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A comparison of fluorescein isothiocyanate and lissamine rhodamine (RB 200) as labels for antibody in the fluorescent antibody technique

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Summary. The relative merits of fluorescein isothiocyanate (FITC) and lissamine rhodamine (RB 200) as labels for antibody in fluorescence microscopy were studied and compared by microphotometry, testing each fluorochrome under its own optimal conditions as far as possible, and at a similar range of dye: protein ratios. The antibody was sheep anti-human globulin, and the tissues stained with it were rat liver sections bearing human anti-nuclear factor on the nuclei. The findings were as follows:

(i) the amount of RB 200 conjugating with protein was strictly proportional to the amount of the sulphonyl chloride derivative added to the reaction mixture; with increasing amounts of FITC in the reaction mixture, however, there was a less than proportional increase in the degree of conjugation.

(ii) Diethylaminoethyl (DEAE)-cellulose chromatography decreased the dye: protein ratio of the conjugates by 40% uniformly for both RB 200 and FITC, regardless of the initial dye: protein ratio.

(iii) When corrections were made for spectral responses of photo-detectors, effects of optimizing the mountants, and benefits to rhodamine of changing from a Xenon to a mercury lamp, it was concluded that RB 200 conjugates could give brighter staining than FITC conjugates at similar dye: protein ratios.

(iv) DEAE-cellulose chromatography greatly improved the contrast of the staining, especially with RB 200 conjugates.

(v) After chromatography, RB 200 consistently gave better contrast than FITC.

(vi) The fluorescence of rhodamine-stained sections did not fade demonstrably when irradiated for several minutes with green light.

(vii) The fluorescence of FITC-stained sections faded rapidly when irradiated with ultra-violet (u.v.) + blue light. The fluorescence appeared to contain two components, one fading with first-order kinetics with a half-life of about a minute under the experimental conditions used and the other not fading at all.

(viii) Raising the pH improved the fluorescence of FITC-stained sections but did not affect rates of fading.

(ix) Narrow-band excitation of FITC-stained sections with blue light instead of u.v. + blue reduced the rate of fading and the fluorescence intensity by equal amounts, an effect presumably due merely to loss of excitation intensity.

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INTRODUCTION

With improvements in modern optical systems it has become doubtful whether we should regard fluores-

cein as the first choice among the various fluorescent labels for antibody. Hiramoto, Bernecky, Jurand & Hamlin (1964), using fluorescein isocyanate, and Lewis & Brooks (1964) using fluorescein isothiocyanate (FITC) were agreed that fluorescein had a clear advantage over both lissamine rhodamine (RB 200) and tetramethylrhodamine isothiocyanate (TRITC) by virtue of its superior brightness, but both these groups were using optical systems designed primarily for fluorescein. By contrast, Hijmans, Schuit, Yamashita & Schechter (1972) reported that with interference filters for excitation, TRITC was more effective than FITC in that it could be detected in smaller amounts in a specimen containing both labels.

Brightness, however, is only one of the merits that needs to be measured. Image contrast rather than brightness is often the factor that limits sensitivity, and this, like brightness may be influenced by the optical system in use. For example, Cormane, Szabo & Hauge (1970) noted that the image contrast of fluorescein-stained tissues could be improved by excitation with narrow-band blue light rather than ultraviolet (u.v.) plus blue, so as to avoid excitation of tissue autofluorescence.

The traditional preference for fluorescein was also eroded by the report of Nairn, Herzog, Ward & de Boer (1969) that the fluorescence of sections labelled with FITC faded more rapidly than those labelled with RB 200 during ultraviolet irradiation. With more intense excitation becoming available, and since fading rate is a function of excitation intensity (Goldman, 1960) it might be thought that this difference in rates of fading could put fluorescein at a considerable disadvantage.

There is, however, some puzzling evidence to the contrary. Kaufman, Nester & Wasserman (1971) used lasers to achieve extremely intense excitation of fluorescein-labelled specimens and found that fading was not a serious problem. Their published graphs show that the fluorescence intensity increased in strict proportion to the excitation energy, as expected, but there was a less than proportional increase in the rate of fading. Also, under constant irradiation energy the fading was fairly rapid at first but slowed down much more than would be expected for an exponential decay. This non-exponential decay is also evident in other published graphs (Goldman, 1960; Nairn *et al.*, 1969). Moreover, the lost fluorescence could be largely restored by allowing the sample to recover in the dark.

Experience in Glasgow (Professor A. S. G. Curtis, personal communication) has also suggested that fading of fluorescein is not a serious problem with argon-ion laser excitation. Some fading was noticed within about the first second of irradiation but was not troublesome thereafter, the image remaining many times brighter than could be obtained with mercury arc excitation.

These findings suggest several testable hypotheses. Does narrow-band blue excitation, the laser being an extreme example, give less photochemical change than irradiation with a broad wavelength band including ultraviolet? This explanation could account for the findings of Brighton & Grulich (1972) that there was much less fading when fluorescein was excited by a mercury lamp containing metallo-halide additives which reduce the ultraviolet line emission at 365 nm and increase the blue continuum emission at 495 nm.

