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quantitative immunofluorescence microscopy

methods and applications

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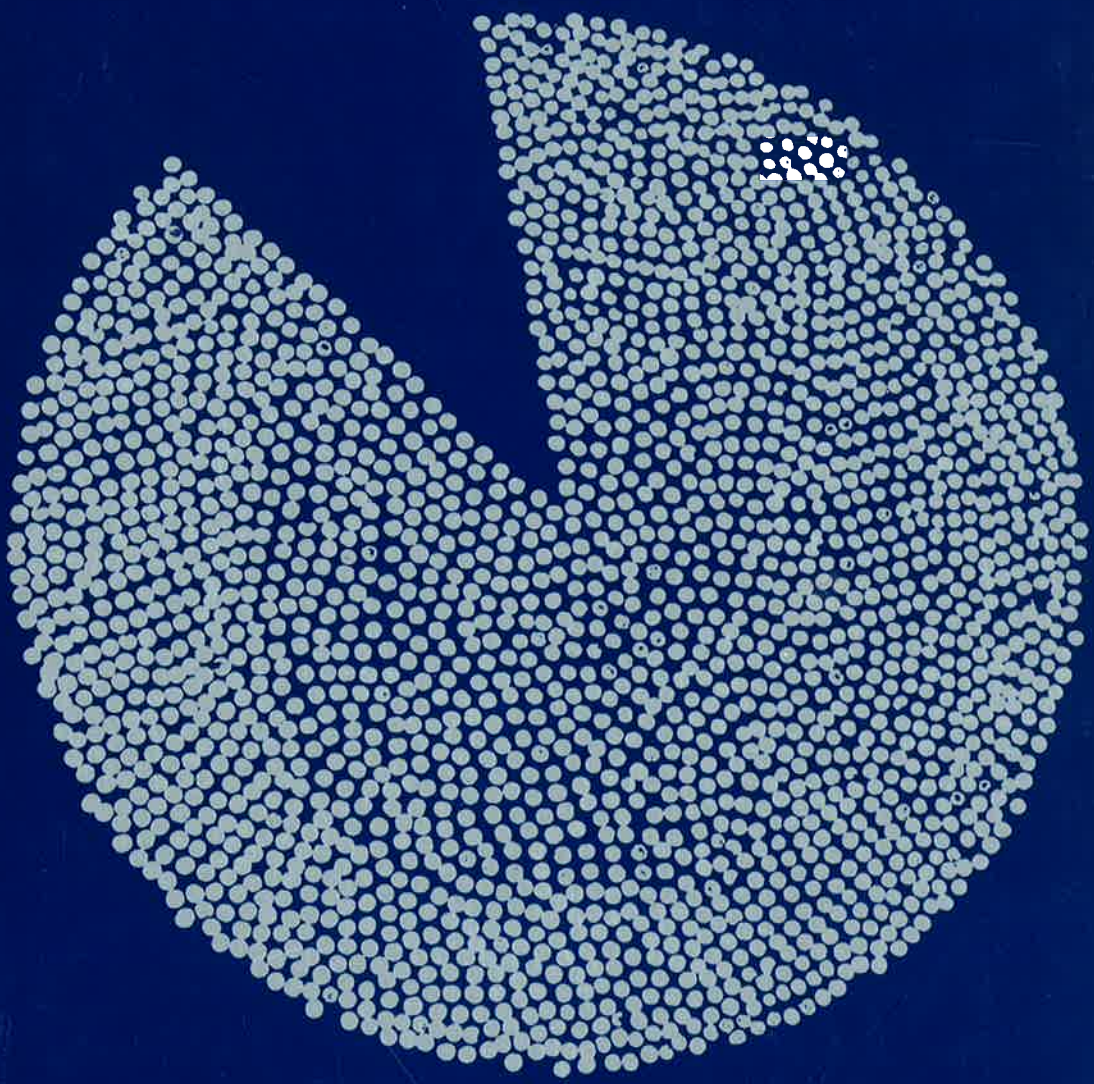

j.j.haaijman

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QUANTITATIVE IMMUNOFLUORESCENCE MICROSCOPY

METHODS AND APPLICATIONS

The work described in this thesis was performed in the Institute for Experimental Gerontology (Director: Prof. Dr. C. F. Hollander), Organization for Health Research TNO, Rijswijk (Z.H.), The Netherlands.

STELLINGEN

I

Slechts een fluorescentiestandaard bestaande uit eenheden van mikroskopische afmetingen is geschikt voor het evalueren van de diverse komponenten van het fluorescentiemikroskoop.

II

Het uitdrukken van fluorescentie intensiteit in candela per m^2, voorbij gaande aan een deugdelijke kalibratie van het gebruikte fluorescentiemeetmikroskoop, leidt slechts tot een schijnbare standaardisatie.

III

Het Sepharose bollen systeem waarin gezuiverde antigenen gekoppeld worden aan Sepharose bollen en dienen als substraat voor immunofluorescentie reakties, maakt het mogelijk de diverse specificiteiten die een antiserum kan hebben te inventariseren, maar biedt alsnog niet de mogelijkheid een algemene uitspraak te doen over de kwaliteit van antisera voor de toepassing van deze antisera op biologisch materiaal.

IV

Fluorescentie mag in een groot aantal klinisch-chemische bepalingen als een volwaardig alternatief voor radioaktiviteit beschouwd worden.

V

Het toepassen van 'zoom'optica in kollektor systemen van lampenhuizen te gebruiken in de fluorescentiemikroskopie, verdient nadrukkelijke aanbeveling.

VI

De plaques van Peyer in de muis bevatten een populatie van antigeen gevoelige cellen die niet *in situ* kunnen differentiëren in antwoord op een antigene stimulus. Het mag aannemelijk worden geacht dat eenmaal gestimuleerde lymfocyten uit de plaques van Peyer migreren naar milt of beenmerg alwaar zij een stimulus tot verdere differentiatie ontvangen. Cellen in een differentiatiestadium tussen B-lymfocyt en plasmacel migreren waarschijnlijk terug naar of de plaques van Peyer of de lamina propria van de darm.

VII

In het netwerkmodel voor het immuunsysteem van vertebrata voorgesteld door Jerne, worden geen randvoorwaarden dusdanig omschreven dat dit model ooit stabiel en eindig zou kunnen zijn.

VIII

De morfologie en kinetiek van transplantabele, door minerale oliën geïnduceerde, plasmocytomen bij BALB/c muizen zijn dermate verschillend van die van het humane multipele myeloom dat zij niet als dier-experimenteel model mogen dienen voor het ontwikkelen van zowel chemo- als radiotherapie van dit myeloom.

IX

Pneumatische komponenten verdienen meer aandacht dan zij tot nu toe ontvangen hebben bij het automatiseren van klinisch-chemische laboratoriumapparatuur.

X

In het kader van de wet op TNO van 1930 zou de stichting van een instituut met als opdracht het ontwikkelen, testen en evalueren van klinisch-chemische apparatuur ten behoeve van instellingen, werkzaam op het gebied van de volksgezondheid, van harte toe te juichen zijn. Tevens zou dit instituut een permanente samenwerking tussen de nijverheids- en gezondheidsorganisatie binnen TNO kunnen waarborgen.

XI

Het verlies van de mogelijkheid in aanmerking te komen voor uitkeringen boven het toegekende pensioen krachtens artikel 14 van het pensioenreglement van de Stichting Centraal Pensioenfonds TNO bij het verlaten van de TNO organisatie vóór het bereiken van de pensioengerechtigde leeftijd, moet gezien worden als een direkte rem op de zo nuttig geachte externe mobiliteit van de individuele wetenschappelijke onderzoeker.

XII

De opbouw van een plasmacellulaire reaktie in het beenmerg van muizen staat onder invloed van een thymus afhankelijke, cellulaire of humorale, faktor.

XIII

De oorzaak van de verminderde immunologische kapaciteit van oude muizen, in die zin dat zij minder antilichamen maken dan jongere dieren, in antwoord op een experimenteel toegediende antigene stimulus, moet in eerste instantie gezocht worden in een disfunktioneren van het regulatoire mechanisme tussen T-cel systeem en B-cel systeem en niet in een verminderde kapaciteit van het B-cel systeem als zodanig.

XIV

Het zien van een reaktie op een maatschappelijke of justitiële onrechtvaardigheid als een bevestiging van de macht van de instelling of persoon die deze onrechtvaardigheid beging of veroorzaakte, kan slechts leiden tot een existentieel minimum.

J. J. Haaijman

februari 1977

QUANTITATIVE IMMUNOFLUORESCENCE MICROSCOPY

METHODS AND APPLICATIONS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE
AAN DE RIJKSUNIVERSITEIT TE LEIDEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS DR. D. J. KUENEN,
HOOGLERAAR IN DE FACULTEIT DER WISKUNDE EN NATUURWETENSCHAPPEN,
VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
TE VERDEDIGEN OP
WOENSDAG 16 FEBRUARI 1977 TE KLOKKE 15.15 UUR

DOOR

JOOST JOHANNES HAAIJMAN
geboren te Eindhoven in 1947

W. D. MEINEMA B.V. – DELFT – 1977

Promotor:
Dr. W. Hijmans

Co-referenten:
Prof. Dr. C. F. Hollander
Dr. J. S. Ploem

Contents

CHAPTER I

Immunofluorescence

Contents:

A. INTRODUCTION

This thesis is devoted to the technique of immunofluorescence (IF) microscopy. This technique was introduced by A. H. Coons and his colleagues in the early forties (Coons, Creech and Jones, 1941). These authors were the first to couple a fluorescent dye to an antibody in order to localize its respective antigen in a microscopical preparation. Since its introduction, IF microscopy has developed into a widely applied diagnostic tool. However, the method entails a large number of variables. This has led to a wide divergence in the success with which it has been applied in the past. It is the purpose of the first part of this thesis to present quantitative data on the two major causes of variation in IF results – namely, the fluorescent reagents and the fluorescence microscope.

In the course of our studies, a number of applications has been worked out using quantitative immunofluorescence techniques. Results of these applications in the fields of gerontology and virology are presented in the second part of this thesis.

The two main elements of IF microscopy will be introduced in this chapter: a) the fluorescent reagents; and b) the fluorescence microscope. In the section on fluorescent reagents, a brief description of the physical basis of fluorescence phenomena will first be given. This will be followed by a review of the different fluorochromes used in IF and the critical role of antisera will be mentioned.

In this introduction, the developments in fluorescence microscope technology will be presented from the point of view of optimization of the image contrast, which depends on the separation of the excitation and emission light. Therefore, two aspects will be dealt with in this chapter: 1) different illumination systems currently in use in fluorescence microscopes; and 2) different types of filters. The last part of this chapter serves as an introduction to the other chapters of this thesis.

B. THE FLUORESCENT REAGENTS

1. *The physical basis of fluorescence phenomena*

Light is a form of electromagnetic radiation which is characterized by a frequency and a wavelength. The energy of light may be described in the form of energy packages, the photons. The energy of individual photons is inversely related to the wavelength of the light.

If light passes through matter, two things may happen: a) there occurs no interaction between the light and matter; or b) the energy of (some of) the photons is absorbed. Upon absorption, the energy of the photons is transferred to electrons of molecules which assume a higher energy level. If this higher energy level is within the same energy band, the energy is dissipated within 10^{-15} seconds. This phenomenon is called Raleigh scattering. With some molecules, photons of certain energies are capable of exciting electrons to levels which are called metastable. After absorption

of a photon by such a molecule, a portion of the energy is liberated within 10^{-15} sec in the form of thermal energy by a return of the electron to the lowest vibrational level of the excited electronic state. The remainder of the energy is then dissipated after typically a few nanoseconds in the form of a light quantum. This process is called fluorescence. If there is a longer time delay (in the order of milliseconds or seconds) between absorption and emission of photons, the process is termed phosphorescence. All photon energies capable of causing fluorescence make up the excitation spectrum. Under normal conditions, the energy of the emitted photons is less than the energy of the exciting photons. Therefore, the emission spectrum is shifted to longer wavelengths compared to the excitation spectrum (Stoke's law; Pesce, Rosén and Pasby, 1971).

A large number of organic molecules show fluorescence phenomena. The relationship between fluorescence properties and molecular structure has been established for only limited classes of compounds. The principles of fluorescence phenomena are extensively treated by Udenfriend (1962, 1969).

2. *Fluorochromes in IF microscopy*

The characteristics required of a fluorochrome for use in IF microscopy are listed in Table I-1. In the first report on IF, Coons, Creech and Jones (1941) used β-anthracene as the fluorescent dye. This dye exhibits a blue fluorescence upon excitation with near ultraviolet (UV) light. This label has a number of disadvantages: a) the human eye is relatively insensitive to blue light (Stiles, 1959; Padmos and van Norren, 1971); b) most tissues already contain natural molecular species which will fluoresce if excited with near UV-light, this autofluorescence may considerably reduce the image contrast; and c) β-anthracene proved to be very liable to photodecomposition. For these reasons, Coons, Creech, Jones and Berliner (1942) introduced fluorescein as label for IF. Fluorescein is maximally excited with blue light and emits green to green-yellow light (Fig. I-1). The fluorescein was originally coupled to protein via the isocyanate intermediate. Fluorescein isocyanate, however, was rather susceptible to hydrolysis and could not be stored for a prolonged period of time. An important step forward in IF was the introduction by Riggs, Seiwald, Burckhalter, Downs and Metcalf (1958) of fluorescein isothiocyanate (FITC), which was obtained through the

TABLE I-1. REQUIREMENTS OF A FLUORESCENT DYE TO BE USED IN IF MICROSCOPY

1. The dye should interfere minimally with the antigen-antibody interaction.
2. The emission maximum of the dye should be within the spectral sensitivity of the human eye.
3. The maximum of the excitation spectrum should preferably be above 360 nm.
4. The excitation and emission spectra should be far enough apart on the wavelength scale that they can be separated from each other.
5. Photodecomposition during excitation should be minimal.
6. The dye should be stable upon storage and available in a highly purified form.

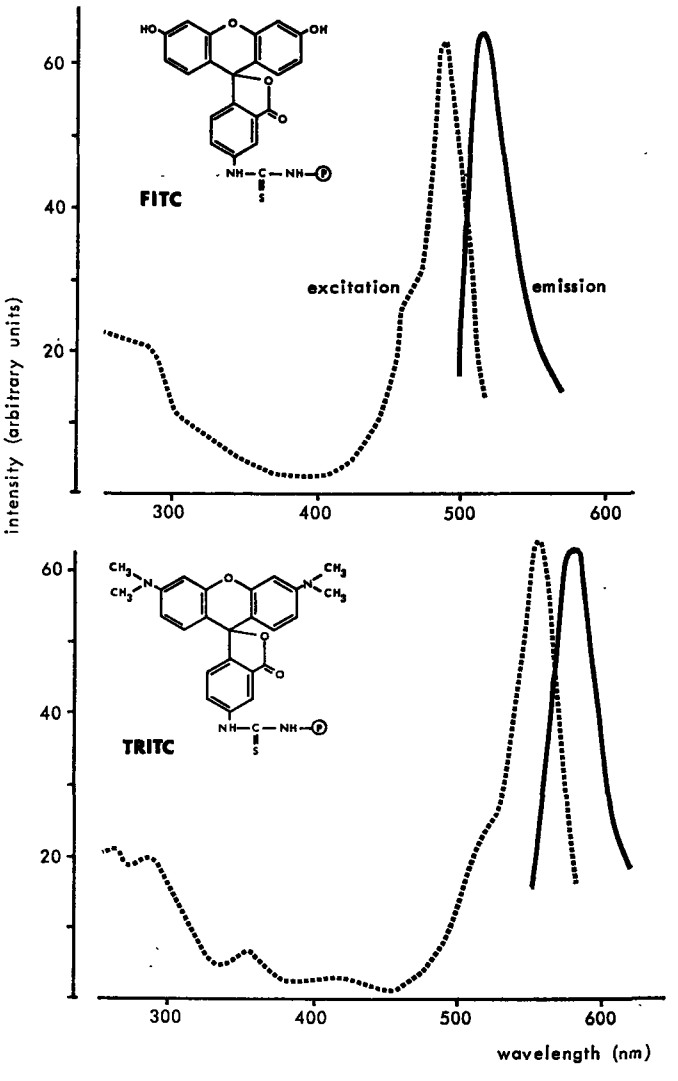

Figure I-1. Structural formula, excitation and emission spectra (redrawn from Hansen, 1964) of the two most commonly used fluorochromes in immunofluorescence microscopy: fluorescein isothiocyanate (FITC; molecular weight 379.39) and tetramethyl rhodamine isothiocyanate (TRITC; molecular weight 431.13). In the formula, P stands for protein.

reaction of aminofluorescein with thiophosgene. The compound can be stored at −20 °C for a considerable period of time. Fluorescein isothiocyanate reacts under weakly alkaline conditions with the amino groups of the lysine residues in proteins. Excess fluorochrome may be removed by either dialysis or with molecular sieves such as Sephadex.

The IF technique has been applied in almost every area of immunology. During its development, the need for more than one fluorochrome was felt. A large number of dyes have been tested (see, for instance, Chadwick, McEntegart and Nairn, 1958) in the past to function as a second fluorochrome in addition to fluorescein. The only two which have found wide application are lissamine rhodamine B (RB 200), introduced by Chadwick, McEntegart and Nairn (1958), and tetramethyl rhodamine which was first reported by Hiramoto, Engel and Pressman (1958) in the form of the isocyanate and by Riggs et al. (1958) in the form of the isothiocyanate. Although RB 200 has the advantage that it is far less expensive than tetramethyl rhodamine isothiocyanate (TRITC), its use in IF has been almost discontinued because of its greater heterogeneity and the necessity of preparing the sulphonyl chloride before coupling. The excitation and emission spectra of TRITC are presented in the lower part of Fig. I-1. TRITC is maximally excited with green light and emits red light.

With the advent of newer microscope illumination systems (see next section), the application of even more than two fluorochromes in the same preparation has become feasible and useful. The emission maximum of the new fluorochrome has to lie in the blue region of the spectrum in order to permit its observation independent of both fluorescein and rhodamine. Up to now, two compounds which may fulfill these requirements have been suggested – namely, stilbene isothiocyanate (Sinsheimer, Stewart and Burckhalter, 1968; Rothbarth, Olthof and Mul, 1975) and 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF; Weigele, DeBernardo, Leimgruber, Cleeland and Grunberg, 1973). No application of three-colour IF microscopy has been reported, however.

3. *Antisera*

Antisera are the pivots on which the whole technique of IF hinges. Antisera are materials of biological origin with inherent variation. A considerable part of this thesis is devoted to methods for the evaluation of the quality of antisera to be applied in IF microscopy.

A good antiserum will react only with its respective antigen. It is this specificity which is one of the assets of IF methods and which explains the explosive growth in the number of IF applications in research and clinical diagnoses.

C. THE FLUORESCENCE MICROSCOPE

Because the intensity of the emission light is always very low compared to that of the excitation light in IF microscopy, they should be separated in order to prevent flooding of the former by the latter. Two aspects of fluorescence microscopes interplay in the realization of this requirement: 1) the optical geometry of the illumination system; and 2) the optical filters in both excitation and emission pathways.

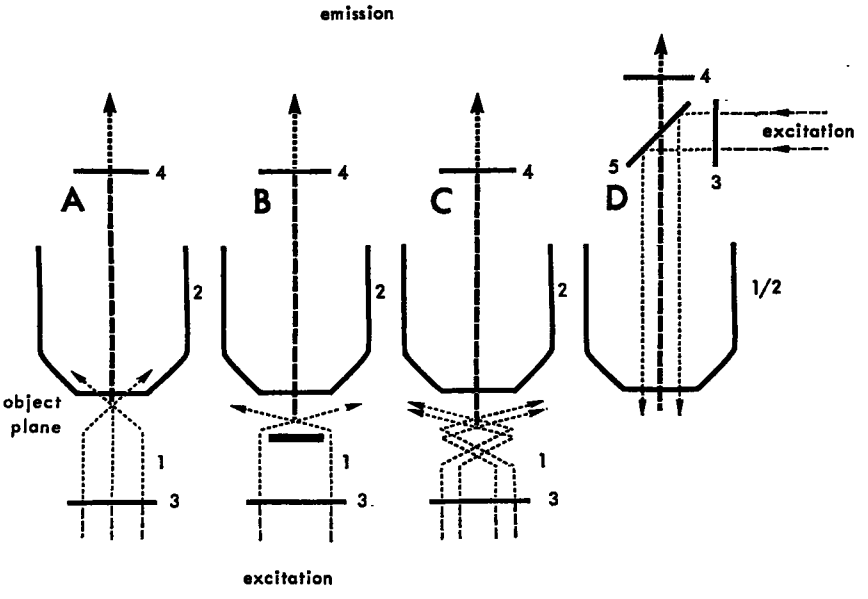

Figure I-2. Schematic representation of different illumination systems in immunofluorescence microscopes.
A. Brightfield diaillumination.
B. Darkfield diaillumination with central stop in the condensor.
C. Darkfield diaillumination with a Tiyoda condensor having a toric lens.
D. Epiillumination. The objective functions as a condensor for excitation light and as an objective for emission light.
1 = condensor; 2 = objective; 3 = excitation filters; 4 = emission filters; 5 = interference dividing plate.

1. *Illumination systems*

The different illumination systems currently applied in fluorescence microscopes are schematically shown in Fig. I-2. In diaillumination, the light of the excitation light source is directed towards the eye of the observer. Filters which have an extremely low transmittance for wavelengths longer than the excitation maximum of the fluorescent dyes are needed to prevent unwanted excitation light reaching the eye. In order to increase the efficiency of diaillumination, special types of darkfield condensors have been developed. Darkfield condensors concentrate the excitation light on the specimen at such an oblique angle that this light does not enter the objective. This condition may be achieved with a central stop in the condensor. With the newer types of filters (see below), high aperture darkfield condensors may be used efficiently with high power objectives. High aperture Tiyoda condensors with a toric lens have the advantage that they concentrate the excitation light effectively on a relatively large area of the preparation and are therefore the most efficient darkfield condensors for diaillumination using low to moderate power objectives at the moment.

In incident- or epiillumination, the excitation light is directed away from the observer's eye. Brumberg (1955, 1959) was the first to place a mirror in the microscope tube for incident near-UV illumination. The mirror reflects the excitation light into the back entrance pupil of the objective, which then functions as a condensor. Ploem (1967) introduced into IF microscopy the use of several dichroic mirrors in a vertical illuminator, permitting epiillumination throughout the whole visible wavelength range. Special dichroic mirrors (also called interference dividing plates) which reflect certain wavelengths and transmit others were used.

The different advantages and disadvantages of dia- versus epiillumination are amply discussed by Pearse (1972). It may be added here that, with diaillumination, rapid switching from one filter combination to the other is hardly possible, a condition which is essential for the application of multiple wavelength IF methodology.

For quantitation in IF microscopy, epiillumination is almost indispensable because a) the uncertainty of the alignment and focusing of the condensor is eliminated; b) only the part of the preparation to be measured is illuminated by the excitation beam and adjacent fields do not suffer from photodecomposition; and c) low power transmitted light using either phase or amplitude microscopy is always available to locate the objects to be measured.

2. *Filters*

Optical filters in excitation and emission pathways of fluorescence microscopes are needed for three reasons: a) prevention of unwanted excitation light reaching the eye and flooding the fluorescence emission; b) selection of optimal wavelengths for excitation; these filters are called the excitation or primary filters; and c) selection of emission wavelengths; these filters are alternatively called the emission, secondary or barrier filters. In Fig. I-2, the location of the different filters in the optical pathways is indicated. Especially in two or more wavelength IF microscopy, judicious selection of the optimal filters is obligatory.

Different types of optical filters are available. Because of the often confusing terminology applied to these different filters, a brief survey is presented here.

a) Coloured glasses. These filters consist of glass mixed with different inorganic salts and are manufactured in two forms:
   1. Filters transmitting certain wavelength bands. These filters are mostly known under an abbreviation indicating their colour properties, followed by a number which represents the factory code. Much in use are the red suppressing BG 38 (German: Blauglas) and the near ultraviolet transmitting UG 1 (German: Ultraviolettglas). The transmission bands of these filters are mostly broad and they are often used in combination with the interference filters to be discussed below.
   2. Filters which transmit wavelengths above a certain wavelength. These filters are alternatively termed:

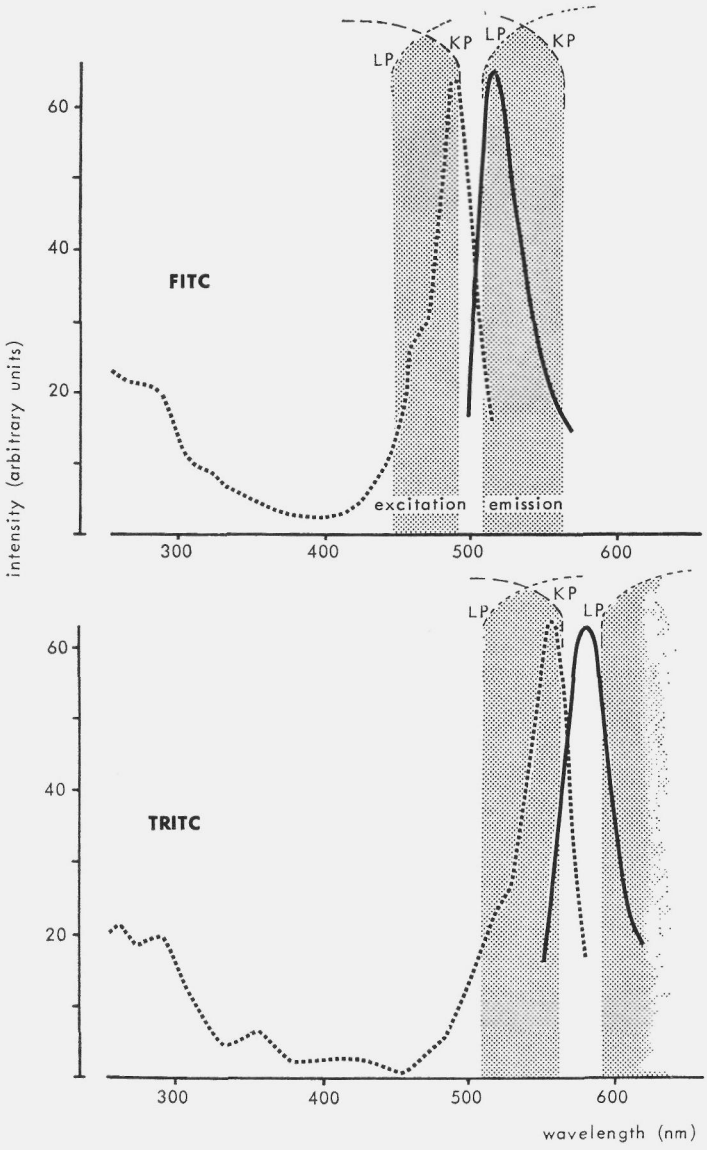

Figure I-3. Schematic representation of the method of window filtering in immunofluorescence microscopy. A wavelength window is constructed with a combination of a highpass filter (type of filter: LP) and a lowpass filter (type of filter: KP). The window for the FITC emission is chosen so as to exclude the emission of TRITC. Only a highpass filter is necessary for TRITC. The excitation and emission filter combinations are superimposed over the excitation (----) and emission (—) spectra of FITC and TRITC.

- K filters (German: Kantenfilter), followed by a number indicating the wavelength in nm of 50% transmission of 3 mm thick filters (examples are: K 510 and K 580);
- GG, OG and RG filters (German for: respectively, Gelbglas, Orangeglas and Rotglas), followed by the 50% transmittance value. The names indicate the colour properties of the filters. Examples of these filters are the much used GG 455, GG 475, OG 515 and RG 610;
- LP (long pass) filters. An example is the LP 520, in which the number stands for the 50% transmission wavelength. The LP 520 functions in the same way as the OG 515 and the K 510, in that they will all transmit most wavelengths above about 525 nm.

b) Interference filters. These filters consist of a glass base on which multilayers of dielectric materials are vacuum deposited. The interference filters may be divided into two classes:
   1. Narrow band interference filters. These filters will transmit wavelength bands typically 15–20 nm in width at 50% transmission. The peak transmission of these filters is in the order of 40-60%. Narrow band filters are known under various names: S, SAL, AL and TAL, followed by the peak transmission wavelength. For example, the S 546 or TAL 546 may be used for the excitation of TRITC and the SAL 525 for the selection of FITC emission.
   2. Interference filters which will transmit wavelengths shorter than a given wavelength. Mostly these filters are termed KP (German: Kurzpass) filters. The abbreviation SP for short pass is sometimes used. The transmittance values of these filters can be as high as 90%.

The excitation energy is one of the factors which influences the intensity of the fluorescence emission. In order to reach the highest sensitivity in IF microscopy, it is therefore indicated to use as much as possible of the energy of the light source. Limits are imposed by the imperfections of the filters.

In double wavelength IF methods, where two fluorescent dyes are present in the same preparation, special measures are necessary to visualize both fluorochromes selectively. Combinations of LP and KP filters, constituting a wavelength window are the best choice for this purpose at present. In Fig. I-3, the method of selective filtering is shown for FITC and TRITC. Stylized filter characteristics have been superimposed on the excitation and emission spectra of these dyes.

Given a set of filters which select only those wavelengths from a light source within the excitation spectrum of a dye and emission filters which block the excitation light effectively, the fluorescence may be observed against a dark background. This contrast, which could be infinite in principle, enables the detection of smaller numbers of dye molecules than is possible with absorption microscopy. The restriction should be made that the molar absorption coefficient of the dyes is comparatively low and the

quantum efficiency of the fluorescent dye is high. These conditions are usually met in IF microscopy. The sensitivity of the IF technique is the second main reason, next to its specificity, why it has attracted so much attention.

D. INTRODUCTION TO THE DIFFERENT CHAPTERS OF THIS THESIS

From the foregoing, it is evident that the eventual results obtained with IF microscopy will depend on the use of optimal equipment and optimal reagents. In the first part of this thesis quantitative data on the influence of different components of fluorescence microscopes on fluorescence emission are presented. Methods for evaluating the quality of antisera and fluorescent conjugates to be used in IF microscopy are subsequently discussed. The second part concerns the application of quantitative IF methods to the determination of antibodies and antigens. A central role in these applications is played by the Sepharose bead immunofluorescence assay (SBIA) introduced by van Dalen, Knapp and Ploem (1973). The principle of this method is the covalent binding of, e.g., antigens to Sepharose beads. These beads may be used as a substrate in IF methods. The reaction of antisera with the Sepharose beads is quantitated with microfluorometry. A number of modifications and extensions of the original method of van Dalen, Knapp and Ploem (1973) are described.

The scope of the different chapters of this thesis may be summarized as follows:

Chapter II. IF microscopy is still often treated as an art rather than as a defined scientific method. The influence of different components of fluorescence microscopes on fluorescence intensity is presented in this chapter. This has been done on the basis of a newly developed fluorescent standard which consists of FITC or TRITC bound to aminoethyl-Sephadex beads (Haaijman and van Dalen, 1974). This standard was used to measure the influence of objectives, eyepieces, light sources and filter combinations on fluorescence intensity. A discussion on standardization in microfluorometry has been incorporated into this chapter.

Chapter III. A fluorescence standard for immunofluorometry is required to behave in the same way to changes in the physicochemical environment as do fluorochromes bound to tissues or cells. Experiments to test whether the fluorescent aminoethyl-Sephadex beads fulfill this requirement are presented in this chapter. Two major characteristics of fluorochromes have been studied, namely their pH-dependent fluorescence and their liability to photodecomposition.

Chapter IV. The quality of the reagents in IF microscopy is of paramount importance. Testing this quality and expressing it in quantitative terms is the subject of this chapter. After reviewing the current trends in reagent standardization, the test systems employing antigens bound to or trapped in artificial substrates are introduced. The Sepharose bead immunofluorescence assay (SBIA) is described in detail. The influence of a number of experimental conditions on the results obtained with the SBIA,

as well as the influence of the chemical composition of the antisera or conjugates, has been studied. Finally, what quantitative measures from the SBIA could be used to predict the behaviour of conjugates in biological systems is discussed.

Chapter V. Several single and multilayer techniques are in use in IF microscopy. The direct and indirect technique are most widely known. In the direct technique, the component of interest is visualized with a fluorescent antiserum. This technique requires only one incubation. In the indirect technique, the preparation is first incubated with unlabelled antiserum which is then detected with fluorescent anti-immunoglobulin antiserum. In this chapter, the prevailing opinion that the direct technique is less sensitive but more specific than the indirect technique is tested. The multilayer techniques which form the basis of the SBIA methods described in chapter VIII are also introduced in this chapter. Finally, the interaction between different reagents directed against different parts of the same molecule is studied. These experiments were necessary to evaluate the validity of double wavelength techniques.

Chapter VI. For the immunoassays to be described, a reliable and rapid microfluorometer is indispensable. In this chapter, the construction of an automated Leitz Diavert inverted microfluorometer with pneumatic microcomponents which is interfaced to a desktop calculator is presented.

Chapter VII. This chapter contains a description of the modified experimental protocol, as compared to that of van Dalen, Knapp and Ploem (1973). The major innovation has been the introduction of flat-bottom microtitration plates in combination with an inverted microfluorometer. The microtitration plates make possible the washing of Sepharose beads without centrifugation and the measurement of individual bead fluorescence without the necessity of preparing individual slides. The development of this technique has considerably increased the efficiency of sample processing.

Chapter VIII. A number of specific examples of the application of the SBIA to the determination of antibodies and antigens are presented. The level of antibodies directed against *Escherichia coli* lipopolysaccharide and human serum albumin has been followed during the course of the immune response in individual mice of various ages. Moreover, the levels of serum IgA, IgM, and the four subclasses of IgG have been determined throughout the life span of CBA mice. In the field of tumour virology, we have applied the SBIA in the following studies: a) determination of naturally occurring antibodies reacting with oncogenic RNA tumour viruses; b) determination of the organ distribution of mouse mammary tumour viral antigens in male mice of different strains and ages; and c) monitoring the production of viral antigens in tissue cultures. In the last part of this chapter, an evaluation of the merits of different tracers currently in use in immunoassays is made.

Chapter IX. One of the major applications of the SBIA in gerontology has been the quantitation of Ig levels throughout the life span of the CBA mouse. This study was

a consequence of our earlier study (Haaijman, Schuit and Hijmans, 1977) in which we enumerated the number of immunoglobulin containing cells (C-Ig cells) in different lymphoid organs of CBA mice of different ages. From this investigation, it became clear that the generally recognized decline of the immunological capacity in senescence (see, for instance, Makinodan and Adler, 1975) is not accompanied by a decline in C-Ig cell numbers. The preceding chapter will have shown that the levels of serum Ig's also do not show an age-related decrease.

In this chapter, qualitative as well as quantitative IF microscopy have been applied to a single biological system. Both the immunological *activity* (as determined by the number of C-Ig cells and the serum levels of the various Ig's) and the immunological *capacity* (measured as the antibody reaction against injected human serum albumin) were determined for CBA mice of different ages. Possible mechanisms for the observed discrepancy between immunological activity and capacity are discussed.

Chapter X. This chapter contains: a) a general discussion of the recent developments in IF microscopy; b) a summary of the results presented in chapters II through IX; and c) a short comment on the developments to be anticipated in IF microscopy.

A number of experimental data forming the basis of this thesis have already been published. The following section of this chapter contains a list of these publications. In this thesis it has been attempted to place the results of our experiments directly in their respective frame of reference rather than to pursue a rigid spatial distinction between literature data and our own results. Experiment details which are not mentioned or discussed in the text are presented either in the references at the end of this chapter or in the legends to the figures.

E. PUBLICATIONS ON WHICH THIS THESIS IS BASED

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Orthoplan microfluorometer using pneumatic components.' J. Immunol. Methods **7**: 255–270.

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CHAPTER II

Equipment

Contents:

A. INTRODUCTION

The fluorescence yield of a given preparation is influenced by a number of technical factors. The influence of different components of the fluorescence microscope is the subject of this chapter.

It is hardly possible to visually compare different microscopical designs. The human eye is very sensitive in the comparison of light intensities if they are presented together. Visual memory for light intensities, on the other hand, is relatively poor. We have, therefore, quantitated the influence of different optical components, using a fluorescence standard. This standard consists of Sephadex-G25 superfine beads which have been provided with amino groups. The amino groups can be reacted with FITC and TRITC, yielding fluorescent beads. This fluorescent standard, together with a discussion of other standards, is the subject of part B of this chapter.

We have used a Leitz Diavert inverted microscope throughout our investigations. The microscope was provided with a Ploemopak illuminator for incident excitation and a MPV I measuring device (Weber, 1965). A number of variables are inherent to the measuring process as such. These variables are discussed in part C. Part D concerns the influence of the different components of the fluorescence microscope. The components which have been studied are indicated in the legend to Fig. II-1. The letters and numbers refer to the subheadings of part C and D of this chapter. Unfortunately, we have not been able to quantitate the influence of monocular versus binocular observation heads. The design of our measuring equipment prevented this.

B. STANDARDS FOR THE MEASUREMENT OF FLUORESCENCE INTENSITY

1. *Introduction*

Quantitation of fluorescence emission is possible only in comparison to a certain standard. In absorption spectrophotometry, the concentration of a given substance in solution is found by comparing the light transmittance of the test solution with that of the diluent. For fluorometry, a 'blank' value as used in absorption measurements does not exist, because the background ideally gives a zero reading. One has to compare, therefore, the fluorescence intensity of a sample with that of a fluorescent standard sample.

Several such standards have been described in the literature for microfluorometry. They can be divided into two main categories: a) macro or conus standards; and b) standard objects of microscopical dimensions.

The first standard proposed by Rigler (1966) consisted of a uranyl glass (GG 17 of Schott and Gen., Mainz, Western Germany). Ploem (1970a, b) proposed the conus standard in which a small volume of a macro object is measured. The volume is determined by the numerical aperture and focal length of the objective. A similar standard was used by Ruch (1970). Jongsma, Hijmans and Ploem (1971) have elaborated on

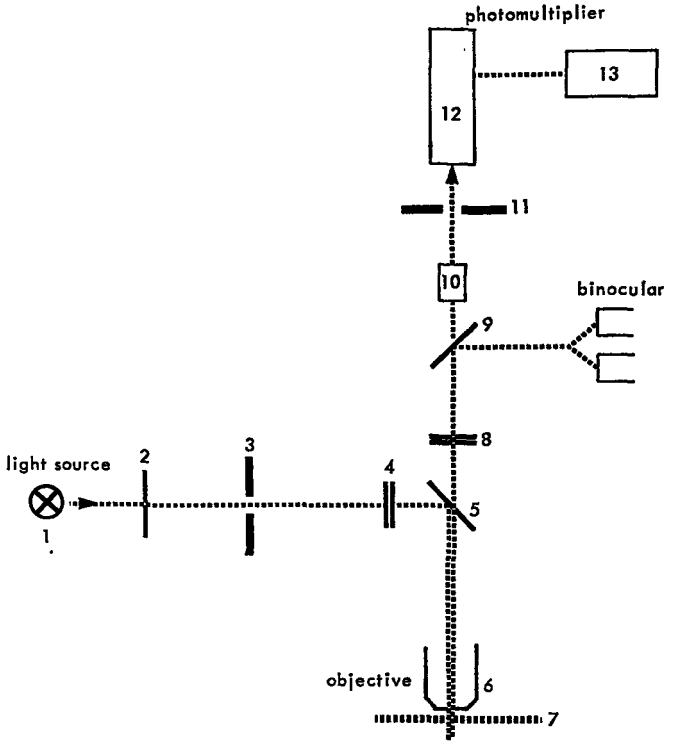


Figure II-1. Simplified diagram of a fluorescence microscope equipped for epiillumination and fluorometry. The different subheadings of this chapter in which the respective components are discussed, have been indicated between brackets. The different components of the diagram are: 1 = light source (D3); 2 = collector lens; 3 = field diaphragm (C2); 4 = excitation filters (D4);5 = interference dividing plate; 6 = objective (D1); 7 = object; 8 = emission filters (D4); 9 = mirror; 10 = measuring eyepiece (D2); 11 = measuring diaphragm (C2); 12 = photomultiplier; 13 = power supply of the photomultiplier (C1).

the use and limitations of this standard. Although the uranyl glass certainly has considerable advantages as a reference during a measuring session, it is not suitable as an absolute standard, first, because all readings are influenced by the position of the collector lens and the diameter of the field (excitation) diaphragm and, secondly, because the fluorescence spectrum of the uranyl glass is not identical to that of FITC. The same holds for the fibres coated with uranyl or europium solutions described by Velapoldi, Travis, Cassatt and Yap (1975) and the ground inorganic crystals mixed with DPX (Walker and Watts, 1970). Another group of standards, namely, the wells filled with fluorochrome solution (Jongsma, Hijmans and Ploem, 1971; Thomson and Hageage, 1975), shares the dependence on collector lens position.

Ploem (1970b) introduced the standard of microscopical dimension in the form of a microdroplet to circumvent these disadvantages. An object of sufficiently small

dimensions to fit within the measuring diaphragm and with a known concentration of fluorochrome molecules will in principle yield a standard which is independent of the type of microscopical design. Goldman (1967) pioneered in this direction with crystals of inorganic fluorophores. This has recently been followed up by Mayer and Thurston (1974). The irregular shape of these crystals, however, made it impossible to use them in an effective calibration method. In the microdroplet method, fluorochrome solutions are mixed with oil and the resulting suspension is put into a modified counting chamber. The fluorescence of individual droplets is measured and, in combination with the volume of the droplet, a standard fluorescence per volume is found. The same approach, although not with the primary aim of standardization, has been followed by Rutili, Arfors and Ulfendahl (1976) using dextran-conjugated FITC and TRITC. The solutions were measured under oil.

A drawback of the microdroplet method is not only that it is rather tedious to work with but also that, up to now, no suitable reference solution for TRITC has been found. For FITC, the microdroplets have been used as calibration standards in a number of quantitative immunofluorescence studies (ten Veen and Feltkamp, 1971; Kolk, Samuel and Rümke, 1974). Microdroplet preparations cannot be stored for a long time; therefore, microcapillaries with a sufficiently small diameter have been introduced by Sernetz and Thaer (1970, 1972) and used by Jongsma, Hijmans and Ploem (1971) as a calibration standard for fluorescence microscopes. Sernetz and Thaer (1973) and Sernetz and von Sengbusch (1975) found that fluorescein liberated by intracellular esterases had the same spectral properties as a mixture of fluorescein and albumin in microcapillaries. From this observation, they conclude that recalculation to numbers of fluorescein molecules is justified. Comparable emission spectra, however, do not imply equal quantum efficiencies.

The expression of the intensity of the fluorescence emission in standard physical units as proposed by Taylor and Heimer (1974, 1975) may lead to confusion. These authors calibrate their equipment with an uranyl glass. Consequently, the readings are influenced by parameters like collector lens position and the diameter of the field diaphragm. The measured intensities, furthermore, are dependent on the aperture of the measuring device. The expression of fluorescence intensity in physical units, without calibration with a fluorescence standard of microscopical dimensions, thus only leads to a false impression of standardization. The same objection holds for the standardization of emission measurements relative to the output of the excitation source (Haskill and Raymond, 1973.)

2. *Aminoethyl-Sephadex standard*

Because of the shortcomings of the standards consisting of microdroplets and microcapillaries we have looked for other possibilities (Haaijman and van Dalen, 1974). The studies of van Duijn and van der Ploeg (1970) on model systems for histochemical reactions formed the basis of this approach. Briefly, Sephadex beads of

microscopical dimensions (G25 – superfine; Pharmacia Fine Chemicals, Uppsala, Sweden) are provided with amino groups by heating them in 2 M NaOH with aminoethyl sulphuric acid. The aminoethyl-Sephadex beads react with both FITC and TRITC under weakly alkaline conditions. A number of variables of the bead preparation which influence the resulting staining, such as concentration of FITC and TRITC, amination and staining time, have already been tested (Haaijman and van Dalen, 1974). The fluorophore was distributed throughout the whole volume of the beads under all tested conditions. The amount of bound fluorescein and tetramethyl rhodamine per individual bead was measured with absorption spectrophotometry. In order to calculate the amount of bound molecules, the molar absorbance coefficients have to be known. Van Dalen and Haaijman (1974) described a method to measure the molar absorbance coefficient of bound tetramethyl rhodamine isothiocyanate based on the earlier study of van Dalen, Ahsmann and van Duijn (1970). An important aspect of this method was the fact that the dyes did not have to be 100% pure.

An estimate of the number of FITC or TRITC molecules which yield a given fluorescence was obtained from absorption measurements on individual beads. The fluorescence over absorption ratio proved to be constant in the range where absorption can be measured with some accuracy.

Aminoethyl-Sephadex beads stained with FITC and TRITC are quite suitable to measure different variations in microscopical design. The measurements to be presented below were carried out with a Leitz Diavert inverted microscope equipped with a MPV I measuring device (Leitz GmbH., Wetzlar, Western Germany; Weber, 1965). The operations necessary for measuring are controlled pneumatically (Haaijman and Wijnants, 1975) and the detailed design is presented elsewhere (chapter VI).

C. INFLUENCE OF SOME VARIABLES OF THE MEASURING EQUIPMENT

We have examined the influence of some parameters inherent to the measuring process before starting the measurement of the influence of different components of the microscopical design, namely, a) the voltage level of the photomultiplier and b) the diameter of the field (excitation) and measuring diaphragms. We have chosen one batch of FITC- and one batch of TRITC-stained aminoethyl-Sephadex beads for these measurements.

1. *Photomultiplier voltage*

In Fig. II-2, the influence of the photomultiplier voltage is shown. Each plotted value represents the average fluorescence per picolitre (fluor/pl) of 15 individual beads. Bars indicate standard deviations. The volume of individual beads is calculated from their diameter which is measured with a calibrated eyepiece micrometer.

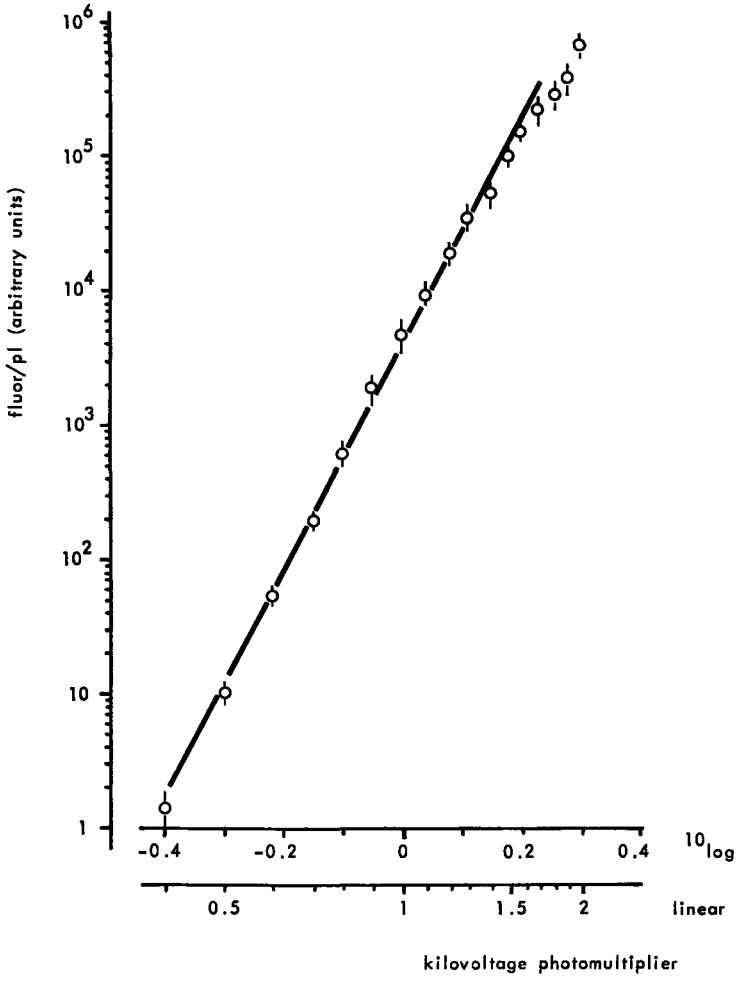


Figure II-2. The influence of photomultiplier voltage level on the fluorescence per picolitre of amino-ethyl-Sephadex beads stained with TRITC. Fluorescence and diameter of individual beads were measured. Each point represents the average fluorescence/picolitre value of 15 individual beads. Bars indicate standard deviations. Objective: 25×/0.60 W; eyepiece: 6.3×.

Photomultiplier voltages from 0.5 to 2.0 kV result in fluor/pl values which differ by a factor of 10^5. The fluor/pl is approximately proportional to the 8th power of the photomultiplier voltage. This result is somewhat below the expectation (Engstrom, 1947), since the photomultiplier tube contains 11 dynodes. This is probably caused by the geometry of the dynodes. A very good correlation between the logarithm of the fluor/pl data and the logarithm of the photomultiplier voltage was observed up to 1.6 kV. The measurements do indicate that a well-stabilized power supply for the

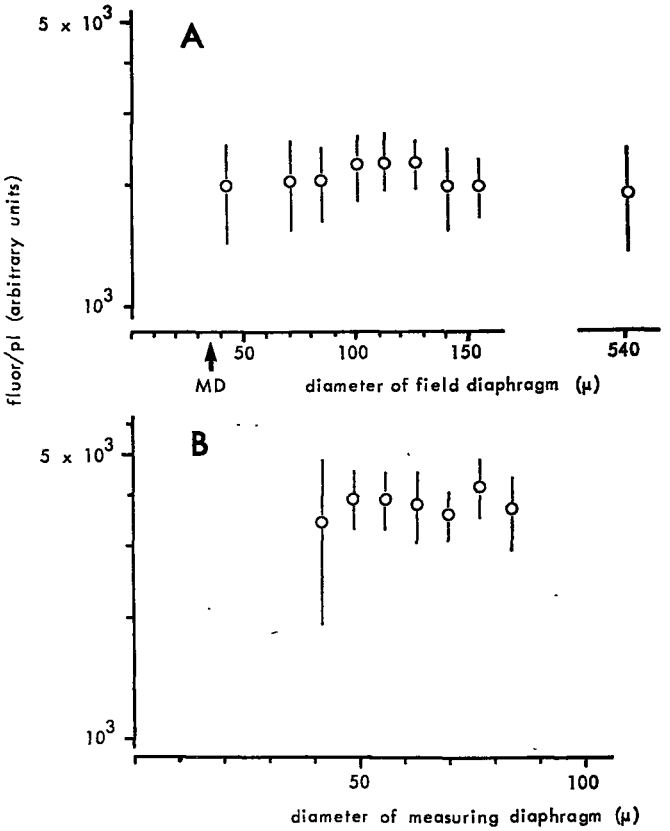


Figure II-3. Influence of the diameter of the field (excitation) diaphragm (A) and measuring diaphragm (B) on the fluorescence/picolitre of aminoethyl-Sephadex beads stained with TRITC. The diameter of the measuring diaphragm (indicated with arrow and MD) was held constant (36 μm) in A. In B, the diameter of the excitation diaphragm was 20 μm larger than the respective diameters of the measuring diaphragms. Each point is the average of 15 individual bead measurements. Bars indicate standard deviations. Objective: 25×/0.60 W; eyepiece: 6.3×.

photomultiplier is a critical part of the instrumentation. We have routinely used photomultiplier voltages between 0.8 and 1.2 kV.

2. *Diameter of field and measuring diaphragms*

The influence of the diameter of the field (excitation) and measuring diaphragms is shown in Fig. II-3. Provided a bead preparation has a high enough fluor/pl value (see below), the diameter of both diaphragms seems not to be critical.

The fluorescence measured from an object is always the sum of the actual fluorescence of the object and the background fluorescence. The background fluorescence is, of course, greatly dependent on the diameter of the measuring diaphragm (Fig.

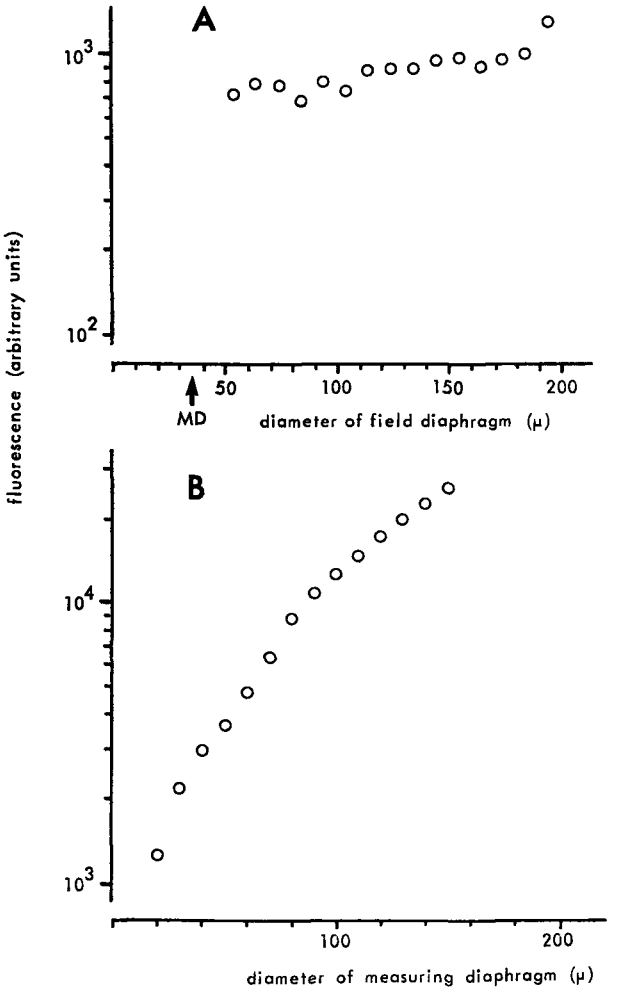


Figure II-4. Influence of the diameter of field (excitation) diaphragm (A) and measuring diaphragm (B) on the fluorescence reading from an empty part of the preparation used in Fig. II-3. In A, the diameter of the measuring diaphragm was 36 μm (indicated with arrow and MD). In B, the diameter of the field diaphragm was adjusted 20 μm larger than the respective diameters of the measuring diaphragms. Each point is the average fluorescence value of 15 separate readings. Objective: 25×/0.60W; eyepiece: 6.3×.

II-4B). Objects with low fluorescence intensity, therefore, will have to be measured with a measuring diaphragm as small as possible in order to minimize the background interference. Regression analysis of the logarithmically transformed values of Fig. II-4B yielded a slope of 1.5. This value probably indicates a not complete integration over the surface of the photocathode. We have not used the five-point measuring method of Böhm and Sprenger (1968) to test this possibility.

From a practical point of view, the influence of the diameter of the excitation diaphragm was of minor importance, as a threefold increase in its diameter resulted in only a 30% increase in the background fluorescence (Fig. II-4A). This finding indicates that the level of unwanted excitation light was very low.

D. THE INFLUENCE OF DIFFERENT COMPONENTS OF THE MICROSCOPE ON THE FLUORESCENCE EMISSION OF FITC OR TRITC BOUND TO AMINOETHYL-SEPHADEX BEADS

1. *Objectives*

Microscope objectives are characterized by a magnification and a numerical aperture (NA). The NA determines the resolving power of the objective. Fluorescent objects emit fluorescence in a solid angle of 2 π rad. The larger the NA of an objective, the more fluorescence is gathered from the same object. In fluorescence microscopy, objectives with as large a NA as possible should be used. Limits to the NA are imposed by the physical dimensions of the front lens of the objectives. Using oil immersion objectives, the highest attainable NA is about 1.40.

TABLE II-1. INFLUENCE OF OBJECTIVE ON FLUOR/PL OF AMINOETHYL-SEPHADEX BEADS STAINED WITH FITC

Type	Magnification	Phase contrast	Immersion	N.A.	Fluor/pl*
	10×	+	−	0.22	5
	10×	−	−	0.25	7
	20×	−	−	0.40	15
	20×	+	+	0.45	60
	22×	−	O+W	0.65	965
PLAN	25×	−	−	0.45	80
	25×	−	−	0.50	133
APO	25×	−	−	0.65	1016
	25×	−	W	0.60	277
	40×	+	−	0.75	292
APO	40×	−	O	1.00	608
PLANAPO	40×	−	O	1.00	573
FL	40×	−	O	1.30	7487
	50×	−	W	1.00	2910
	54×	−	O	0.95	4925
	63×	+	O	1.30	8818
FL	70×	−	O	1.30	10855
	90×	+	O	1.15	3701
NPL	100×	+	O	1.30	6260

* fluorescence per picolitre.

Each fluor/pl value is the average of 15 individual bead measurements. Bead diameters were measured with an eyepiece micrometer which was recalibrated with an objective micrometer for each objective. The light source (100 W mercury arc) was adjusted to optimally fill the entrance pupils of the different objectives.
Eyepiece: 6.3×; filter combination: no. 4, Table II-5.

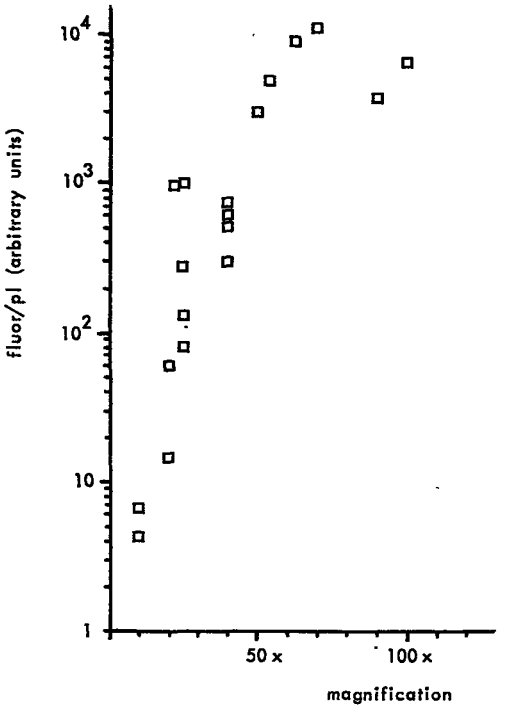


Figure II-5. The influence of the magnification of objectives on the fluorescence per picolitre of aminoethyl-Sephadex beads stained with FITC. Fluorescence and diameter of individual beads were measured. Each value represents the average of 15 individual bead measurements. The light source (CS 100 W) and collector lens were adjusted for each objective in order to fill the back entrance pupil of the objectives optimally. Eyepiece: 6.3×.

Classically, only objectives with a high magnification could have a large NA. Recently, medium power objectives with NA's of about 1.30 have also been constructed (Ploem, 1975). These objectives are constructed for maximum fluorescence yield.

In order to test the influence of the magnification and NA of objectives, the fluorescence per picolitre (fluor/pl) of aminoethyl-Sephadex beads stained with FITC was measured with different objectives ranging in magnification from 10× to 100×. For each objective, optimal filling of the entrance pupil was sought by careful adjustment of the collecting lens of the lamp housing. Data are presented in Table II-1 and in Figs. II-5 and II-6. In Fig. II-5, the logarithm of the fluor/pl is plotted versus the magnification and, in Fig. II-6, versus the logarithm of the numerical aperture. A correlation coefficient of 0.96 was found between the logarithms of the fluor/pl and NA. The slope of the regression line was 4.25. An increase in the fluorescence with the NA to the fourth power was to be expected in an epiillumination system in which both the intensity of excitation and emission light are increasing with the second power of the NA (Pearse, 1972; Nairn, 1976).

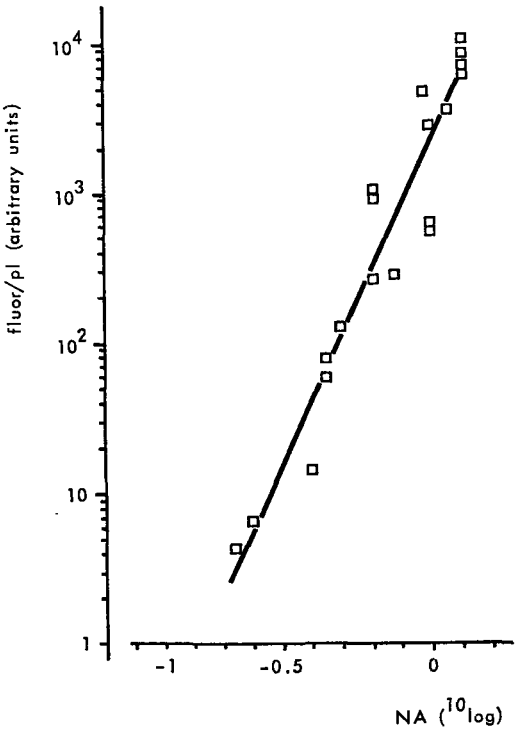


Figure II-6. The influence of the numerical aperture (NA) of objectives on the fluorescence per picolitre of aminoethyl-Sephadex beads stained with FITC. The fluorescence/picolitre data from Fig. II-5 are plotted versus the logarithm of the numerical aperture of the objectives. The drawn line represents the least squares fit with the equation: $y = 3.34 + 4.25x$, in which y stands for the logarithm of the fluorescence/picolitre and x for the logarithm of the numerical aperture. Each value represents the average of 15 individual bead measurements. Eyepiece: 6.3×.

The data of Table II-1 may be used as guidelines for choosing objectives based on the fluorescence yield. The data do not give an impression of image quality. For instance, the recently introduced FL 40×/1.30 suffers from some spherical aberration which makes it less suitable for microphotography. For each type of investigation, the optimal objective in combination with the optimal eyepieces, should be selected.

2. *Eyepieces*

The image plane of the objective is the object plane of the eyepiece. High magnifying eyepieces spread the image of a given object over a larger surface of the retina of the observer than do low power eyepieces. They collect less energy of the objective intermediate image per unit of surface. The influence of eyepiece magnification has been measured with aminoethyl-Sephadex beads stained with TRITC. The diameter of the beads was measured with an eyepiece micrometer. If this micrometer is cali-

TABLE II-2. THE INFLUENCE OF EYEPIECE MAGNIFICATION ON THE FLUORESCENCE OF AMINOETHYL-SEPHADEX BEADS STAINED WITH TRITC

Type	Magnification	Fluor/pl *
PERIPLAN	6.3×	417
KPL	8.0×	464
PERIPLAN GF	10.0×	439
PERIPLAN	12.0×	389
KPL	12.5×	327

* fluorescence per picolitre.

Each fluor/pl value is the average of 15 individual bead measurements. The diameter of the beads was measured with an eyepiece micrometer, recalibrated for each eyepiece.
Objective: 25×/0.60 W; filter combination: no. 13, Table II-5.

brated with an object micrometer, the calculated volume of the beads remains the same. The total fluorescence per bead also remains the same. Therefore, the data in Table II-2 give only an indication of the transmittance of the different eyepieces. No systematic influence of eyepiece magnification on the transmittance was found. In Table II-3, the eyepiece micrometer has not been calibrated for eyepiece magnification. The fluorescence is then calculated per apparent volume. The logarithm of the fluorescence per apparent volume is plotted in Fig. II-7 as a function of the logarithm of the eyepiece magnification. The calculated slope of the regression line was −3.09. The third power is introduced because we determined the fluorescence per unit of volume. This result indicates that the fluorescence per unit of area, as in qualitative IF, will decrease with the square root of the eyepiece magnification.

The conclusion from these measurements is that as low as possible magnifying eyepieces as the objects under study will allow should be used.

