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Technical Considerations in the Preparation of Fluorescent-Antibody Conjugates

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ABSTRACT

LEWIS, VESTER J. (Communicable Disease Center, Atlanta, Ga.), WALLIS L. JONES, JOHN B. BROOKS, AND WILLIAM B. CHERRY. Technical considerations in the preparation of fluorescent-antibody conjugates. Appl. Microbiol. **12**:343–348. 1964.—A comparison was made of $(NH_4)_2SO_4$, HCl, ethodin, and ethanol for fractionation of rabbit antiserum prior to conjugation with fluorescein isothiocyanate. Fractionation with the salt was found to be the method of choice from the standpoints of simplicity and recovery of antibody effective in conjugates prepared from the fractions. Effects of pH, temperature, dye-protein ratio, and molarity and type of buffer upon conjugation were studied. These technical factors were adjusted to produce conjugates for *Corynebacterium diphtheriae* which possessed higher specific titers than did reagents obtained by previously employed techniques.

Few basic modifications of the technique for conjugation of antibody with fluorescein have occurred since the development of the procedure by Coons and Kaplan (1950). The most widely accepted major modification has been replacement of the isocyanate of fluorescein by the isothiocyanate (Riggs et al., 1958).

For efficient utilization of valuable antiserum, the method employed for fractionation should yield most of the antibody originally present, and conjugation of this material should produce reagents of high specific titer. Methods of fractionation and conjugation should be simple, economical, and rapid.

Recently McKinney, Spillane, and Pearce (*personal communication*) studied the effect of temperature, pH, and salt concentration on the rate of reaction of fluorescein isothiocyanate (FITC) with normal serum proteins.

In the present report, selected procedures for fractionation of antiserum prior to conjugation were compared. The results obtained by McKinney, Spillane, and Pearce with normal serum proteins were confirmed in a study of the reactivity of FITC with immune globulins, and the effects of certain additional factors on this reaction were analyzed.

MATERIALS AND METHODS

Fractionation of antisera. All antisera were produced in rabbits. Portions of a pool of *Klebsiella pneumoniae* antiserum were fractionated by: (i) salt precipitation, in which $(NH_4)_2SO_4$ solutions of various molarities were added slowly to constantly stirred antiserum maintained at 4 C; (ii) acid fractionation, carried out by the addition of 9 volumes of 0.0027 N HCl to 1 volume of antiserum at 4 C (Fife and Muschel, 1959); (iii) ethodin (2-ethoxy-6,9-diaminoacridine-lactate; Winthrop Laboratories, New York, N.Y.) fractionation consisting of the addition of five parts of a 0.4% aqueous solution of the acridine to one part of antiserum, a modification of the method of Frommhagen and Martins (1963); and (iv) ethanol fractionation according to Nichol and Deutsch (1948).

Antisera to group A streptococci and *Escherichia coli* were fractionated at 4 C by addition of $(NH_4)_2SO_4$ solution to a final concentration of 1.95 M. This technique was employed for fractionation of antiserum to *Corynebacterium diphtheriae*, except that the final concentration of $(NH_4)_2SO_4$ in the antiserum was 1.56 M.

Characterization of fractions. All supernatant liquids and precipitates from each fractionation procedure were examined. Electrophoresis was carried out on cellulose acetate strips with a Shandon Universal Electrophoresis apparatus (Consolidated Laboratories, Inc., Chicago Heights, Ill.). The strip area which was occupied by each band after staining with light green SF dye (Allied Chemical and Dye Corp., New York, N.Y.) was determined by planimeter. For immunoelectrophoresis, 0.5% agar prepared with 0.0375 M barbital buffer of pH 8.6 was used on glass slides. Protein concentrations were determined by the biuret reaction (Gornall, Bardawill, and David, 1949). Hemagglutination tests with sensitized sheep red blood cells were performed by use of the technique described by Neter, Bertran, and Rabesman (1952). For agglutination tests, 0.2 ml of dilutions of the antiserum fractions in physiological saline were mixed with 0.2 ml of antigen suspension. Readings were taken after 4 hr at 37 C and again after standing overnight at 4 C.

