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Conjugation of Fluorescein Isothiocyanate to Antibodies

II. A REPRODUCIBLE METHOD

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Summary. A reproducible method for conjugation of fluorescein isothiocyanate to antibodies, based on previous experiments, is described in detail. The negative electrical charge of antibodies was found to increase by conjugation. This negative charge may cause the non-specific fluorescence of acidophilic tissue components if overlabelled antibodies are used. Even non-specific nuclear fluorescence was observed when the substrate was previously washed in phosphate buffer. This was probably caused by acid extraction. Underlabelled antibodies showed a weak specific fluorescence. Antibodies with molecular fluorescein/protein ratios between 1 and 4 or 'optical density ratios' ($E^1_{495} : E^1_{280}$) between 0·3 and 0·95 were found to be optimal. However, even antibodies conjugated to an optimal degree may demonstrate non-specific fluorescence if used in high concentrations. Restricted preservation testing of reconstituted lyophilized conjugates revealed a negative effect of exposure to daylight at room temperature.

INTRODUCTION

In the previous paper (The and Feltkamp, 1970) experiments were described regarding (1) the preparation of IgG globulins from antisera, (2) the actual conjugation procedure, and (3) the final selection of fractions labelled to an optimal degree. These experiments led to a conjugation method which proved to be reproducible. The method, which will be described in detail in the present study, allowed examination of non-specific fluorescence. Further materials and methods have been described previously (The and Feltkamp, 1970).

METHOD

Isolation of IgG

To antiserum at 0° an equal volume of saturated $(NH_4)_2SO_4$ at 0° is gradually added, under continuous stirring. After standing for 1 hour at 0° the mixture is centrifuged at 28,000 ***g*** at +0° for 10 minutes. The sediment is dissolved in half of the original volume and dialysed against phosphate buffered saline (PBS) (0·01 M phosphate, pH 7·2). The nearly albumin free serum fraction is put on a DEAE Sephadex A 50 medium column equilibrated with PBS, from which all fine Sephadex particles have been previously removed. (A second DEAE Sephadex A 50 column of half the volume is prepared at the same time, to be used for purification of the conjugate.) The column is eluted with PBS at a minimal flow rate of 1·5 ml/min. All fractions from the protein peak consisting of IgG with $OD_{280} > 0{\cdot}1$ are pooled and concentrated by ultrafiltration to a protein concentration of at least 25 mg/ml.

Conjugation of FITC to IgG

A standard solution of 1 mg FITC per ml 0·15 M $Na_2HPO_4 . 2H_2O$ solution (pH 9·0) is made for immediate use. About* 10 ml of the FITC solution per gram protein is added to the IgG solution while stirring at room temperature. The pH is adjusted to 9·5 by addition of 0·1 M $Na_3PO_4 . 10H_2O$ while stirring. The reaction is continued for 60 minutes at room temperature, while pH is maintained at 9·5. The conjugate is put on a 'Sephadex G 25 coarse' column, equilibrated and eluted with PBS, and the free dye is removed. The average F/P ratio of the resulting conjugate should be about 2·5 (The and Feltkamp, 1970).

Purification of the conjugate

The conjugate freed from unbound dye is put on a DEAE Sephadex A 50 medium column equilibrated with PBS. Elution with PBS results in the separation of a light coloured band. A gradient elution is then achieved by connection with a closed mixing chamber filled with half the column volume of PBS, linked to an open vessel with high molar phosphate buffer (1 M NaCl, 0·01 M phosphate, pH 7·2). Fractions are collected in the cold. F/P ratios are determined from the respective OD_{280} and OD_{495} values, using the nomogram presented in Fig. 1. Fractions with optimal F/P ratios are sampled, dialysed against PBS, and concentrated by ultrafiltration to 10 mg/ml. Ampoules containing 1 ml are freeze dried and vacuum sealed. Reopened and redissolved ampoules are tested for protein concentration, mol. F/P ratio, and optimal dilution for immunofluorescent technique.

