relative concentrations of serum immunoglobulins change at various stages of growth and abnormalities are found in many disease states. These abnormalities generally fall into three categories: (i) decreased levels in two or more immunoglobulins as found in immunologic deficiency diseases; (ii) increased levels of two or more immunoglobulins as in cases of chronic infections; and (iii) large increase in the level of one of the immunoglobulins as

may occur in cases of myeloma .

Human IgG comprises 75 per cent of the total serum immunoglobulins with a mean adult serum concentration of 990 - 1300 mg dl^{-1} depending on the geographical factors. Its concentration is found to be raised in patients suffering from autoimmune diseases (Sunderman 1964; Sherlock 1970) and in cases of IgG myelomas (Cohen 1968; Nah 1971). Decreased levels in serum IgG are encountered in cases of congenital and acquired hypogammaglobulinemia, some malabsorption syndrome and during excessive protein loss (Cohen 1968). Maternal IgG is the only class of immunoglobulin that is normally transferred across the placenta to the foetus. IgG is, however, the last of the immunoglobulins to be produced after an antigenic challenge or immunization.

Sensitive techniques which have already been developed for the quantitation of IgG in human serum and other biological fluids are summarized in Table 7.1.

Table 7.1 Techniques for IgG quantitation

Technique	References
Radial immunodiffusion	Mancini et al (1965); Fahey et al(1965).
Electroimm noassay	Laurell (1966;1972); Perry et al (1974).
Immunoelectrophoresis	Ghanta and Hiramoto (1974).
Radioimmunoassay	Salmon et al (1969); Cawley et al(1974); Woo et al (1979)
Nephelometry	Killingsworth and Savory (1972); Markowitz et al (1972); Deaton et al(1976).
Enzymeimmunoassay	Engvall and Perlmann (1971); Halliday and Wisdom (1978).
Fluorescence immunoassay	Blanchard and Gardner (1978).

Human immunoglobulin A, with a concentration of 200 mg % in normal serum (Hobbs 1971), constitutes approximately 20 % of the serum immunoglobulins. As the major immunoglobulin in body secretions such as saliva, mucus, colostrum, etc., it provides protection for linings of the digestive, respiratory, excretory and reproductive passages (Gabl and Wachter, 1961). Secretory IgA contains an additional structural unit called the secretory piece (Hansen and Johanssen, 1967).

Serum IgA concetration is found to be raised in patients suffering from Wiscott-Aldrich syndrome and IgA multiple myeloma (Cohen 1968; Nah 1971), in some stages of autoimmune disease, and in many cases of hepatic cirrhosis (Sunderman, 1964). Decreased levels of serum IgA are found in cases of nephrotic syndrome, gastrointestinal disease associated with severe protein loss and certain leukemias. Extremely low concentrations of IgA have also been encountered in cases of ataxia telangiectasia and congenital agammaglobulinemia (Cooper et al, 1973).

7.1.2 Development of fluorescence immunoassay methods for immunoglobulin G and A.

This chapter describes the FIA methods that have been developed in this work for the quantitation of IgG and IgA. The fluorescamine-fluorescein label pair are again applied to the development of a direct ETIA for IgG and an indirect (sandwich) ETIA for IgA. The methodology used in the direct energy transfer assay for IgG is as previously described for nortriptyline and transferrin (Chapter 5 and 6 respectively). In the sandwich assay for IgA, separate portions of the rabbit anti-IgA antibodies are labelled with fluorescamine (donor) and fluorescein (acceptor)

respectively. Addition of unlabelled IgA molecules causes the aggregation of the separately labelled antibodies. The mean distance between the donor and acceptor groups within the "sandwich" immune complex formed should be sufficient for resonance energy transfer to occur. The consequent quenching of the donor and/or enhancement of the acceptor emission intensities can be related to the amount of the IgA present. In this method, the use of purified or enriched antigens is not necessary. Studies on the fluram and MDPF enhancement effects of IgG conjugates show that such effects can also be applied to the development of fluorescence assays for the determination of IgG.

7.2 Experimental

Quantitative fluorimetric measurements were performed in thermostatted 10 mm path length silica cuvettes using the MPF-44B Spectrofluorimeter. All determinations were made at 35 C after reagents had been incubated for one hour at the same temperature. Corrections were made for fluorescence background contributed by the buffer (0.01M phosphate, pH 7.2, containing 0.145M NaCl) and added proteins including standard serum and test serum samples. The fluorescence background due to the added labelled antibodies was also subtracted from all measurements in the quantitative study of the energy transfer effects between IgG-fluorescamine conjugates and added antibody-fluorescein molecules. All spectra were recorded in the corrected mode. For the determination of IgG concentrations in test serum samples, the sera were diluted 400-fold prior to analysis by the energy transfer and the fluram enhancement assay methods.

7.3 Results and Discussion

7.3.1 FIA methods for IgG determination

Experiments were first performed to study the fluram and MDPF enhancement effects of IgG-fluorescamine and IgG-MDPF conjugates. Figures 7.1 and 7.2 show respectively the changes in the emission spectra of a solution of IgG-FL conjugate (7.85×10^{-8}M; label:protein ratio, 6.6:1) and of a solution of IgG-MDPF conjugate (2.3×10^{-8}M; label:protein ratio, 6.8:1) when increasing amounts of unlabelled rabbit anti-IgG antibodies were added. A notable feature of these spectra is that, except for a general enhancement of the fluorescence in the 400 - 600 nm range, no significant shift in the emission maxima at 495 nm was evident. The magnitude of the fluram and MDPF enhancement effects is also very dependent on the fluorophore:protein ratios of the conjugates, with heavily-labelled conjugates exhibiting the largest effects (see Figure 7.3). This finding had previously been noted when such effects were encountered in transferrin conjugates (Chapter 6). The specificity of antibody binding of the fluorescent IgG conjugates was illustrated by the reversal of the enhancement effects on combining unlabelled IgG molecules (in pure solutions and in serially diluted standard serum) with (a) 1.1×10^{-8}M IgG-FL conjugate (F:P ratio, 10.8:1) solution containing a 12.4-fold molar excess of unlabelled antibodies - see Figure 7.4 ; and (b) 9.5×10^{-9}M IgG-MDPF conjugate (F:P ratio, 9.9:1) solution containing a 14.3-fold molar excess of unlabelled antibodies - see Figure 7.5. These standard graphs permit the quantitation of IgG in pure solutions and diluted serum samples at nanomolar levels and below.

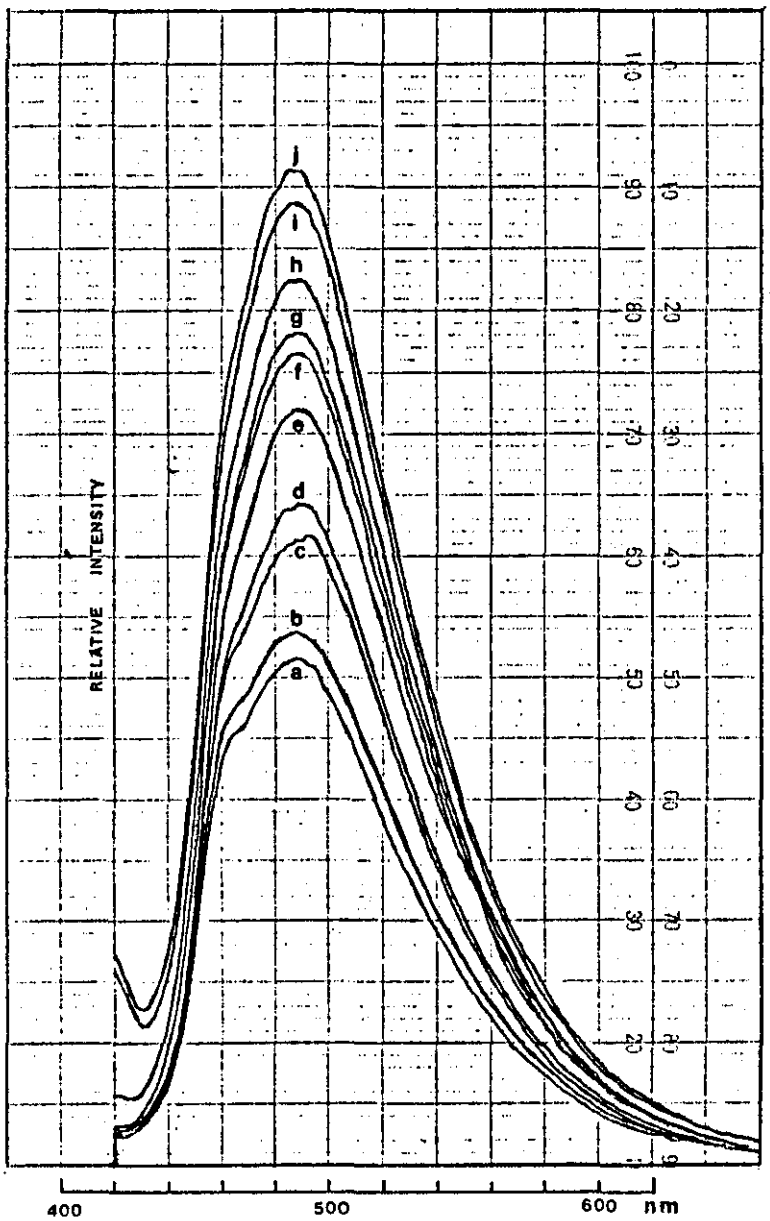

Figure 7.1

Spectra showing the changes in the fluorescence intensity of a solution of IgG-FL conjugate (7.85×10^{-8}M; label:protein ratio, 6.6:1) upon adding increasing amounts of unlabelled rabbit anti-IgG antibodies:

(a) without antibody; (b) Ab:IgG-FL molar ratio, 0.65:1 ; (c) 1.3:1 ; (d) 1.95:1 ; (e) 2.6:1 ; (f) 3.25:1 ; (g) 3.9:1 ; (h) 6.55:1 ; (i) 9.82:1 ; (j) 13.1:1 .

Note the overlapping Raman scatter peak at ca. 465 nm which appeared as a shoulder in spectra (a) - (d). Excitation wavelength, 390 nm; bandwidths, 8 nm.

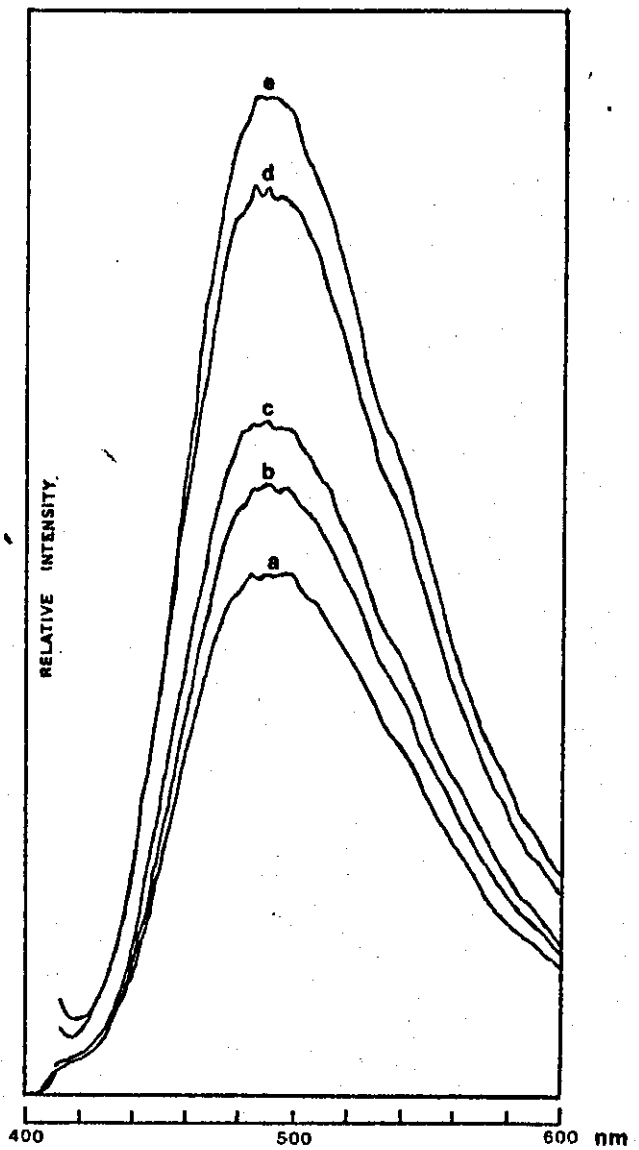

Figure 7.2

Spectra showing the changes in the fluorescence intensity of a solution of IgG-MDPF conjugate (2.3×10^{-8}M; label:protein ratio, 6.8:1) upon adding increasing amounts of unlabelled rabbit anti-IgG antibodies:
(a) without antibody; (b) 1.5:1 ; (c) 2.0:1 ; (d) 8.9:1 ; (e) 14.8:1.

Spectra were recorded with "blank" (ie. added unlabelled antibodies) subtractions. Hence the absence of the overlapping Raman scatter peak at 465 nm in all the spectra. Cf. Figure 7.1. Excitation wavelength, 390 nm; bandwidths, 8 nm.