Conjugation of serum proteins. The FITC content of the lot of fluorochrome employed for conjugation was 80%, as shown by infrared spectrophotometric determinations performed by R. M. McKinney of the Communicable Disease Center. Accordingly, 1.25 mg of the lot were used for each milligram of FITC desired. The amounts of FITC stated in the report are of pure FITC, rather than of the total weight of dye used.

Except where otherwise stipulated, conjugation was carried out by the technique of Riggs et al. (1958), with

the modification that acetone was excluded from the reaction mixture and the FITC was added to the reaction flask as a slurry in the buffer. Conjugates were freed from unreacted fluorescent material by passage through columns of Sephadex G-25 (Pharmacia, Uppsala, Sweden), a technique which preliminary experiments showed to be as effective as dialysis.

Characterization of conjugates. Fluorescein concentrations of the conjugates were determined by absorbance at a wavelength of 485 mμ. Fluorescein amine was used in construction of the standard curve relating absorbance to concentration. For the determination of the F-P ratios, the concentration of fluorescein amine in the conjugate was expressed as FITC, in accord with the suggestion of McKinney and Pearce (*personal communication*). This was done by dividing the concentrations of fluorescein amine found from the curve by 0.89, the ratio of the molecular weight of aminofluorescein to that of FITC. No correction was made for possible difference between the extinction coefficients of the conjugated dye and the unconjugated standard. The protein content of the conjugates was found by the biuret test, reading absorbance of the biuret-protein complex at a wavelength of 560 mμ. The F-P ratio represents micrograms of FITC per milligram of protein in the conjugate.

Fluorescent-antibody (FA) titers were obtained by staining fixed bacterial smears for 30 min with dilutions of the conjugates, rinsing in 0.01 M phosphate-buffered 0.85% saline of pH 7.2 for 10 min, gently blotting, and mounting with buffered glycerol-saline and a cover slip. The stained smears were examined with a fluorescence assembly employing an Osram HBO-200 mercury arc lamp and a Schott BG-12 (3 mm) primary filter coupled with a Schott OG-1 (2 mm) barrier filter. The observer was unaware of the history of the conjugates under test. Unless otherwise stated, conjugates were adjusted to the same final volume and the pH was brought to 7.3 before comparisons of staining titers were made.

RESULTS

Comparison of serum fractionation techniques. Less than 60% of the γ-globulin in the antiserum to *K. pneumoniae* was recovered by fractionation with $(NH_4)_2SO_4$ at final concentrations of 0.98, 1.17, and 1.37 M. Greater concentrations of the salt gave higher yields. Only 75% of the amount of γ-globulin recovered from antiserum containing 1.95 M $(NH_4)_2SO_4$ was obtained when the molarity of the salt was 1.56 (Table 1). Conversely, contamination of the globulin fraction by albumin was reduced fivefold upon decrease of the salt molarity from 1.95 to 1.56 M. Conjugates obtained from samples of antiserum which were 1.56, 1.77, and 1.95 M with respect to final concentrations of $(NH_4)_2SO_4$ possessed identical FA titers when adjusted to the same concentration of γ-globulin. A 1.95 M solution was produced by the addition to serum of an equal volume of an aqueous solution of $(NH_4)_2SO_4$ that was saturated at 4 C.

Euglobulin was separated from pseudoglobulin by dialysis of redissolved $(NH_4)_2SO_4$ precipitates against distilled water. It was found that agglutination, hemagglutination, and FA activities were not restricted to water-soluble pseudoglobulin, but also occurred in the euglobulin fraction.