TABLE II-3. INFLUENCE OF EYEPIECE MAGNIFICATION ON FLUORESCENCE OF TRITC STAINED AMINOETHYL-SEPHADEX BEADS

Type	Magnification	Fluorescence
	5.0×	430
C	5.0×	480
MOBIMI	5.0×	455
B	6.0×	284
PERIPLAN	6.3×	233
B	8.0×	125
KPL	8.0×	101
PERIPLAN	10.0×	60
PERIPLAN GF	10.0×	52
PERIPLAN	12.0×	33
KPL	12.5×	25

Each fluorescence value is the average of 15 individual bead measurements. The apparent diameter of the beads was measured with an eyepiece micrometer. No calculation to actual bead diameter was performed in order to determine the fluorescence per unit of apparent volume.
Objective: 25×/0.60 W; filter combination: no. 13, Table II-5.

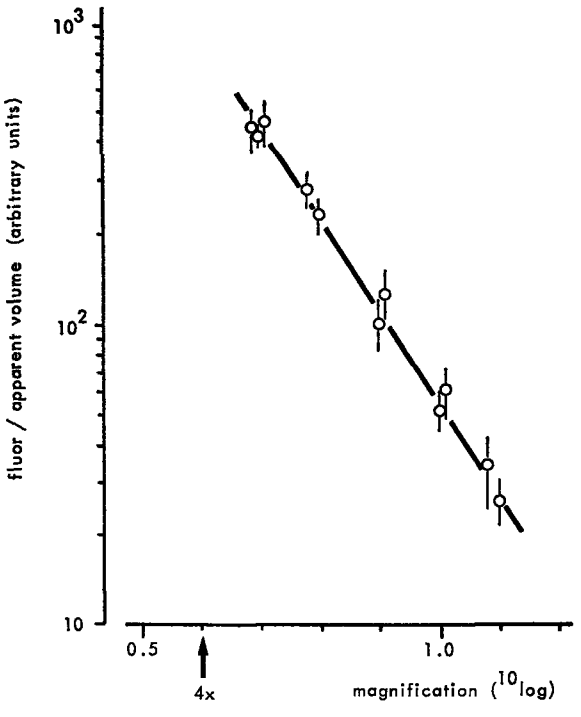


Figure II-7. The influence of eyepiece magnification on the fluorescence of aminoethyl-Sephadex beads stained with TRITC. The fluorescence and diameter of individual beads were measured with different eyepieces. The apparent volume of the beads was used (see text). Each point is the average of 15 individual bead measurements. Bars indicate standard deviations. The drawn line represents the least squares fit. The position of the 4× wide-angle eyepieces is indicated on the abscissa with an arrow. Objective: 25×/0.60 W.

We have not been able to measure the fluorescence yield with the newly introduced 4× wide-angle eyepieces because these are only suited for microscopes with a 32 mm tube diameter. The position of the 4× magnifying eyepieces on the logarithmic scale of Fig. II-7 is indicated for convenient extrapolation.

3. *Light sources*

Originally, only the carbon arc generated light intense enough to be suitable for immunofluorescence (Gottschewski, 1954; Goldman, 1968). These bulky lamps which emit very strongly in the ultraviolet region have later been mainly replaced by compact mercury arcs. The spectral output of the mercury arc is a line spectrum superimposed on a continuum (Nairn, 1976). The line at about 546 nm makes the mercury arc ideally suitable for TRITC excitation. There is no special mercury line in the spectral range ot maximal FITC absorbance. For this reason, some investigators have preferred the xenon high pressure arcs, which emit a continuous spectrum with some-

what more energy in the FITC absorbance region (Koch, 1971). Brighton and Grulich (1972) studied the applicability of a mercury-halogen lamp (CSI-250 W). This lamp gives less ultraviolet light than do pure mercury arcs. CSI arcs have not been often used in immunofluorescence. With regard to the power of the mercury arcs, the 200 W variety has been favoured for a long time (Price, 1965; Goldman, 1968; Nairn, 1976). The power of a light source, however, is not the discriminatory criterion. The light flux in a certain solid angle is more important. The 200 W mercury arc has a cathode-anode distance which is comparatively large (2.2 mm). This source is suitable for systems in which a large entrance pupil has to be filled, e.g., a dark field condensor or low power objectives in an epiillumination system. Low (50 W) and medium (100 W) power mercury arcs have a much smaller cathode-anode distance (0.2 mm) and their radiance per solid angle is much stronger. The most suitable light source for a given set-up and experiment is not easily chosen, because other factors such as the design of the collector of the lamp housing may play as important a role as the mechanical distance of this collecting lens to the entrance pupil of the condensing system.

The type of power supply is of importance in microfluorometry. The 50, 150, and 200 W mercury arcs are AC arcs, whereas the 100 W is a DC arc. DC power supplies can be better stabilized with respect to output current resulting in better arc stability than AC supplies (see, for instance, Irvine, Chan and Williamson, 1969). DC arcs have been mostly used, therefore, in microfluorometry (Jongsma, Hijmans and Ploem, 1971).

Mercury and xenon arcs are relatively expensive and their life span is rather limited. The introduction of multicoated interference filters with high transmittance values (Kraft, 1970; Ploem, 1971; Rygaard and Olson, 1971; Ploem, 1975) made the use of less intense light sources feasible. A number of studies has been devoted to this subject (Tomlinson, 1967, 1972; Young and Armstrong, 1967; Larché, 1969; Koch, 1971; Heimer and Taylor, 1972).

Measurement of light source performance is a hazardous undertaking. First of all, the number of burning hours should be the same. Secondly, it is our experience that the variation between different lamps of the same manufacturer can be considerable. Thirdly, the design of the collector and lamp housing may differ from one microscope design to another and this may make the results of only limited value.

With these reservations in mind, we have compared the fluorescence of amino-ethyl-Sephadex beads stained with FITC or TRITC using 6 different light sources (Table II-4).

New specimens of each kind of light source were employed. The high pressure sources were allowed to form a stable arc by burning them vibration-free overnight. Two oil-immersion objectives, namely, a 22×/0.65 and a FL 70×/1.30 objective, were used. With both objectives, optimal filling of the objective back entrance pupil was achieved.

The 100 W variety proved to yield the brightest fluorescence from the mercury arcs. The difference with the other sources was the most marked with the 70× objective. Within the mercury group, the CS 100 W gave, moreover, the smallest dif-

TABLE II-4. THE INFLUENCE OF THE LIGHT SOURCE ON THE FLUORESCENCE OF AMINOETHYL-SEPHADEX BEADS STAINED WITH EITHER FITC OR TRITC

	FITC [1] Fluor/pl		TRITC [2] Fluor/pl		70×/22× [3]		TRITC/FITC [4]	
Light source	22×/0.65 [5] O+W [6]	FL 70×/1.3 O	22×/0.65 O+W	FL 70×/1.3 O	FITC	TRITC	22×	70×
HBO 50 W (mercury)	1014 [7]	24488	3649	97952	24.2	26.8	3.6	4.0
CS 100 W-2 (mercury)	5383	203163	13891	453519	37.8	32.6	2.6	2.2
CS 150 W (mercury)	545	12037	1720	41100	22.1	23.9	3.2	3.4
HBO 200 W (mercury)	1349	26379	5031	90580	19.6	18.0	3.7	3.4
XBO 75 W/2 (xenon)	342	9124	250	5857	26.7	23.5	0.7	0.6
Halogen 12 V 100 W (iodine)	114	2539	117	3028	22.4	26.9	1.0	1.2

[1]) Aminoethyl-Sephadex beads stained with FITC; [2]) Aminoethyl-Sephadex beads stained with TRITC; [3]) Ratio of fluor/pl values obtained for FITC and TRITC beads with the 70× and 22× objectives; [4]) Ratio of fluor/pl values of FITC and TRITC beads obtained with the 22× and 70× objectives; [5]) Objective; [6]) Type of immersion: O = oil, W = water; [7]) Each value is the average fluorescence per picolitre of 15 individual beads.

ference in fluorescence between TRITC and FITC beads (last two columns of Table II-4). It was confirmed that TRITC is exceptionally well excited by the mercury arc. The 75 W xenon high pressure arc performed less efficiently than did the CS 100 W. The instability of our particular XBO 75 W arc was a secondary point. The arc proved to be very susceptible to forced air circulation, caused by an air conditioner, resulting in considerable fluctuations.

The 12 V, 100 W halogen lamp proved inferior in fluorescence yield to all high pressure arcs. The small difference between TRITC and FITC fluorescence was not expected, as the emission spectrum of halogen lamps contains more green than blue light (Larché, 1969). This finding points to an exceptionally high quantum efficiency of FITC as compared to TRITC. In the emission spectrum of mercury arcs, the strong mercury emission line of 546 nm contains at least ten times more energy than the wavelength region of FITC absorbance. It might be that, by this condition, the fluorescence efficiency of TRITC in IF is overestimated. In the emission spectrum of halogen lamps, about 2 times more green light than blue light is present. Nevertheless, TRITC is not better excited than is FITC. The FITC and TRITC fluorescence values can only be compared relative to each other because the photomultiplier response is wavelength-dependent.

Thomson and Hageage (1975) recently studied a number of light sources. They used a conus standard, which makes comparison with our results difficult. The fluorescence yield was optimal with a XBO 450 W (xenon) arc. They reported that the fading of

FITC with this source was less than that with the 100 W mercury arc. The authors do not give an explanation for the fact that the HBO 100 W has a five times larger luminous density than the XBO 450 W but still yields less fluorescence. In contrast to our results (Table II-1), Thomson and Hageage (1975) did not demonstrate a significant difference between the fluorescence obtained with a 100×/1.32 and 40×/0.74 objective.

Caution should be exercised in drawing conclusions regarding the best light source for routine immunofluorescence from the data of Table II-4. We have adjusted all individual light sources 'optimally'. Adjustment of an arc in practice is done by eye and is liable to variations. Secondly, if an arc is used for qualitative immunofluorescence, an even light distribution is mostly sought over the whole image field by applying a Köhler type illumination, in which the image of the arc is focused at the position of the back aperture diaphragm of the objective. In a microfluorometer, the field (excitation) diaphragm is adjusted to about 1.5 times the diameter of the measuring diaphragm. In our experiments, the measuring diaphragm covers only about 1/10th of the image field. The highest excitation intensity with a small field (excitation) diaphragm is obtained with a semicritical illumination; this means that the arc is almost focused in the object plane of the objective. The illumination cannot be exactly critical, because an even light distribution over the excitation area in the object plane is necessary. This type of illumination is quite possible with mercury arcs which have a rather symmetrically shaped arc. Xenon arcs present more difficulties because the shape of the arc is very pointed (Goldman, 1968).

4. *Filter combinations for FITC and TRITC*

The function of optical filters in IF microscopy is to prevent the excitation light from reaching the eye and to make the emission light visible. For this purpose, the transmission of excitation filters should be minimal for wavelengths longer than the excitation maximum of the dye to be traced.

In double wavelength IF methodology, the emission should be filtered in such a way that the observer can decide unambiguously which dye is fluorescing. For example, if FITC and TRITC are used in IF microscopy, optimal excitation of the former (blue excitation) will result in some excitation of the latter. Without selective emission filters, this will lead to erroneous interpretation.

We have used in this section either FITC or TRITC bound to aminoethyl-Sephadex beads to measure the fluorescence yield of both fluorochromes with filter combinations currently in use. It had already been shown (Haaijman and van Dalen, 1974) that these beads are an appropriate model system for this study, in that the dyes bound to them show comparable excitation and emission spectra with the respective dyes bound to cells. The absolute values presented cannot be compared directly to results obtained visually, because of the different spectral sensitivity of the human eye and the photomultiplier.

TABLE II-5. THE INFLUENCE OF DIFFERENT FILTER COMBINATIONS ON THE FLUORESCENCE OF FITC AND TRITC

No.	Excitation filter(s)	Mirror	Emission filter(s)	FITC fluor/pl	TRITC fluor/pl	F/T
1	2×2 mm UG 1	TK 430	K 430	361	375	1.0
2	3 mm BG 3 + S 405	TK 455	K 460	114	133	0.9
3	AL 481	TK 510	SAL 525	134	10	13.4
4	AL 481	TK 510	SAL 525 + K 515[4])	82	4	20.5
5	AL 481	TK 510	KP 560 + K 515	294	8	38.7
6	2×KP 490[1])	TK 510	SAL 525 + K 515	557	171	3.3
7	2×KP 490 + BG 38[2])	TK 510	SAL 525 + K 515	391	104	3.7
8	2×KP 490 + BG 38 + GG 455[3])	TK 510	SAL 525 + K 515	241	51	4.7
9	2×KP 490	TK 510	KP 560 + K 515	1812	902	2.0
10	2×KP 490 + BG 38	TK 510	KP 560 + K 515	1524	673	2.3
11	2×KP 490 + BG 38 + GG 455	TK 510	KP 560 + K 515	970	259	3.7
12	2×KP 490	TK 510	K 515	1042	394	2.8
						T/F
13	2 mm BG 36 + S 546	TK 580	K 580	9	320	34.7
14	1 mm BG 36 + KP 560 + K 515	TK 580	K 580	38	456	12.1
15	KP 560 + K 515	TK 580	K 580	78	634	8.1

[1]) The 2×KP 490 are always combined with 2 mm BG 38; [2]) 2 mm; [3]) 1 mm [4]) 2 mm.
Aminoethyl-Sephadex beads were stained with FITC or TRITC. The fluorescence per picolitre (fluor/pl) of both preparations was measured with different filter combinations. Each fluor/pl value is the average of 15 individual bead measurements. For each filter combination, the excitation diaphragm was focused optimally in the object plane. F/T and T/F stand for ratios between, respectively, FITC and TRITC and TRITC and FITC fluorescence. Objective: 25×/0.60W; eyepiece: 6.3×.

Table II-5 summarizes the results. UG 1 and BG 3 + S 405 excite FITC and TRITC to the same extent. The narrow band filter, S 405, gives a lower fluorescence yield than the broad band filter, UG 1. These filters cannot be used in the double wavelength method of Hijmans, Schuit and Hulsing-Hesselink (1971), because both fluorochromes are excited and no provision has been made for selective filtering of the emission. The next nine combinations (3-11 in Table II-5) are meant for the selective visualization of FITC. The AL 481 is a narrow band filter of the interference type with a relatively low peak transmission. Combined with a narrow band emission filter (SAL 525), a comparatively low fluorescence yield is obtained. The last column of Table II-5 presents the ratio between FITC and TRITC fluorescence (F/T) of the particular bead preparations used in this experiment. Adding a high pass filter (K 515) to the SAL 525 results in a 30% decrease in the FITC and in a 60% decrease in the TRITC fluorescence values. In combination 5 of Table II-5, a broad band filter combination is used for the emission pathway – namely, the high pass filter K 515 and the low pass filter KP 560. With this emission combination, almost the entire energy content of the FITC emission spectrum is transmitted (Trapp, personal communi-

cation; Kraft and Koch, 1974; Ploem, 1975). This combination gives a 3.4 times improvement in fluorescence over the SAL 525.

In the next 7 rows of Table II-5 (nos. 6–12), the excitation is provided by the low pass filter KP 490 (Kraft, 1970). Because a single KP 490 transmits too much red light, 2 KP 490 filters are mostly combined with 2 mm BG 38. This set is sometimes confusingly called KP 500. The F/T ratio with this kind of excitation filters is greatly reduced. In combination 7, it was attempted to increase the F/T ratio by inclusion of an extra 2 mm BG 38 filter in the excitation pathway. The extra BG 38 gives a 30% reduction of the FITC fluorescence and 45% of the TRITC fluorescence. Adding a GG 455 high pass filter in the excitation pathway (combination 8) has a more beneficial effect: FITC fluorescence is reduced by a factor of 1.6 but TRITC by a factor of 2.3. Narrow band or broad band emission filters (compare combinations 6–8 with 9–11) give the same result with respect to changes in the excitation filters, and data of both have been used to calculate the reduction factors.

In the last three rows of Table II-5, the results with three combinations for the selective excitation of TRITC are shown. In essence, the same picture was observed as with FITC although not as clear-cut: broad band excitation gives higher fluorescence values but less discrimination between TRITC and FITC fluorescence than narrow band excitation. Inclusion of a 1 mm BG 36 filter is advisable to improve the T/F ratio.

The results obtained with the FITC and TRITC bound to aminoethyl-Sephadex beads cannot be immediately translated into a choice for a particular filter combination for qualitative immunofluorescence microscopy. Experience has shown that, for qualitative immunofluorescence, the FITC/TRITC discrimination of combinations 8, 11 and 14 (Table II-5) is acceptable. Other factors are of importance in this respect. Cells, but especially tissues, exhibit strong autofluorescence if illuminated with high intensities of short wavelengths (Pearse, 1972; Gianetti and Cormane, 1973). The glass in the microscope optics provides an efficient high pass filter with a cut-off wavelength around 360 nm; however, it is mostly advisable to limit the excitation wavelengths further on the lower side of the wavelength scale. The kind of material under investigation determines whether a GG 455 or even a GG 475 is the best choice (Ploem, 1969; Walter, 1968). Next to the image contrast, by which is meant the ratio between desired specific fluorescence and background fluorescence (or autofluorescence), the intensity of the fluorescence is of importance. As strong as possible fluorescence is mostly sought for visual evaluation of slides, whereas this is of less importance in quantitative immunofluorescence microscopy.

E. CONCLUSIONS

For immunofluorescence microscopy, the following components of the equipment are of importance:

a) Objectives with as high as possible numerical apertures should be used for the

highest fluorescence yield. This may involve some concessions to image quality for lower magnifications.

b) The magnification of the eyepieces should be as low as possible. Of course, an optimal combination of objective and eyepieces should be chosen for each single application.

c) The 100 W mercury arc operated on a DC power supply is best suited as an excitation light source for all applications. The 50 and 200 W AC mercury arcs are the second best choices.

d) As filter combination for FITC is recommended:
   1) 1 mm GG 455 or GG 475 + 2 × KP 490 + 4 mm BG 38 for excitation and K 515 + KP 560 for emission. This combination seems optimal for qualitative IF microscopy. The choice of the GG 455 or the GG 475 depends on the degree of autofluorescence of the tissue or cells under study.
   2) AL 481 for excitation and K 515 + KP 560 for emission if the highest discrimination between FITC and TRITC is sought as in quantitative IF microscopy. Also the 2 × KP 490 can be used however.

e) As filter combination for TRITC is recommended: K 515 + KP 560 + 1 mm BG 36 for excitation and K 580 for emission. The S 546 + 2 mm BG 36 which gives lower fluorescence values but a better discrimination between TRITC and FITC will be chosen often in quantitative work. It should be mentioned here that narrow band filters are less expensive than the multicoated interference filters of the KP type.

The results presented in this chapter may serve as a general guideline for choosing the optimal equipment for IF microscopy. By no means our investigation has been exhaustive nor has it led to the description of *the* fluorescence microscope. Rather, it has been the aim to draw the attention of the IF microscopist to the improvement of image contrast and the increase of fluorescence intensity, which may result from a careful choice of the available microscope components.

CHAPTER III

The behaviour of FITC and TRITC bound to aminoethyl-Sephadex beads compared to the behaviour of FITC and TRITC bound to cells in the form of conjugates

Contents:

A. INTRODUCTION

A fluorescence standard for IF microscopy should react in the same way to physicochemical changes in the environment as does the fluorochrome in a conjugate bound to cells or tissues.

In principle, the quantum efficiency (QE) of cell-bound conjugate has to be compared to that of the IF standard, but no methods are available at the moment to measure the QE of single cells. Quantitative autoradiography using tritium or ^{14}C labelled fluorochrome may prove a possibility (Entingh, 1974); however, this technique needs as much standardization as does immunofluorescence.

We have attempted to answer the question of comparable QE's of FITC and TRITC bound to aminoethyl-Sephadex beads and in cells in an indirect way.

In order to compare the fluorescence yield of different bead preparations the amount of fluorochrome coupled to the beads has to be known. Haaijman and van Dalen (1974) described a method to determine the amount of bound fluorochrome which is based on absorption spectrophotometry. In the experiments described in this chapter, ^{14}C labelled FITC was used. The validity of the latter method was checked in the same way as was done earlier for the absorption method: aminoethyl-Sephadex beads were reacted with different concentrations of radioactive and nonradioactive FITC. Both the fluorescence per picolitre and the radioactivity of these preparations were measured. The results are presented in part B of this chapter.

The chemical environment of the fluorochrome attached to the aminoethyl sulphuric acid residue on the Sephadex matrix is not the same as that of the fluorochrome attached to protein via the epsilon amino group of lysine. We have determined the influence of the nature of the amino-group-containing spacer between beads and fluorochromes on the fluorescence yield in part C. Diamino ligands of different carbon chain length were coupled to Sepharose beads. The terminal NH_2-groups were then reacted with FITC. The influence of the spacer length on the fluorescence emission is an indication of whether the Sephadex matrix of aminoethyl-Sephadex standard beads is also likely to exert influence on the fluorescence characteristics of bound fluorochrome.

The influence of the attachment of FITC to protein on fluorescence has been determined with ovalbumin-FITC-^{14}C attached to Sepharose beads (Seph-OVA-FITC-^{14}C).

The fluorescence of FITC is susceptible to changes in the pH of the medium. In part D of this chapter, the fluorescence of FITC coupled directly to Sepharose beads and FITC coupled via OVA to Sepharose beads has been determined as function of pH. The curves are compared to those reported in the literature for conjugates either in solution or bound to cells.

The fading of FITC and TRITC is the subject of part E. The fluorescence emission of fluorochromes decreases with the duration of the excitation. An explanation for

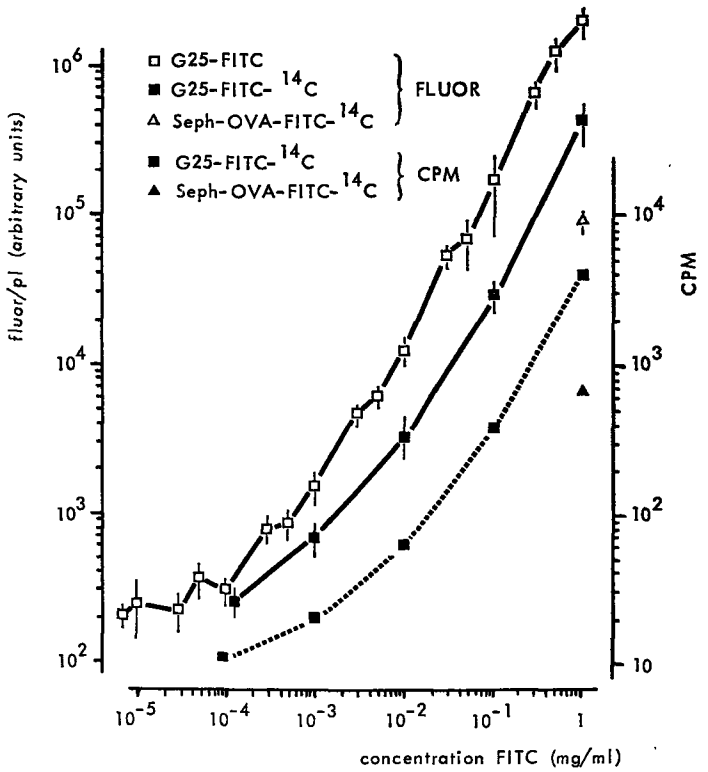


Figure III-1. The reaction of FITC and FITC-^{14}C with aminoethyl-Sephadex-G25 beads. Aminoethyl-Sephadex beads were incubated with different concentrations of FITC and FITC-^{14}C. The average fluorescence/picolitre of 15 individual beads is plotted. Bars indicate standard deviations. The radioactivity of the different FITC-^{14}C preparations was measured in a liquid scintillation counter. The volume of packed beads per counting vial was standardized (200 µl). The fluorescence/picolitre and radioactivity of Sepharose beads coupled with ovalbumin (OVA) and incubated with FITC-^{14}C are also indicated. G25- and Seph- respectively stand for aminoethyl-Sephadex-G25 and Sepharose-4B beads.

the fading characteristics is sought after having demonstrated a difference between the fading of fluorochromes bound to aminoethyl-Sephadex beads and the fading of conjugates bound to cells.

B. QUANTITATION OF FITC BOUND TO AMINOETHYL-SEPHADEX BEADS WITH FITC-^{14}C

Haaijman and van Dalen (1974) applied absorption spectrophotometry to measure the number of FITC or TRITC molecules bound to aminoethyl-Sephadex beads. The use of narrow band filters with a maximum transmission wavelength not exactly corresponding to the absorption maxima of the dyes introduced a source of inaccuracy.

Another method for measuring the amount of bound fluorochrome is illustrated in

Fig. III-1. A batch of aminoethyl-Sephadex-G25 beads was reacted with different concentrations of FITC or FITC-^{14}C (Johnson and Brighton, 1971a, b), the latter kindly provided to us by Dr. W. D. Brighton (National Institute for Biological Standards and Control, London, England). The fluorescence per picolitre (fluor/pl) of both radioactive and nonradioactive beads was measured. The radioactivity of a standardized number of beads was measured in a liquid scintillation counter. The ^{14}C label is equally distributed over fluorescein isothiocyanate and fluorescein carbonate as a consequence of the procedure of synthesis, according to Dr. Brighton. Both compounds have the same absorption characteristics. The specific radioactivity of the preparation was 3.1×10^3 cpm/μg. The data of Fig. III-1 show that the staining of aminoethyl-Sephadex beads with FITC-^{14}C is concentration-dependent in the same way as the staining with nonradioactive FITC as measured with microfluorometry. The carbonate impurity evidently does not disturb the reaction. The curve of the radioactivity data of FITC-^{14}C beads in Fig. III-1 runs parallel to the fluorescence per picolitre curve. The same phenomenon had been observed for absorption and fluorescence per picolitre. We may conclude that there is a fixed relationship between the number of FITC molecules and the fluorescence of these molecules. This observation justifies the extrapolation of the fluorescence per number of molecules value to fluorescence data in the range where the radioactivity measurements are unreliable, e.g., below a FITC concentration of 0.01 mg/ml. Radioactivity measurements are used in the subsequent section to quantitate the amount of bound FITC after various coupling procedures.

C. INFLUENCE OF THE LENGTH AND NATURE OF DIAMINO SPACERS BETWEEN SEPHAROSE BEADS AND FITC

Aminoethyl-Sephadex beads stained with FITC or TRITC can be used to express the fluorescence of objects like cells in terms of FITC molecules bound to the Sephadex (Haaijman and van Dalen, 1974). The quantum efficiency (QE) of FITC bound to Sephadex beads needs not to be the same as that of FITC conjugated to proteins and bound to cells. This provision makes it impossible to estimate with certainty the number of molecules present in a given cell from fluorescence measurements alone. As outlined in the introduction to this chapter, QE's of single beads or cells cannot be measured. In order to still gain an impression of the value of the fluorescent aminoethyl-Sephadex beads as a standard for IF, we have studied a number of variables inherent to the standard as such and to the fluorochromes used in IF.

In this section, experiments in which the influence of the proximity of the bead matrix on the fluorescence characteristics of FITC has been investigated by inserting carbon chains of different lengths between the matrix and FITC are described. Sepharose beads which were activated with cyanogen bromide (CNBr) according to March, Parikh and Cuatrecasas (1974) were used for this experiment. The activated beads

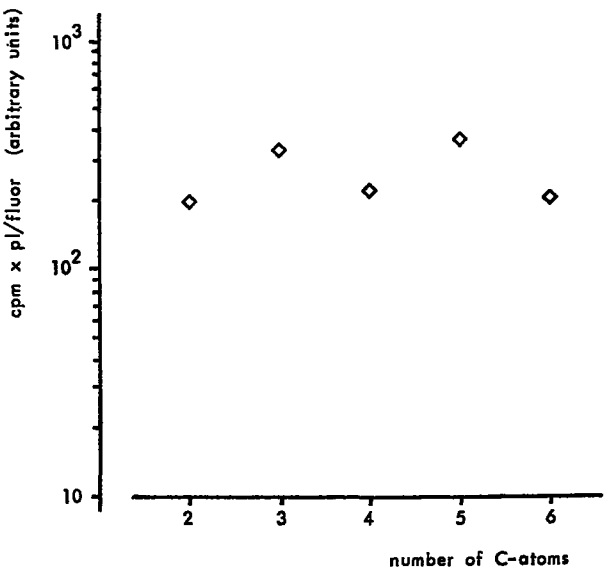


Figure III-2. The influence of spacers on the fluorescence of FITC. Sepharose beads were activated with cyanogen bromide. The activated Sepharose was reacted with 0.5 M diaminoethane, diaminopropane, diaminobutane, diaminopentane and diaminohexane. These beads were reacted subsequently with FITC-^{14}C after deactivation of the residual active groups with ethanolamine. Fluorescence per picolitre and the radioactivity of the different bead species were measured.
Ordinate: the ratio of radioactivity (in cpm per 200 µl packed beads) and average fluorescence/picolitre (in arbitrary units); abscissa: number of carbon atoms in diamino ligand used for coupling.

were reacted with diamines of different carbon chain length. After deactivation of the residual active groups with ethanolamine, the beads were stained with FITC-^{14}C and the fluorescence per picolitre and the radioactivity were determined. In Fig. III-2 the ratio between radioactivity and fluorescence/picolitre of the different bead preparations has been plotted. No systematic influence of spacer length was observed. We have assumed that this finding will also apply to the IF standard beads in which Sephadex is the matrix. We have reacted ovalbumin coupled to Sepharose beads (van Dalen, Knapp and Ploem, 1973) with 1 mg/ml FITC-^{14}C in order to see whether the relationship fluorescence/number of molecules is altered after conjugation of the fluorochrome to protein. Both fluorescence per picolitre and radioactivity are indicated in Fig. III-1. The ratio of these two parameters was found to be almost the same for Sepharose-OVA-FITC-^{14}C as for aminoethyl-Sephadex FITC-^{14}C.

We have concluded from the above experiments that the proximity of the Sephadex matrix nor the absence or presence of protein grossly changes the quantum efficiency of FITC.

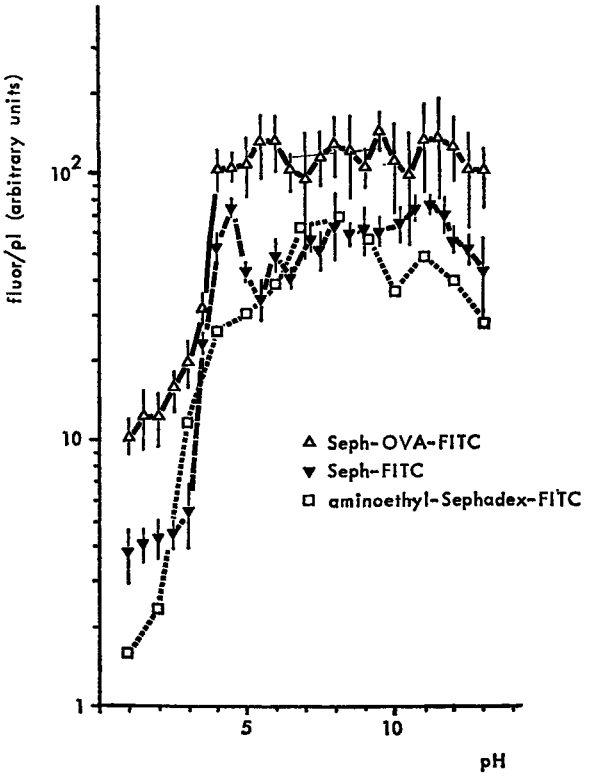


Figure III-3. The influence of pH on the fluorescence/picolitre of Sepharose-FITC, Sepharose-OVA-FITC and aminoethyl-Sephadex stained with FITC. Sepharose was activated with cyanogen bromide and stained with FITC (Seph-FITC). A portion of the activated Sepharose was coupled with ovalbumin and subsequently stained with FITC (Seph-OVA-FITC) after deactivation of the residual active groups with ethanolamine. Each point represents the average of at least 5 individual bead measurements. Bars indicate standard deviations. The data for the aminoethyl-Sephadex beads stained with FITC are from Haaijman and van Dalen (1974).

D. INFLUENCE OF pH ON THE FLUORESCENCE OF FITC BOUND TO SEPHAROSE BEADS

The pH of the embedding medium strongly influences the fluorescence of FITC (Klugerman, 1966; Chen, 1969; Jongsma, Hijmans and Ploem, 1971; Steinbach, 1975; Nairn, 1976; Steinbach and von Mayersbach, 1976). This phenomenon has recently been used to monitor intracellular pH changes (Thomas and Johnson, 1975). We have shown earlier (Haaijman and van Dalen, 1974) that the fluorescence of FITC bound to aminoethyl-Sephadex beads at pH 1 and 2 is greatly reduced. A clear increase in fluorescence was observed between pH 3 and 7. In the experiments of Jongsma, Hijmans and Ploem (1971), the fluorescence of FITC conjugated to an antiserum showed the strongest pH dependency between pH 5 and 7. The influence of

pH on FITC bound to Sephadex beads seems to have shifted to lower pH values. We have tested the hypothesis that this effect is caused by the absence of protein in the standard beads. The effect of pH on Sepharose-FITC and on Sepharose-OVA-FITC is shown in Fig. III-3. Sepharose-FITC was prepared by reacting CNBr activated Sepharose beads directly with FITC. Most probably, an isourea bond is formed. Our earlier data on FITC bound to aminoethyl-Sephadex beads have been included in the figure. The pH-dependent behaviour of Sepharose-FITC and Sepharose-OVA-FITC are not conspicuously different. We believe, therefore, that the absence of protein in the fluorescence standard is not the main cause of the shift in pH dependency.

FITC bound to Sepharose beads showed a somewhat different behaviour than when bound to aminoethyl-Sephadex beads. Maximum fluorescence of Sepharose-FITC was reached at pH 5 and the fluorescence between pH 5 and 13 was almost constant. Aminoethyl-Sephadex-FITC showed maximum fluorescence at pH 8 and higher pH values caused a decrease in fluorescence. We have no explanation for this phenomenon. The fact that FITC in cell-bound conjugates is more susceptible to pH changes in the range of 6–8 than is aminoethyl-Sephadex-bound FITC makes rigid control of the pH in IF slides obligatory. We do not think, however, that this finding makes the fluorescent aminoethyl-Sephadex beads unsuitable as IF standards, because the QE of Sepharose-OVA-FITC and aminoethyl-Sephadex-FITC seems to be quite comparable, at least in the physiological pH range (see previous section).

E. FADING OF FITC AND TRITC

Fading is the phenomenon where the fluorescence emission of a given object decreases with increasing duration of the excitation. It is a property of most fluorochromes. This phenomenon is of special importance if extended excitation times are required, such as sometimes in microphotography of IF slides (Schuit, 1970) and in microfluorometry (Nairn, Herzog, Ward and de Boer, 1969). Results of fading measurements on a number of different preparations have been included in this chapter, because they may give clues to the interaction of FITC and TRITC with their physicochemical surroundings.

The process of fading after the onset of the excitation light can be divided into two phases: the first few seconds in which the fluorescence decreases rapidly and a phase in which the decrease in fluorescence is more gradual. Enerbäck and Johansson (1973) reported 50% fading within the first two seconds after the onset of the excitation. They used a rapid sampling device triggered by a camera shutter. We have not looked at these early phenomena.

No unambiguous explanation of fading in IF has yet been given. Two possibilities have been mentioned: 1) shattering of fluorophore molecules themselves; and 2) capture of excited electrons by electrophilic centres near to the fluorophore. The second, but not the first phenomenon may be reversible. Schauenstein, Wick, Herzog and Steinbatz (1975) and Wick, Schauenstein, Herzog and Steinbatz (1975) have studied

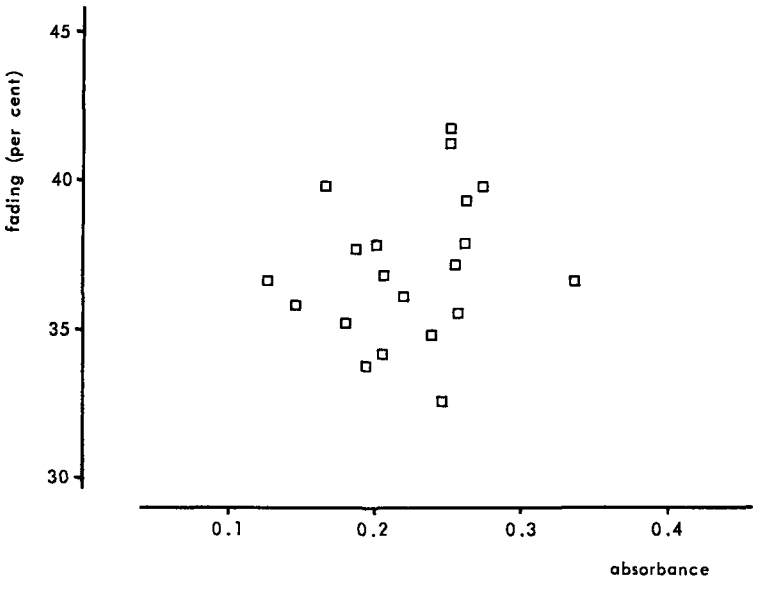


Figure III-4. Fading of FITC bound to aminoethyl-Sephadex beads. The degree of fading and the absorbance of individual beads were measured. The fading is expressed as the percentage of the initial fluorescence value remaining after 20 consecutive measurements (taking 120 seconds). The absorbance of individual beads was measured as described by Haaijman and van Dalen (1974). The absorbance is directly proportional to the amount of FITC present on the beads.

the influence of extended periods in the dark on the recovery of the fluorescence of FITC after excitation with a laser beam. Using laser flashes of 1/50 sec and dark intervals of 20 seconds or more, an appreciable recovery of fluorescence was observed. Rundquist and Enerbäck (1976) measured the fading of a number of fluorescent dyes in the first few seconds after the onset of laser excitation. They found appreciable fading in the first 10 milliseconds with berberine sulphate, acriflavin and acridine orange. Fluorescein isothiocyanate did not show such an initial rapid fading. The phenomena occurring with laser excitation, however, need not be comparable to those seen with conventional light sources, because of the extreme intensity.

The amount of fading per time unit has been described as a function of the excitation intensity (Goldman, 1960; Jongsma, Hijmans and Ploem, 1971). Herzog, Albini and Wick (1973) confirmed this observation. These authors compared the effect of different filter combinations on the fading of FITC. Those filters with the highest transmission caused the most severe fading.

The first hypothesis regarding fading, destruction of fluorochrome molecules, would imply a fixed relation between the amount of fading and the number of irradiated molecules. We have plotted the fading of individual aminoethyl-Sephadex-FITC beads against the absorption of the same beads (Fig. III-4). No correlation was

observed between the amount of FITC per bead and fading. This result showed that fading cannot be regarded as a random photodecomposition process. West, Golden, Menter and Love (1976) reported second order kinetics for the fading of acridine orange in mast cells. We have performed no experiments to confirm their data for FITC or TRITC.

Haaijman and Wijnants (1975) compared the fading of aminoethyl-Sephadex-bound FITC and TRITC with the fading of the fluorescence of plasma cells stained with either FITC or TRITC anti-immunoglobulin conjugates. In their experiments, FITC behaved in the same way whether bound to Sephadex or present in cells. A discrepancy was noted for the fading of TRITC fluorescence. The fluorescence of TRITC bound to aminoethyl-Sephadex beads showed hardly any fading, whereas the fluorescence of plasma cells stained with a TRITC conjugate showed the same pattern as FITC.

We have extended this line of investigation by comparing the fading characteristics of a number of bead preparations. The summarized results are in Table III-1. G25- stands for Sephadex beads aminated with aminoethyl sulphuric acid and 4B-stands for CNBr activated Sepharose beads. The small variation between the fading of individual beads per preparation is evident from Table III-1. Fading appears to be a very reproducible phenomenon.

TABLE III-1. FADING OF FITC AND TRITC BOUND TO BEADS

Beads	% fading in 120″ [1])
G25-FITC [2])	44.2±7.8 [3])
4B-FITC [4])	66.4±3.3
4B-2C-FITC	88.9±4.5
4B-6C-FITC	76.3±2.6
4B-OVA-FITC pH 1.0	14.4±3.6
4B-OVA-FITC pH 4.5	65.9±2.4
4B-OVA-FITC pH 10.0	81.6±1.0
4B-FITC pH 1.0	24.0±5.0
4B-FITC pH 4.5	79.5±2.4
4B-FITC pH 10.0	83.2±1.3
4B-GASiSV/p28-TCF-GASiSV/p28-FITC	95.7±5.4
G25-TRITC	9.1±3.8
4B-OVA-TRITC	28.6±3.8
4B-RAM/IgM-NMS-GAM/Ig-TRITC	16.6±2.2

[1]) Beads were exposed to 120″ of irradiation with a 25×/0.65 W objective using the filter combination no. 11 of Table II-5 for FITC and no. 14 for TRITC. The fading is expressed as the percentage of the initial fluorescence that disappeared. Eyepiece: 6.3×.

[2]) G25-: aminoethyl-Sephadex-G25 beads.

[3]) Average fading with standard deviation of at least 5 individual beads.

[4]) 4B-: Sepharose-4B activated with CNBr.

Explanations of different bead abbreviations are given in the text.

FITC coupled to Sepharose beads faded about 20% more than FITC coupled to aminoethyl-Sephadex beads. It was not possible to reduce this influence of the Sepharose matrix by introducing a spacer of either 2 or 6 carbon atoms between the matrix and the FITC. Actually, the fading of both 4B-2C-FITC and 4B-6C-FITC is 10–20% more than that of 4B-FITC.

We have compared the pH-dependency of the fading of both 4B-OVA-FITC and 4B-FITC in order to test the hypothesis that the presence of electrophilic groups near to the FITC moiety will influence its fading behaviour. At pH 1.0, all amino and carboxyl groups of the protein will be hydrogenated. 4B-OVA-FITC should fade more rapidly in low than in high pH according to the above-mentioned hypothesis. The reverse was observed, however. Our finding is in disagreement with those of Nairn et al. (1969). The absorbance of FITC at pH 1.0 is greatly reduced as compared with pH 4.5 or 10 (Nairn, 1976). This will lead to less fading. The experiment was done, however, to study the influence of pH on the fading in the presence or absence of protein. The similarity of the pH influence on the fading of 4B-OVA-FITC and 4B-FITC fluorescence indicates that fading is a characteristic of the fluorophore itself and is not caused by the presence of electrophilic centres.

Beads coupled with a goat antiserum directed against a protein (p28) of simian sarcoma virus (GASiSV/p28) and incubated subsequently with the supernatant of a SiSV infected tissue culture (TCF) and GASiSV/p28-FITC, showed the most severe fading. Almost all fluorescence disappeared in the course of 2 minutes of excitation. No explanation was found for the difference between these beads and 4B-OVA-FITC beads with regard to fading.

The following conclusions seem warranted. Fading of FITC is not influenced by electrophilic centres in the protein to which it is coupled. It is a property of the molecule itself. The amount of fading is influenced, however, by the matrix to which FITC has been coupled. The FITC coupled to Sepharose in one way or the other fades more rapidly than FITC coupled to aminoethyl-Sephadex beads. No relationship between the number of FITC molecules exposed to irradiation and the degree of fading could be demonstrated for the latter kind of beads. This finding excludes a first order photodecomposition effect as the basis for fading of FITC.

In the lower part of Table III-1, the fading of TRITC stained beads is shown. The filter combination K 515 + KP 560 + 1 mm BG 36 (see Table II-5) was used for excitation. Beads stained with TRITC faded much less under excitation than beads stained with FITC. 4B-OVA-TRITC faded more rapidly than G25-TRITC. This is the same phenomenon as observed for FITC. The influence of the matrix and the protein was not separately tested with TRITC. The following experiment was done to study the influence of more protein layers between the Sepharose matrix and TRITC. Beads coupled with the IgG fraction of a rabbit antiserum directed against the Fc part of murine IgM (RAM/IgM) were incubated with normal mouse serum (NMS) and subsequently with a fluorescent goat antiserum directed against mouse

TABLE III-2. FADING OF FITC AND TRITC BOUND TO BEADS AND CELLS

Preparation	% fading in 120″ [1]
G25-TRITC [2]	39.3±1.4 [3]
PBC-RAHu/Br-GAR/Ig-TRITC	64.5±2.8
Murine bone marrow – RAM/IgA-TRITC	68.7±1.6
Murine bone marrow – RAM/IgM-FITC	98.0±2.5
G25-FITC	83.6±2.6

[1]) Beads and cells were irradiated for 120″ with a 100×/1.30 objective using the same filter combination as in Table III-1. Fading is expressed as the percentage of the initial fluorescence that disappeared.

[2]) G25-: aminoethyl-Sephadex-G25 beads; further abbreviations are explained in the text.

[3]) Average fading in per cent±standard deviation of at least 5 individual beads.

Ig's (GAM/Ig-TRITC, lot no. 2-773; Nordic Immunological Laboratories, Tilburg, The Netherlands). The fading of the fluorescence of these beads was less than that of 4B-OVA-TRITC. This phenomenon again indicates that the protein is not the major determinant for the degree of fading.

The fluorescence of TRITC labelled beads, in both the presence and absence of protein, showed considerably less fading than their FITC counterparts under our conditions of excitation. This is in agreement with our earlier observations. The discrepancy between the fading of the fluorescence of TRITC bound to beads and the fading of the fluorescence of a TRITC conjugate bound to cells was overestimated by Haaijman and Wijnants (1975) because two different objectives were used. A 100×/1.30 objective was used for cells, while beads were measured with a 22×/0.65 objective. The intensity of the excitation in the former case is four times higher than in the latter (Fig. II-6).

We have compared the fading of membrane-bound as well as intracytoplasmic conjugate with the corresponding aminoethyl-Sephadex beads in Table III-2. The same 100×/1.30 objective was used. The fading of G25-TRITC is increased up to 40% with this objective, thus confirming the relationship between fading and excitation intensity. Human peripheral blood cells (PBC) incubated with a rabbit antiserum raised against human brain (RAHu/Br) and subsequently with a TRITC-labelled goat antiserum directed against rabbit Ig's (GAR/Ig-TRITC, lot no. 774; Nordic Immunological Laboratories) showed 25% more fading in 2 minutes than TRITC stained aminoethyl-Sephadex beads. The same was found with intracytoplasmically-bound conjugate. For this experiment, fixed mouse bone marrow cells were incubated with a TRITC-conjugated rabbit antiserum directed against mouse IgA (RAM/IgA-TRITC, prepared by Dr. J. Radl of the Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands). The fluorescence of the plasma cells faded by 70% during 2 minutes of excitation.

With the 100×/1.30 objective, comparable FITC preparations were more susceptible to fading than TRITC. Almost 100% of the fluorescence of plasma cells stained with RAM/IgM-FITC was lost after 2 min.

Haaijman and Wijnants (1975) reported a fading of only 18% in 2 min for plasma cells stained with a FITC conjugate. The difference may be partly due to the difference in excitation filters. They used an AL 481 filter with comparatively low transmittance, while the combination 1 mm GG 455 + 2 mm BG 38 + 2 × KP 490 (Table II-5) was used in the present measurements. Furthermore, the curves of Haaijman and Wijnants (1975) were recorded by taking successive measurements of the same bead or cell. The measurement cycle time of their equipment was 6 seconds. During these 6 seconds, the excitation shutter was opened for only about 2 seconds. After 2 min of measuring, or 20 measurements, the objects had only received about 40 seconds of excitation. In this report, we illuminated the beads and cells for 2 minutes. A more serious discrepancy with the results presented in this chapter is the fact that in the experiments of Haaijman and Wijnants (1975) the fluorescence of FITC bound to cells faded as fast as the fluorescence of FITC bound to aminoethyl-Sephadex beads. We have not been able to confirm this observation.

FITC or TRITC bound to aminoethyl-Sephadex beads faded less rapidly than cell-bound conjugates. FITC is more liable to fading than TRITC. We favour the hypothesis that the Sephadex matrix in some way or another protects the fluorophores from photodecomposition. Rundquist and Enerbäck (1976) reported no fading of fluorescent polystyrene microspheres when excited with laser light. The chemical nature of the particles, however, is not made clear by the authors.

TABLE III-3. USEFULNESS OF AMINOETHYL-SEPHADEX BEADS STAINED WITH FITC OR TRITC AS STANDARDS IN IMMUNOFLUORESCENCE MICROSCOPY

Optical variables:	Objectives	Same influence	
	Eyepieces	Same influence	The beads react in the same
	Light sources	Same influence	way to changes in these
	Filters	Same influence	components as do cells
	Field (excitation) diaphragm	Same influence	stained with FITC or
	Collector lens position	Same influence	TRITC conjugates
	Light source alignment	Same influence	
Physicochemical variables:	pH	pH dependency of aminoethyl-Sephadex beads stained with FITC is shifted to lower pH values	
	Duration of excitation	Beads fade less rapidly than do cells	
Practical assets standard beads:	Storage	Dry	
	Stability	Excellent	
	Preparation and handling	Easy	
	Dimensions	Small enough for most purposes	
	Inclusion in IF slides for direct comparisons	Possible	

F. CONCLUSIONS

A prerequisite for an immunofluorescence standard is that it exhibits the same behaviour under different conditions as that of conjugates bound to cells or tissues. We have collected in Table III-3 a number of variables which will influence fluorescence emission. The components of the equipment have been discussed in chapter II. The influence of these variables is the same on the standard beads as on cells; first, because it is a standard of microscopical dimensions and, secondly, because the excitation and emission spectra of FITC and TRITC bound to aminoethyl-Sephadex beads are very comparable to those of FITC and TRITC bound to cells (Haaijman and van Dalen, 1974). The results of chapter III are summarized in the middle part of Table III-3, while the lower part summarizes the practical assets of the fluorescent aminoethyl-Sephadex standard beads.

In conclusion, we think that aminoethyl-Sephadex beads or amino derivatives of Sepharose sufficiently fulfill the requirements of a standard for immunofluorescence microscopy. It should be possible then to use these beads for the determination of the absolute number of fluorochrome molecules in or bound to cells.

CHAPTER IV

Standardization of reagents for immunofluorescence microscopy

Contents:

A. INTRODUCTION

The reagents used in immunofluorescence microscopy are of biological origin and the quality of these reagents is of paramount importance. In chapter II, the influence of instrumental factors on IF results has been discussed. The purpose of the present chapter is to discuss different approaches to the standardization of IF reagents, notably, antigen preparations, antisera and fluorescent conjugates.

Standardization of IF reagents has been reported on during five international congresses (London, 1966; Florence, 1968; London, 1968; Stockholm, 1970 and Leiden, 1974). Reviews on this topic have been written by Beutner (1971b), Faulk and Hijmans (1972), Pillot (1972), Cherry and Reimer (1973), Nairn (1976), and Fagraeus and Bergquist (1975).

A classification of the different approaches to standardization of IF reagents should take into account the different motives for standardization in different fields of application. Consistency of inter- and intralaboratory results is of prime importance in clinical IF microscopy. This aim has been pursued by a number of investigators. Their results are discussed in part B of this chapter. In their trials, aliquots of the same antiserum or conjugate batches were distributed to different laboratories and the results obtained with these reagents were compared. All variables from preparation of the substrate to the visual assessment of the final slides are taken together in this way.

Reports describing the influence of the antiserum or conjugate can be divided into three main categories: 1) reports in which the chemical specification of antisera in terms of antibody content and number of fluorochrome molecules per antibody molecule (F/P ratio) is emphasized; 2) reports in which the performance of conjugates with selected biological substrates is stressed; and 3) reports in which a quantitative measure of the suitability of antisera for the application to biological systems employing antigens bound to artificial supports is sought.

The chemical specification of antisera is discussed in part C of this chapter, the performance testing in part D and the experimental results obtained with the artificial substrates in part E. The last approach is discussed in more depth, because of our own involvement.

Attention is focused on the Sepharose bead immunofluorescence technique, in which antigens bound covalently to Sepharose beads are used as the substrate for IF reactions. Experimental conditions and variations in the Sepharose bead system are discussed in Part F. Part G describes the titration of antisera with Sepharose beads. Attention is paid to the different reactivities of antisera and by which means these reactivities may be distinguished. The influence of a number of antiserum or conjugate parameters not related to the immunological specificity of these antisera forms the subject matter of part H. Discussed are the fractionation of antisera and the F/P ratio of conjugates. Part I of this chapter deals with the quantitative evaluation of the specificity of conjugates and antisera. The aim of this evaluation is to arrive at a

quantitative measure of the acceptability of conjugates or antisera for application in biological systems. For this purpose, the behaviour of a set of conjugates in both the Sepharose bead system and a biological test system has been compared.

B. STANDARDIZATION BY INTERLABORATORY TRIALS

A large number of factors influences the result of an IF procedure. A number of technical factors has already been mentioned in the previous chapters. In clinical IF microscopy, intra- as well as interlaboratory consistency of results is obligatory (Anderson, Addison and Dixon, 1971; Cherry and Reimer, 1973). The use of different microscope set-ups, different substrate preparations, different preparative techniques and the inherent subjectivity of visual screening seem to have made 'defined IF microscopy' something like a fata morgana. The subcommittee on standardization of biological materials of the World Health Organization has distributed aliquots of antisera and conjugates to different laboratories, together with very elaborate experimental protocols. Beutner (1971a) and Fagraeus and Bergquist (1975) have reported on the progress in this field. It appeared that interlaboratory comparisons will continue to represent a very difficult point in the future. The establishment of reference laboratories in various countries which can keep in contact with the individual laboratories may improve this situation. Quantitation in this respect of antinuclear factor (ANF) results produced by different antisera and conjugates by means of microfluorometry has been undertaken by Hijmans, Schuit, Jongsma and Ploem (1970), ten Veen and Feltkamp (1971), ten Veen, Kuivenhoven and Feltkamp (1971) and Wachsmuth and Woodhams (1974). This approach is very promising, but has not yet been followed up sufficiently. It has already been pointed out in chapters II and III that microfluorometry data can only be compared with each other on the basis of a reliable fluorescence standard.

C. STANDARDIZATION BY CHEMICAL SPECIFICATION OF ANTISERA AND CONJUGATES

Beutner (1971b) and Wick (1972) reviewed the progress in attempts to relate staining properties of conjugates to antibody content and the fluorochrome/protein (F/P) ratio (see also Hebert, Pittman and Cherry, 1967, 1971; Beutner, Sepulveda and Barnett, 1968). Beutner (1971b) assumes that the *d*esired *s*pecific *s*taining (DSS) is directly related to the specific antibody content of an antiserum and that the number of fluorochrome molecules per antibody molecule determines the *nons*pecific *s*taining (NSS). The degree of DSS and NSS is estimated visually in his system after checkboard titration (indirect technique, see chapter V). Bergquist and Norberg (1976) elaborated on this system and determined radiometrically the specific antibody content of antisera.

The basic work of Emmart (1958), McKinney, Spillane and Pearse (1964a, b, and

1966) and, more recently, Jobbágy and Jobbágy (1973a, b) has been essential for the determination of fluorochrome concentration. These authors studied the purity and physicochemical characteristics of FITC. The National Committee for Clinical Laboratory Standards has published recommendations based on McKinney's work and on that of Wells, Miller and Nadel (1966) for the procedure to be used to measure the labelling efficiency and F/P ratio of FITC conjugates. T.H. The (1967, 1970a and b) investigated the conjugation between FITC and IgG in great detail. As did Beutner (1971a, b), The used the ANF system to test the influence of the F/P ratio. Preparations with different F/P ratio's were obtained by ionexchange chromatography and those with F/P ratio's between 1 and 3 gave optimal results in The's experiments.

Standardization of TRITC conjugates has received less attention than has the standardization of FITC conjugates. Since Hiramoto, Engel and Pressman (1958) introduced tetramethyl rhodamine as the second label in IF microscopy, it has found many applications. TRITC (Riggs et al., 1958) cannot as yet be manufactured in highly purified form, which makes the estimation of F/P ratios difficult. McKinney and Spillane (1975) and McKinney, Thacker and Hebert (1976) studied several commercial preparations with IR spectroscopy and found a number of them unsatisfactory with regard to purity and labelling efficiency. The conjugation properties of TRITC decrease if it is purified to a crystalline form (Amante, Ancona and Forni, 1972). Several rhodamine derivatives have been tested for IF by Brandtzaeg (1973, 1975). He based his judgement for suitability on visual observation of endpoint titres. Tetramethyl rhodamine isothiocyanate and lissamine rhodamine B (RB 200) gave the best results. The solubility of TRITC is by far not as good as that of FITC. Bergquist and Nilsson (1974) circumvented this disadvantage by adding TRITC to protein solutions, dissolved in a small volume of dimethylsulphoxide.

D. PERFORMANCE TESTING OF ANTISERA AND CONJUGATES

Performance testing of a conjugate is the best way to circumvent all problems related to standardization of equipment, experimental conditions, etc., if biological substrates of sufficient purity are available. By performance testing is meant that the reactions of a conjugate or antiserum with different specified biological substrates are evaluated under the same conditions as the eventual system in which the conjugate is to be applied. Examples of performance testing are: a) the monoclonal human bone marrow technique developed by Hijmans, Schuit and Klein (1969) for antihuman immunoglobulin conjugates; b) monocultures of bacteria for the testing of antibacterial conjugates; and c) virus infected and noninfected cells for antivirus antisera. We will limit this discussion to the bone marrow system. The difficulty with techniques based on performance testing is in fact the circularity of the reasoning. For example, monoclonal bone marrow cells are monoclonal because they only react with certain specific antisera. New and unknown antisera may then be tested for specificity by assuming monoclonality of the cells. Van Camp, Schuit, Hijmans and Radl (1977)

described the existence of biclonal myelomas in which the cells contain more than one immunoglobulin class. These myelomas have to be excluded from the performance testing as soon as they are detected. Another aspect of using malignant cells for performance testing is the presumed uniqueness of their product. This implies that antisera raised against Ig molecules have to be tested on a number of different myeloma preparations per heavy chain specificity (Chantler and Haire, 1972). Notwithstanding these difficulties, performance testing is still the classical way of assessing the specificity of conjugates, because the test is done on material which is directly comparable with the material on which the conjugate is to be used as a diagnostic tool.

In performance testing techniques, only those reactivities present in the antiserum which are directed against antigens contained within the target cells or tissues are appraised. Other reactivities do not interfere with the testing and may go unnoticed (see also part J of this chapter).

TABLE IV-1. LITERATURE SURVEY OF METHODS EMPLOYING ANTIGENS BOUND TO, OR TRAPPED IN, INSOLUBLE CARRIERS FOR THE TESTING OF FLUORESCENT ANTISERUM SPECIFICITY

Reference	Test system	Comment
Brandtzaeg (1972, 1975)	Hu/Ig's incorporated in polymerized rabbit serum	Visual evaluation
Case, Lussier and Mannik (1975)	Sections of agarose beads coupled with Hu/Ig's	Visual evaluation
Mawhinney and Shirodaria (1973)	Sections of agar blocks containing lactoglobulins or casein	Visual evaluation
Scales, Jacobs and Skaggs (1975)	Hu/Ig's coupled to agarose beads	Visual evaluation
Camargo and Ferreira (1970)	Antigens bound covalently to cellulose particles	Cellulose irregular shaped
Toussaint and Anderson (1965)	Filter papers soaked in antigen solutions	Not quantitative
Allen (1963)	Ig's incorporated into agar	Not quantitative
Paronetto (1963)	Antigens on cellulose acetate strips	Not quantitative
Fey and Jost (1973)	Antibodies bound to Sepharose beads	Not quantitative
Holubar, Bergquist, Lesser and Beutner (1973), Bergquist and Kreisler (1974), Bergquist and Nilsson (1975)	Hu/Ig's polymerized into compact spheres by glutaraldehyde	Antigen requirement high, quantitative
Van Dalen, Knapp and Ploem (1973)	Antigens coupled to Sepharose beads	Quantitative
Knapp, Haaijman, Schuit, van den Berg, Ploem and Hijmans (1975)	Antigens coupled to Sepharose beads	Evaluation anti-Hu/Ig conjugates
Bloemmen, Radl, Haaijman, van den Berg, Schuit and Hijmans (1976)	Antigens coupled to Sepharose beads	Evaluation anti-M/Ig conjugates

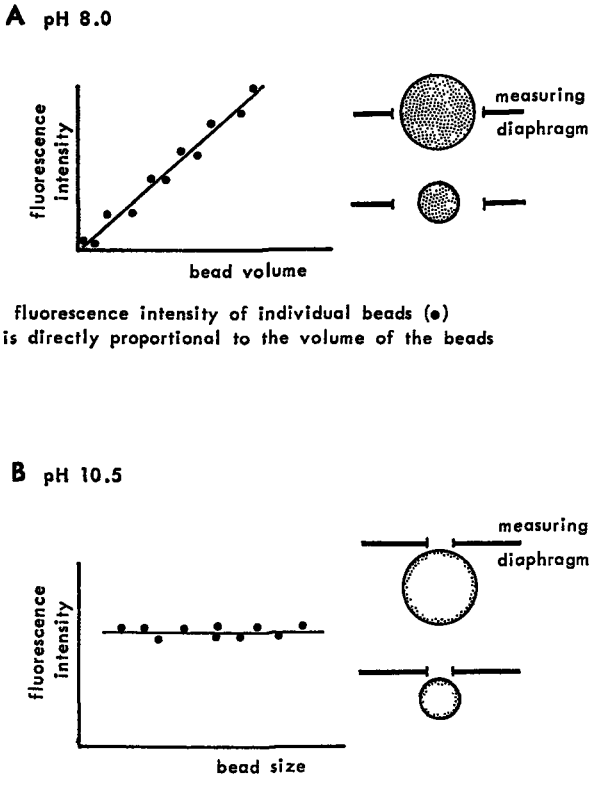


Figure IV-1. The difference in the distribution of antigens after coupling them to Sepharose activated with cyanogen bromide at pH 8.0 (A) or at pH 10.5 (B). Beads are coupled with antigen and subsequently incubated with fluorescent antibodies.
A: Sepharose beads are activated according to van Dalen, Knapp and Ploem (1973). The antigen becomes distributed throughout the whole volume of the beads. The fluorescence and the diameter of individual beads are measured. The diagram shows the relationship between bead size and measuring diaphragm and a stylized curve representing the fluorescence of individual beads versus their volume.
B: Sepharose beads are activated according to Capel (1974). The antigen is concentrated in the direct vicinity of the bead surface. Only a fixed area of the bead surface is measured. The diameter of the measuring diaphragm is smaller than the diameter of the beads. The curve shows that the fluorescence of individual beads is independent of their respective size.

