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Conjugation of Fluorescein Isothiocyanate to Antibodies

I. EXPERIMENTS ON THE CONDITIONS OF CONJUGATION

T. H. THE AND T. E. W. FELTKAMP

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Immunopathology, University of Amsterdam, Amsterdam

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Summary. In the present study experiments concerning the conjugation of fluorescein isothiocyanate (FITC) to antibodies, which were previously performed by Goldstein, Slizys and Chase (1961), McKinney, Spillane and Pearce (1964) and Wood, Thompson and Goldstein (1965) are compiled, reproduced, extended and discussed.

The results of the experiments indicate that if a relatively pure IgG, obtained by DEAE Sephadex chromatography, is conjugated with FITC of high quality, a maximal molecular fluorescein/protein (F/P) ratio is reached in a short time if reaction temperature, pH and protein concentration are high. Maximal labelling was obtained in 30–60 minutes at room temperature, pH 9·5 and an initial protein concentration of 25 mg/ml. The separation of optimally labelled antibodies from under- and over-labelled proteins may be achieved by gradient DEAE Sephadex chromatography. Electrophoretically distinct IgG molecules proved to have about the same affinity for FITC. A correlation between the activity of antibodies in fluorescent and precipitation techniques was found.

INTRODUCTION

The objective of the present study was to compile experiments performed by Goldstein Slizys and Chase (1961) who suggested the use of pure IgG antibodies, by McKinney, Spillane and Pearse (1964) who studied kinetics of conjugation, and by McDevitt, Peters, Pollard, Harter and Coons (1963) and Wood, Thompson and Goldstein (1965) who selected antibodies conjugated to an optimal degree. These experiments are reproduced, confirmed and extended. The authors feel that while only a few new findings are presented, presentation is justified since former authors devoted only special attention to certain aspects of conjugation for immunofluorescence.

MATERIALS AND METHODS

Antisera were prepared by immunization of rabbits, sheep and horses with human IgG, IgM, IgA, complement and mixtures of the mentioned immunoglobulins, while also rabbit immunoglobulins were used as antigens in horses and sheep.

Ammonium sulphate precipitation was performed by addition of one part of a saturated solution (707 g $(NH_4)_2SO_4$, aqua ad 1000 ml) at 0° to one part of antiserum at 0° under continuous stirring. After standing for 1 hour at 0° the mixture was centrifuged at 28,000 ***g***

at 0° for 10 minutes. The sediment was dissolved in saline and dialysed against phosphate buffered saline (PBS) (0·01 M phosphate, pH 7·2).

Gel filtration was performed on columns filled with Sephadex G 25 coarse (Pharmacia, Uppsala) equilibrated for at least 4 hours with PBS.

DEAE chromatography was performed with DEAE Sephadex A 50 medium (Pharmacia, Uppsala). The particles were swollen at room temperature for at least 18 hours in distilled water, brought to pH 7·2 by addition of 0·1 N NaOH. The suspension was washed five times with PBS which removed the fine particles. The remaining particles sedimented in a few minutes with a sharp border. Chromatography was performed on columns equilibrated for at least 16 hours with PBS.

Concentration was performed by ultrafiltration in a Diaflo apparatus (Amicon Corp., Lexington, Mass., U.S.A.). Protein concentrations were determined by spectrophotometry using the formula: IgG concentration = $E^1_{280}/1{\cdot}4$ mg/ml. This formula was based on the extinction coefficient of human IgG.

Conjugation of IgG with FITC was performed according to McKinney *et al.* (1964) by the addition of a calculated amount of an FITC stock solution to the concentrated IgG protein under continuous stirring. The stock solution was prepared by dissolving a calculated amount of chromatographically pure crystalline isomer I FITC (Baltimore Biological Laboratories, U.S.A.) in 0·15 M Na_2HPO_4. $2H_2O$ (pH 9). To avoid considerable hydrolysis the solution was used within 3 hours. The temperature was kept constant during the conjugation procedure, and the pH was continuously adjusted by addition of 0·1 M Na_3PO_4. $10H_2O$ (pH 11). When the conjugation time was up, unbound dye was separated by Sephadex G 25 gel filtration.