Other possibilities are that the surprisingly long-lived fluorescence observed with lasers depends on a stable, non-fading, fluorescent, minor component, or a photodecomposition product of fluorescein conjugates, or depends on regeneration of fluorescein from a decomposition product, similar to the regeneration that is said to occur spontaneously in the dark (Kaufman *et al.*, 1971). A further possibility arises from Goldman's discovery (1960) that fading of fluorescein was faster at higher fluorescein concentrations. This suggests that the photodecomposition may obey second (or higher) order kinetics, as would be expected if the reaction depended on interaction of two or more fluorescein molecules. The above findings leave many questions to be answered. Which fluorochrome gives the brightest fluorescence when tested under its own optimal conditions? Which fluorochrome gives the best contrast and what are the best conditions for attaining it? How rapidly do different fluorochromes fade in the experimental conditions most commonly used? Can fading be reduced by narrow-band blue excitation? Do the kinetics of fading support the idea of a radiation-resistant impurity or photochemical degradation product of fluorescein? How much does the balance of advantage between fluorochromes depend on the mountant in which they are viewed, and the photodetector used, be it an eye, a film or a photomultiplier?

We have attempted to answer all six of these questions for FITC- and RB 200-labelled specimens. RB 200 was chosen because we have had good results with it in the past, and the data of Lewis & Brooks (1964) suggested that it might be marginally better than TRITC.

MATERIALS AND METHODS

Preparation of fluorescein conjugates

A pool of sheep anti-human gamma globulin (anti-HGG) was obtained by mixing several hyperimmune sheep sera, and a preparation of immune globulin was obtained from this serum pool by adding sodium sulphate at 20° to give a final concentration of 18% w/v of Na_2SO_4, and dialysing the precipitate against 0·15 M saline. Five fluorescein conjugates were prepared as follows: 50 mg of globulin in 4 ml of saline at 20° were mixed with 1·4, 1·0, 0·7, 0·5 or 0·35 mg of fluorescein isothiocyanate isomer I (BDH Chemicals Ltd, Poole, Dorset) dissolved in 0·75 ml of 1·0 M carbonate buffer at pH 9·0. Within 20 min the mixtures were transferred to a cold room at 4° and left to react for 18 hr. They were then chromatographed on Sephadex G25 in phosphate-buffered saline at pH 7·8, the first coloured fraction being collected.

For some experiments another conjugate, conjugate 330, known to give good staining, was used for comparison. It had been prepared from goat anti-human IgG antiserum by reacting 50 mg of ammonium-sulphate precipitated serum globulin with 0·75 mg of FITC in 5 ml carbonate buffer, 0·5 M at pH 9·0 for 19 hr at 4°, chromatographing first on Sephadex as described above, and then on diethylaminoethyl (DEAE)-cellulose as described below. Its absorbance ratio $A_{280\ nm}/A_{495\ nm}$ was 2·0.

Preparation of rhodamine conjugates

The dye used was a sample of lissamine rhodamine provided by ICI Ltd. and dated 9 June 1965.

By infrared spectroscopy and by thin-layer chromatography it appeared to be identical in every respect to the product supplied by G. Gurr, Ltd, London, and to two very old samples provided by Dr J. H. Humphrey and Dr J. E. Fothergill which were reputed to give good results. The material contains equal weights of dye and dextrin.

The globulin used was the same as for the fluorescein conjugates. The conjugation method was adapted from that of Chadwick, McEntegart & Nairn (1958). The dye-dextrin mixture, 74 mg, was ground for 20 min with twice its weight of phosphorus pentachloride in a mortar. The product was extracted by further brief grinding with 3·7 ml of acetone previously dried over anhydrous sodium sulphate. The soluble extract, containing rhodamine sulphonyl chloride, was decanted.

For each conjugate, 50 mg of globulin in 4 ml of saline was mixed with 0·75 ml of 1·0 M carbonate buffer at pH 9·0, and a measured volume of the acetone solution, 0·06, 0·09, 0·13, 0·18 or 0·25 ml was added over a period of 1 min with mechanical stirring at 20°. Thus the amount of dextrin-free dye used ranged from 1·2% to 5% of the mass of globulin. The reaction mixtures were removed to a 4° cold room within 10 min, and after 2 hr they were chromatographed on Sephadex G25 in phosphate-buffered saline at pH 7·2, the first coloured fraction being collected.

Chromatography of conjugates on DEAE-cellulose

Each of the conjugates was chromatographed on a 300 mm × 10 mm column of Whatman DE22 DEAE-cellulose (Whatman Biochemicals Ltd, Springfield Mill, Maidstone, Kent) previously equilibrated with 0·15 M NaCl containing 0·005 M KH_2PO_4 and 0·005 M K_2HPO_4, the ionic strength 0·17, pH 7·20. Each sample applied to the column contained 35 mg of conjugated protein in 6 ml of the same buffer. It was eluted with the same buffer, only the first main fraction being collected.

Estimation of dye:protein molar ratios

The fluorescein:protein molar ratio was estimated by the formula

$$\text{F:P molar ratio} = \frac{4{\cdot}8}{\frac{A_{280}}{A_{495}} - 0{\cdot}36}$$

and the rhodamine:protein molar ratio from the formula

$$\text{R:P molar ratio} = \frac{3{\cdot}2}{\frac{A_{280}}{A_{575}} - 0{\cdot}22}$$

The numerator of each formula was derived by measuring the optical absorbance of a solution of a weighed sample of the free dye at pH 7·2 and by assuming that the labelled globulin has an average mol. wt of 160,000 Daltons and an extinction of 1·4 at 280 nm in a 1 mg/ml solution. The constants of 0·36 and 0·22 in the formulae are the ratios of the absorbance of the free dyes at 280 nm to their absorbance at their own respective maxima. The formula for FITC contains an allowance for the fact that its absorbance is reduced by 25% on conjugation with protein (Jobbagy & Kiraly, 1966; Wells, Miller & Nadel, 1966) but no allowance has been made for any change that may take place in the ratio A_{280}/A_{495} on conjugation with protein.