E. TESTING OF ANTISERA AND CONJUGATES WITH ARTIFICIAL ANTIGEN SUBSTRATES

The major impetus for the development of test systems based on purified proteins coupled to inert supports has been threefold: 1) the need for a quantitative expression for conjugate specificity; 2) to make test systems available to those who do not have access to the equivalent of the monoclonal bone marrow system (e.g., conjugate

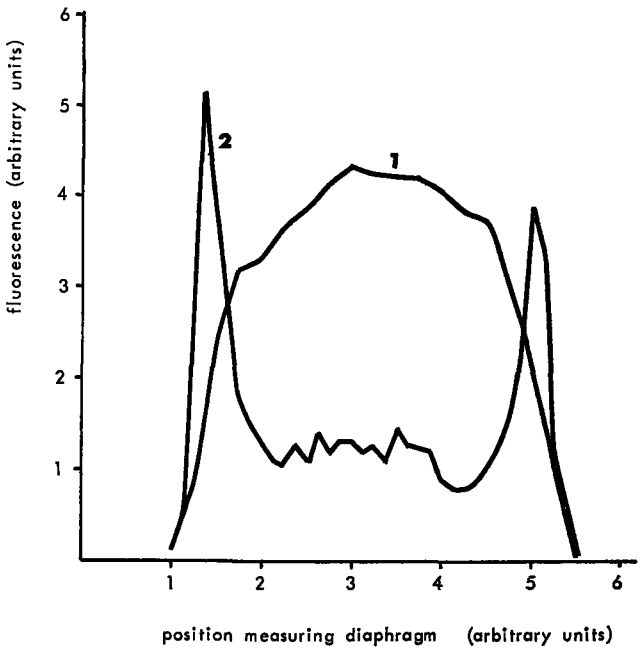


Figure IV-2. Surface and volume staining after activation of Sepharose beads according to van Dalen, Knapp and Ploem (1973). Sepharose beads were activated with cyanogen bromide at pH 8.0. Human IgG (Hu/IgG) was coupled to the activated beads. These beads were then incubated with a TRITC labelled rabbit antiserum directed against Hu/IgG (RAHu/IgG-TRITC) and afterwards with a FITC labelled horse antiserum directed against rabbit Ig's (HAR/Ig-FITC). The TRITC conjugate was diluted 1:2 and the FITC conjugate 1:50.
The fluorescence of both TRITC and FITC present on the same bead was measured along the diameter of that bead, using a small measuring diaphragm and a 54×/0.95 O objective. The RAHu/IgG-TRITC (curve 1) is distributed throughout the whole volume of this particular bead, while the HAR/Ig-FITC (curve 2) is present only on the surface of the bead.

manufacturers); and 3) to devise a system that may be applied to a variety of antigen-antibody systems. A number of systems has been described for this purpose. A summary of references and systems is presented in Table IV-1.

The initial approach of van Dalen, Knapp and Ploem (1973) was based on the earlier work of Camargo and Ferreira (1970). The former authors coupled purified human immunoglobulins to cyanogen-bromide-activated Sepharose beads. Fluorescent conjugates were incubated with beads coupled with a variety of antigens. The fluorescence of individual beads was measured with a microfluorometer. The authors recognized Sepharose beads as being a more suitable substrate for IF reactions than cellulose powder particles, as used by Camargo and Ferreira (1970), for two reasons: 1) Sepharose is transparent for excitation as well as emission light used in FITC and TRITC IF microscopy; and 2) Sepharose beads have a spherical shape. The cyanogen bromide (CNBr) activation of Sepharose according to Axén, Porath and Ernback

(1967) and Porath, Axén and Ernback (1967) yielded rather high background values. Van Dalen, Knapp and Ploem (1973), therefore, modified the activation conditions. They stressed the use of a pH of 8 during CNBr activation of the Sepharose beads and continuous rotation of the bead suspensions during the coupling and the deactivation stages of the procedure. The even distribution of antigen throughout the whole volume of the beads was a disadvantage of their method. The fluorescence of individual beads was a function of their volume (Fig. IV-1A). Capel (1974) was the first to revert to the original method of Porath, Axén and Ernback (1967), namely, activation of the Sepharose with CNBr at a pH of 10.5. Antigens primarily bind to the direct vicinity of the surface of the Sepharose beads with this activation procedure. It thus became possible to measure a fixed area of the bead surface (plug method) after incubation of these beads with fluorescent antisera. The fluorescence intensity is then independent of bead size (Fig. IV-1B). The difference between the two methods is not essential in practice. It was established (Fig. IV-2) that surface staining also occurred in conditions of antigen excess with the original method of van Dalen, Knapp and Ploem (1973). Fluorescence per volume was measured in the first report of van Dalen, Knapp and Ploem (1973). These measurements were much more time-consuming than plug measurements, because not only the fluorescence but also the diameter of the individual beads had to be determined.

F. EXPERIMENTAL CONDITIONS FOR THE SEPHAROSE BEAD IMMUNOFLUORESCENCE SYSTEM

A literature review concerning experimental conditions in the bead system will be given in this section.

The procedure may be divided into four successive stages:

1. Activation of Sepharose beads
2. Coupling of protein to the activated matrix
3. Deactivation of residual active groups
4. Incubation of antisera or conjugates with antigens bound to Sepharose beads

1. *Activation of Sepharose beads*

The basic step in the Sepharose bead immunofluorescence system is the coupling of proteins to Sepharose beads. The effect of pH during activation with CNBr has already been mentioned. Deelder, Snoijink and Ploem (1975a) studied the influence of the concentration of CNBr. There was some differential effect on specific and nonspecific staining, in that high CNBr concentrations tended to result in relatively higher nonspecific reactions. March, Parikh and Cuatrecasas (1974) introduced a simplified method for the activation of Sepharose with CNBr. CNBr is added dissolved in acetonitrile to Sepharose beads suspended in 2 M carbonate buffer. The high buffering capacity makes the adjustment of the pH with NaOH during activation un-

necessary. Other coupling methods have been described for coupling proteins to solid supports, e.g., bifunctional reagents like glutaraldehyde (Cambiaso, Goffinet, Vaerman and Heremans, 1975), and p-benzoquinone (Ternynck and Avrameas, 1976) for coupling proteins to aminated supports, the introduction of amino group reactive aldehydic groups on the Sepharose matrix with 4-aminobutyraldehyde diethyl acetal (Korpela and Hinkkanen, 1976), direct coupling of protein to periodate-oxidized Sephadex (Wilson and Nakane, 1976), and poly-L-lysine (Pachmann and Leibold, 1976) for the adsorption of proteins to polyacrylic beads. However, with exception of the last method, these have not been used in immunofluorescence applications. Cuatrecasas (1970) reported on a large number of chemical modifications of Sepharose for the binding of a variety of ligands. He also stressed the use of spacers (see also Caron, Faure and Cornillot, 1976). The role of spacers seems unquestionable in the retaining of high specific activity of immobilized enzymes. Spacers have not been used frequently, however, in antigen-antibody studies employing immunofluorescence, with exception of the use of aminocaproic acid by Hämmerling, Chin and Arny (1974) for the labelling of mouse alloantisera. Coupling proteins via their $-COO^-$ terminal to amino derivatives of Sepharose with carbodiimides did not meet with success in our experiments, due to a high degree of nonspecific staining. A number of authors (e.g., Gottlieb, Seide and Kindt, 1975) reported favourable results with these compounds in the preparation of solid immunoadsorbents.

Beads other than Sepharose have been suggested as solid supports for the coupling of proteins, e.g., polyacrylamide beads (Ekman and Sjöholm, 1975), aminopropyl glass beads (Chin and Wold, 1974), latex particles (Moklay, Dreyer, Rembaum and Yen, 1975), hydroxyalkyl methacrylate beads (Tlaskalová, Tucková, Krivaková, Rejnek and Coupek, 1975) and polyacrylic plastic beads (Pachmann and Leibold, 1976). The commercial immunofluorescence assay kit of BioRad (Richmond, Cal., USA) employs their own brand of polyacrylamide beads. In the directions for use of this kit, it is suggested that the fluorescence of the incubated beads can be measured with a normal (macro)fluorometer. In order to prevent heterogeneity of the bead suspensions during measuring, the beads should be of much smaller dimensions than are Sepharose beads. No practical application of the BioRad kit has yet been reported in the literature.

Only Sepharose-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) have been used in our experiments. These beads have a diameter of between 80 and 140 μm and are supplied in the form of a suspension.

2. *Coupling of proteins to the activated matrix*

Proteins are reacted with activated Sepharose in a buffer of pH 9.5. Coupling has mostly been done during 8 hr. March, Parikh and Cuatrecasas (1974) reported a reduction in the coupling percentages in reactions prolonged for more than 24 hr. Spontaneous deactivation of the active groups takes place during the incubation.

Wilchek, Oka and Topper (1975) studied the chemical nature of the Sepharose-protein bond. An isourea bond is most probably formed. Under some conditions, the isourea bond may be susceptible to solvolysis leading to detachment of protein from the Sepharose beads. Schnapp and Shalitin (1976) have proposed the CNBr activation of amino-Sepharose, which would lead to the more stable guanidino bond. Under our experimental conditions, we have never observed protein detachment from the Sepharose beads to any significant extent.

Different results have been reported regarding the optimal amount of protein per ml of activated beads. Up to 20 mg of ligand is capable of coupling to 1 ml of packed beads (Sharma and Slaunwhite, 1975); however, Capel (1974, 1975) reported a decrease in the stainability of IgG Sepharose beads with a fluorescent anti-IgG conjugate if more than 1 mg of IgG was coupled to 1 ml of Sepharose. Knapp, Menzel and Steffen (1974) observed the same effect when working with a collagen/anticollagen system. Knapp and Ploem (1974) showed that at least 5 mg of OVA may be coupled to 1 ml of beads without loss of stainability with fluorescent anti-OVA. In our experiments, we have normally used between 1 and 5 mg protein per ml of activated beads.

The amount of coupled protein is usually estimated by taking the difference in protein content of the supernatant before and after completion of the coupling reaction. The protein content can conveniently be measured either spectrophotometrically with the Lowry method (Lowry, Rosebrough and Randall, 1951) or with fluram (Udenfriend, Stein, Böhlen, Dairman, Leimgruber and Weigele, 1972; see also chapter VII, part D). Other more sophisticated methods have been reported: amino acid analysis of the acid hydrolysate of coupled beads (Axén and Ernback, 1971), direct measurement of the tryptophane fluorescence of protein present on the beads (Barel and Roosens, 1974), Lowry determination of beads coupled with protein (Koelsch, Lash, Marquardt and Hanson, 1975), spectrophotometry after solubilization of the protein coupled beads with picryl sulphonic acid (Failla and Santi, 1973) and the determination with fluram after alkaline hydrolysis (Naoi and Lee, 1974). These techniques are rather time-consuming, with the exception of the Lowry and fluram methods. The coupling percentages need only be known, moreover, with moderate precision.

Proportional coupling is a very important aspect in the coupling of mixtures of proteins. Are all proteins of the mixture coupled to the same extent? To our knowledge this has not been tested rigorously. Especially in the application of the Sepharose bead immunofluorescence system to the field of virology (chapter VIII, parts D and E) is proportional coupling a major concern. Preparations of disrupted virus contain proteins which may differ considerably in characteristics. Polyacrylamide electrophoresis of the protein mixture before and after the coupling reaction might provide an answer to the question of proportional coupling. Radioactively labelled virus preparations or antisera specific for the different proteins in the mixture may be used to establish whether the percentage of coupling is the same for all components of a mixture.

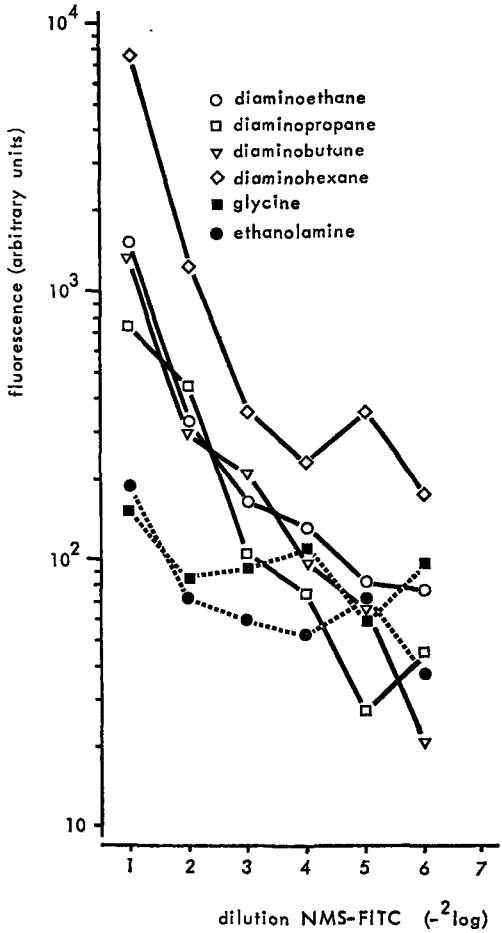


Figure IV-3. Influence of different deactivators on the nonspecific reaction. Sepharose beads were activated with cyanogen bromide. Activated beads were incubated with 0.5 M of the indicated compounds for 8 hr. The beads were incubated subsequently with different dilutions of a FITC conjugated normal mouse serum (NMS-FITC) in order to estimate the degree of nonspecific adherence with the different bead species. Each point is the average fluorescence intensity of at least 5 individual beads.

3. *Deactivation of residual active groups*

Residual active groups on the Sepharose matrix have to be deactivated after coupling of the protein. This was initially accomplished by keeping the beads for a prolonged time at pH 10, which causes spontaneous hydrolysis of the active groups (van Dalen, Knapp and Ploem, 1973).

Reagents containing amino groups are used in more recent reports. Ethanolamine is most widely employed. Equally good results have been obtained, however, with

glycine or diaminoethane. Deactivation with aliphatic diamino compounds with more than 2 C-atoms increases the nonspecific binding of antisera and conjugates (Fig. IV-3), confirming the results of Capel (1975). Deelder, Snoijink and Ploem (1975a) tested a large number of different deactivators. They employed the *Schistosoma mansoni* (*S.mansoni*)/anti*S.mansoni* system. Aminobutyric acid was found op timal with regard to the ratio between specific and nonspecific binding. The authors mention, however, the possibility that different antigen/antibody systems will have their own optimal deactivator. In our own experiments, almost exclusively ethanolamine and sometimes glycine has been used with a variety of antigen-antibody systems. No systematic differences were observed between results obtained with the two deactivators. Freshly prepared solutions of ethanolamine should be used. The presence of polymers, evident by a yellow colour of aged solutions, will result in high nonspecific binding of proteins.

4. *Incubation of antisera or conjugates with antigens bound to Sepharose beads*

Antisera or conjugates were at first diluted in phosphate buffered saline (PBS) for incubation with Sepharose beads. Deelder and Ploem (1975a) observed a large improvement if 2% bovine serum albumin (BSA) in PBS was used as the diluent. The nonspecific reaction was relatively more reduced than the specific reaction. Bloemmen, Radl, Haaijman, van den Berg, Schuit and Hijmans (1976) found that haemoglobin prevented the nonspecific binding of proteins to Sepharose beads even better than did BSA. An explanation for this phenomenon may be sought in the difference in charge between haemoglobin and BSA. If nonspecific staining is caused by binding of positively charged IgG molecules, the positively charged haemoglobin might compete better in this interaction than the negatively charged BSA.

The beads should be washed free of excess conjugate after incubation. Capel (1974) introduced PBS with 0.65 M NaCl to reduce the nonspecific binding, based on the observation of Palmer (1972) that IgG tends to stick with nonspecific ionic forces to antibodies bound to the Sepharose matrix and that this bond may be disrupted by high concentrations of salt. The influence of NaCl is not as pronounced as shown by Capel (1974), according to Deelder, Snoijink and Ploem (1975a) and our own experiments. It was used, however, throughout the experiments. Urea and Tween-20 were tested for their ability to reduce nonspecific staining, but these substances were of no avail in our antigen-antibody systems. Capel and Aalberse (1975) applied the detergent Tween-20 successfully in the Sepharose bead system for the determination of antiallergen antibodies as did Ruitenberg, Steerenberg, Brosi and Buys (1976), working with the enzyme-linked immunosorbent assay (ELISA).

Evaluation of all the variables mentioned above has led to the experimental protocol described in detail in chapter VII.

G. TITRATION OF ANTISERA WITH SEPHAROSE BEADS

1. *Definitions*

The different reactivities of an antiserum may be defined as follows (Beutner, 1971b):
a) desired specific staining (DSS); b) undesired specific staining (USS); c) cross-reactive specific staining (XSS); and d) nonspecific staining (NSS).

a) Desired specific staining (DSS)

This is the staining that an antiserum or conjugate is meant to perform. Examples will be given from the applications of anti-IgA conjugates. The DSS of an anti-IgA conjugate is the staining of cells containing IgA. This reaction is also called the homologous reaction.

b) Undesired specific staining (USS)

The observed reaction is caused by a specific immunological reaction, but it is not desired. Two types of USS may be distinguished: 1) USS caused by antibodies also causing the DSS; and 2) USS caused by antibodies directed against antigens other than the homologous antigen. USS type 1 is encountered, for instance, if an anti-IgA conjugate is applied to cryostat sections of gut tissue for the visualization of IgA containing cells. The DSS of IgA containing cells may sometimes be obscured by USS type 1 caused by the presence of serum IgA around the cells. This kind of USS will be more obvious with anti-IgG conjugates than with anti-IgA conjugates, because of the higher concentration of serum IgG. USS type 2 is caused by impurities in the antigen preparation used for raising the antiserum. USS type 2 is, for instance, the reaction of an anti-IgA conjugate with IgG containing cells due to the presence of anti-IgG heavy chain antibodies.

c) Cross-reactive specific staining (XSS)

The observed reaction is caused by the presence of antigenic determinants on other proteins which resemble those determinants on the homologous antigen. The antibodies causing DSS react with the determinants on the other proteins, causing the XSS. Two types of XSS are encountered: 1) XSS caused by the presence of the same molecular structure within a group of proteins, within one species. An example is the XSS caused by anti-Ig conjugates due to cross-reaction via anti-light-chain determinants; 2) XSS caused by the presence of molecular structures which resemble each other in proteins of different species. An example of this kind of XSS may be found in antisera directed against leukaemia viruses. An antiserum directed against mouse leukaemia virus will cross-react with cat or gibbon ape leukaemia virus. Another example of this type of XSS is the reaction of antisera directed against the Fc part of mouse IgG with IgG of the rabbit.

d) Nonspecific staining (NSS)

NSS is caused by nonimmunological reactions. These reactions may be of very diverse origin. The binding of antibody molecules to cells or glass due to excessive fluorochrome loading has been called NSS, as well as autofluorescence of cells or tissues due to suboptimal excitation filters. A distinction has sometimes been made between unavoidable and avoidable NSS. In our opinion, this distinction is confusing.

2. *Titration of antisera and conjugates with Sepharose-bound antigens*

An ideal conjugate should exhibit only DSS. However, in addition to the DSS, antisera always show NSS and sometimes XSS and USS. In the Sepharose bead system the antiserum is incubated in different dilutions with homologous antigen beads and a number of heterologous control beads to ascertain the relationship between these different antiserum reactivities. The homologous beads are coupled with the antigen against which the antiserum was raised. A number of heterologous beads is normally chosen: 1) beads coupled with the most likely contaminants to which antibodies may be present in the antiserum; 2) beads coupled with antigens likely to give cross-reactions; and 3) beads intended to demonstrate NSS. The NSS control consists either of plain beads or beads coupled with an unrelated antigen. Table IV-2 gives a number of examples for a variety of antisera.

The distinction between XSS and USS may become confused in the example of the antimouse immunoglobulin antisera. Reactions with heterologous beads due to the presence of anti-Fc antibodies will normally belong to the USS, whereas the reactions due to the insufficient removal of anti-Fab reactivity will belong to the XSS.

TABLE IV-2. TESTING OF ANTISERUM SPECIFICITY IN THE SEPHAROSE BEAD IMMUNOFLUORESCENCE ASSAY

Antiserum to be tested	Homologous beads	Heterologous beads for testing		
		USS	XSS	NSS
RAOVA	OVA	Conalbumin	BSA HSA	Hu/IgG
RAMTV	MTV	NMS RLV MG	A-particle protein	OVA
GASiSV	SiSV	NRS RaE	RLV GaLV	OVA
RAM/IgG_{2b}	M/IgG_{2b}	M/IgG_{2a} M/IgG_1 M/IgG_3 M/IgA M/IgM	NRS	OVA

Examples for four antisera are presented. Beads coupled with different proteins are used to test the various reactivities of an antiserum or conjugate.

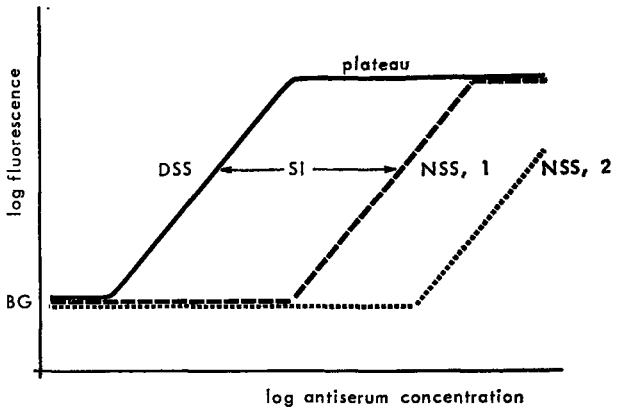


Figure IV-4. Idealized titration curves of antisera reacting with antigens bound to Sepharose beads. Beads coupled with the homologous antigen are used to measure the desired specific staining (DSS). Beads coupled with heterologous antigens serve to determine the undesired specific staining (USS), cross-reactive specific staining (XSS) or nonspecific staining (NSS). The antiserum is incubated with the different bead species in a number of dilutions. NSS 1 and 2 refer to two hypothetical antisera. Abbreviations: BG = background staining; SI = specificity interval.
Ordinate: logarithm of average bead fluorescence in arbitrary units; abscissa: logarithm of antiserum concentration.

Idealized titration curves of an antiserum with different beads are shown in Fig. IV-4. The logarithm of the average bead fluorescence is plotted against the logarithm of the antiserum concentration. Hardly any fluorescence will be detectable at very low antiserum concentrations. The observed fluorescence in this range is called the background (BG). The logarithm of the fluorescence is linearly related to the logarithm of the antiserum concentration within a certain range of antiserum concentrations. The available binding sites on the beads are saturated above this range, resulting in a plateau. A similar result is obtained for both the homologous reaction (labelled: DSS in Fig. IV-4) and the heterologous one. The difference between the concentrations at which the same fluorescence is observed for DSS and NSS constitutes the specificity interval (SI). The titration curves for two hypothetical antisera are shown in Fig. IV-4. Both antisera showed the same DSS but one also showed appreciable NSS (NSS-1) and one had a much lower NSS activity (NSS-2). A result like NSS-1 for the USS or XSS and NSS-2 for the NSS could be envisaged in other cases. It has been shown to be an essential point in the assessment of different conjugates that the NSS and USS curves as functions of concentration are parallel to the DSS curve, but shifted to higher concentrations.

The influence of the affinity of the antibodies on the slope of the curve in the concentration-dependent range is not clear. We have observed quite comparable slopes with a large variety of antigen-antiserum combinations. Complete stoichiometry of the reaction would predict a slope of 1.0 for the curve on double logarithmic paper. This slope is never obtained, possibly because of incomplete mixing of the incubation ingredients or because of steric hinderance at high antiserum concentrations.

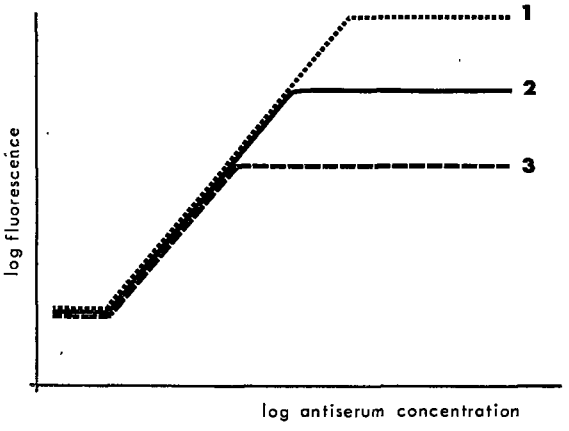


Figure IV-5. The influence of the amount of antigen coupled to Sepharose on the titration curve with an antiserum. The stylized curves indicate that the amount of antigen bound to the Sepharose beads influences only the height of the plateau and not the slope of the curve in the range where the fluorescence depends on the antiserum concentration. The numbers 1–3 refer to decreasing amounts of the same antigen coupled to Sepharose beads (1 is the highest amount of antigen).
Ordinate: logarithm of average bead fluorescence in arbitrary units; abscissa: logarithm of antiserum concentration.

The level of the plateau in Fig. IV-4 is determined for a given antiserum by the number of available binding sites present on the beads. The effect of increasing the amount of antigen per bead is schematized in Fig. IV-5. The slope of the curve will not change, only the plateau level. Haaijman, Bloemmen and Ham (1975) gave experimental evidence for this model, using a Sepharose-ag * ab * ag-FITC system.

In an indirect technique (see also chapter V), the concentration of added conjugate (anti-Ig) may also be limiting, resulting in a plateau. Such effects may be excluded by the titration of the conjugate and antiserum in the indirect technique. Fig. IV-6 (from Haaijman and Brinkhof, 1977) shows a comparison between beads coupled with the IgG fraction of a rabbit antiserum directed against mouse IgG_{2b} (Seph-RAM/IgG_{2b}) incubated with different dilutions of normal mouse serum (NMS) and subsequently with either GAM/Ig-TRITC diluted 1:20 or 1:50. All of the values obtained with the 1:20 conjugate dilution proved to be higher than the comparable values with the 1:50 diluted conjugate. This phenomenon indicates that the beads are participating in the equilibrium reaction. The fact that the logarithm of the staining of beads loaded with small amounts of mouse IgG_{2b} (M/IgG_{2b}) reacted to the same degree on the increase of conjugate concentration as those beads with larger amounts of M/IgG_{2b} is in agreement with the law of mass action. We reached the same conclusion with another system. Fluorescent rabbit antigoat/Ig (RAG/Ig-FITC) was incubated in different dilutions with Rauscher leukaemia virus coupled to beads (Seph-RLV) and Seph-OVA which had been incubated with either a high concentration (1:2) or a low concentration (1:128) of a goat antiserum directed against RLV (GARLV). Results

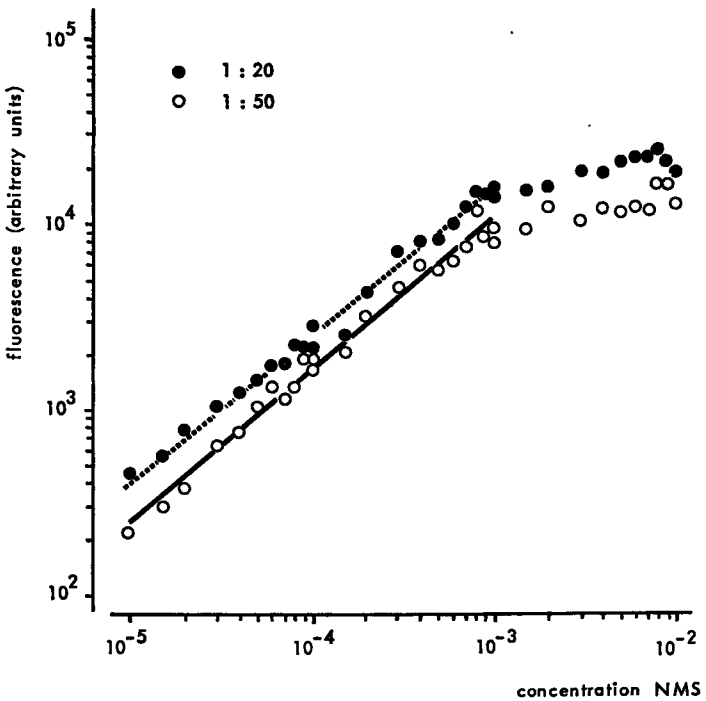


Figure IV-6. The influence of the concentration of fluorescent anti-Ig conjugate on the titration curve. Sepharose beads were coupled with the IgG fraction of a rabbit antiserum directed against IgG_{2b} of the mouse. These beads were incubated with different dilutions of normal mouse serum (NMS). They were subsequently incubated with a fluorescent goat antiserum directed against mouse Ig's (GAM/Ig-TRITC) diluted either 1:50 or 1:20. Each point is the average of at least five individual bead measurements. Concentration of undiluted NMS was taken as 1.

are presented in Fig. IV-7. The curves are essentially parallel and do not show a clear plateau. Interestingly, Wachsmuth and Woodhams (1974) did not observe this phenomenon with chicken erythrocyte nuclei fixed on slides and incubated with ANF followed by different concentrations of fluorescent antihuman/IgG.

A certain paradox has to be mentioned with respect to saturation in the Sepharose bead immunofluorescence system. Whereas saturation is easily obtained in an indirect system, this seems to be very difficult in direct techniques. These data confirm those of Sundqvist (1973) who applied microfluorometry to cells stained vitally with direct and indirect techniques. A number of incubations is listed in Table IV-3, together with an indication of whether saturation was obtained with the indicated components. Only Knapp and Ploem (1974) and Knapp, Menzel and Steffen (1974) obtained clear saturation with a direct technique. It is unlikely that this is due only to the concentration of antibodies, because conjugates prepared from pure antibodies also did not give saturation in the experiments of Knapp, Haaijman, Schuit, van den Berg, Ploem and

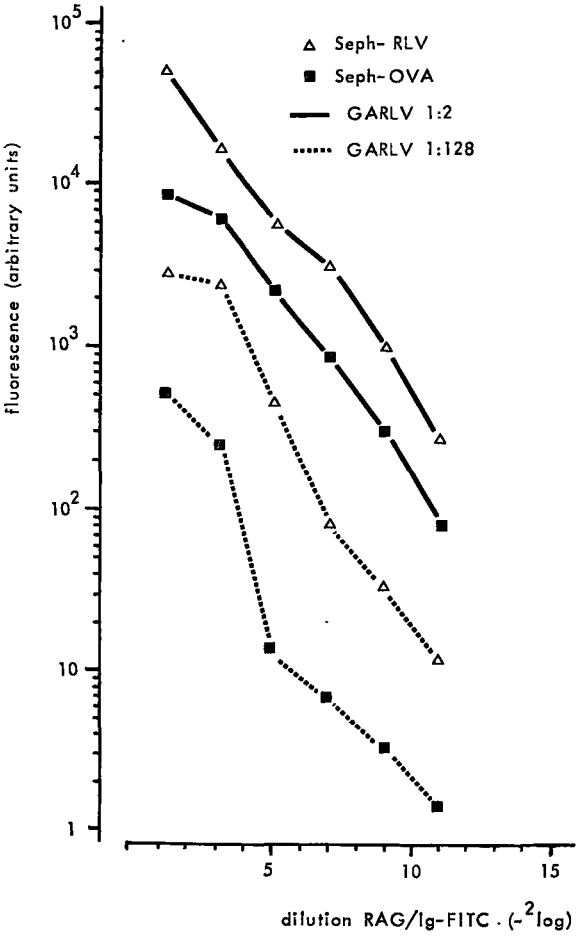


Figure IV-7. Titration of the fluorescent conjugate in the anti-Ig technique. Sepharose beads coupled with Rauscher leukaemia virus (Seph-RLV) and ovalbumin (Seph-OVA) were both incubated with a goat antiserum directed against RLV (GARLV) diluted either 1:2 or 1:128. These beads were subsequently incubated with different concentrations of a fluorescent rabbit antiserum directed against goat Ig's (RAG/Ig-FITC). Each point is the average of ten individual bead measurements.

Hijmans (1975, see their Table 2). The explanation that not all unlabelled antibodies, binding to an antigen bead, are accessible to the anti-Ig conjugate molecules due to steric hindrance is more likely (Linder and Miettinen, 1976). Alternatively, the number of anti-Ig molecules binding to an Ig molecule is concentration-dependent.

The nature of the NSS in the Sepharose bead system is not exactly known. One possibility would be the poor-fit theory (see Roitt, 1975) which states that, in high concentration, molecules will also be bound which do not precisely fit the antigen binding site of an antibody. Richards and Koningsberg (1973) and Richards, Konings-

TABLE IV-3. COMPARISON BETWEEN THE DIRECT AND SEVERAL INDIRECT TECHNIQUES, WITH RESPECT TO SATURATION

Beads	First layer	Second layer	Saturation with first	Saturation with sec.	Reference
Hu/IgM	RAHu/IgM-FITC		−		Knapp, Haaijman, Schuit, van den Berg, Ploem and Hijmans (1975) *
M/IgM	RAM/IgM-FITC		−		Bloemmen, Radl, Haaijman, van den Berg, Schuit and Hijmans (1976)
S. mansoni	HuA*S. mansoni*	SwAHu/IgG-FITC	+		Deelder and Ploem (1975a)
S. mansoni	HuA*S. mansoni*	SwAHu/IgG-FITC		−	Deelder, Snoijink and Ploem (1975a)
ShAHu/IgE	Hu/IgE	ShAHu/IgE-FITC	+		Capel and Aalberse (1975)
M/IgG_{2b}	RAM/IgG_{2b}	HAR/Ig-FITC	+		Haaijman, Bloemmen and Ham (1975)
RAM/IgM	M/IgM	GAM/Ig-TRITC	+	−	Haaijman and Brinkhof (1977)
Hu/IgG	RAHu/IgG	HAR/Ig-FITC	−		Capel (1974)
HAHu/IgG	Hu/IgG	HAHu/IgG-FITC	+		Capel (1974)
Hu/IgG	RAHu/IgG-FITC		+		Knapp and Ploem (1974)
OVA	RAOVA-FITC		±		
OVA	RAOVA	GAR/Ig-FITC	+	−	
OVA	RAOVA	OVA-FITC		+	Haaijman, Bloemmen and Ham (1975)
RLV	GARLV	GAR/Ig-FITC	+	−	
MTV	RAMTV	GAR/Ig-FITC	+	−	
RAMTV	MTV	RAMTV-FITC	+		
Hu/collagen	RAHu/coll-FITC		+		Knapp, Menzel and Steffen (1974)
Hu/collagen	RAHu/collagen	GAR/Ig-FITC	+		Knapp, Menzel and Steffen (1974)

Examples which have not been referenced are unpublished observations.

* The contrast plateau obtained between dilutions 1:8 and 1:40 with this conjugate (their Fig. 2) was due to an increase in the USS and not to saturation of the DSS (c.f.their Table 2).

berg, Rosenstein and Varga (1975) have given evidence for this mechanism as being the basis for the cross-reactivity of antibodies with haptens closely related as to spatial characteristics. We have attempted to test this hypothesis in the following way. If the poor-fit theory also holds for antiprotein antibodies, the ratio between specific and nonspecific staining (contrast) will increase if the antiserum is confronted with a mixture of both homologous and heterologous beads rather than with each kind of beads separately. In the experiment (Table IV-4), human IgG coupled to Sepharose beads (Seph-Hu/IgG), ovalbumin coupled to beads (Seph-OVA) and a mixture of the two were incubated with a mixture of a FITC labelled rabbit antiserum directed against human IgG (RAHu/IgG-FITC) and a TRITC labelled rabbit antiserum directed against ovalbumin (RAOVA-TRITC). Both the FITC and TRITC fluorescence were measured. The TRITC fluor/FITC fluor (simulating the contrast) is given in Table

TABLE IV-4. TEST FOR THE INDEPENDENCE OF HOMOLOGOUS AND HETEROLOGOUS STAINING IN THE SEPHAROSE BEAD IMMUNOFLUORESCENCE ASSAY

Beads	Incubation	Fluor TRITC/fluor FITC
Seph-OVA	RAOVA-TRITC+RAHu/IgG-FITC	53.5±9.5
Seph-Hu/IgG	RAOVA-TRITC+RAHu/IgG-FITC	0.0060±0.0022
Seph-OVA+Seph-Hu/IgG	RAOVA-TRITC+RAHu/IgG-FITC	47.7±16.7 0.0053±0.0009

A mixture of two conjugates with different fluorochromes was incubated with either homologous beads alone or with a mixture of both. After incubation, both the FITC and TRITC fluorescence of 10 individual beads were measured and averaged. The ratio between TRITC and FITC fluorescence with standard deviation is indicated in the table. After incubation of the mixture of the two conjugates with the mixture of the two beads, two clearly distinct populations of ratios were observed, representing the Seph-OVA and Seph-Hu/IgG. Both ratios are indicated.

IV-4. The ratio did not change if a mixture of beads was added to the mixture of conjugates instead of the separate beads. The conclusion is that the presence of the homologous beads does not prevent the binding of antibody molecules to the heterologous beads.

The observation that BSA (Deelder and Ploem, 1975a) and haemoglobin (Bloemmen et al., 1976) may inhibit the nonspecific binding to a certain extent suggests an adsorption phenomenon. This would imply that, in every incubation, a certain proportion of the antibodies will be bound nonspecifically. Preabsorption of antisera with Seph-OVA reduces the nonspecific binding (see below p. 85). This latter phenomenon indicates that only a certain fraction of the antiserum is likely to be adsorbed to the heterologous beads.

H. INFLUENCE OF ANTISERUM FRACTIONATION AND F/P RATIO IN THE SEPHAROSE BEAD IMMUNOFLUORESCENCE SYSTEM

Antisera or conjugates have to be tested not only for their DSS but also for their USS and XSS properties. It is important, therefore, to evaluate some seemingly trivial aspects of the Sepharose bead immunofluorescence test, which may influence and even obscure the specificity spectrum of antisera.

1. *Fractionation of antisera*

The chemical composition of the antiserum influences the apparent specificity spectrum in the Sepharose bead system. Unfractionated serum has a much greater tendency to bind nonspecifically to Sepharose beads than do globulin or IgG fractions. This phenomenon is evident after comparing Fig. IV-8A and -B. An antiserum raised in a rabbit against disrupted mouse mammary tumour virus (MTV) was tested in these experiments. The antiserum was tested for contaminants directed against normal

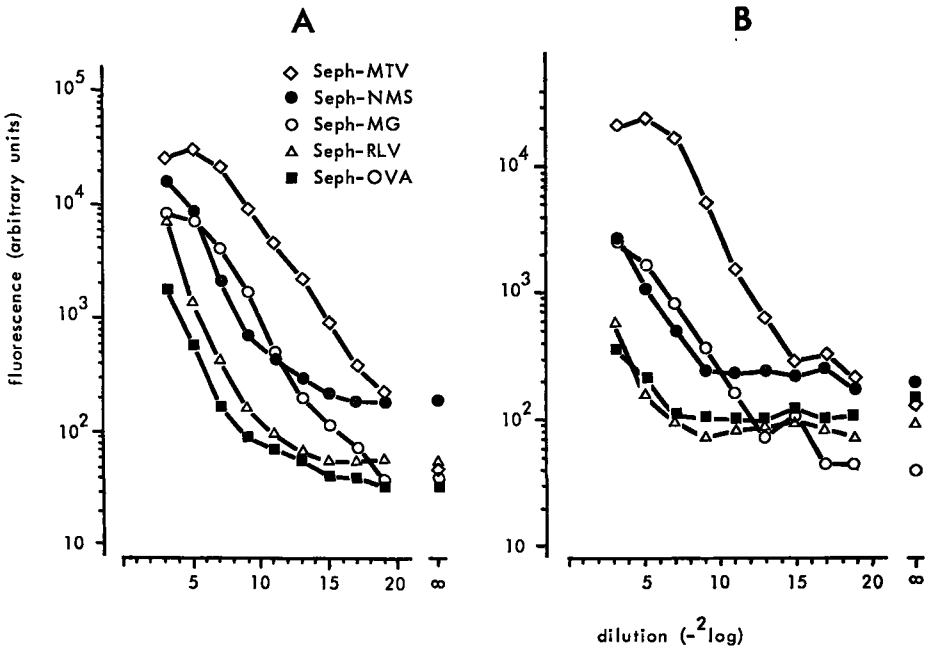


Figure IV-8. The influence of the fractionation of antisera on their specificity in the Sepharose bead immunofluorescence system. An unfractionated (left hand figure) rabbit antiserum directed against mouse mammary tumour virus (RAMTV) and the globulin fraction (right hand figure) of the RAMTV were incubated in different dilutions with Sepharose beads coupled with mouse mammary tumour virus (Seph-MTV), extract of lactating mammary glands (Seph-MG), normal mouse serum (Seph-NMS), Rauscher leukaemia virus (Seph-RLV) and ovalbumin (Seph-OVA). The antiserum had been absorbed beforehand *in vitro* with Seph-NMS and Seph-MG. The beads were subsequently incubated with GAR/Ig-FITC, absorbed with Seph-NMS. Each point is the average of at least 5 individual bead measurements. ∞ represents the conjugate control, in which the different beads are incubated only with GAR/Ig-FITC.

mouse serum (NMS), lactating mammary gland extract (MG) and Rauscher leukaemia virus (RLV). Seph-OVA was included for the estimation of NSS. The NSS of this antiserum was already substantially reduced by taking only the globulin fraction (Fig. IV-8B) in stead of total serum. The reason for this phenomenon can be twofold, in our opinion: a) in unfractionated serum, albumin with a tendency to adsorb FITC (Andersson, Rehnström and Eaker, 1973) and to form complexes with immunoglobulins (Hauptman and Sobczak, 1976) is responsible for NSS; and b) complement factors present in unfractionated serum adsorb to the Sepharose matrix. These adsorbed complement components are then stained by anticomplement antibodies in the fluorescent conjugate.

Direct evidence for either of these two hypotheses is not available. The repeated observation that unfractionated serum but not globulin or IgG fractions tend to show a prozone effect is perhaps in line with the first possibility. The prozone effect has

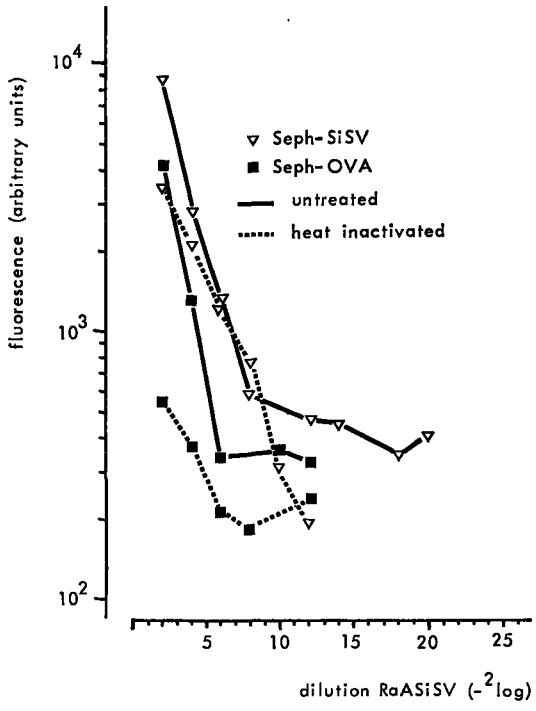


Figure IV-9. The influence of heat inactivation of antisera on the NSS. Sepharose beads coupled with disrupted simian sarcoma virus (Seph-SiSV) and Seph-OVA were incubated with a rat antiserum directed against SiSV (RaASiSV) either untreated or heat inactivated. Heat inactivation took place for 30 min at 56 °C. The beads were subsequently incubated with a 1:50 diluted fluorescent goat antiserum directed against rat Ig's (GARa/Ig-FITC, lot no. 7-1093; Nordic Immunological Laboratories). Each point represents the average fluorescence intensity of at least 5 individual beads.

been explained by either the formation of soluble antigen-antibody complexes (Zweibaum, Halpern, Palou and Veyre, 1966; Johnson and Holborow, 1973) or to steric hindrance of antibody molecules (Bellon and Druet, 1974; Linder and Miettinen, 1976). It is also possible, in our opinion, that adsorption of too much albumin from total serum to Sepharose beads inhibits the specific binding of antibodies. Wachsmuth and Woodhams (1974) did not observe a prozone effect in their quantitative ANF systems. Complement binds spontaneously to IgG linked covalently to Sepharose beads (Bing, 1971), to native Sepharose and to Sephadex (Knapp, 1974). It has been observed that treatment of certain sera for 30′ at 56 °C reduces the NSS (Fig. IV-9). In this system, the NSS is measured as the reactivity towards a nonrelated antigen. The evidence presented above is of course circumstantial. The presence of anticomplement antibodies in anti-Ig conjugates has not been demonstrated.

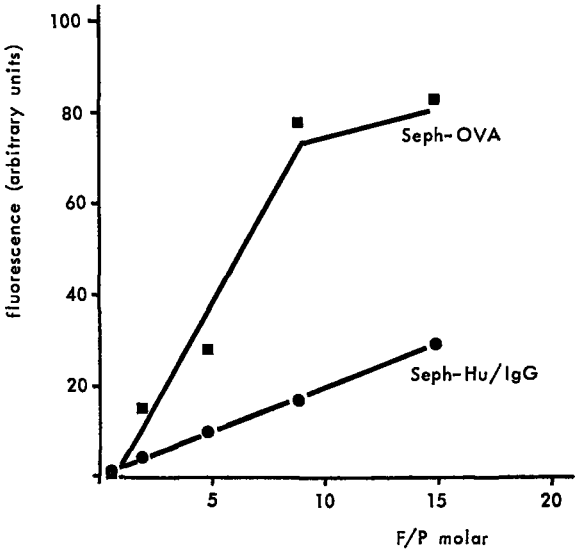


Figure IV-10. The influence of F/P ratio in the Sepharose bead immunofluorescence system. Beads coupled with ovalbumin (Seph-OVA) and human IgG (Seph-Hu/IgG) were incubated with a rabbit antiserum directed against OVA (RAOVA). The antiserum was conjugated with different amounts of TRITC. The RAOVA preparation consisted of pure antibodies isolated by means of affinity chromatography. The molar F/P ratio was determined spectrophotometrically. Each point is the average fluorescence intensity of at least 10 individual beads. The fluorescence of Seph-OVA and Seph-Hu/IgG incubated with a conjugate with a F/P ratio of 0.5 were taken as 1.0. All conjugates had the same protein content and were diluted 1:20.

2. *Influence of F/P ratio*

The number of fluorochrome molecules per molecule of protein (F/P ratio) has been held to be responsible for the NSS properties of conjugates (Hebert, Pittman and Cherry, 1967, 1971; Beutner, 1971b; Wick, 1972). We have tested the influence of the F/P ratio in the Sepharose bead system (Fig. IV-10). Different aliquots of the same antibody preparation of RAOVA were conjugated with different amounts of fluorochrome (TRITC). Conjugate and free dye were separated on a Sephadex-G50 column. The protein content of the different conjugates was equalized and the F/P ratios were determined. The molar absorbance of TRITC, as determined by van Dalen and Haaijman (1974), was used. Seph-OVA and Seph-Hu/IgG beads were incubated with the same amount of conjugates. The contrast (DSS/NSS: reaction with Seph-OVA divided by the reaction with Seph-Hu/IgG) increased up to a F/P ratio of 9. The antigen binding sites seem to be affected in the conjugate with a F/P ratio of 15, resulting in decreased DSS. The NSS increased linearly up to a F/P ratio of 15. This latter observation is in accordance with the previously mentioned reports (see also Pillot, 1972). It is evident, however, that the contrast measured in the Sepharose bead system is not directly comparable to the visual image contrast to be obtained in quali-

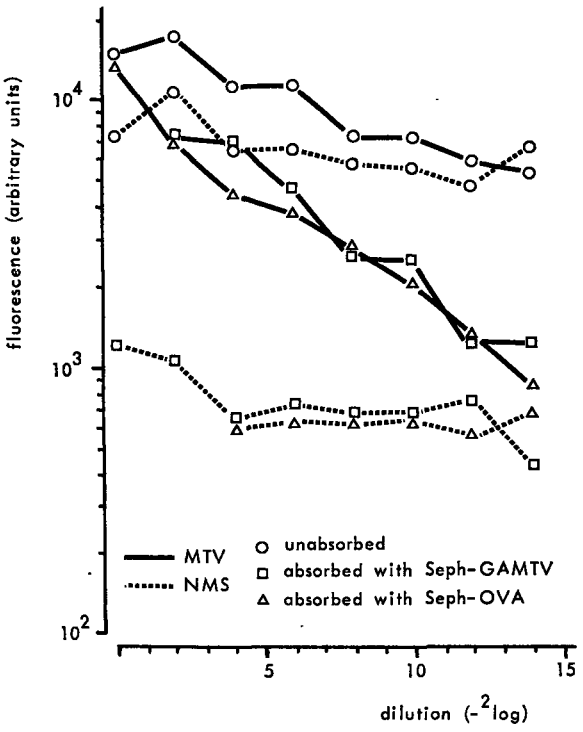


Figure IV-11. The influence of the absorption of the indicator conjugate in an indirect technique. Sepharose beads were coupled with a goat antiserum directed against mouse mammary tumour virus (Seph-GAMTV). These beads were incubated with different dilutions of MTV and NMS and subsequently with a fluorescent rabbit antiserum directed against MTV (RAMTV-FITC) either untreated, preabsorbed with Seph-GAMTV, or preabsorbed with Seph-OVA. The MTV and NMS samples contained the same amount of protein/ml. Each point is the average fluorescence of at least 5 individual beads.

tative immunofluorescence microscopy, as the influence of the F/P ratio on the NSS of conjugates with cells is certainly different from the NSS with Sepharose beads. In qualitative IF microscopy, F/P ratios around 1.0 are mostly sought. Interestingly, Bergquist and Nilsson (1975) reported a lower fluorescence intensity of glutaraldehyde polymerized antigen beads incubated with a conjugate with a F/P ratio of 5.3 than with a F/P ratio of 1.2. These authors attributed this phenomenon to fluorescence quenching. We have not been able to confirm their results. If the interpretation of Bergquist and Nilsson (1975) is correct, it reflects the very high antigen density in their model system as compared to that in the Sepharose bead immunofluorescence system.

3. *Influence of reactivities of the fluorescent anti-Ig for the testing of antisera by the indirect technique*

Fig. IV-11 shows a specificity test for a goat antiserum raised against mouse mam-

mary tumour virus (GAMTV) coupled to Sepharose beads. The GAMTV beads were first incubated with different dilutions of MTV and NMS and subsequently with a fluorescent rabbit antiserum directed against MTV (RAMTV-FITC). Without absorbing the conjugate hardly any contrast between MTV and NMS is seen. The lack of contrast gives the impression that the GAMTV present on the beads is higly nonspecific. However, preabsorption of the indicator conjugate (RAMTV-FITC) with either Seph-GAMTV or Seph-OVA reduced the nonspecific binding in such a way that the specificity of GAMTV for MTV could be established beyond doubt. From this experiment it is clear that, in an indirect system, not only the specificity of the first layer but also that of the second layer is of importance.

I. ANTISERUM AND CONJUGATE EVALUATION WITH ANTIGENS BOUND TO SEPHAROSE BEADS

A typical result of an antiserum test as reported by Haaijman and Brinkhof (1977) is shown in Fig. IV-12. Beads were coupled with different purified mouse Ig's including the Fab fragments of heterogeneous IgG. These beads were incubated with different dilutions of a rabbit antiserum directed against IgG_{2b} of the mouse (RAM/IgG_{2b}, prepared by Dr. J. Radl). They were subsequently incubated with a FITC conjugated goat antiserum directed against rabbit Ig's (GAR/Ig-FITC, lot no. 10-273; Nordic Immunological Laboratories). The GAR/Ig-FITC conjugate had to be preabsorbed with Seph-NMS in this particular experiment in order to remove cross-reactive antibodies. Seph-OVA served as a control for the NSS properties of RAM/IgG_{2b}. The RAM/IgG_{2b} reacted most strongly, as was expected, with the M/IgG_{2b} beads. A plateau was observed up to a $-^5$log dilution of 3. In higher antiserum dilutions, the fluorescence decreased with dilution. The fluorescence values presented in Fig. IV-12 are the average fluorescence values of 10 individual beads. The values are always corrected for instrumental background fluorescence. The USS (or XSS, see page 72) properties of this particular RAM/IgG_{2b} antiserum were quite acceptable. It is evident, however, that the antiserum reacts more strongly with the other mouse Ig beads than with the OVA beads. The conjugate controls in which the beads were incubated only with GAR/Ig-FITC indicated that this phenomenon is due to residual cross-reactivity of the fluorescent conjugate.

In an immunological system for testing the specificity of antisera, the question as to whether an observed reaction is due to impurity of the antigen or of the antiserum is most important. The bead system is one in which this has been studied extensively (Knapp et al., 1975; Bloemmen et al., 1976). An answer to the question of antigen or antiserum impurity can be obtained by cross-absorptions. This approach is summarized in Table IV-5.

Possibilities 1 and 2 are usually the most likely ones to be met in practice. Table IV-6 gives an example of the results obtained with this system using a rabbit antiserum directed against MTV. The data in Table IV-6 suggest that: 1) RAMTV contained a

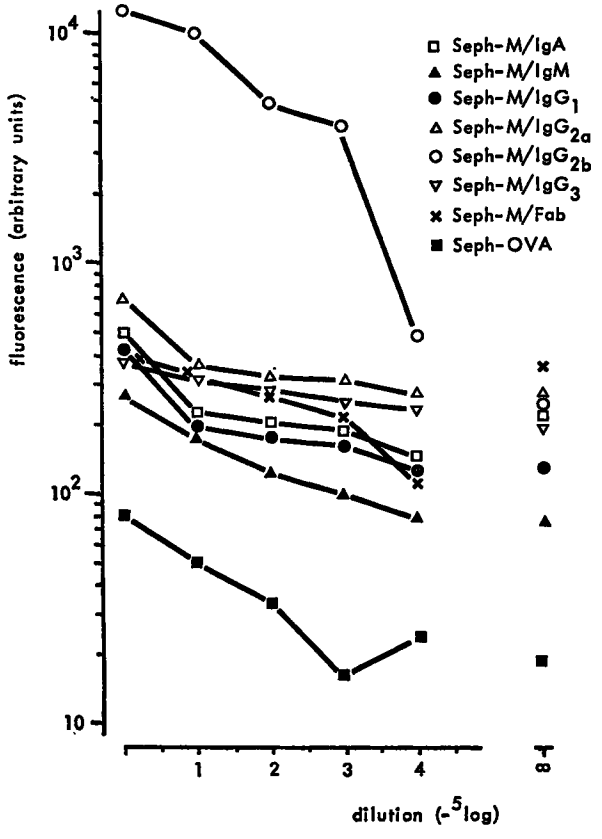


Figure IV-12. Specificity test for a rabbit antiserum directed against IgG$_{2b}$ of the mouse (RAM/IgG$_{2b}$). Sepharose beads were coupled with IgA, IgM, IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$, Fab fragments of heterogeneous IgG of the mouse and ovalbumin. These beads were incubated with different dilutions of RAM/IgG$_{2b}$ and subsequently with a fluorescent goat antiserum directed against rabbit Ig's (GAR/Ig-FITC). The GAR/Ig-FITC was preabsorbed with Seph-NMS. Plotted values represent the average fluorescence intensity of at least 5 individual beads. ∞ represents the conjugate control. Dilution zero represents an antiserum dilution of 1:50.

component causing NSS, which could be removed with Seph-OVA as well as with native Sepharose; 2) RAMTV contained antibodies against an antigen present in the lactating mammary gland extract (MG); 3) RAMTV contained antibodies against an antigen present in both the MG extract and normal mouse serum (NMS); and 4) the MTV coupled to Sepharose beads and used to determine the DSS did not contain antigens common to MG or NMS. In other words, the MTV was free of contaminants according to the criteria used in this test. Had the MTV contained, for instance, proteins also present in NMS, then the reaction of RAMTV with MTV should have decreased upon the absorption of the antiserum with Seph-NMS.

TABLE IV-5. ABSORPTION SCHEME FOR AN IMAGINARY ANTISERUM OR FLUORESCENT CONJUGATE DIRECTED AGAINST ANTIGEN A

Observation: Conjugate anti-A reacts strongly with Seph-A, less with Seph-B and hardly with Seph-C.

Hypothesis	Absorption with		Test after absorption	
	Seph-A	Seph-B	Seph-A	Seph-B
1. anti-A contains[1]) anti-B	−[2])	−	+++[3])	+++
	+[4])	−	↓↓↓[5])	=
	−	+	=	↓↓↓
2. B contains A	+	−	↓↓↓	↓↓↓
	−	+	↓	↓↓↓
3. anti-A contains anti-B	+	−	↓↓↓	↓
B contains A	−	+	↓	↓↓↓
4. anti-A contains anti-B	+	−	↓↓↓	↓↓↓
A contains B	−	+	↓↓	↓↓↓

[1]) Contains means impurity (small in comparison to the major component).
[2]) Not absorbed.
[3]) Observed reaction.
[4]) Absorbed with Seph-A.
[5]) ↓↓↓: strong reduction, ↓↓: intermediate reduction, ↓: little reduction, =: no reduction, upon absorption.

The antiserum is incubated for 1 hr with the indicated absorption beads. After incubation, the antiserum is separated from these beads and incubated with the test beads. For the indirect technique, this incubation is followed by an incubation with fluorescent conjugate. The fluorescence of the individual beads is measured after washing and compared with the fluorescence values obtained without absorption. Seph-C serves as the NSS control.

TABLE IV-6. THE ABSORPTION OF RAMTV

Absorption *				Fluorescence values			
Seph	Seph-OVA	Seph-NMS	Seph-MG	Seph-MTV	Seph-MG	Seph-NMS	Seph-OVA
−	−	−	−	9128	4517	6943	993
+	−	−	−	8034	3014	6443	271
−	+	−	−	10072	3791	6603	320
+	+	+	−	8427	1794	1369	227
+	+	−	+	9462	1429	5073	228
+	+	+	+	9320	1438	931	151

Values represent the average fluorescence of 10 individual beads.
* 300 µl of 1:60 diluted RAMTV was incubated for one hour with 50 µl of packed beads of the indicated varieties. After separating the antiserum from the beads, the antiserum was incubated with, respectively, Seph-MTV, Seph-MG, Seph-NMS and Seph-OVA. Bound antibodies were detected with GAR/Ig-FITC preincubated with Seph-OVA (1 ml of 1:20 diluted conjugate per 400 µl of packed beads).

J. THE RELATIONSHIP BETWEEN THE RESULTS OBTAINED WITH CONJUGATES IN THE SEPHAROSE BEAD IMMUNOFLUORESCENCE SYSTEM AND THE BEHAVIOUR OF CONJUGATES IN BIOLOGICAL SYSTEMS

The development of test systems based on the coupling of antigens to artificial substrates was prompted by the lack of suitable biological substrates for the testing of antisera against mouse Ig's (Bloemmen et al., 1976). To evaluate the results obtained with the model system, it was necessary to 'calibrate' the results of the model system on the results obtained with the same antisera in a biological test system. For this purpose, Knapp et al. (1975) compared the behaviour of six conjugates in both the model system and the monoclonal bone marrow system (Hijmans, Schuit and Klein, 1969). The aim of this study was to express the quality of a given conjugate in terms of the reactivities of that conjugate with beads coupled with homologous and heterologous antigens.

What factors will influence the suitability of conjugates in biological systems? The observer of an IF slide will appreciate a number of variables: 1) the brightness of the desired specific staining (DSS); 2) the amount of USS type 2, if substrates to test this USS are available; 3) the amount of XSS, if this is important; and 4) the amount of NSS. He will possibly make a distinction between the NSS of cells or tissue and that of empty parts of the slide. The relationship between the variables is important in this respect. Conjugates or antisera exhibiting a strong DSS are allowed to have a greater degree of NSS or USS/XSS properties than weaker antisera. The image contrast is the parameter which counts most for visual evaluation.

Because the DSS is a fixed parameter inherent to the antiserum, much effort has been exerted to reduce the amount of NSS. The group of von Mayersbach (von Mayersbach, 1972; Arnold, Kalden and von Mayersbach, 1975) has approached the NSS problem from the substrate side. They investigated the influence of fixation, embedding and washing techniques on the degree of NSS caused by certain conjugates. Different techniques for immunohistochemical processing were reviewed by Feltkamp-Vroom (1975).

A special form of NSS is caused by autofluorescence; it has no relationship to the conjugate as such. Autofluorescence is a poorly understood phenomenon. It may be caused by different tissue or cell constituents. Prolonged storage of preparations sometimes increases this kind of NSS (Nairn, 1976). Appropriate and selective filtering of the excitation light for the fluorochrome to be traced is often the only way to reduce this kind of NSS.

NSS may be increased from the conjugate side of the system by free dye present in the conjugate (Beutner, 1971b; Nisengard and Beutner, 1972), a too high F/P ratio (Hebert, Pittman and Cherry, 1967; The, 1967; Calcagno, Sweeney and Oels, 1973) and the presence of overlabelled antibodies (Pittman, Hebert, Cherry and Taylor,

1967; Arnold and von Mayersbach, 1972; Forsum, 1972; Lyerla and Hierholzer, 1975). More trivial aspects of the preparation of substrates can also induce NSS. It was observed by us, e.g., that batches of glycerol kept for too long at room temperature induced strong NSS confined to the cell nuclei with some conjugates, This NSS can be so strong in the monoclonal bone marrow system that reliable conjugate evaluation is no longer possible. We now keep the stock solution of glycerol at 4 °C and new batches of buffered glycerol are prepared weekly.

USS phenomena are of major concern in the evaluation of antiserum or conjugate specificity. USS type 2 is caused by antibodies in the antiserum which are directed against impurities in the antigen preparation used for immunization. The word impurity already suggests that it is to be regarded as a minor component of the antigen preparation. This condition does not necessarily imply that the antibodies in the antiserum produced by the animal against the impurity also constitute a minor part of the antiserum. Some impurities can be much more immunogenic than the antigen against which the antiserum was intended to be raised.

The means of removing USS, if it is not stronger than the DSS, are dilution of the antiserum or further purification. The technique of affinity chromatography has been of great importance in IF microscopy (Avrameas and Ternynck, 1969; Radl, van den Berg, Voormolen, Hendriks and Schaefer, 1974) for the removal of antibodies to known impurities. The logical step further to purify antibody preparations, by eluting them from antigen immunoadsorbent columns, has not given the great advantages one would have anticipated. Several reasons may account for this: 1) The long-term stability of antibody conjugates seems not as good as that of, for instance, IgG preparations. It is often necessary to add stabilizing proteins and the conjugates should be stored at −70 °C or lower. 2) Elution of bound antibodies by either acid pH, urea or other chaotropic compounds causes denaturation which is difficult to control (Murphy, Imam, Hughes, McGucken, Buchanan, Conlon and Elmore, 1976). 3) The preparation of antigen columns of sufficient capacity and sufficient purity is often difficult. 4) Widespread application of commercial antibody conjugates will be limited due to financial costs. This means that they are not likely to be used in clinical IF microscopy. 5) Other factors, a number of which has been mentioned above, play a role in the eventual conjugate quality. The special efforts which are inherent in the preparation of antibody conjugates will be reserved for special cases.