After addition of the conjugate to a DEAE Sephadex column and a previous elution with PBS a gradient elution was achieved by connection of a closed mixing chamber filled with 250 ml PBS linked to an open vessel filled with high molar phosphate buffered saline (1 M NaCl, 0·01 M phosphate, pH 7·2).

FITC concentration of a conjugate was estimated photometrically at 495 nm. The extinction coefficient found for chromatographically pure crystalline isomer I FITC was used as a reference. Although the actual extinction coefficient for protein bound FITC is somewhat lower, this difference was neglected (McKinney *et al.*, 1964). Therefore we used: conc. FITC = $E^1_{495}/200$ mg/ml.

Protein concentration of conjugated IgG was $(E^1_{280} - 0{\cdot}35 \times E^1_{495})/1{\cdot}4$ mg/ml according to Wood *et al.* (1965). We used this formula assuming that the extinction coefficient of IgG was not altered by conjugation.

Molecular fluorescein/protein (F/P) ratio was calculated assuming a molecular weight for IgG of 160,000 and of 390 for FITC.

$$\text{Mol F/P} = \frac{16 \times 10^4}{390} \times \frac{\text{conc. FITC mg/ml}}{\text{conc. IgG mg/ml}}$$

$$= 410 \times \frac{E^1_{495} : 200}{(E^1_{280} - 0{\cdot}35 \times E^1_{495}) : 1{\cdot}4}$$

$$= \frac{2{\cdot}87\, E^1_{495}}{E^1_{280} - 0{\cdot}35 E^1_{495}}$$

RESULTS

I. ISOLATION OF THE IgG ANTIBODY FRACTION

A sheep anti-rabbit IgG serum was fractionated by $(NH_4)_2SO_4$ precipitation. The crude globulin fraction was purified by DEAE Sephadex chromatography. Two protein peaks were obtained by elution. The first peak contained slow moving IgG, and the second peak consisted of fast IgG and some transferrin. Precipitating antibodies (titre 1 : 32) were only found in the fast IgG fraction, while a passive haemagglutination test (Boyden, 1951) revealed agglutinins to a titre of 1 : 64 in both the slow and the fast IgG fraction. If conjugated in the same way to the same degree, a very strong activity in the immunofluorescent technique was found for the fast IgG fraction, while slight if any activity was demonstrated with the slow moving IgG fraction.

A rabbit anti-human Ig serum was treated in the same way. DEAE Sephadex chromatography resulted in only one peak, followed by a plateau. The peak consisted of IgG, while the end of the plateau was contaminated with some transferrin. The individual fractions of the eluted proteins revealed a gradually increasing electrophoretic mobility of the IgG molecules. Precipitating activity was found in all fractions, but mainly in the slow moving IgG.

With a horse anti-human Ig serum results comparable to those with the rabbit serum were obtained.

Comparable results were obtained with another three sheep, seven rabbit and four horse antisera.

II. CONJUGATION WITH FITC

The kinetics of the conjugation of FITC to IgG molecules were studied by measuring the influence of some variables in the reaction conditions on the degree of labelling (mol. F/P ratio). The time between mixing of the constituents and the separation of unbound FITC by Sephadex gel-filtration was considered as 'the conjugation time'.

Influence of IgG concentration

The slow IgG fraction of a horse anti-rabbit immunoglobulin serum was conjugated with FITC. Three samples of the horse IgG were brought to a protein concentration of 29·3, 2·9 and 0·58 mg/ml respectively. After addition of the FITC solution, the protein concentration in the three final reaction mixtures was 13·5, 2·3 and 0·55 mg/ml respectively, (further reaction conditions: 12 mg FITC per gram IgG; pH 9·5; room temperature). After 30, 90, 120, 180, 240 and 270 minutes respectively 1 ml samples were taken and after gel filtration the mol. F/P ratio was measured. The samples with protein concentration of 13·5 mg/ml reached an F/P ratio of 2·75 after 30 minutes and a top level of 3·5 after 60 minutes. For the samples with 0·55 mg protein per ml an F/P ratio of only 0·8 was found after 30 minutes. After 120, 180, 240 and 270 minutes F/P values of 1·7, 2·0, 2·3 and 2·4 were found. The samples with a protein concentration of 2·3 mg/ml produced intermediate figures. We therefore demonstrated that with high protein concentrations higher F/P ratios are reached in a shorter time than with low protein concentrations.