Fluorescent staining

The anti-HGG-FITC and anti-HGG-RB 200 conjugates were tested on sections of normal rat liver, treated with anti-nuclear factor (serum, Reference Number 176/0404/0699 supplied by the Department of Pathology, Western Infirmary, Glasgow) from a patient with systemic lupus erythematosus. Fresh rat liver was rapidly frozen by immersion in isopentane at the temperature of liquid nitrogen. Sections of the liver 5 μm thick were cut in a cryostat at $-20°$. After drying in air, the sections were fixed in absolute methanol for 10 min at room temperature.

The sections were hydrated in phosphate-buffered saline (PBS), 0·01 M phosphate, 0·15 M NaCl, pH 7·2 and covered with a drop of the human serum known to be strongly positive for anti-nuclear factor, diluted 1 in 5 with PBS. The sections were incubated in a moist chamber for 20 min at room temperature. They were then washed in several changes of PBS and covered with one drop of the test conjugate. The slides were incubated as previously for 20 min at room temperature and washed in PBS for a further 30 min. The sections were mounted in PBS-glycerol 85:15 v/v pH 7·2. In one experiment the buffer composition was varied to give mountants with pH values of 8·2 and 8·8.

Microphotometry

The sections were viewed with a Leitz Ortholux microscope fitted with an Osram XBO75 high-pressure Xenon light source, and a Leitz MPV microscope photometer containing an EMI 9658B photomultiplier tube, as part of a Knott Electronic light measuring device, type MFLK. This was fed with a stabilized voltage of 570 V and the output current led through a 100 kΩ resistor connected in parallel with a Solartron A200 digital voltmeter of input resistance 10^{10} Ω. Thus each 10 μV measured on the voltmeter represented an output current of 0·1 nA from the photomultiplier.

Comparison of the XBO 75 Xenon arc with an Osram HBO 200 mercury arc revealed that the Xenon lamp gave a much more stable intensity of illumination than the mercury arc. Hence the Xenon lamp was chosen for all subsequent investigations.

The microscope was arranged for epi-illumination through the microscope objective. For FITC the excitation filters were a heat filter KG1/2 mm (Schott and Genossen, Mainz, Germany), a BG12/1·5 mm (Schott and Genossen, Mainz, Germany) which transmits blue plus near ultraviolet, a KP490 (Schott and Genossen, Mainz, Germany) interference filter which transmits near ultraviolet and blue light up to 490 nm, and a TK510 (Barr and Stroud Ltd, Glasgow) dichroic beam-splitting mirror; the suppression filters were Leitz coloured glass filters K515 and K530. For RB 200 the excitation filters were a heat filter KG1 (Schott and Genossen, Mainz, Germany), a green glass filter BG36/2 mm (Schott and Genossen, Mainz, Germany), a green narrow-pass band interference filter KP546 (Schott and Genossen, Mainz, Germany) and a TK580 (Barr and Stroud Ltd, Glasgow) dichroic mirror; the suppression filters were Leitz K580 and K610 orange-red glass filters. For some experiments with FITC the excitation path included a GG475/2 mm (Schott and Genossen, Mainz, Germany) filter, which absorbs violet and ultraviolet while transmitting blue light to provide narrow-band excitation.

The slides were observed using a 54 × oil immersion objective (n.a. 0·95) and a 6·3 × eyepiece. The photometer field-stop diaphragm was adjusted so that the diameter was just wide enough for a large nucleus to be included within the field viewed by the photometer. This diameter was then kept constant for all further observations. In practice this means that with smaller nuclei, the readings obtained through the diaphragm represented the excitation from the nucleus itself together with a small area of background cytoplasm.

For FITC measurements, a stopwatch was started every time a particular field began to be irradiated. A nucleus was selected at random within the field and steered by the stage controls into the area delimited by the diaphragm. When the alignment was completed, the fluorescent emission from this area was passed into the photomultiplier unit and a measurement taken of the output voltage together with the time interval since initial excitation. With practice this interval was usually of the order of 15 s. Further voltage measurements were made at 15 s intervals over a 1 or 2 min period. After this, the fluorescence of an adjacent area of background not yet exposed to the incident radiation was measured in the same way. These fluorescence data were plotted and extrapolated to zero time to estimate the fluorescence emitted *on initial excitation* by both nuclei and backgrounds.

For measurements of RB 200 conjugates it was not necessary to take repeated readings, as the fluorochrome exhibited negligible fading over the 1 min exposure period. One measurement, once the nucleus was aligned, was sufficient to indicate the fluorescence at the initial exposure.

For each experimental condition, at least six nuclei with their adjacent backgrounds were measured in this manner.

RESULTS

Degree of labelling attained, as a function of the amounts of dye used and DEAE-cellulose chromatography

It can be seen from Fig. 1 that the rhodamine:protein raio attained in the conjugate was almost exactly proportional to the amount of rhodamine used in the reaction mixture, regardless of whether the measurements were made before or after DEAE-cellulose chromatography. Each time the amount of dye used was increased by a factor of 1·4, the degree of labelling increased by the same factor.

With fluorescein, however, a different relationship was found. Each time the amount of fluorescein used was increased by a factor of 1·4, the degree of labelling increased by a factor of only 1·27, but again this relationship persisted after DEAE-cellulose chromatography. Possibly the reaction of fluorescein isothiocyanate is limited by competition for the most reactive sites on the protein. Rhodamine, by contrast, showed no evidence of competition, perhaps because the sulphonyl chloride group is less discriminating and can react equally well with a large number of different sites.