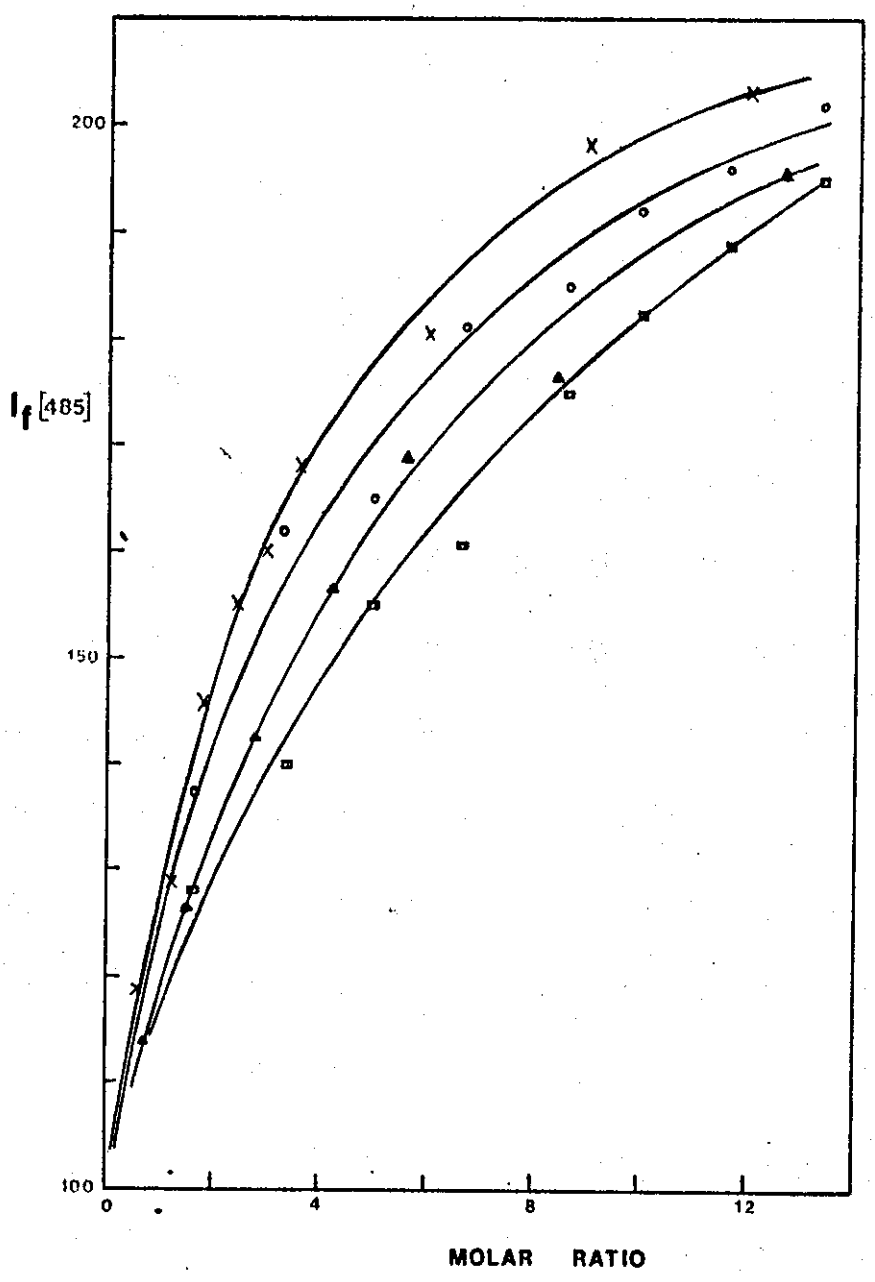

Figure 7.3

Changes in the fluorescence intensity at 485 nm of mixtures containing a fixed concentration of IgG-FL or IgG-MDPF conjugates and increasing amounts of unlabelled anti-IgG antibodies.

1.9×10^{-8}M IgG-M conjugate (label:protein ratio, 9.9:1)(-□-□-);
3.4×10^{-8}M IgG-M conjugate (label:protein ratio, 16:1) (-▲-▲-);
1.9×10^{-8}M IgG-FL conjugate (label:protein ratio, 9.3:1)(-o-o-);
2.2×10^{-8}M IgG-FL conjugate (label:protein ratio, 16.7:1)(-X-X-).

Pure solutions of fluorescent IgG conjugates have arbitrary emission intensity of 100. Excitation wavelength, 390 nm; bandwidths, 8 nm.

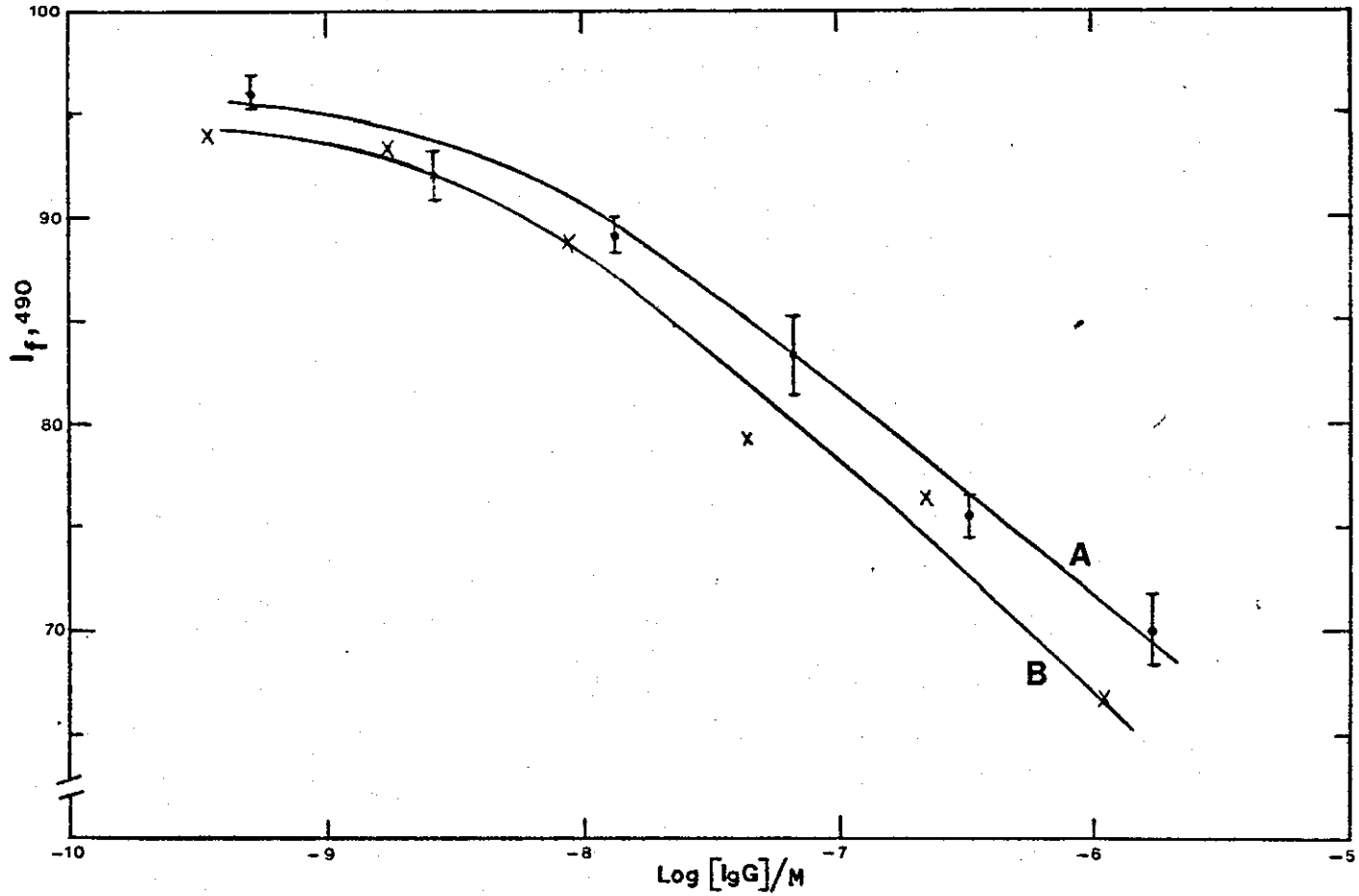

Figure 7.4 Fluorescence intensity changes at 490 nm upon combining IgG molecules in pure solutions (A) and in serially diluted standard serum (B) with 1.1 x 10^{-8}M IgG-FL conjugate (F:P ratio, 10.8:1) and 1.4 x 10^{-7}M anti-IgG molecules. Reagent mixture without any added unlabelled IgG has arbitrary fluorescence intensity of 100. Error bars represent standard deviations. Excitation wavelength, 390 nm; bandwidths, 12 nm.

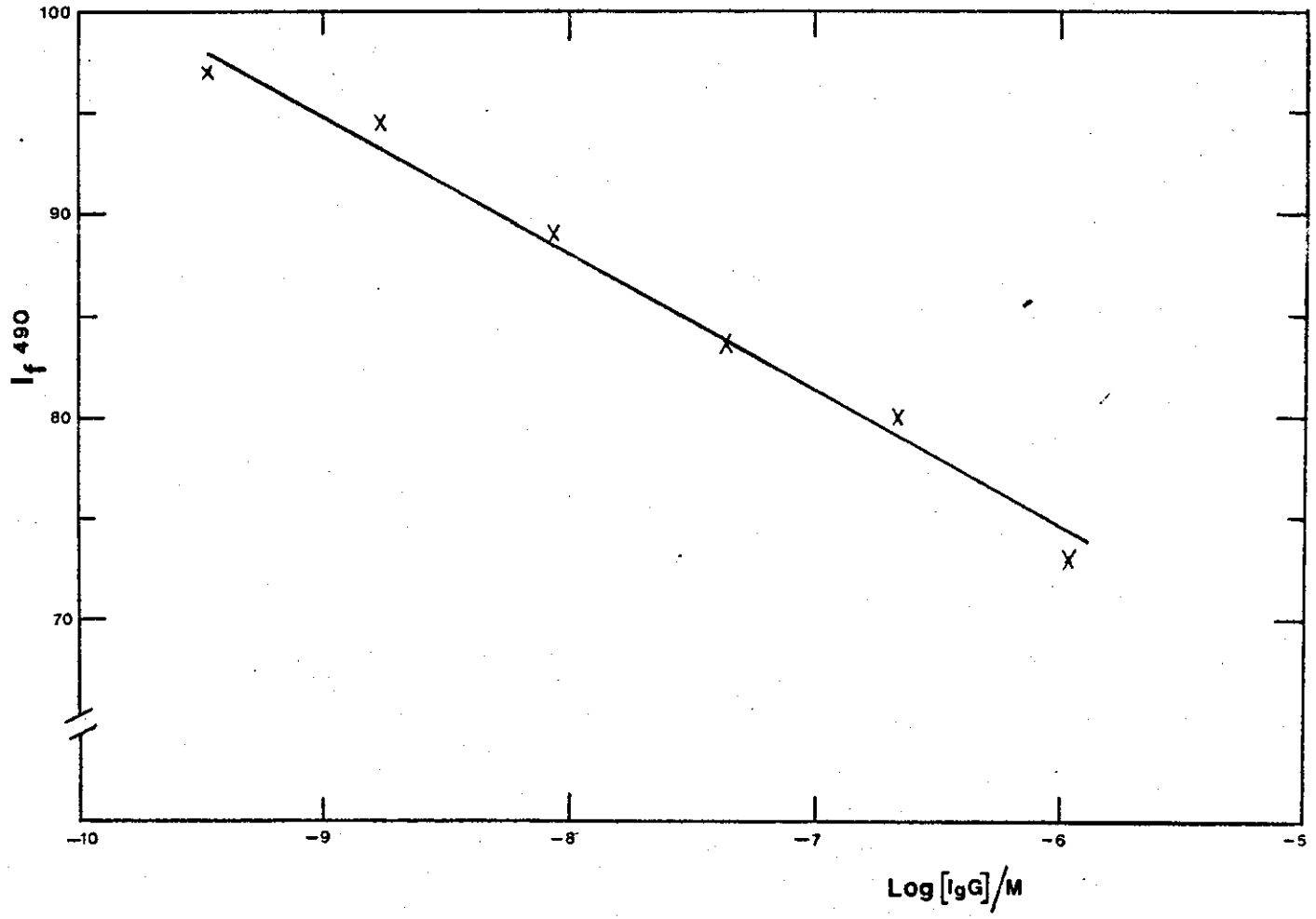

Figure 7.5 Fluorescence intensity changes at 490 nm upon combining unlabelled IgG molecules in pure solutions with 9.5×10^{-9}M IgG-MDPF conjugate (F:P ratio, 9.87:1) and 1.4×10^{-7}M anti-IgG molecules. Other details as in Figure 7.4.

The corrected spectra of Figure 7.6 show once again that there is excellent spectral overlap between the fluorescamine (donor) emission and the fluorescein (acceptor) excitation spectra. An efficient energy transfer between IgG-fluorescamine and antibody-fluorescein molecules can therefore be expected to occur within the antigen-antibody complex. Studies of the energy transfer effects were performed by adding increasing amounts of antibody-fluorescein molecules to solutions containing a fixed concentration of IgG-fluorescamine conjugate. The magnitude of the transfer effects was assessed by measuring the decreases in the fluorescamine emission intensity at 485 nm and the simultaneous increases in the fluorescein emission intensity at 530 nm (excitation wavelength, 390 nm). Results obtained are summarized in Figures 7.7(a), (b) and (c). The general conclusions to be drawn from these results are similar to those already discussed in Chapter 6. In particular, the observed disproportionality between the quenching and enhancement effects can again be related to the counteracting fluram enhancement effect which partially offset the quenching of the fluorescamine emission by energy transfer. The optimum conditions required for the development of an energy transfer assay for IgG are also similar to those found for the assays of albumin (Chapter 3) and transferrin (Chapter 6). These include the use of a heavily-labelled IgG-fluorescamine conjugate (label:protein ratio, 12:1) and a lightly-labelled antibody-fluorescein conjugate (label:protein ratio, $< 2:1$). Examples of standard graphs for the assay of IgG in pure solutions and in serum samples are shown in Figure 7.8.