Conjugates prepared from IgG fractions of antisera and absorbed for impurities on solid immunoadsorbents seem to be suitable for most systems. Solid immunoadsorbents have several advantages over conventional absorption techniques for conjugate purification: a) Conjugates may be mixed without risking the precipitation of either conjugate with the added proteins meant for absorption. Mixing of conjugates is convenient in the double wavelength technique because of time saving. b) Only adding absorbing protein in an optimal quantity will completely remove the unwanted specificities from an antiserum. Too much or too little will result in soluble

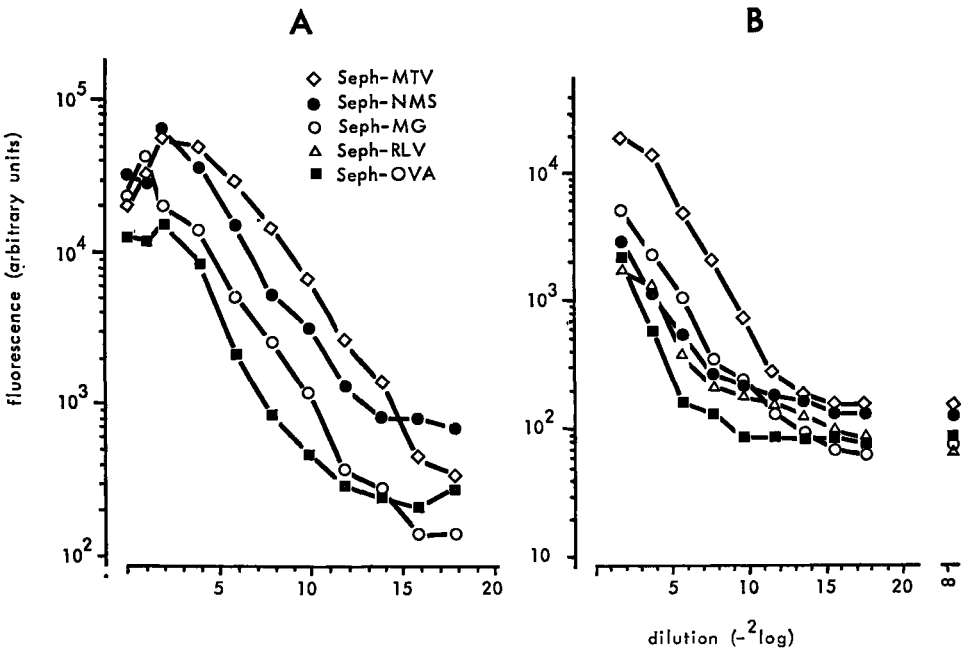


Figure IV-13. *In vivo* absorption of a rabbit antiserum directed against mouse mammary tumour virus (RAMTV). Sepharose beads coupled with MTV, MG, NMS, RLV and OVA were incubated in different dilutions with untreated RAMTV (A) and RAMTV absorbed *in vivo* (B). Beads were subsequently incubated with GAR/Ig-FITC diluted 1:50. One ml of untreated RAMTV was injected i.p. into a BALB/c mouse for the *in vivo* absorption. Serum was collected 24 hr later. Each point is the average fluorescence intensity of at least 5 individual beads. ∞ represents the conjugate control. In A the Seph-RLV was not used.

immune complexes which may still cause USS. c) A good immunoadsorbent may be reused indefinitely. d) A practical point concerning the conventional absorption with liver powder for the removal of both USS and NSS (Coons and Kaplan, 1950), is the vulnerability of conjugates treated in this way to microbial contamination. Sterilizing the conjugates by Millipore filtration results in a substantial loss of antibody concentration. e) Another method is the *in vivo* absorption. This method has been applied quite often in the preparation of antivirus antisera (Daams, Hageman, Calafat and Bentvelzen, 1973) and recently also in a very elegant procedure to remove unwanted antibodies from rabbit antimouse brain antisera (Loor and Roelants, 1975). An example of *in vivo* adsorption is given in Fig. IV-13. A rabbit antiserum raised against MTV from which antibodies directed against mouse proteins had to be removed was injected intraperitoneally into mice. The complexes formed between antibodies and antigens are presumably removed by the macrophages. The mice were bled twenty-four hours later. Unabsorbed RAMTV (total serum) was tested in the experiment represented in Fig. IV-13A. Fig. IV-13B shows the result after *in vivo* absorption. The reactivity towards Seph-MG was, interestingly, not as effectively removed *in vivo* as

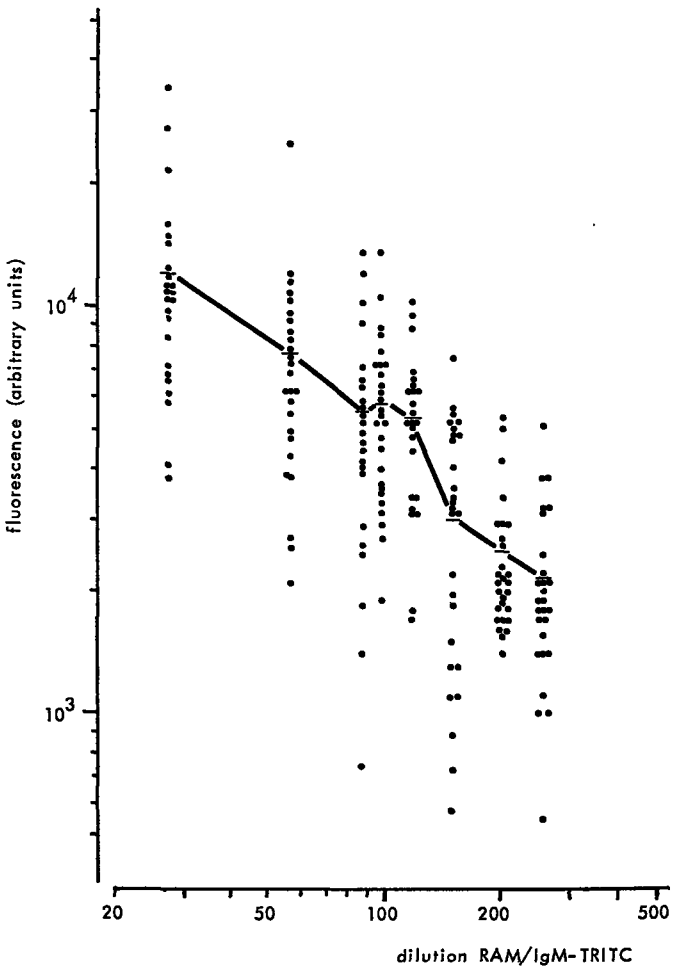


Figure IV-14. Quantitation of intracellulary bound fluorescent antiserum. Cytocentrifuge slides were prepared from a cell suspension of mouse spleen. The cells were fixed with acid ethanol. The slides were incubated with different dilutions of a fluorescent rabbit antiserum directed against IgM of the mouse (RAM/IgM-TRITC). Individual fluorescent plasma cells were located with a 100 W halogen lamp. The fluorescence of individual cells was measured, using a 100 W mercury arc. Individual fluorescence values are indicated together with the averages.

was the anti-NMS activity. A drawback of the *in vivo* absorption is the reduction in DSS. For other reasons mentioned above, we have avoided *in vivo* absorption for our antivirus antisera and have relied on *in vitro* methods.

Two conditions should be taken into account when comparing reactions of anti-human Ig antisera or conjugates in the Sepharose bead immunofluorescence system and in the monoclonal bone marrow system:

a) The relationship between the concentration of Ig's in cells and the concentration

of the conjugate in the working dilution (Knapp et al., 1975) is different from the concentration relationship normally encountered in the Sepharose bead immunofluorescence system. The working dilution of a conjugate is determined by titration of the conjugate on a suitable substrate. The highest dilution of a conjugate at which the DSS is still scored visually as '+ +' is determined and the working dilution is taken to be a 1 step less diluted conjugate. The concentration difference covered in one step depends on the concentration range. A conjugate, e.g., whose last '+ +' dilution is 1:60 will be used at 1:40, whereas one which can be diluted 1:16 will be used at 1:12. The conjugate is applied in working dilution to a number of monoclonal test substrates for testing the conjugate specificity.

We have attempted to quantitate this 'visual plateau' in Fig. IV-14. Cytocentrifuge slides of mouse spleen cells were incubated with different dilutions of a TRITC conjugate of a rabbit antiserum directed against mouse IgM (RAM/IgM-TRITC). A low intensity light source (100W halogen lamp) was used to locate the fluorescent plasma cells. The fluorescence of the cells was measured, however, with a 100W mercury arc. In Fig. IV-14, the individual values and the averages per dilution are plotted versus dilution. Tenfold differences in the fluorescence intensities of individual cells were encountered within single preparations treated with one dilution of conjugate. The working dilution of this particular conjugate for qualitative IF had been determined to be 1:60. No clear plateau is observed in Fig. IV-14. It cannot be argued that a number of the measured cells in Fig. IV-14 was below the visual threshold, because the cells were located with a halogen lamp. This implies that the fluorescence of these cells is clearly above the visual threshold, even more so if a 100W mercury arc is used.

It must be concluded that quantitation of the fluorescence of cells treated with different concentrations of conjugate does not give indications about the working dilution of that conjugate for qualitative immunofluorescence microscopy. This conclusion was not reached by Albini, Herzog and Wick (1972) who reported a steep drop of the measured fluorescence intensity for conjugates diluted further than the visual endpoint titre. An explanation for the discrepancy between their observations and ours may be that we have used a direct technique which shows usually less distinct saturation phenomena (see part G of this chapter).

Our observations make it difficult to select a single dilution of conjugate to characterize the specificity of that conjugate in a model system. The parameter independent of dilution in the bead system is the specificity interval (SI) indicated in Fig. IV-4. The determination of the SI value of a number of conjugates may be difficult. Firstly, because the estimation requires a lot of conjugate and, secondly, because, with some good conjugates, the NSS or USS of undiluted conjugate is still very low. If the possibility exists to measure the SI value, it gives more information than the concentration-dependent parameters of contrast (DSS/NSS) and %S (100 [USS-NSS]/[DSS-NSS]) introduced by Knapp et al. (1975) and Bloemmen et al. (1976). The %S value is a measure of the degree to which an antiserum

reacts with heterologous beads. Bloemmen et al. (1976) attempted to set standards for the amount of %S of conjugates which may be tolerated of conjugates intended for qualitative immunofluorescence. They applied different dilutions of a conjugate to cytocentrifuge preparations and counted the number of fluorescent cells per slide. Above certain conjugate dilutions, the number of positive cells began to decrease per cytocentrifuge slide. The dilution of conjugate at which this occurred was applied to homologous beads. They compared the fluorescence of the beads incubated with this dilution with beads incubated with the working dilution of the conjugate. The criterion of Bloemmen et al. (1976) of 40% reduction in the number of visible fluorescent cells seems arbitrary. It has to be taken into account, however, that the NSS of a conjugate is also concentration-dependent. It is quite possible that, with a NSS occurring at the working dilution of the conjugate, the cells Bloemmen et al. (1976) saw at a conjugate dilution of 1:800 would have been invisible. In other words, the %S values for the acceptability of a given conjugate mentioned in the report of Bloemmen et al. (1976) are either under- or overestimated. A way to avoid this problem is to add increasing amounts of, e.g., RAM/IgM-TRITC to a preparation stained optimally with, e.g., RAM/IgA-TRITC. The number of positive cells is then counted. The amount of added RAM/IgM-TRITC resulting in a significant increase in the total number of fluorescent cells can be considered as the limit of acceptability. This mixture of RAM/IgM- and RAM/IgA-TRITC is then tested on beads coupled with IgM and IgA to give the wanted %S value. A complication of this approach is the need to correct for the F/P ratio of the RAM/IgM-TRITC. Only antibody preparations can be used for this experiment.

b) Unexpected results may be obtained in the comparison of the behaviour of conjugates with beads and cells if the antigen on the beads contains impurities not normally present in the biological test system. As an example, IgG preparations coupled to the beads might contain traces of fibrinogen and antisera might contain antifibrinogen antibodies (Antoine and Neveu, 1968). These antisera may prove perfectly acceptable for the tracing of IgG cells in bone marrow preparations, whereas they will seem unacceptable when tested on the beads. The other possibility may also occur: the antiserum contains antibodies against proteins not coupled to the beads. The impurities can make the antisera unsuitable to work with biological substrates, whereas they behave perfectly in the Sepharose bead immunofluorescence system. This latter pattern has been repeatedly observed with goat antisera raised against RNA tumour viruses. These sera show highly nonspecific reactions with cells for some unknown reason. They can be quite acceptable in the Sepharose bead immunofluorescence system. A major contaminant of these sera may be the antibodies directed against tissue antigens. The purification of tissue antigens for coupling them to beads has not yet been attempted.

K. CONCLUSIONS

1) A model system like the Sepharose bead system is a very valuable tool for making an inventory of the specificities of an antiserum.
2) The suitability of a conjugate or an antiserum for working with biological material can best be assessed by taking the specificity interval (SI) into consideration. The specificity interval is the difference in antiserum concentration giving the same DSS and USS or NSS (Fig. IV-4).
3) The apparent specificity of antisera in the bead system is influenced by: a) concentration; b) F/P ratio; c) anti-Ig conjugate, in the indirect technique; and d) chemical composition. The apparent specificity increases in the order: total serum, globulin fraction, IgG fraction, pure antibodies.
4) Quantitative measures have not yet been satisfactorily determined for conjugate quality in qualitative immunofluorescence microscopy.
5) Direct comparison between conjugate specificity in the Sepharose bead system and in the performance test on biological substrates is hampered by the following factors: a) Inadequate data reduction of the results obtained with the bead system. A solution for this difficulty could be the introduction of the SI value for a number of conjugates; b) Quantitation of the reaction of antisera with cells remains difficult (Fig. IV-14); c) The mechanism of non-specific staining in biological systems is still poorly understood.

CHAPTER V

Preparative procedures in immunofluorescence microscopy with emphasis on the application of single and multilayer techniques in the Sepharose bead immunofluorescence system

Contents:

A. INTRODUCTION

A number of incubation schemes based on different immunological phenomena is in use or has been described for immunofluorescence microscopy. In this chapter, we will review these different methods and discuss in more detail those which are used in the fluorescence immunoassays of chapter VIII.

In immunofluorescence studies, a distinction is classically made between direct and indirect techniques. In the direct technique (Coons, Creech and Jones, 1941), the component of interest is demonstrated after incubation with a fluorescent tracer. A number of incubations are required in the indirect technique. The best known indirect technique is the antiglobulin or anti-Ig method, first described by Weller and Coons (1954). The direct and indirect (anti-Ig) methods are schematized in Fig. V-1.

Two forms of the direct technique are known: the fluorescent antibody method and the fluorescent antigen method. The first is used for the tracing of antigens in tissues or cells and the second for the tracing of specific antibodies.

In the anti-Ig method, the preparation is first incubated with an unlabelled antiserum directed against the antigen of interest. The binding of the unlabelled antibodies is demonstrated after a second incubation with a fluorescent antiserum directed against the Ig molecules of the animal species in which the unlabelled antiserum was raised.

Many discussions have been devoted to the respective merits of the direct and the indirect techniques in IF microscopy. The direct technique has been advocated for its specificity and the indirect one for its sensitivity. In part B of this chapter, a number of experiments in which the direct and the indirect techniques are compared using the Sepharose bead immunofluorescence system is reported.

In part C, indirect techniques other than the anti-Ig method are discussed. Emphasis is laid on two sandwich techniques which have proved to be of value in fluorescence immunoassays.

Double staining techniques have been shown to represent a major advance in immunofluorescence microscopy. Proteins are conjugated with either FITC or TRITC and used on the same preparation. Technical requirements for discriminating between the fluorescence of these two fluorochromes have been discussed in chapter II, part D4. In part D of this chapter, the double staining technique is first introduced and then the interaction between antibodies directed against different parts of the same molecule is studied with the Sepharose bead immunofluorescence system. These experiments were necessary to demonstrate the validity of double staining procedures.

B. COMPARISON OF THE SPECIFICITY AND SENSITIVITY OF THE DIRECT TECHNIQUE AND THE ANTIGLOBULIN TECHNIQUE, USING ANTIGENS BOUND TO SEPHAROSE BEADS

The following experiments were done to test the prevailing opinion that the direct

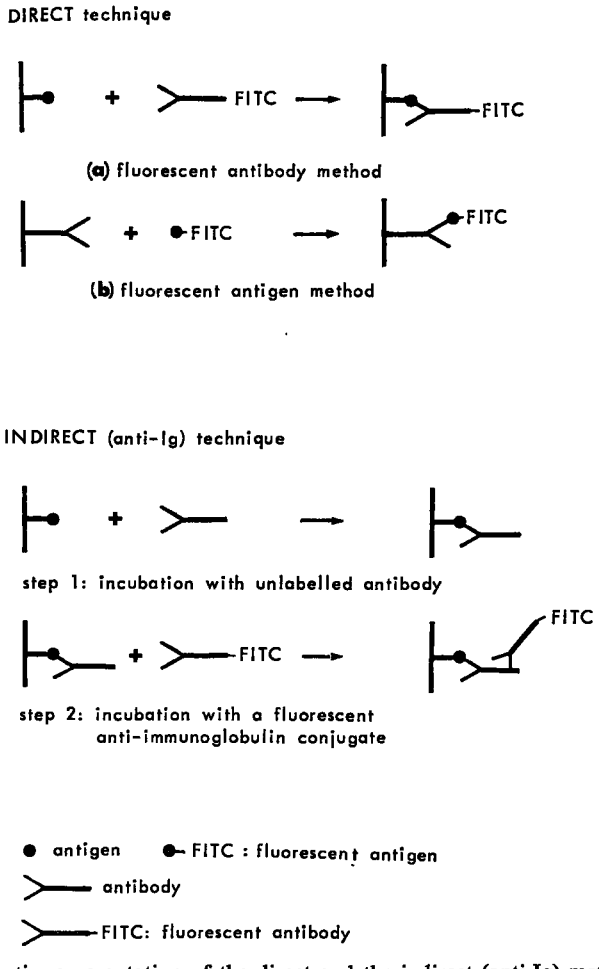


Figure V-1. Schematic representation of the direct and the indirect (anti-Ig) methods in immunofluorescence microscopy.
Two versions of the direct technique are shown: the labelled antibody and the labelled antigen technique. The direct technique requires one incubation, whereas two incubations are employed in the indirect technique.

technique is more specific and the indirect one is more sensitive (Table V-1). In the experiments a) and c) of Table V-1, beads coupled with the homologous antigen were incubated with either a fluorescent antiserum (direct technique) or first with an unlabelled antiserum and subsequently with a fluorescent anti-Ig conjugate (indirect technique). Different dilutions of the fluorescent and the unlabelled antiserum were applied in order to obtain an estimation of the sensitivity of both techniques. The specificity was assessed by using beads coupled with a nonrelated antigen (NSS control). The titration curves and the contrast curves (DSS/NSS) of both experiments are presented in Figs. V-2 and V-3.

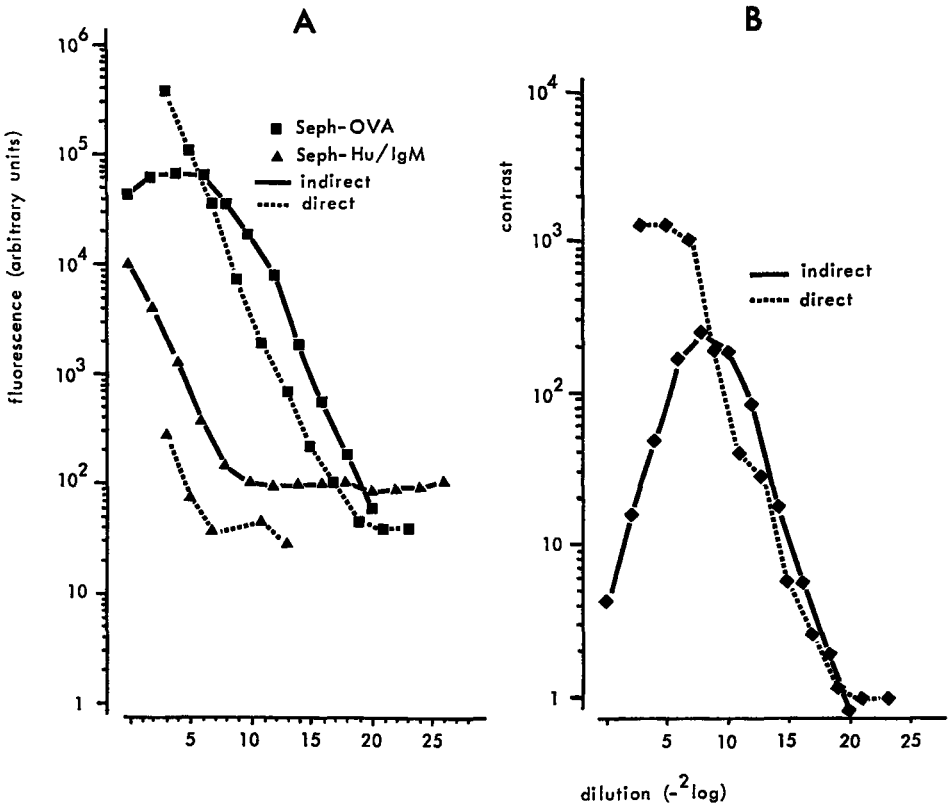


Figure V-2. Comparison of the direct and anti-Ig methods with the Sepharose bead immunofluorescence system. Sepharose beads coupled with ovalbumin (Seph-OVA) and human IgM (Seph-Hu/IgM) were incubated with either a labelled or an unlabelled globulin fraction of a rabbit antiserum directed against OVA (RAOVA). Labelled and unlabelled preparations contained the same amount of protein/ml. Incubation with RAOVA-FITC is a direct technique. The beads incubated with unlabelled RAOVA are subsequently incubated with a fluorescent horse antiserum directed against rabbit Ig's (HAR/Ig-FITC) diluted 1:50 and absorbed with Seph-OVA (indirect technique). Each point in the left hand figure is the average fluorescence of at least 5 individual beads. In the right hand figure, the ratio between the fluorescence of Seph-OVA and Seph-Hu/IgM (contrast) is plotted for the direct and the indirect technique.

TABLE V-1. EXPERIMENTS TO COMPARE THE DIRECT AND INDIRECT (ANTI-Ig METHODS IN IF MICROSCOPY WITH THE SEPHAROSE BEAD IMMUNOFLUORESCENCE SYSTEM

Method	Beads	First layer	Second layer	Control
a) Direct	Seph-OVA	RAOVA-FITC		Seph-Hu/IgM
Indirect	Seph-OVA	RAOVA	HAR/Ig-FITC	Seph-Hu/IgM
b) Direct/indirect	Seph-OVA	RAOVA-TRITC	HAR/Ig-FITC	Seph-Hu/IgG
c) Direct	Seph-RLV	GARLV-FITC		Seph-OVA
Indirect	Seph-RLV	GARLV	RAG/Ig-FITC	Seph-OVA

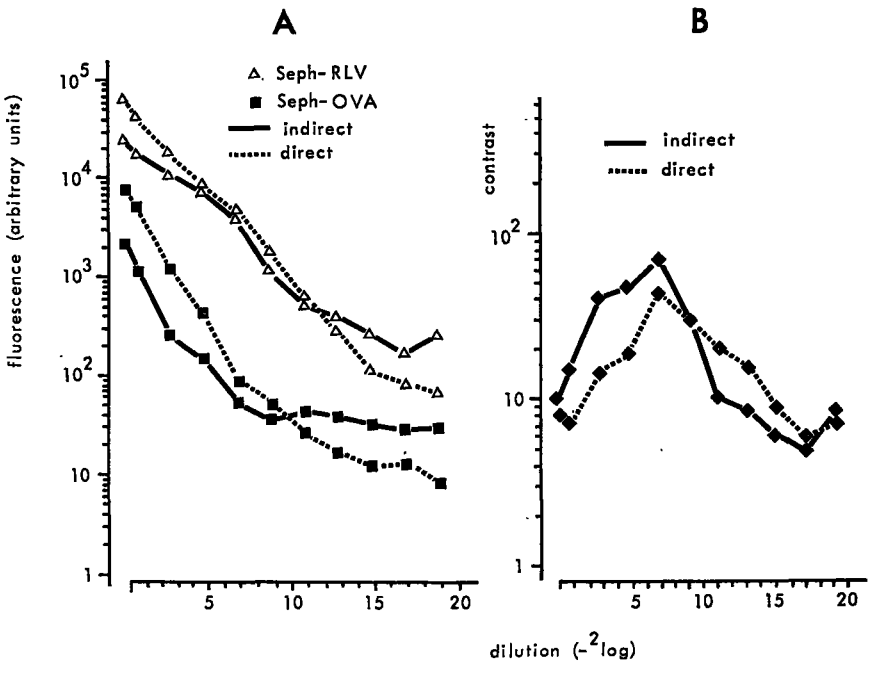


Figure V-3. Comparison of the direct and the indirect technique with the Sepharose bead immunofluorescence system. Sepharose beads coupled with Rauscher leukaemia virus (Seph-RLV) and ovalbumin (Seph-OVA) were incubated with a goat antiserum directed against RLV (GARLV) either labelled or unlabelled. Beads incubated with the latter were subsequently incubated with 1:50 diluted RAG/Ig-FITC (lot no. 5-774, Nordic Immunological Laboratories). Further details as in Figure V-2. Note the small difference in this experiment between direct and indirect technique with respect to saturation (see chapter IV-G2).

For a comparison of the two techniques, the labelled and the unlabelled antiserum should contain the same amount of specific antibody. The first layer in Figs. V-2 and V-3 were the globulin fractions of the antisera. The protein content of both labelled and unlabelled fractions had been equalized in both incubation procedures. The specificity interval (see Fig. IV-4) in Fig. V-2 for the direct technique was about one ^{2}log unit larger than for the indirect technique. The reverse was true, however, in the experiment shown in Fig. V-3.

In the rabbit antiovalbumin (RAOVA) system, the indirect technique was about four times as sensitive as the direct technique. No reliable estimate of the sensitivity could be made for the goat antiRauscher leukaemia virus (GARLV) system, because the homologous curve did not intercept the heterologous one. The use of different antiserum preparations for the comparison between the direct and the indirect techniques was circumvented in experiment b) of Table V-1. Sepharose beads coupled with ovalbumin (Seph-OVA) and coupled with human IgG (Seph-Hu/IgG) (NSS control) were incubated with different dilutions of a TRITC labelled rabbit antiserum

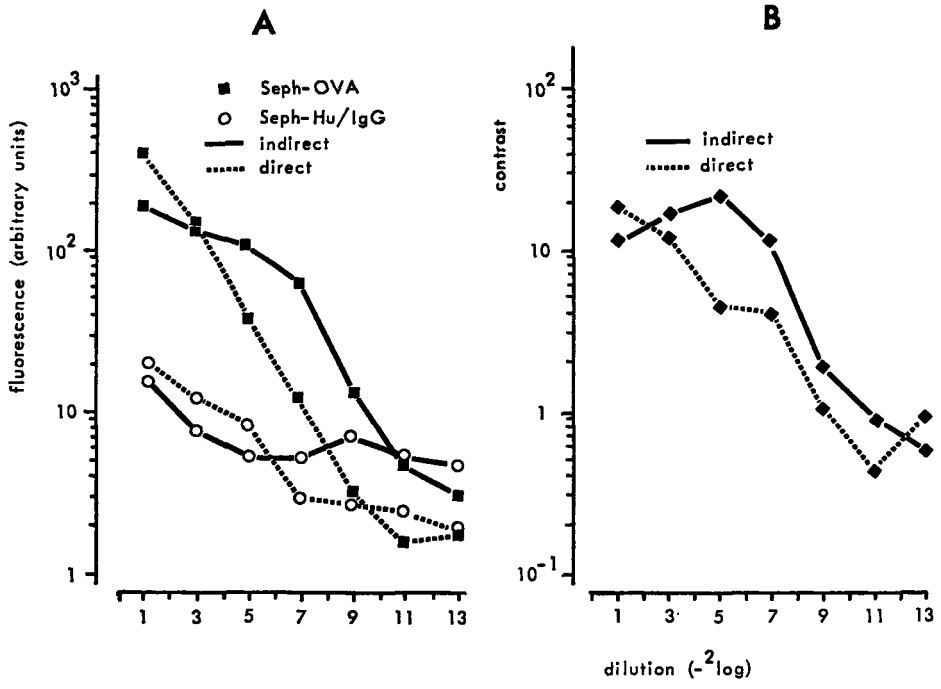


Figure V-4. Comparison of the direct and indirect technique with the Sepharose bead immunofluorescence system. Seph-OVA and Seph-Hu/IgG were incubated with different dilutions of RAOVA-TRITC. The beads were then incubated with 1:50 diluted HAR/Ig-FITC. The fluorescence of TRITC and FITC was measured from at least 10 individual beads. The average fluorescence values are plotted in the left hand figure. The TRITC fluorescence represents the direct technique, the FITC fluorescence the indirect technique. The ratio between the fluorescence of Seph-OVA and Seph-Hu/IgG beads (contrast) is shown for both the direct and indirect techniques in the right hand figure.

directed against ovalbumin (RAOVA-TRITC, direct technique). A FITC labelled horse antiserum directed against rabbit/Ig (HAR/Ig-FITC, lot no. 17-2-F3, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) was used in a second incubation to simulate the indirect technique. Both TRITC and FITC fluorescence of the individual beads were measured. The results are presented in Fig. V-4. The contrast curves (Fig. V-4B) can only be compared because the operational quantum efficiencies of FITC and TRITC differ from each other. The presence of saturation in the indirect technique and the absence of saturation in the direct technique is striking (see chapter IV, p. 75). The sensitivity of the two methods in this experiment differed by a factor of 4. An objection against the above described experiment is the fluorescent label attached to the RAOVA molecules. This situation might influence the number of determinants recognizable by the HAR/Ig-FITC, which would bias the experiment in favour of the direct technique.

Only antigen controls are necessary to establish the specificity of the observed

reactions in the direct technique. The antigen control will also suffice, in most instances, to establish the specificity of an antiserum in an indirect technique. The normal (or, preferably, preimmune) serum is another control. With Seph-Hu/IgM in Fig. V-2 and Seph-OVA in Fig. V-3, the NSS activity of the antiserum and that of the fluorescent conjugate are measured. The normal serum control makes it possible to distinguish between immunological and adsorption reactions. A major advantage of the normal or preimmune serum control is the use of only one substrate for the determination of the specificity. Haaijman, Bloemmen and Ham (1975) gave an example of the different results obtained with different controls in their Figure 2.

For a just comparison of the direct and indirect technique in immunofluorescence, other than quantitative aspects also have to be taken into account. The conjugates intended for direct use will be made, in practice, at least from globulin fractions of antisera. Mostly, however, purified IgG fractions will be used. In indirect techniques the antiserum as such will often be used. This explains why indirect methods are more likely to give nonspecific results in the practice of IF microscopy than are direct methods (see also chapter IV, part H). A few other assets of the two techniques should also be mentioned here: a) a limited number of fluorescent conjugates are required for a large variety of antigen/antibody systems with the indirect technique; b) the direct technique is not suited for diagnostic purposes, involving serum antibodies; c) double wavelength methods (Hijmans, Schuit and Hulsing-Hesselink, 1971) are far more easily applied in direct systems than in indirect systems.

The difference in sensitivity between the direct and the indirect technique was about fourfold in our experiments. This finding is in agreement with measurements of Wachsmuth and Woodhams (1974) on erythrocyte nuclei, incubated successively with antinuclear factor (ANF) serum and either one or two anti-Ig conjugates.

Whether the measured sensitivity data can be translated into sensitivity differences obtained visually will depend on the relationship between DSS and NSS encountered in different systems. The anti-Ig conjugate reacts with bound antibody molecules. This phenomenon is the same for specifically bound as well as nonspecifically bound antibodies. Under conditions where, by using the multilayer techniques, the nonspecific fluorescence is augmented without reaching the visual threshold, the gain in specific fluorescence is a true sensitivity gain. Increasing the fluorescence intensity still further will result in more fluorescence light but will not increase the information for the observer.

In the literature, different values have been reported for the relative sensitivity of the two techniques. Coons (1956) estimated a tenfold sensitivity gain with the anti-Ig method, and Pressman, Yagi and Hiramoto (1958) a four to twelvefold increase in sensitivity. These results were obtained by visual evaluation of endpoint titres. Knapp and Ploem (1974) reported the indirect technique to be 10 times as sensitive as the direct technique when using the Sepharose bead immunofluorescence system. They compared, however, different antigen-antibody systems.

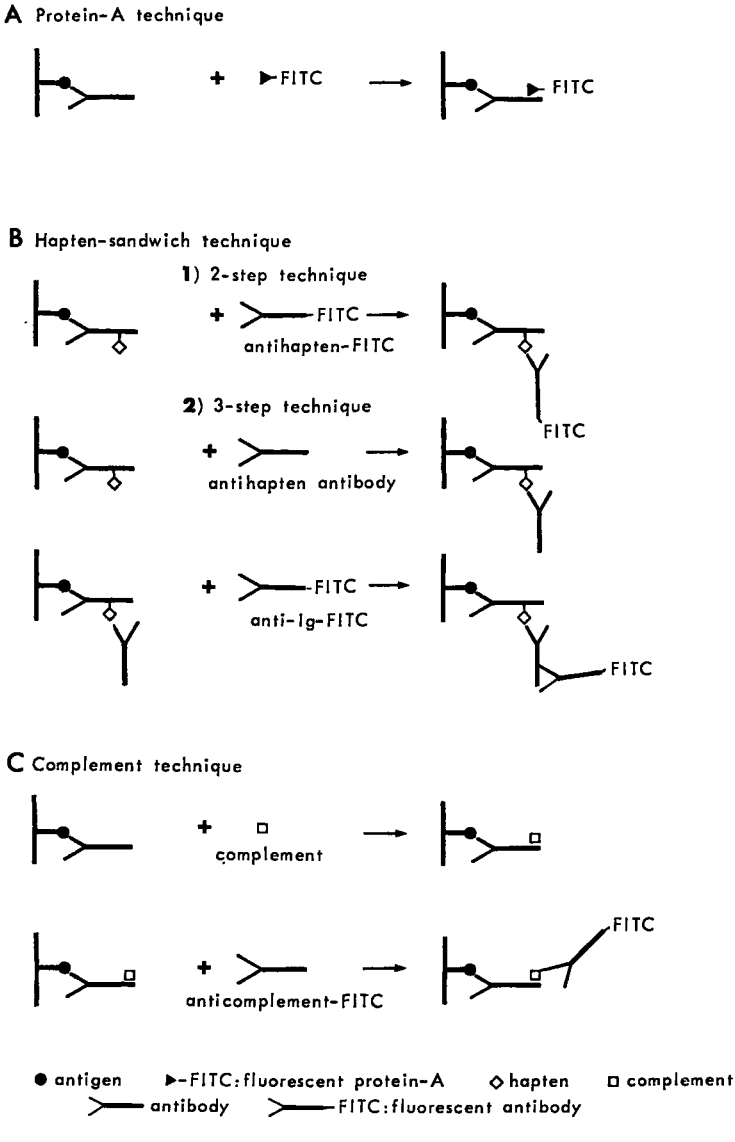


Figure V-5. Indirect immunofluorescence techniques other than the anti-Ig method for the demonstration of the binding of antibodies to antigens. Diagrams of A) protein-A method, B) hapten-sandwich method and C) complement technique are presented. Two variations of the hapten-sandwich method are shown: 1) 2-step technique, and 2) 3-step technique.

C. INDIRECT TECHNIQUES OTHER THAN THE ANTI-Ig TECHNIQUE

1. *Introduction*

A number of immunofluorescent techniques have been described for the detection

of the binding of antibodies to antigens. Diagrams of these techniques are presented in Fig. V-5.

Protein-A is a component of the cell wall of *Staphylococcus aureus*. It binds specifically to the Fc part of most IgG's. Fluorescent protein-A has been used as a substitute for anti-Ig conjugates (Dorval, Welsh and Wigzell, 1974; Ghetie, Fabricius, Nilsson and Sjöquist, 1974; Biberfeld, Ghetie and Sjöquist, 1975).

Anti-Ig conjugates cannot be used in certain systems. The use of alloantisera to detect membrane markers on lymphoid cells is an example of such a system. Wofsy, Baker, Thompson, Goodman, Kimura and Henry (1974) have introduced the hapten sandwich technique for such systems. Haptens are conjugated to the alloantisera. The binding of these antisera to cells can then be demonstrated either with a fluorescent antiserum directed against the hapten or alternatively, bound unlabelled antihapten antiserum can be detected by a fluorescent anti-Ig conjugate.

In a number of studies (e.g., Goldwasser and Shepard, 1958; Diaz-Gill, Beutner and Eisenberg, 1973), the capacity of antigen-antibody complexes to bind complement factors has been employed. Fluorescent antisera directed against the components of the complement system are used to detect complement binding. The complement-fluorescent anticomplement system has the advantage that a large number of different antigen-antibody reactions can be studied with only one fluorescent conjugate. This fact should provide an impetus for standardization. The same holds true also for the anti-Ig technique. The complement techniques have not been used widely in immunofluorescence microscopy. Four reasons may account for this: a) A large number of controls is necessary to establish the specificity of the observed reactions (Goldman, 1968). b) Not all Ig classes and subclasses are capable of binding complement. c) The application of double wavelength techniques (see part D of this chapter) is not possible. d) Complement techniques cannot normally be used with living cells for the detection of membrane markers because of cytotoxic reactions. This may be avoided by the use of serum of rabbit or guinea pig inbred strains deficient in the C4 or C6 complement factors (Nairn, 1976).

2. *The antigen-antibody-fluorescent-antigen sandwich*

This incubation scheme (see Fig. V-6) was introduced by Beutner, Holborow and Johnson (1965) for the demonstration of antinuclear factor. The technique has not found many applications in biological work, mainly because of the requirement for pure and labelled antigen. It is based on the observation that antibody molecules have basically two antigen binding sites.

The phenomenon of 'capping' in membrane IF suggests that, with hyperimmune sera applied to cells in optimal dilution, both antigen binding sites of the cell-bound antibody molecules are occupied. Under these conditions antiserum molecules bound to cells will thus have no free binding sites available.

In the Sepharose bead immunofluorescence system, the density of antigen per unit

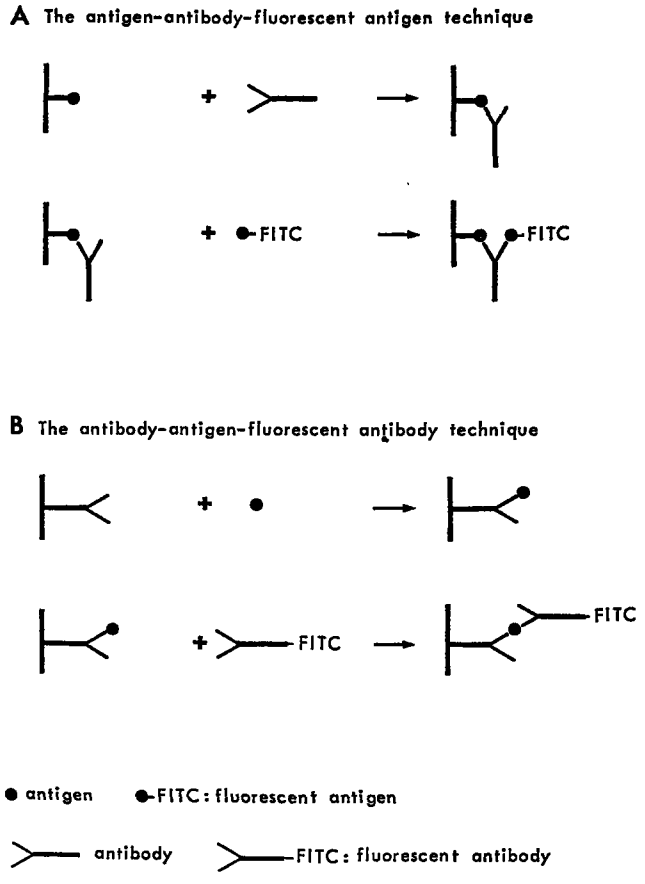


Figure V-6. Schematic representation of the antigen-antibody-fluorescent-antigen sandwich (A) and the antibody-antigen-fluorescent-antibody sandwich (B).

of surface is relatively small. At least 45 grams of OVA would have to be coupled to 1 ml of packed Sepharose beads in order to bring two individual OVA molecules within the reach of one IgG molecule. Haaijman, Bloemmen and Ham (1975) have shown that a competition fluorescence immunoassay can be devised with the antigen-antibody-fluorescent-antigen system. In their experiments, ovalbumin bound to Sepharose beads (Seph-OVA) was first incubated with a fixed dilution of a rabbit antiserum raised against OVA (RAOVA). The unoccupied binding sites were subsequently titrated with FITC labelled OVA (OVA-FITC). The optimal concentration of RAOVA had been determined in preliminary experiments. For the competition assay, the concentration of OVA-FITC was kept constant. Unlabelled OVA competes for the available binding sites if added to the beads together with OVA-FITC. Seph-Hu/IgG served as the control in the immunoassay.

Different information may be obtained if RAOVA in a fixed concentration and

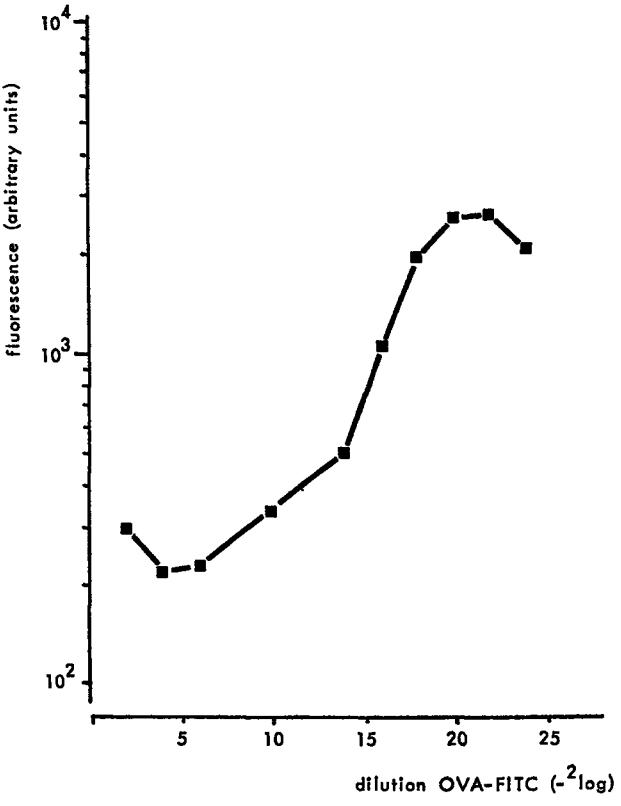


Figure V-7. Competition between Sepharose-bound ovalbumin (Seph-OVA) and free OVA-FITC for the binding sites of a rabbit antiserum directed against OVA (RAOVA). Seph-OVA was incubated with a mixture of a fixed amount of RAOVA and varying amounts of OVA-FITC.
Undiluted OVA-FITC contained 8.6 mg protein/ml. The RAOVA was diluted 1:4096. The fluorescence of at least 5 individual beads was measured. The individual bead values were averaged and plotted.

OVA-FITC in various concentrations are incubated simultaneously with Seph-OVA. The two binding sites of a portion of the RAOVA incubated together with high concentrations of OVA-FITC will be saturated in solution with OVA-FITC. These saturated RAOVA molecules will not bind to the Seph-OVA beads. With decreasing concentrations of OVA-FITC, the chance that one binding site of the RAOVA molecules will combine with the OVA on the beads while the other binding site is occupied with OVA-FITC will increase. Still lower concentrations of OVA-FITC will yield less fluorescent beads. This system is promising as an alternative for quantitative precipitation assays. It should be possible with this technique to also quantitate the number of binding sites per antigen molecule in nonprecipitating systems.

The kinetics of the system including a solid and a soluble component are certainly worth further studies. Experimental results are given in Fig. V-7. The undiluted OVA-

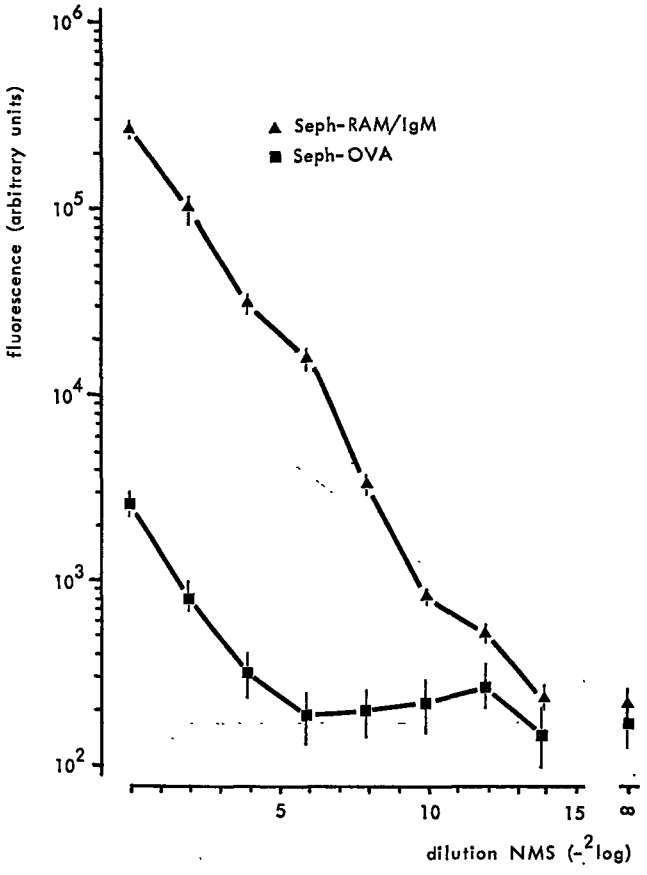


Figure V-8. The antibody-antigen-fluorescent-antibody technique.
Sepharose beads were coupled with the IgG fraction of a rabbit antiserum directed against IgM of the mouse (Seph-RAM/IgM) and with ovalbumin (Seph-OVA). Seph-RAM/IgM and Seph-OVA were incubated with different dilutions of normal mouse serum (NMS). The -^{2}log dilution of zero stands for 1:100 diluted NMS. Beads were subsequently incubated with fluorescent RAM/IgM (RAM/IgM-TRITC, diluted 1:100). The plotted values are the average fluorescence of at least 5 individual beads. Bars indicate standard deviations. ∞: conjugate control.

FITC contained 8.6 mg protein/ml. This means that the $-^2$log dilution of 21 contained about 4 ng OVA-FITC/ml. The maximum fluorescence intensity is probably caused by the complex Seph-OVA/RAOVA/OVA-FITC. The bound OVA and the OVA-FITC in solution had the same chance to encounter an anti-OVA molecule. The concentration of bound OVA was about 20 μg/ml as calculated from the approximate number of beads used in the experiment. The difference in the concentrations of the two OVA moieties implies that the bound OVA cannot be treated in kinetic equations as a free diffusing molecule (see for an extensive discussion of this topic in histochemistry, van Duijn, 1976).

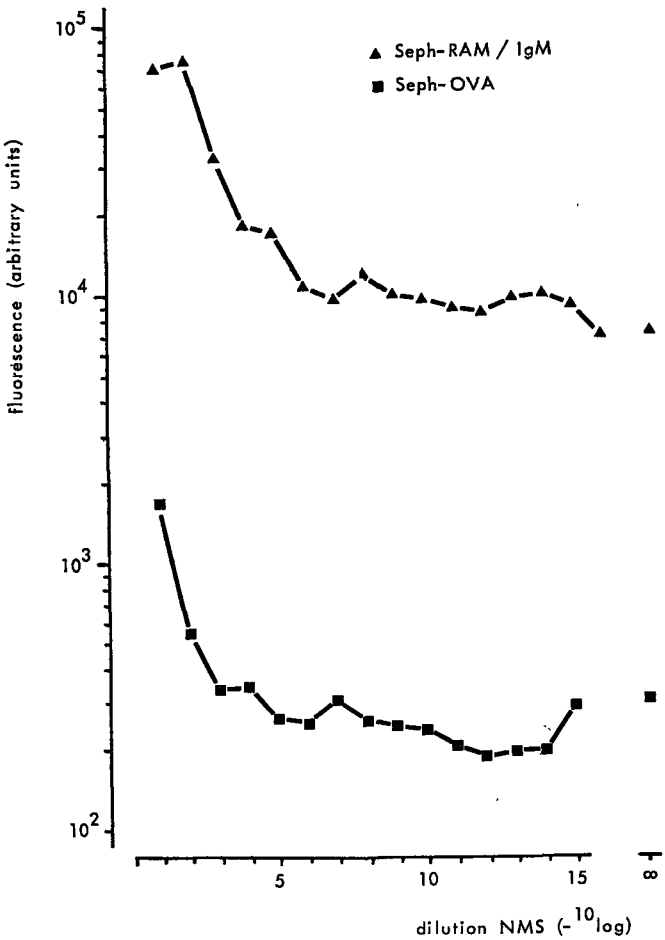


Figure V-9. The antibody-antigen-fluorescent-antibody technique.
Seph-RAM/IgM and Seph-OVA were incubated with NMS as in Fig. V-8. A fluorescent goat antiserum directed against mouse Ig's (GAM/Ig-TRITC, diluted 1:50) was used instead of the RAM/IgM-TRITC. The $-^{10}$log dilution of zero stands for undiluted NMS. ∞: conjugate control.

Matthews, de Kretser and Nairn (1975) have used a system similar to the one described above to simulate the influence of blocking antigen and antibodies in tumour immunology.

3. *The antibody-antigen-fluorescent-antibody sandwich*

A diagram of this sandwich is shown in Fig. V-6. It has been used to detect the synthesis of certain antibodies in plasma cells. Hijmans, Schuit, Yamashita and Schechter (1972) used such an incubation scheme, for instance, to study whether

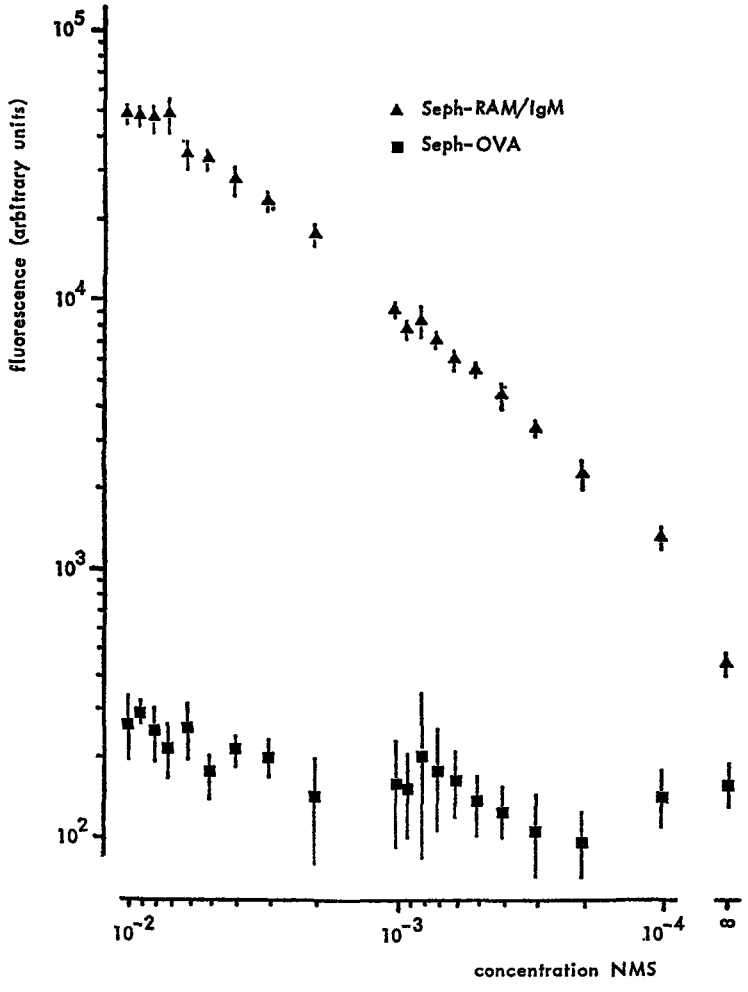


Figure V-10. The antibody-antigen-fluorescent-antibody technique.
The conjugate from Fig. V-9 was absorbed with Sepharose beads coupled with normal rabbit serum (NRS) in order to remove possible cross-reactive antibodies. Another dilution scheme was used than in the previous figures. The concentration of undiluted NMS was taken as 1. Each point represents the average of at least 5 individual bead measurements. Bars indicate standard deviations. ∞: conjugate control.

single plasma cells in rabbits injected with a carrier-hapten complex contained both anticarrier and antihapten antibodies; the plasma cells did not.

This incubation scheme has also been the basis of: the IgE and IgG determinations of Capel and Aalberse (1975), the Ig determination with the BioRad commercial fluorescence assay kit (see chapter IV, part F1), and our determination of serum Ig levels in mice. In addition, the methods for the determination of B- and C-type RNA virus proteins in milk and tissues of mice have been developed with this scheme.

The specificity of the assay is determined by the specificity of the antiserum coupled to the beads as well as by the specificity of the fluorescent conjugate. The greatest specificity is obtained by using a conjugated and an unconjugated preparation of the same specific antiserum. A single example of such a technique is shown in Fig. V-8. Sepharose beads coupled with the IgG fraction of a rabbit antiserum directed against mouse IgM (Seph-RAM/IgM) and ovalbumin (Seph-OVA) were incubated with different dilutions of normal mouse serum (NMS) and subsequently with fluorescent RAM/IgM (RAM/IgM-TRITC). The $-^2\log$ dilution of 14 in Fig. V-8 represents about 3 ng IgM/ml.

The use of a polyvalent fluorescent conjugate to determine the amount of bound antigen is often more economical. In the example of Fig. V-8, GAM/Ig-TRITC (Nordic Immunological Laboratories) could be used. The result of doing this is shown in Fig. V-9. The same beads from Fig. V-8 were first incubated with different dilutions of NMS and subsequently with the polyvalent fluorescent conjugate. The GAM/Ig-TRITC showed a strong cross-reactivity with the rabbit Ig's on the beads. This reactivity of the conjugate could be completely removed by a preincubation of the conjugate with Sepharose beads coupled with normal rabbit serum (Seph-NRS). The result after such a preincubation is shown in Fig. V-10.

The system can be made more sensitive if pure antibodies are coupled to the beads. In one instance, a sensitivity of 0.1 ng M/IgG per ml was achieved. Further details of this system for the estimation of mouse serum Ig levels, including experiment details, are presented in chapter VIII, parts F and G.

D. DOUBLE STAINING TECHNIQUES

1. *Introduction*

Double staining techniques using two fluorochromes conjugated to different antisera in the same preparation have been used very frequently since the introduction of TRITC by Hiramoto, Engel and Pressman (1958). The technical requirements for discriminating between these two fluorochromes have been discussed by Hijmans, Schuit and Klein (1969), Hijmans, Schuit and Hulsing-Hesselink (1971), Faulk and Hijmans (1972), Kraft (1975) and in chapter II. The technique has been shown to be most valuable for the performance testing of antihuman Ig conjugates with monoclonal bone marrow preparations.

The fluorochromes should not interact with each other for proper functioning of the technique. A possible interaction would be the absorption of FITC emission by TRITC. The absorption spectrum of TRITC conjugates suggests this possibility. It clearly shows two optima, one at 555 nm and one around 520 nm. The 520 nm absorption peak lies in the region of maximum FITC emission. The 520 nm optimum seems to be present only in conjugates prepared with amorphous TRITC (Amante, Ancona and Forni, 1972). According to McKinney and Spillane (1975), the relative height

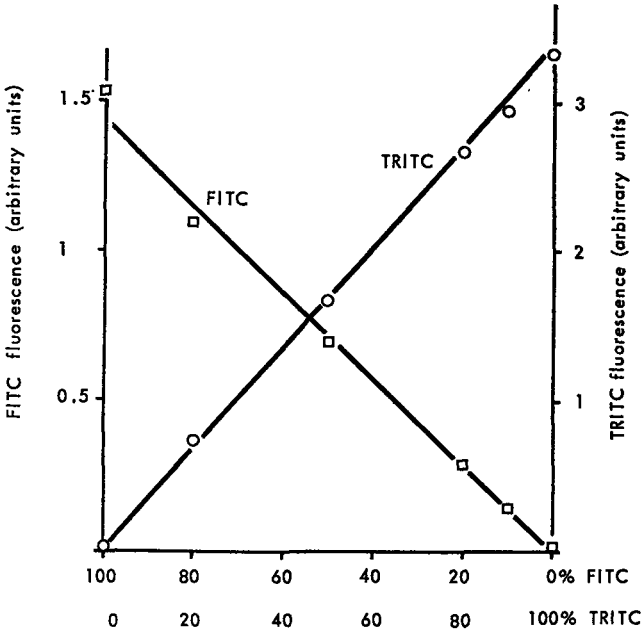


Figure V-11. Test for the interference with FITC emission by TRITC in aqueous solutions. Solutions of 0.1 mg/ml TRITC and 0.1 mg/ml FITC in 0.05 M carbonate buffer pH 9.5 were mixed in different proportions. The fluorescence of FITC and TRITC was measured in a microcuvette using a 3.2×/0.07 objective.

of the two absorption maxima is determined by the conjugation parameters. Brandtzaeg (1975) mentions the possibility that the 520 nm component is likely to cause NSS. The fact that the excitation spectrum of TRITC (Porro, Dadik, Green and Morse, 1963; Hansen, 1964) shows only one maximum around 555 nm is interesting. Discrepancies between excitation and absorption spectra of organic dyes are relatively rare (Birks and Dyson, 1963).

We have attempted to measure the interference of TRITC with FITC fluorescence in the following experiment. Solutions of FITC and TRITC were mixed in different proportions (Fig. V-11). The fluorescence of FITC and TRITC was measured in microcuvettes with 1 mm path length with the microscope using a 3.2×/0.07 objective. No significant influence of TRITC concentration on FITC emission was observed. The same holds for FITC and TRITC present on the same bead (see Fig. V-12). We have concluded that the FITC emission is not absorbed to a significant extent by TRITC. The same conclusion was reached by Pachmann and Killander (1976) using antigens adsorbed to polyacrylic beads. Another view is held by Ullman, Schwarzberg and Rubinstein (1976) who recently reported an immunoassay based on the energy transfer between FITC and TRITC, if these were coupled to, respectively, antigens and antibodies. According to these authors the degree of quenching

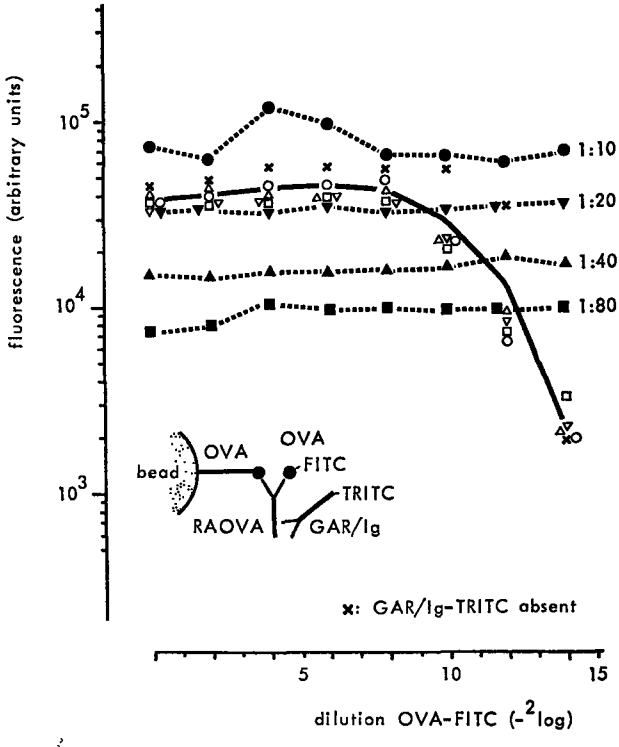


Figure V-12. Test for the interference between the binding of different molecules to the Fab and Fc parts of immunoglobulins.
Seph-OVA was incubated with RAOVA diluted 1:20. These beads were incubated with different dilutions of OVA-FITC. Undiluted OVA-FITC contained 8.6 mg protein/ml. The subsequent incubation consisted of GAR/Ig-TRITC. Four dilutions of GAR/Ig-TRITC were used: 1:10, 1:20, 1:40, and 1:80. Both FITC fluorescence (open symbols) and TRITC fluorescence (closed symbols) were measured per individual bead. If no GAR/Ig-TRITC was added only FITC fluorescence was measured. The solid line connects the averages of the FITC fluorescences. The inset shows a diagram of the complex which is most probably formed.

of FITC fluorescence by TRITC is inversely proportional to the sixth power of the distance between the two molecules. It is likely, therefore, that this phenomenon is only of significance if the fluorophores are present in the direct proximity of the antigen-antibody binding site. It is of interest that significant quenching of FITC fluorescence was observed only with antibodies carrying more than two TRITC residues.

2. *Tests for the interference between the binding of antigen to the Fab part of an immunoglobulin molecule and the binding of anti-Fc reagents*

A prerequisite for the application of the double staining technique is the strong antigen-antibody bond. Should this not be the case, conjugates could easily displace each other.

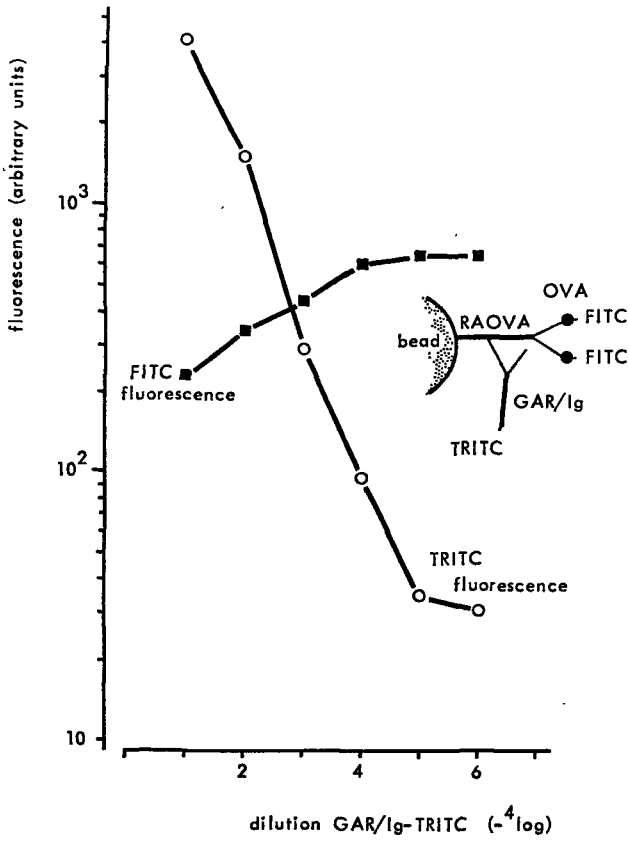


Figure V-13. Test for the interference between the binding of different molecules to the Fab and Fc parts of immunoglobulins.
The globulin fraction of RAOVA was coupled to Sepharose beads. The Seph-RAOVA was incubated with a fixed dilution of OVA-FITC, followed by an incubation with different dilutions of GAR/Ig-TRITC. Both FITC and TRITC fluorescence from individual beads were measured. The average of at least 10 individual bead measurements is plotted. The inset shows a schematic drawing of the complex which is presumably formed.

We have tested the interference in a number of systems, of different conjugate molecules directed against the same antigen molecule. The aim of these experiments was to study the validity of double staining techniques employed in qualitative immunofluorescence microscopy in a model system. The influence of the binding of antigens to the Fab part of Ig's on the stainability with anti-Ig conjugates (see, for instance, also Emmings and Genco, 1974) was studied in the following three experiments:

a) Seph-OVA was incubated with a fixed dilution of RAOVA. Different dilutions of OVA-FITC were added to those beads. The beads were next incubated with different dilutions of a TRITC labelled goat antiserum directed against rabbit Ig's (GAR/Ig-

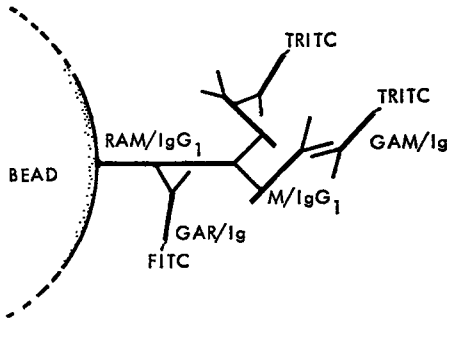


Figure V-14. Test for the interference between the binding of different molecules to the Fab and Fc parts of immunoglobulins.
Diagram of the complex which is presumably formed in the experiment presented in Table V-2. Sepharose beads coupled with the IgG fraction of a rabbit antiserum directed against M/IgG_1 (Seph-RAM/IgG_1) were first incubated with different dilutions of NMS in order to bind M/IgG_1, then with GAM/Ig-TRITC diluted 1:50 and subsequently with GAR/Ig-FITC diluted also 1:50.