In $(NH_4)_2SO_4$ fractionation of serum in this laboratory prior to the present study, centrifugation of the precipitate formed by the initial addition of $(NH_4)_2SO_4$ to the serum was delayed routinely until the next day. Experiments were performed to determine whether the delay in centrifugation resulted in increased recovery of antibody. Samples of a pool of antiserum to *C. diphtheriae* were fractionated at a final concentration of 1.56 M $(NH_4)_2SO_4$. Samples were centrifuged for 45 min at 1,500 × *g* in a conical tube immediately and 2, 4, 6, and 21 hr after addition of the salt. Recovery of antibody was as complete when centrifugation was begun 4 hr after addition of the $(NH_4)_2SO_4$ solution as when centrifugation was delayed 21 hr, as judged by the amount of protein recovered and by the FA titers of conjugates prepared from the fractions.

Precipitation of globulin with HCl was inefficient. Only 0.2 g of γ-globulin was recovered per 100 ml of antiserum, but this was almost free from albumin (Table 1). Conjugates prepared from the supernatant fluid showed substantial FA titers, indicating the presence of unprecipitated antibody.

After treatment of antiserum with ethodin to precipitate protein other than γ-globulin, the latter was recovered from the supernatant fluid by addition of an equal volume of 3.90 M $(NH_4)_2SO_4$ solution. Cellulose acetate strip elec-

TABLE 1. *Comparison of fractionation methods using rabbit antiserum for Klebsiella pneumoniae*

Fractionation	Yield per 100 ml of serum		Specific staining titer of conjugates[a]
	Albumin	Globulin	
	g	g	
Unfractionated serum	4.1	1.0	1:2
$(NH_4)_2SO_4$			
1.56 M[b]	0.02	0.6	1:8
1.77 M[b]	0.04	0.7	1:8
1.95 M[b]	0.10	0.8	1:8
HCl, 0.0027 M	0.02	0.2	1:4
Ethodin[c]	0.00	0.4	1:4
Ethanol[d]			
Precipitate C-1[e]	0.00	0.4	1:4
Precipitate C-2[f]	0.00	0.2	1:16

[a] Conjugates adjusted to γ-globulin concentration of 0.2% before titration.

[b] Final concentration in serum.

[c] The fractionation with ethodin was a modification of the technique of Frommhagen and Martins (1963).

[d] Technique of Nichol and Deutsch (1948).

[e] Precipitate C-1 should be largely γ_1-globulin (Nichol and Deutsch, 1948).

[f] Precipitate C-2 should be largely γ_2-globulin (Nichol and Deutsch, 1948).

trophoresis (CASE) revealed a 40% recovery of the antiserum γ-globulin (Table 1).

Upon ethanol fractionation, precipitate C-1 contained 40% of the γ-globulin present in the original antiserum, and precipitate C-2 yielded 20% (Table 1). Only γ-globulin was detected in these fractions by CASE. According to Nichol and Deutsch (1948), precipitate C-1 from antisera fractionated by their technique contains predominately γ_1 globulin and precipitate C-2 is largely γ_2 globulin. $Gamma_1$ was not differentiated from $gamma_2$ by the CASE employed in the present study.

Agglutination titers of 33 antiserum fractions were compared with the specific FA titers of conjugates prepared from these fractions. Supernatant fluids as well as precipitates obtained by the four fractionation techniques employed in this study were used in making the comparison. Table 2 reflects the observation that the correlation was not close between the agglutination titers of the various antiserum fractions and the FA titers of the corresponding conjugates. Hemagglutination titers, although higher than agglutination titers, generally paralleled the latter.

FA activity was associated with γ-globulin as expected. Invariably, FA staining was obtained only with conjugates from antiserum fractions that contained γ-globulin detectable by CASE. In contrast, 12 of the fractions that possessed FA activity upon conjugation lacked α-globulin, and no β-globulin was demonstrated in 3 of these 12 fractions.