The effect of the DEAE-cellulose chromatography was remarkably uniform: it reduced the dye:protein molar ratio by about 40% in almost every conjugate, regardless of the initial molar ratio and regardless of whether it was a rhodamine or a fluorescein conjugate. This is not at all what would be expected if the function of DEAE-cellulose were to remove heavily-labelled proteins, leaving the more moderately-labelled molecules of the same protein species. The results are more consistent with a role of removing selected protein species which have a propensity to become heavily labelled and which are always present in the same proportions, leaving other protein species such as immunoglobulins, which do not take up so much label.

Staining properties of the conjugates

Intensity of fluorescence. The ten conjugates were all used to stain fresh rat liver sections previously treated with the same anti-nuclear factor. The fluorescence of a field containing one nucleus and the fluorescence of the background were measured with the photomultiplier tube before and after DEAE-cellulose chromatography, and the measurements were corrected for fading of fluorescein, as described earlier, and then averaged over six fields. The means and standard errors are shown in Table 1 in arbitrary units of 10 μV.

Before DEAE-cellulose chromatography the nuclear fluorescence obtained with fluorescein, as judged by the photomultiplier, tended to be about two to three times as intense as the fluorescence obtained with

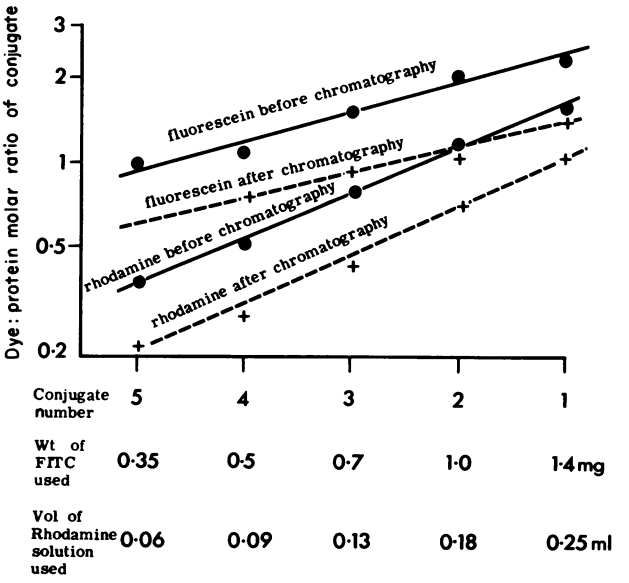

Figure 1. Dye:protein molar ratio attained in FITC and RB 200 conjugates as a function of the amount of dye used in the reaction mixture, shown both before and after chromatography on DEAE-cellulose.

Table 1. Comparison of the conjugates for intensity of staining of rat liver sections treated with anti-nuclear factor

	Before DEAE chromatography			After DEAE chromatography		
	Mean fluorescence (units of 10 μV) of			Mean fluorescence (units of 10 μV) of		
Conjugate	field containing one nucleus	background	dye:protein molar ratio	field containing one nucleus	background	dye:protein molar ratio
F1	194 ± 14	121 ± 17	2·26	185 ± 7	68 ± 6	1·42
F2	121 ± 5	76 ± 2	1·93	162 ± 10	71 ± 6	1·06
F3	148 ± 4	85 ± 5	1·50	115 ± 6	58 ± 3	0·92
F4	114 ± 7	65 ± 1	1·08	91 ± 5	51 ± 3	0·75
F5	86 ± 4	56 ± 2	0·97	100 ± 2	47 ± 3	1·01
R1	52 ± 2	34 ± 1	1·59	105 ± 12	33 ± 2	1·01
R2	36 ± 1	26 ± 1	1·15	42 ± 4	16 ± 1	0·67
R3	34 ± 1	20 ± 1	0·77	47 ± 7	18 ± 2	0·42
R4	26 ± 1	19 ± 1	0·51	40 ± 4	19 ± 2	0·28
R5	21 ± 1	15 ± 0	0·37	21 ± 1	13 ± 1	0·21

Each fluorescence figure is the mean for six microscopic fields and is accompanied by its standard error. The data for fluorescein conjugates are corrected for fading. None of the data are corrected for the spectral response of the photomultiplier tube.

rhodamine, when compared at similar degrees of labelling. After DEAE-cellulose chromatography the difference in fluorescence between the two labels was rather less marked, the fluorescein-stained nuclei being more fluorescent by a factor of about 1·5. These data on the brightness advantage of fluorescein, however, are useful only when considered in conjunction with data on the spectral response of the photomultiplier tube and the spectral response of the light detectors that are most commonly used in fluorescence microscopy, namely the human eye and photographic film. Some data on these spectral responses are shown in Fig. 2, which is derived from data supplied by EMI Electronics Ltd (1970), Kodak Ltd, (1971) and the