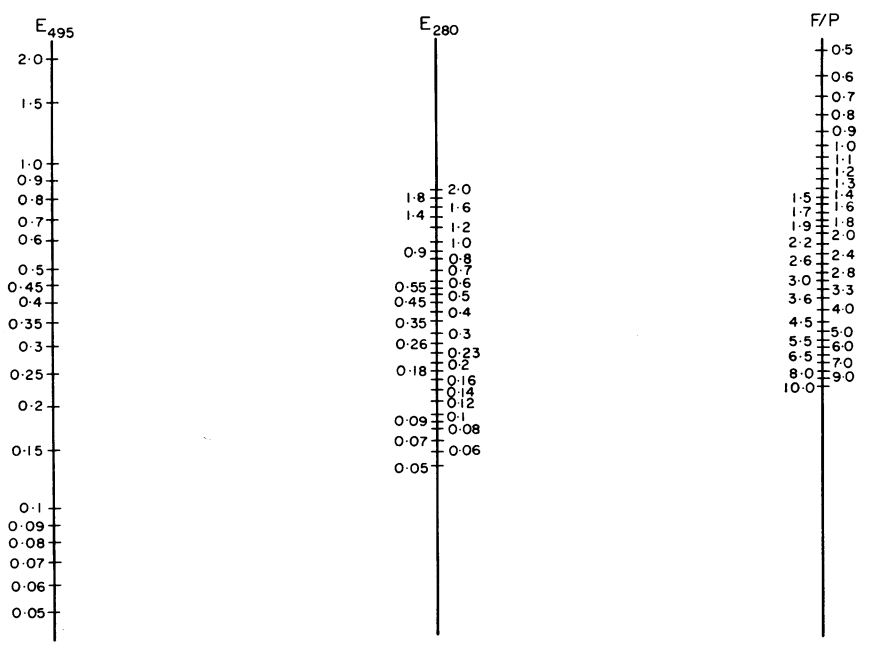

FIG. 1. Nomogram for calculating F/P ratio.

* Since small variations in the batches of FITC are possible the optimal FITC/IgG ratio is adjusted by conjugation of a preliminary series using small amounts.

RESULTS

Horse IgG antibodies against rabbit Ig were conjugated as mentioned above. Immunoelectrophoresis of seven fractions, taken at regular intervals during the last DEAE chromatography, revealed that increase of negative electrical charge of conjugated IgG molecules is correlated with the degree of labelling, as presented in Fig. 2.

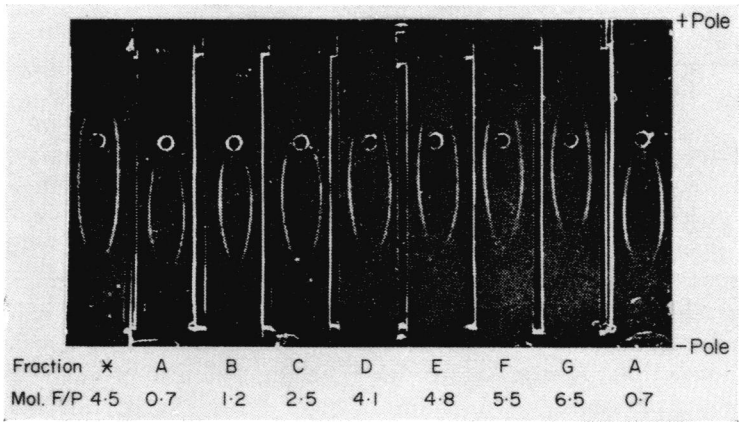

FIG. 2. Relation between electrical charge and mol. F/P ratio demonstrated by immune electrophoresis. The troughs contain an anti-whole horse serum. Wells A to G contain fractions of a horse anti-rabbit Ig conjugate obtained after DEAE Sephadex chromatography. Well * contains the unfractionated conjugate. (Fraction A was studied twice to control the homogeneity of the electrical field.)