FITC reacts more rapidly with albumin than with the globulins (McKinney and Pearce, *personal communication*). When albumin is present as a contaminant of γ-globulin solutions, it may be expected to combine with a disproportionate amount of FITC by virtue of its greater affinity for the dye. An experiment was performed to determine how high an albumin-globulin ratio was required during conjugation to depress the specific staining titer of the resulting conjugate. γ-Globulin of greater than 95% purity was obtained by ethanol fractionation (Nichol and Deutsch, 1948) of rabbit antiserum for *E. coli*. The fractionation was performed through the courtesy of Kent Miller, New York State Health Department. Normal rabbit albumin of comparable purity was obtained commercially (Pentex, Inc., Kankakee, Ill.). The albumin and globulin were used to prepare solutions containing varying ratios of these proteins. All solutions contained the same amount of γ-globulin and varying amounts of albumin, to give a final protein concentration of 1%. Half of each solution was conjugated at a FITC-protein ratio of 1:20, and half at a ratio of 1:40.

Results of FA titration of the conjugates are shown in Table 3. The specific staining titer was depressed at an albumin-globulin (AG) ratio of 2:4 when the FITC-protein ratio during conjugation was 1:20. The depression became greater as the AG ratio was increased. Competition of albumin with globulin for FITC was apparent at lower AG ratios when the FITC-protein ratio during conjugation was decreased to 1:40.

Comparison of conjugation techniques. A pool of antibacterial serum for *C. diphtheriae* was fractionated by $(NH_4)_2SO_4$ at a final concentration of 1.56 M. After adjustment of the globulin fraction to 1% protein, conjugates were prepared from samples by four techniques. Method I was based on a conjugation technique developed by McKinney and Pearce (*personal communication*), and consisted of the addition of 4 ml of 0.15 M phosphate buffer (pH 9) to 10 ml of the globulin solution, followed by 4 ml of 0.1 M phosphate buffer (pH 8) that contained sufficient freshly dissolved FITC to produce a FITC-protein ratio of 1:20 for conjugation. The pH was adjusted to 9 with 0.1 N NaOH, after which the volume was brought to 20 ml with 0.15 M NaCl. All solutions were warmed to 25 C before use, and the conjugation reaction was allowed to proceed for 21 hr at this temperature. Method II was that of Marshall, Eveland, and Smith (1958), the FITC being added as a

TABLE 2. *Relationship of agglutination titers of antiserum fractions* to Klebsiella pneumoniae to the FA titers of the corresponding conjugates*

Agglutination titers of fractions	No. of conjugates staining at a dilution of						
	Undiluted		1:2	1:4	1:8	1:16	1:32
	Negative	Positive					
<1:2	6	1		2		1	
1:2	2						
1:4	2				1		
1:8	2						1
1:16	1				1	2	1
1:32		1					2
1:64				2	2		1
1:128					1		
1:256						1	

* Fractions (including supernatant fluids) obtained by four fractionation methods.

TABLE 3. *Effect upon the FA titer of the albumin-globulin ratio during conjugation of antiglobulin for Escherichia coli*

Amt of FITC present per mg of protein during conjugation	Albumin-globulin ratio for conjugation	FA titer of product	F-P ratio of product*
mg			
0.050	0:4	1:256	21
0.050	1:4	1:256	24
0.050	2:4	1:128	25
0.050	3:4	1:64	24
0.050	4:4	1.64	24
0.025	0:4	1:256	11
0.025	1:4	1:128	13
0.025	2:4	1:32	12
0.025	3:4	1:32	13
0.025	4:4	1:32	13

* F-P ratio represents micrograms of conjugated FITC per milligram of protein.

dry powder to the reaction mixture. Method III consisted of conjugation by the procedure described by Riggs et al. (1958). Method IV was the technique of Riggs et al. (1958), modified in three details: (i) the 1% globulin solution was not diluted further before conjugation, (ii) the FITC was added to the buffered globulin as a slurry in carbonate-bicarbonate buffer instead of being added in acetone, and (iii) no acetone was employed in the conjugation mixture. Method IV was employed routinely to prepare conjugates for *C. diphtheriae* in this laboratory prior to the present investigation (Moody and Jones, 1963).