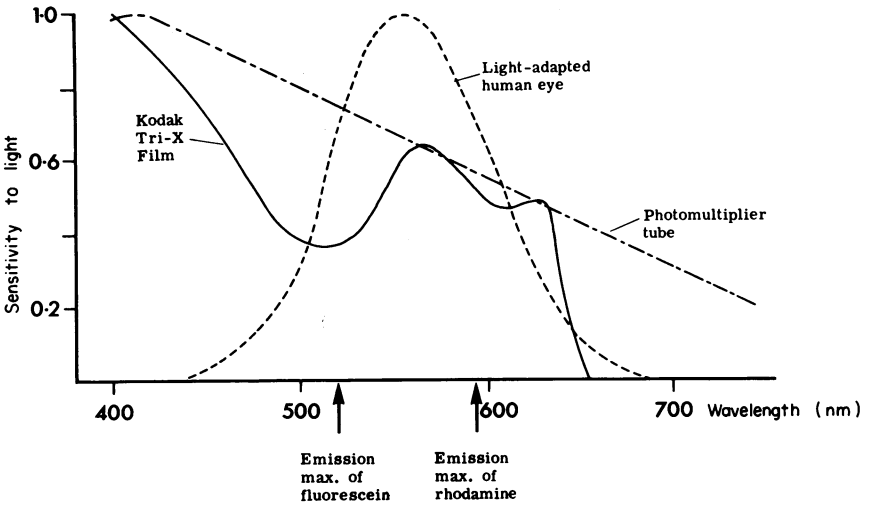

Figure 2. Spectral response curves of three sensors used to detect fluorescence. Each is plotted with its own scale of arbitrary units of sensitivity, all expressed as response to unit energy flux.

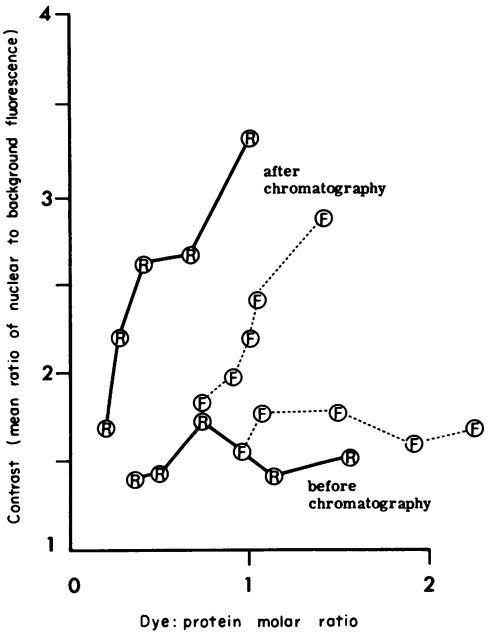

Figure 3. Mean ratio of the fluorescence of a field containing one stained cell nucleus to that of a field containing none, shown as a function of dye:protein molar ratio for fluorescein (--- Ⓕ ---) and rhodamine (—— Ⓡ ——) conjugates before and after chromatography on DEAE cellulose.

Commission Internationale d'Eclairage (1970, quoted by Keitz, 1971).

It can be seen from Fig. 2, for example, that the ratio of sensitivity at 520 nm (emission maximum of fluorescein) to sensitivity at 595 nm (emission maximum of rhodamine) is 1·33 for the photomultiplier, but only 1·02 for the human eye and only 0·73 for Kodak Tri-X film. To obtain a fair comparison of rhodamine and fluorescein brightness as seen by the human eye therefore, the fluorescence measurements of rhodamine in Table 1 would need to be multiplied by 1·31, and for users of Tri-X film the fluorescence figures given for rhodamine would need to be multiplied by 1·82.

Another factor that must be considered along with these data on brightness is the effect of changing the light source. To avoid fluctuation in brightness, we have used the Xenon XBO75 arc lamp for all measurements, but in doing so we have underestimated the merits of rhodamine. On changing from the XBO75 Xenon arc to an HBO200 mercury arc while viewing individual nuclei we find with our optical system that the fluorescence emission from rhodamine-stained nuclei increases by a factor of 1·6 and the contrast with the background is slightly improved, whereas the fluorescence of fluorescein-stained nuclei decreases by a factor of 0·95, and the contrast with the background is reduced. Thus if each fluorochrome were used with the lamp that best suits it, the brightness measurements for rhodamine would need to be multiplied further by a factor of 1·6 to obtain a fair comparison with fluorescein, or for users of microscopes fitted with mercury sources only, the multiplication factor would be about 1·7.

The brightness of fluorescein must also be evaluated in the light of its relatively rapid rate of fading as seen below, and in the light of the more recent observation (McKay, unpublished) that by using glycerol or butan-1-ol as a mounting medium instead of the PBS-glycerol 85:15 mixture that we have used here, the quantum yield of rhodamine can be increased substantially.

Contrast. Probably of more direct value are data on the contrast obtained, i.e. the ratio of nuclear brightness to background brightness. The fluorescence intensity of each field containing one nucleus was divided by the fluorescence intensity of an adjacent field in the same section, and the ratios then averaged over six pairs of fields. Since each field was rather larger than a nucleus these ratios underestimate the true contrast obtained, but are valid for comparing different dyes and degrees of labelling. The results are shown in Fig. 3.

It can be seen that before DEAE-cellulose chromatography rhodamine showed a distinct optimum dye:protein molar ratio of about 0·8. In other words, conjugate R3 gave significantly better contrast than the others as judged by the two-tailed t test ($P < 0{\cdot}05$).

With the fluorescein conjugates before DEAE-cellulose chromatography, however, there was no more than a suggestion of an ill-defined optimum at a dye:protein ratio of about 1 or 1·5.

The effect of DEAE-cellulose chromatography was pronounced. In contrast with the uniform and undiscriminating effect of chromatography on the dye:protein ratio, namely a 40% reduction across the board, the effect on the contrast was much greater with heavily labelled than with moderately labelled conjugates. Figure 3 leaves no doubt that DEAE-cellulose treatment improved contrast more than could be achieved by merely reducing the dye to protein ratio by adjusting for example, the composition of the reaction mixture. Although the chromatographic conditions were originally chosen for fluorescein conjugates, the improvement of the rhodamine conjugates on chromato-

graphy was at least as great as for the fluorescein conjugates.