TRITC). A schematic representation of the formed complex, together with the titration curves, is shown in Fig. V-12. The binding of OVA-FITC did not influence the binding of GAR/Ig-TRITC. The TRITC fluorescence values were independent of the FITC fluorescence values. It appears that GAR/Ig-TRITC displaces some OVA-FITC. The FITC fluorescence values after the incubation with GAR/Ig-TRITC were somewhat lower than without this incubation. The action of GAR/Ig-TRITC was not clearly concentration-dependent, as the beads incubated with either 1:10, 1:20, 1:40 or 1:80 diluted GAR/Ig-TRITC gave the same results. This phenomenon implies that FITC fluorescence is not influenced to a major extent by TRITC present on the same bead. The GAR/Ig conjugate most probably binds to the Fc part of the RAOVA. It is at least evident that the binding of OVA-FITC to the Fab part of RAOVA does not render the Fc part unaccessible to GAR/Ig-TRITC.

b) RAOVA was coupled directly to Sepharose beads. These beads were incubated successively with a fixed concentration of OVA-FITC and different dilutions of GAR/Ig-TRITC. FITC and TRITC fluorescence of individual beads were measured. The result is shown in Fig. V-13. In this experiment, the OVA-FITC was displaced to a certain extent by the GAR/Ig molecules. The phenomenon was dependent on the concentration of the GAR/Ig-TRITC.

c) Ovalbumin is a relatively small protein compared to Ig's. It could be that the binding of these larger Ig molecules to the Fab of anti-Ig antisera interferes with the stainability of the Fc fragments of these antisera. We have employed the model that is schematized in Fig. V-14 for testing this possibility. Beads coupled with the IgG fraction of a rabbit antiserum directed against mouse IgG_1 (Seph-RAM/IgG_1) were first incubated with NMS and then with GAM/Ig-TRITC. The beads were incubated afterwards with GAR/Ig-FITC. An incubation with PBS instead of NMS served as a control. Results of both FITC and TRITC fluorescence are presented in Table V-2.

TABLE V-2. TEST FOR THE INTERFERENCE BETWEEN THE OCCUPATION OF THE Fab PART OF AN IMMUNOGLOBULIN AND THE STAINABILITY OF THE Fc PART OF THAT IMMUNOGLOBULIN WITH A FLUORESCENT ANTI-Ig ANTISERUM

Bead	NMS	Fluor TRITC	Fluor FITC
RAM/IgG_1	1:5	28984	20440
	0 [1])	104	12422
RAM/IgG_{2a}	1:5	26255	18912
	0	98	22960
RAM/IgG_{2b}	1:5	20561	16957
	0	110	12841

Beads were coupled with the IgG fractions of rabbit antisera directed against the Fc part of mouse IgG_1, IgG_{2a} and IgG_{2b}. These beads were either incubated with 1:5 diluted NMS or with PBS. TRITC labelled goat antimouse/Ig, diluted 1:50 was used to demonstrate the binding of mouse Ig's after the former incubation. The rabbit Ig's coupled to the beads were stained with a 1:50 diluted fluorescent goat antiserum directed against rabbit Ig's (GAR/Ig-FITC). [1]) incubation with PBS.
Values represent the average TRITC and FITC fluorescence of at least five individual beads.

No appreciable influence on the GAR/Ig-FITC fluorescence was observed, whether the Fab parts of the antiserum coupled to the beads were occupied or not.

We have concluded from these experiments that the binding of antigen to the Fab part of an antibody influences the accessibility of the Fc part to anti-Fc reagents to only a minor extent.

E. CONCLUSIONS

Different single and multilayer techniques for IF microscopy have been discussed in this chapter. A distinction was made between the direct (single layer) and indirect (multilayer) techniques. The choice of a certain technique depends first of all on the field of study.

The anti-immunoglobulin and complement techniques have the advantage of requiring only a limited number of fluorescent reagents. These reagents may be used in a large variety of systems. The time saved in the preparation of fluorescent conjugates, however, is paid back dearly in the increased number of incubations.

The number of controls necessary to establish the specificity of a certain reaction increases exponentially with the number of incubations (Goldman, 1968). The non-specific reactions are amplified with successive incubations in the same way as are the specific ones. Only in special cases can the increase in sensitivity of multilayer techniques, as compared to more simple systems, be made of real advantage. We think that, where possible, the direct technique is to be preferred for its simplicity and unambiguous results. The commercial manufacturing of reliable and tested fluorescent conjugates for application in the direct technique will make this method also available for clinical immunofluorescence microscopy.

With the Sepharose bead immunofluorescence system, it was established that the anti-Ig method is about four times as sensitive as the direct method. Some conditions prevailing in qualitative IF microscopy, which may prevent the full exploitation of the sensitivity gain of the indirect technique are discussed.

In the Sepharose bead immunofluorescence system, antibodies are bound to the antigen on the bead at only one antigen binding site. Evidence was presented with an antigen-antibody-fluorescent-antigen system that the kinetics of antibodies in solution reacting with antigens bound to Sepharose beads are not the same as the reaction of these antibodies with the antigen in solution. For the double wavelength technique, independence of FITC and TRITC fluorescence is essential. Experimental data were presented which showed that FITC and TRITC do not interfere with the fluorescence properties of each other, neither when together in solution nor when present on the same Sepharose bead. Finally, it was established that occupation of both antigen binding sites of an antibody molecule does not shield its Fc part to a major extent from fluorescent anti-Fc reagents.

CHAPTER VI

The construction of automated versions of the Leitz Diavert inverted microfluorometer

Contents:

A. INTRODUCTION

Fluorescence immunoassays have been developed for the determination of a variety of antigens and antibodies. These assays are based on the covalent binding of proteins to Sepharose beads and are described in detail in chapter VIII of this thesis. The measurement of the fluorescence of individual Sepharose beads is the final step in the assays. The use of a microfluorometer is indicated for these measurements, as the beads range in diameter between 40 and 150 μm. The fluorescence intensity distribution of individual beads within one preparation follows a normal distribution. The fluorescence intensity of a number of different beads will consequently have to be determined and averaged.

Beads were incubated in small plastic tubes in the original paper of van Dalen, Knapp and Ploem (1973). The beads had to be washed after every incubation, using relatively time-consuming centrifugations. Capel (1974) used a multichannel dispenser and aspirating device to increase the efficiency in the processing of samples. Knapp and Ploem (1974) introduced an ingenious apparatus in which the beads were incubated in tubes, sealed with a nylon mesh. The beads could be washed without centrifugation.

We have incubated the Sepharose beads in microtitration plates. The microtitration plates contained 96 flat-bottomed wells. An extensive experimental protocol of our method is presented in chapter VII. The advantages of this method are twofold: a) beads may be washed without centrifugation; and b) the fluorescence of individual beads can be measured through the bottom of the microtitration plates. The preparation of individual slides for each sample is made unnecessary.

The introduction of the microtitration plates made the use of an inverted microscope obligatory. Initially, we attempted to fix beads on the bottom of the microtitration plates. One hundred μl of warm 3% agar was pipetted into each well for this purpose. The object of these trials was to use a normal upright microfluorometer and invert the plate. The trials proved unsuccessful, however. The larger beads tended to sink slowly away and became out of focus. A Leitz Diavert inverted microscope was therefore introduced.

It is the purpose of this chapter to report on the construction of automated Leitz Diavert inverted microfluorometers. An automated version of this type of microscope is not available commercially. We want to report, moreover, on the progress which has been made in the control of the different shutters since the report on the automation of the Leitz Orthoplan microfluorometer of Haaijman and Wijnants (1975). In part B, the construction and position of the different shutters are discussed. Two types of control over the shutter sequence have been devised. The pneumatic control and the control by a desktop calculator are the subjects of part C of this chapter. Fluorescence intensities are measured with a photomultiplier. The output of the photomultiplier is determined with a voltmeter. The handling of the voltmeter readings by a calculator is presented in part D. The first section of part E contains a

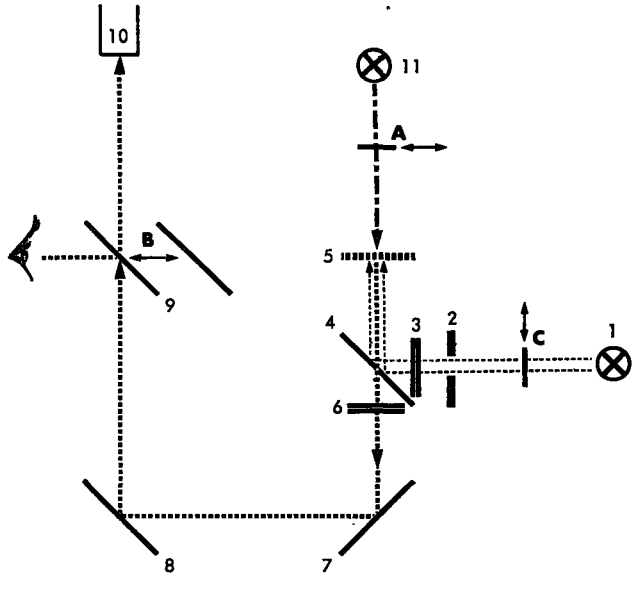


Figure VI-1. Schematic representation of the optical pathways in the Leitz Diavert inverted microscope equipped for epiillumination and fluorometry.
Components: 1 = mercury arc (CS 100W); 2 = field diaphragm; 3 = excitation filters; 4 = interference dividing plate; 5 = object; 6 = emission filters; 7 = mirror; 8 = mirror; 9 = prism; 10 = photomultiplier; 11 = light source for diaillumination.
Components 2–4 and 6 are contained in the Ploemopak 2.2. A = shutter in the diaillumination pathway; B = shutter for the photomultiplier; C = shutter in the excitation pathway.

list of the different parts of the electronic and optical equipment which has been used in the fluorescence immunoassays of chapter VIII. The second section concerns the operation of the automated inverted microfluorometer. An appraisal of the use of pneumatic components in the automation of microfluorometers compared to other designs is given in part F of this chapter.

B. CONSTRUCTION OF THE SHUTTERS IN THE LIGHT PATHS OF THE INVERTED MICROSCOPE

The position of the three shutters, A, B and C, is indicated in the diagram of the different light paths of the Leitz Diavert inverted microscope in Fig. VI-1. Shutter A controls the light path of the diaillumination. This light path is used to locate the different beads and to position them relative to the measuring diaphragm. Fig. VI-2a shows the simple design of shutter A. The shutter is fastened to the lamp housing. It consists of a small double acting cylinder with a stroke of 40 mm (DG-6-40, Festo-Maschinenfabrik G. Stoll, Berkheim-Esslingen, Western Germany). A shutter blade of brass is fastened to the cylinder piston. The blade is guided by two stainless steel

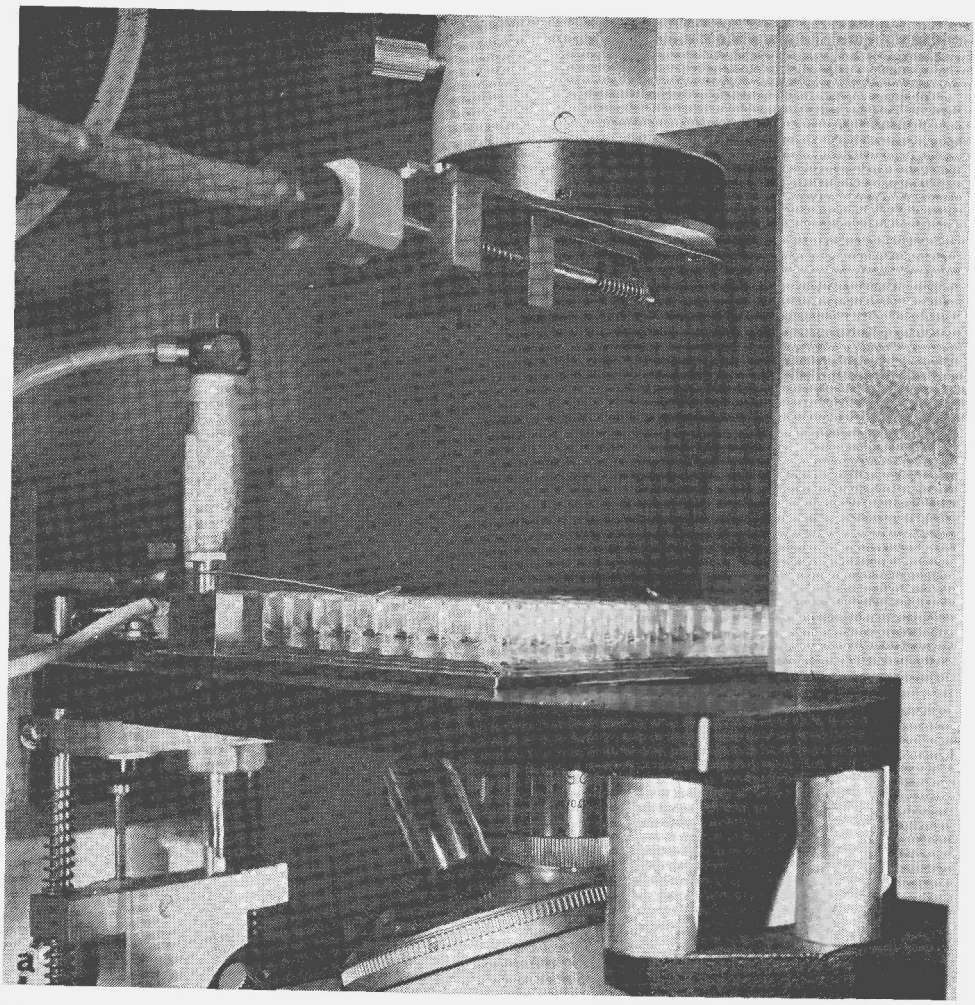


Figure VI-2a. Construction of an automated microfluorometer.
Construction of shutter A (see Fig. VI-1) in the diaillumination pathway. The cylinder is a DG-6-40 (Festo-Maschinenfabrik G. Stoll).

rods. The rods provide the possibility of including springs to prevent excessive bumps.

The prism in the binoculars (indicated as shutter B in Fig. VI-1) or the mirror of the pilot lamp (labelled 2 in Fig. VI-3) functions as the shutter for the photomultiplier. In the original design of Haaijman and Wijnants (1975), a cylinder moved the photomultiplier lever. This construction is shown diagrammatically in Fig. VI-3. An advantage of this construction was the fact that the measuring diaphragm could be observed together with the object image. Careful positioning of objects within the measuring diaphragm was possible. A number of disadvantages of this design, how-

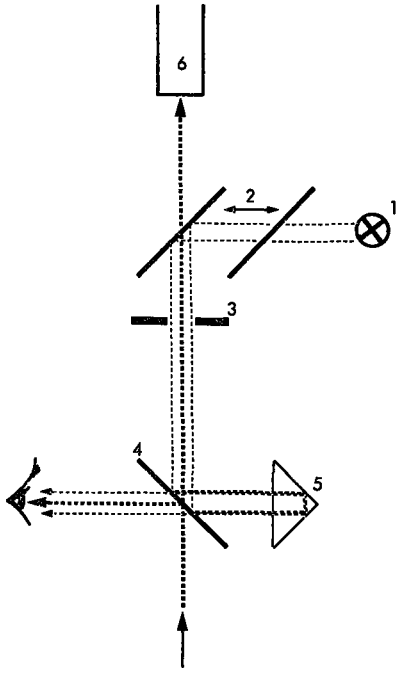


Figure VI-3. Construction of an automated microfluorometer.
Alternative construction of the shutter B in Fig. VI-1. The lever of the mirror reflecting the light of the pilot lamp is moved instead of the prism in the binoculars. For a technical description, see Haaijman and Wijnants (1975), their Fig. 5a.
Components: 1 = pilot lamp; 2 = movable mirror; 3 = measuring diaphragm; 4 = dividing plate; 5 = inverting prism; 6 = photomultiplier.

ever, prompted us to seek another solution. The disadvantages were: a) one extra shutter was needed to exclude the possibility of the entering of ambient light into the photomultiplier; b) the field of view of the monocular viewing piece was smaller than that of the binoculars; c) the screw thread of the photomultiplier lever showed signs of wearing after two years of operation; and d) the dividing mirror reduced the light intensity reaching the photomultiplier by a factor of about 2.

The more recent construction of the photomultiplier shutter is shown in Fig. VI-2b. The prism of the binoculars is used as the shutter. It is moved with a double acting cylinder (DG-12-25) fastened to the original lever. The forward motion is damped with a silicone stopper glued to the inside of the housing. The silicone material has a very high shock absorbing quality, acting over a relatively short distance. The inward movement of the piston is damped by a spring around the piston itself. The present shutter does not have a number of the disadvantages of the previous one. Moreover, binocular viewing induces less fatigue than monocular viewing. The disadvantage is the loss of the simultaneous observation of the object to be measured and the mea-

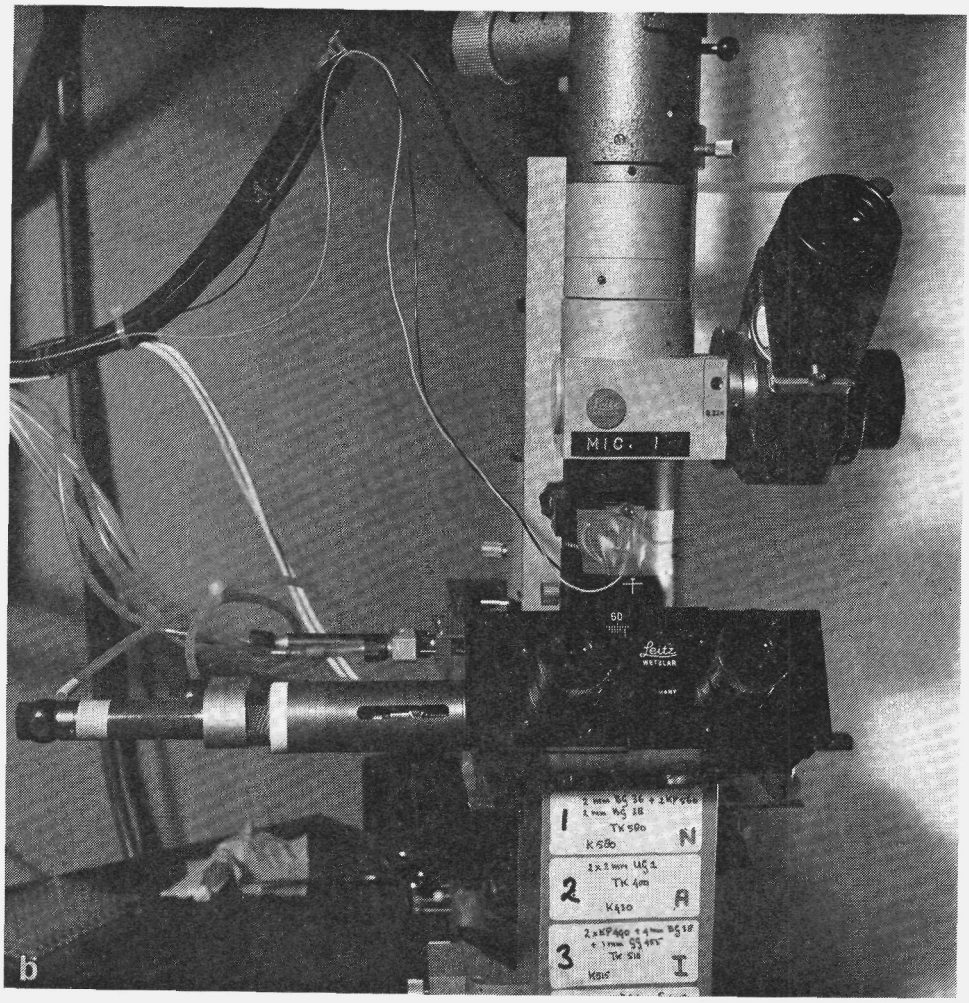


Figure VI-2b. Construction of shutter B (see Fig. VI-1) for the photomultiplier. The prism in the binoculars is moved with a DG-12-25 cylinder.

suring diaphragm. This disadvantage has been overcome as much as possible by inclusion of a centering plate in one of the eyepieces of the binocular. The method of plug measurements in the Sepharose bead immunofluorescence assays (see chapter IV) introduced by Capel (1974) makes the positioning of the beads relative to the measuring diaphragm less critical.

The shutter in the excitation pathway was of the guillotine type (Fig. VI-2c). Two stainless steel rods ($\varnothing$ 4 mm) hold two brass plates. The lower brass plate is fastened to the base of the microscope. Two rubber rings are placed between the brass plate and the base, in order to avoid the transmittance of bumps. The upper brass plate

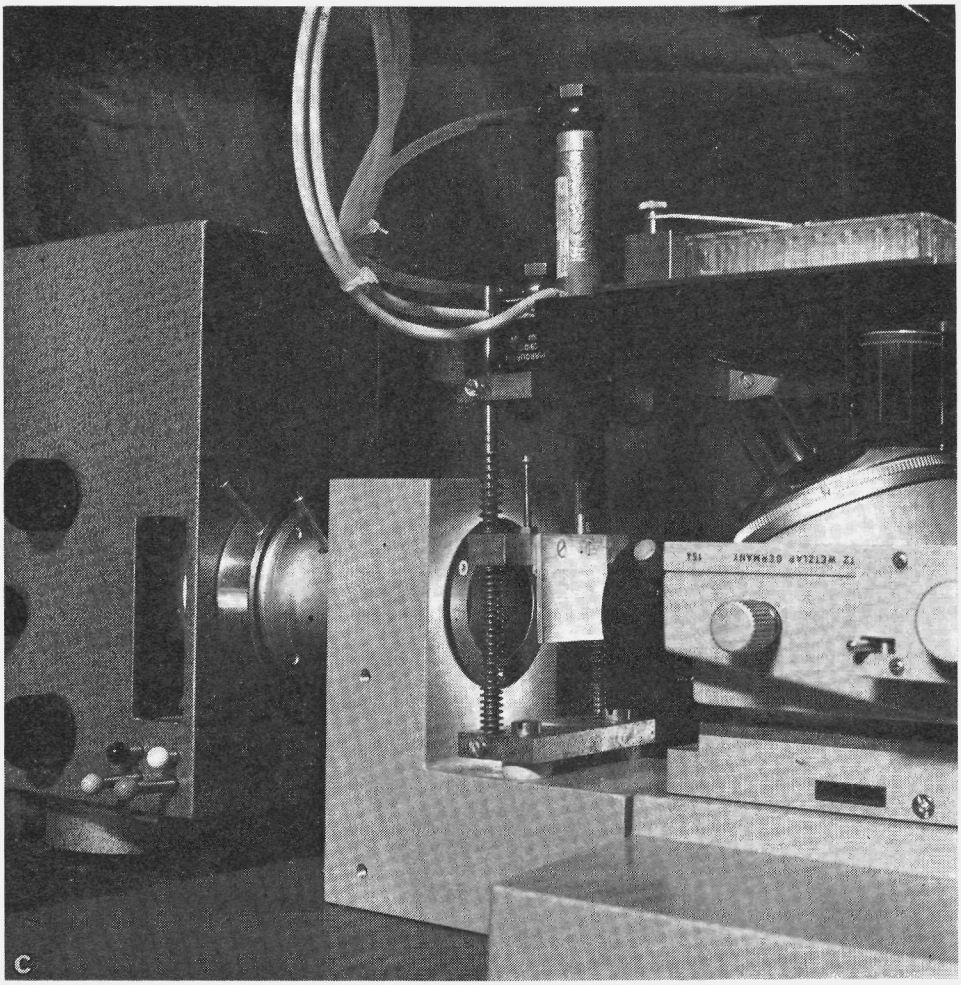


Figure VI-2c. Construction of shutter C (see Fig. VI-1) in the excitation pathway. The cylinder is a DG-12-25.
The adjustable screw on the shutter blade presses against the microswitch if the shutter is fully opened.

holds a double acting cylinder (either a DG-12-25 or DG-6-40) with a 25 or a 40 mm stroke. The shutter blade is guided by the steel rods and connected to the piston. The upper brass plate also holds a microswitch which is activated if the shutter is fully opened. This signal provides the link between the pneumatics and the electronics (see part D of this chapter). Fig. VI-2d presents an overview of the automated Leitz Diavert inverted microscope set up.

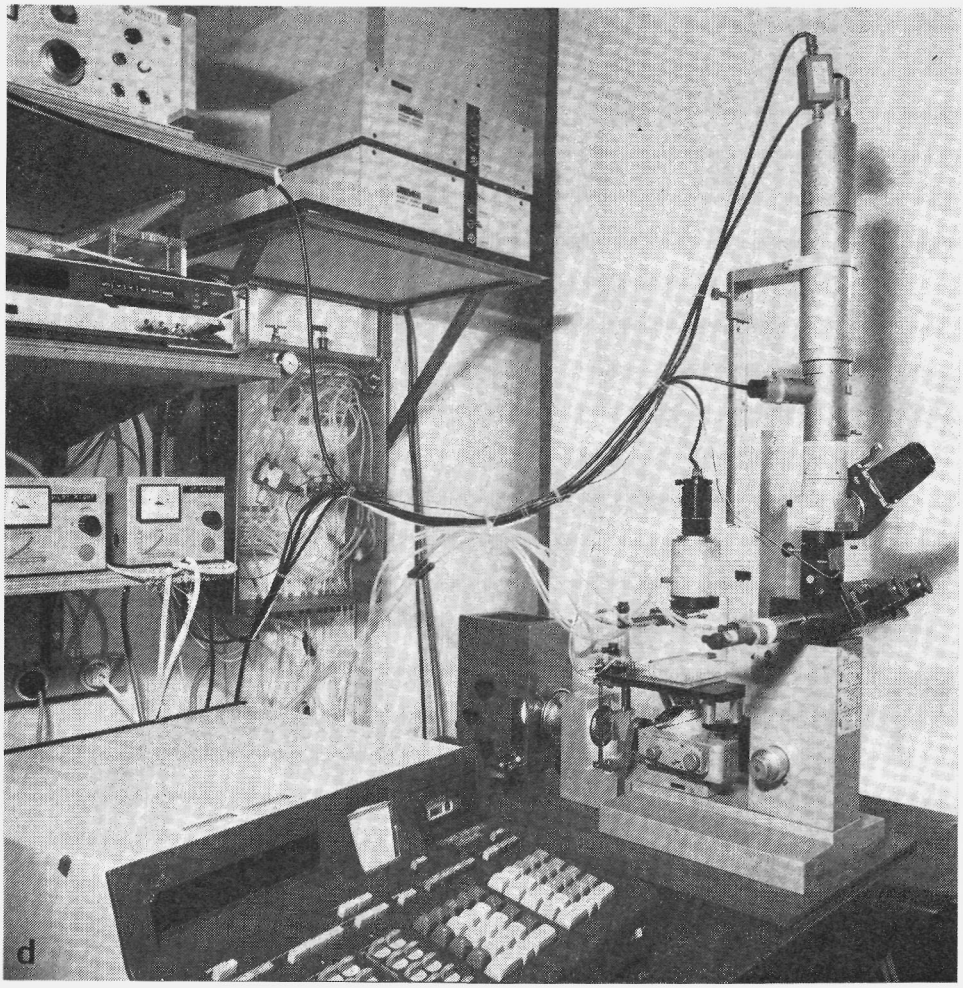


Figure VI-2d. General view of the automated Leitz Diavert inverted microfluorometer.

C. CONTROL OVER THE SEQUENCE OF THE SHUTTER MOVEMENTS

The fluorescence intensity of an individual bead is measured in the following sequence of operations:

a) A bead to be measured is located with diaillumination. The bead is positioned relative to the measuring diaphragm.
b) The diaillumination pathway is closed (shutter A in Fig. VI-1).
c) The pathway to the photomultiplier is opened (shutter B).
d) The excitation pathway is opened (shutter C).
e) As soon as shutter C is opened, the voltmeter is triggered.
f) The reading of the voltmeter is collected.

g) Shutters C and B resume their starting positions.

h) Shutter A opens and the next bead is located.

Haaijman and Wijnants (1975) established the automated control over the sequence of the shutters with an electromagnetic programme switch. This programme switch consisted of a capstan driven by an electromotor. The capstan was provided with adjustable cams which activated a set of three valves at a preset time. A measuring sequence was completed within one revolution of the capstan. There were two reasons to look for an alternative for this system: a) The switching of the electromotor tended to interfere with the rest of the electronic equipment. b) The speed of operation did not fulfill our requirements. Increasing the number of revolutions per minute of the capstan proved to be no attractive solution.

Two new control systems have been developed. The first is a completely pneumatic control which was constructed according to the scheme provided by Mr. F. Eykenhout of Festo Pneumatics B.V. (Delfgauw, The Netherlands). In the second control system, electromagnetic valves are activated by a desktop calculator.

1. *Pneumatic control*

Cascade programming has been applied in the pneumatic control. This possibility was already anticipated (Haaijman and Wijnants, 1975). Cascade programming means that the termination of the movement of one cylinder activates the movement of the next cylinder.

The measuring sequence is started with the activation of a valve in a foot pedal. The shutters A, B and C are activated one after the other. The microswitch in shutter C is connected to the calculator (see part D of this chapter), which instructs the voltmeter to take a reading of the photomultiplier output. The shutters resume their respective starting positions in the sequence C, B and A. The cycle time, that is the time needed for one measurement, is reduced with the pneumatic control to about 0.8 second, as compared to 6 seconds in our earlier design. Flow regulator valves in the lines to the cylinders determine the speed of the shutter movements. The air pressure is another important factor in the speed regulation. The pressure should be not less than about 2.5 atmospheres to guarantee proper operation of the pneumatic logics.

The majority of the data presented in this thesis have been collected with the pneumatically controlled inverted microscope. Only very recently we have adopted another approach: calculator control over pneumatically driven cylinders.

2. *Calculator control*

There were three reasons for constructing a second microscope under calculator control: a) In the pneumatic control, the excitation shutter (C) has to be open long enough to permit the voltmeter to take a reading. The pneumatic control is independent of the electronics. The time that the excitation shutter is opened has to be ad-

justed on the safe side, which means a loss in speed. b) The calculator has to check whether the shutter has been open long enough (see part D of this chapter). c) The sequence of the shutter movements can only be changed by retubing the lines to the cylinders.

Calculator control over the pneumatic cylinders was achieved by introduction of a Hewlett Packard 9821A desktop calculator (Hewlett Packard, Palo Alto, Cal., USA). The calculator was provided with a 11202A I/O TTL interface. This interface is connected to the calculator through one (of four) of the I/O slots in the back of the calculator. The 11202A interface has 8 input and 8 output lines. The logic status of the output lines can be varied by the calculator through the peripheral control ROM (Read Only Memory). Logic '1' of an output line can switch a relay. Switching of a relay results in the activation of an electromagnetic valve connected to the cylinders described in part B of this chapter. We have chosen 24V DC electromagnetic valves with a low power consumption (5W) in order to prevent interference with other parts of the electronics. The sequence of the different shutters may be programmed in the calculator with simple statements.

D. DATA ACQUISITION AND HANDLING SYSTEM

The desktop calculator is the core of the data acquisition and handling system. The calculator is interfaced via a HP/IB (interface bus) to a 3490A voltmeter (Hewlett Packard). The voltmeter is remotely controlled via the HP/IB. The status of the microswitch in the excitation pathway is 'read' by the calculator either via the 11202A I/O TTL interface or via the 11203BCD input interface. The latter is used if the 11202A interface is employed to control the relays. The moment the microswitch in the excitation shutter is activated, the calculator instructs the voltmeter to take a reading of the photomultiplier output. The voltage is measured over a 100 kOhm resistance through which the photomultiplier current is fed. The voltmeter is instructed to output its reading to the calculator at the termination of a measurement. The data of the voltmeter are stored by the calculator in its memory.

The background is measured from an empty part of a well. Three of these readings are usually taken per well of a microtitration plate. The calculator calculates the standard deviation and the standard error of the three background measurements. A standard error exceeding 10% of the average background fluorescence value is signalled by the calculator. In that case, three new background measurements must be performed. The fluorescence of at least 5 individual beads is measured after completion of the background measurements. The standard error is calculated after the fifth measurement and after each additional measurement. If the standard error becomes smaller than 7% of the average fluorescence value, the calculator prints out: a) the label of the well which has been measured (see below), b) the average fluorescence of the beads in that well, c) the standard deviation, d) the standard error of the measurement, and e) the percentage of the average which the standard error

constitutes. A maximum number of ten beads is measured per microtitration well. If the standard error after 10 bead measurements lies between 7 and 10% of the average fluorescence, the calculator gives the same output as mentioned above. The calculator prints the fluorescence values of all individual beads if the standard error exceeds 10%, so that errors may easily be detected and corrected.

Two microscopes can be connected to the calculator at the same time. Data are put on the magnetic tape of the calculator, together with codes identifying the microscope and the well number. This procedure has been chosen to make the waiting time for both microscope operators as short as possible.

E. PRACTICAL MICROFLUOROMETRY ON SEPHAROSE BEADS IN MICROTITRATION PLATES

The fluorescence of individual beads can be measured through the bottom of the microtitration plates. A special holder had to be constructed to hold the plates with 96 wells on the stage of the microscope. The range of the mechanical stage of the microscope is, unfortunately, not large enough to cover a whole plate. We always measure, therefore rows of 6 out of 8 wells. These wells are numbered 11 through 16, 21 through 26, etc. This code is also followed in the calculator print-out. The fluorescence of individual beads is measured with a 25 ×/0.60 water immersion objective. No leakage of water occurs if a small collar of adhesive tape is placed around the distal end of the objective. The working distance of this objective is 1.57 mm. The focus of the objective needs not to be changed for the location of successive wells. The water droplet used for immersion is carried along with the objective, probably because of the slightly hydrophobic nature of the plastic plates.

1. *Details of the equipment*

Microscope: Leitz Diavert inverted microscope equipped for epiillumination with Ploemopak 2.2 and with a holder for a 100W mercury arc. The 100/100 trinocular head held a MPV I measuring outfit (all from Leitz GmbH., Wetzlar, Western Germany) with a 5 × or 6.3 × eyepiece and a 3.2 mm measuring diaphragm.

Light source: CS 100W-2 mercury arc (Philips Nederland B.V., Eindhoven, The Netherlands) fed by an IREM E1-XH5P/L DC power supply, obtained through Leitz GmbH..

Photomultiplier: either 9558/S20 or 6094/S11 (EMI Electronic Ltd., Hayes, Middlesex, England) fed by stabilized power supplies A3.4K-40R (Oltronix, Vällingby, Sweden) or NSHM BN 600 S/N 397 (Knott Elektronik A.G., Munich, Western Germany). The photomultipliers were mostly used with 1.0 kV. The current of the photomultiplier was fed through a 100 kOhm resistance. The voltage drop over this resistance was measured.

Voltmeter: 3490A Multimeter (Hewlett Packard).

Calculator: 9821A (Hewlett Packard) with the interfaces 59405A (HP/IB), 11202A I/O TTL, 11203BCD input interface and the ROM's: PC II and Mathematics.

The filter combinations 8 and 11 (Table II-5) were commonly used for FITC stained beads and the combinations 13 and 14 for TRITC stained beads.

2. *Alignment of the microscope*

The field (excitation) diaphragm was focused into the object plane and adjusted to 1.5 times the diameter of the measuring diaphragm. The diaphragms were aligned concentrically. This procedure was done separately for each filter combination. The ring determining the width of the field diaphragm was secured with adhesive tape. The mercury arc was adjusted optimally using strongly fluorescent beads. Optimal fluorescence is obtained with a semi-critical illumination (see chapter II, part D 3). The mirror in the lamp housing was also adjusted in order to give optimal fluorescence. It was checked whether the centering aid in the eyepiece was in line with the measuring diaphragm.

F. CONCLUSIONS

No automated version of an inverted microfluorometer has been made commercially available up to now.

The Leitz Diavert inverted microscope lends itself, due to its open design, to automation with pneumatic components. The major advantage of pneumatics is the reliability. At least half-a-million measurements have been taken with the pneumatically controlled version of the inverted microscope without technical difficulties. This figure compares favourably with other microscopes equipped with electronic shutters.

The direct interfacing of the calculator to the voltmeter means a large improvement over the earlier design of Haaijman and Wijnants (1975). With the automated microscopes, it is possible to measure a thousand beads distributed over 96 wells of a microtitration plate in the course of 1 to 1½ hours. A second microscope, provided with calculator controlled shutters, has been recently developed. The software needed for its operation is negligible. The commercial manufacturing of this type of microfluorometer may promote the wider application of fluorescence immunoassays.

CHAPTER VII

Experimental protocol for fluorescence immunoassays with proteins bound to Sepharose beads

Contents:

A. INTRODUCTION

The fluorescence immunoassays, to be described in chapter VIII, require a rigid experimental protocol. A number of variations in the preparation of Sepharose beads coupled with proteins and the possible alternatives has already been discussed in chapter IV, part F.

The protocol we used for the immunoassays is described in this chapter. The first step in the procedure is the activation of Sepharose beads with cyanogen bromide (CNBr). A complete description of the method for the activation and subsequent coupling of proteins to the activated beads is given in part B of this chapter. It has been attempted to indicate which steps are critical and which are less critical. Incubation and washing procedures are described in part C of this chapter.

Methods for standardization and day-to-day calibration are fully discussed, with the relevant applications, in chapter VIII.

B. COUPLING OF PROTEINS TO SEPHAROSE BEADS ACTIVATED WITH CYNANOGEN BROMIDE

The method of March, Parikh and Cuatrecasas (1974) was used for the CNBr activation of Sepharose and the subsequent coupling of protein. Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were washed with a large volume of distilled water in order to remove the preservative added by the manufacturer. One volume of washed Sepharose consisting of equal volumes of beads and water was added to one volume of 2 M sodium carbonate. The mixture was then slowly stirred. The rate of stirring was increased and 0.05 volume of a solution of cyanogen bromide (Fluka A.G., Buchs, Switzerland) in acetonitrile (2 gr CNBr/ml acetonitrile) was added. The stirring was continued for 1–2 min. The gel was then washed on a coarse-sintered glass funnel with 5–10 volumes of the following solutions: a) 0.1 M sodium bicarbonate, pH 9.5; b) distilled water; and c) 0.2 M sodium bicarbonate, pH 9.5. The gel was sucked almost dry after the last wash and transferred to a tube containing one volume of 0.2 M sodium bicarbonate, pH 9.5, and one volume of the protein usually dissolved in phosphate buffered saline (PBS). One to five mg of protein were incubated with one ml of packed activated beads. The coupling was carried out for 8–12 hr on a rollerbank at 4 °C. The residual active groups were subsequently deactivated by overnight incubation with 2 volumes of 0.5 M ethanolamine or glycine. The beads were washed after coupling and deactivation with 20 volumes of the following solutions: a) 0.1 M sodium acetate, pH 4.0; b) 2 M urea; and c) 0.1 M sodium bicarbonate, pH 9.5. All washing solutions also contained 0.65 M NaCl. The washing was performed with a sintered glass funnel when a large volume of beads was used. For small volumes, a centrifuge tube was employed. Beads were finally stored as a 1:10 suspension in PBS (1 volume beads + 9 volumes PBS) at either 4 °C or

−20°C. If the beads were stored at 4°C, 20 µl of a 0.5% merthiolate solution was added per ml of bead suspension.

Comments. a) The CNBr solution in acetonitrile is stored at −20°C. It can be used for several months. Dimethylformamide and N-methylpyrrolidone have been successfully used as alternative solvents for CNBr (Nishikawa and Bailon, 1975; Bloemmen, personal communication). b) The washing solutions are stored at 4°C. No special measures were taken to further control the temperature. This is different from most reports, which stress the use of ice baths during the whole activation procedure. c) The protein was added as quickly as possible after termination of the activation and washing procedures. d) The following substances are often present in antigen or antibody preparations and should be removed by dialysis before coupling: NaN_3, glycine, Tris and diaminoethane. Dialysis is usually performed against PBS.

C. INCUBATION AND WASHING PROCEDURES

The 1:10 bead suspensions were diluted tenfold with PBS. Fifty µl aliquots of these 1:100 suspensions were dispensed into the wells of M220-29ART microtitration plates (System Cooke; Greiner, Würtingen, Western Germany) with 96 flat-bottom wells. Normally, 50 µl dropper bottles (Microdel; System Cooke, no. M49) were used. In very large experiments, the 1:100 suspension was stirred slowly with a magnetic stirrer and 50 µl aliquots were delivered with disposable pipettes (Cooke, M36). Attention should be paid to the homogeneity of the bead suspension.

Antisera and conjugates were diluted in 2% bovine haemoglobin (Sigma, St. Louis, Md., USA) in PBS. Incubation was carried out for 1 hr at room temperature or overnight at 4°C under continuous agitation (Cooke Microshaker, AM 69).

The beads were washed with a home-made apparatus after incubation. This apparatus combines an eight-channel dispenser and an eight-channel aspirating manifold. The dispenser consisted of a pressure vial held at 3.5 kg/cm^2, the outlet of which is fed through a magnetic valve which could be activated with a foot pedal. A laboratory vacuum line was used to remove the supernatant. A valve ensures that only vacuum was applied if necessary. The washing fluid consists of 0.65 M NaCl in PBS. The microtitration plate was left standing for 4 min after adding approximately 100 µl of washing fluid to each well. The supernatant may then be aspirated without disturbing the beads lying on the bottom of the wells. About 50 µl of fluid remained in the wells. Successive incubations could be carried out in the same plate. During incubation, the plates were sealed with adhesive tape (77-021-01; Titertek, Flow Laboratories, Rockville, Md., USA).

Absorptions of antisera or conjugates were done in plastic tubes of appropriate capacity. The tubes were rotated head-over-head during absorption. The antiserum

was separated from the absorption beads by either filtering the suspension over a sintered glass filter in a disposable minicolumn (737–1230; Bio Rad, Richmond, Cal., USA) or forcing the suspension with centrifugal force through nylon mesh. It is essential, of course, to remove all beads used for absorption.

Plates with incubated beads may be stored frozen for at least one week before measuring without appreciable adverse effects. We have prepared plates filled with 50 µl of appropriate bead suspensions for large series and stored them frozen for one month before starting the incubations. No loss of antigen binding capacity was observed.

Comment. The introduction of the microtitration plates and the washing device has considerably reduced the time needed for sample processing. The preparation of the sample before incubating it with the appropriate beads is the time-limiting factor in most experiments (see also chapter VIII, part K).

D. THE MEASUREMENT OF PROTEIN CONCENTRATION WITH FLURAM

The observed reactions in the Sepharose bead immunofluorescence system are strongly concentration-dependent (see for instance Fig. V-2). The total protein content of the samples is the only method to standardize the results in some cases, where functional or other tests are lacking or too time-consuming. Examples of such cases are the determination of the concentration of viral proteins in milk and organ extracts (see chapter VIII, parts H and I). Spectrophotometric methods cannot be used in these cases due to the presence of the detergent Nonidet P40 (NP40; Shell Oil Co., New York, N.Y., USA) and the lack of molar absorbance data. We have adopted the fluram (fluorescamine) protein assay (Udenfriend, Stein, Böhlen, Dairman, Leimgruber and Weigele, 1972) for our purposes.

Ten µl of protein solution in PBS is mixed with 140 µl of 0.1 M borate buffer, pH 9.0. Fifty µg of fluram (Hoffman-La Roche A.G. Diagnostica, Basel, Switzerland) in 50 µl of acetone is added under vigorous stirring. Aliquots of 150 µl of the mixture are transferred to a flat-bottom microtitration plate after 10 min at room temperature. The fluorescence is measured with the inverted microscope using an UG 1 excitation filter and a K 430 emission filter (combination 1, Table II-5). A good linearity was observed with transferrin as the standard protein in the range of 0.1–5 mg/ml protein. Up to 1% of the NP40 detergent did not interfere with the reaction.

Comment. The fluram reacts with primary as well as secondary amino groups in the protein. The reaction proceeds to completion in a matter of seconds. Fluram molecules which do not react with amino groups, hydrolyse rapidly in aqueous solution.

The fluram solution should be prepared freshly before starting a determination on a series of samples. Each introduction of water into the fluram solution, e.g., with the tips of pipettes, will result in hydrolysis of the fluram. The appearance of hydrolysed fluram is evident by a slight yellow discolouration of the acetone solution.

CHAPTER VIII

Application of the Sepharose bead immunofluorescence assay (SBIA)

Contents:

A. INTRODUCTION

The Sepharose bead immunofluorescence system can be used for the detection and quantitation of almost any compound, provided that there is an antiserum directed against that compound available. A number of specific applications is presented in this chapter. One of the most important applications has already been discussed in chapter IV, namely, the evaluation of the quality of antisera and conjugates.

The examples given in this chapter may be divided into different categories according to different criteria. From a technical point of view, examples B through E deal with the detection of antibodies by using antigens bound to Sepharose beads. Antibodies are used bound to Sepharose beads for the detection of antigens in examples F through J.

Another classification of the applications in this chapter can be made according to the biological field of interest.

The age-related changes in the immunological *capacity* of mice to react to experimentally injected antigens are the subjects of parts B and C. Mice of different ages have been stimulated with either a lipopolysaccharide (LPS) isolated from *Escherichia coli*, or human serum albumin (HSA). For the antibody response to HSA, interaction between T-cells (thymus-derived cells) and B-cells (cells processed in mammals by the equivalent of the Bursa of Fabricius in birds) is necessary. LPS is considered to be a T-cell-independent antigen. With the Sepharose bead immunofluorescence assay, the production of both anti-LPS and anti-HSA antibodies in individual mice has been measured throughout the course of the immune response against these antigens.

The variations in the immunological *activity* with age are discussed in parts F and G. The immunological *activity* is the reaction of the immune system observed against not experimentally injected antigens. The definitions of immunological *capacity* and *activity* are of course operational. These studies on the age-related immunological activity were an extension of the investigations reported by Haaijman, Schuit and Hijmans (1977). They estimated the number of cells containing cytoplasmic immunoglobulin (C-Ig cells) irrespective of the antigens to which this Ig was directed in different lymphoid organs of CBA mice of different ages. These mice were not intentionally stimulated with antigens. The immunological *activity* rather than the *capacity* to react to certain antigens is therefore measured. In parts F and G, the products of the C-Ig cells are analyzed, namely, the serum immunoglobulins. In collaboration with Dr. J. Radl and Miss P. van den Berg of the Institute for Experimental Gerontology TNO, we were able to prepare a set of Sepharose beads coupled with the IgG fractions of rabbit antisera specifically directed against the Fc parts of IgA, IgM and the four subclasses of IgG. The technical details of the determination of Ig levels with these beads are presented in part F of this chapter; the results obtained with this system are given in part G.

A more thorough discussion of the data on the number of C-Ig cells, the levels of

Ig classes and subclasses and the ability (capacity) to react to HSA as a function of age in CBA mice will be presented in a separate chapter (chapter IX).

Tumour virology is the next field of interest to which the bead immunofluorescence technique has been applied (applications D, E and H through J). Every mouse harbours endogenous RNA tumour viruses. The DNA copy of the genome of endogenous RNA tumour viruses (oncornaviruses) is integrated into the genome of the host and is consequently transmitted vertically. Two types of RNA tumour viruses receive attention in this chapter: the B- and C-type RNA viruses. The B-type viruses contain a spherical excentric core (nucleoid) and are associated with mammary tumours in mice. The C-type viruses have a central polygonal core and are associated with leukaemia in, among others, mice, cats and gibbon apes.

One of the most interesting immunological aspects of these viruses is the fact that they elicit antibodies in their hosts, although they may be regarded as 'self' components. Measurement of this natural immunity against endogenous viruses is the subject of parts D and E. We have been able to demonstrate naturally occurring antibodies reacting with simian sarcoma virus and Rauscher leukaemia virus in rats. Sepharose bead immunofluorescence techniques have failed, however, in the detection of natural immunity against mammary tumour virus in mice (part E).

Endogenous viruses are transmitted vertically. Male mice also receive the genetic information for the expression of mammary tumour viral antigens, although they lack the target organ. Parts H and I deal with the expression of these antigens in different organs of male mice of different strains and ages. We have divided this application into a technical section (H) and a section containing the results (I).

The techniques for the detection and quantitation of C-type viral proteins have

TABLE VIII-1. APPLICATIONS OF THE SEPHAROSE BEAD IMMUNOFLUORESCENCE ASSAY TO THE DETERMINATION OF PROTEINS

Reference	Application or determination of:
Capel (1974)	RAHu/IgG and Hu/IgG
Knapp, Menzel and Steffen (1974)	Hu anticollagen
Capel (1975)	Hu/IgE, Hu antiallergen, complement binding to Seph-IgG and β-lipoprotein
Capel and Aalberse (1975)	Hu antiallergen, Hu/IgE
Deelder and Ploem (1975a)	Hu and M anti*Schistosoma mansoni* antibodies
Deelder and Ploem (1975b)	Hu anti*Fasciola hepatica* R anti*F. hepatica*
Deelder, Snoijink and Ploem (1975a)	Hu anti*S. mansoni*
Deelder, Snoijink and Ploem (1975b)	Hu anti*S. mansoni*
Haaijman, Bloemmen and Ham (1975)	Hu/IgG, M/IgM and OVA
Sernetz and von Sengbusch (1975)	Esterase activity
Streefkerk, Deelder, Kors and Kornelis (1975)	Hu anti*S. mansoni*
Knapp and Ludwig (1976)	Hu antithyroglobulin
Sernetz, Puchinger, Couwenbergs and Ostwald (1976)	Esterase activity

been elaborated along the same lines as those techniques for the mammary tumour viral proteins. A single application is reported (J), namely, the monitoring of the production of viral proteins by infected cells in tissue cultures.

It will be attempted in this chapter to outline the biological significance of the different applications in the respective sections. In the last part of this chapter (K), a general discussion on the alternatives for the bead immunofluorescence technique is presented.

A number of papers have already been published applying the Sepharose bead immunofluorescence system to a variety of antigen-antibody systems. Some of them, concerning the evaluation of antiserum or conjugate specificity, have already been listed in chapter IV, Table IV-1. References and applications of the bead system to the quantitation of proteins (antigens or antibodies) are listed in Table VIII-1.

B. MEASUREMENT OF THE LEVELS OF CIRCULATING ANTIBODIES DURING THE COURSE OF THE IMMUNE RESPONSE AGAINST LPS IN INDIVIDUAL MICE

Since the classical experiments of Makinodan and co-workers (Makinodan and Peterson, 1964, 1966a, 1966b), it has been generally recognized that the immunological capacity of mice declines with age. These authors injected sheep red blood cells (SRBC) into BC3F1 mice of different ages and measured the number of plaque-forming cells (PFC's) in the spleen. A tenfold reduction in the number of PFC's in aged mice was reported as compared to young mice. The secondary immune response towards SRBC was measured in mice primed at 3 months of age. A 90% reduction in the secondary response was found in old mice. This experiment is, however, subject to objections. In the experimental design of Makinodan and Peterson (1966b), the reduction of the number of memory cells rather than the reduction in secondary response potential is measured. It should be mentioned that Finger, Beneke, Emmerling, Bertz and Plager (1972) were not able to demonstrate an age-related decline in secondary immune capacity towards sheep red blood cells in NMRI mice.

The increase in variability among individuals with age is one of the most evident gerontological phenomena (see Kohn, 1971; and, for example, Radl, Sepers, Skvaril, Morell and Hijmans, 1975; Makinodan, Albright, Good, Peter and Heidrick, 1976; Burek and Hollander, 1977; Haaijman, van den Berg and Brinkhof, 1977;). This phenomenon makes it often difficult to draw conclusions based on transsectional data (Gore, 1973).

We have followed the immune response in individual animals with the bead immunofluorescence technique. Two antigens have been used: lipopolysaccharide (LPS) and human serum albumin (HSA). LPS isolated from *E. coli* is a potent immunogen for

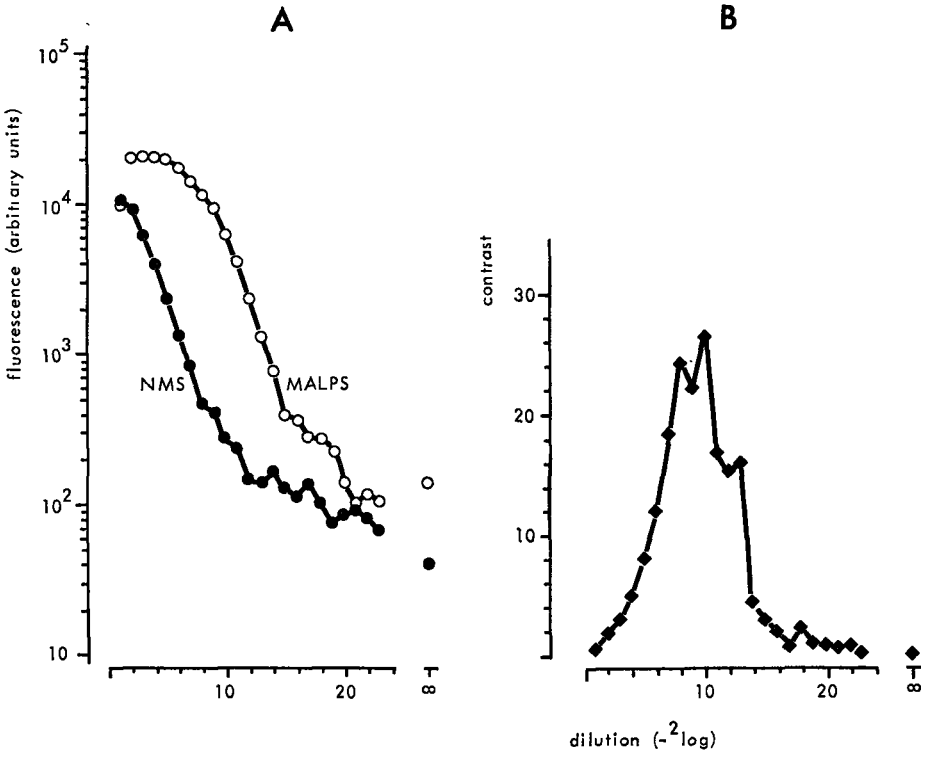


Figure VIII-1. The reaction of a normal mouse serum (NMS) and a BALB/c mouse antiserum directed against LPS (MALPS) with *E. coli* lipopolysaccharide bound to Sepharose beads (Seph-LPS). NMS was obtained from nonimmunized mice. A TRITC labelled rabbit antiserum directed against mouse IgM (RAM/IgM-TRITC) was used in a second incubation. The conjugate was diluted 1:100 and absorbed with Seph-LPS before use. Each point in the left hand figure is the average fluorescence of 10 individual beads. Right hand figure: ratio (contrast) between the average fluorescence obtained with MALPS and NMS as a function of dilution. ∞: conjugate control.

mice. The antibody response towards LPS is T-cell-independent in mice (Andersson and Blomgren, 1971). Blankwater, Levert and Hijmans (1975) showed that 19- to 21-month-old BALB/c mice reacted slightly better to LPS than did 2- and 4-month-old mice, as measured with the plaque-forming cell (PFC) assay. Smith (1976) reported a peak in the PFC response to LPS around 40–45 weeks of age. His data on the haemagglutination titre in serum, however, did not show an optimum.

We have used Sepharose beads coupled with LPS to measure the amount of circulating anti-LPS antibodies after a single i.v. injection of LPS. These experiments were done in collaboration with G. Scheurkogel (Leiden University, The Netherlands). LPS (Difco Laboratories, Detroit, Mich., USA) was coupled to Sepharose beads under standard conditions (see chapter VII, part B). Coupling presumably takes place via the glucosamine residues of the LPS. We obtained a pool of BALB/c mouse

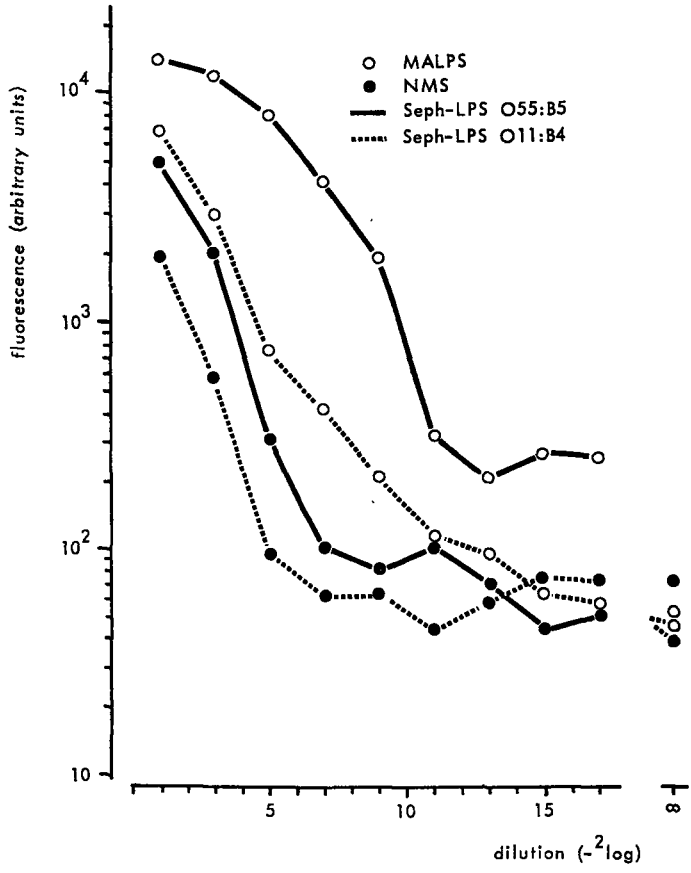


Figure VIII-2. Cross-reactivity of the BALB/c mouse antiserum directed against *E. coli* lipopolysaccharide LPS O55:B5 (MALPS) with LPS O11:B4. Sepharose beads were coupled with LPS O55:B5 and LPS O11:B4. Both bead preparations were incubated with MALPS(O55:B5) and NMS (from nonimmunized mice). The second incubation was with RAM/IgM-TRITC (see Fig. VIII-1) diluted 1:100. Each value represents the average fluorescence of 10 individual beads.

serum with a high titre of haemagglutinating anti-LPS antibodies. This serum will be referred to as MALPS. MALPS was used to demonstrate the feasibility of the assay for circulating antibodies (Fig. VIII-1). Seph-LPS was incubated with MALPS and normal mouse serum (NMS). The NMS was taken from mice which were not immunized. Antibodies bound to the LPS beads were stained in a subsequent incubation with a fluorescent rabbit antiserum directed against mouse IgM (RAM/IgM-TRITC). This antiserum was selected because it is known that LPS induces only IgM antibodies. A clear reaction of the Seph-LPS with MALPS was observed. The reaction was concentration-dependent. This makes it possible to estimate the amount of serum antibodies against LPS. The highest contrasts between MALPS and NMS (Fig. VIII-1, right hand side) was obtained with $-^2$log dilutions of MALPS between 8 and 10. For

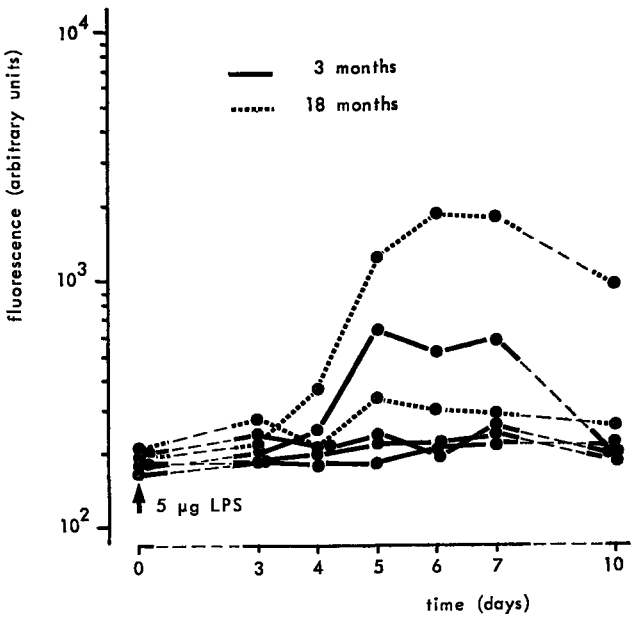


Figure VIII-3. The immune response towards *E. coli* lipopolysaccharide (LPS) in individual C57BL mice. Two animals were 18 months, the others 3 months of age. The animals received 5 µg of LPS in 0.5 ml of PBS at day 0. Serum samples were taken from the tail vein at indicated days and stored frozen in haematocrit tubes. The anti-LPS reactivity was measured using 1 µl of serum, diluted 500-fold. Diluted sera were incubated with Seph-LPS and the antibodies bound to the beads were quantitated after incubation with RAM/IgM-TRITC (see Fig. VIII-1). Each value represents the average fluorescence of 10 individual beads.

the estimation of the anti-LPS activity in different serum samples, the samples have to be diluted more than 256-fold ($-^2$log dilution of 8). The nature of the reaction of NMS with Seph-LPS was not further investigated.

The specificity of the MALPS serum was further tested on two different preparations of LPS. The serum had been raised against LPS O55:B5 (Difco Laboratories). It reacted only weakly with another LPS preparation, O11:B4 (Fig. VIII-2), also from Difco Laboratories.

Individual mice were injected i.v. with 5 µg LPS (O55:B5) in 0.5 ml of PBS. Serum samples of two to three µl were obtained from the tail vein at daily intervals. The samples were stored frozen in sealed haematocrit tubes. Samples of one µl were diluted 500-fold and incubated with Seph-LPS (O55:B5). The beads were washed and subsequently incubated with the conjugate RAM/IgM-TRITC diluted 1:100. The fluorescence of individual beads was measured after washing the beads free of excess conjugate. The averaged fluorescence intensities are shown in Fig. VIII-3.