Results of the analyses of 3 test serum samples by the energy transfer assay and the fluorescence enhancement assay methods are summarized in Table 7.2 : good agreement between the two methods is obtained.

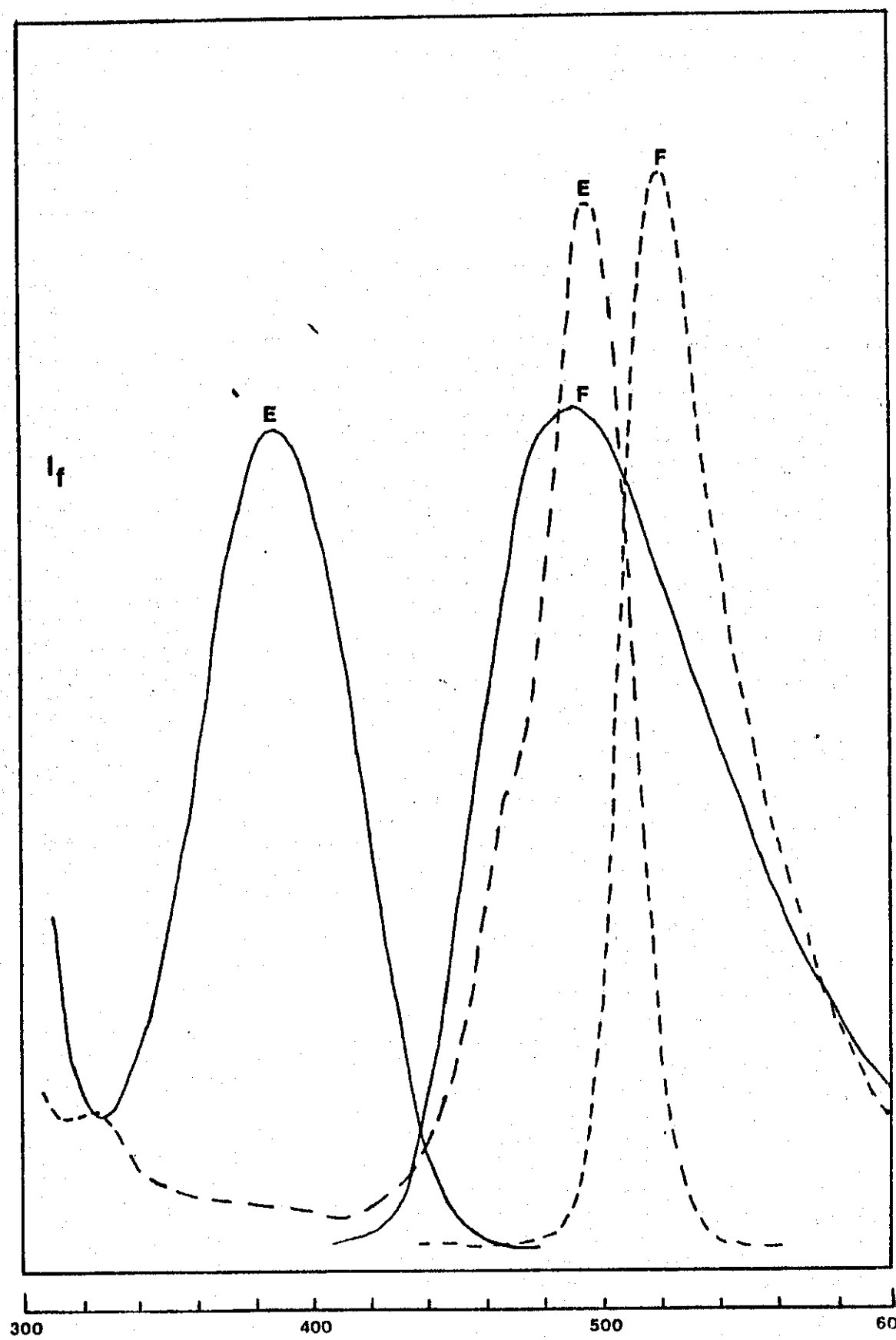

Figure 7.6 Corrected excitation (E) and fluorescence (F) spectra of an IgG-fluorescamine conjugate (label:protein ratio, 10.8:1) (————), and of an antibody-fluorescein conjugate (label:protein ratio,1.5:1) (— — — —).

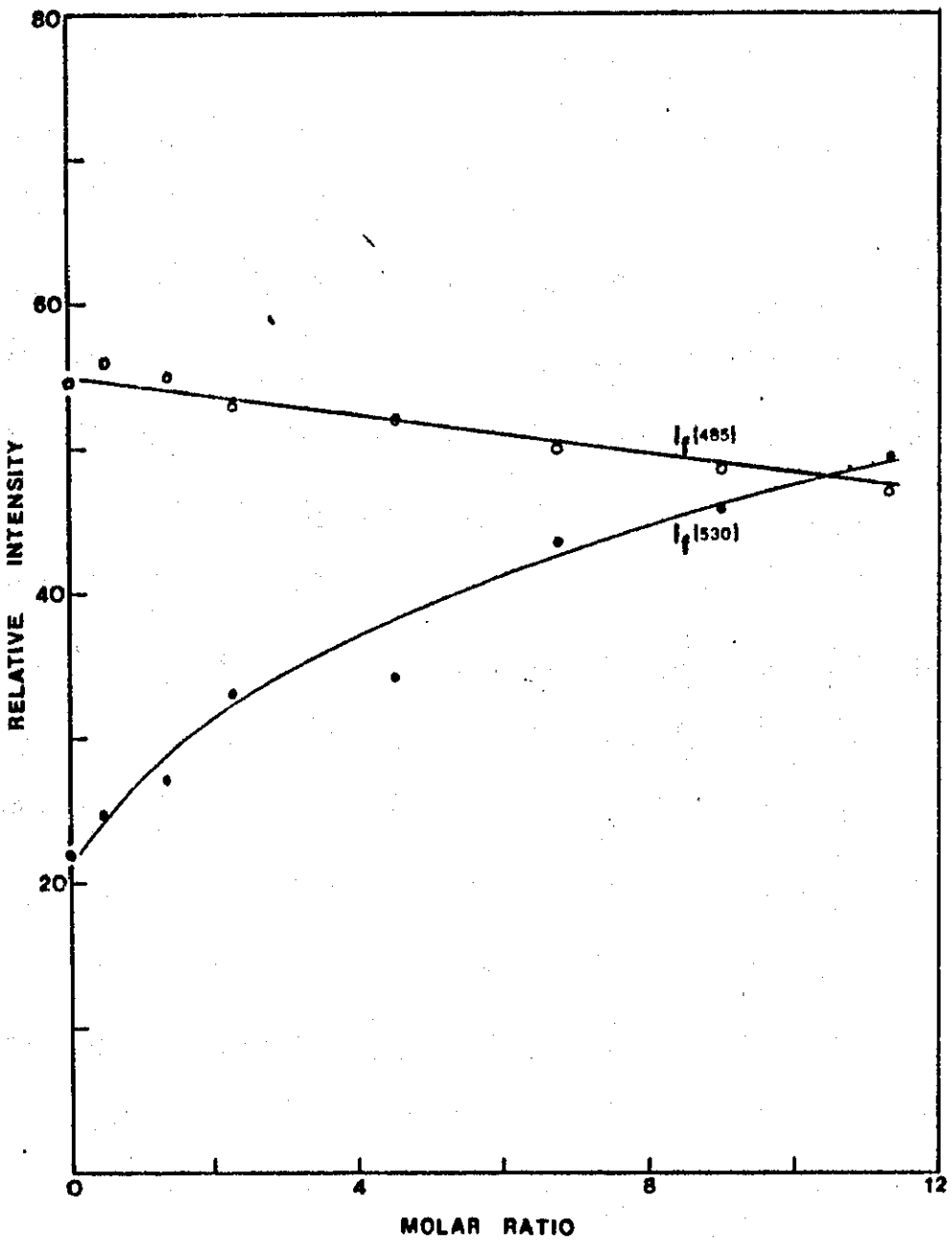

Figure 7.7 (a)
Enhancement of fluorescein fluorescence (530 nm) and quenching of fluorescamine fluorescence (485 nm) when increasing amounts of fluorescein-labelled antibody conjugate (F:P ratio, 1.5:1) are added to solutions containing a fixed concentration of IgG-FL conjugate (9.2×10^{-8}M; F:P ratio, 10.8:1).
Excitation wavelength, 390 nm; bandwidths 10 nm.

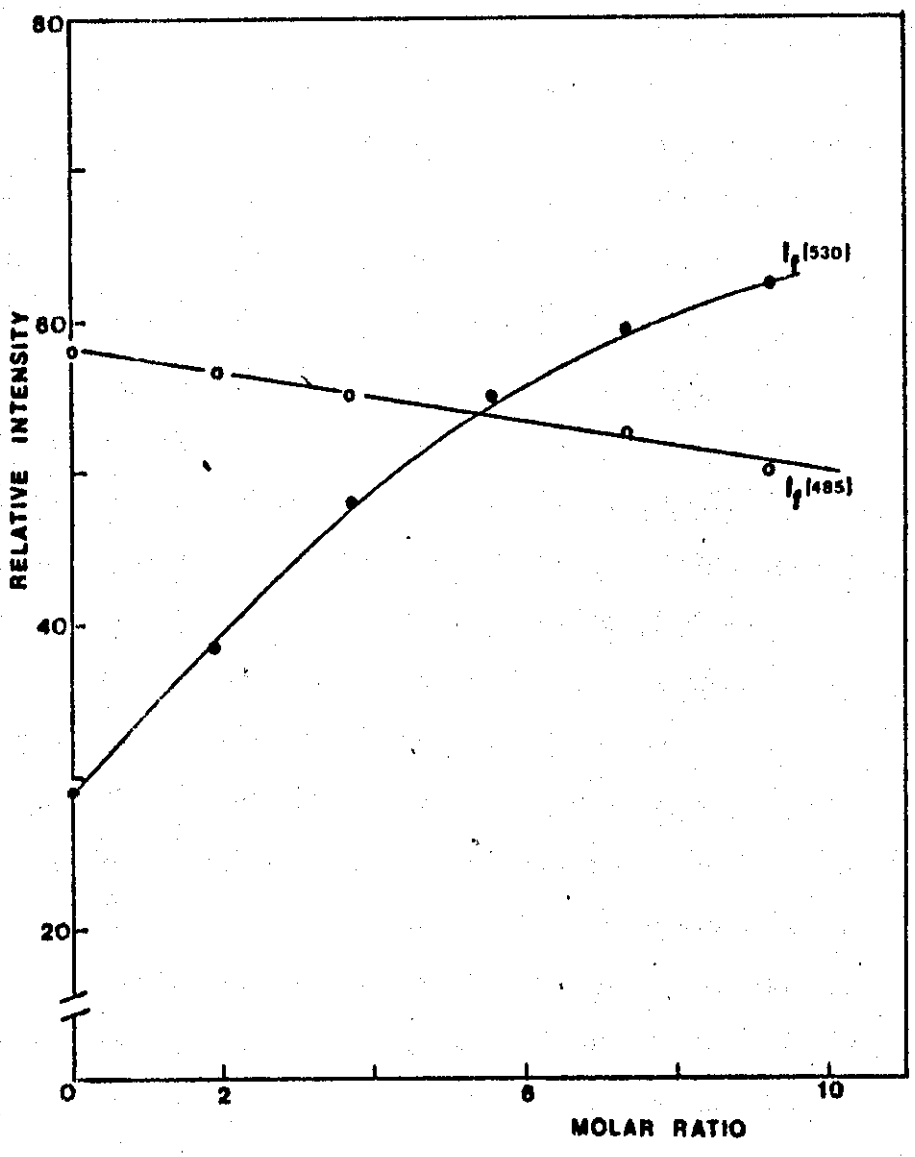

Figure 7.7(b)

As in Figure 7.7(a) except that antibody-fluorescein molecules with label:protein ratio, 2.5:1 are added to 2.2×10^{-8}M IgG-fluorescamine conjugate (label:protein ratio, 9.3:1)