Influence of the IgG type

A rabbit anti-human immunoglobulin serum was fractionated into slow, medium and fast moving IgG. Each fraction initially containing 14·4 mg protein per ml was conjugated with

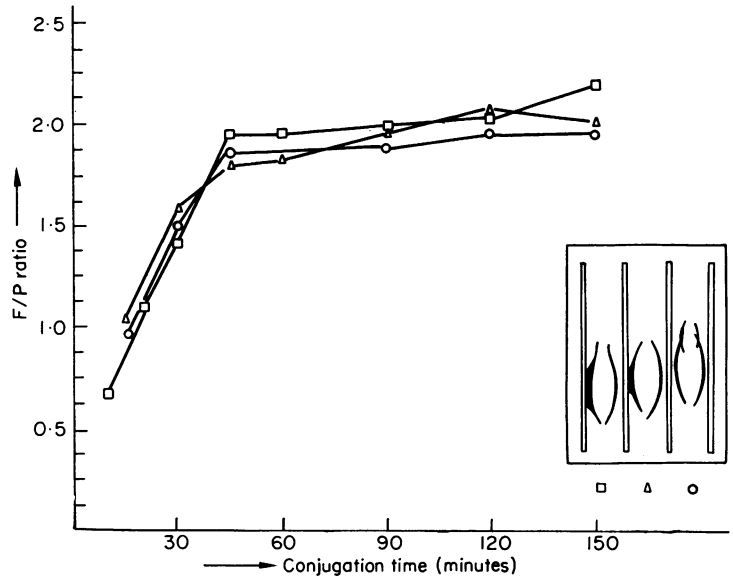

FIG. 1. Conjugation of FITC to slow (□-□-□); medium (△-△-△) and fast moving (○-○-○) rabbit-IgG (anti-human-Ig). Amount of FITC: 8 mg per gram IgG. Further reaction conditions: pH 9·5, temperature 22°, IgG concentration 14·4 mg/ml, reaction time 15, 30, 60, 120 and 150 minutes after correction of pH.

8 mg FITC per gram of protein at room temperature and pH 9·5. The results are presented in Fig. 1. After 45 minutes the maximal F/P ratio was reached for all three IgG samples having all about the same value. Comparable results were obtained for the slow, medium and fast moving IgG fractions of a horse anti-human immunoglobulin serum if these were conjugated in the same way. However, when fractions of a sheep anti-rabbit immunoglobulin serum were conjugated the F/P ratios reached at 45 minutes were 2·5, which is higher than the F/P ratios of rabbit and horse IgG. No differences between the distinct sheep IgG fractions could be demonstrated.

Influence of the pH

Slow IgG from a horse anti-rabbit immunoglobulin serum with an initial protein concentration of 29·3 mg/ml was conjugated with 8 mg FITC per gram protein at room temperature. Four samples of the reaction mixture were studied in which the pH was brought to 7·7, 8·8, 9·5 and 10·0 respectively. A mol. F/P ratio of about 2 was reached in 30 minutes if the pH was 10. The same ratio was obtained at 60 minutes if the pH was 9·5 and at 240 minutes if the pH was 8·8. With a pH of 7·7 an F/P ratio of only 1·3 was reached after 240 minutes. No harmful effect to the fluorescent antibodies could be demonstrated in the immunofluorescent technique, even if the pH was kept at 10·0 for 3 hours.

Influence of temperature

Three samples of a horse slow IgG fraction were conjugated. The temperature of the protein solutions was brought to 0°, 25° and 37° respectively (further reaction conditions: 8 mg FITC per gram IgG; pH 9·5; protein concentration 29·3 mg/ml). The results are presented in Fig. 2. At a temperature of 37°, a relatively high mol. F/P ratio of 2·8 is reached after 30 minutes, while at 25° a mol. F/P ratio of 2·3 is obtained in that time. At 0°, even after 180 minutes, only a mol. F/P ratio of 1·4 is reached.