Six individual C57BL mice were followed longitudinally for 10 days. The data for two 18-month-old and four 3-month-old animals are shown. Serum from one of the

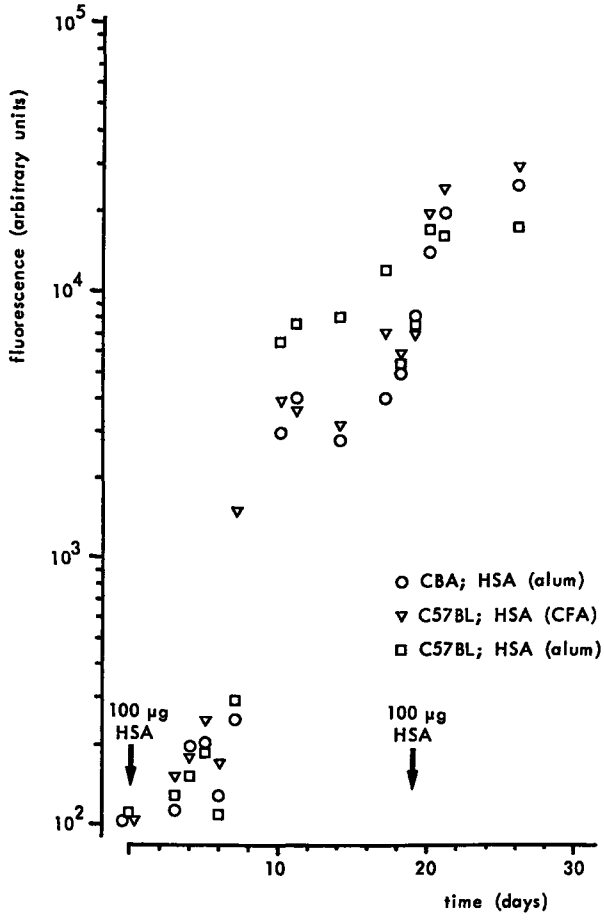


Figure VIII-4. The antibody response of 12-week-old mice to human serum albumin (HSA). Five male CBA mice and C57BL mice were immunized at day zero and at day 19 i.p. with 100 µg HSA either alum-precipitated or emulsified in complete Freund's adjuvant (CFA). Serum samples were collected from the tail vein on indicated days and stored frozen in haematocrit tubes. Anti-HSA reactivity was measured with 1 µl samples, diluted 150-fold. The diluted serum samples were incubated with HSA coupled to Sepharose beads (Seph-HSA) and these beads were subsequently incubated with a 1:50 diluted fluorescent goat antiserum directed against mouse Ig's (GAM/Ig-TRITC). From each preparation, the fluorescence of 10 individual beads was averaged. Plotted values are the averages of the fluorescence obtained with the individual sera per group. Data have been calibrated relative to a pool of mouse anti-HSA serum.

18-month-old animals reacted strongly and one reacted weakly. Three out of the four young animals did not respond at all. The fourth young mouse showed an intermediate reaction. The immune response towards LPS is genetically determined (di Pauli, 1971). The C57BL strain proved to be a poor responder to LPS. Five 3-month-old BALB/c mice which were used as controls in the same experiment all reacted very strongly. The great differences among the responses of individual C57BL mice (Fig. VIII-3)

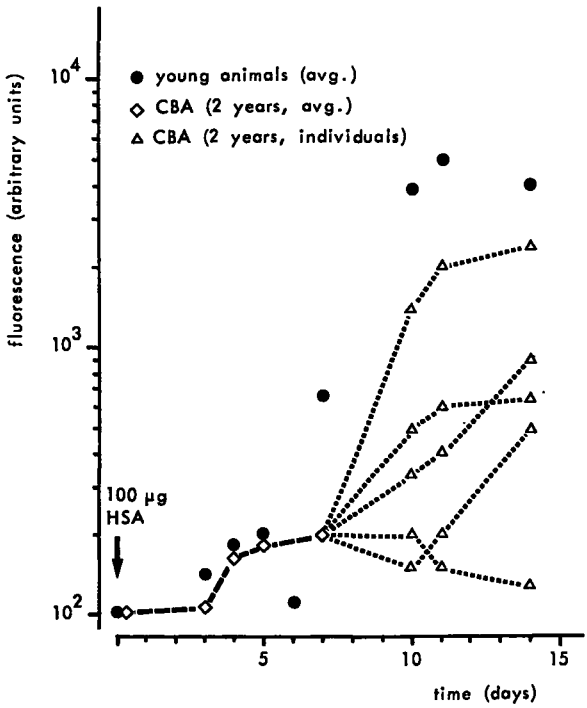


Figure VIII-5. Antibody formation against human serum albumin (HSA) in 2-year-old male CBA mice. Five mice were immunized i.p. with 100 µg alum-precipitated HSA. The anti-HSA reactivity was measured as in Fig. VIII-4. The data obtained with sera of the individual mice up to day 8 after immunization have been averaged. From that day on the individual mice showed such a divergence in their anti-HSA response that individual data have been plotted. The averaged data from Fig. VIII-4 obtained with young mice are shown for comparison.

resemble the results of Blankwater, Levert and Hijmans (1975) obtained with the NZB mouse strain.

C. MEASUREMENT OF THE LEVELS OF CIRCULATING ANTI-HSA ANTIBODIES IN MICE

As earlier mentioned, LPS is a T-cell-independent antigen which elicits antibodies of only the IgM class. We were interested in the influence of age on immune responses involving the T-B-cell interaction. We have studied therefore the response of mice of different ages to human serum albumin (HSA) as antigen. Mice are known to respond poorly to HSA alone. For this reason, we have injected HSA i.p. either adsorbed on an alum precipitate or emulsified with complete Freund's adjuvant (CFA). The monitoring of the immune response of individual mice longitudinally was of first importance. A more detailed study on the influence of age on the immune response to HSA is presented in chapter IX.

Three groups of 5 male mice were immunized: a) 12-week-old CBA mice with 100 μg alum-precipitated HSA; b) 12-week-old C57BL mice with 100 μg HSA emulsified in CFA; c) 12-week-old C57BL mice with 100 μg alum-precipitated HSA. Each mouse received 0.2 ml of the antigen preparation. Serum samples were collected from individual mice as described in part B for the experiment involving LPS. For the determination of the level of anti-HSA antibodies, individual sera were diluted 150-fold, as was indicated by preliminary experiments. The sera were incubated with Sepharose beads coupled with HSA. Bound antibodies were stained with a 1:50 diluted TRITC labelled goat antiserum directed against mouse Ig's. The fluorescence of individual beads was measured after they were washed.

The results for the individual mouse sera closely resembled each other. This is in contrast to the results obtained with LPS in C57BL mice. The averaged data for 5 individual mice per antigen treatment are presented in Fig. VIII-4. The reaction of the sera with the Seph-HSA showed an increase around the 7th day after immunization and reached a plateau around the 10th day. A second injection of 100 μg HSA was given 19 days after the first. A slight reaction to this second injection was observed. The manner in which the antigen was presented to the mice did not yield systematic differences in the antibody response. CBA and C57BL mice reacted in much the same way.

In the next experiment, we injected five 2-year-old CBA mice i.p. with alum-precipitated HSA (Fig. VIII-5). Striking differences were found between the different individuals of this group. One animal reacted almost as strongly as did the young animals, whereas three animals reacted very little or the reaction was delayed. One animal showed no reaction. Unfortunately, all mice died by day 16 after immunization, perhaps due to the stress involved in the daily blood collection.

The increase in variability with age is a recurrent phenomenon in ageing research. It is sometimes regarded as the most general attribute of ageing in general (Kohn, 1971).

The problem of day-to-day standardization arose in the experiments illustrated in Figs. VIII-4 and VIII-5. The measurement of a standard preparation each day is an obvious solution. Two standards may be used: a) the aminoethyl-Sephadex beads stained with TRITC as discussed at length in chapters II and III; and b) Seph-HSA incubated with a fixed dilution of a selected pool of serum and incubated with fluorescent conjugate. The first standard compensates for changes in the microfluorometry equipment, but not for differences in preparation technique. These differences include small variations in the concentration of the fluorescent conjugate, the introduction of newly prepared bead suspensions, etc. We have chosen, therefore, to calibrate our daily measurements on Seph-HSA beads incubated with a pool of anti-HSA serum. The control is handled in the same plate as the test samples. The variation in the dilution of the calibration pool is a difficulty with this approach. A sufficient number of independent estimates have to be made in order to obtain a reliable calibration factor. This will be further discussed in sections F and H.

D. DETECTION OF NATURALLY OCCURRING ANTIBODIES REACTING WITH ONCORNAVIRAL ANTIGENS IN RATS

A number of animal species harbour endogenous RNA tumour viruses. A virus is endogenous if a DNA copy of the viral genome is present in the cellular DNA of the host and is thus transmitted vertically. It has been established in a number of reports (Ihle, Yurkonic and Hanna, 1973; Aaronson and Stephenson, 1974; Nowinski and Kaehler, 1974; Ihle, Denny and Bolognesi, 1976; Oldstone, Del Villano and Dixon, 1976; Schwartz, Donnelly, Melief and Louie, 1976) that, at least in mice, antibodies are generated against these endogenous viruses, although the viruses, immunologically-speaking, belong to the 'self' components. We have attempted to determine the presence of natural antibodies against oncornaviral antigens in the serum of rats. Sepharose beads were coupled with disrupted simian sarcoma virus (SiSV), Rauscher leukaemia virus (RLV) and mouse mammary tumour virus (MTV). The SiSV and RLV isolates were obtained through the Office of Program Resources and Logistics, Viral Oncology Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md., USA. The MTV was isolated from a homogenate of BALB/cfC3H mammary tumours. Beads coupled with ovalbumin were used as the NSS control. The results for only two rat sera will be shown as an example for an anti-SiSV and an anti-RLV positive as well as negative serum. Seph-RLV,Seph-SiSV, Seph-MTV and Seph-OVA were incubated with different dilutions of the serum of one germfree WAG/Rij rat and one conventionally raised BROFO rat. The beads were washed and incubated subsequently with a FITC labelled goat antiserum directed against rat Ig's (GARa/Ig-FITC). The results of the fluorescence measurements are shown in Figs. VIII-6A and -B. The presence of components in the serum of the WAG/Rij rat reacting specifically with Seph-RLV as well as Seph-SiSV is clear. The serum of the BROFO rat reacted weakly only with the RLV beads. No reaction was observed with the SiSV beads. It was not surprising that antibodies as in the WAG/Rij serum react with both kinds of beads because SiSV and RLV share antigenic determinants (Geering,

TABLE VIII-2. ABSORPTION OF WAG/Rij SERUM

Serum absorbed with	Reaction with			
	Seph-SiSV	Seph-RLV	Seph-MTV	Seph-OVA
Unabsorbed	1549	13400	340	200
SiSV	450	1000	280	550
RLV	500	1250	200	250
MTV	1400	10500	400	350

Serum of a 6-week-old germfree male WAG/Rij rat was tested either unabsorbed or absorbed with the indicated viruses before being tested, diluted 1:4, on the different beads. Each value is the average fluorescence of at least 5 individual beads. Viruses were pelletted in a Beckman SW60 rotor at 121,000 g for 60 min. The pellet (1 mg) was resuspended in 0.1 ml of 0.1 M lithium diiodosalicylate in distilled water. Undiluted serum (0.1 ml) was added and incubated with the viral proteins for 2 hr. After absorption, any microprecipitates were spun down at 18,000 g for 5 min.

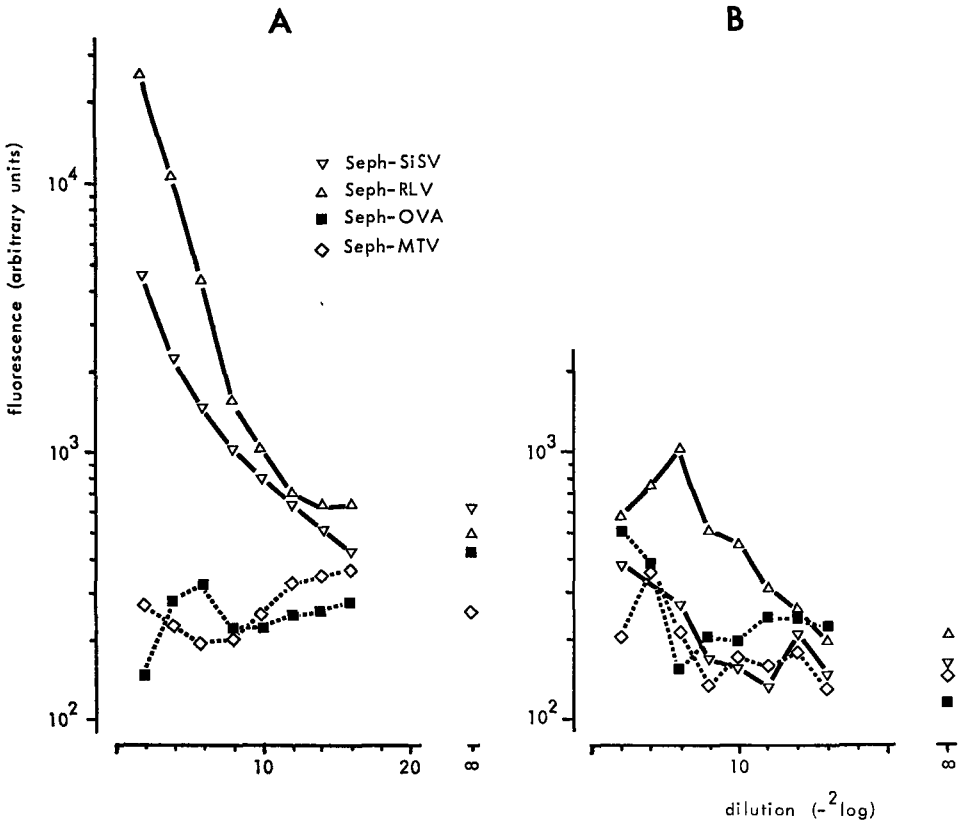


Figure VIII-6. The detection of naturally occurring antibodies against C-type oncornaviral antigens in rats. The heat-inactivated sera (30', 56 °C) of one 6-week-old male WAG/Rij rat, raised under germ-free conditions (left hand figure, A), and one 28-week-old female BROFO rat, raised conventionally (right hand figure, B), were incubated in different dilutions with beads coupled with disrupted simian sarcoma virus (Seph-SiSV), Rauscher leukaemia virus (Seph-RLV), mouse mammary tumour virus (Seph-MTV) and ovalbumin (Seph-OVA). In order to demonstrate binding of rat Ig's, the beads were washed and incubated with a 1:50 diluted fluorescent goat antiserum directed against rat Ig's (GARa/Ig-FITC) previously absorbed with Seph-OVA. Each value is the average fluorescence of at least 5 individual beads. ∞: conjugate control.

Aoki and Old, 1970; Gilden, Oroszlan and Huebner, 1971). It is not an indication of the presence of two different species of antibodies. This may be corroborated by absorption experiments *in vitro* (see chapter IV, part I). The serum of the WAG/Rij rat of Fig. VIII-6A was absorbed *in vitro* with solubilized SiSV, RLV and MTV and subsequently tested on Seph-SiSV, Seph-RLV, Seph-MTV and Seph-OVA (Table VIII-2). Absorption with SiSV evidently also reduces the reactivity of the serum with Seph-RLV and vice versa. This suggests that SiSV and RLV contain antigenic determinants which are very much alike. We have assumed that the reactivity of the sera with virus beads is due to antivirus antibodies, although no definite proof can be

presented (see also Fischinger, Ihle, Bolognesi and Schäfer, 1976). It should be excluded, e.g., that the reaction is caused by strongly adsorbing (glyco)proteins in the virus preparations, causing a nonimmunological adherence of the rat proteins. The low conjugate controls in Fig. VIII-6A and B reduce, however, the likelihood of this possibility.

The presence of the same or closely related antigenic determinants on the proteins of a variety of leukaemia viruses makes it plausible to assume that the antibodies in the WAG/Rij rats are directed against an endogenous rat C-type oncornavirus. The existence of an endogenous C-type virus in the rat has been shown by, among others, Verwoerd and Sarma (1973) and Rasheed, Bruszewski, Rongey,Roy-Burman, Charman and Gardner (1976).

Horizontal transmission of SiSV or RLV virions under the germfree rats can be excluded with a high degree of certainty. Experiments are in progress to study the influence of ageing on the presence of natural antibodies against oncornaviruses in different strains of rats.

E. DETECTION OF NATURAL ANTIBODIES AGAINST MAMMARY TUMOUR VIRUS IN MICE

In addition to leukaemia viruses, mammary tumour viruses (MTV) are also endogenous in mice (Bentvelzen, 1968). The presence of cellular immunological reactivity against MTV has been shown by Blair (1965), Blair and Lane (1975) and Lane, Roubinian, Slomich, Trefts and Blair (1975). Naturally occurring antibodies against MTV have been detected with a radioimmunoprecipitation (RIP) assay (J. N. Ihle and D. L. Fine, Frederick Cancer Research Center, Frederick, Md., USA, personal communication) and with immunofluorescence in mice infected with MTV through the milk (Müller and Zotter, 1973; Zotter and Müller, 1973). Some authors (Charney and Moore, 1971; Priori, Seman, Dmochowski, Gallager and Anderson, 1971; Müller, Zotter and Kemmer, 1976) have reported a cross-reaction between antibodies in the serum of human breast cancer patients and murine MTV antigens. Newgard, Cardiff and Blair (1976) warned, however, against oversimplification. These authors discussed a number of factors which caused false positive results in the RIP assay.

We have done the same type of experiments as described above for the rat antibodies against C-type viruses for the detection of naturally occurring antibodies against MTV antigens in the mouse. Seph-MTV and Seph-OVA were incubated with sera of the following mouse strains: CBA, C57BL, GR (young), GR (mammary tumour bearing) and BALB/cfC3H. The subsequent incubation was with GAM/Ig-TRITC. No specific reaction with the MTV beads was found.

This result may have been due to the following: a) the proteins of MTV to which the putative antibodies are directed are not coupled to the CNBr activated Sepharose

beads; and b) the detergent NP40 which was used to disrupt the viral particles before coupling the viral proteins to the beads selectively destroys the antigenic determinants which provoke the antibody response *in vivo* (J. N. Ihle, personal communication).

We prepared IgG fractions from a number of the mouse sera and coupled them to Sepharose beads in order to test the former possibility. A negative result was obtained after incubation of these beads with NP40-disrupted MTV followed by RAMTV-FITC (for application of this kind of sandwich see also part H of this chapter).

Mouse antibodies against injected antigens and naturally occurring antibodies in rats against RLV and SiSV were easily detected with the Sepharose bead immunofluorescence system. We believe, therefore, that the failure to detect naturally occurring antibodies in mice against MTV proteins is not due to an intrinsic defect in the Sepharose bead immunofluorescence system, but that it is confined to these particular antigens. Experiments with different incubation schemes (e.g., Seph-GAM/Ig * NMS * MTV * RAMTV-FITC) and different detergents (for instance, lithium diiodosalicylate; Ihle, Denny and Bolognesi, 1976) are in progress to solve the problems with the detection of the murine anti-MTV antibodies. These experiments have not yet yielded definite results.

THE DETERMINATION OF ANTIGENS WITH ANTIBODIES BOUND TO SEPHAROSE BEADS

F. DETERMINATION OF IMMUNOGLOBULIN CLASS AND SUBCLASS LEVELS IN MURINE SERUM: TECHNICAL ASPECTS

In experimental gerontology, there is a need for methods which may be applied in longitudinal studies. The amount of serum which can be obtained from a mouse regularly without risk is limited. We have applied the Sepharose bead immunofluorescence system to the estimation of serum Ig levels in the mouse.

Details of the method are described elsewhere (Haaijman and Brinkhof, 1977). Results obtained for the CBA mouse strain during its life span have already been presented (Haaijman, van den Berg and Brinkhof, 1977). No longitudinal follow-up of individual mice has been attempted as yet, because the feasibility of the method had to be first shown with available serum samples. The principle of the method will be outlined here. The reader is referred to the mentioned publications for further details. Results obtained with the system are the subject of part G of this chapter.

The IgG fractions of rabbit antisera directed against the Fc part of mouse IgA, IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 were coupled to Sepharose beads. With the exception of the RAM/IgG_{2a}, the antisera were raised and purified by Dr. J. Radl and Miss P. van den Berg. The RAM/IgG_{2a} antiserum was obtained from Litton Bionetics (Kensington, Md., USA) and further purified with an IgG_3 immunoadsorbent. The

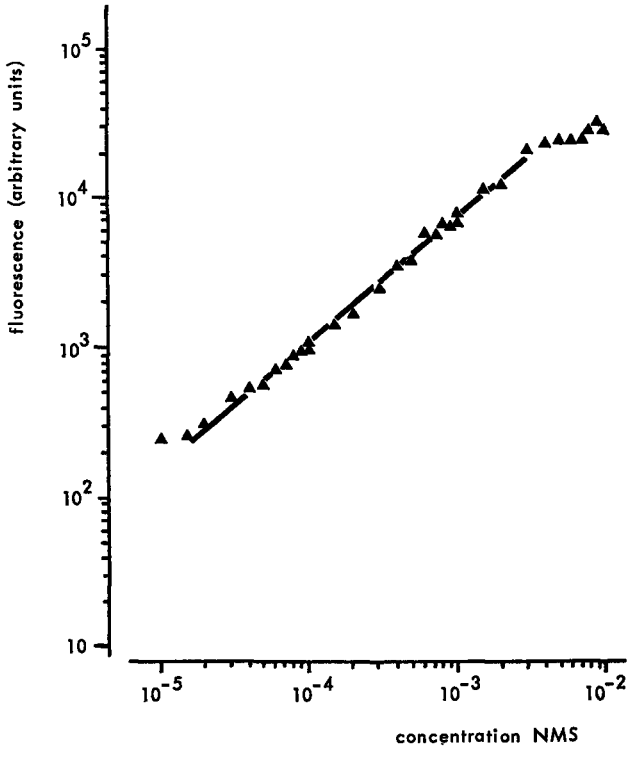


Figure VIII-7. Calibration curve for IgM in mouse serum with Sepharose beads coupled with the IgG fraction of a rabbit antiserum directed against mouse IgM (Seph-RAM/IgM). Seph-RAM/IgM was incubated with different dilutions of a serum pool of 1-year-old CBA mice (NMS). Eleven dilutions were chosen to cover one ^{10}log concentration unit. The beads were washed and subsequently incubated with a 1:50 diluted fluorescent goat antiserum directed against mouse Ig's (GAM/Ig-TRITC) absorbed beforehand with Sepharose beads coupled with normal rabbit serum (Seph-NRS) in order to remove cross-reactive antibodies. After washing again, the fluorescence of individual beads was measured per preparation. The plotted values represent the average fluorescence of at least 5 individual beads. The equation for the solid line was calculated with the least squares method. The concentration of undiluted NMS was taken as 1.

antisera were tested either unlabelled or in conjugated form for their specificity as described by Bloemmen et al. (1976); see also Fig. IV-12.

The beads will bind their respective immunoglobulin during incubation with mouse serum. Bound Ig's can be quantitated after an incubation with a fluorescent goat antiserum directed against mouse Ig's (GAM/Ig-TRITC). The idea of the method was to construct a calibration curve for each Ig class or subclass with a pool of serum. The calibration curves provide the relationships between the fluorescence of the different anti-Ig beads and the concentration of the pool serum. These relationships could then serve to calculate from the fluorescence obtained with test samples, the respective Ig concentrations in these samples relative to the concentrations of the Ig's in the pool of serum.

In order to obtain a sufficiently reliable estimate of a titration curve with a pool of NMS for the calculation of Ig levels in the test samples, we have employed a dilution scheme in which a tenfold dilution was covered by 11 different dilutions. One example of such a titration which was obtained with RAM/IgM beads is shown in Fig. VIII-7.

The titration curve can be approximated with a straight line within a certain range, although it is essentially sigmoid. The range in which the logarithm of the fluorescence is linearly related to the logarithm of the pool serum concentration was about the same for all antiserum beads (Table VIII-3). The parameters *a* (intercept) and *b* (regression coefficient) of the equations for the curves were calculated with linear regression analysis.

For estimating a given Ig level, an individual serum sample was diluted, e.g., 10^4-fold, and incubated with the respective antiserum beads in the same way as was the pool serum. The beads were subsequently incubated with the fluorescent conjugate. The logarithm of the averaged fluorescence value of the beads incubated with the unknown sample has to be substituted in the regression equation of the calibration curve obtained with those particular beads, in order to obtain a measure of the concentration of the Ig class or subclass in the sample relative to the concentration of that Ig in the pool serum.

Day-to-day calibration of the microfluorometry equipment is of importance in the measuring of Ig levels with our method. Only the parameter *a* of the regression line (ordinate intercept) but not *b* (slope of the curve) can change with the variations in the equipment, provided the concentration of the different Ig's in the pool serum does not change with time. It is not necessary, therefore, to remeasure over the whole calibration curve in every measuring session. It is sufficient to measure only the

TABLE VIII-3. CALIBRATION CURVES FOR DIFFERENT IMMUNOGLOBULINS

Ig	a	b	r	Range
IgA	6.1326	0.7712	0.998	10^{-5}–10^{-3}
IgM	6.3557	0.8294	0.998	1.5×10^{-5}–3×10^{-3}
IgG_1	6.9224	0.8346	0.987	10^{-5}–10^{-3}
IgG_{2a}	7.4849	0.9292	0.993	10^{-5}–6×10^{-4}
IgG_{2b}	7.0586	0.9566	0.989	10^{-5}–10^{-3}
IgG_3	6.1655	0.7449	0.986	10^{-5}–10^{-3}

The IgG fractions of RAM/IgA, RAM/IgM, RAM/IgG_1, RAM/IgG_{2a}, RAM/IgG_{2b} and RAM/IgG_3 were coupled to Sepharose beads. The beads were first incubated with different dilutions of a serum pool of CBA mice and subsequently with GAM/Ig-TRITC, diluted 1:50. The conjugate was absorbed with Seph-NRS. Individual bead fluorescence was measured with a microfluorometer. The curve representing the logarithm of the average fluorescence per preparation as a function of the serum concentration shows a lower plateau (background) and a higher plateau (saturation). Values falling within the serum concentration-dependent range were analyzed with the least squares method. The equation for the regression line is $y = a+bx$, in which y is the logarithm of the fluorescence intensity observed with the logarithm of the serum concentration x; a is the intercept, b is the regression coefficient; r is the correlation coefficient.

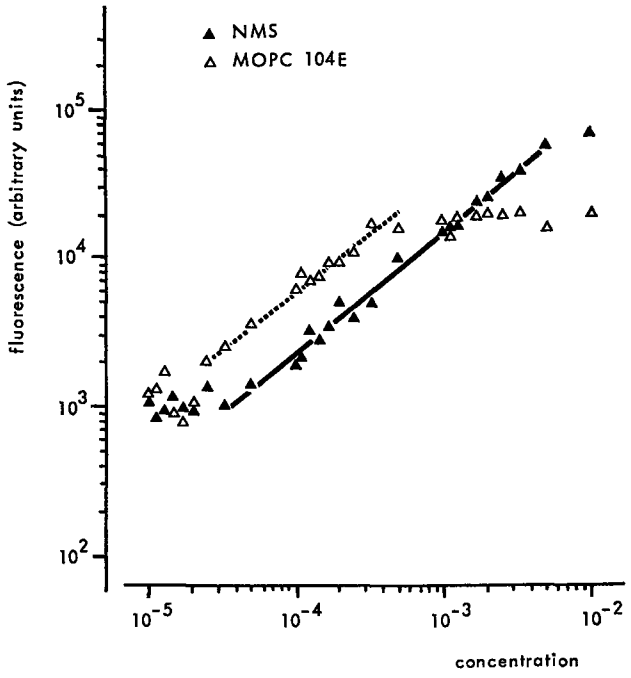


Figure VIII-8. Determination of the concentration of IgM in CBA serum. Seph-RAM/IgM beads (see Fig. VIII-7) were incubated with different dilutions of a pool of CBA serum collected from 1-year-old animals (NMS) and with different concentrations of purified MOPC 104E IgM paraprotein. The undiluted solution of IgM paraprotein contained 2.58 mg/ml. The equations for the solid and dashed lines were calculated with the least squares method. Substitution of the values obtained with the CBA pool in the MOPC 104E equation gave an IgM content of the pool of 0.8 mg/ml. The concentrations of undiluted NMS and paraprotein solution were taken as 1 on the abscissa.

fluorescence obtained with one dilution of pool serum. A correction factor for the daily measurements can thus be calculated. Haaijman and Brinkhof (1977) chose a 10^4-fold dilution of the serum pool to standardize their equipment. Each microtitration plate with diluted individual serum test samples contained 6 wells filled with 10^4 diluted pool serum. The average fluorescence of the beads in these six wells was compared with the fluorescence value calculated from the respective regression equation in order to give the correction factor. All subsequent measurements from that microtitration plate were then multiplied by this factor. The corrected fluorescence values of individual samples were substituted in the regression equation in order to calculate the respective levels of Ig, relative to that of the pool.

In an attempt to reduce the error introduced by repeated dilution of the pool serum, batches of 10^4 diluted serum were stored at –70 °C. The volume of these batches was sufficient for one series of six microtitration plates, each containing one species of antiserum beads. This has not been a good policy when considered in retrospect,

because it appeared that the measurable Ig content of the diluted pool serum was reduced considerably after one month of storage. This phenomenon has not influenced our results, however, because we used the diluted serum in the course of three consecutive days. It is highly unlikely that a considerable change in the Ig levels of the pool serum will have occurred within these three days, although, it could be that the relative Ig levels of the test samples are somewhat overestimated in our results.

It would have been better to use a pool serum dilution that yields a plateau staining for the establishment of the correction factor. The reestimation of the fluorescence values to be obtained with the pool serum would then be less susceptible to variations in the respective Ig concentrations caused either by variations in the dilution or by degradation upon storage.

Expression of Ig levels in individual samples relative to a certain pool is not completely satisfactory. The data of different laboratories cannot be directly compared without standardization of the different pools. No standard normal mouse serum comparable to the WHO standard for human Ig's exists at this moment. The only possibility was to standardize our own pool of normal mouse serum with purified preparations of different mouse Ig's. This is shown in Fig. VIII-8 for M/IgM. Seph-RAM/IgM beads were incubated with different dilutions of the standard serum pool as well as with dilutions of a purified MOPC 104E IgM plasmocytoma paraprotein. Chemically induced plasmocytomas of the BALB/c mouse producing highly homogeneous Ig's (Potter, 1972) are for most Ig classes and subclasses the only practical means for obtaining sufficient quantities of purified mouse Ig's. An exception is IgM, which may be obtained, although in relatively small quantities, as a cryoglobulin from NZB mice (Bloemmen et al., 1976). Two aspects are evident from the titration curves of Fig. VIII-8: a) the titration curve of the MOPC 104E IgM paraprotein runs parallel to that of the pool serum; and b) the plateau reached with the paraprotein, is considerably lower than that found with the pool serum.

The RAM/IgM coupled to the beads was raised against the IgM isolated from the cryoglobulin from NZB mice. This IgM seems to be heterogeneous, as judged from electrophoretic data. The RAM/IgM on the beads apparently recognizes more determinants of the heterogeneous IgM present in the serum pool than of the plasmocytoma IgM protein. Nevertheless, a reliable estimation of the IgM content of the pool can be obtained by comparing the concentration-dependent ranges. Under these conditions, the antiserum reacts with the determinants shared by heterogeneous and homogeneous IgM.

We have not made this kind of calibration for the other Ig classes and subclasses, because we did not have enough purified material of all of the IgG subclasses and because antisera other than RAM/IgM were raised against paraproteins. In these circumstances, the pool serum can be calibrated only for those determinants it shares with the respective paraprotein used for a test, such as is shown in Fig. VIII-8. The chance

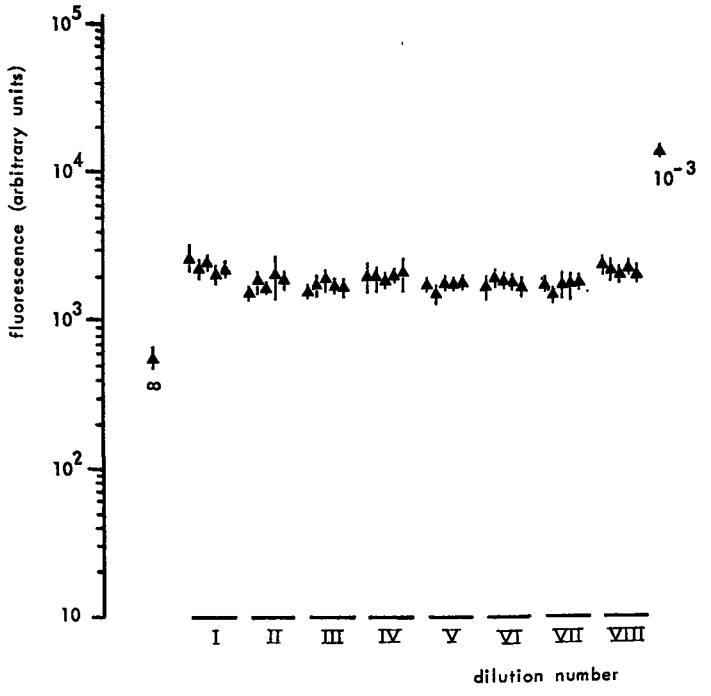


Figure VIII-9. Testing the reproducibility of the Ig level determination with the Sepharose bead immunofluorescence assay. Eight independent 10^4-fold dilutions (numbered I through VIII) were prepared from a pool of CBA mouse serum. Five aliquots from these dilutions were incubated with Sepharose beads coupled with the IgG fraction of a rabbit antiserum directed against mouse IgG_3 (Seph-RAM/IgG_3). The beads were subsequently incubated with GAM/Ig-TRITC, absorbed with Seph-NRS. The fluorescence of at least 5 individual beads was measured and averaged. Bars indicate standard deviations. The fluorescence measured with 10^3-fold diluted serum and the bead fluorescence after incubation with conjugate alone are indicated also in the figure.

exists, consequently, that, with some antisera, the true concentration of the respective (heterogeneous) Ig's in serum will be underestimated. Plasmocytoma paraprotein preparations need not lack antigenic determinants, compared to their heterogeneous counterparts. Antisera raised against plasmocytoma proteins of a certain Ig (sub)class should always recognize the constant regions of the Fc part of all members of the heterogeneous family of that particular (sub)class, if it were possible to isolate the paraproteins in their native form and if the paraproteins do not lack specific determinants. It is possible, however, that some conformational determinants are lost during purification. The preparative procedures involved in the purification of heterogeneous Ig's may also destroy certain determinants. The heterogeneity in itself, however, makes it unlikely that one determinant will be altered in all molecules of a preparation. The heterogeneity of an antigen preparation is difficult to assess and which determinants of an antigen an antiserum will recognize cannot be predicted. The different possibilities have to be checked, therefore, with every new antiserum.

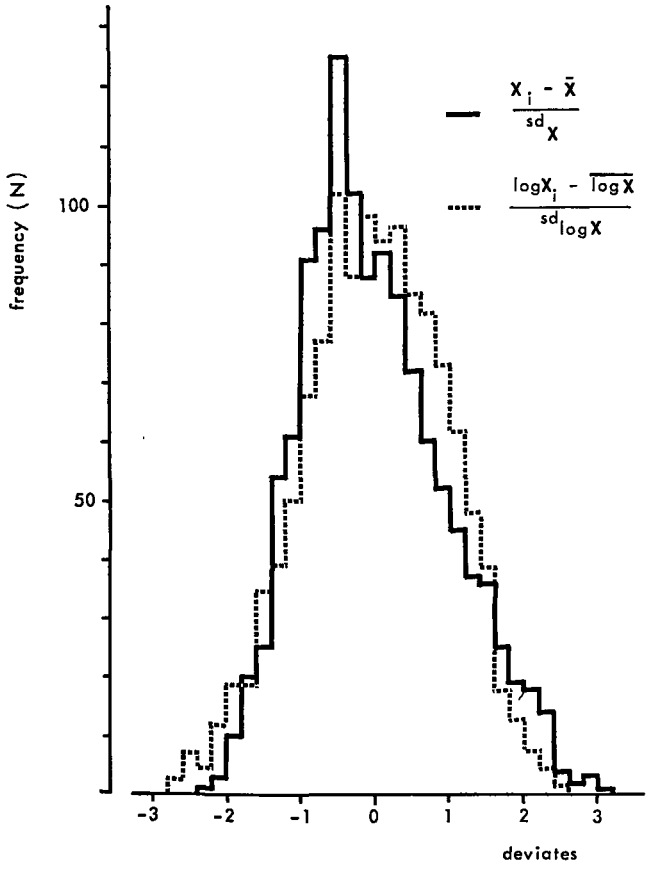


Figure VIII-10. The distribution of Ig levels in CBA mice as measured with the SBIA. The levels of IgA, IgM and the four subclasses of IgG have been determined in individual mice of different ages, as described in part F of this chapter. In order to test the hypothesis that the Ig levels of different animals are normally distributed, we have plotted the frequency distribution of the deviates $(x_i - \bar{x})/sd_x$ (drawn histogram) in which x_i stands for the individual Ig level within an age group and $\bar{x}$ and sd_x, respectively, for the average Ig level and standard deviation within that age group. The same has been done after logarithmic transformation, that is, the distribution of the deviates $(\log x_i - \overline{\log x})/sd_{\log x}$ (dashed histogram). The two distributions were not statistically different when tested with Student's t-test.

The reproducibility of the Ig level determination with the antisera coupled to beads has been amply discussed (Haaijman and Brinkhof, 1977). Fig. VIII-9 represents their Figure 5. Five wells of a microtitration plate with antiserum beads were incubated with 50 µl aliquots from each of eight independent 10^4 dilutions of pool serum. The error within dilutions was not conspicuously larger than the error of the fluorescence measurements per well. The greatest error was caused by dilution. The coefficient of variation (CV%: 100 sd/average) of the data in Fig. VIII-9 was 7.3%. The CV% increased up to 20% if only one dilution per serum was tested. The only way to

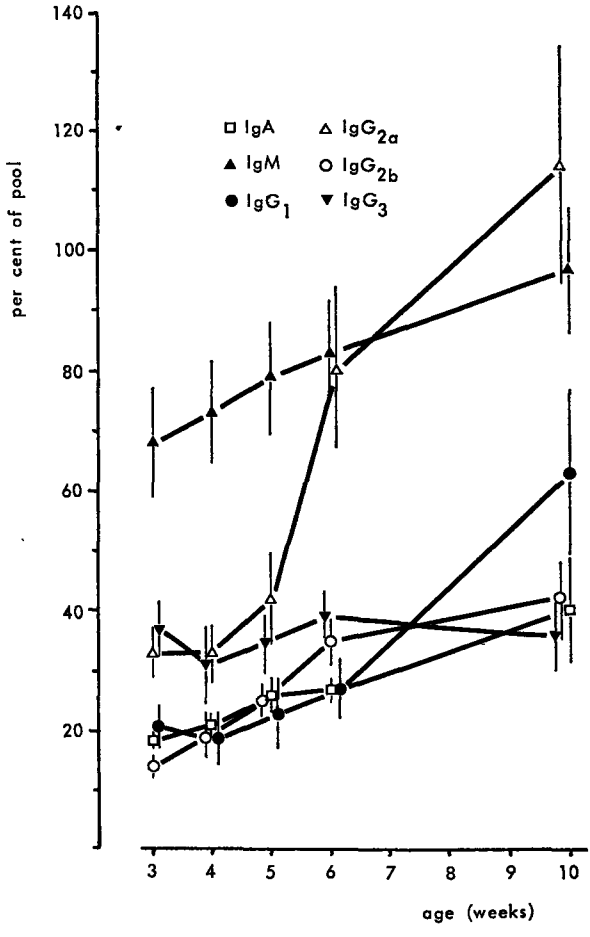


Figure VIII-11. The serum levels of IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 in male CBA mice between 3 and 10 weeks of age. The levels of the different Ig's were determined in sera of individual mice according to the methods described in part F of this chapter. The respective Ig levels are expressed as a percentage of those levels present in a serum pool of CBA mice around 1 year of age. Each value is the arithmetic average of the levels of 15 individual animals. Bars indicate 95% confidence limits of the averages.

reduce the CV% is the use of the average fluorescence obtained with a number of independent dilutions. This procedure will daunt many investigators in practice, because of the enormous increase in workload.

G. IMMUNOGLOBULIN CLASS AND SUBCLASS LEVELS IN CBA MICE THROUGHOUT THEIR LIFE SPAN

The method described in the preceding section was used to measure the levels of the different Ig classes and subclasses during the life span of CBA mice. The CBA strain

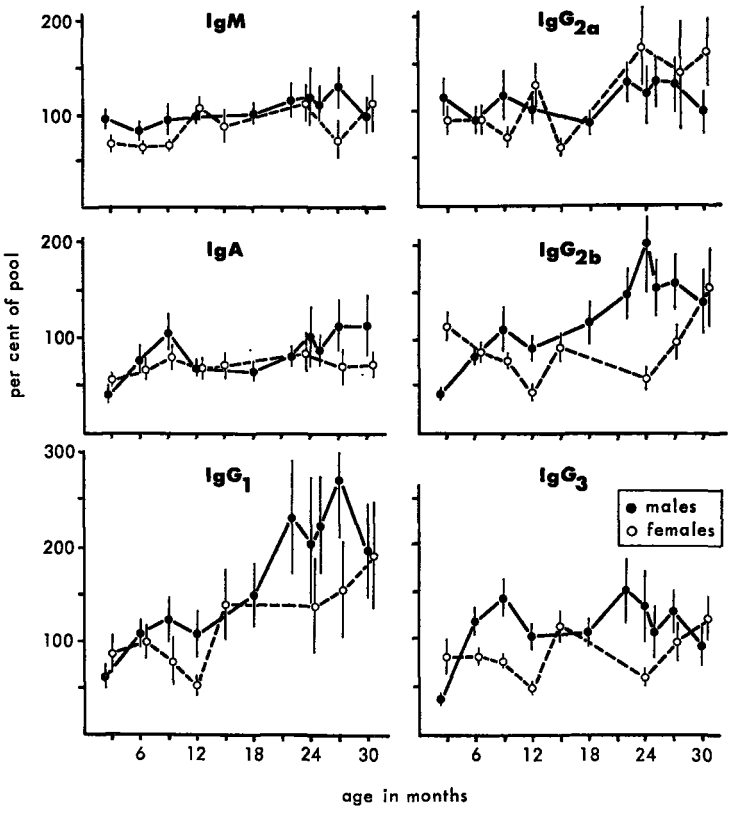


Figure VIII-12. The serum levels of IgM, IgA and the four subclasses of IgG throughout the life span of female and male CBA mice. The method for determination of the different Ig's is described in part F of this chapter. Fifteen animals were tested per age group. Each value represents the average Ig level within an age group. Bars indicate 95% confidence limits of the averages.

was chosen because it is a long-lived mouse strain with no known tendency to age-related pathology of the immune system itself. Data obtained with this strain may serve as a reference for studies with mouse strains which are prone to such a specific pathology. Examples of these strains are the NZB with autoantibodies (Stutman, 1972), the C3H and C57BL with idiopathic paraproteinaemia and lymphoreticular malignancies (Radl and Hollander, 1974) and the RFM with glomerulonephritis.

We did not intentionally stimulate the animals of our study with antigens. We have studied in this way the immunological activity versus age rather than the immunological capacity (see introduction of this chapter).

CBA/Rij mice were bred under conventional conditions at the Radiobiological Institute TNO (Rijswijk, The Netherlands). At the age of 9–15 weeks, cohorts of animals born in the same week were transferred to the animal quarters of the Institute for Experimental Gerontology TNO. The mice were housed in plastic cages and received

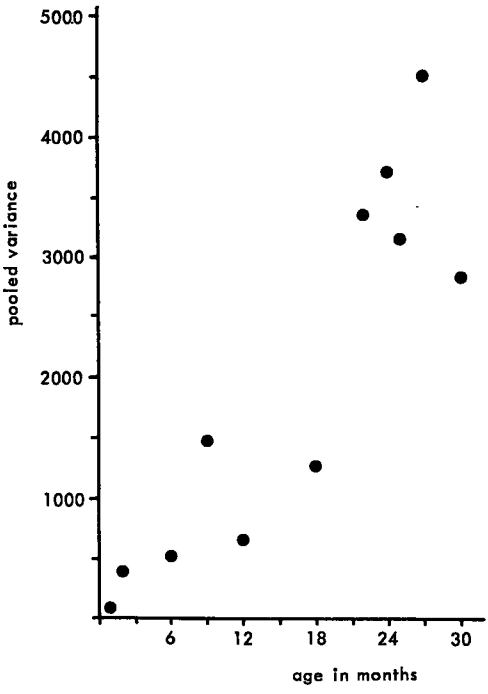


Figure VIII-13. Variation in Ig levels among individual mice of the same age as a function of age. The variance (sd_x^2) observed within age groups in Fig. VIII-12 was pooled for the six Ig's. The pooled variance is plotted versus age. The variances of the age groups 24, 25, 27 and 30 months are not significantly different from each other, as tested with the Fisher F-test for the equality of variances. All variances of this group differ significantly from those of the younger age groups (a complete list of the Fisher F-values is given by Haaijman, van den Berg and Brinkhof, 1977).

food and water *ad libitum*. Our CBA stock has a sigmoid survival curve with 50% survival at 30 months of age and 10% survival at 32 months of age (C. Zurcher, personal communication). Serum samples were taken from the tail vein or from the retro-orbital plexus in the case of moribund animals.

Fifteen animals per age group were used for the estimation of Ig levels. Each serum sample was diluted 10^4-fold and incubated with the different antiserum beads. The average fluorescence values were corrected for day-to-day variations as already described. The fluorescence values were converted into Ig levels by substituting them in the respective regression equations from Table VIII-3.

Immunoglobulin levels of individuals within age groups are often averaged geometrically. We have tested the necessity of the logarithmic transformation by plotting the frequency distribution of $(x_i - \bar{x})/sd_x$ before and after logarithmic transformation (Fig. VIII-10); x_i stands for the individual Ig level within an age group, $\bar{x}$ for the average Ig level and sd_x for the standard deviation of that age group. The two frequency distributions were not significantly different when tested with the Student's

t-test. We have plotted therefore the arithmetic means in our figures. Bars indicate the 95 % confidence limits of the averages.

The development of Ig levels in male CBA mice from 3 weeks up to 10 weeks of age is shown in Fig. VIII-11. The level of IgM is already appreciable at 3 weeks of age and increases slowly up to adult levels in the period shown. The level of IgA remains low during this period. The development of the levels of the subclasses of IgG was not synchronous. The levels of IgG_{2b} and IgG_3 remain low in the first weeks of life, whereas the IgG_1 level rises around the 10th week of age and the IgG_{2a} even earlier.

From 6 months of age on to almost the end of their life span, there were no major changes in the levels of the respective Ig's, with the exception of the IgG_1 and IgG_{2b} levels which showed an increase with age (Fig. VIII-12). Data for female and male mice are presented in Fig. VIII-12. Differences between the two curves were observed at certain ages and for certain subclasses. No general trend, however, was found. From Fig. VIII-12, it is clear that the variation within age groups increases with age. We have pooled the variance (sd_x^2) per age group for the six immunoglobulins and plotted this pooled variance as a function of age in Fig. VIII-13. It should be mentioned that the greater part of the age-related increase in variance is due to the increase in the variation in IgG_1 levels. The level of IgM did not show an increased variance. This finding is different from that reported by Radl et al. (1975) obtained in men. It may be that the level of IgM in mice is more constant than in humans due to the chronic stimulation which the mice receive from the environment.

According to our results of the Ig level determinations, the life span of the CBA mouse may be divided into three main periods: a) from birth up to about 0.5 year of age; b) from 0.5 up to about 2 years of age; and c) older than 2 years. The adult levels of the respective Ig's are reached during the first period. In the second period, the levels of the different Ig's remain more or less constant, except for IgG_1 and IgG_{2b} which rise. The third period, in which the survival curve drops, has as its most important feature an increase in variation among individuals.

The data on the Ig levels in the CBA mouse agree with the observations of Haaijman, Schuit and Hijmans (1977) on the number of C-Ig cells in CBA mice. Neither study indicates diminishing immunological activity in the CBA mouse with age. The relevance of these findings for immunological theories of ageing is discussed in more detail in chapter IX.

H. DETERMINATION OF MOUSE MAMMARY TUMOUR VIRAL ANTIGENS: TECHNICAL ASPECTS

The expression of mammary tumour viral antigens in mice is under genetic control. In collaboration with Dr. P. Bentvelzen of the Radiobiological Institute TNO, we have developed a fluorescence immunoassay for the detection of mammary tumour

viral antigens in milk and organ extracts of mice. A summary of the results of these studies is presented in part I of this chapter. The purpose of the present section is to draw attention to the technical aspects of these assays. In viral immunology, the available amounts of viral material are usually small. The purification of the different viral proteins has been achieved only recently (Parks, Howk, Scolnick, Oroszlan and Gilden, 1974; Noon, Wolford and Parks, 1975; Sarkar and Dion, 1975; Westenbrink, Koornstra and Bentvelzen, 1977) and the preparation of antisera against single viral proteins is still in its infancy. In most of our studies, we have worked with antisera raised against disrupted preparations of whole virus. The mammary tumour virus (MTV) is isolated in most cases from mammary tumour material. The isolation procedure consists of multiple ultracentrifugation steps. Inevitably, proteins of non-viral origin are associated with the virus preparations. Antisera raised with this kind of material will contain antibodies against a variety of mouse proteins. It is of importance, therefore, to absorb these antisera vigorously. The absorbed antisera are tested for their specificity in the manner described in chapter IV, part I.

The isolation of mammary tumour virus from tissue cultures of mammary tumour cells has been reported (Sykes, Whitescarver and Briggs, 1968; Young, Cardiff and Ashley, 1975; Kimball, Boehm-Truitt, Schochetman and Schlom, 1976; Kimball, Michalides, Colcher and Schlom, 1976). These methods, which have been used successfully with leukaemia viruses, are most promising, in that they usually yield virus isolates which are far less contaminated with nonviral proteins than are tumour isolates.

The assay for MTV proteins was worked out along the same lines as described for the mouse Ig's. The globulin fraction of a rabbit antiserum directed against MTV (RAMTV) was coupled to Sepharose beads. These beads were incubated with different dilutions of disrupted MTV, followed by an incubation with RAMTV-FITC. The RAMTV and the RAMTV-FITC have been thoroughly tested for their MTV specificity with beads coupled with MTV, Rauscher leukaemia virus (RLV), extract from lactating mammary gland (MG), normal mouse serum of young BALB/c mice (NMS) and ovalbumin (OVA). The MTV used for coupling was passed over a Sepharose immunoadsorbent column of a goat antiserum directed against mouse serum proteins (GAM-Ielfo, lot no.: 771; Nordic Immunological Laboratories). Results of these tests have already been presented in Fig. IV-8 and IV-13 and in Table IV-6.

A titration curve of two virus preparations with Seph-RAMTV beads is presented in Fig. VIII-14. The protein content of the two preparations was equalized. The first virus preparation was a MTV isolate purified from the milk of the RIII mouse strain; the other was an ultrapellet of a BALB/cfC3H mammary tumour homogenate, which was first cleared from debris with low-speed centrifugation. NMS was used for the NSS control.

It is clear from Fig. VIII-14 that the RIII sample contained more viral protein per

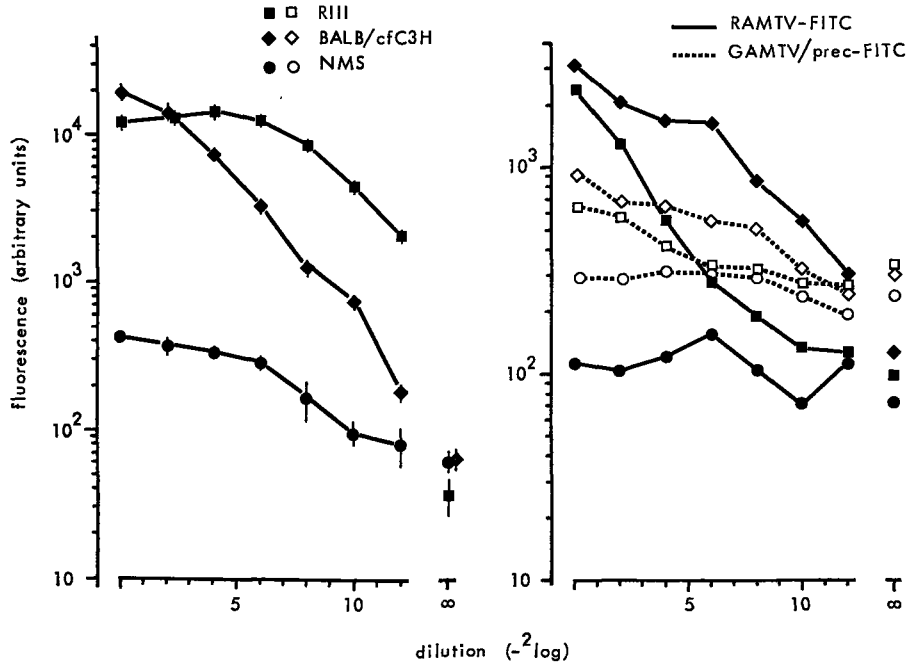


Figure VIII-14. The comparison of two mammary tumour virus (MTV) samples with Sepharose beads coupled with the globulin fraction of a rabbit antiserum directed against MTV (RAMTV). Highly purified MTV from milk of the R III mouse strain, an ultrapellet from a BALB/cfC3H mammary tumour homogenate and NMS were incubated in various dilutions with Seph-RAMTV. The protein content of all of these preparations was equalized. Undiluted preparations contained 0.5 mg protein/ml. The beads were incubated subsequently with RAMTV-FITC. Each plotted value is the average fluorescence intensity of at least 5 individual beads. Bars indicate standard deviations. ∞: conjugate control.

Figure VIII-15. The influence of different indicator conjugates on the results obtained with Sepharose beads coupled with a goat antiserum directed against a presumably single protein of MTV (Seph-GAMTV/prec). Seph-GAMTV/prec was incubated with the same R III milk MTV isolate, BALB/cfC3H MTV and NMS from Fig. VIII-14 containing undiluted 0.5 mg protein per ml. After washing, the beads were incubated with either GAMTV/prec-FITC or RAMTV-FITC both diluted 1:50. Each value is the average fluorescence intensity of at least 5 individual beads, ∞ represents the conjugate control. Symbols as in Fig. VIII-14.

mg protein than did the BALB/cfC3H pellet. It is essential, furthermore, that the titration curves of the two samples are almost parallel.

The same two virus isolates have been incubated with beads coupled with another antiserum, namely, GAMTV/prec. This antiserum was raised by Dr. J. Ouwehand of the Radiobiological Institute TNO. One precipitation line (of four) which arose between a MTV isolate and a polyvalent GAMTV antiserum in a macroimmunodiffusion plate was cut out. The agar containing the precipitation line was washed and injected emulsified in Freund's complete adjuvant into a goat. Smith, Gallop and

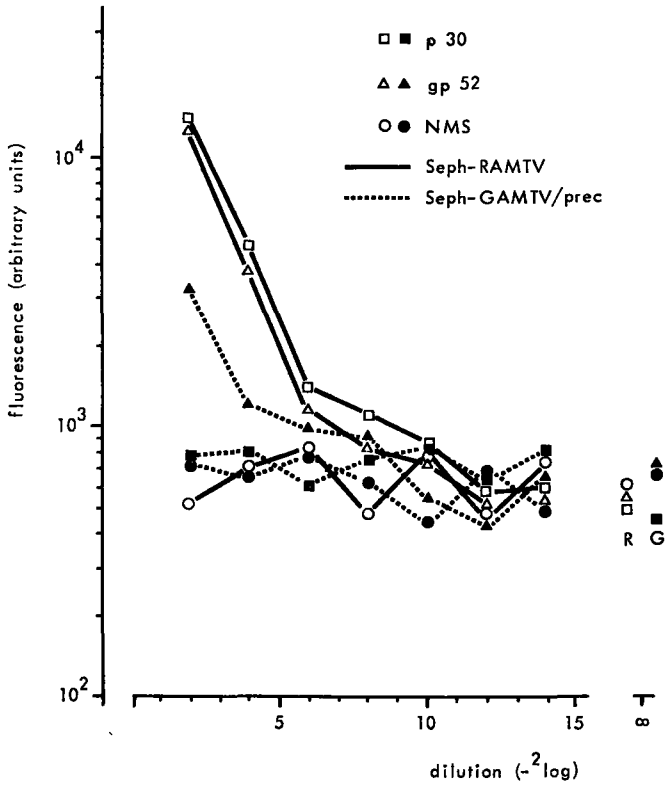


Figure VIII-16. The reactivity of RAMTV and GAMTV/prec coupled to beads, as tested with purified gp52 and p30. NMS served as the control. Undiluted samples contained 200 ng protein/ml. Bound protein was stained with RAMTV-FITC diluted 1:100. Each value is the average fluorescence intensity of at least 5 individual beads. ∞: conjugate control. R and G represent, respectively, the rabbit and goat antiserum beads.

Tozer (1964) first described this method for artificial protein mixtures. Goudie, Horne and Wilkinson (1966) described the method for obtaining antisera against single proteins from biological materials.

The globulin fraction of the GAMTV/prec antiserum was tested for its specificity as described for the RAMTV and subsequently absorbed with Seph-NMS and -MG and coupled to Sepharose beads. The same virus preparations plus NMS from Fig. VIII-14 were incubated in different dilutions with the Seph-GAMTV/prec beads. Either GAMTV/prec-FITC or RAMTV-FITC was used as the conjugate (Fig. VIII-15). Two phenomena may be observed in the data from Fig. VIII-15, in comparison with Fig. VIII-14: a) the BALB/cfC3H preparation reacts more strongly with the GAMTV/prec beads than does the RIII preparation, despite the fact that the latter contained relatively more viral proteins; and b) the RAMTV-FITC conjugate gives a far better contrast between MTV and NMS than does the GAMTV/prec-FITC.

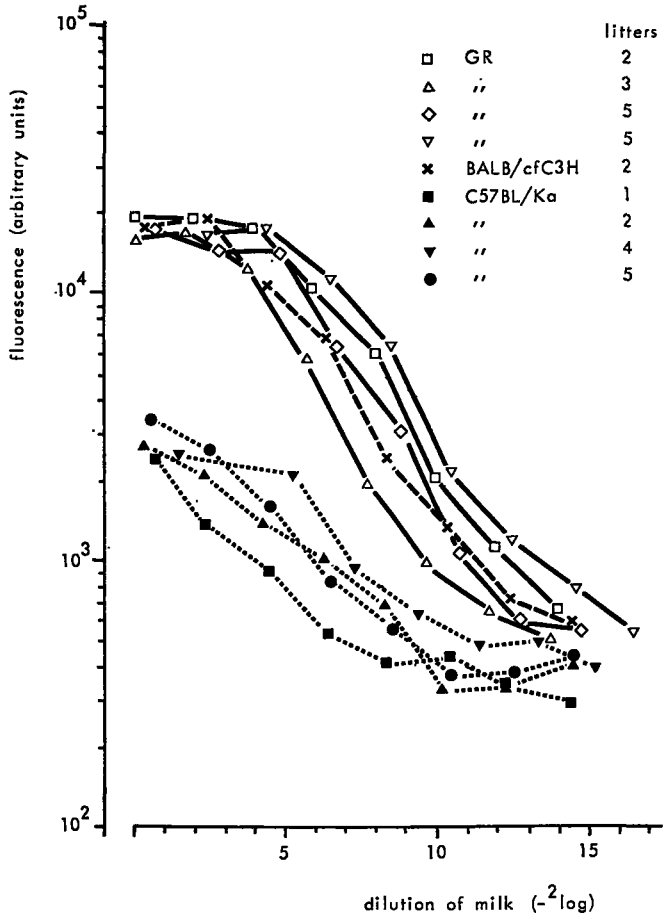


Figure VIII-17. The demonstration of MTV proteins in the milk of different strains of mice. Beads coupled with the globulin fraction of RAMTV were incubated with different dilutions of milk. Milk was skimmed before incubation. The protein content was determined with fluram (see chapter VII, part D). All curves are shifted along the abscissa so that dilution zero stands for 1 mg protein/ml. Bound MTV proteins were stained with RAMTV-FITC. Each value represents the average fluorescence intensity of at least 5 individual beads.

It may be argued that the antiserum GAMTV/prec recognizes only a very limited number of antigenic determinants and that it contains a strong type-specific component. In this kind of investigations, type refers to MTV isolates of different inbred strains of mice. With molecular hybridization techniques, Ringold, Blair, Bishop and Varmus (1976) were not able to demonstrate nucleotide sequence differences between mammary tumour virus isolates from different strains. Michalides and Schlom (1975) reported, on the other hand, a 25% difference between MTV-L (low oncogenic MTV, endogenous in the C3Hf mouse strain) and a number of MTV-S (standard strain of

MTV, exogenous in the C3H strain) isolates. Recently Friedrich, Morris, Goodman, Bishop and Varmus (1976) showed differences in the nucleotide sequences of MTV-S and MTV-P (endogenous MTV from the GR strain). More experiments are needed to decide whether our results are really due to type specificity of the GAMTV/prec antiserum.

The two most important proteins of MTV (Dickson and Skehel, 1974) are gp52 (glycoprotein with a molecular weight of 52,000) and p30 (protein with a molecular weight of about 30,000). The specificity of GAMTV/prec for either of these two proteins was tested with purified protein preparations obtained through the courtesy of Dr. W. P. Parks (National Institutes of Health, Bethesda, Md., USA). Seph-GAMTV/prec as well as Seph-RAMTV were incubated with different dilutions of gp52 and p30 (Fig. VIII-16) and subsequently with RAMTV-FITC. It was observed that GAMTV/prec reacts only with gp52, whereas RAMTV reacts with both proteins to about the same extent. The $-^2\log$ dilution of 2 in Fig. VIII-16 contained 50 ng protein/ml. This indicates that the lowest detectable amount of protein in this system was in the order of 0.3 ng protein/sample.

MTV antigens were readily detected in milk of GR as well as BALB/cfC3H mice with the Seph-RAMTV beads (Fig. VIII-17; see also Noon, Wölford and Parks, 1975). They were detected in very low concentration in the milk of multiparous C57BL mice. This is of considerable interest as C57BL mice are known to have a very low mammary tumour incidence in old age. The reaction of the milk of uniparous C57BL females can be considered to be due to nonspecific adherence of proteins. In section D of this chapter it was mentioned that it is difficult to establish beyond doubt the nature of the protein reacting with the beads. Studies to solve this problem by eluting bound proteins and electrophoresing them on polyacrylamide gels are in progress.

The system described above for the detection of MTV antigens in milk has been applied to the study of the genetic factors which influence the appearance of MTV in the milk of different strains of mice (Bentvelzen, Brinkhof and Haaijman, 1977). The results obtained so far confirm those of van Nie and Verstraeten (1975) using a radioimmunoassay.

An assay for the quantitation of the amount of MTV proteins in organ extracts resembling that described for the measurement of Ig levels was worked out. Sepharose beads were coupled with the globulin fraction of a rabbit antiserum specifically directed against mammary tumour virus (Seph-RAMTV). These beads were incubated with different dilutions of a purified preparation of MTV (Fig. VIII-18) and subsequently with fluorescent RAMTV (RAMTV-FITC). A linear relationship was found between the logarithm of the fluorescence and the logarithm of the MTV concentration in the range of 4.2×10^{-3} to 1.4×10^{-4} mg MTV/ml. The MTV was an isolate from BALB/cfC3H mammary tumours.