Kinetics of fading

Some of the stained nuclei and backgrounds were observed for several minutes and the decay of fluorescence recorded. With rhodamine there was little if any fading. The decline in fluorescence measured over 2 min varied from 0% to 4%, and could not be clearly distinguished from instrumental error, decay of autofluorescence and variations in the intensity of irradiation.

With fluorescein the fluorescence decayed markedly under irradiation, and appeared to reach a plateau level. The fluorescence measurements could best be interpreted as the sum of two components, one of which decayed exponentially while the other remained constant. This was illustrated as follows. For each cell nucleus or background the plateau level was first estimated by a common method of extrapolating to infinite time: a graph of fluorescence against the reciprocal of irradiation time (t) was drawn and extrapolated to $1/t=0$, where it intercepted the fluorescence intensity axis to give an estimate of the final plateau level. This plateau level, representing the luminous component resistant to fading, was then subtracted from each fluorescence measurement to give the fluorescence component which does fade. When this latter component was plotted on a logarithmic scale against time on a linear scale as in Fig. 4 a straight line was obtained for each of three nuclei and three backgrounds, showing that the fading obeyed first-order kinetics with a half-life of just under 1 min under the conditions of irradiation being used. The initial fluorescence intensity of the component subject to fading, can be estimated by extrapolation of the lines in Fig. 4 to zero time. The final (plateau) intensities estimated as described above for the nuclei stained with conjugates F3, F4 and F5 were 65, 58 and 45 u. respectively, and the final (plateau) intensities of the corresponding background fields were 51, 48 and 38 u.

It is shown in Fig. 5 that the component of luminance that did not fade appeared to be a function of the component that faded. This is one reason for suspecting that the non-fading component did not consist entirely of unwanted excitation light and autofluorescence of tissue.

In some more recent preliminary experiments on the mechanism of fading, the fading was allowed to proceed for about six half-lives and then the intensity of excitation was, in effect, reduced by opening and closing the excitation shutter alternately. It was found, for example, that a nucleus initially giving a fluorescence measurement of 270 u. faded after 6 min to give a reading of 64 u., and on extrapolation to infinite time

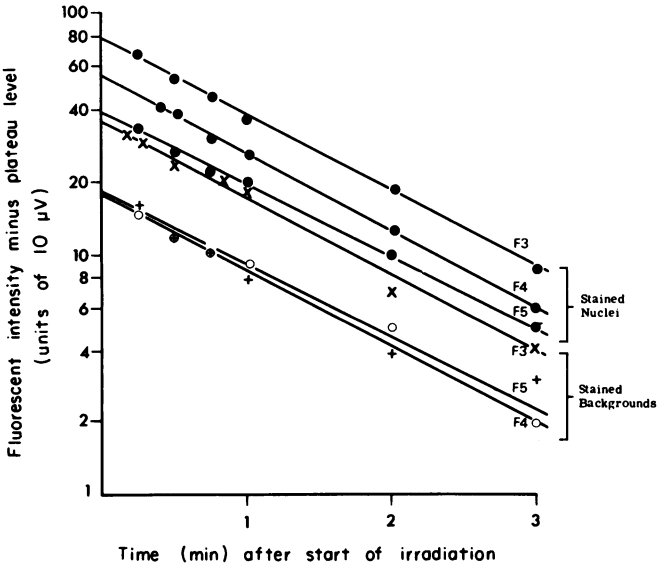

Figure 4. Kinetics of fading of three cell nuclei (•) and three adjacent backgrounds stained with conjugates F3 (×), F4 (+) and F5 (o). The final plateau levels of fluorescence subtracted before plotting the graphs were 65, 58 and 45 u. for F3, F4 and F5 conjugates, respectively, and 51, 48 and 38 u. for the corresponding three backgrounds.

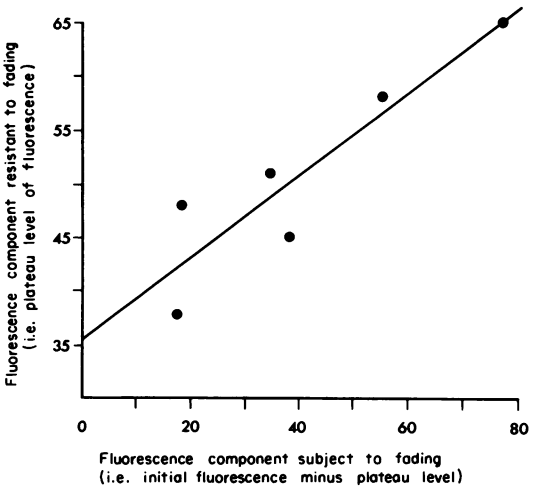

Figure 5. The plateau level of luminance of three cell nuclei and three backgrounds, shown as a function of the fluorescence component that is susceptible to fading. The ordinate includes unwanted excitation light that has passed through the optical system.

as described above it appeared to be approaching a plateau of 50 u. When, however, the excitation was reduced by opening the shutter for only 3 s every minute, the readings obtained increased for 5 min and then remained constant at 86 u. When the excitation was further reduced by opening the shutter for only 3 s every 5 min the luminance increased again to 99 u. This apparent recovery of fluorescence was probably not an electronic artefact, because it could not be obtained with unstained specimens.