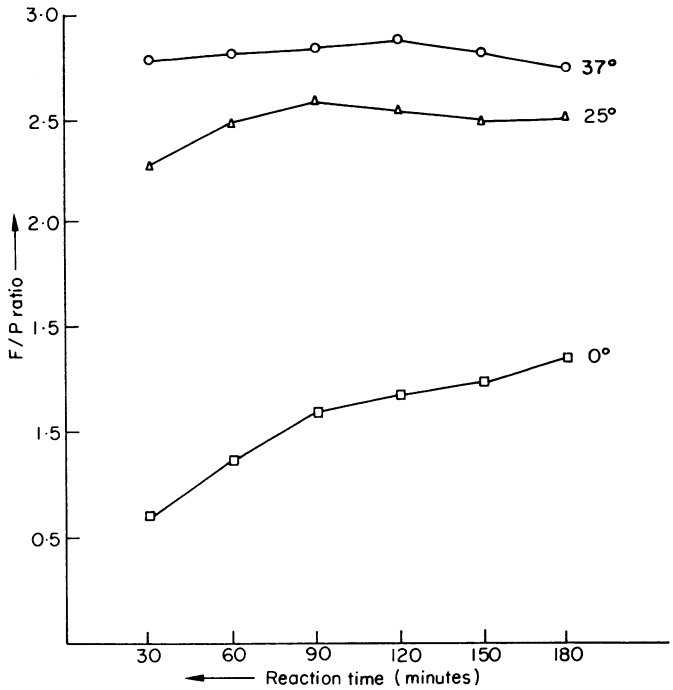

FIG. 2. Conjugation of FITC to horse IgG (anti-rabbit-Ig). Influence of reaction temperature; 0° (□-□-□); 25° (△-△-△) and 37° (○-○-○). Amount of FITC 8 mg per gram IgG. Further reaction conditions: pH 9·5, IgG concentration 29·3 mg/ml (original solution), reaction time 30, 60, 90, 120, 150 and 180 minutes after correction of pH.

Influence of the amount of FITC

Six samples of a slow IgG fraction of a horse anti-rabbit immunoglobulin serum were conjugated for 1 hour with 4, 8, 16, 25, 40 and 60 mg FITC per gram protein respectively (further reaction conditions: IgG concentration 10·8 mg/ml; pH 9·5; room temperature). The results, presented in Table 1, show that the more FITC is added the higher the obtained mol. F/P ratios are. However, this correlation is not linear, as the percentage of bound FITC decreases if increasing amounts of FITC are added.

TABLE 1

INFLUENCE OF AMOUNT OF FITC ON THE PERCENTAGE OF BOUND FITC

Amount of † FITC added to protein (mg/g)	Finally bound FITC protein (mg/g)	Mol. F/P ratio	Percentage* of bound FITC
4	2·8	1·3	70
8	5·5	2·3	68
16	10·6	4·4	66
25	13·9	5·8	55
40	15·9	6·5	40
60	17·8	7·3	30

* $\frac{\text{mg bound FITC : g protein}}{\text{mg originally added FITC : g protein}} \times 100\%$.

† to 'slow' horse IgG (final concentration 10·8 mg protein/ml). Further reaction conditions: pH 9·5; room temperature; conjugation time 1 hour.