Small aliquots of the undiluted MTV isolate were stored at $-20°C$ in order to

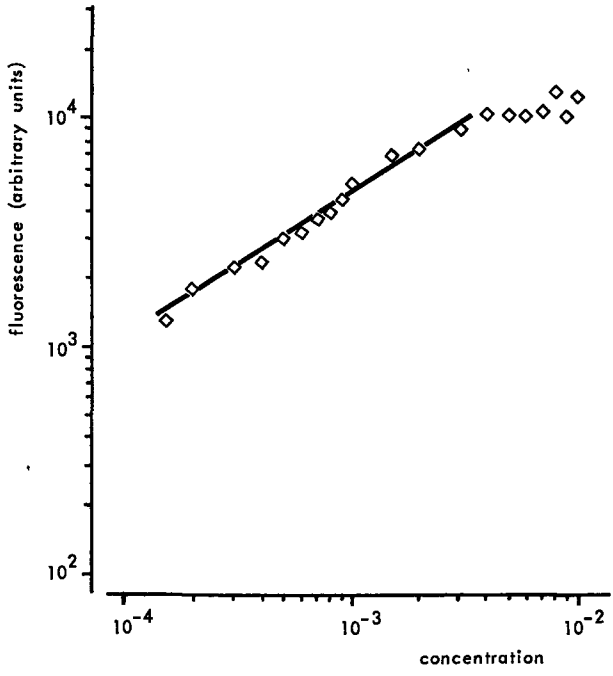


Figure VIII-18. Calibration curve for mammary tumour viral antigens with Sepharose beads coupled with RAMTV (Seph-RAMTV). MTV was isolated from a BALB/cfC3H mammary tumour homogenate. The undiluted preparation contained 1.5 mg protein/ml. Seph-RAMTV was incubated with different dilutions of the MTV preparation and subsequently with RAMTV-FITC. Each value represents the average fluorescence intensity of at least 5 individual beads. The equation for the solid line was calculated with the least squares method. The concentration of the undiluted sample was taken as 1 on the abscissa.

avoid repeated freezing and thawing of the preparation which may lead to denaturation. A number of controls were added to each microtitration plate containing samples: six wells were filled with 10^3-fold diluted MTV. In addition two wells were filled with 10^2-fold diluted virus and four wells were used for the estimation of the fluorescence of the conjugate control. Freshly prepared dilutions were used only (see part F). The average fluorescence value of the wells with 10^3-fold diluted virus was used to calculate a correction factor for day-to-day changes in the microfluorometry equipment. The corrected fluorescence values of the beads incubated with test samples were substituted in the regression equation of the MTV calibration line (Fig. VIII-18) in order to give the amount of viral protein per sample.

I. ORGAN DISTRIBUTION OF MAMMARY TUMOUR VIRAL ANTIGENS IN MALE MICE OF DIFFERENT STRAINS AND AGES

From the point of view of gene regulation, it seemed of interest to study the

expression of mammary tumour viral antigens in different organs of male mice, which lack the target organ of this particular virus.

Extracts of the organs listed in Table VIII-4 were prepared. The tissue was homogenized on ice with a teflon Potter-Elvehjem homogenizer in PBS containing 0.03% NP40. The homogenate was centrifuged in the cold at 4,000 g for 30 min and the supernatant was collected and stored at −70 °C. The protein content of the homogenate was estimated by the fluram method (chapter VII, part D) and adjusted to 1 mg of protein/ml. Immediately after the protein determination and dilution the samples were incubated with Seph-RAMTV beads.

The organs of five animals per age group were tested for the presence of MTV proteins with the assay described in the previous section. In Table VIII-4 the average amount of MTV protein in nanograms per mg of tissue protein is listed per organ.

The following mouse strains have been investigated: CBA, known for its low mammary tumour incidence and with no known endogenous MTV; C57BL, also a low mammary tumour strain, but with its own type of endogenous MTV (MTV-Y); BALB/c, a low mammary tumour strain with endogenous virus (MTV-O) and a high susceptibility to the milk-borne exogenous MTV-S produced by the C3H strain; BALB/cfC3H, BALB/c animals foster-nursed by C3H females, resulting in a high incidence of mammary tumours; GR, a high mammary tumour strain with the endogenous MTV-P. For extensive reviews of the biology of the different MTV strains, see Bentvelzen (1972, 1974).

Three age groups of the CBA, C57BL and BALB/c strains have been studied: 6-week-old and 1- and 2-year-old animals. For the BALB/cfC3H and GR strains, unfortunately, only the indicated age groups were available, since these strains have not been included in the cohort system of the Institute for Experimental Gerontology (see page 154).

The values in Table VIII-4 have been corrected for the conjugate control fluorescence which was equivalent to 20 ± 7 ng virus/mg protein. The extreme variation in the MTV content of the organs of individual mice within the same group makes it difficult to draw definite conclusions from the data in Table VIII-4. We have considered 20 ng MTV/mg protein (three times the standard deviation of the conjugate control) as a significant indication of the presence of MTV. The largest amounts of MTV protein were observed in the epididymis of CBA and BALB/ cfC3H and in the prostate of BALB/cfC3H mice. The presence of MTV-S in the epididymis of high cancer strains has already been described (Mühlbock, 1952; Schlom, Michalides, Kufe, Hehlmann, Spiegelman, Bentvelzen and Hageman, 1973). The organs of the urogenital tract have been shown to also contain relatively large amounts of gp69/71, the structural glycoprotein of murine leukaemia viruses (Lerner, Wilson, Del Villano, McConahey and Dixon, 1976). B-type viral particles were found in seminal vesicles of wild mice by Rongey, Abtin, Estes and Gardner (1975). Small amounts of protein were found in our study in tongue, liver, kidney, gut and pancreas. With the exception of the CBA strain, the spleen also showed low amounts of MTV protein. Bentvelzen and

TABLE VIII-4. NANOGRAMS MTV PROTEINS PER MG OF PROTEIN IN EXTRACTS OF ORGANS OF DIFFERENT MOUSE STRAINS AND MICE OF DIFFERENT AGES

Strain	Age	BM [1]	Lung	Epid.[2]	Spleen	Pancreas	Kidney	Saliv. gland [3]	Gut	PP [4]	Liver	Tongue	Prostate
CBA	6 weeks	20±7	46±19	73±17	16±15	16±13	28±10	48±53	47±17	91±27	18±12	30±33	49±21
	1 year	59±22	49±20	160±196	70±18	65±48	62±25	35±17	37±25	83±42	47±40	38±16	77±40
	2 years	59±34	49±32	44±27	42±31	9±11	20±23	19±20	21±14	36±27	12±13	14±14	22±14
C57BL	6 weeks	14±5	37±16	15±15	21±20	12±13	7±5	15±5	20±10	18±9	10±14	14±8	18±14
	1 year	24±14	18±7	26±22	31±11	3±5	15±11	13±9	3±8	30±15	5±9	3±5	3±4
	2 years	17±17	21±13	21±21	22±13	3±3	11±5	15±10	4±7	7±10	2±4	3±6	11±14
BALB/c	6 weeks	2±3	27±23	16±16	31±28	4±9	19±20	28±28	43±26	111±95	13±18	1±2	25±27
	1 year	42±31	18±7	20±7	32±20	4±3	16±12	3±3	14±16	43±29	9±8	4±3	19±29
	2 years	18±14	8±11	6±8	1±2	0	1±1	1±2	3±5	1±1	3±4	7±8	0
BALB/cfC3H	6 weeks	50±36	60±51	108±182	17±9	10±10	22±24	23±9	40±43	79±48	37±27	17±14	200±176
	1 year	89±18	12±13	129±198	38±27	43±39	20±16	16±17	4±6	61±71	17±13	15±13	99±82
GR	6 weeks	9±13	22±21	35±17	33±15	22±12	19±10	35±20	44±39	32±13	19±30	4±6	22±18

The average of five animals with standard deviation is presented. Each value has been corrected for the conjugate control.
[1]) bone marrow; [2]) epididymis; [3]) salivary gland; [4]) Peyer's patches.

Brinkhof (1976) used the mammary-tumour-susceptible BALB/c mice to test *in vivo* for the presence of mammary tumour inducing agents in different organs of male BALB/cfC3H mice.

These authors made a distinction between the oncogenic potential transferred by live cells and that transferred by cell-free extracts. The latter is thought to be due to the presence of MTV virions. Only cell-free extracts from salivary gland, kidney, testis and epididymis induced mammary tumours. In our experiments, only the epididymis was found to contain significant levels of MTV proteins. Whereas Bentvelzen and Brinkhof (1977) were unable to demonstrate oncogenic potential in viable lung cells of the BALB/cfC3H, Parks, Gillette, Blackman, Verna and Sibal (1972) reported the expression of MTV proteins in this organ of C3H mice. Our results tend to confirm these latter observations, especially in young animals. Gillette, Robertson, Brown and Blackman (1974) demonstrated the presence of MTV coded membrane antigens on lymphoid cells, notably the bone marrow derived lymphocytes. Possibly, this is an explanation for the MTV antigens we detected in bone marrow, spleen and Peyer's patches.

In relation to age, the average amount of MTV antigens was highest in the CBA strain at one year of age in most organs tested. Again, the great variation between individual animals makes statistical comparison meaningless. In the BALB/c strain, all of the organs of the 2-year-old animals were negative. This observation is at variance with the results of Schlom et al. (1973) using molecular hybridization techniques. Our observation is of interest because this strain is known for the appearance of virus-induced mammary tumours in late age. The same picture was observed, however, for the C57BL strain. A number of organs of the BALB/cfC3H animals were positive in the 6-week-old as well as in the 1-year-old animals. In the GR strain, a number of tissues showed small amounts of MTV proteins.

The mammary tumour system is an extremely complex one. The presence of endogenous virus has been proven for many mouse strains. In addition to the endogenous group of MTV's a milk-borne exogenous MTV is produced by the C3H strain. There are great differences in the biology of the tumours induced. It is noteworthy that the tumours caused by MTV-P of the GR strain are hormone-dependent. This condition might explain why no MTV proteins were found in the male GR mice. Another aspect is the susceptibility of some strains like the BALB/c for the induction of mammary tumours by exogenous MTV-S. Evidently, the immune system of BALB/c is not capable of coping with the MTV-S introduced with the milk. This effect is not due to neonatal tolerance induction, as MTV injected in adult BALB/c animals also leads to rapid development of mammary tumours. The factors regulating the expression of MTV in male animals are not known at present. It should be investigated for instance, whether the appearance of MTV proteins in the low cancer strain CBA at one year of age and its disappearance at two years of age is the result of the release of a genetic block, followed by a vigorous immune response.

We have not yet been able to collect a sufficient quantity of the proteins binding to the Seph-RAMTV to be able to establish beyond doubt their MTV origin. Without this proof, there is a possibility that not MTV but another highly cross-reacting protein is in fact measured. The likelihood of this possibility, however, is slight in view of the following observations: 1) only a selective number of organs gave consistently positive indications for the presence of materials reacting with the RAMTV beads; 2) considerable differences were observed among individual animals; 3) the organ distribution of the material reacting with the Seph-RAMTV beads was in essence comparable for different inbred strains; and 4) the Seph-RAMTV system combined with the RAMTV-FITC did not show reaction with normal mouse serum, even when concentrations of over 20 mg/ml protein were used.

The combination of these facts makes it unlikely that the reactions observed with our system are due to materials other than MTV or proteins directly related with MTV.

J. MONITORING THE PRODUCTION OF C-TYPE VIRAL PROTEINS BY CELL CULTURES

C-type viral particles are isolated either from serum and tissues of leukaemic mice or from tissue cultures infected *in vitro* with virus preparations. The tissue culture method yields preparations which are less contaminated with interfering proteins than does the former. Some viruses grow easily in cultured cells, while others do not. It was important, therefore, to establish an assay system for monitoring the production of viral proteins.

The Sepharose bead system was adapted for this application in collaboration with G. Koch of the Radiobiological Institute TNO. The IgG fractions of specific goat antisera were coupled to Sepharose beads. The antisera were obtained through the Office of Program Resources and Logistics, Viral Oncology, Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, Md., USA). The antisera were directed against p30 and gp71 of Rauscher leukaemia virus (RLV); p30 is the main core protein of RLV and gp71 is the major component of the viral coat (see, for instance, August, Bolognesi, Fleissner, Gilden and Nowinski, 1974). A polyvalent antiserum against total RLV (GARLV) was used for comparison. This antiserum was produced by us by immunization of a goat.

3T3 mouse fibroblasts were infected with RLV. The supernatant of the cultures was collected and spun for 60 min at 35,000 rpm (35N rotor, Beckman Instruments Inc., Palo Alto, Cal., USA). The pellet was resuspended in 0.25 M sucrose in 1.5 M Tris buffer and spun for 3 hr at 25,000 rpm (SW27 rotor, Beckman Instruments Inc.) on a linear sucrose gradient. The opalescent band around a density of 1.15 to 1.17 g/ml was collected and viral particles were disrupted by repeated freezing and thawing. The supernatant of uninfected BALB/c 3T3 cells was processed in exactly the same way. This preparation served as the control. Both preparations were incubated with

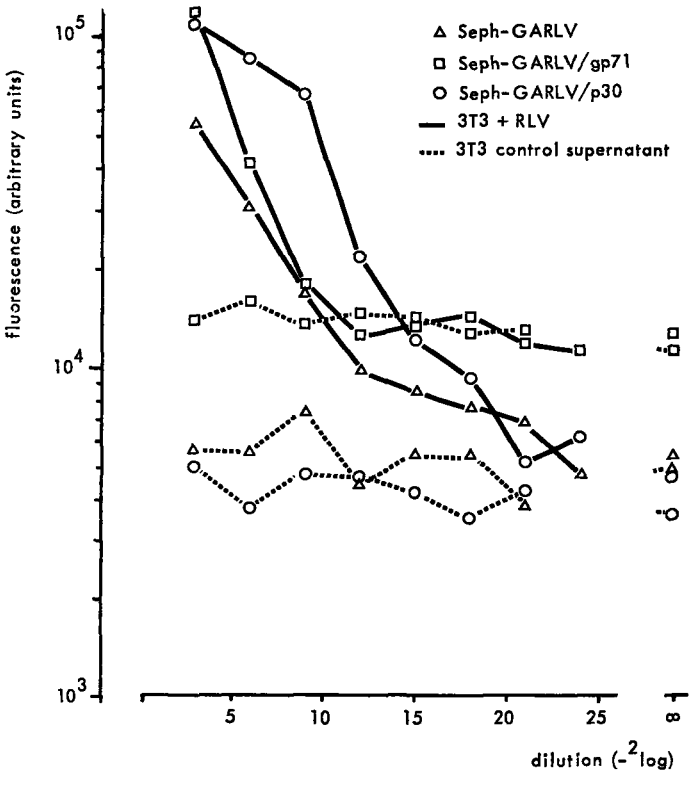


Figure VIII-19. Monitoring the production of viral proteins with the SBIA. Beads were coupled with the IgG fractions of goat antisera directed against RLV, p30 of RLV and gp71 of RLV. These beads were incubated with the supernatant of RLV-infected BALB/c 3T3 cells and the supernatant of non-infected 3T3 cells. Both preparations were equalized with regard to protein content. Bound RLV proteins were stained with fluorescent GARLV (GARLV-FITC). Each value is the average of at least 5 individual bead measurements. ∞ represents the conjugate control.

the three kinds of beads mentioned above. The beads were washed and incubated afterwards with fluorescent GARLV. The fluorescence data are presented in Fig. VIII-19. The supernatant of the infected cells reacted much more strongly with the antiserum beads than did the control supernatant. The polyvalent GARLV antiserum was not as strong as the GARLV/p30 and the GARLV/gp71. This experiment shows the possibility of monitoring the production of virus in cell cultures with the Sepharose bead immunofluorescence system.

The preparation of virus isolates from cell culture supernatants has many advantages over isolation of these viruses from tumours. A technical problem with the former isolation is the processing with ultracentrifugation of large volumes of supernatant. Several methods have been described to solve this problem. One of them is

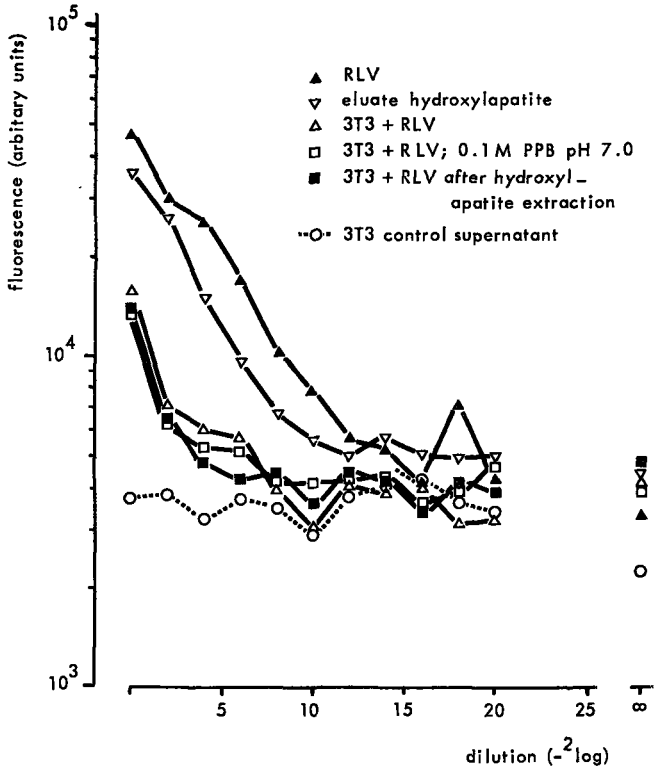


Figure VIII-20. The purification of Rauscher leukaemia virus (RLV) by hydroxylapatite chromatography. The supernatant of a BALB/c 3T3 cell culture infected with RLV was passed over a hydroxylapatite column. The passed fraction after hydroxylapatite extraction as well as the fraction eluted with 0.1 M potassium phosphate (PPB) were incubated with Seph-GARLV (see Fig. VIII-19). The controls were the following: the supernatant of RLV infected 3T3 cells without and with 0.1 M PPB, a purified RLV preparation and the supernatant of uninfected 3T3 cells. The protein content of all preparations was equalized. The RLV proteins bound to the Seph-GARLV were stained with GARLV-FITC. Each value represents the average fluorescence intensity of at least 5 individual beads. ∞ represents the conjugate control.

hydroxylapatite chromatography in which the supernatant is passed over a column of hydroxylapatite. Viral particles as well as a large number of serum proteins are bound. Selective elution of the virions is sought with solutions of increasing ionic strength.

We have tested this method for the purification of RLV as shown in Fig. VIII-20. Sepharose beads coupled with the IgG fraction of GARLV were incubated with: a) the supernatant of BALB/c 3T3 cells infected with RLV; b) the same supernatant after passage over a hydroxylapatite column; c) the fraction that was eluted from the column with 0.1 M potassium phosphate; c) the supernatant of BALB/c 3T3 cells infected with RLV+0.1 M potassium phosphate to test the influence of this salt concentration in the Sepharose bead immunofluorescence system; e) a purified RLV

preparation isolated by means of ultracentrifugation; and f) the supernatant of unin fected 3T3 cells. All preparations were equalized with regard to protein content. Fluorescent GARLV was used to demonstrate the binding of RLV proteins to the beads. With the hydroxylapatite, a 64-fold concentration of RLV may be obtained (Fig. VIII-20), however, the method is not efficient in the removal of all RLV from the supernatant. The supernatant not bound to the column reacted almost as strongly as the untreated supernatant. We have, therefore, discontinued the use of hydroxylapatite for the purification of RLV. Instead, we purify virus preparations with repeated ultracentrifugation in discontinuous and continuous sucrose gradients.

K. COMMENTS AND CONCLUSIONS

Almost any compound can be detected with the Sepharose bead immunofluorescence assay (SBIA), provided that there is an antiserum against it available. Whether the SBIA will be the technique of choice in a given investigation depends on the nature and objective of that investigation.

A very large number of other methods has been described for the quantitation of antigens and antibodies. These methods will not be discussed exhaustively here. Rather, a selected number of techniques will be treated in more detail with respect to three assets of the SBIA, namely: versatility, sensitivity and the possibility for automation.

Versatility

With the SBIA, it is possible to quantitate antigens as well as antibodies in biological fluids. The antibodies may be of a precipitating or nonprecipitating nature. This provides an advantage over assays in which the formation of a precipitate in agar gels (immunodiffusion techniques, according to Mancini, Carbonara and Heremans, 1965) or in solution (nephelometry, according to Ebeling, 1973; Prellwitz, Kapp and Müller, 1974; Cambiaso, Masson, Vaerman and Heremans, 1974) is essential. It should be mentioned that the incorporation of precipitation enhancing substances such as polyethylene glycol (Adams and Jerry, 1974) or dextran (Hyslop and Cochrane, 1974) into the agar of diffusion plates has considerably increased the applicability of the diffusion techniques.

A number of methods for antibody quantitation relies on properties of antibodies other than the binding of their respective antigens. The haemolysis and cytotoxicity assays are widely known. These techniques are only applicable, however, to complement binding antibodies.

In the haemagglutination and haemadsorption assays (Fagraeus and Espmark, 1961; Wood and Barth, 1975), the erythrocytes are coated with antigens and antibodies. These techniques may be applied to a large variety of antigen-antibody systems. The possibility of screening the results with the unaided eye is of course a major advantage of these techniques for application in field work. A drawback to the

haemagglutination techniques is that they require a considerable amount of experience. The fragility of the indicator erythrocytes makes them less suitable for long term storage and a number of methods have been described for stabilizing the erythrocyte membrane (Suzuki, Tanaka and Kawanishi, 1974). Simple adsorption of antigens to the erythrocyte membrane is possible only with a selected number of substances. Other procedures for coupling a variety of antigens to erythrocytes, notably chromium chloride (Kishimoto, Tsuyugachi and Yamamura, 1968), glutaraldehyde (Lemieux, Avrameas and Bussard, 1974), para-benzoquinone (Ternynk and Avrameas, 1976) or binding after CNBr activation, have been described. Erythrocytes coated with antigens have been used as substrate for an immunofluorescence procedure by Killander, Levin, Inoue and Klein (1970). These authors quantitated the amount of bound antibodies microfluorometrically. The versatility of the radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA) is the same as that of the SBIA. Those techniques will be discussed later.

Sensitivity

The sensitivity of the SBIA for a number of antigen-antibody systems has been determined in experiments described in this thesis. The detection limit is generally in the order of some nanograms of protein per ml of sample. This sensitivity compares favorably with that of diffusion techniques. In routine use, the sensitivity of the single radial immunodiffusion (SRID) technique is in the order of a few μg protein per ml (Kalff, 1970). Modifications of the SRID technique in order to increase its sensitivity have been reported. Centifanto and Kaufman (1971) used fluorescent anti-Ig conjugates to visualize precipitation rings. Davis, Stone and Glazier (1974) used ultraviolet photography and fluorescent antibodies in immunoelectrophoresis. Guesdon and Avrameas (1974) employed peroxidase labelled anti-Ig's to develop their diffusion plates. Autoradiography of plates which were incubated with radioactive anti-Ig's, was reported by Rowe (1969). The latter method increases the sensitivity of the SRID assay substantially. The method is rather time-consuming and this may explain why it has been used only sparingly.

Radioimmunoassays (RIA's) and enzymatic assays (see, e.g., Deelder, Ruitenberg, Kornelis and Steerenberg, 1977) have a sensitivity which is better or at least comparable to that of the SBIA. Especially the RIA has found many applications.

^{125}I and ^{131}I are the most commonly used nuclides in RIA's. The molecules may be introduced into proteins by a number of methods. The method of Hunter and Greenwood (1964) uses the oxidant chloramine-T. This method yields radioactive conjugates of very high specific activity. The high specific radioactivity of iodine conjugates makes them the logical choice for those systems in which the highest sensitivity is needed. In other cases, tritium or ^{14}C may form a substitute for the iodine. These labels may be introduced into proteins with either radioactive formaldehyde (McMillen and Consigli, 1975) or phenyl isothiocyanate (Levy and Dawson, 1976). A disadvantage of the chloramine-T method is the (variable) denaturation of proteins which may

occur with this compound. More gentle methods have been described for the introduction of iodine into proteins, such as the peroxidase (Thorell and Larsson, 1974) and iodine-chloride methods (Helmkamp, Goodland, Bale, Spar and Mutschler, 1960). Recently, Bolton and Hunter (1973) reported the use of the ester N-succinimidyl-3-(4-hydroxyphenyl) proprionate into which the iodine label may be incorporated. The iodinated ester reacts under mild conditions with the terminal amino groups of lysine residues in proteins. A similar approach was taken by Wood, Wu and Gerhart (1975) using the imido ester methyl parahydroxybenzimidate-HCl which may also be iodinated before the reaction with proteins. Conjugation of antisera with fluorochromes does not seem to affect their antigen binding capacity to a significant degree (see, however, Arnold and von Mayersbach, 1972). On the other hand, the functional properties attributed to the Fc part of immunoglobulins may become severely impaired upon conjugation (Thrasher, Bigazzi, Yoshida and Cohen, 1975).

RIA's have been developed for a large variety of antigens and antibodies. In essence, three types of RIA's are most commonly used.

1. In the sandwich method, the amount of bound antigen or antibody is quantitated with a radioactive antibody. The methods described in this thesis for the quantitation of mouse Ig's and MTV antigens are directly comparable to these sandwich RIA's.
2. In the competition RIA, unlabelled antigen is presented together with labelled antigens to a limited number of antibody binding sites. The displacement of the label relative to a control preparation is a measure for the amount of unlabelled antigen.
3. In saturation assays, unlabelled antigen is first reacted with antibody and the remaining free binding sites are saturated with labelled antigen. The more unlabelled antigen is added, the fewer are the free binding sites.

The distribution of radioactivity between the bound and free fractions of antibody or antigen is the parameter to be measured in RIA's. The separation between these two fractions presented difficulties, particularly in the case of protein determinations. Solid phase techniques have been developed for such RIA's.

The competition RIA is the most widely used type, at least in the field of virology (for MTV see, Cardiff, 1973; Verstraeten, Hageman and Kwa, 1973; Noon, Wolford and Parks, 1975; Verstraeten, van Nie, Kwa and Hageman, 1975). The major advantages of the competition RIA's are their sensitivity and the fact that the displacement curves obtained with different proteins give clues about the antigenic relatedness between these proteins. A disadvantage of the competition RIA is the liability of this assay to false positive results. False positives may occur due to proteolysis of the radioactive probe. It is known that substituted proteins are more susceptible to proteolytic degradation than native proteins (Goldberg and Dree, 1974). Proteolysis of the probe results in less radioactivity in the bound fraction, imitating a displacement. In the SBIA and the sandwich RIA's, mentioned above, proteolytic enzymes can cause

only false negative results. Theoretically, more rapid degradation of proteins in the reference sample as compared to that in the test samples would lead to false positive results. For the Ig determination presented in parts F and G of this chapter, this would result in overestimation of the Ig levels in the samples. The likelihood of this happening is reduced if the reference and test samples are of the same nature and stored and handled in the same way.

A direct comparison of the sensitivities of RIA and SBIA has been presented by Capel and Aalberse (1975) for the determination of human IgE. These authors concluded that the SBIA is 10-fold less sensitive than the RIA. The same conclusion was reached by Haaijman, Bloemmen and Ham (1975). The results presented in this chapter indicate, however, that the detection limit of the SBIA can be in the order of a few nanogram per ml. The sensitivity can be even better in some antigen-antibody systems. The reason for this discrepancy is most probably the recently introduced reduction in the number of Sepharose beads in the incubation mixture, which was made possible by the use of the microtitration plates.

Recently, Cukor, Woehler, Persiani and Fermin (1976) compared the use of ^{125}I and the fluorescent label 2-methoxy-2,4-diphenyl-3(2H) furanone (MDPF) in the allergosorbent test. These authors concluded that this fluorescent label cannot replace ^{125}I because it causes too high background fluorescence. On the other hand, Parkinson and Kalmakoff (1976) found only a twofold difference in sensitivity between radioactivity measurement and fluorescence using a doubly labelled fluorescein – ^{125}I antibody on virus infected cells. The authors compared visual endpoint titres with the total radioactivity bound to monolayers of cells. It may be questioned whether this comparison is valid, because in IF microscopy the individual cells are assessed and in the RIA the average degree of binding is measured.

The sensitivity of the enzyme-linked immunoassays (see, for instance, Avrameas and Guilbert, 1972) is comparable to that of the SBIA and RIA. In the ELISA (Enzyme-Linked ImmunoSorbent Assay), enzymes are covalently coupled to antibodies. A variety of enzymes and substrates has been used (Scharpé, Cooreman, Blomme and Laekeman, 1976). Antigens are adsorbed onto a plastic surface, mostly tubes (Engvall and Perlmann, 1971, 1972; Engvall, Jonsson and Perlmann, 1972) or microtitration plates (Voller, Bidwell and Bartlett, 1976). Antibodies binding to these antigens are measured with an enzyme-anti-Ig conjugate. The amount of liberated product is proportional to the amount of bound antibody. Maiolini and Masseyeff (1975) compared the ELISA and RIA for rat and human alphafoetoprotein. With the ELISA, 5 ng and with the RIA, 1 ng of the protein could be measured. The reaction product is normally measured colourimetrically in ELISA's. A new development in the ELISA techniques is the use of fluorogenic substrates. The product of these substrates is fluorescent, while the substrate itself is not. Kato, Fukui, Hamaguchi and Ishikawa (1976) and Kato, Hamaguchi, Fukui and Ishikawa (1976) developed an assay for human IgG using an enzyme-antiserum complex consisting of a rabbit antiserum

directed against human IgG and β-D-galactosidase. The fluorogenic substrate methylumbelliferyl galactoside was employed. With this assay, amounts of human IgG in the order of 0.1 ng could be detected.

Automation

A large proportion of the sample processing may be automated in the SBIA when microtitration plates are employed. The number of samples, which may be handled each day, can be increased about tenfold compared to the original assay described by van Dalen, Knapp and Ploem (1973). The same kind of automation is possible for the RIA and ELISA. A drawback of the SBIA in comparison with the other assays is the necessity to measure the fluorescence of individual Sepharose beads. These measurements require the attendance of an operator at the microfluorometer. Radioactive measurements are still much easier to perform and there are several systems for the automatic measurement of colourimetric data in the ELISA. It should be mentioned, however, that the time needed for measurements in the SBIA is short compared to the time needed for the preparation of samples in most cases.

A fast automated inverted microfluorometer is an indispensable part of our equipment for the SBIA. Unfortunately, this instrument is not marketed commercially. The introduction of pneumatic microcomponents into the design of an automated microfluorometer (see chapter VI) offers a cheap and reliable solution. Calculator control over electromagnetic valves with small power consumption constitutes a versatile and simple system. The software needed for the shutter sequence programming and data handling could be easily supplied with the microscope.

In conclusion, we think that, with the present degree of automation in our SBIA system, about 200 samples may be routinely handled per day by one person. A larger sample processing capacity can be realized only with relatively costly investments.

COMPARISON BETWEEN DIFFERENT LABELS IN IMMUNOASSAYS

Stability of reagents

The short half-life time of ^{125}I and also ^{131}I makes radioactive conjugates unstable. Normally, they have to be used within about one month after preparation. Ceska, Berglund, Lundkvist and Grossmüller (1972) reported storage of ^{125}I conjugates for more than one year. The counting time per sample was increased to one hour by that time, however. Antibody-enzyme conjugates seem to be quite stable. Engvall and Perlmann (1971) reported storage of such a conjugate for one year at 4 °C without a noticeable loss of antigen binding capacity. If stored at −70 °C or lower, the preservation of fluorescent conjugates is excellent (Roberts, Miller, Pringle and Binnings, 1968; Green, Gray and Knox Harrell, 1976). In our laboratory, undiluted conjugates have been stored for more than 10 years without loss of activity. It was mentioned in chapter IV that this condition holds for conjugates prepared from IgG fractions of antisera. Purified antibodies seem to be more liable to denaturation. It is clear,

however, that the fluorescent label does not change the behaviour of antisera in this respect. The long shelf-life of enzyme and fluorescent conjugates increases the possibilities for standardization in immunoassays.

Double label techniques

The use of different fluorochromes makes it possible to determine at least two different substances in one sample, or two different antigenic structures of the same molecules (see chapter V, part D). In enzyme histochemistry, double label experiments are made possible by using different enzymes and substrates. The low contrast between the colours of the different precipitates is perhaps the reason why these techniques have not been applied frequently. Pachmann and Killander (1976) reported the simultaneous use of ^{125}I and ^{131}I in a model system. Differential counting of these two isotopes is possible, but requires considerable correction of the data due to the overlap in the energy spectra.

Sensitivity

The sensitivity of the assays employing fluorescent reagents approaches that of the RIA's employing radioactive iodine. The efficiency of tritium and ^{14}C in RIA's is only about 1% of that of ^{125}I (Hunter, 1973). The sensitivity of ELISA's in which the product is measured colourimetrically is comparable to the RIA and the SBIA. The introduction of fluorogenic substrates holds the promise of an even higher sensitivity.

Hazard

Radioactivity represents an environmental and personal hazard. Radioactive iodine is especially notorious in this respect, due to the low but consistent sublimation of the iodine (Bognadove and Strash, 1975). Special facilities have to be used for the handling of isotopes. Some substrates which have been used in enzyme histochemistry have

TABLE VIII-5. COMPARISON OF RADIOACTIVITY, ENZYMES AND FLUORESCENCE AS LABELS IN IMMUNOASSAYS

	RIA		SBIA	ELISA	
	^{125}I, ^{131}I	^{14}C, ^{3}H		colourimetric [1]	fluorometric [2]
Denaturation of antibodies during labelling	++, − [3]	−	−	−	−
Stability of labelled compound	−	+	++	+	+
Sensitivity of assay	++	±	++	++	+++
Possibility for double label experiments	±	++	++	?	+
Personal hazard	++	+	−	±	−
Environmental hazard	++	+	−	−	−
Automation of preparative procedures	+	+	+	+	+
Automation of measurements	+	+	−	+	+
Applicability to epidemiology	−	−	−	+	+

[1]) Amount of coloured reaction product is measured in an absorption spectrophotometer.
[2]) A nonfluorescent substrate is converted by the enzyme to a fluorescent product.
[3]) Dependent on the method used for the introduction of the iodine label.

proved to be carcinogenic. The production of benzidine has been discontinued for this reason in the USA. The most commonly used fluorescent markers, fluorescein and rhodamine, do not seem to be hazardous to animals or men.

In view of the increasing demand for immunoassays in clinical chemistry, it seems worthwhile to pursue alternatives for radioactivity in the future (Challand, Goldie and Landon, 1974). The different pros and cons of the different immunoassay labels and techniques are summarized in Table VIII-5.

The feasibility of fluorescence immunoassays for a number of different antigen-antibody systems has been demonstrated in this chapter. Emphasis has been placed on the aspects of standardization and calibration which are inherent to the measurement of fluorescence. In the area of antibody measurements, it has been shown that the assay is sensitive enough to follow longitudinally the immune response to LPS as well as HSA in individual mice. Naturally occurring antibodies reacting with RLV and SiSV were demonstrated in germfree WAG/Rij rats. A number of experiments has been devoted to the detection of naturally occurring antibodies to MTV in mice. These experiments were unsuccessful. Possible explanations for this phenomenon were suggested.

The measurement of IgA, IgM and the four subclasses of IgG in the sera of mice and the measurement of mammary tumour viral antigens in milk of female mice and organ extracts of male mice were accomplished by the use of an antibody/antigen/fluorescent-antibody sandwich.

The Ig level assay has been applied to sera of CBA mice of various ages. Briefly, the levels of IgA, IgM, IgG_{2a} and IgG_3 did not show a clear trend with age, whereas the levels of IgG_1 and IgG_{2b} increased. The most prominent phenomenon, however, was the increase of the variability among the Ig levels of individual animals with advancing age.

Mammary tumour viral antigens were readily detected in the milk of uniparous and multiparous GR female mice. No clear relationship between age and viral protein concentration was observed. Preliminary evidence was presented for the presence of small amounts of MTV proteins in the milk of multiparous C57BL mice. This strain is known for its low mammary tumour incidence. The presence of MTV proteins was demonstrated in various organs of male mice. Relatively high concentrations were found in the organs of the urogenital tract. The relationship between age and the expression of MTV proteins in the different organs of various mouse strains did not result in a consistent picture.

Finally, the possibility of monitoring the production of C-type oncornaviruses in tissue cultures with the SBIA was shown.

In comparing the SBIA with the RIA and ELISA, it became clear that the SBIA offers certain advantages over the RIA in laboratory or small-scale operations and that the third method, especially with fluorogenic substrates, is more suited for large-scale applications.

CHAPTER IX

The application of qualitative and quantitative immunofluorescence microscopy to a single biological system: the influence of age on the immunological activity and capacity of the CBA mouse

Contents:

A. INTRODUCTION

The incidence of a number of diseases increases with age. Some of these diseases are associated with an age-related dysfunction of the immunological system, especially autoimmune diseases (Good and Yunis, 1974). This led Walford (1969, 1974) to the formulation of his immunological theory of ageing, in which deterioration in the distinction between 'self' and 'non-self' is seen as the primary event in the ageing process. The role of the immune system in oncogenesis has attracted much attention. Although the theory of immune surveillance (see Burnet, 1970) has not been generally accepted (Klein, 1975), it has been a major impetus for the study of the immune system in ageing.

The capacity of the immune system to react against experimentally given antigens was extensively investigated by Makinodan and co-workers (Makinodan and Peterson, 1964, 1966a, 1966b; Albright, Makinodan and Deitchman, 1969; Price and Makinodan, 1972a, 1972b; Nordin and Makinodan, 1974). The authors reported a progressive decline in the number of plaque-forming cells with age in BC3F1 mice after injection with sheep red blood cells. The decline was observed in the primary as well as in the anamnestic immune response. With cell transfer experiments, it was established that the major part of the age-related decline in immunological capacity was to be attributed to the cells of the immunological system rather than to the changes in the environment in which these cells had to function. Reviews on this subject have been given by Makinodan, Perkins and Chen (1971), Micklem, Ogden and Payne (1973), Makinodan and Adler (1975), Adler, Jones and Nariuchi (1977), and Hijmans and Hollander (1977).

The possible mechanisms involved in an age-related decline of the immune capacity will be introduced here under three subheadings: stem cells, B-cell system and T-cell system.

1. *Stem cells*

Goodman and Makinodan (1975) demonstrated with a limiting dilution assay a twofold reduction in the number of cells in the spleen of BC3F1 mice which were able to generate an immune reactive clone in irradiated recipients. Harrison and Doubleday (1975) reconstituted lethally irradiated CBA/CaJ mice with bone marrow and spleen cells of young and old mice of the histocompatible CBA/HT6J strain. Recipients reconstituted with both types of cells of either 3- or 10-month-old donors reacted equally well to antigenic challenge. These results indicate that, at least at 10 months of age, no change had occurred in the number of immunocompetent unit precursor cells in this strain. An immunocompetent unit has been defined by Heidrick and Makinodan (1972) as: 'the minimum configuration of interacting cells (accessory cells and T-cells and B-cells) which upon T-dependent antigen challenge will give a

positive antibody response.' Coggle, Gordon, Proukakis and Bogg (1975) studied the spleen colony forming ability of bone marrow and spleen cells of 1- and 24-month-old SAS/4 mice. While the number of CFU-S in the spleen decreased with age, it remained constant in the bone marrow. Vacek, Bartonícková and Tkadlecek (1976) reported also no decrease in the femoral stem cell compartment for the Wistar rat. It seems likely from the available data that, although in some strains of mice an age-related decline in the number of stem cells may occur, this is not a consistent phenomenon and, therefore, cannot be regarded as the primary event in immunological ageing.

2. *B-cell system*

The declined immunological competence in senescence may be either due to a dysfunctioning of the B-cell or the T-cell system, or to both.

Jaroslow, Suhrbier and Fritz (1974) first presented results indicating that the aged B-cell system is not always less responsive to antigens than the young B-cell system. These authors fractionated the dose of antigen to be administered and injected several small doses at short intervals. With the bacteriophage T4 as antigen and this immunization schedule, aged beagle dogs reacted as efficiently with respect to antibody formation as young ones. If the same amount of antigen was given in one injection, the aged dogs responded poorly and with large variations among individuals relative to the young dogs. This phenomenon of increased variation among aged individuals has already been described in chapter VIII, part C, for the antibody formation by aged mice against HSA. Fractionation of the antigen dose might have the same effect on the immune system, as have adjuvants (Herbert, 1966). It is indicative that Finger et al. (1972) reported no difference in antibody response of young and aged mice if the antigen was given in combination with the adjuvant *Bordetella pertussis*. The experiments of Jaroslow, Suhrbier and Fritz (1974) indicated that the B-cell compartment of the immune system is not primarily affected in senescence. Corroborative evidence was reported by Blankwater, Levert and Hijmans (1975) and Smith (1976) who reported that BALB/c mice did not show an age-related decline in the response towards the T-cell-independent antigen LPS (see also chapter VIII, part B). Gerbase-DeLima, Wilkinson, Smith and Walford (1974) observed, however, some decline in antibody response towards LPS in aged (C57BL/6J $\times$ 129J)F_1 mice as compared to young-adult mice. The onset of the decline seemed to take place later in life than that of the decline in the T-dependent immune functions.

From literature data, it is thus likely that the primary defect in the immune system in senescence cannot be attributed to the B-immune system in the first place.

3. *T-cell system*

Direct measurements of the capacity of the T-immune system to respond to an

antigenic challenge are difficult because of the large diversity of T-cell functions. An age-related reduction in the number of theta-bearing cells as well as in the amount of theta antigen per individual T-lymphocyte was reported by Brennan and Jaroslow (1975). The change in the number of T-lymphocytes may very well be strongly strain-dependent, as it was not observed in C57BL/6J mice (Kishimoto, Shigemoto and Yamamura, 1973) or in CBA mice (Stutman, 1974). An age-related decline has been reported for phytohaemagglutinin stimulation of T-cells in mice (Hori, Perkins and Halsall, 1973; Gerbase-DeLima, Liu, Cheney, Mickey and Walford, 1975), in rats (Kruisbeek, 1976) and in man (Roberts-Thomson, Whittingham, Youngchaiyud and Mackay, 1974; Weksler and Hütteroth, 1974). An age-related decline in cell-mediated immunity as expressed in the ability of splenocytes to lyse tumour target cells and the ability to reject skin allografts was shown for C57BL/6J mice by Menon, Jaroslow and Koesterer (1974). Konen, Smith and Walford (1973) reported a significant decline in the mixed lymphocyte reaction of spleen cells from C57BL/6J mice as did Hirano and Nordin (1976) for the NZB and DBA/2 strains of mice. The same phenomenon was found with lymph node cells of the longlived hybrids (C57BL/6J × BALB/cJ)F_1 and (C57BL/6J × 129J)F_1 (Meredith, Tittor, Gerbase-DeLima and Walford, 1975). Thymus cells, on the contrary, showed an age-related increase in the ability to respond to allogeneic spleen cells. Adler, Takiguchi and Smith (1971) stressed the genetic influences on the results obtained with the mixed lymphocyte reaction (MLR). While the MLR of C57BL/6J and CBA spleen cells declined after 20 weeks of age, that of spleen cells of the A/J strain remained constant up to one year of age. Friedman, Keiser and Globerson (1974) showed that, in their *in vitro* graft versus host system, cells of old (C3H/eb × C57BL)F_1 mice were less active than cells of young mice. These authors attributed the defect in T-cell function of old mice to inadequate activity of the putative thymus hormone in senescence.

Popp (1975) was not able to demonstrate an age-related difference between young and old mice regarding the activation of T-cells by histocompatibility antigens, as estimated from the appearance of pyroninophilic blast cells. Walters and Claman (1975) assayed the T-cell function in young and aged mice with four different systems. No difference with regard to contact sensitivity was found. On the other hand, cells of old BALB/c mice were unable to elicit a graft versus host reaction and were far less responsive to phytohaemagglutinin and concanavalin A. In a mixed lymphocyte reaction test, the spleen cells of old BALB/c mice reacted as well or better than the spleen cells from young BALB/c mice, as both stimulator and responder cells.

The results from the literature thus indicate that, although some T-cell functions may become deficient in old age, this is certainly not the case for all functions.

The immunological response towards experimentally given antigens is superimposed on the ongoing immunological activity. This activity is a result of antigens which enter continuously from the environment. The overall immunological activity at a given age may be assayed, for example, by estimating the number of immuno-

globulin containing cells (C-Ig cells, Haaijman, Schuit and Hijmans, 1977) or by measuring the levels of serum Ig's, irrespective of the antigens to which these molecules are directed. Measurement of the immunological activity as a function of age may give an indication as to whether a general decline occurs in the defence mechanisms of ageing individuals under physiological conditions.

We define the immunological capacity as the ability of the immune system to react to experimentally injected antigens with the production of detectable antibodies. The definitions of activity and capacity are operational.

Immunological activity and capacity are compared in this chapter within one mouse strain. The levels of different Ig's in the serum of CBA mice during their life span have already been discussed in chapter VIII, part G. In part B of the present chapter, the results of the estimation of the number of C-Ig cells in different lymphoid organs of the CBA mouse at different ages are presented. In part C the results are presented of the determination of the immunological capacity of our particular stock of CBA mice to respond to human serum albumin as a function of age.

A discussion of the data on immunological activity and capacity is presented in part D.

B. IMMUNOLOGICAL ACTIVITY

1. *Enumeration of the number of immunoglobulin containing cells (C-Ig cells) as a function of age in different lymphoid organs of the CBA mouse*

Hijmans, Schuit and Klein (1969) described the technique for the visualization of Ig containing cells in great detail. Briefly, cell suspensions are prepared with a Borel type wire mesh in PBS (pH 7.6) containing 5% bovine serum albumin (BSA; Poviet, Amsterdam, The Netherlands) and 0.5% EDTA (Titriplex; Merck A.G., Darmstadt, Western Germany).

Bone marrow cells are obtained as a suspension by flushing the two femurs with PBS-BSA. Benner and van Oudenaren (1975,1976) showed that, in the mouse, the antibody forming potential of the femoral bone marrow compartment is representative for the total bone marrow, as measured with the plaque assay. From their results, we have assumed that, also for the C-Ig cell count, the femoral bone marrow may be regarded as a representative for the total bone marrow. Cell suspensions were washed once with 15 ml of PBS-BSA. After centrifugation in the cold (10 min, 800 g), the pellet was resuspended in a suitable volume. The number of nucleated cells was counted in a Bürker chamber and adjusted to 4×10^6 cells/ml. Fifty µl of the cell suspensions were spun down in a cytocentrifuge according to Vossen (1975) and Vossen, Langlois van der Bergh, Schuit and Hijmans (1976). The slides prepared in this way may be stored at $-20\,^{\circ}C$ for prolonged periods of time, if wrapped in cellophane. For visualization of C-Ig cells, cytocentrifuge slides were fixed for 15 min at

−20 °C in acid ethanol (5 parts of glacial acid to 95 parts of ethanol). Slides were then washed three times in PBS for 10 min each and incubated for 30 min with fluorescent antisera in a humid chamber. Excess conjugate was removed with one wash of PBS. Preparations were mounted in buffered glycerol (9 parts of glycerol, p.a.; Merck A.G.) and sealed with paraffin. Buffered glycerol was freshly prepared once a week (see chapter IV, part J).

A FITC conjugated goat antiserum directed against mouse Ig's (GAM/Ig-FITC, lot no. 2-873; Nordic Immunological Laboratories) was used to count the total number of C-Ig cells per slide. From the number of nucleated cells per slide and per organ, one can then determine the total number of C-Ig cells per organ. Following Benner (1975), we have adopted a conversion factor of 7.8 to calculate from the numbers found in two femurs, the number of C-Ig cells in the total bone marrow.

The Ig class distribution of the C-Ig cells was determined according to Hijmans, Schuit and Klein (1969). Slides were stained with the following combinations of antisera:

a) TRITC conjugated rabbit antimouse IgA (RAM/IgA-TRITC) combined with FITC conjugated rabbit antimouse IgM (RAM/IgM-FITC);
b) RAM/IgA-TRITC combined with RAM/IgG-FITC;
c) RAM/IgG_2-TRITC combined with RAM/IgG_1-FITC;
d) RAM/IgG_2-TRITC combined with RAM/IgG_3-FITC.

The antisera were prepared, purified and conjugated by Dr. J. Radl and Miss P. van den Berg. The antisera met all specificity criteria described at length in chapter IV. A minimum of fifty positive cells were counted per combination. From the first two slides, the IgA:IgM:IgG distribution was deduced and from combinations c) and d) the relation IgG_1:IgG_2:IgG_3. The absolute number of C-Ig cells of a certain class per organ was calculated from the class distribution and the total number of C-Ig cells. No distinction was made between the IgG_{2a} and IgG_{2b} subclasses, because the antiserum against IgG_{2a} was not yet optimal when this series of experiments was started. The IgG_{2a} and IgG_{2b} C-Ig cells were designated arbitrarily as IgG_2 cells in this study. C-Ig cells which showed double staining were not taken into account in the calculations. These cells will be treated separately.

Slides were examined with a Zeiss standard 18 microscope which was provided with an incident light illuminator (type IV/F; Zeiss, Oberkochen, Western Germany). The illuminator was equipped with a 50W, AC mercury arc and the filter combinations 11 and 14 from Table II-5 for, respectively, FITC and TRITC. A Leitz FL 70 ×/1.30 oil immersion objective in combination with 6.3 × eyepieces was used.

Results of the estimation of the C-Ig cell number versus age in the different lymphoid organs of the CBA mouse have already been presented elsewhere (Haaijman, Schuit and Hijmans, 1977). In the present report, only new observations extending the previous results will be discussed.

Data are presented in Figures IX-1 and IX-2 on the number of C-Ig cells containing IgA, IgM or IgG in different lymphoid organs of male CBA mice as a function of age.

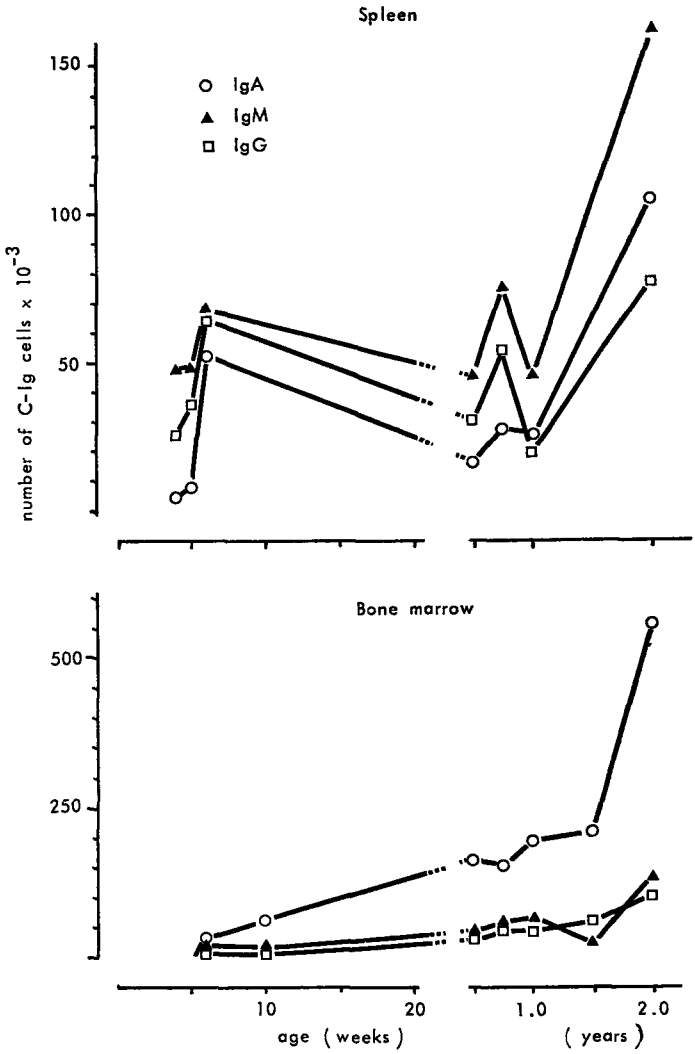


Figure IX-1. The numbers ($\times 10^{-3}$) of IgA, IgM and IgG C-Ig cells as a function of age in the spleen (upper figure) and bone marrow (lower figure) of male CBA mice. Note the two age scales and the different ordinates for spleen and bone marrow.

The results for spleen and bone marrow are shown in Fig. IX-1, while those for the mesenteric lymph nodes and Peyer's patches are presented in Fig. IX-2.

In the spleen, the number of IgA, IgM and IgG cells increased markedly around 6 weeks of age. The variation among the numbers found in individual animals was considerable. Ten male animals of 6 weeks of age were sacrificed to obtain a reliable estimate of the average number of C-Ig cells. The same was done for animals of 2 years of age. The averaged data of these two groups varied considerably from those ob-

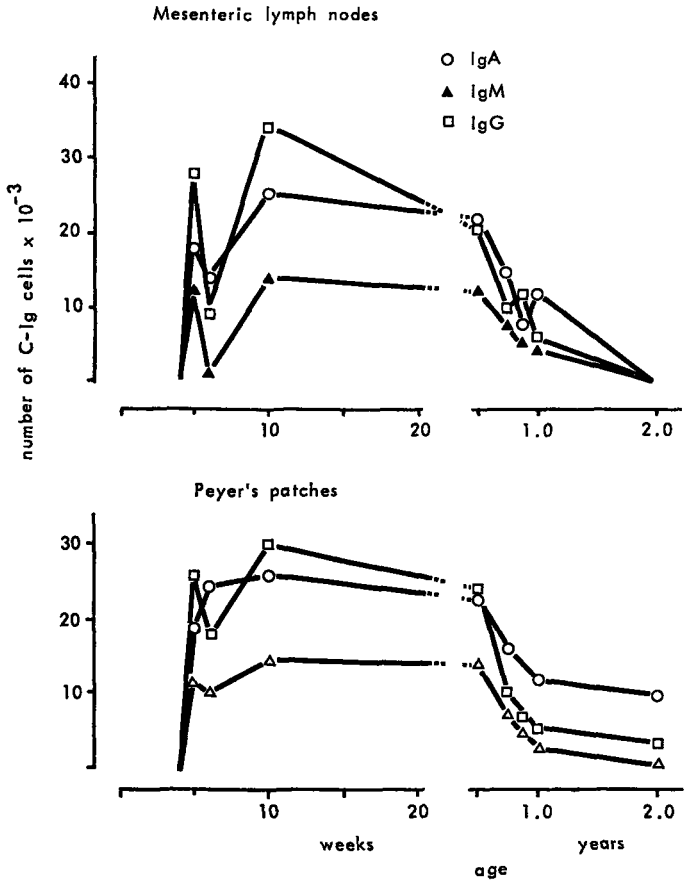


Figure IX-2. The numbers ($\times 10^{-3}$) of IgA, IgM and IgG C-Ig cells as a function of age in the mesenteric lymph nodes (upper figure) and Peyer's patches (lower figure) of male CBA mice. Note the two age scales. The open triangles in the lower figure represent IgM C-Ig cells.

tained previously by Haaijman, Schuit and Hijmans (1977) in some cases. These discrepancies will be noted where relevant. In Figs. IX-1 and IX-2, the averaged data from the two groups of ten animals, viz., of 6 weeks and 2 years of age, were included.

The number of C-Ig cells in bone marrow increased steadily with age up to one year (Fig. IX-1, lower part). A preponderance of IgA cells was observed. Haaijman, Schuit and Hijmans (1977) reported a substantial difference between female and male animals, in that the former showed a considerably increased number of C-Ig cells after one year of age, whereas the number remained about constant in the latter. This phenomenon was not confirmed as such in our later experiments, but a bimodality was observed in the data obtained from the ten male animals of two years of age (Table IX-1). In the bone marrow of five of these animals, C-Ig cell numbers as high as or higher than those observed earlier for female mice were found. In the bone marrow

TABLE IX-1. ABSOLUTE NUMBERS OF Ig POSITIVE CELLS ($\times 10^{-3}$) IN THE BONE MARROW OF YOUNG AND OLD MALE CBA MICE

Age	No.	IgA	IgM	IgG_1	IgG_2	IgG_3	Total
6 weeks	1	44.1	24.4	7.1	9.9	0.6	86.2
	2	48.9	28.0	3.5	16.9	0.0	97.2
	3	48.3	10.5	14.2	11.7	0.7	85.3
	4	5.0	1.6	2.1	1.9	0.0	10.5
	5	11.9	7.0	6.6	3.7	0.2	29.3
	6	23.5	14.0	8.2	4.8	0.1	50.6
	7	15.9	7.4	5.6	8.6	0.0	37.5
	8	13.0	15.6	3.3	4.4	0.0	36.3
	9	122.3	26.4	17.2	13.4	0.0	179.3
	10	29.6	41.8	4.6	4.9	0.0	80.8
2 years	1	914.3	198.1	100.6	97.5	20.5	1331.1
	2	782.0	91.4	23.7	88.9	0.0	986.1
	3	336.1	61.8	4.3	39.7	35.5	477.4
	4	298.4	129.7	0.4	13.2	4.5	446.2
	5	191.3	67.3	44.6	38.6	1.5	343.4
	6	1048.9	99.2	81.2	97.8	9.9	1337.1
	7	250.9	67.4	16.4	30.0	3.8	368.4
	8	857.5	291.2	48.9	70.8	8.5	1276.9
	9	48.0	22.0	8.7	14.3	0.6	93.5
	10	856.7	311.5	19.2	172.6	0.0	1360.1
Avg. 6 weeks	$\bar{x}$	36.2	17.7	7.2	8.0	0.2	69.3
	sd	34.2	12.3	4.9	4.9	0.3	48.4
	se	10.8	3.9	1.5	1.5	0.1	15.3
Avg. 2 years	$\bar{x}$	558.4	134.0	34.8	66.3	8.5	802.0
	sd	365.5	100.0	33.8	49.6	11.4	502.2
	se	115.6	31.6	10.7	15.7	3.6	158.8

Technical details of the determination of C-Ig cell numbers and Ig class distribution are discussed in the text.

of the five remaining animals, numbers of C-Ig cells were found comparable to those described earlier. Whether this bimodality has a functional significance is the subject of ongoing investigations, which have not yet given definite clues. Despite the large variation, the data on IgA, IgM and IgG cells have been averaged and plotted in Fig. IX-1 for the bone marrow of 2-year-old male mice. The actual data and standard deviations are in Table IX-2 and will be discussed below.

In the gut-associated lymphoid organs (mesenteric lymph nodes and Peyer's patches, Fig. IX-2) hardly any C-Ig cells were observed in animals younger than 5 weeks of age. At 5 weeks of age, however, considerable numbers of C-Ig cells of all three Ig classes were present. Up to six months of age, a more or less constant number of these cells was found in both lymphoid organs in which IgG and IgA cells predominated. Between six months and one year of age, the immunological activity of the two organs decreased, as measured from their C-Ig cell content. Significant numbers of C-Ig cells were no longer observed in the mesenteric lymph nodes of two-year-old

animals; there were only some IgA cells in the Peyer's patches. These results confirm our earlier observations.

For a further discussion of the changes in immunological activity of male CBA mice with age, we have concentrated on the two already mentioned groups of ten animals of 6 weeks and 2 years of age.

In Table IX-2, the absolute numbers with standard deviations of IgA, IgM, IgG_1, IgG_2 and IgG_3 C-Ig cells are presented together with the total number of C-Ig cells observed in the spleen, bone marrow, mesenteric lymph nodes and Peyer's patches. At the lower end of the Table, the total numbers of C-Ig cells of the different (sub)-classes, summed over the four studied organs are given. Due to the large numbers of C-Ig cells found in both the spleen and bone marrow, there is a fourfold increase in the total number of C-Ig cells summed over Ig classes and subclasses and the four

TABLE IX-2. ABSOLUTE NUMBERS OF C-Ig CELLS PER ORGAN ($\times 10^{-3}$) IN YOUNG AND OLD CBA MICE

Age	Organ	IgA	IgM	IgG_1	IgG_2	IgG_3	Total C-Ig cells
6 weeks	SPL	52.9 [1]	68.8	17.8	45.3	2.1	168.9
		37.9 [2]	47.0	10.3	44.1	1.7	110.2
	BM	36.2	17.7	7.2	8.0	0.2	69.3
		34.2	12.3	4.9	4.9	0.3	48.4
	ML	14.0	1.0	3.4	5.2	0.2	23.8
		17.6	1.4	5.7	8.1	0.3	31.5
	PP	24.3	9.9	4.8	11.2	1.2	51.4
		21.0	7.9	5.7	12.9	2.1	47.4
2 years	SPL	110.0	163.3	19.4	57.5	10.5	360.7
		49.2	69.6	13.0	55.8	11.9	154.7
	BM	558.4	134.0	34.8	66.3	8.5	802.0
		365.5	100.0	33.8	49.6	11.4	502.2
	ML	– [3]	–	–	–	–	–
	PP	9.8	1.6	0.8	1.8	0.2	14.2
		13.0	1.9	0.8	2.5	0.4	17.9
6 weeks	Total [4]	127.4	97.4	33.2	69.7	3.7	313.4
2 years		678.2	298.9	55.0	125.6	19.2	1176.9

[1]) Average number of C-Ig cells based on 10 individual male animals.
[2]) Standard deviation.
[3]) Number of C-Ig cells too small to make analysis feasible.
[4]) Total number of C-Ig cells summed over the four indicated organs.
Abbreviations: SPL = spleen; BM = bone marrow; ML = mesenteric lymph nodes; PP = Peyer's patches.

lymphoid organs from 6 weeks to 2 years of age. This is in direct contrast to our earlier observations (Haaijman, Schuit and Hijmans, 1977, see their Figure 4).

The distribution of the different classes and subclasses within the four lymphoid organs at 6 weeks and 2 years of age is shown in Table IX-3. Only cells containing one Ig class or subclass have been taken into account (see below). A remarkable finding was that the Ig class distribution of different individuals was much less liable to variation than were the absolute numbers. In the spleen of 6-week-old animals, almost half of the total number of C-Ig cells was of the IgM class. About equal proportions of IgA and IgG cells were found. Within the IgG class, the IgG_2 cells were predominant (see also below). The Ig (sub)class distribution pattern of the spleen C-Ig cells did not change with age. In the bone marrow, about 50% of all C-Ig cells were of the IgA class at 6 weeks of age. In the older group, this percentage was increased to almost 70%, with a concomitant reduction in the percentage of both IgM and IgG cells. Three out of four C-Ig cells in the mesenteric lymph nodes of the young animals were of the IgA class. In the 2-year-old group, too few C-Ig cells were observed

TABLE IX-3. Ig CLASS DISTRIBUTION WITHIN ORGANS AS A PERCENTAGE OF C-Ig CELLS IN YOUNG AND OLD CBA MICE

Age	Organ	IgA	IgM	IgG_1	IgG_2	IgG_3	Total IgG [1]
6 weeks	SPL	28.0 [2]	40.0	9.4	21.4	1.3	32.1
		9.6 [3]	14.0	3.6	14.2	1.2	
	BM	47.5	26.5	12.6	13.1	0.2	35.9
		9.8	12.6	6.2	5.1	0.3	
	ML	75.3	3.2	7.9	13.1	0.4	21.4
		19.7	4.0	8.6	10.1	0.5	
	PP	49.6	20.2	8.4	20.0	1.8	30.2
		7.3	6.8	2.8	6.7	1.1	
2 years	SPL	32.5	46.0	5.4	13.5	2.6	21.5
		14.1	12.2	3.0	9.2	2.8	
	BM	66.9	18.1	4.9	8.8	1.3	15.0
		8.7	6.9	4.1	3.5	1.2	
	ML	– [4]	–	–	–	–	–
	PP	68.4	12.0	7.5	10.7	1.3	19.5
		7.7	3.1	3.1	3.4	1.5	

[1]) Sum of IgG_1, IgG_2 and IgG_3 C-Ig cells.
[2]) Average value based on 10 individual male animals.
[3]) Standard deviation.
[4]) Number of C-Ig cells too small to make analysis feasible.
Abbreviations: SPL = spleen; BM = bone marrow; ML = mesenteric lymph nodes; PP = Peyer's patches.