These and 42 other fractions of the conjugated horse IgG proteins all brought to a concentration of 0·5 mg/ml were tested for non-specific fluorescence, using cryostat sections of different tissues (Feltkamp and van Rossum, 1968), which were previously fixed in acetone for 10 minutes and washed in PBS for 30 minutes, were incubated for 20 minutes with conjugate and finally washed again for 15 minutes. After this treatment, which took place at room temperature, the sections were microscopically read as previously described (Feltkamp and van Rossum, 1968). The results (Table 1) show that non-specific

TABLE 1

RELATION BETWEEN THE mol. F/P RATIO AND NON-SPECIFIC STAINING (GRADED −, ±, +, ++, +++) OF 49 CHROMATOGRAPHICALLY ISOLATED FRACTIONS OF A HORSE IgG CONJUGATE ALL WITH A PROTEIN CONCENTRATION OF 0·5 mg/ml

Mol. F/P ratio of the fraction	0·6 to 2	2 to 3	3 to 4	4 to 5	5 to 6	>6
Number of fractions investigated	7	16	10	6	7	9
Substrate						
human thyroid	−	−	−	−	+	++
rat liver	−	−	−	±	+	++
rat kidney	−	−	−	±	+	++
human adrenal	−	−	−	±	+	++
human parotid gland	−	−	−	±	+	++
human stomach	−	−	−	+	+	++
rat stomach	−	−	±	+	++	++
calf thymus	−	−	±	+	++	+++
rat diaphragm	−	−	±	+	++	+++
rat heart	−	−	±	+	++	+++

TABLE 2

CELLS OR PARTS OF THE STUDIED TISSUES WITH A HIGH DEGREE OF NON-SPECIFIC STAINING AFTER INCUBATION WITH CONJUGATES WITH F/P RATIOS > 4 AND A PROTEIN CONCENTRATION OF 0·5 mg/ml

Rat diaphragm	(mainly A-bands (Strauss and Kemp, 1967)
Rat heart	(mainly sarcolemma and intercalated discs)
Rat stomach	(mainly parietal cells, smooth muscle and eosinophilic leucocytes)
Rat kidney	(mainly glomerular basal membranes and tubular cells)
Human thyroid	(mainly epithelial cells and colloid)
Human adrenal	(mainly cortical cells)
Rat liver	(mainly parenchymal cells)
Human parotid gland	(mainly salivary duct cells)

fluorescence was not present in any tissue tested if the mol. F/P ratio was below 3, while non-specific fluorescence was present with all tissues if the mol. F/P ratio was higher than 5. Eosinophilic tissues or cells especially were stained non-specifically. These are often cells known to contain antigens for autoantibodies (Table 2).

Non-specific nuclear fluorescence was also observed (Fig. 3). However, this type of non-specific fluorescence was weaker if washings were performed with barbitone buffer (sodium barbitone 2·06 g; 0·1 N HCl 80·6 ml; NaCl 8·5 g; aqua ad 1 L; pH 7·2), while it was absent if the sections were not washed before incubation with conjugate. This finding correlates with the observation that after haematoxylin-eosin staining nuclei appear blue if the cryostat sections are not previously washed, faint blue if washed in barbitone buffer, and unstained if washed in PBS.

FIG. 3(a). Non-specific nuclear fluorescence. PBS washed and acetone fixed rat liver sections were incubated directly with a horse anti-human Ig conjugate (mol. F/P ratio 6·8, protein concentration 0·5 mg/ml) ×250.