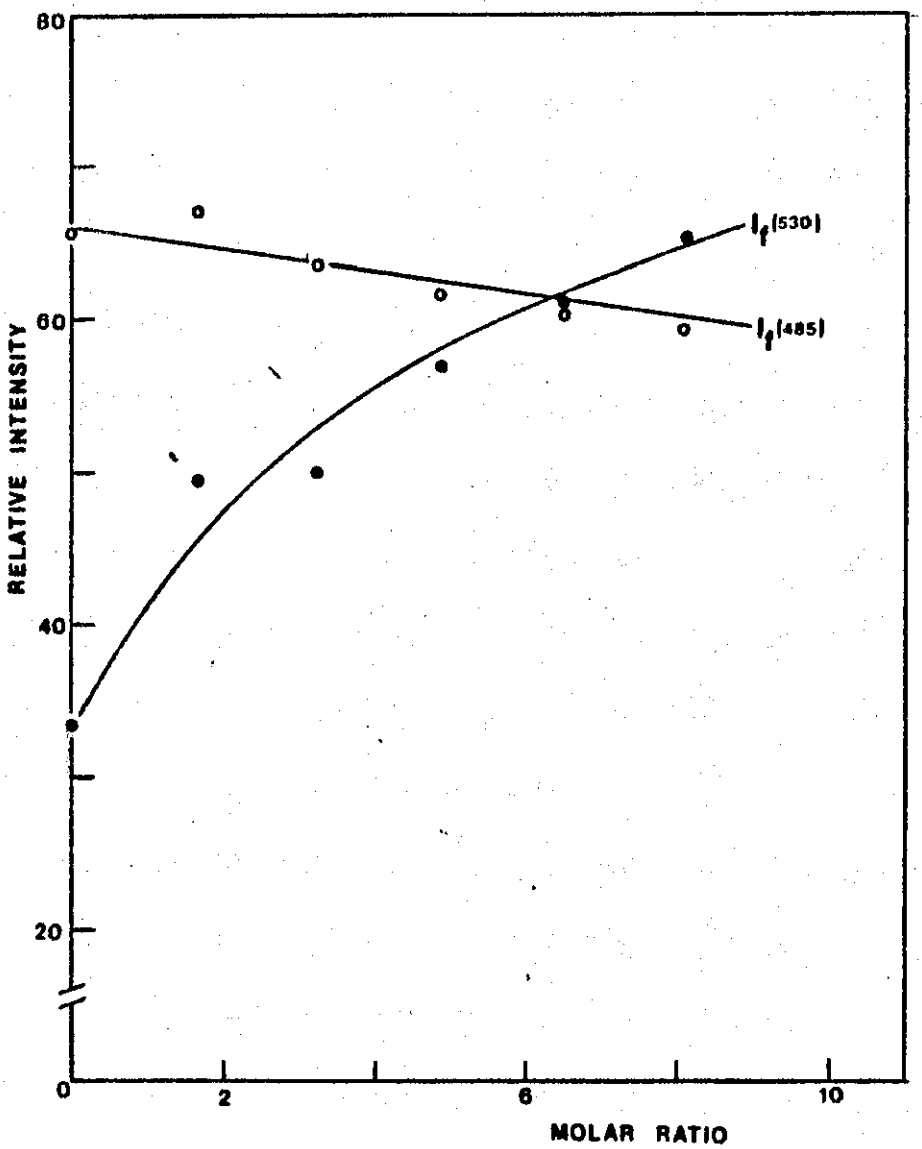

Figure 7.7(c)

As in Figure 7.7(a) except that antibody-fluorescein molecules with label:protein ratio, 2.5:1 are added to 2.2×10^{-8}M IgG-fluorescamine conjugate (label:protein ratio, 16.7:1).

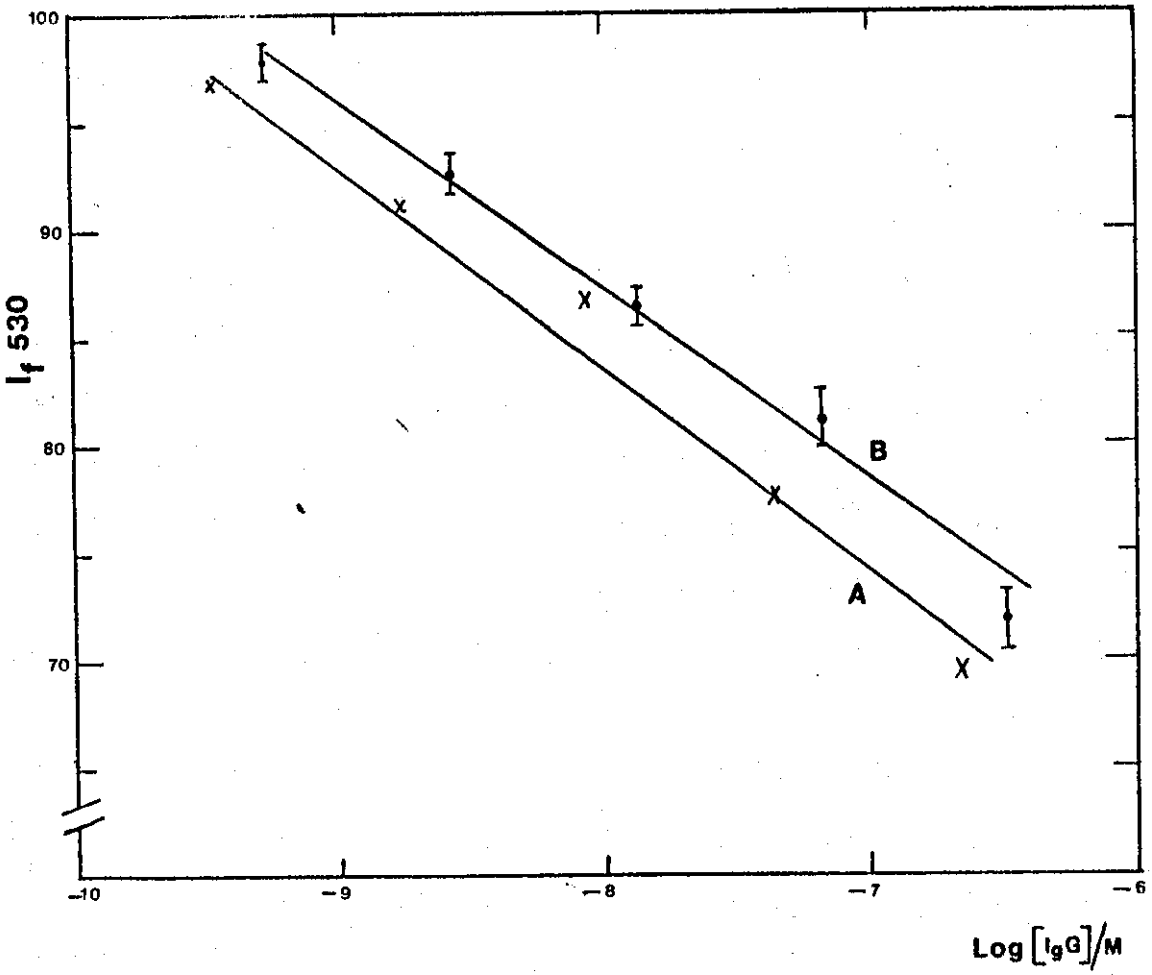

Figure 7.8 Standard graphs for IgG determination
Changes in the fluorescein fluorescence (530 nm) when unlabelled IgG molecules in pure solutions (A) and in serially diluted standard serum (B) are combined with 2.2 x 10^{-8}M IgG-FL conjugate (F:P ratio, 10.8:1) and 1.0 x 10^{-7}M antibody-fluorescein conjugate (F:P ratio, 2.5:1). Fluorescence intensity of reagent mixture without any added unlabelled IgG is assigned an arbitrary value of 100. Error bars represent standard deviations. Excitation wavelength, 390 nm; bandwidths, 8 nm.

Table 7.2 Determination of IgG

	Fluorescence immunoassay methods	
Sample	ETIA	"Fluram" enhancement assay
A	1260 ± 106 (5)	1195 ± 96 (5)
B	1078 ± 93 (5)	1020 ± 80 (4)
C	1130 ± 98 (4)	1200 ± 92 (4)

Concentrations in mg dl^{-1}. Figures in brackets refer to the number of determinations.

Experiments were also performed in an attempt to develop a solid-phase energy transfer assay for IgG based on one of the reaction schemes outlined below.

Scheme I. Competitive binding assay with labelled antigen (IgG^*) on solid-phase

$$\vdash IgG^* + IgG + Ab^{**} \rightleftharpoons \vdash IgG^*—Ab^{**} + IgG—Ab^{**}$$

Scheme II. Noncompetitive sandwich assay with Ab^* on solid-phase.

$$\vdash Ab^* + IgG \rightleftharpoons \vdash Ab^*—IgG \xrightarrow{Ab^{**}} \vdash Ab^*—IgG—Ab^{**}$$

Scheme III Competitive binding assay with Ab^{**} on solid-phase.

$$\vdash Ab^{**} + IgG^* + IgG \rightleftharpoons \vdash Ab^{**}—IgG^* + \vdash Ab^{**}—IgG$$

$\vdash$ = solid-phase

$*$ = donor label (eg. fluorescamine)

$**$ = acceptor label (eg. fluorescein).

Attempts to immobolize the fluorescent protein conjugates onto the IMMUNOBEADS (the solid-phase) have so far not yielded

satisfactory results. The observed fluorescence signal from the immobilized conjugates was found to be too feeble to permit its use in the development of a solid-phase energy transfer assay.

7.3.2 Sandwich ETIA for IgA determination

In the sandwich ETIA method for IgA determination, separate portions of the rabbit anti-IgA antibodies were labelled with fluorescamine and fluorescein respectively. The corrected excitation and fluorescence spectra of these conjugates are shown in Figure 7.9. Since the IgA molecule is multivalent (MW of monomeric IgA = 180000), the reaction of the differently labelled antibody fractions with the antigen should bring the donor and acceptor label groups within close proximity, and therefore resonance energy transfer can be expected to occur within the molecular dimension of the sandwich immune complex formed. In order to first establish the optimum conditions required for the development of a sandwich energy transfer assay for IgA, a number of preliminary experiments were performed.

The change in the fluorescence intensity upon adding unlabelled IgA molecules to antibody-fluorescamine (Ab-FL) conjugate was first investigated. Results are summarized in Table 7.3. Addition of IgA to a solution of Ab-FL conjugate (2.4×10^{-8}M; label:protein ratio, 14.7:1) resulted in a small enhancement of the fluorescence up to a maximum of 19 per cent. This enhancement effect, though small in magnitude, is nevertheless analogous to the fluram enhancement effect encountered in previous studies of fluorescamine-labelled antigens.

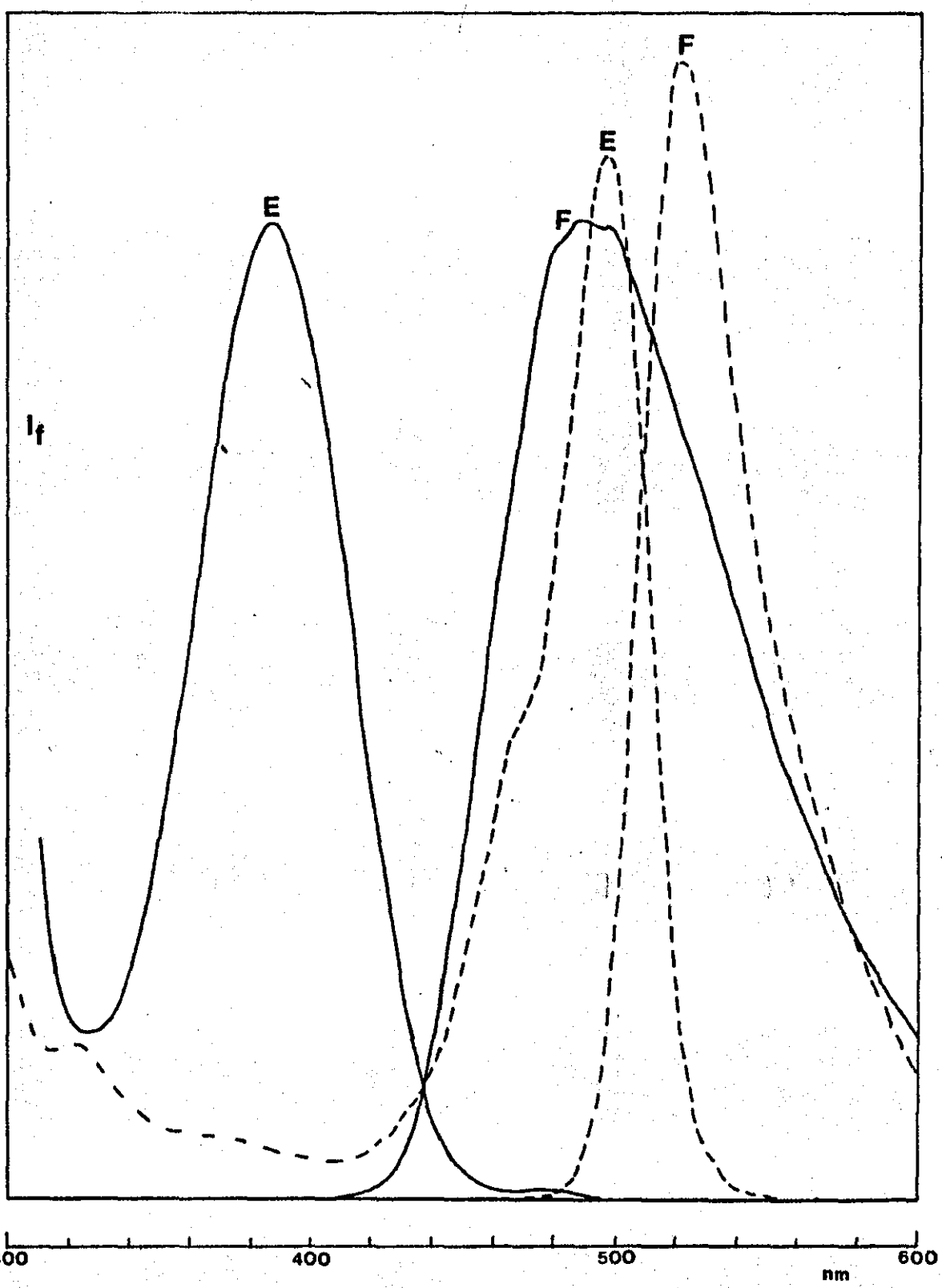

Figure 7.9 Corrected excitation (E) and fluorescence (F) spectra of a fluorescamine-labelled anti-IgA conjugate (label:protein ratio, 14.7:1) (————), and of a fluorescein-labelled anti-IgA conjugate (label:protein ratio, 2.3:1) (- - - - -).