After dilution of all conjugates to the same volume, titration demonstrated that method I yielded the most potent FA reagent (Table 4). Substitution of carbonate-bicarbonate buffer for phosphate buffer in method I failed to improve the product.

The effect of pH on conjugation of fractionated antiserum to the diphtheria bacillus was examined. Method I (above) was employed, with phosphate buffers of appropriate pH values substituted for the pH 9 buffer. Reaction mixtures were adjusted to the following pH values immediately before the flasks were placed in the 25 C water bath: 2, 3, 4, 5, 6, 7, 8, 9, 9.5, 10, 10.5, 11, and 12. In Table 5 are depicted the results of representative experiments. Conjugation within the pH range of 8 to 11 produced reagents having the highest specific staining titers and highest F-P ratios. The titer of the conjugate made at pH 12 was much lower, although the F-P ratio was high.

Temperatures of 25, 37, and 56 C were used with method I for conjugation of samples of antiglobulin for *C. diphtheriae*. No temperature tested was markedly superior, as judged by specific titers and F-P ratios. Likewise, variation of the molarity of pH 10.5 phosphate buffer from 0.05 to 0.5 M for conjugation by method I failed to affect greatly the titer or F-P ratio of the product.

The FITC-protein ratio for conjugation by method I at pH 10.5 was varied from 1:5 to 1:80. The final protein concentration of antiglobulin for *C. diphtheriae* in the reaction flasks was 0.5%. No increase in specific titer was apparent when the FITC-protein ratio during conjugation exceeded 1:20, although the F-P ratio of the products continued to increase (Table 6).

The possibility of simplification of the method I conjugation technique was explored. The specific titers for *C. diphtheriae* and the F-P ratios of conjugates prepared by the simplified technique described below were at least as high as those of conjugates prepared from the same antibody pool by any of the other methods described in this report. (i) Dye was dissolved in 0.1 M phosphate buffer (pH 10.5) to a final concentration of 0.625 mg of FITC per ml. (ii) An 8-ml amount of the freshly prepared solution of FITC was added dropwise with agitation to 10 ml of a solution of antiglobulin (1% protein) for *C. diphtheriae*. (iii) The pH of the conjugation mixture was adjusted to 10.5 with 0.1 N NaOH. (iv) The volume was brought to 20 ml with 0.15 M NaCl solution. (v) Conjugation was carried out at 25 C, the temperature to which the globulin and the other reagents were adjusted prior to conjugation. Agitation of the conjugation flask during labeling was found to be unnecessary, since it did not improve the product.

The velocity of the labeling reaction was studied by use of the above procedure, and the FA titers of conjugates for

TABLE 4. *Comparison of conjugation techniques using antiglobulin for Corynebacterium diphtheriae*

Method	Conjugation technique*	FA titer†
I	25 C, phosphate buffer, dye added in buffer	1:128
II	4 C, carbonate buffer, dye added as dry powder	1:32
III	4 C, carbonate buffer, dye added in acetone	1:16
IV	4 C, carbonate buffer, dye added in buffer	1:64

* All conjugations were allowed to proceed for 21 hr.

† All conjugates were adjusted to the same volume before titration.

TABLE 5. *Effect of pH during conjugation* of antiglobulin for Corynebacterium diphtheriae on the FA titers and the F-P ratios of the products*

pH of conjugate	FA titer of product	F-P ratio of product†
2.0	4	1
3.0	16	2
4.0	4	2
5.0	16	8
6.0	4	6
7.0	64	8
8.0	128	18
9.0	128	22
9.5	128	24
10.0	256	24
10.5	256	25
11.0	128	30
12.0	16	21

* Conjugation carried out for 21 hr (25 C) at a final protein concentration of 0.5% in phosphate-buffered saline; 0.05 mg of FITC offered per mg of protein for conjugation.

† F-P ratio represents micrograms of conjugated FITC per milligram of protein.