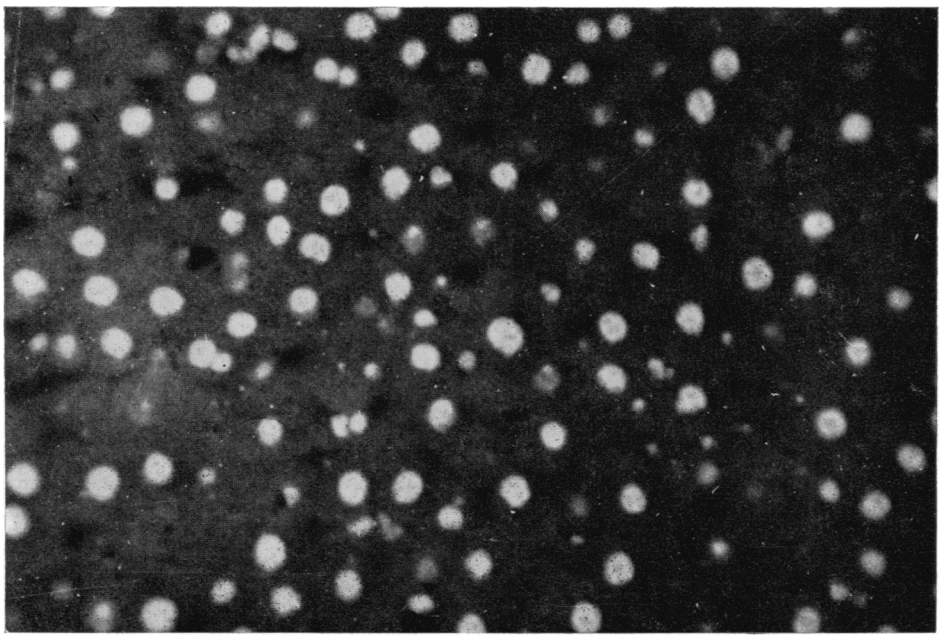

FIG. 3(b). Specific nuclear fluorescence. Sections treated in the same way now incubated with a human serum containing anti-nuclear factors followed by a horse anti-human Ig conjugate (mol. F/P ratio 2·5, protein concentration 0·016 mg/ml) ×250.

The relation between mol. F/P ratio and specificity of immunofluorescence was further tested with the fractions all brought to a protein concentration of 0·5 mg/ml from the horse anti-rabbit Ig conjugate. Acetone-fixed and PBS washed rat liver sections were subsequently incubated with a human serum (diluted 1 : 25) containing anti-nuclear factors (ANF), a rabbit anti-human Ig serum (diluted 1 : 25) and the conjugate to be tested. The results of this experiment summarized in Table 3 revealed that fractions with mol. F/P ratios between 1 and 4 were optimal, as they presented a maximal specific staining in the absence of interfering non-specific fluorescence. Analogous results were obtained in the study of conjugated IgG fractions of two sheep anti-rabbit Ig sera and three rabbit anti-human Ig sera. For the study of the latter sera the non-conjugated anti-human Ig layer was of course omitted.

Besides the mol. F/P ratio the FITC or protein concentration is also of importance for the appearance of non-specific fluorescence. This was demonstrated by incubation of rat liver sections with a twofold dilution series of five different conjugates of equal protein concentrations (8 mg/ml) and varying mol. F/P ratios (1·3–8·7) (Table 4). The results

TABLE 3

RELATION BETWEEN mol. F/P RATIO, SPECIFIC FLUORESCENCE AND NON-SPECIFIC FLUORESCENCE OF 63 ISOLATED FRACTIONS OF A HORSE ANTI-RABBIT Ig CONJUGATE ALL BROUGHT TO A PROTEIN CONCENTRATION OF 0·5 mg/ml

Mol. F/P ratio	F/P < 1	1 < F/P < 3	3 < F/P < 4	F/P > 4
No. of fractions studied	9	27	9	18
Specific fluorescence	± to +	++	+++	+++ to ++
Non-specific fluorescence	negative	negative	acceptable	very marked

TABLE 4

INFLUENCE OF PROTEIN CONCENTRATION AND MOL. F/P RATIO ON NON-SPECIFIC FLUORESCENCE OF RAT LIVER

	Twofold dilutions of five conjugates all with initial protein concentration of 8 mg/ml							
Mol. F/P ratio	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
1·3	+	±	−	−	−	−	−	−
2·4	+	+	±	−	−	−	−	−
3·6	+	+	+	±	−	−	−	−
4·5	+	+	+	+	±	−	−	−
8·7	+	+	+	+	+	+	±	−

+ = Strong non-specific fluorescence. ± = Weak non-specific fluorescence. − = Absence of non-specific fluorescence.

obtained after 30 minutes incubation followed by a 10 minute washing with PBS revealed that conjugates with low mol. F/P ratios may give non-specific fluorescence at high concentrations and that heavily labelled IgG molecules have to be strongly diluted to avoid non-specificity.