TABLE IX-4. THE NUMBERS OF C-Ig CELLS ($\times 10^{-3}$) CONTAINING TWO Ig CLASSES OR SUBCLASSES IN 6-WEEK-OLD MALE CBA MICE

		Combination [1]						Combination [1]			
No.	Organ	A+M	A+G	G_1+G_2	G_2+G_3	No.	Organ	A+M	A+G	G_1+G_2	G_2+G_3
1	SPL	– [2]	1.4	1.0	0.8	6	SPL	–	0.5	12.6	–
	BM	0.8	1.6	0.3	–		BM	–	–	1.1	0.1
	ML	–	–	–	–		ML	–	–	–	–
	PP	0.3	–	–	–		PP	–	–	0.4	0.3
2	SPL	–	–	76.2	–	7	SPL	2.2	–	93.1	–
	BM	–	1.4	3.5	–		BM	–	1.3	0.9	0.3
	ML	–	0.9	12.6	–		ML	–	7.4	0.4	–
	PP	–	–	15.3	–		PP	0.2	0.3	1.3	0.2
3	SPL	2.1	0.7	6.2	–	8	SPL	–	–	1.0	–
	BM	0.7	2.7	1.2	–		BM	–	–	1.1	0.1
	ML	–	0.1	–	–		ML	–	–	1.9	–
	PP	0.9	3.1	–	0.2		PP	–	–	0.9	–
4	SPL	–	–	1.8	–	9	SPL	2.4	–	30.0	–
	BM	–	–	0.1	–		BM	7.2	–	17.2	–
	ML	–	–	–	–		ML	–	–	33.2	–
	PP	0.4	0.3	0.1	–		PP	–	–	2.4	–
5	SPL	–	–	0.1	–	10	SPL	–	–	23.7	–
	BM	–	–	1.0	–		BM	1.0	0.7	0.6	–
	ML	–	0.2	0.3	–		ML	–	–	–	–
	PP	0.6	–	0.8	–		PP	–	–	0.8	–

Numbers 1 through 10 refer to individual animals. The cytocentrifuge preparations of the indicated organs were incubated with four combinations of two fluorescent antisera as described in the text. The values represent the number of C-Ig-cells ($\times 10^{-3}$) staining with two conjugates.

[1]) A = IgA; M = IgM; G = IgG; G_1 = IgG_1; G_2 = IgG_2: G_3 = IgG_3.

[2]) No double staining cells were observed.

Abbreviations: SPL = spleen; BM = bone marrow; ML = mesenteric lymph nodes; PP = Peyer's patches.

to make the class distribution analysis feasible. One-half of the total number of C-Ig cells in the Peyer's patches were of the IgA class in the young group. In the older group, the percentage of IgA cells increased up to about 70%.

Four combinations of fluorescent conjugates were used in the Ig class distribution analysis of the C-Ig cells. In Tables IX-4 and IX-5, the number of cells containing two Ig classes or subclasses have been listed. The data for the young animals are in Table IX-4 and those of the 2-year-old animals in Table IX-5. Of course, these data are not complete, because not all possible combinations of antisera have been tested. In some preparations of the spleen of young animals (numbers 2, 7, 9 and 10 in Table IX-4), large numbers of primarily IgG_1+IgG_2 C-Ig cells were observed. With the exception of animal no. 9, no appreciable numbers of double Ig producing cells were found in the bone marrow. The specificity of our conjugates is proven by the absence of double staining cells in a large number of preparations. Hardly any cells were seen to

TABLE IX-5. THE NUMBERS OF C-Ig CELLS ($\times 10^{-3}$) CONTAINING TWO Ig CLASSES OR SUBCLASSES IN 2-YEAR-OLD MALE CBA MICE

		Combination						Combination			
No.	Organ	A+M	A+G	G_1+G_2	G_2+G_3	No.	Organ	A+M	A+G	G_1+G_2	G_2+G_3
1	SPL	–	2.8	–	–	6	SPL	–	–	0.9	–
	BM	–	–	–	5.6		BM	–	–	3.7	–
	ML	–	–	–	–		ML	–	–	–	–
	PP	–	–	–	–		PP	–	–	–	–
2	SPL	–	–	–	–	7	SPL	–	–	5.0	1.0
	BM	–	–	–	–		BM	–	3.7	1.1	–
	ML	–	–	–	–		ML	–	–	–	–
	PP	–	–	–	–		PP	–	–	–	–
3	SPL	–	–	–	–	8	SPL	–	–	–	–
	BM	–	–	–	0.7		BM	–	19.7	–	1.4
	ML	–	–	–	–		ML	–	–	–	–
	PP	–	0.1	–	–		PP	–	–	–	–
4	SPL	–	–	0.7	4.3	9	SPL	–	–	0.6	–
	BM	–	–	–	–		BM	0.8	0.8	0.5	–
	ML	–	–	–	–		ML	–	–	–	–
	PP	–	–	–	–		PP	–	–	–	–
5	SPL	–	–	–	–	10	SPL	–	–	–	–
	BM	–	3.1	2.6	–		BM	–	–	–	–
	ML	–	–	–	–		ML	–	–	–	–
	PP	–	–	–	–		PP	–	–	–	–

Numbers 1 through 10 indicate individual animals. Abbreviations as in Table IX-4.

contain both IgG_2 and IgG_3. This observation suggests a closer relationship between IgG_1 and IgG_2 in comparison to IgG_3. Far fewer instances of double producing cells were observed in older animals (Table IX-5). Only in the bone marrow of one animal (no. 8) was a significant number of cells containing both IgA and IgG found.

The close resemblance between the class distribution in bone marrow and Peyer's patches in the young as well as in the older animals is striking. An explanation for this phenomenon could be that lymphocytes receiving their antigenic stimulus in the Peyer's patches migrate to the bone marrow (see also Pierce and Gowans, 1975). In the bone marrow, the lymphocytes proliferate and mature to such an extent that the Ig class is determined under the influences prevailing at that moment in the bone marrow. Having been determined for their Ig class or subclass, these plasma cell precursors may then migrate back to the Peyer's patches or to the lamina propria of the gut. Circumstantial evidence for such a hypothesis was presented earlier (Haaijman, Schuit and Hijmans, 1977). Direct evidence may be obtained by using labelled cell techniques or by using parabiotic mice consisting of a young and an old member.

Although the majority or at least half of the C-Ig cells in mesenteric lymph nodes and Peyer's patches is of the IgA class, cells of other classes were also present in appreciable numbers. This observation confirms the data of Allen and Porter (1973)

TABLE IX-6. IgG SUBCLASSES AS A PERCENTAGE OF THE TOTAL IgG C-Ig CELLS IN YOUNG AND OLD MALE CBA MICE

Age	Organ	IgG_1	IgG_2	IgG_3
6 weeks	SPL	27.3	69.5	3.2
	BM	46.8	51.9	1.3
	ML	38.6	59.1	2.3
	PP	27.9	65.1	7.0
2 years	SPL	22.2	65.8	12.2
	BM	31.8	60.5	7.8
	ML	– *	–	–
	PP	28.6	64.3	7.1

Abbreviations: SPL = spleen; BM = bone marrow; ML = mesenteric lymph nodes; PP = Peyer's patches.
* Number of C-Ig cells too small to make analysis feasible.

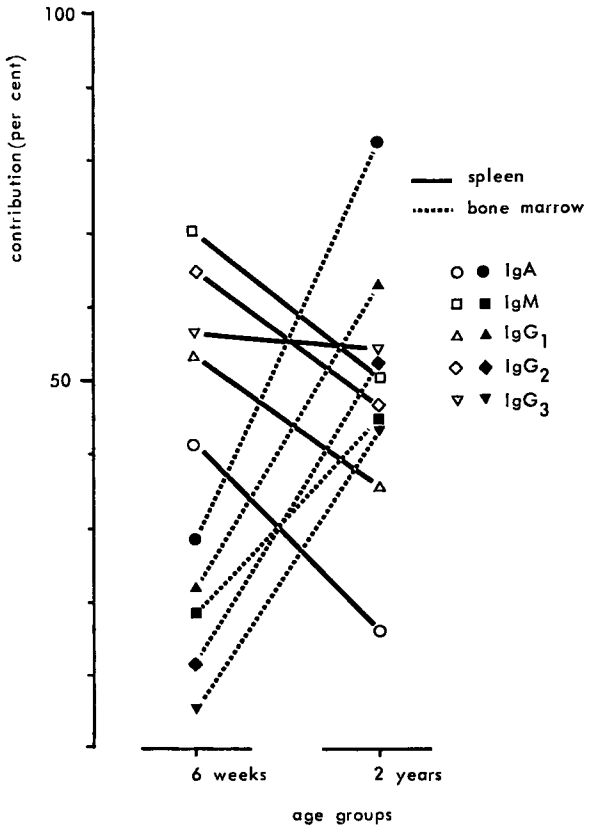
obtained in pigs, but does not agree with those of Crabbé, Nash, Bazin, Eyssen and Heremans (1970) and of Craig and Cebra (1971), who reported over 90% of IgA C-Ig cells in gut-associated lymphoid organs of mice. An explanation for this discrepancy might be that, in the reports mentioned, tissue sections rather than cytocentrifuge slides of cell suspensions were employed. In the tissue sections, the background staining caused by serum IgG trapped between the cells might very well obscure the presence of IgG C-Ig cells (see also chapter IV, part G1b).

The preponderance of IgA cells in the mouse (cf. Table IX-3), especially in the older animals, is in contrast with the results obtained in men by Hijmans, Schuit and Hulsing-Hesselink (1971) and Turesson (1976). The C-Ig cells in the bone marrow of adult, healthy, human individuals are approximately for 37% of the IgA, 12% of the IgM and 51% of the IgG class. Investigations on chimpanzees (Hijmans and Schuit,

TABLE IX-7. CONTRIBUTION IN PER CENT OF DIFFERENT LYMPHOID ORGANS TO THE TOTAL NUMBER OF C-Ig CELLS IN YOUNG AND OLD MALE CBA MICE

Age	Organ	IgA	IgM	IgG_1	IgG_2	IgG_3	Total
6 weeks	SPL	41.5	70.6	53.6	65.0	56.8	53.9
	BM	28.4	18.2	21.7	11.5	5.4	22.1
	ML	11.0	1.0	10.2	7.5	5.4	7.6
	PP	19.1	10.2	14.4	16.1	32.4	16.4
2 years	SPL	16.2	50.5	35.3	45.8	53.7	30.6
	BM	82.3	44.8	63.3	52.8	44.3	68.1
	ML	0	0	0	0	0	0
	PP	1.4	0.5	1.1	1.7	1.0	1.2

The absolute number of C-Ig cells found in SPL, BM, ML and PP were summed and the contribution of these four organs to the total number was calculated in per cent.
Abbreviations: SPL = spleen; BM = bone marrow; ML = mesenteric lymph nodes; PP = Peyer's patches.



FigureIX- 3. The changing role of spleen (open symbols, solid lines) and bone marrow (closed symbols, dashed lines) as major C-Ig cell organs as a function of age. Two groups of ten male CBA mice of, respectively, 6 weeks and 2 years of age were compared. The numbers of C-Ig cells were determined in spleen, bone marrow, mesenteric lymph nodes and Peyer's patches. The relative contribution as a percentage of the summed number per individual Ig class and subclass was calculated for spleen and bone marrow (see also Table IX-7).

unpublished observations) indicate that the differences in Ig class distributions between mice and men are not a reflection of the less favorable hygienic and sanitary circumstances encountered by conventional mice.

In order to better compare the IgG subclass distribution within different organs of the young and old animals, the numbers of IgG_1, IgG_2 and IgG_3 C-Ig cells have been calculated relative to the total number of IgG cells in Table IX-6. The majority of the IgG C-Ig cells were IgG_2 cells, which is in accordance with the predominance of IgG_{2a} plus IgG_{2b} in the serum of mice (Fahey, Wunderlich and Mishell, 1964a, b). The IgG_3 C-Ig cells constituted only a minor percentage of the total number of IgG cells. In relation to age, only a slight increase in the number of IgG_2 relative to the IgG_1 cells in the bone marrow was observed.

The relative contribution of the four lymphoid organs studied, to the total number of C-Ig cells is shown in Table IX-7 for the different Ig classes and subclasses. The data from Table IX-7 confirm those of Haaijman, Schuit and Hijmans (1977, see their figure 3) that the bone marrow in older animals takes over from the spleen the role of principal C-Ig cell organ. The data for spleen and bone marrow from Table IX-7 are plotted graphically in Fig. IX-3. With the exception of the IgG_3 C-Ig cells, there was a decrease in the contribution of the spleen with a concomitant increase in the contribution of the bone marrow when comparing the young and older animals. In the two-year-old animals, 80% of the IgA, 60% of the IgG_1 and around 50% of the IgM, IgG_2 and IgG_3 C-Ig cells were found in the bone marrow.

The finding that the IgG cells are equally distributed over spleen and bone marrow does not need to invalidate our earlier hypothesis regarding the changing roles of these two organs. It was postulated that the take over as the major C-Ig cell organ by the bone marrow from the spleen reflected adaptation of individual mice to their antigenic surroundings, in such a sense that in older age secondary type responses, which have an important bone marrow component, prevail. During the early phases of secondary type responses there is, however, also a substantial contribution from the spleen (Benner, 1975). Important is that the contribution of the bone marrow to the total number of C-Ig cells increases with age, for all separate Ig (sub)classes. Taking also into consideration the predominance of the IgA C-Ig cells in the bone marrow (see Table IX-3) and the substantial age-related increase in the contribution of the bone marrow to the total number of IgA C-Ig cells (Table IX-7 and Fig. IX-3), we have felt no need to change our hypothesis that the increase of the number of C-Ig cells with age in the bone marrow is a reflection of the preponderance of secondary type responses to environmental antigens in older animals.

What reasons may account for the virtual disappearance of C-Ig cells from the organs around the intestinal tract remain obscure. It might be that the presence of C-Ig cells in mesenteric lymph nodes and Peyer's patches of young animals should be regarded as the result of primary and local first phase secondary responses. In later stages of the response, and in general with advancing age, the bone marrow could be the source of circulating antibodies.

2. *Immunoglobulin levels in the serum of CBA mice as a function of age*

The changes with age in the serum levels of IgA, IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3, as measured with the Sepharose bead immunofluorescence assay, have already been described in chapter VIII, part H. Only details relevant to the subject of the present chapter will now be discussed. The difference in the numbers of C-Ig cells of the different classes and subclasses (Table IX-2) between 6-week- and 2-year-old animals is reflected roughly in differences in the respective average serum Ig levels (see Figs. VIII-11 and VIII-12).

The serum levels of the IgA, IgM and the four subclasses of IgG have also been

determined in the sera of the two groups of mice of the preceding section. Surprisingly, no correlation was observed per individual between the C-Ig cell numbers of a given Ig class or subclass and the serum level of that Ig. This finding is in direct conflict with the results obtained in humans by Vossen (1975) and Turesson (1976). The lack of correlation between serum levels and numbers of C-Ig cells in our study might, on the one hand, be caused by the large differences in the Ig content of individual C-Ig cells (see figure IV-14). However, it is not clear as yet whether these differences in Ig content also reflect differences in the synthetic rate of these cells. On the other hand, the discrepancy may be due to the fact that the authors mentioned, observed a much wider range in both the C-Ig cell counts and the serum levels in the groups of the human individuals they studied. This wider range, in the order of a 10- to 1000-fold difference between the lowest and the highest values, facilitates the establishment of a correlation between the two variables.

Fig. VIII-12 shows that, from the age of 6 months up to 2½ years of age, the levels of IgA, IgM, IgG_{2a} and IgG_3 remain almost constant. The levels of IgG_1 and IgG_{2b} tend to increase. These results strengthen the hypothesis that the activity of the B-immune system of old animals is as active as that of young adult animals, at least with respect to total Ig production.

C. IMMUNOLOGICAL CAPACITY: THE ANTIBODY RESPONSE OF CBA MICE TO HUMAN SERUM ALBUMIN AS A FUNCTION OF AGE

The immunological capacity to respond to experimentally given antigens generally declines with age according to the literature mentioned in the introduction of this chapter. In order to test whether this phenomenon also occurs in our stock of CBA mice, we have conducted a limited number of experiments using human serum albumin (HSA) as the antigen. The technique for measuring antibodies against HSA with the Sepharose bead immunofluorescence system has already been described in chapter VIII, part C. Diluted sera of immunized animals are incubated with Seph-HSA. Bound antibodies are quantitated by means of microfluorometry after a second incubation with a fluorescent goat antiserum directed against mouse immunoglobulins (GAM/Ig-TRITC). In the first experiment, 9 male animals of 3 months and 2½ years of age were injected intraperitoneally with 100 μg HSA in 0.2 ml containing 0.1 ml complete Freund's adjuvant. The serum of these animals was collected at day 11 after injection. None of the sera of the 2½-year-old animals showed reactivity specific for HSA in a dilution of 1:20, whereas substantial reactivity was found in the sera of all 3-month-old animals. The values obtained with the individual sera and Seph-HSA are indicated in the rectangles of Figs. IX-4 and IX-5. Two representative sera from both the 3-month- and 2½-year-old groups were incubated in various dilutions with Seph-HSA, Seph-BSA and Seph-OVA in order to exclude the possibility that no anti-HSA activity was observed with the sera of the older mice due to a dilution effect. The results obtained with the sera of the young animals are shown in Fig. IX-4

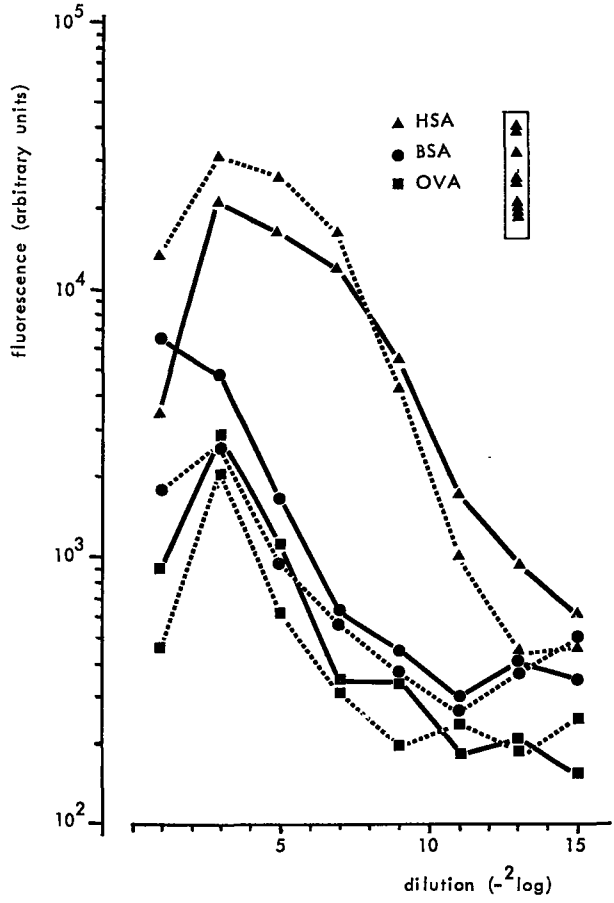


Figure IX-4. Antihuman serum albumin (HSA) antibodies in CBA mice. Nine male CBA mice of 3 months of age were immunized i.p. with 100 μg HSA in 0.2 ml containing 0.1 ml complete Freund's adjuvant. Animals were bled 11 days after the injection. Two typical sera (dashed, respectively, solid lines) were incubated in various dilutions with Seph-HSA, Seph-BSA and Seph-OVA. Bound antibodies were stained with GAM/Ig-TRITC diluted 1:50 and absorbed with Seph-BSA. Each point is the average fluorescence of at least 5 individual beads. The fluorescence values observed with the nine individual sera, diluted 1:20, and incubated only with Seph-HSA are shown in the rectangle.

and those of the old animals in Fig. IX-5. A considerable prozone effect was observed with all four sera. The sera of the old animals showed no clear positive anti-HSA reaction in any of the dilutions.

In the next experiment, female animals of 6 weeks, 3 months, 6 months, 1 year, 2 years and 3 years of age were injected intraperitoneally with 100 μg HSA in 0.2 ml containing 0.1 ml complete Freund's adjuvant. Seven out of the 10 animals of 3 years of age died one day after the injection. All but one of the animals of 6 weeks of age died within one week after the injection. No adverse effects were observed within the

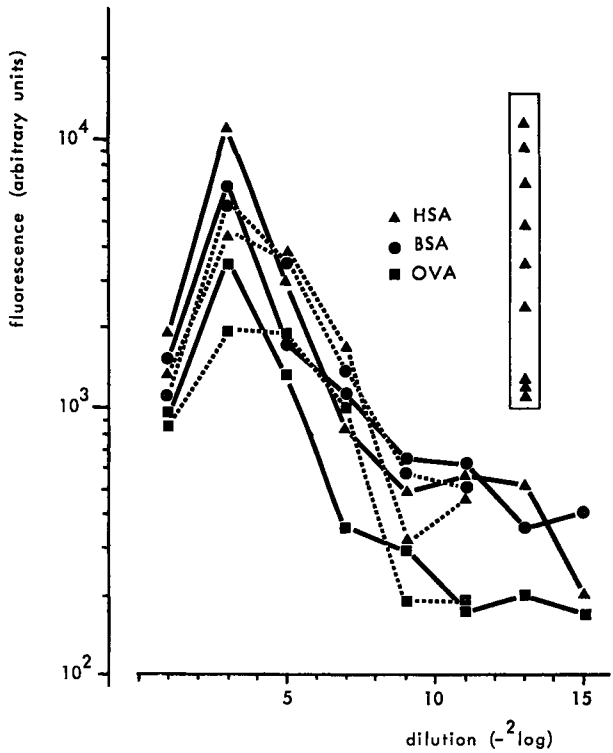


Figure IX-5. Antihuman serum albumin (HSA) antibodies in CBA mice. Nine 2½-year-old male CBA mice were immunized i.p. with HSA as in Fig. IX-4. Serum was collected 11 days after immunization. The reaction of Seph-HSA with 1:20 diluted individual sera and subsequent incubation with GAM/Ig-TRITC is shown in the rectangle. Two typical sera (dashed, respectively, solid lines) were incubated in different dilutions with Seph-HSA, Seph-BSA and Seph-OVA as described in the preceding figure. Each point is the average fluorescence of at least 5 individual beads.

other groups of animals. Surviving animals received a second i.p. injection of 100 µg HSA, in 0.1 ml containing 0.05 ml of complete Freund's adjuvant, 15 days after the first one. One 3-year-old animal died one day after this second injection. Plasma samples were collected from individual animals in haematocrit tubes just before the first injection and one day before and 7 days after the second injection. Individual sera were stored in the haematocrit tubes at −70 °C. Sera were diluted 200-fold in order to avoid the prozone effects (Fig. IX-4) and tested in the usual way for their reactivity towards Seph-HSA. Results are presented in Fig. IX-6. The reaction of the preimmune sera with Seph-HSA increased from 6 weeks to 6 months of age. After 6 months, they showed a slight decreasing trend. For the fluorescence values obtained with sera collected at day 14 after the first injection, a clear age-related decline was observed. Two-year-old mice generally showed no reactivity. Also clear was the increased variability as early as one year of age. The response after the booster dose of

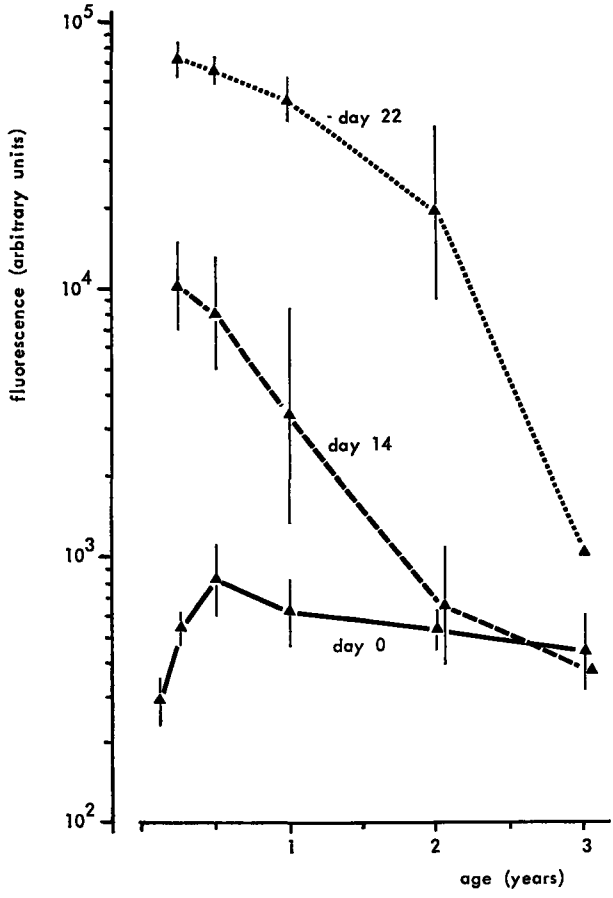


Figure IX-6. The influence of age on the antibody response of female CBA mice to human serum albumin (HSA). Ten animals each of the indicated ages received i.p. 100 μg HSA in 0.2 ml containing 0.1 ml complete Freund's adjuvant (CFA) at day zero and 100 μg HSA in 0.1 ml containing 0.05 ml CFA at day 15. Plasma samples were collected in haematocrit tubes at days 0, 14 and 22. One μl aliquots were diluted 200-fold and incubated with Seph-HSA. Bound antibodies were stained with GAM/Ig-TRITC. Each point is the geometric average of the fluorescences observed with the individual sera. The fluorescence with individual sera was the average of at least five individual beads. Bars indicate 95% confidence limits. Only two 3-year-old animals were available after completing the experiment (see text). For this age group no confidence limits were calculated, except for the day zero values.

HSA was less susceptible to alteration with age than was the primary response, thus confirming the data of Morton and Siegel (1969) for the NZB strain and those of Finger et al. (1972) for the NMRI strain. For two-year-old animals the increased variability of the booster response was evident. Unfortunately, only two 3-year-old animals survived the experiment. A very slight reactivity after the booster dose was observed in both animals.

D. DISCUSSION

The immunological apparatus is a complex system consisting of many interacting elements. Apparent age-related changes in the immune system can be appraised only against the background of this complexity (Hijmans and Hollander, 1977). This discussion will be focused on the age-related function of the B-immune system.

The B-cells are the antigen-sensitive cells which may differentiate upon antigenic stimulation into antibody synthesizing cells. The interrelationship with the T-cell system can be of a varied nature. On the one hand, T-cells are involved in the triggering of B-cells (T-helper cells), on the other hand, they may suppress the activity of B-cells (T-suppressor cells). The T-cell system exerts an important regulatory influence on the B-cells. In cell-mediated reactions, the T-cells show a number of other activities as well. It seems likely that the different T-cell functions are exercised by different subpopulations of cells. Recently, Jerne (1974) postulated a regulatory mechanism within the B-cell system itself which is based on the reaction of lymphocytes to the differentiation products of other lymphocytes.

A second level in the organization of the lymphoid system is represented by the lymphoid organs. After an antigenic stimulus, lymphocytes cease circulating and show preferential homing to secondary lymphoid organs. During the primary immune response, the spleen is the major site of antibody production. Benner and collaborators (Benner, Meima and van der Meulen, 1974; Benner and van Oudenaren, 1975) showed that, during the late primary response, B- and T-memory cells which migrate to the bone marrow are generated. During the early secondary response, the larger proportion of antibody producing cells is found in the spleen. In the later phases of this response, however, the bone marrow takes over the role of major antibody producing organ. The presence of memory cells is necessary for the antibody production in bone marrow. Haaijman, Schuit and Hijmans (1977) argued that a preponderance of the secondary type responses to frequently encountered environmental antigens is to be expected with advancing age. This mechanism would explain the increasing number of C-Ig cells in the bone marrow in contrast to the spleen.

What factors regulate the Ig producing cells in the bone marrow is not clear. Benner and van Oudenaren (unpublished observations) found that the bone marrow of nude mice is barely capable of antibody formation to the thymus-independent antigen LPS, in contrast to their heterozygous littermates. Furthermore, the number of C-Ig cells in the bone marrow of both germfree and conventionally raised nude mice appeared to be much lower than in the normal littermates. This deficiency of nude mice was much less prominent in the mesenteric lymph nodes and Peyer's patches. Both the antibody forming capacity of the bone marrow and the number of C-Ig cells, could be restored by transplantation of a 300 rad irradiated neonatal thymus under the kidney capsule of the nude mice. These experiments suggest that T-lymphocytes are directly involved in the antibody formation in the bone marrow.

The possibility that also the amount of circulating Ig's serves as a feed-back signal

seems to be borne out after comparing Figs. VIII-12 and IX-1. Whereas, for instance, the number of IgA C-Ig cells increases steeply in the bone marrow after one year of age, the IgA serum level is more or less constant.

An organ-specific regulating influence on the differentiation of B-lymphocytes into plasma cells containing different classes of Ig has to be concluded from the observation that, while the absolute number of C-Ig cells is subject to large fluctuations (see Tables IX-1 and IX-2), the Ig class distribution is much less variant (Table IX-3). We postulate that the above-mentioned influence is a maturation process, regarding the presence of cells synthesizing more than one class and subclass of Ig, especially in young animals (Tables IX-4 and IX-5).

The capacity of CBA mice to respond to HSA decreases with age. This finding confirms the data of many other authors (see the introduction of this chapter). From the above discussion it is likely that the primary defect in the response to HSA lies in the spleen. From the observation that primary and secondary response patterns are differently affected during ageing, it might be concluded that two subpopulations of T-cells are responsible for respectively T-helper function and the generation of memory cells. Corroborative evidence for this hypothesis is twofold: a) Emmerling, Beneke and Finger (1972) observed a normal secondary response to sheep red blood cells in NMRI mice in which the primary response was inhibited with cyclophosphamide; b) Benner and van Oudenaren (see above) showed that, in nude mice, the bone marrow does not participate after a booster dose in the antibody production against the T-independent antigen LPS, whereas a clear primary response was observed in the spleen. During ageing, the T-helper function seems primarily affected. This hypothesis is in accordance with the observations of Chused, Steinberg and Parker (1973), who reported a relative increase in the number of T-suppressor cells with age (see also Jaroslow, Suhrbier, Fry and Tyler, 1975). Recently, Hirokawa, Albright and Makinodan (1976) showed that the response of spleen cells of old BC3F1 and B6D2F1 hybrid mice to phytohaemagglutinin could be restored by a combined graft of young thymus and bone marrow. This observation might indicate that changes in the splenic microenvironment are not primarily responsible for the decline in T-B cell interaction. This conclusion was already reached by Price and Makinodan (1972b) employing a cell-transfer system.

A gradual age-related deterioration in the regulatory influence of the T-cell system on the B-cell system could be an explanation for the increase in bone marrow C-Ig cells (Fig. IX-1). This phenomenon proved to be of a very variable nature (Table IX-1). We have not been able as yet to correlate a declining immune response in individual animals with an increased number of C-Ig cells. It is indicative, however, that, on a transsectional basis, the increase in the number of mainly the IgA C-Ig cells is not accompanied by an increase in the serum level of IgA.

What factors should be held responsible for the age-related decline of T-cell function remains totally obscure. The involution of the thymus as such is not a sufficient explanation, although it might be the primary event (Greenberg and Yunis, 1972). What factors do cause the involution of the thymus then remains to be explained. Animals and humans are able to live 'normally' after neonatal thymectomy. It is the enormous flexibility of living organisms and the complexity of the immune system which will have to be taken into account in every theory on immunological ageing.

CHAPTER X

General discussion and conclusions

Contents:

A. INTRODUCTION

The still increasing popularity of the immunofluorescence (IF) technique is based on three assets which are offered, namely, sensitivity, specificity and the possibility of localization of compounds in microscopical preparations. Without knowledge of the influence of different variables involved, however, it may lead to erroneous or at least to suboptimal results. It is our belief that only the quantitative evaluation of the variables will eventually lead to a better use of the immunofluorescence technique in, e.g., qualitative diagnostic applications. Two major sources of variation in IF techniques can be distinguished: a) the equipment for visualization of the fluorescence; b) the immunological reagents which are used.

With regard to the equipment, we have described the influence of the most important components in chapter II of this thesis. This has been tested by using a microscopical fluorescence standard consisting of aminoethyl-Sephadex beads stained with either fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate. Our investigations have not led to a description of *the* fluorescence microscope. The varied areas of applications of immunofluorescence microscopy makes this almost impossible. Rather, the aim has been to draw the attention of the IF microscopist to the great increase in the image contrast and the fluorescence intensity which may result from a careful choice of available microscope components. As such, the results may serve as guidelines for new investments to be made in IF microscopes.

The recent introduction of comparatively simple and relatively inexpensive fluorescence microscopes featuring the latest advances in IF technology by the major microscope manufacturers may raise the standards of IF microscopy in general.

The second important variable in IF is the antiserum. Antisera are biological products and therefore not easily standardized. An advance in fluorescent conjugate evaluation has been the introduction of the Sepharose bead immunofluorescence assay (SBIA) by van Dalen, Knapp and Ploem (1973). This made it possible to express the different reactivities of an antiserum or conjugate in quantitative terms.

Quantitation in a model system has significance only if the results are compared with the behaviour of the tested reagents in the systems for which they are intended. Knapp et al. (1975) and Bloemmen et al. (1976) elaborated on this aspect of the quantitative evaluation of conjugate quality. A discussion of this theme is given in chapter IV. The primary aim of all endeavours in conjugate evaluation should eventually be the quality control of commercially available IF reagents. In this respect, the bead immunofluorescence assay is most promising.

The development of the SBIA for conjugate evaluation has led to the introduction of quantitative IF techniques in the determination of antigen and antibody concentrations in biological fluids. With regard to sensitivity, the SBIA is competitive with the radioimmunoassay. The ever increasing demand for sensitive assays in clinical

chemistry may make fluorescence an attractive alternative to radioactivity in the long run, because of the biohazards inherent to the latter.

B. SUMMARY OF THE CHAPTERS II THROUGH IX

Chapter II

Fluorescence intensities can only be measured relative to a certain standard. A new standard of microscopical dimensions which has several advantages over earlier reported standards is described. It consists of fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) bound to aminoethyl-Sephadex-G25 beads. FITC and TRITC are the most commonly used fluorochromes in IF microscopy. The standard beads have been used to measure the influence of different microscope components on fluorescence intensity. First, the influence of variables in the measuring process as such, including the photomultiplier voltage and the diameters of field (excitation) and measuring diaphragms is discussed. The fluorescence was found to be proportional to the eighth power of the photomultiplier voltage. The objective of the fluorescence microscope proved to be of prime importance when using epiillumination. The fluorescence varies with the fourth power of the numerical aperture of the objective. Although already anticipated theoretically by a number of authors, this relationship was confirmed experimentally here for the first time. From measurements on standard beads, it is concluded that the fluorescence intensity of flat objects per unit of area varies with the square root of the eyepiece magnification. A number of the most current light sources was tested for their ability to excite FITC and TRITC. The 100W DC mercury arc was found to give the highest fluorescence intensities. The differences between quantitative and qualitative IF microscopy are stressed for the choice of the optimal light source. In the selection of the optimal filter combinations for FITC and TRITC, a compromise has to be found between the highest fluorescence intensity and the desired contrast between the two dyes. The newly developed window filtering technique, employing combinations of high and low pass filters, proved to give the best results.

Chapter III

A standard for IF microscopy should react in the same way to changes in the physicochemical environment as do fluorochromes bound to cells or tissues. In order to evaluate the influence of a number of variables on fluorescein bound to aminoethyl-Sephadex, a new method is introduced to measure the amount of FITC bound to Sephadex or Sepharose beads using FITC-^{14}C. No influence of the length of the spacer between FITC and the Sephadex matrix nor of the presence of protein on the fluorescence was observed. A difference was found in the influence of pH on the fluorescence of FITC bound either to beads or to cells in the form of a conjugate. FITC and TRITC bound to aminoethyl-Sephadex beads fade less rapidly than fluorescent conjugates. FITC fades more rapidly than TRITC. It was established that the degree

of fading is not proportional to the number of irradiated molecules. Fading seemed not to be caused by electron capture by the proteins to which the fluorochromes are coupled. The hypothesis is forwarded that the Sephadex matrix of the standard beads protects the fluorophores from photodecomposition in a yet unknown way.

Chapter IV

A number of possibilities for the standardization of IF reagents is discussed in this chapter. Emphasis is placed on the quantitative evaluation of conjugate quality with the Sepharose bead immunofluorescence assay (SBIA). In this assay, antigens are coupled covalently to cyanogen-bromide-activated Sepharose beads. These beads are then used as the substrate in IF procedures. The fluorescence per individual bead is measured microfluorometrically.

Experimental conditions which influence the eventual testing of conjugates in the SBIA, namely, the pH during activation of the Sepharose and the washing and incubation procedures, are discussed. Fractionation of antisera, first into a globulin fraction then into IgG and finally into purified antibodies, affects the nonspecific binding characteristics of the antisera, and consequently their apparent specificity in the SBIA considerably. The fluorochrome loading (F/P ratio) of a conjugate influences its nonspecific staining properties. It is concluded that the influence of the F/P ratio in the SBIA is not comparable to that in qualitative IF. Whereas, in the SBIA an optimum contrast was observed between specific and nonspecific staining with a F/P ratio as high as 9, the optimum F/P ratio of conjugates applied to cells is about 1. In indirect techniques, in which the beads are first incubated with unlabelled antisera and subsequently with fluorescent anti-immunoglobulin conjugates, the nonspecific reactivities of the fluorescent conjugates must be investigated before the antisera are tested. In the actual specificity test, antisera or conjugates are incubated in various dilutions with different kinds of beads: a) the homologous beads coupled with the antigen to which the antiserum was raised; b) a collection of heterologous beads to test the different possible immunological reactivities of the antiserum; and c) a control for nonimmunological nonspecific staining. After the appropriate incubation(s), the fluorescence of the individual beads is measured. The relationship between the different observed reactions proved highly dependent on the concentration of antiserum or conjugate. The concept specificity interval (SI) is introduced to describe the specificity of an antiserum, independent of its dilution. The SI is the difference in the dilutions of an antiserum, yielding the same fluorescence with both homologous and heterologous beads. Finally, the application of the quantitative data obtained with the SBIA to quality control of conjugates intended for use in biological systems is discussed. It was concluded that the SBIA is an excellent tool for the inventory of the different antiserum or conjugate specificities, but that quantitative measures for acceptability in biological applications cannot yet be given. The main reason for the latter conclusion is insufficient knowledge about the mechanisms which cause nonimmunological nonspecific staining of both Sepharose beads and biological substrates.

Chapter V

A number of single and multi-layer techniques currently in use in IF microscopy is compared. It was established that the anti-Ig technique, also called the indirect technique, is about four times as sensitive as the direct technique. It is questioned, however, whether this difference in sensitivity will always be exploited in practice, because, in the indirect technique, the antisera are used mostly unfractionated. The antigen-antibody-fluorescent-antigen and the antibody-antigen-fluorescent-antibody sandwiches in the SBIA are discussed, as a preamble to a discussion of the application of fluorescence immunoassays in chapter VIII. A system for studying the kinetics of soluble and Sepharose bound antigens, employing the first-mentioned sandwich is described. The antibody-antigen-fluorescent-antibody sandwich is used for the determination of antigen concentrations. A number of procedures for testing the interference between binding of antigen molecules to the Fab part of an immunoglobulin and the accessibility of the Fc part of the same Ig molecule for anti-Fc reagents is given. The interference proved to be minimal. These experiments provided proof of the validity of double staining techniques.

Chapter VI

For the application of the SBIA in routine determinations, a rapid and reliable microfluorometer is indispensable. The construction of an automated version of the Leitz Diavert inverted microscope is described in this chapter. Air driven cylinders were used for the construction of shutters in the diaillumination pathway, the pathway to the photomultiplier and the pathway of the excitation light. Two types of control over the sequence of shutter movements are described: a) total pneumatic control based on the cascade principle; b) desktop-calculator control over electromagnetic valves. Attention is also paid to the on-line processing of data by a calculator and the practical aspects of microfluorometry.

Chapter VII

A detailed experimental protocol for the SBIA is presented in this chapter. The use of flat-bottom microtitration plates for the incubation of Sepharose beads is described. These plates have considerably increased the sample processing efficiency, first, because no centrifugation is needed for washing of the beads and, second, because the fluorescence of the individual beads can be measured through the bottom of these plates by use of the inverted microscope. The preparation of slides for each individual sample is thus circumvented.

Chapter VIII

A variety of applications of the SBIA other than the testing of antiserum specificity is the subject of this chapter. The determination of the level of serum antibodies,

either experimentally induced or naturally occurring, is reported. With respect to the former, the course of the immune response to the T-independent antigen *E.coli* lipopolysaccharide (LPS) and the T-dependent antigen human serum albumin (HSA) was followed in individual animals. For LPS, it was shown that, within the low responding C57BL strain, great individual differences exist in the degree of antibody formation. With HSA as the antigen, it was demonstrated that the variation among the responses of individual animals was small in young animals and excessive in old animals. The number of animals was too small, however, to study the influence of age on the immune response in detail. Naturally occurring antibodies against Rauscher leukaemia and simian sarcoma viral antigens were detected in sera of germfree WAG/Rij rats and, to a much lesser degree, and only against Rauscher leukaemia viral antigens, in sera of conventionally raised BROFO rats. No naturally occurring antibodies against mammary tumour viral antigens could be found in the sera of a variety of mouse strains. The possible reasons for this negative finding are discussed.

The use of the SBIA for the determination of the serum levels of IgA, IgM and the four subclasses of IgG in the mouse, based on the antibody-antigen-fluorescent-antibody sandwich is reported. Technical details and the application of the method to sera of CBA mice throughout their life span are presented. Based on the Ig levels, the life span of these animals was divided into three main periods: a) the first period up to about 6 months of age, in which the respective Ig levels reach their adult level; b) the second period stretching from 6 months to about 24 months of age, during which no major changes in the Ig levels were observed, with the exception of the levels of IgG_1 and IgG_{2b}, showing a clear age-related increase; and c) the third period, from 24 months onwards, which is the period that is characterized by a considerable increase in the variation among Ig levels of individual animals and also the period during which the survival curve starts to decline.

The same kind of SBIA as used for the Ig level determination has been applied to the determination of mouse mammary tumour viral (MTV) antigens in milk samples of different strains and to organ extracts of male mice of both low and high mammary cancer strains. MTV antigens were readily detected in milk of GR and BALB/cfC3H mice. Suggestive evidence that milk of multiparous C57BL female mice contains low concentrations of MTV proteins, in spite of the fact that this strain shows a negligible incidence of mammary tumours in old age, was obtained. Finally, an example of the application of the SBIA to the monitoring of cell cultures for the production of viral proteins is given. It was possible with the SBIA to detect the release of C-type viral antigens and furthermore to follow the purification of these viruses with hydroxylapatite chromatography.

In the discussion of this chapter, an appraisal of different labels currently in use in immunoassays is made. The conclusion was reached that fluorescence might be a valuable alternative to radioactivity in the future. The development of immunoassays employing enzyme-antibody complexes and fluorogenic substrates for large scale epidemiological applications in various areas of health research was anticipated.

Chapter IX

The *capacity* of animals to respond immunologically with detectable antibody formation to experimentally administered antigens decreases with age. On the other hand, it was shown that neither the number of immunoglobulin containing cells (C-Ig cells) nor the levels of serum Ig's decrease with age. These two parameters are used to estimate the immunological *activity* at a given age. With immunological activity is meant the ongoing immunological reaction against antigens which are not experimentally introduced. Numbers of C-Ig cells and serum Ig's are determined irrespective of the antigens to which the Ig's are directed.

In this chapter, both the immunological activity and capacity are studied as a function of age in inbred mice of the CBA strain. The CBA strain was chosen because it is a long-lived strain which is not prone to age-related pathology of the immune system as such. The immunological activity was characterized by both the number of C-Ig cells in spleen, bone marrow, mesenteric lymph nodes and Peyer's patches and the levels of circulating serum Ig's, in not-intentionally stimulated animals. With respect to the C-Ig cells, it was shown that the absolute number in individual animals is subject to great variation. In young animals, the majority of the C-Ig cells is found in the spleen, whereas the bone marrow contains the larger numbers in old animals. This pattern was found for all Ig classes and for the IgG_1, IgG_2 and IgG_3 subclasses, but was most marked for the IgA class. Instead of a decrease with age, the total number of C-Ig cells, notably of the IgA class, showed a clear increase after one year of age. A decrease in the number of C-Ig cells was observed for the mesenteric lymph nodes and Peyer's patches.

The immunological capacity of animals was assessed as a function of age by injecting human serum albumin (HSA) emulsified in complete Freund's adjuvant and measuring the level of anti-HSA antibodies with the SBIA. The antigen was injected twice, on day 0 and day 15. The reactivity of serum samples with Sepharose-coupled HSA was measured at day 0, day 14 and day 22. A substantial influence of age was observed on the reactivity in the sera taken 14 days after the first injection. The response was already decreased at one year of age as compared to 3- and 6-month-old animals and was absent in two-year-old animals. After the second injection, a clear reaction was seen in animals up to two years of age. In three-year-old animals, only a slight reaction to this booster injection was observed. The variation in response among individual animals was markedly increased in one-year-old animals for the primary response and only in the two-year-old animals for the booster response. An explanation for the increasing number of C-Ig cells in the bone marrow, the relative constancy of the serum Ig levels and the decrease in the primary and secondary response capacity is sought in a deterioration of the regulatory influence of the T-cell system over the B-cell system, with ageing.

C. PROSPECTS

Since the introduction of the IF technique by A. H. Coons and colleagues in 1941 (Coons, Creech and Jones, 1941), tremendous advances have been made in both the equipment and the production of high quality reagents. With respect to equipment, the method of vertical illumination according to Ploem (1967) was a major improvement. This system made it possible to utilize the full numerical aperture of objectives to full advantage. Moreover, it has made microfluorometry a feasible endeavour. The numerical aperture of objectives, especially developed for IF microscopy has been raised to almost the physical limit (Ploem, 1975). Eyepieces with low magnification and a maximum of fluorescence collecting power have been developed. It would be of benefit if these eyepieces were constructed not only for large research microscopes but in future also for the smaller microscopes intended for routine use. The introduction of comparatively small and inexpensive fluorescence microscopes has met the need of an evergrowing interest in IF microscopy for application in research and for diagnostic purposes. In some of these small microscopes, extra attention has been paid to the design of the collector lens, which has improved the fluorescence yield considerably. Unfortunately, the design of some of the smaller microscopes does not permit the use of 100W mercury arcs, which, up to now, may be considered as optimal. The design of collectors which are optimally suited for IF for the larger research microscopes is also badly needed. At present, these large microscopes cannot compete with their small successors for fluorescence yield. It would be of interest to investigate the possibility of introducing zoom optics into the collecting system. Zoom optics would have the advantage that the dimensions of the arc image can be varied without changing the distance between image and collecting lens. With such a system optimal filling of the back entrance pupil of every objective could be accomplished.

From the point of view of practical IF microscopy, the mechanics of the fluorescence microscope with its accessories has not yet reached the degree of sophistication found in other areas of clinical instrumentation.

The introduction of the multilayered interference filters of the KP type covering almost the whole visual spectrum has clarified much of the mystification associated with the older sets of filters. It can only be hoped that the designation of these filters by different manufacturers will be unified in the near future.

The use of more than one fluorochrome in one preparation after the introduction of rhodamine by Hiramoto, Engel and Pressman (1958) has been a major step forward in IF microscopy. Although some candidates have already been described as the third fluorochrome (Sinsheimer, Stewart and Burckhalter, 1968; Rothbarth, Olthof and Mul, 1975), they have not yet found wide application. Eventually, however, IF microscopy will definitely require a third or even a fourth fluorochrome. It is questionable, however, whether conditions can be worked out under which the human observer is able to distinguish unambiguously more than three colours.

In microfluorometry, the introduction of reasonably priced tunable lasers is eagerly

awaited. Mansberg and Kusnetz (1966) have already anticipated this development. These authors estimated that a 10^3 increase in fluorescence intensity could be achieved with lasers in comparison to mercury sources. Experiments of Kaufman, Nester and Wasserman (1971), Kaufman and Nester (1972), Vozelj, Rajver and Vrenko (1972), Bergquist (1973), Bergquist and Nilsson (1975), Schauenstein et al. (1975), Wick et al. (1975), and Rundquist and Enerbäck (1976) have shown the extreme fluorescence intensity which may indeed be achieved with these laser light sources. It may be questioned, however, whether this increase in excitation intensity will not increase the background readings proportionally, although a narrow energy band for excitation may give intriguing results. Lasers will probably be eventually employed in microscopes provided with scanning stages. Application of a scanning microscope together with a pattern recognition system for automatic measuring of Sepharose bead immunofluorescence would make the SBIA a serious competitor for the RIA.

It is more realistic, although, to anticipate the introduction of comparatively simple and inexpensive microfluorometers. The calculator-controlled microscope described in chapter VI might provide an attractive and reliable solution.

Because the use of radioactivity cannot increase forever due to serious environmental hazards, we expect an ever increasing interest in the technique of fluorescence immunoassays.

Summary

With the technique of immunofluorescence (IF) microscopy, localization of antigens in microscopical preparations of cells or tissues using antisera labelled with fluorescent dyes is pursued. The technique combines the specificity of the antigen-antibody bond with the sensitivity of fluorescence detection.

This thesis consists of two parts. In the first part (chapters II through IV), the influence of a number of variables inherent to the IF technique, i.e. the fluorescence microscope and the reagents, is discussed. Because visual evaluation of the different variables was not satisfactory, use has been made of microfluorometry. The intensity of fluorescence can only be measured relative to a certain standard. Several standards had already been described for this purpose, but they all suffered from a number of disadvantages to a greater or lesser extent. In this thesis, a new standard for IF microfluorometry which has a number of advantages over those earlier reported is described. It consists of aminoethyl-Sephadex-G25 beads labelled with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). The beads are of microscopical dimensions and can be stored dry for extended periods of time. The new standard has been used to quantitate the influence on the intensity of fluorescence emission, of several components of fluorescence microscopes, such as objectives, eyepieces, light sources and filter combinations for FITC and TRITC.

Next to the fluorescence microscope, the second major source of variation in IF microscopy is the fluorescent reagent. The Sepharose bead immunofluorescence assay (SBIA), introduced by van Dalen, Knapp and Ploem (1973), was used to study the possibility of expressing the quality of antisera and conjugates in quantitative terms. In the SBIA, IF reactions are carried out with antigens or antibodies covalently linked to Sepharose beads. The eventual reaction product on individual beads is quantitated microfluorometrically. The results obtained with this model system are evaluated with respect to their predictive value for the behaviour of antisera with biological substrates. It was concluded that, although the SBIA proved to be an excellent tool for the inventory of antiserum specificities, no absolute criteria could be formulated, as yet, for the acceptability of antisera to be employed in biological studies. This fact was attributed to insufficient knowledge of the nature of the nonspecific staining properties of antisera in both model and biological systems.

The second part of this thesis describes how the SBIA has been applied to various fields of study. In order to increase the number of samples that could be handled efficiently, the original method of van Dalen, Knapp and Ploem (1973) had to be modified. These modifications and the construction of a reliable automated microfluorometer, interfaced to a desktop-calculator, are described.

With the SBIA, the levels of IgA, IgM and the four subclasses of IgG in the serum of CBA mice were measured throughout their life span. The methods for day-to-day

standardization are emphasized. The life span of these animals may be divided into three main periods according to the immunoglobulin levels: 1) from birth up to about 6 months of age, in which the different immunoglobulins attain their respective adult levels; 2) from 6 months up to 2 years of age, during which the levels of the immunoglobulins do not show substantial changes, except for the levels of the IgG_1 and IgG_{2b} subclasses, which increase; and 3) from 2 years of age till death, characterized by a pronounced increase in the variation in immunoglobulin levels among individual animals of the same age.

Mammary tumour virus (MTV) is endogenous in mice and thus transmitted vertically. The distribution of MTV antigens over different organs of male mice of a variety of strains and ages has been determined with the SBIA. Significant amounts of MTV antigens were detected in organs of the urogenital tract of male mice of both high and low mammary cancer strains. No consistent age-related pattern for the expression of MTV antigens was observed.

The capacity of the immune system to produce serum antibodies against injected antigens declines with age. We have confirmed this already reported phenomenon for the reaction of CBA mice to human serum albumin (HSA). It was observed that the booster response towards HSA is affected later in life than is the primary response. The immunological activity in the absence of experimental antigenic stimulation, as defined by: a) the number of cells containing cytoplasmic immunoglobulins (C-Ig cells) and, b) the levels of serum immunoglobulins irrespective of the antigens to which these immunoglobulins are directed, was found not to decrease with age but rather to increase. An explanation for the differential influence of age on immunological capacity versus activity was sought in a deteriorating T-cell regulation of the B-immune system in senescence.

Samenvatting

In immunofluorescentie (IF) mikroskopie wordt getracht antigenen te lokaliseren in mikroskopische preparaten van cellen of weefsels met behulp van antisera gemerkt met fluorescerende kleurstoffen. De techniek maakt gebruik van zowel de specificiteit van de antigeen-antilichaam interaktie als van de gevoeligheid van fluorescentiedetektie.

Dit proefschrift bestaat uit twee delen. In het eerste deel (hoofdstukken II t/m IV) wordt de invloed van een aantal variabelen behorende bij de IF techniek beschreven, namelijk de fluorescentiemikroskoop en de reagentia. Omdat de visuele beoordeling van de verschillende variabelen niet bevredigend was, is gebruik gemaakt van mikrofluorometrie. De intensiteit van fluorescentielicht kan alleen relatief ten opzichte van een bepaalde standaard bepaald worden. Een aantal van zulke standaards voor IF zijn al beschreven. Deze standaards vertonen echter allen in meer of mindere mate een aantal nadelen. In dit proefschrift wordt een nieuwe fluorescentiestandaard beschreven die duidelijk minder beperkingen heeft dan de eerder beschreven standaards. De nieuwe standaard bestaat uit aminoethyl-Sephadex bolletjes die gemerkt zijn met fluoresceïne isothiocyanaat (FITC) of tetramethyl rhodamine isothiocyanaat (TRITC). Deze stoffen zijn de meest gebruikte fluorescerende kleurstoffen in IF mikroskopie. De gekleurde Sephadex bolletjes hebben mikroskopische afmetingen en kunnen droog, zeer lang bewaard worden. De nieuwe standaard werd gebruikt om de invloed na te gaan van verschillende onderdelen van de fluorescentiemikroskoop op de intensiteit van de fluorescentie emissie. Onderzocht werden objektieven, oculairen, lichtbronnen en filterkombinaties voor FITC en TRITC.

De tweede belangrijke bron van variaties in IF mikroskopie is het fluorescerend reagens. De Sepharose bol immunofluorescentie techniek (SBIT), die geïntroduceerd werd door van Dalen, Knapp en Ploem (1973), werd gebruikt om na te gaan of het mogelijk is de kwaliteit van antisera en conjugaten uit te drukken in kwantitatieve eenheden. Sepharose bollen waaraan covalent antigenen of antilichamen zijn gebonden worden in de SBIT gebruikt voor het uitvoeren van IF reakties. Het uiteindelijke reaktieprodukt wordt bepaald met behulp van mikrofluorometrie aan individuele bollen. Getoetst is of de resultaten verkregen met de SBIT van nut kunnen zijn voor het voorspellen van het gedrag van antisera met biologische substraten. Geconcludeerd werd, dat de SBIT uitnemend geschikt is voor het bepalen van de verschillende specificiteiten die een antiserum kan bezitten, maar dat nog geen absolute maat kan worden gegeven voor de kwaliteit die een antiserum moet hebben voor het toepassen op biologisch materiaal. Dit resultaat werd toegeschreven aan het nog onvoldoende inzicht in de aard van de niet-specifieke reakties die antisera kunnen vertonen in zowel het modelsysteem als in biologische systemen.

In het tweede deel van dit proefschrift worden toepassingen van de SBIT in ver-

schillende onderzoekgebieden beschreven. De oorspronkelijke techniek van van Dalen, Knapp en Ploem (1973) moest gemodificeerd worden om de behandeling van een groter aantal monsters mogelijk te maken. Deze modifikaties en de konstruktie van een betrouwbare mikrofluorometer verbonden met een tafelrekenmachine worden beschreven.

Met de SBIT werden de niveaus van IgA, IgM en de vier subklassen van IgG gemeten in het serum van CBA muizen van verschillende leeftijden. Nadruk werd gelegd op methodes voor de standaardisatie van de dag-tot-dag variaties. Het leven van de CBA muis kan met het oog op de niveaus van de verschillende immunoglobulinen verdeeld worden in 3 perioden: 1) van de geboorte tot ongeveer een half jaar, waarin de verschillende immunoglobulinen hun volwassen niveau bereiken; 2) van een half jaar tot ongeveer twee jaar, waarin de niveaus van de verschillende immunoglobulinen geen opzienbarende veranderingen vertonen met uitzondering van IgG_1 en IgG_{2b}, die stijgen; 3) vanaf twee jaar, een periode die gekenmerkt wordt door een opvallend toenemen van de variatie in de immunoglobuline-niveaus tussen verschillende dieren van dezelfde leeftijd.

Borstkankervirus komt in muizen endogeen voor en wordt dus genetisch overgedragen. De SBIT is gebruikt om het voorkomen te bestuderen van borstkankervirus antigenen in verschillende organen van mannelijke dieren van diverse ingeteelde muizenstammen op diverse leeftijden. Aanzienlijke hoeveelheden van deze antigenen konden worden aangetoond in organen van de tractus urogenitalis. Dit gold zowel voor muizenstammen met een hoge als met een lage borstkankerincidentie. Het voorkomen van borstkankervirus antigenen was niet op een duidelijke manier leeftijdsafhankelijk.

De reaktie van het immuunsysteem op geïnjekteerde antigenen met de aanmaak van antilichamen (immunologische kapaciteit) neemt af met de leeftijd. Dit reeds eerder beschreven verschijnsel hebben we bevestigd voor de reaktie van CBA muizen op ingespoten menselijk serum albumine. De reaktie op één injektie bleek sneller af te nemen met de leeftijd dan de reaktie na twee injekties gegeven met een interval van 14 dagen. De immunologische aktiviteit aanwezig zonder opzettelijke stimulatie, werd bepaald aan de hand van zowel het aantal cellen dat immunoglobulinen in het cytoplasma bevat, als aan de hand van de serumniveaus van immunoglobulinen, ongeacht tegen welke antigenen deze immunoglobulinen waren gericht. De op deze wijze bepaalde immunologische aktiviteit bleek in CBA muizen eerder te stijgen dan te dalen met de leeftijd. Een verklaring van de verschillende invloed die de leeftijd heeft op de immunologische aktiviteit en kapaciteit werd gezocht in het verslechteren met de leeftijd van de regulatoire invloed van T-cellen op het B-immuunsysteem.

Abbreviations

Generally accepted abbreviations in immunology, such as IgG, Fc and Fab are not included in this list. For abbreviations used in the designation of optical filters, see chapter I, part C2.

Antisera and conjugates have been abbreviated as in the following example:

RAM/IgM-TRITC

in which
- R = rabbit, the animal in which the antiserum was raised
- A = anti
- M/IgM = the antigen to which the antiserum has been raised. The character preceding the dash stands either for the animal from which the antigen was isolated (in the example M = mouse) or for the entity from which the compound after the dash is a part (as in RLV/p30 which denotes the protein with a molecular weight of 30,000 isolated from Rauscher leukaemia virus)
- -TRITC = fluorochrome with which the antiserum has been labelled (in this case tetramethyl rhodamine isothiocyanate)

The abbreviation RAM/IgM-TRITC thus stands for a tetramethyl rhodamine isothiocyanate labelled rabbit antiserum directed against mouse IgM. The different animals used for immunization and the antigens are included in the list below.

ab	antibody
AC	alternating current
ag	antigen
ANF	antinuclear factor
avg	average
4B-	Sepharose-4B beads
BM	bone marrow
Br	brain
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
CFU-S	colony forming unit - spleen
C-Ig cells	cells containing cytoplasmic immunoglobulins
CNBr	cyanogen bromide
coll	collagen
cpm	counts per minute
CV%	coefficient of variation in per cent
DC	direct current
DPX	factory code for an embedding material
DSS	desired specific staining

E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
F. hepatica	*Fasciola hepatica*
FITC	fluorescein isothiocyanate
fluor	fluorescence
fluor/pl	fluorescence per picolitre
F/P ratio	number of fluorochrome molecules per molecule of protein
F/T	ratio between fluorescence of FITC and TRITC
G	goat
G25-	aminoethyl-Sephadex-G25 superfine beads
GaLV	gibbon ape leukaemia virus
gp	glycoprotein
H	horse
HSA	human serum albumin
Hu	human
IF	immunofluorescence
Ig	immunoglobulin
i.p.	intraperitoneally
i.v.	intravenously
kV	kilovolt
LPS	*E. coli* lipopolysaccharide
M	mouse
MD	measuring diaphragm
MDPF	2-methoxy-2,4-diphenyl-3(2H)-furanone
MG	mammary gland extract
ML	mesenteric lymph nodes
MOPC	mineral oil induced plasmocytoma
MTV	mouse mammary tumour virus
NA	numerical aperture
NMS	normal mouse serum
NP40	nonionic detergent P40
NRaS	normal rat serum
NRS	normal rabbit serum
NSS	nonspecific staining
O	oil immersion; in the designation of objectives
OVA	ovalbumin
p	protein
PBS	phosphate buffered saline
PFC	plaque-forming cell
PP	Peyer's patches
PPB	potassium phosphate buffer
QE	quantum efficiency

R	rabbit
Ra	rat
RaE	rat embryo fibroblasts
RB200	lissamine-rhodamine
RIA	radioimmunoassay
RIP	radioimmunoprecipitation
RLV	Rauscher leukaemia virus
S. aureus	*Staphylococcus aureus*
SBIA	Sepharose bead immunofluorescence assay
SBIT	Sepharose bol immunofluorescentie techniek (Dutch)
sd	standard deviation
se	standard error
Seph-	Sepharose-4B beads
Sh	sheep
SiSV	simian sarcoma virus
S. mansoni	*Schistosoma mansoni*
SPL	spleen
SRBC	sheep red blood cells
SRID	single radial immunodiffusion technique
Sw	swine
TCF	tissue culture fluid (supernatant)
T/F	ratio between TRITC and FITC fluorescence
TRITC	tetramethyl rhodamine isothiocyanate
USS	undesired specific staining
W	water immersion; in the designation of objectives
XSS	cross-reactive specific staining

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Curriculum vitae

In 1964 eindexamen HBS-B aan het Lorentz Lyceum te Eindhoven. Van 1964 tot 1971 studie in de biologie aan de Rijksuniversiteit te Utrecht. Doctoraal examen met hoofdvak Algemene Zoölogie bij Prof. Dr. Ch. P. Raven (onderzoek naar het thymidine metabolisme tijdens de vroege klievingen van *Lymnaea stagnalis* L. eieren, onder leiding van Dr. J. A. M. van den Biggelaar).
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Sinds 1972 werkzaam als wetenschappelijk medewerker van het Instituut voor Experimentele Gerontologie GO-TNO waar het onderzoek werd gedaan dat ten grondslag ligt aan dit proefschrift.