Effect of pH on fluorescence intensity, contrast and rates of fading

Since the fluorescence of fluorescein varies with pH, it was thought that some of the advantage of fluorescein over rhodamine might have been missed by mounting the sections at a suboptimal pH. To assess this effect two fluorescein conjugates, F3 ex-Sephadex and conjugate 330 ex-DEAE-cellulose were used to stain rat liver sections treated with anti-nuclear factor by the same method as used above, and the sections were mounted in phosphate-buffered 15% v/v glycerol at pH 7·2, pH 8·2 and pH 8·8. For each section six fields containing a nucleus and six adjacent backgrounds were chosen and their fluorescence measured at 15, 30, 45 and 60 s after the start of irradiation. The initial fluorescence intensity F_0 and the rate constant k of fading were estimated from the fluorescence measurements F and the irradiation time t by fitting an exponential decay curve $F=F_0e^{-kt}$ to the data by the method of least squares. This simple model of the decay curve was adequate for extrapolation of readings taken during the first minute. The results are shown in Table 2.

Intensity of fluorescence. It can be seen that with conjugate F3, a 23% increase in nuclear fluorescence could be achieved by raising the pH progressively from 7·2 to 8·8. This progressive increase of fluorescence was statistically significant ($P<0·01$) as judged by analysis of variance. With conjugate 330, only a 6% increase was seen and this did not satisfy the same statistical criterion. The background fluorescence measurements showed no significant change, except that with conjugate F3 there was a sharp decrease in brightness at pH 8·2, a result which, though statistically significant, was unexpected and inconsistent with the three other trends, and should be interpreted with caution.

Contrast. The contrast, as measured by the ratio of

Table 2. Effect of pH on fluorescence intensity, contrast and rate of fading of sections stained with two fluorescein conjugates

Conjugate	pH of mountant	Initial fluorescence (units of 10 μV) of: field containing one nucleus	Initial fluorescence (units of 10 μV) of: adjacent background	Nucleus to background fluorescence ratio	Exponential rate constant of fading (units of 10^{-4} sec^{-1}) for: nucleus	Exponential rate constant of fading (units of 10^{-4} sec^{-1}) for: background
F3 ex-Sephadex	7·2	77·0 ± 4·3	58·5 ± 3·2	1·33 ± 0·08	33·3 ± 6·3	25·0 ± 5·9
	8·2	90·7 ± 2·1	48·2 ± 1·7	1·89 ± 0·07	30·5 ± 3·9	28·3 ± 8·4
	8·8	94·5 ± 3·8	61·7 ± 2·8	1·55 ± 0·09	40·3 ± 5·4	30·7 ± 5·3
330 ex-DEAE-cellulose	7·2	222 ± 21	94 ± 12	2·42 ± 0·24	36·8 ± 3·1	34·3 ± 3·3
	8·2	229 ± 12	98 ± 9	2·39 ± 0·15	39·2 ± 4·1	34·3 ± 7·6
	8·8	236 ± 14	101 ± 7	2·39 ± 0·19	32·8 ± 3·6	34·8 ± 8·6

Table 3. Effect of narrow-band excitation on fluorescence-intensity, fading rate and contrast of sections stained with fluorescein conjugates

Conjugate and filter	Initial nuclear fluorescence, F_0 (units of 10 μV)		Fading rate constant k during first minute (10^{-4} s^{-1})	F_0/k	Initial contrast nuclear/F_0 background
F3 ex-Sephadex without GG475 filter	Mean	92·5	36·8	2·53	1·66
	SE	3·9	1·7	0·14	0·21
F3 ex-Sephadex with GG475 filter	Mean	48·0	19·2	2·83	1·96
	SE	0·9	2·8	0·48	0·08
330 ex-DEAE without GG475 filter	Mean	241	28·2	9·38	3·13
	SE	9	2·8	1·71	0·20
330 ex-DEAE with GG475 filter	Mean	153	17·8	8·46	3·77
	SE	13	1·9	1·29	0·42

fluorescence of a field containing a nucleus to that of a nearby field containing none, appeared to be insensitive to pH. The only apparent effect of pH was the increased contrast obtained at pH 8·2 conjugate F3, a result which satisfies statistical criteria ($P < 0·001$ by one-way analysis of variance) but is still unconvincing as it clearly arises from the suspiciously low background fluorescence.

Rate of fading. The fading rates were virtually the same for both the fluorescein conjugates, for nuclei as well as backgrounds, and for all pH values. Among the twelve fading rates listed in Table 2, no two of them, differ significantly as judged by the two-tailed unpaired t test ($P > 0·078$ in all sixty-six t tests).

Effect of narrow-band excitation on fading rate, fluorescence intensity and contrast

To test whether the use of blue-light excitation alone instead of ultraviolet plus blue would give less fading, as it is reputed to do, or better contrast, a series of tests were carried out on sections stained with conjugate F3 before DEAE-cellulose chromatography, and with conjugate 330 after DEAE-cellulose chromatography. The initial fluorescence intensities and rate constants of fading of nuclei and backgrounds were determined as before, but with a GG 475 filter (which absorbs light below 475 nm) alternately in and out of the exciting beam.