Table 7.3

Changes in the fluorescamine fluorescence (at 490 nm) upon binding IgA molecules with antibody-fluorescamine conjugate (label:protein ratio, 14.7:1). Excitation wavelength, 390 nm.

	$I_f(490)$
2.4×10^{-8}M Ab-FL	50.0
IgA : Ab-FL (molar ratio)	
2.3	54.0
4.6	60.0
6.8	60.0
9.1	60.0
11.4	58.4

The Ab-FL concentration was then varied while keeping the concentration of unlabelled IgA constant at at 2.73×10^{-9}M and 5.45×10^{-8}M. Fluorescence measurements (at 490 nm) were made both without the inclusion of unlabelled antibody (Figure 7.10) and with the addition of an amount of unlabelled antibody sufficient to maintain a constant <u>total</u> antibody concentration of 2.4×10^{-8}M (see Figure 7.11). In both the cases (Figures 7.10 and 7.11) it was found that the enhancement produced by the IgA molecules became significant only in very low concentrations (4.8×10^{-9}M) of Ab-FL containing a large excess of the IgA molecules (5.45×10^{-8}M). In the absence of unlabelled antibody, no significant enhancement in the fluorescence was evident in the case when the IgA concentration is low (2.73×10^{-9}M) - see curve B in Figure 7.10. However, in the presence of the unlabelled antibody, a small

increase (ca. 8 %) in the fluorescence intensity was noted at low concentrations of Ab-FL containing 2.73×10^{-9}M IgA - see curve B in Figure 7.11.

The above experiments were repeated using fluorescein-labelled antibody (Ab-F) conjugate (label:protein ratio, 2.3:1) in place of the unlabelled antibody and with the concentration of IgA kept constant at 5.46×10^{-9}M. Fluorescence measurements at 490 nm (fluorescamine emission) and at 530 nm (fluorescein emission) were made. The results are shown in Figure 7.12. In contrast to the enhancement in fluorescence (at 490 nm) noted above, a reduction in the fluorescamine emission at 490 nm and a simultaneous enhancement in the fluorescein emission at 530 nm were observed as the Ab-F/Ab-FL molar ratio was varied from 0.7 to 4.0. There did not seem to be any significant change in the fluorescence intensities when the Ab-F/Ab-FL molar ratio < 0.7. On the basis of these results, 1.5/1 and 4.0/1 mixtures of Ab-F and Ab-FL were incubated with increasing amounts of IgA and the fluorescence intensities at 490 nm and 530 nm were recorded. These results are summarized in Figures 7.13 and 7.14. At very low concentrations of the IgA (< 55 nM), an increase in the fluorescein emission at 530 nm was accompanied by a reduction in the fluorescamine emission at 490 nm. However, as the IgA concentration was increased, not only was there an enhancement in fluorescein emission but the reduction in the fluorescamine emission appeared to be offset by a counter-acting enhancement effect (cf. fluram enhancement effect). Addition of comparable amounts of nonspecific bovine serum albumin (BSA) and bovine gammaglobulin(BGG) to the labelled antibody mixtures resulted in only a small enhancement in fluorescence which can be attributed to the viscosity effect.

An explanation for these observations seems likely to be that, as the IgA concentration in the assay mixture is increased, the fluram enhancement effect (refer Table 7.3) becomes so pronounced to the extent that it can more than offset the quenching of the fluorescamine emission caused by energy transfer. In practice this would mean that the assay must utilize only the fluorescein emission measurements. In the assays developed, IgA at nanomolar concentrations can be detected. However, a serious drawback is the narrow concentration range in which these assays are applicable. It is proposed as suggestions for further work that experiments be performed to optimize the assay conditions along the lines described above.

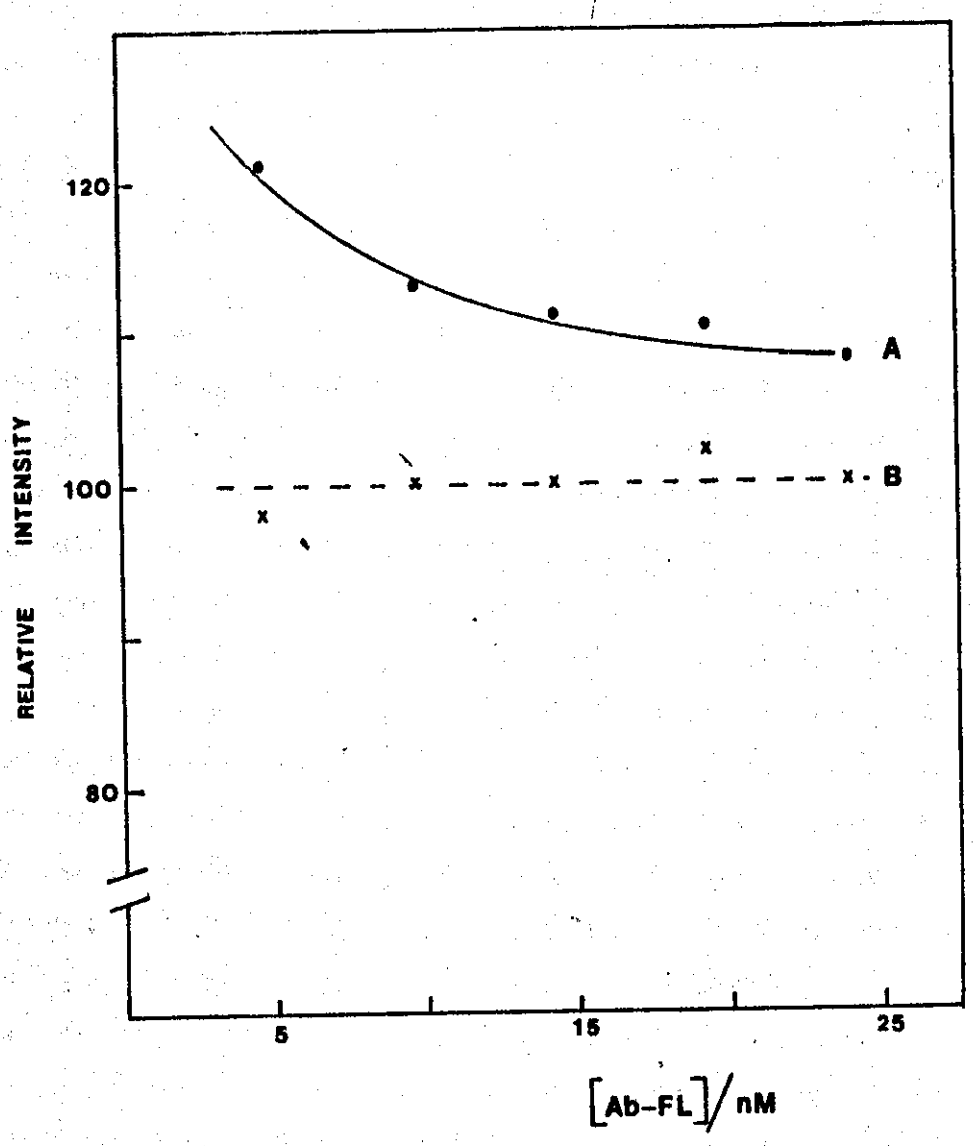

Figure 7.10

Ratio of the fluorescence intensities (at 490 nm) of Ab-FL conjugate (label:protein ratio, 14.7:1) solutions measured in the presence and absence of IgA whose concentration is kept constant at (A) 54.5 nM ; and (B) 2.73 nM.
Excitation wavelength, 390 nm; bandwidths, 6 nm.

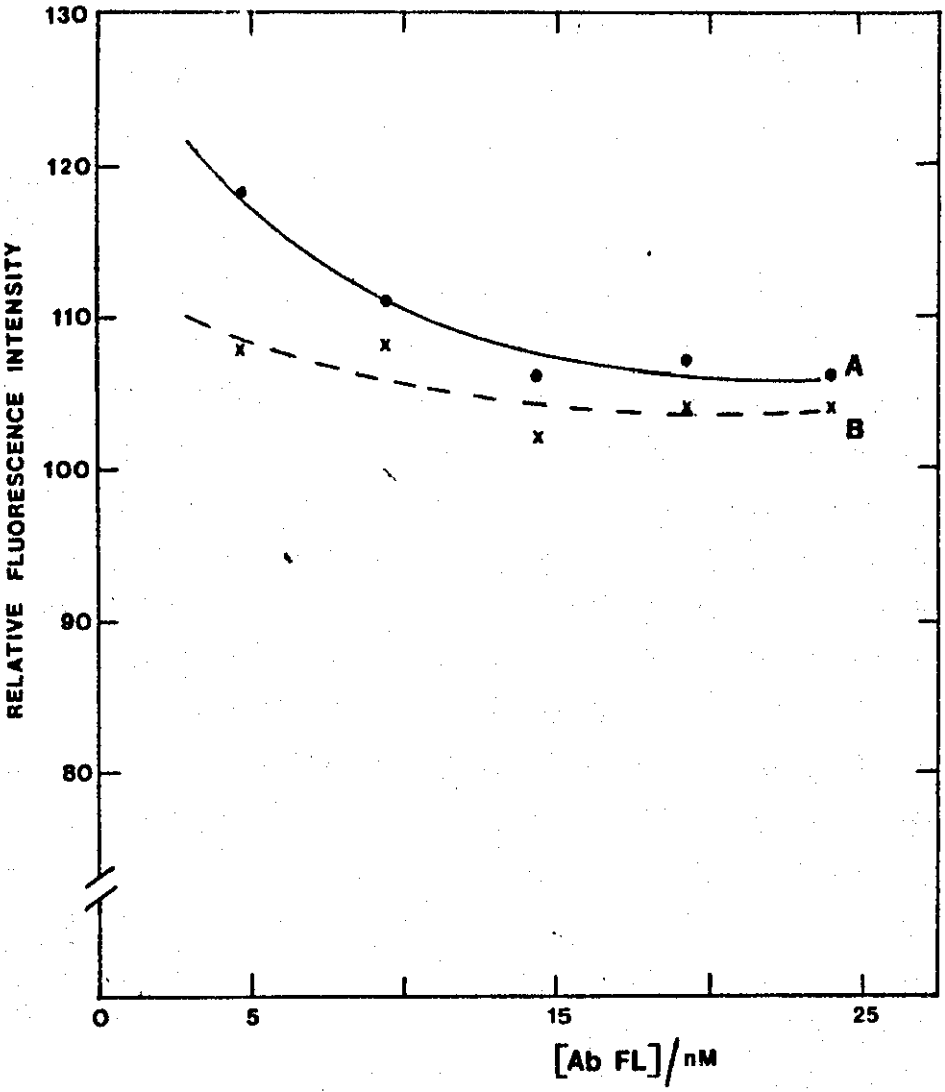

Figure 7.11

Ratio of the fluorescence intensities (at 490 nm) of Ab-FL conjugate (label:protein ratio, 14.7:1) solutions measured in the presence and absence of (A) 54.5 nM IgA and (B) 2.73 nM IgA plus sufficient unlabelled antibody to maintain a <u>total</u> constant antibody concentration of 2.4×10^{-8}M.

Excitation wavelength, 390 nm; bandwidths, 6 nm.

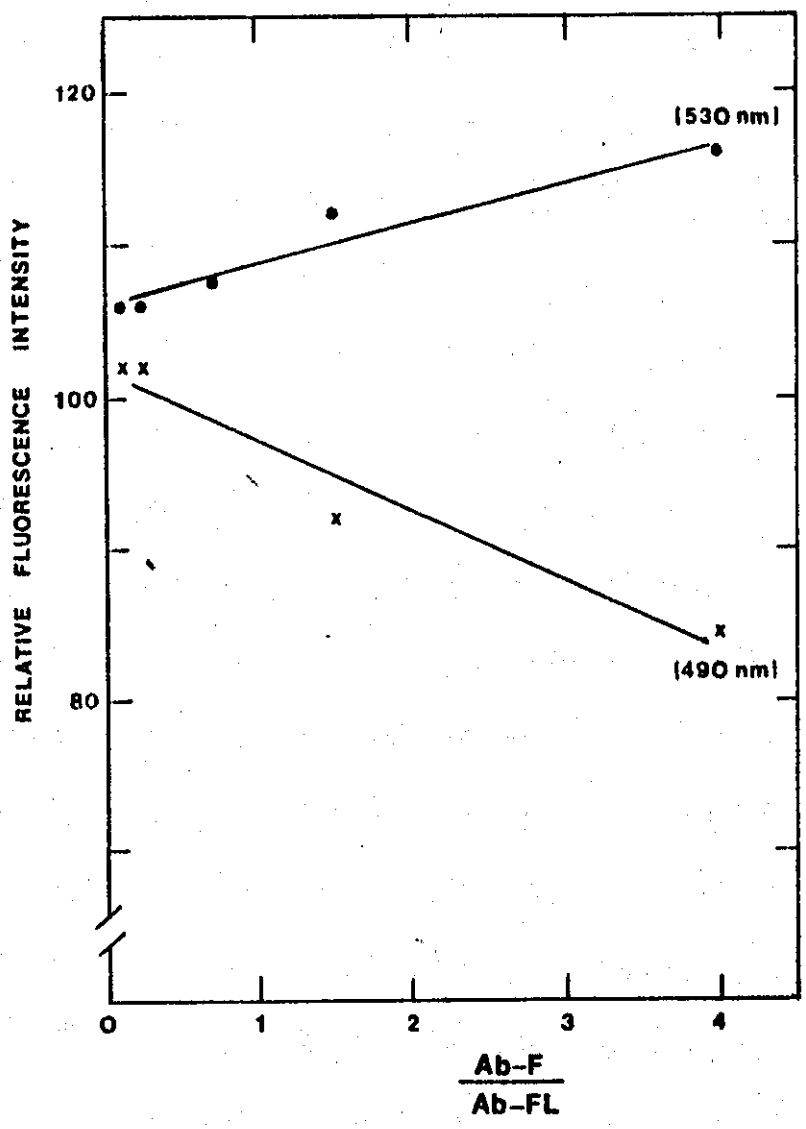

Figure 7.12

Ratio of the fluorescence intensities (at 490 nm and 530 nm) of mixtures containing Ab-FL conjugate (label:protein ratio, 14.7:1) and Ab-F conjugate (label:protein ratio, 2.3:1) in the presence and absence of 5.46 nM IgA. The composition of the separately labelled antibody fractions is varied but the total antibody concentration is kept constant at 2.4×10^{-8}M. Excitation wavelength, 390 nm; bandwidths, 6 nm.