TABLE 6. *Results of labeling* antiglobulin for Corynebacterium diphtheriae at various fluorescein isothiocyanate-protein ratios*

Amt of FITC present per mg of protein during conjugation	Titer of product	F-P ratio of product†
mg		
0.2000	1:128	70
0.1000	1.64	44
0.0500	1:128	26
0.0250	1:8	15
0.0125	1:2	8

* Conjugation carried out for 21 hr (25 C) at a final protein concentration of 0.5% in phosphate-buffered saline.

† F-P ratio represents micrograms of conjugated FITC per milligram of protein.

C. diphtheriae were found to be 1:50 at 2 hr, 1:75 at 4 hr, and 1:100 at 6, 21, and 48 hr.

Microbial growth can occur in the reaction flask when conjugation is carried out at 25 C for 21 hr. This was prevented by the addition of Merthiolate. Neither the specific titer nor the F-P ratio of the product was adversely affected by the presence of this preservative at a final concentration of 1:1,000, the highest tested.

It was necessary to learn whether cross-staining was increased in the conjugates prepared by the simplified procedure. Accordingly, conjugates were prepared from samples of a pool of fractionated antiserum for *C. diphtheriae* by both the 25 C simplified technique detailed above and by method IV (4 C) described earlier. A 1:80 dilution of the conjugate labeled at 25 C and a 1:12 dilution of the conjugate labeled at 4 C stained six strains of *C. diphtheriae* equally well and failed to stain five diphtheroid strains. The diphtheroid cultures were chosen as test strains, because previously they were stained by low dilutions of conjugates for the diphtheria bacillus.

For further comparison of the two conjugates with respect to the degree of undesirable staining, pooled washings from normal throats were centrifuged, and the sedimented organisms were suspended in a small volume of Heart Infusion Broth. Serial dilutions of strains of *C. diphtheriae* were added to tubes containing the concentrated normal throat flora. After thorough mixing, replicate smears were prepared. These were stained with the 4 C conjugate (1:12 dilution) and the 25 C conjugate (1:80 dilution). The smallest number of diphtheria bacilli detectable in the presence of normal throat flora and the degree of nonspecific fluorescence were identical with both conjugates.

The stability of conjugates that were prepared at a pH of 10.5 was compared with the stability of conjugates labeled at pH 9.0. Samples of an antiglobulin pool for *C. diphtheriae* were labeled at these pH values. Sephadex chromatography removed unreacted fluorescent material from the conjugates and brought the pH to 7.2. The specific FA titers of portions of each conjugate were determined; Other portions were stored at 4 C and −20 C for 50 days. At the end of this time, no conjugate had decreased in titer.

The simplified labeling procedure with phosphate buffer was compared with the usual method for preparation of conjugates specific for enteropathogenic *E. coli* (Thomason et al., 1961). Reagents prepared by the two methods from samples of antiglobulin for *E. coli* were equal in both FA titer and F-P ratio.

Streptococcal conjugates prepared by the simplified technique were compared with conjugates made from the same antiserum pool according to standard procedures (Moody et al., 1963). No significant difference existed between the two conjugates in either specific staining titer or F-P ratio.

DISCUSSION

A satisfactory method for fractionation of antiserum prior to conjugation should yield a product which contains most of the antibody of the original serum and which is relatively free from nonantibody protein. These requirements were fulfilled best by $(NH_4)_2SO_4$ fractionation, a method which also was simple and inexpensive.

The higher $(NH_4)_2SO_4$ concentrations employed increased the per cent recovery of γ-globulin, but also increased contamination with other proteins. The presence of labeled albumin in conjugates to be used on tissues may increase nonspecific staining. In such instances, 1.56 M $(NH_4)_2SO_4$ fractionation may be preferable, with the sacrifice of some γ-globulin in exchange for increased purity. However, the globulin fraction obtained from rabbit antiserum with 1.95 M $(NH_4)_2SO_4$ was adequate for conjugates employed to stain bacterial smears in the present study.