III. CHROMATOGRAPHIC PURIFICATION OF FLUORESCENT IgG CONJUGATES

To study the chromatographic purification of IgG conjugates, experiments were performed using isolated IgG from sheep, rabbits and horses, conjugated with different amounts of FITC. After gel filtration on Sephadex G 25 the fluorescent preparations were put on DEAE Sephadex columns equilibrated with PBS. By elution with the same buffer a light coloured band migrated downwards and became separated from a more heavily coloured band. A salt gradient elution was then started. With increasing concentration (0·15 M to 1·0 M) the heavily coloured IgG molecules migrated downwards. The eluted proteins were collected in fractions. The results, presented in Table 2, are summarized as follows:

TABLE 2

RECOVERY OF FLUORESCEIN LABELLED IgG FROM DEAE SEPHADEX A 50 COLUMNS

Conjugate*		mg IgG conjugated (= 100%)	mg FITC per g IgG	mg IgG present in fractions with F/P ratios of			mg IgG recovered
				F/P<1	1≤F/P<4	F/P≥4	
				mg = %	mg = %	mg = %	mg = %
Horse	A	127	5	32 = 25	65 = 51	12 = 9	109 = 86
	B	127	7·5	17 = 13	78 = 61	15 = 12	110 = 87
	C	127	10	8 = 6	62 = 49	27 = 21	97 = 76
	D	127	12·5	6 = 5	52 = 41	36 = 28	94 = 74
	E	127	15	3 = 2	42 = 33	39 = 31	84 = 66
	F	127	20	2 = 2	7 = 6	72 = 57	81 = 64
Sheep	G	72	6	16 = 22	47 = 65	4 = 6	67 = 93
	H	72	8	8 = 11	48 = 67	8 = 11	64 = 89
	I	72	10	3 = 4	35 = 49	20 = 28	58 = 81
Rabbit	J	112	9	16 = 14	69 = 62	19 = 17	98 = 87·5

* Reaction conditions were: initial IgG concentration 25–30 mg/ml; pH 9·5; 1 hour at R.T.

1. DEAE Sephadex chromatography of the conjugates indicated in Table 2 as A-J, resulted in the separation of two protein peaks. The first peak consisted of unlabelled or lightly conjugated IgG with mol. F/P ratios <1. The second peak showed fractions with gradually increasing mol. F/P ratios.

2. The amount of FITC used had a significant influence on the appearance of the elution curve of the IgG conjugate. The more FITC was used, the smaller the first peak and the larger the second.

3. When less than 10 mg FITC per gram IgG was used (conjugates A, B, G, H and J in Table 2) the second peak was fairly symmetrical, narrow and high, and the optical density at 280 nm was, in general, higher than the OD_{495}. This implies that the fractions have a mol. F/P ratio <4·5.

4. When more than 10 mg FITC per gram IgG was used an increasing asymmetry of the second peak was observed (conjugates C, D, E, F and I in Table 2). Many fractions with such peaks had F/P ratios of >4·5.

Recovery of labelled IgG antibody

Table 2 shows that when less than 10 mg FITC per gram IgG was used for conjugation nearly all the proteins (90 per cent) were eluted. The recovery in the range of optimal F/P ratios (1<F/P<4) was 50–75 per cent.

DISCUSSION

Because IgG antibodies are known to have generally a good precipitating activity, while IgM antibodies do not contribute much to the strength of specific fluorescence and easily show non-specific fluorescence (Brooks, Lewis and Pittman, 1965), we preferred, like most authors in this field, IgG antibodies for labelling studies. Goldstein *et al.* (1961) advised anion exchange chromatography for the purification of IgG antibodies. In the present study their method was slightly modified using DEAE-Sephadex instead of DEAE-cellulose while phosphate buffered saline was used for elution. This resulted in a higher IgG recovery, sometimes allowing a separation in slow and fast moving IgG. Brighton (1966) pointed to the importance of the use of chromatographically pure FITC, and McKinney *et al.* (1964) and Klugerman (1965) to the danger of weighing errors, if small amounts of FITC had to be used. We therefore followed their indications.

The present study demonstrated, in agreement with the findings of McKinney *et al.* (1964) and Klugerman (1965), that maximal molecular F/P ratios were reached in the shortest time if the reaction temperature, the pH and the protein concentration were high. In practice optimal conditions are found at room temperature, pH 9·5 and an initial protein concentration of 25 mg/ml respectively. These require a reaction time of 30 to 60 minutes, after which no significant increase of the F/P ratio was found.