The stability of conjugates was tested by exposing opened and redissolved ampoules of a horse anti-human Ig conjugate without any preservative to:

(a) ten times freezing at −20° and thawing at room temperature;
(b) storage at 4, 16 or 37° in the dark for 1 week;
(c) storage at room temperature in daylight for 1 week.

This was performed with the undiluted conjugate (protein concentration 7 mg/ml) and in dilutions of 1 : 16 and 1 : 32. The latter dilution was the optimal performance dilution. If tested as described above a detrimental effect was only observed on storage in daylight. Diluted conjugate proved to be especially sensitive for this treatment.

DISCUSSION

The main purpose of the present study was the presentation of a method for conjugation of FITC to antibodies. The method described proved to be satisfactory and reproducible. It offers the possibility of selecting antibodies conjugated to an optimal degree.

Goldstein, Slizys and Chase (1961), McDevitt, Peters, Pollard, Harter and Coons (1963) and Wood, Thompson and Goldstein (1965) considered fractions with a molecular F/P ratio between 0·86 and 1·5 as optimal. However, McDevitt *et al.* (1963) were not sure about the strength of the specific fluorescence obtained with antibodies labelled to such a low degree. Also, Fothergill (1964) observed a dull fluorescence with such conjugates, Pittman, Hebert, Cherry and Taylor (1967) pointed to the substrate as being of great importance for the determination of the range of optimal F/P ratios. Recently Beutner, Holborow and Johnson (1967) described optimal results with conjugates with mol. F/P ratios between 1 and 4. Although their conjugation method differed from the method described in the present study, we also found antibodies conjugated to this degree to be optimal. The 'optical density ratios' ($E^1_{495} : E^1_{280}$) of the conjugates which react optimal range between 0·3 and 0·95. This is in accordance with Brighton, Taylor and Wilkinson (1967) who suggested an optimal 'optical density ratio' of 0·5 to 1·0 for the final product.

Underlabelled antibodies might have a 'blocking effect' and are therefore better removed. Overlabelled antibodies might have lost antibody activity, although a greater danger is that they may cause non-specific fluorescence.

Non-specific fluorescence was noticed with the introduction of the immunofluorescent technique by Coons, Creech, Jones and Berliner (1942). The mechanism was further

studied by Hughes and Louis (1959), Louis (1958, 1960) and von Mayersbach and Schubert (1960). The latter authors pointed to the increased negative electrical charge of conjugates, which is confirmed by the present study. In highly labelled conjugates this might cause a behaviour analogous to acid dyes, e.g. eosin. The present study confirmed these observations. If nuclear acids are extracted by washing in phosphate buffer non-specific nuclear fluorescence due to overlabelling may still result (von Mayersbach, 1967). Washing with barbitone buffer instead of phosphate buffer decreases this cause of non-specific nuclear fluorescence. The present study confirms the observation of Beutner *et al.* (1967) that even antibodies labelled to a low degree may cause non-specific fluorescence if used in high concentration, while on the other hand over-labelled antibodies lose their non-specific staining capacities at very high dilutions. Hebert, Pittman and Cherry (1967) were able to demonstrate that non-specific fluorescence is directly related to the FITC concentration of the conjugate and is independent of the protein concentration.

Fluorescent IgG fractions of antibodies, labelled to about the same degree allow better chemical characterization. This in turn allows better standardization of the immunofluorescent technique. Standardized antigens, together with improved fluorescent microscope equipment which allows measurement of the intensity of fluorescence might stimulate further progress in this field (ten Veen and Feltkamp, 1969).

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