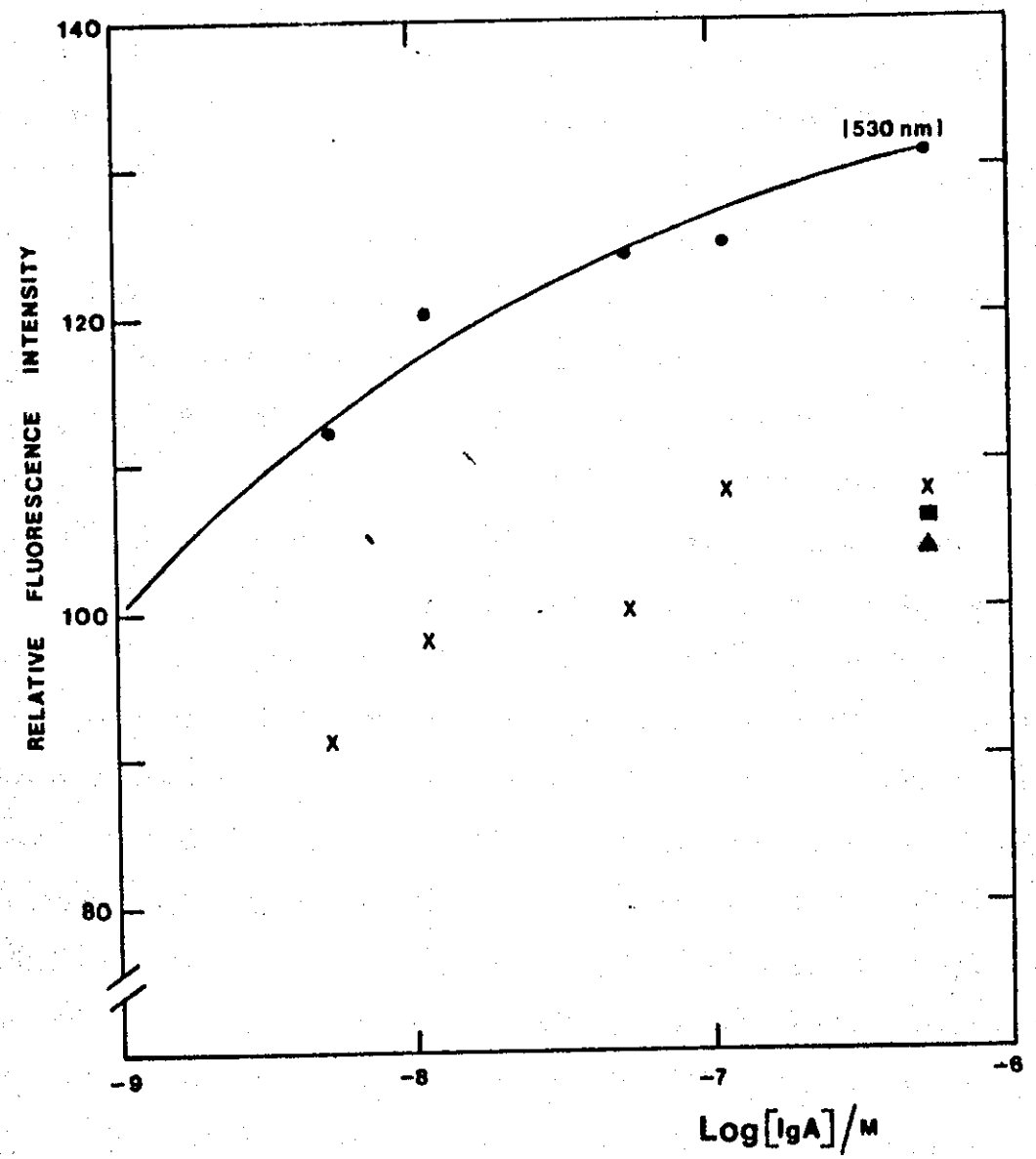

Figure 7.13

Effect of IgA on the fluorescence of a 1.5/1 mixture of Ab-F conjugate (label:protein ratio, 2.3:1) and Ab-FL conjugate (label:protein ratio, 14.7:1) having total antibody concentration = 4.8×10^{-8}M. Intensity changes at 530 nm are indicated by (-•-•-); at 490 nm by (x x x). Effect of nonspecific bovine serum albumin and gammaglobulin on the fluorescence of the mixture is indicated by (■) and (▲) respectively. Excitation wavelength, 390 nm; bandwidths, 6 nm.

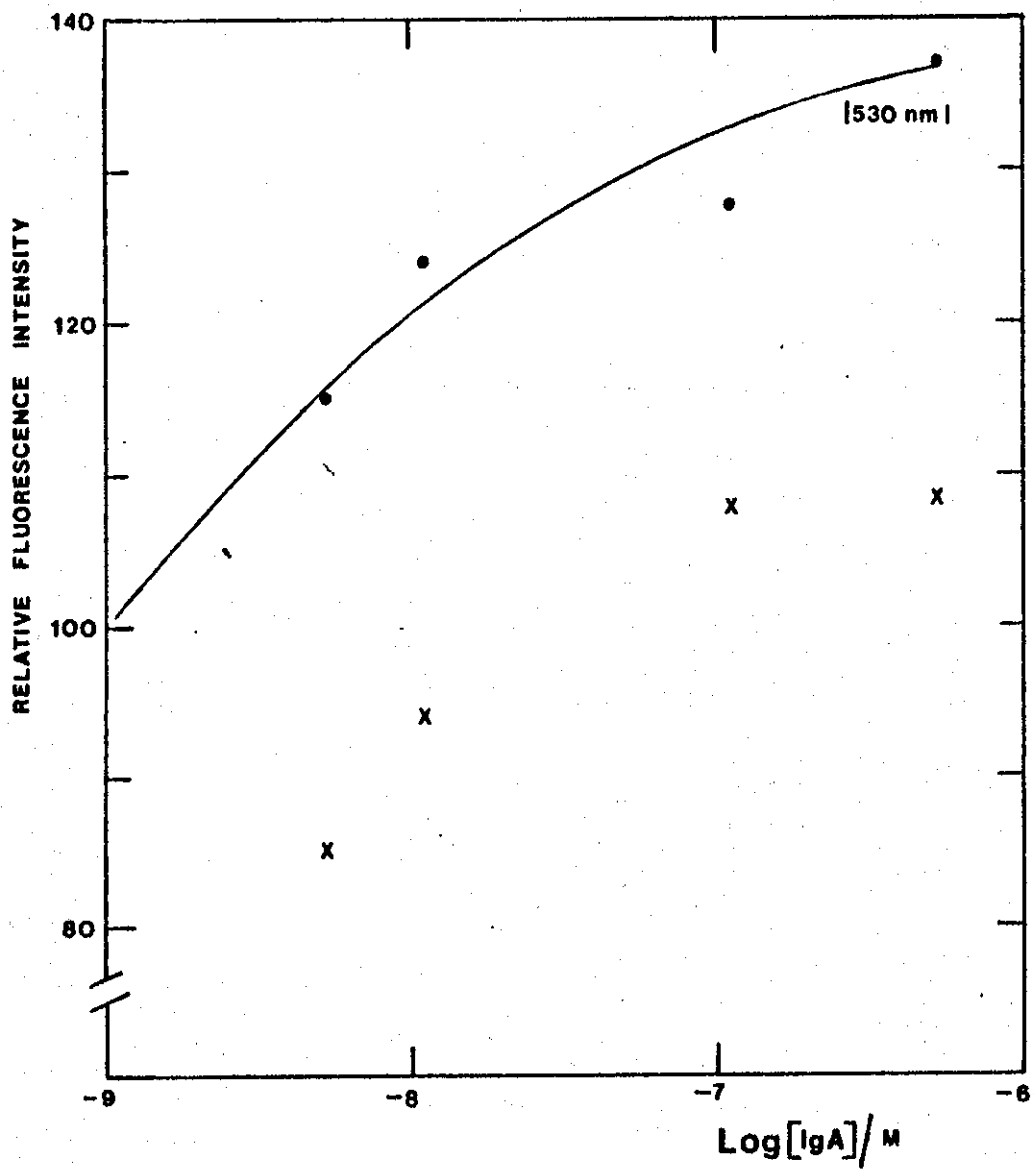

Figure 7.14

As in Figure 7.13 except that a 4/1 mixture of Ab-F and Ab-FL conjugates is used. Other details same.

CHAPTER 8 AUTOMATION OF AN ENERGY TRANSFER IMMUNOASSAY BY USING STOPPED-FLOW INJECTION ANALYSIS WITH MERGING ZONES

8.1 Introduction

Since its introduction in 1974 by Ruzicka and Hansen (1975), and by Stewart et al (1974), flow injection analysis has been applied to the automation of a number of determinations (reviewed by Betteridge,1978). These analyses have been performed by injecting precisely measured sample zones into a reagent-containing carrier stream; by varying the design of the flow system, a wide range of interfacial gradients can be exploited. This approach is less well-suited to analyses in which the reagent is a biological macromolecule such as an antibody or enzyme, since such reagents may be costly and are often available only in small quantities: published applications include the use of readily-available enzymes to the analysis of glucose (Hansen et al,1977) and urea (Ruzicka et al,1979b) in serum. Two additional principles recently applied to flow injection analysis have, however, opened up new potential applications of the technique. It has been shown (Ruzicka and Hansen,1978) that "stopped flow" analyses are feasible ie. that the dispersion of a sample zone in the carrier stream will remain constant if the flow rate of the stream is reduced to zero. This principle is of great value in kinetic assays (Ruzicka and Hansen, 1978 & 1979a) and in analyses where an incubation period is required before the sample zone reaches the detector. In addition, economies of sample and of reagent can be achieved by using the "merging zone" approach, in which small volumes of sample and reagent are injected into inert carrier streams

and merge before reacting and being carried to the detector. The merging zone principle has been applied to a number of analyses (Bergamin et al, 1978; Zagatto et al, 1979) including, in conjunction with the stopped-flow principle, the enzymatic assay of serum glucoes (Ruzicka and Hansen,1979a).

This chapter describes the application of flow injection analysis in the field of immunoassay. The stopped-flow and merging-zone principles are applied to the automation of a homogeneous energy-transfer immunoassay for albumin described in Chapter 3. In this assay fluorescein and rhodamine were used as the donor-acceptor fluorescent labels, though as pointed out in Chapter 3, these groups are far from ideal. Since the phenomenon of energy transfer involves a change in the fluorescence properties of one or both of the labels on antigen-antibody binding, the analysis is homogeneous (ie. no separation step is required) and very suitable for automation. Previous studies have shown that a fluorimetric detector can be combined successfully with flow injection analysis in the determination of albumin by a dye-binding procedure using anilinonaphthalene (Braithwaite and Miller, 1979). A modification of that assay using the merging zone principle will also be described.

8.2 Experimental

Flow injection analysis was performed using the arrangement shown in Figure 8.1. The pump was a Gilson Minipuls HP-4 8-channel instrument and the double-injection valve (Figure 8.2) was obtained from BIFOK (Sollentuna, Sweden) through E.D.T. Research Ltd., London (Cf. Ruzicka and Hansen,1979a).

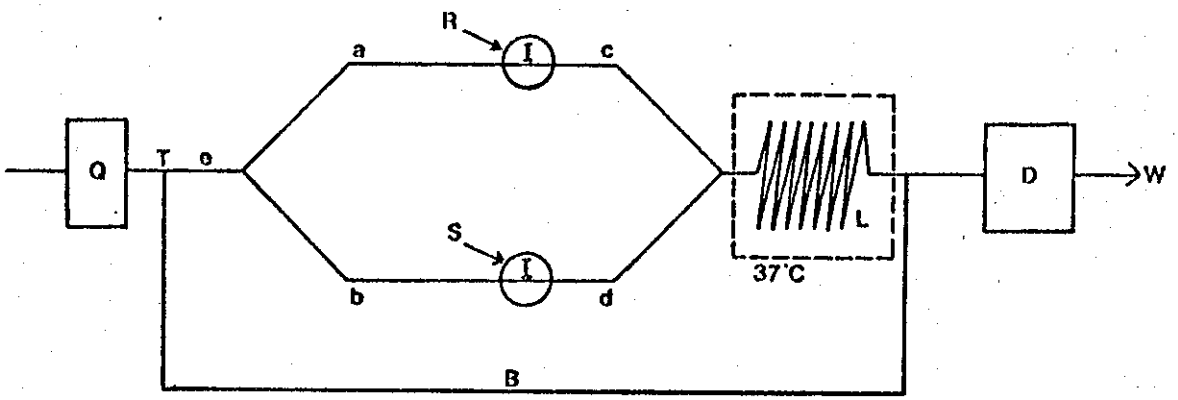

Figure 8.1

Flow-injection analysis arrangement. (B) Bypass; (D) fluorimetric detector; (I) injector valve; (L) reaction coil; (Q) pump; (R) reagent; (S) sample; (T) 3-way valve; (W) waste.