As Yagi, Maier and Pressman (1962), Benacerraf, Ovary, Bloch and Franklin (1963) and Pondman, van Es and Schuurs (1965) have shown that antibodies directed against one antigen, even within the IgG subclass, can be separated into immunoglobulins with different immunological properties, the question arose whether electrophoretically distinct IgG molecules are equally coupled with FITC molecules. The results of the present study seem to show that electrophoretically different IgG molecules have about the same binding affinity for FITC.

If the reaction conditions were standardized, the level of the final F/P ratio only depends on the amount of FITC used. However, this relation is not a linear one, as more heavily coupled IgG molecules apparently had a lower affinity for FITC than still unlabelled and the more lightly coupled IgG molecules. This finding supports the supposition of Tokumaru (1962).

After the conjugation reaction the conjugated antibody molecules are not labelled to the same degree. For the separation of the optimally labelled antibodies from under- and over-labelled proteins Goldstein *et al.* (1961), McDevitt *et al.* (1963) and Wood *et al.* (1965) advised the use of anion-exchange columns. However, Fothergill (1962), White (1960) and von Mayersbach (1966) were not satisfied with this method. In the present study no disadvantages were noted if DEAE Sephadex A 50 columns were used for the final purification. Reproducible results were obtained, leading to a recovery of 50–75 per cent of the antibodies with F/P ratios between 1 and 4. These results were obtained by a gradient elution as advised by Goldstein *et al.* (1961). Although this elution method is time consuming, it has advantages over the stepwise elution method described by McDevitt *et al.* (1963) and Wood *et al.* (1965) since minor differences between diverse antibodies to be labelled and their actual conjugation procedure may lead to diversities of the electrical charge of the conjugate to purify, which are sufficient to disturb the reproducibility of stepwise elution procedures. The use of DEAE Sephadex A 50 chromatography for both the purification of the antibodies to be labelled and the purification of the conjugated antibodies implies that the recovery of the optimal labelled antibodies by the final gradient

elution procedure depends only on the degree of conjugation. An F/P ratio of about 2·5 of the unfractionated conjugate was found to have a maximal yield in the optimal F/P ratios. This was achieved by conjugation with much less FITC than described by Marshall, Eveland and Smith (1958). This might be due to differences in purity of the FITC used.

For studies concerning fluorescent conjugation, F/P ratios are an important criterion for the characterization of the final product. However, the exact determination of fluorescein and protein concentration of a conjugate is far from simple. This resulted in different formulas for F/P determination as used by different authors (Wood *et al.*, 1965; Wells, Miller and Nadel, 1966; Jobbagy and Kiraly, 1966). In view of the need for standardization it is advisable to use 'optical density ratios', expressing $E^1_{495} : E^1_{280}$, instead of molecular F/P ratios, if comparison with other reagents is desired (Brighton, Taylor and Wilkinson, 1967). The 'optical density ratios' from the present study may be achieved by the use of the following formula: $\text{OD ratio} = \frac{\text{mol. F/P ratio}}{2{\cdot}87 + 0{\cdot}35 \times \text{mol. F/P ratio}}$

Although it was not the purpose of the present study to examine the relation between the activity of antibodies in immunofluorescent and precipitation techniques, the experience with the slow and fast moving IgG fractions of a sheep anti-rabbit immunoglobulin serum strongly suggest such a correlation. The slow moving IgG fraction revealed agglutinins to tanned red cells coated with rabbit IgG, while no activity in the immunofluorescent and precipitation technique was observed. The fast IgG fraction, however, if conjugated in the same way to the same degree, revealed strong precipitating antibody activity and a very strong activity in the immunofluorescent technique, while the agglutinating antibodies to tanned red cells coated with rabbit IgG were of the same titre as those present in the slow IgG fraction.

This finding supports the correlation between the activity of antibodies in fluorescent and precipitation techniques described by Beutner, Holborow and Johnson (1967). Precipitation methods are therefore probably a reliable test method for the selection of sera for the immunofluorescent technique, while agglutination methods are possibly of less value for this purpose.

The results of the present study led to a standard conjugation method which will be described and discussed separately (The and Feltkamp, 1970).

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