Both the amount and specific FA titers of globulins recovered by $(NH_4)_2SO_4$ fractionation were higher than by ethodin treatment. Frommhagen and Martins (1963) also observed that conjugates from $(NH_4)_2SO_4$-fractionated antisera stained more intensely than did conjugates from globulins obtained by fractionation with the acridine. These investigators suggested that antibody recovery was greater upon fractionation with the salt.

Fractionation by use of HCl or ethanol offered no advantages over $(NH_4)_2SO_4$. Likewise, separation of the water-soluble from the water-insoluble globulin of the salt-fractionated antiserum was not helpful, antibody being present in both components.

When the amount of FITC available for conjugation of a mixture of albumin and γ-globulin was below a critical level, combination of the FITC with antibody was restricted by the competitive reaction of FITC with albumin. The albumin-globulin ratio in antiserun fractionated by 1.95 M $(NH_4)_2SO_4$ was 1:8 (Table 1). This was safely below the ratio at which the competition of albumin with globulin for available FITC during conjugation was shown to lower the FA titer of the product. Therefore, the competitive reaction presented no problem when the amount of FITC employed for conjugation was 0.025 mg or more per mg of protein precipitated by 1.95 M $(NH_4)_2SO_4$ (Table 3).

The concentration of $(NH_4)_2SO_4$ used to fractionate antiserum is expressed commonly in the literature in terms of per cent saturation of an aqueous solution with this salt. The temperature must be specified, because the solubility of this salt is temperature-dependent. For accuracy, the concentration of $(NH_4)_2SO_4$ should be stated as molarity after addition to the antiserum.

FA titers were not paralleled closely by the agglutination titers of the serum fractions. This lack of agreement can be accounted for partly by the imprecision inherent in measurement of agglutination and FA activity. The precision of tube agglutination titers under optimal conditions does not exceed a factor of 2 (Kabat and Mayer, 1961). FA titrations are subject to errors of dilution and to

errors resulting from subjective impressions of fluorescence intensity. Complete correlation between FA staining titers and titers obtained by other serological tests would be surprising in view of the clear evidence that the antibodies detectable by the FA test and other tests are not always the same (Nairn, 1962).

Definition of the optimal conditions for the reaction of FITC with globulin revealed that conjugates for *C. diphtheriae* produced in the presence of phosphate buffers at 25 C possessed higher specific titers than did those made by the usual techniques. When more than 0.05 mg of FITC per mg of protein was present during conjugation, an increase was observed in the F-P ratios of the products, but FA titers were not increased concomitantly (Table 6). Possibly antibody lost ability to combine with antigen when heavily labeled by high concentrations of FITC.

For successful conjugation, the conditions of the reaction must allow adequate combination of FITC with antibody before the FITC is degraded to a form incapable of labeling protein and before the antibody has been denatured. These requirements were satisfied by the simplified conjugation procedure for antiglobulin to *C. diphtheriae*; otherwise, conjugates prepared by the simplified technique would have been unsatisfactory. A pH of 10.5 and a temperature of 25 C were employed in this technique. Although Frommhagen and Spendlove (1962) observed that FITC was degraded rapidly in saline at pH 9.0 or higher and that the rate of degradation was greater at 37 C than at 4 C, these observations are not necessarily in conflict with results of the present study. The velocity of the labeling reaction may have been increased by pH 10.5 and 25 C more than was the degradation of the FITC. Also, the offering of 0.05 mg of FITC per mg of protein for conjugation may have allowed a sufficient quantity of FITC to escape degradation and remain available for labeling, since Frommhagen and Spendlove (1962) found that only 10 to 25% of the FITC was degraded after 16 hr at a pH of 9 or above.

Further study may reveal that a specified F-P ratio and protein concentration are characteristic of conjugates possessing the characteristics of maximal specific and minimal nonspecific staining in reference to a given antigen-antibody system. It should prove possible to produce conjugates of predetermined F-P ratios by control of the conditions used for labeling (McKinney and Pearce, *personal communication*).

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