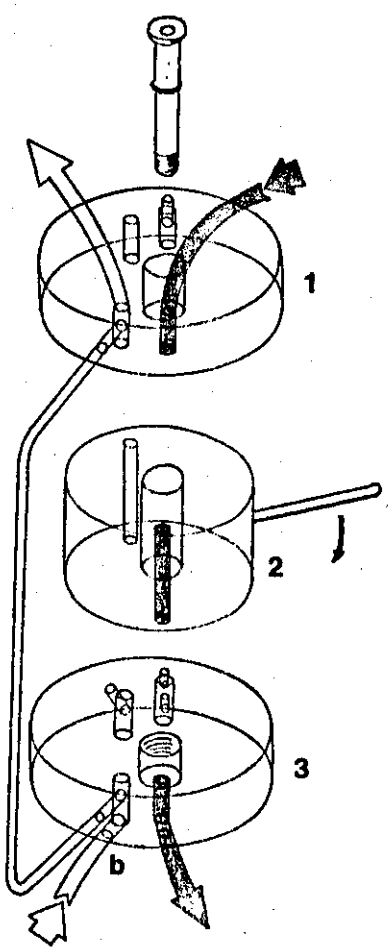

Figure 8.2 Double-injection valve

The rotor (2) of the double-injection valve is sandwiched between two stators (1 and 3) and the whole system is clamped together and held in place by a bolt. Two volumteric bores in the rotor allow precisely measured volumes of the sample and reagent to be introduced into the carrier stream. The diagram shows a volumetric bore of the rotor filled with the sample solution (shown shaded) before sampling; the carrier stream enters through the bottom stator at "b" and bypasses the rotor through a shunt. During sampling the rotor moves in the direction indicated by the shaded arrow and introduces the precisely measured sample zone into the carrier stream. The shunt is being bypassed during sampling because of its higher hydrodynamic flow resistance.

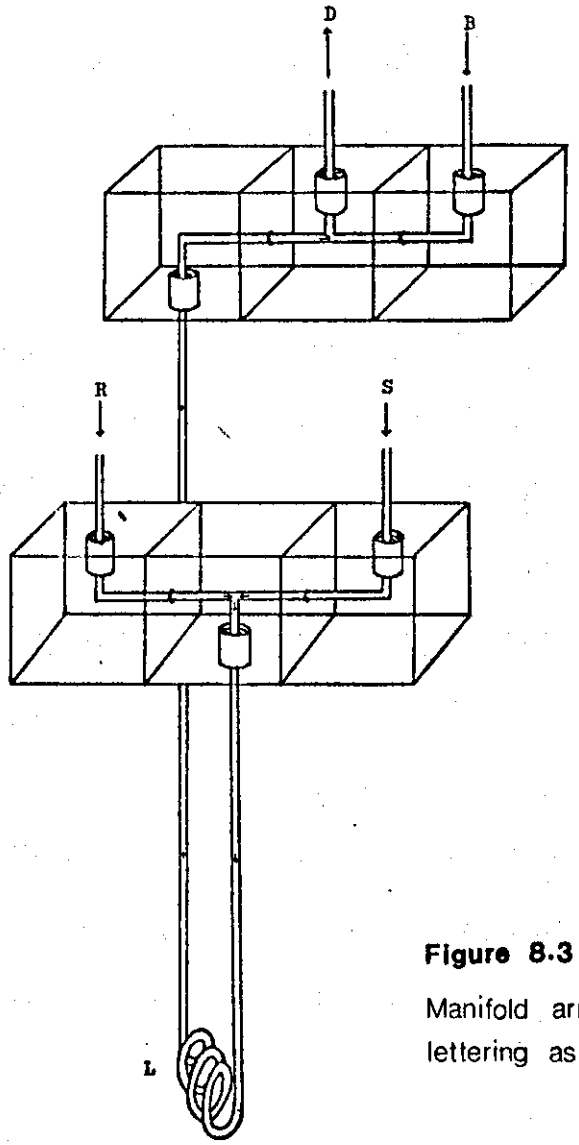

Figure 8.3

Manifold arrangement ; lettering as in Fig. 8.1

Polyethylene tubing was used throughout and was connected using the manifold system shown in Figure 8.3. The lengths and diameters of the tubing used were as follows: a = b = 250 mm. long, 0.5 mm. i.d. ; c = d 150 mm. long, 0.5 mm. i.d. ; e = 100 mm. long, 0.7 mm. i.d. ; bypass (B) = 320 mm. long, 0.7 mm. i.d. The reaction coil, L, was made of 0.7 mm. i.d. tubing and was 650 mm. long for the dye-binding assay and 950 mm. long for the immunoassay. The detector was a Perkin-Elmer Model 1000M filter fluorimeter, with a flow cell specially adapted with silica tubing of 1 mm. i.d. (Figure 8.4).

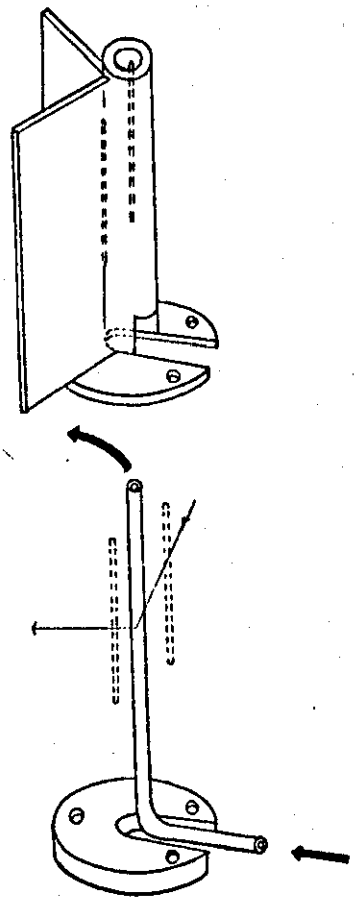

Figure 8.4 Flow cell

The illuminated volume of the cell was ca. 16 µl . Excitation and emission wavelengths were 364 and 470 nm respectively for the dye-binding assay, and 470 and 541 nm respectively for the immunoassay. The fluorimeter was connected to an Omniscribe 10 mV recorder (Houston Instruments).

The carrier stream was a phosphate buffer (0.067M, pH 7.0 for the dye-binding assay; 0.01M, pH 7.2, containing 0.145M NaCl for the immunoassay). The 8-anilino-1-naphthalene sulphonic acid (ANS: Sigma) was used at a concentration of 15 mg l^{-1}. Pure human serum albumin and purified rabbit anti-albumin antibodies were labelled with fluorescein isothiocyanate and rhodamine isothiocyanate respectively as described in

Section 2.3.1. Electroimmunoassay was done on "Cellogel" cellulose acetate membrane (Section 2.3.2). Individual serum samples were obtained from healthy laboratory workers.

8.3 Results

8.3.1 ANS binding procedure

This assay, previously performed using conventional flow-injection procedures (Braithwaite and Miller, 1979), was successfully adapted to the merging zone approach. A flow rate of 1.03 ml min^{-1} was used. The volumes of sample and reagent injected (determined by injecting an air-bubble into the carrier stream) were 10.4 µl. This represents a substantial saving in reagent compared with the previous method. The merge time, sampling time, and residual time (non-stop) were 9.8 s, 3.0 s, and 23.6 s respectively. A linear relationship was obtained between the fluorimeter response (fluorescence of protein-bound ANS) and albumin concentration in the range 0 - 200 mg dl^{-1} (Figure 8.5)

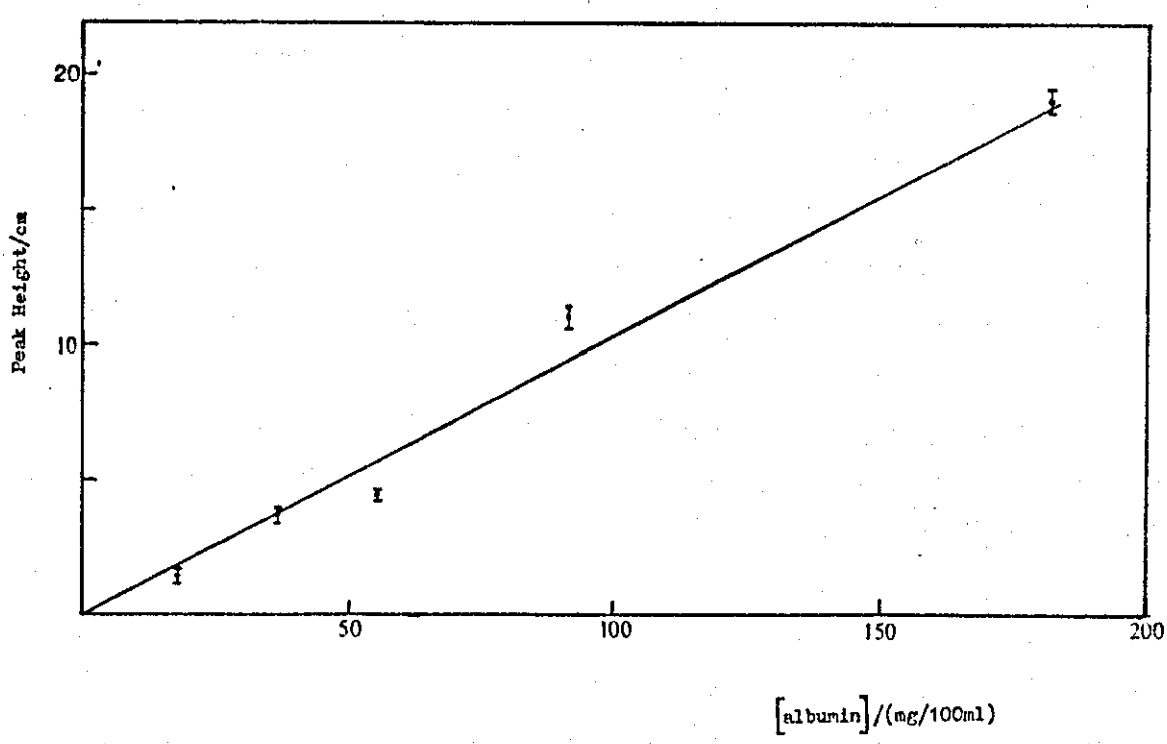

Figure 8.5 Standard graph for albumin determination using ANS binding procedure.

Using diluted standard serum the coefficient of variation was was 2.3 % at 18 mg dl^{-1} and 2.1 % at 180 mg dl^{-1}. The coefficients of variation using diluted test sera were somewhat higher, averaging 4.5 %. The albumin concentrations of four test sera were determined, the results being given in Table 8.1.

8.3.2 Energy Transfer Immunoassay

Initial attempts to automate this assay using conventional flow injection procedures gave unsatisfactory results. Not only was the consumption of labelled albumin and labelled antibody unacceptably high, but the high background fluorescence of the carrier stream (a mixture of the labelled species) severely restricted the precision and the sensitivity of the assay. These objections were overcome using the stopped-flow, merging zone method. The injected reagent was a solution containing 2.4 x 10^{-7}M fluorescein-labelled albumin (fluorescein:albumin ratio, 1.4:1) and 4 x 10^{-6}M rhodamine-labelled antibody (rhodamine:antibody ratio, 16.5:1). Previous studies (Chapter 3) had demonstrated that this combination of lightly-labelled antigen and heavily-labelled antibody, with a large excess of the latter, produced optimum results when the assay was performed without automation. Diluted serum samples were injected at S (Figure 8.1). The flow rate used was 3.8 ml min^{-1}, giving a merge time of 3.2 s, a non-stop residual time of 16 s and sample and reagent volumes of 36 µl. A stopped-flow incubation time of 6 minutes was used: during this period, the three-way valve, T, was used to divert the carrier stream through the bypass, and the recorder chart was switched off. The recorder was re-started as T was turned to re-start the flow of the sample.

In the analysis of unlabelled (sample) albumin

concentrations, the fluorescence of the fluorescein label was determined (the fluorescence of the rhodamine label was too feeble in practice to provide a sensitive assay - see Chapter 3). Fluorescence intensities were related to the emission from a 2.4×10^{-7}M labelled albumin solution, arbitrarily assigned a value of 100. The results obtained in this way were satisfactory. Readily-measurable fluorescence peaks were obtained (Figure 8.6): the coefficient of variation of the results using diluted standard sera was 2.5 % at an albumin concentration of 5×10^{-5}M, and 2.3 % at a concentration of 10^{-7}M. The standard curve obtained (Figure 8.7) showed that albumin concentrations of 10^{-7}M and below could be determined. The values obtained from four test sera are shown in Table 8.1, along with the results of the ANS binding and electroimmunoassay techniques: agreement between the three methods was generally good.