The results are summarized in Table 3. For both conjugates the use of the GG475 filter did indeed give a significant reduction in the nuclear fading rate constant ($P < 0·01$ by the t test), amounting to 48% for F3 and 37% for conjugate 330. Unfortunately, the nuclear fluorescence intensities were also reduced by almost exactly the same amounts (48% and 36·5% for conjugates F3 and 330, respectively). Furthermore, the ratio of nuclear brightness to nuclear fading rate was not significantly affected by the GG475 filter for either of the two conjugates. Thus the GG475 filter reduced fading rate and fluorescence emission by equal amounts, and this occurred with each conjugate. One cannot but suppose that the two effects simply represent a loss of excitation intensity. The results are exactly what one would expect of a neutral-density filter. The slower decay of fluorescence could never compensate completely for the lack of initial brightness, not even in a long photographic time exposure.

The use of the filter for improving the contrast is a practice that is neither justified nor condemned by the data in Table 3. The contrast appeared to increase by 18% and 20% for the two conjugates respectively, but the increase was not statistically convincing ($P = 0·10$ in each case, according to Student's single-tailed t test).

DISCUSSION

Our findings tend to favour lissamine rhodamine RB 200, compared with FITC as an agent for labelling antibody. Fluorescein-stained sections did appear to the photomultiplier tube to be more fluorescent than sections stained with rhodamine conjugates at a similar dye:protein ratio, but this advantage of fluorescence

intensity of fluorescein was both modest in magnitude and dubious in its relevance. The apparent brightness advantage of fluorescein averaged a factor of about 2·5 before, and about 1·5 after DEAE chromatography. If we regard these figures as experimental deviants from a common mean of about 2·0 and correct for differences in spectral responses of photomultiplier, human eye and photographic film, we find that the fluorescence advantage of fluorescein is reduced to a factor of 1·53, not very noticeable to the eye, or 1·10, barely detectable by a photographic film. On excitation with the optical system we have used, these factors are reduced to 1·00 within 40 s for the eye or within 10 s for the film, on account of the fading of fluorescein.

We used a Xenon arc to ensure a stable intensity of excitation, but as a result have further underestimated the merits of rhodamine relative to fluorescein. The mercury arc, having an intense green-line emission at 546 nm, is particularly well suited to the excitation of rhodamine. For instance, Ploem (1971) found that the ratio of RB 200 fluorescence to residual unwanted excitation light was four to six times better with mercury lamps than with Xenon lamps. On changing from the XBO 75 Xenon arc to the HBO 200 mercurcy arc while viewing individual cell nuclei we have found that the fluorescence of rhodamine-stained nuclei increased by a factor of 1·6 while that of fluorescein-stained nuclei decreased by a factor of 0·95. It is clear that whether we view each fluorochrome with the light source that best suits it, or whether we follow the common practice of using a mercury source only, the brightness estimates of rhodamine reported above have to be multiplied by a further substantial factor, namely 1·6 or 1·7 to obtain a fair comparison with fluorescein. This is sufficient to swing the balance of advantage modestly in favour of rhodamine.

Finally it is necessary to consider the effects of the mountant. It was found that the fluorescence of FITC-stained sections could be improved at best by 23% by raising the pH from 7·2 to 8·8. This is reasonably compatible with the work of Nairn *et al.*, (1969). Eleven percent might be a more realistic figure, based on the data of Fothergill (1969). In recent experiments with rhodamine, however (McKay, unpublished) it was found that by replacing our 15% glycerol with a more favourable mountant such as pure glycerol or butanol, the quantum yield of RB 200 in solution could be approximately doubled and the intensity of fluorescence of stained slides could be increased by about 50%.

When all these factors are given due weight it seems that the balance of advantage in respect of brightness rests decisively with rhodamine. Further, the data of Fig. 3 indicate that rhodamine conjugates after DEAE cellulose chromatography were superior to fluorescein conjugates of similar dye:protein ratio in respect of contrast.

There are, however, two considerations that might in some circumstances favour fluorescein. One is the possibility that fluorescein-stained specimens might sometimes be viewed with dark-adapted human eyes, which have a maximum sensitivity at 507 nm, rather than light-adapted eyes, which are most sensitive at 554 nm. We have based our calculations on the properties of light-adapted eyes, i.e. of retinal cones rather than the rods because in our experience the image visible in the microscope has always been sufficiently bright to allow clear perception of colour and good visual acuity; colour perception being a property solely of cones and visual acuity depending upon the fovea which contains no rods. We cannot, however, be certain that light-adapted eyes and retinal cones are used by all fluorescence microscopists.

Another consideration arises from the intriguing evidence, both from the kinetics of fading described above and elsewhere (Goldman, 1960; Nairn *et al.*, 1969) and from published descriptions of laser-excited fluorescein-stained specimens (Kaufman *et al.*, 1971), that the fading of fluorescein-stained specimens may *not* be total and irreversible and does not increase inexorably in proportion to the intensity of excitation.

Our own data do not prove convincingly that the fading is incomplete: the plateau level reached by the fluorescence measurements is certainly due partly to unwanted excitation light that has passed through the optical system, partly to autofluorescence of the tissue and partly to the dark current of the photomultiplier. Nevertheless, there are some grounds for supposing that the plateau may also be due partly to fluorescence of fluorescein. Firstly the plateau level of luminance appeared to be a function of the fluorescence component that fades as shown in Fig. 5. Secondly the image seen after prolonged fading (about six half-lives) still appeared green to the eye, though very faint. Thirdly, the plateau was at a slightly higher level when the intensity of excitation was in effect reduced by closing and opening the excitation shutter alternately. This may indicate that there is a residual fluorescence that depends on a dynamic balance between photodestruction and spontaneous regeneration of fluorescein. The residual fluorescence, though too dim to be useful

with most light sources, may well be the main reason for the success reported with lasers.

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