8.4 Discussion

The results demonstrate clearly the suitability of stopped-flow merging zone flow injection analysis for the automation of homogeneous immunoassays: they also confirm the usefulness and sensitivity of a fluorimeter as a detector for flow injection methods. In the present assay the flow injection approach had several substantial advantages over the static analysis described in Chapter 3. These included smaller samples, a considerable economy in labelled reagents and a shorter incubation period. The incubation period of six minutes was perfectly adequate in the albumin assay, but there would be no technical objections to the use of longer periods if required in other assays. The use of a flow cell of narrow bore precluded any interference by inner filter effects, and the background

fluorescence of the diluted serum was negligible compared with the fluorescein emission. The detection limit of the assay was more than adequate for the determination of albumin in diluted serum samples and in urine (Cf. Woo et al, 1978). Improved detection limits could almost certainly have been achieved by the use of a spectrofluorimeter, rather than a filter fluorimeter, as the detector: detailed studies of the fluorescein-rhodamine energy-transfer pair show that larger quenching and enhancement effects occur at narrow spectral bandwidths (Chapter 3).

The relatively low sampling rate available in this stopped-flow assay can be improved upon by modifications to the manifolds (Ruzicka and Hansen, 1978) and in any event provides a faster throughput of samples than the manual method. Two further small problems were noted, the first being the need to carry out frequent checks (by injecting air-bubbles) to ensure synchronous merging of the sample and reagent: very small adjustments in the flow rate could be achieved by means of a screw clip attached to a short length of thin-walled tubing inserted in one of the channels (eg. in tube "a" in Figure 8.1). The second problem was the tendency of air bubbles to form in the carrier streams: this effect could be avoided by equilibrating the samples and the reagents at 37 C before beginning an analysis.

A wide variety of other homogeneous immunoassays are suitable for automation using flow injection analysis, including fluorescence quenching, enhancement and polarization assays. The present energy transfer approach can also be applied to the analysis of many other macromolecules and low molecular-weight species such drugs and hormones. Flow injection techniques will increasingly used in this branch of clinical and biochemical analysis.

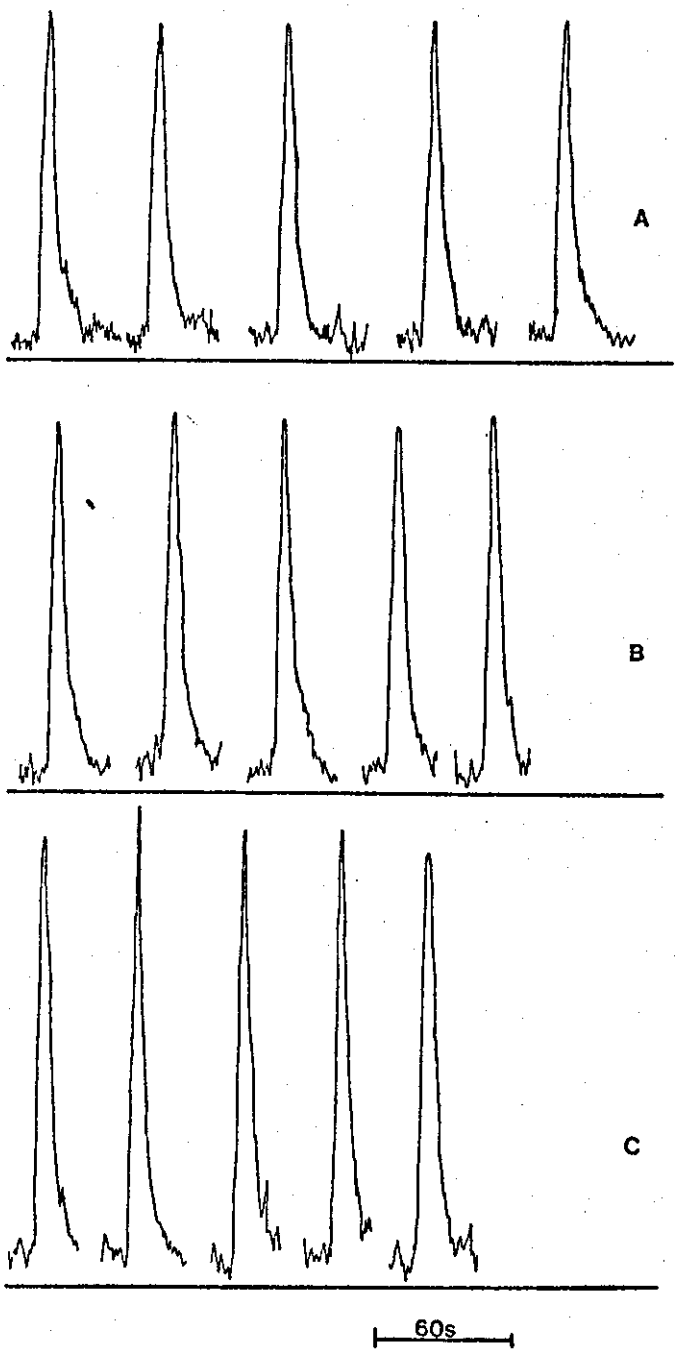

Figure 8.6 Examples of recorder responses obtained during the energy transfer immunoassay of serum albumin in diluted standard serum. The albumin concentrations were: (A) 1.1×10^{-6}M; (B) 5.5×10^{-6}M; (C) 1.1×10^{-5}M.

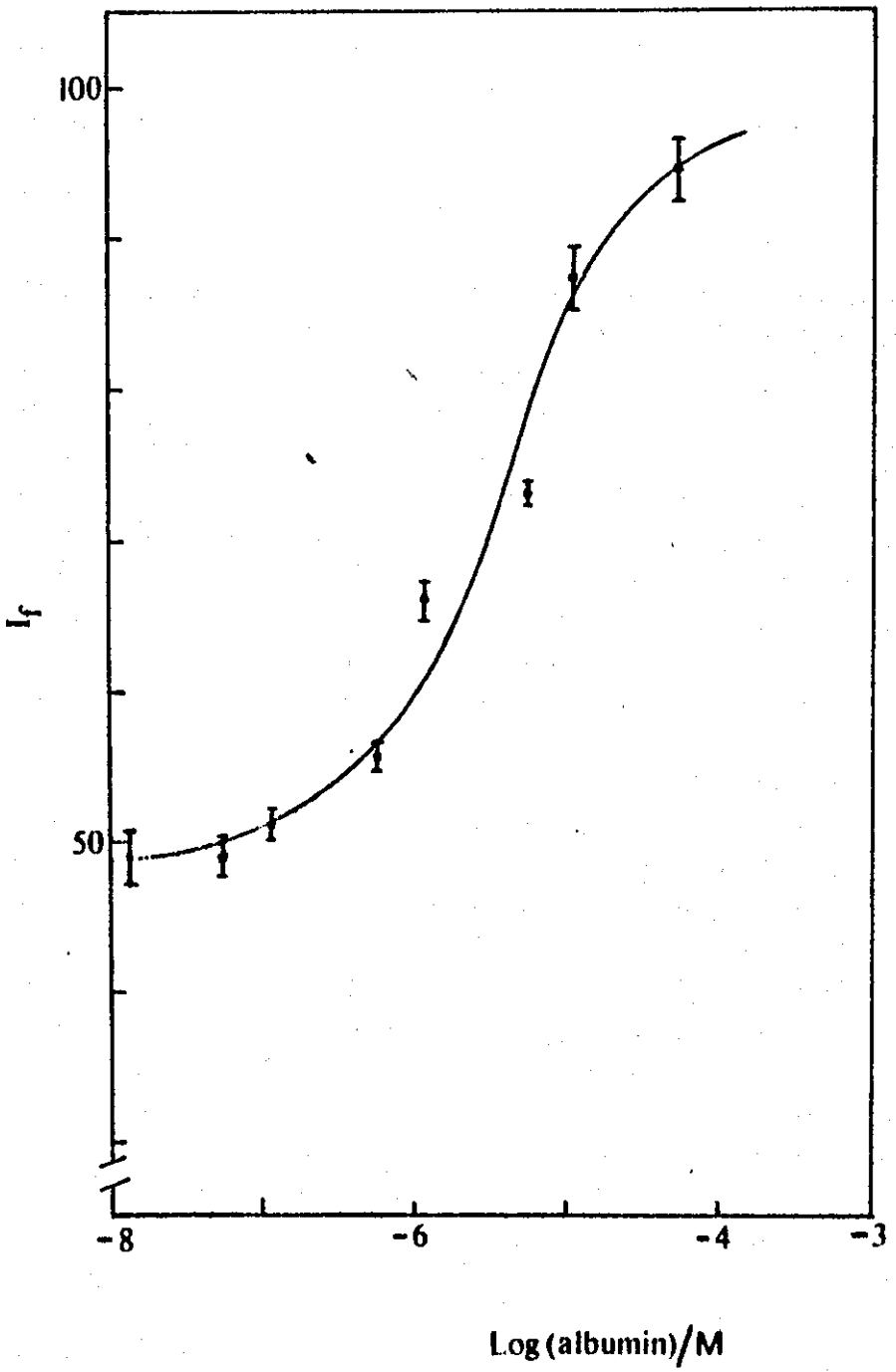

Figure 8.7

Standard curve for the energy transfer immunoassay for serum albumin. Error bars represent standard deviations.

Table 8.1

Determination of albumin in serum samples

Sample	ANS-binding FIA* method	Energy-transfer immunoassay - FIA* method	Electroimmunoassay method
1	(100) 3700 ± 180 [10]	(100) 3860 ± 200 [8]	(150) 3800 ± 140 [5]
2	(100) 3600 ± 170 [10]	(500) 3320 ± 170 [8]	(150) 3680 ± 80 [8]
3	(40) 4380 ± 150 [10]	(500) 4810 ± 150 [8]	(150) 4500 ± 140 [7]
4	(75) 3675 ± 200 [8]	(100) 3460 ± 220 [4]	(150) 3080 ± 80 [5]

All concentrations in $mg.dl.^{-1}$

FIA* : Flow injection analysis

Figures in round brackets are the factors by which serum samples were diluted before study; figures in square brackets are numbers of measurements used in calculating standard deviations.

CHAPTER 9 CONCLUSION

In contrast to other fluorescence immunoassay (FIA) methods, energy transfer immunoassay (ETIA) requires the simultaneous use of two different types of fluorescent labels, the donor and acceptor, attached respectively to the antigen and antibody in the direct assay method, or to separate portions of the antibody in the indirect (or sandwich) assay method. The direct and indirect assays that have been developed and described in this thesis demonstrate clearly that ETIA is a general immunochemical method applicable to the analysis of both haptens (eg. nortriptyline) and macro-molecular species (eg. IgG, IgA, transferrin, etc). The principal merits of the energy transfer assay technique can be summarized as follows.

(a) Sensitivity

In the assays developed in this work, detection limits in the sub-nanomolar range were attainable. For maximum assay sensitivity, it has been found necessary to optimize a given assay system with respect to a number of experimental variables including the degrees of labelling of antigen and antibody, the total concentration and the relative composition of the labelled antibody and antigen in the assay, the fluorimeter spectral bandwidth, etc.

(b) Specificity

The simultaneous observation of the quenching and enhancement effects of the energy transfer phenomenon permits specific antigen-antibody combination to be distinguished from spurious environmental effects on the fluorescence intensity.

(c) Wide applicability

The same donor-acceptor labels may be applied to

the analysis of a wide range of materials. The instrumentation required in this case is a relatively simple fluorimeter with filters providing fixed exciting and emitting wavelengths.

(d) Speed of analysis

Relatively short incubation times are required (cf. immunoprecipitation methods). An incubation time of one hour at a temperature of 35 C had been found to be adequate for the analyses performed in the present study. Furthermore, the assay is homogeneous and hence can dispense with the troublesome separation step required for the heterogeneous assays. This has permitted the successful application of the flow injection analysis principles to the automation of an energy transfer immunoassay for albumin (Chapter 8). With the trend towards increasing use of microprocessor-based technology in analytical instrumentation and the recent introduction of image detection in fluorimetry (Talmi et al, 1978), it seems likely that the next few years will witness rapid developments in the field of fluorescence immunoassay automation which will in turn contribute to increasing the speed of analysis by the ETIA method.

(e) Stability of labelled reagents

The labelled immune reactants were found to be generally stable both under measurement conditions and on storage. In the case of fluorescamine-labelled antigens, the preparation of the conjugates can be easily performed immediately prior to the analysis.

All the applications of ETIA so far described in this thesis as well as in the published works have utilized only the

fluorescein-rhodamine and the fluorescamine-fluorescein label pairs. These pairs of labels are by no means ideal or adequate for the wide applicability of which the ETIA technique is capable. There is therefore a need to develop new fluorescent labels specially designed for specific applications in energy transfer assays.

A principal requirement of the ETIA technique is the need to use purified homogeneous antigens and antibody with a high degree of specificity in order to avoid the excessive fluorescence background due to the non-specific fluorescence-labelled proteins. Recent advances in in vitro mouse myeloma cell fusion techniques have made possible the increasing commercial availability of monoclonal antibodies in gram quantities (Koprowski et al, 1977). It can be expected that the use of these highly enriched immune reactants in the assay system will further increase the sensitivity and specificity of the ETIA technique.

Attempts to develop a solid-phase ETIA method have so far not yielded satisfactory results primarily because the fluorescence signal from the immobilized fluorescent conjugates was too feeble to permit its application in an assay. Much work is therefore needed to overcome this problem before a number of possible solid-phase ETIA's can be developed based on the reaction schemes outlined on page 175.

Detailed studies of the fluram and the analogous MDPF enhancement phenomena have revealed that such effects might also be encountered with many other fluorescamine- and MDPF- labelled antigens. A more thorough theoretical understanding of the mechanisms underlying these phenomena is required in order to exploit these effects for the development of a generally-applicable fluorescence enhancement assay.

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