Immunochemical Study of the Diphtheria Toxin-Fluorescent Antitoxin System

By W. F. HEAD, Jr.†

Six fluorescent diphtheria antitoxins were prepared. These products were then examined for excess adsorbed fluorescein, chemically bound fluorescein, and reactivity toward diphtheria toxin and toxoid. Particular attention was paid to antitoxic potency loss inasmuch as it was desired to find optimum methods of preparation wherein maximum labeling and, therefore, higher sensitivity could be obtained with maximum retention of activity. The fluorescence of the floccules formed at the equivalence point in the immunochemical titration of toxin with fluorescent antitoxin was shown to be directly proportional to the biological potency of the toxin.

COONS and Kaplan (1) were the first to demonstrate the fluorescent antibody technique by determining the localization of specific antigens in tissues using the homologous fluorescent antibody. Many similar diagnostic tests have appeared in the literature (2–7) since that time; however, only empirical data are available for preparation of fluorescent antibodies and quantitative information concerning the antigen-fluorescent antibody reactions is lacking entirely.

The excellent sensitivity afforded by fluorescence measurements combined with the high degree of specificity involved in antigen-antibody reactions would seem to justify examination of quantitative data for possible application as an analytical method. The principle of such a quantitative procedure rests upon the proportionality of the biological potencies of various toxins or toxoids to the weights of antigen-antibody complexes at the equivalence points in their titrations. It is also necessary that the ratio of antigen to antibody at these points be constant. If these requirements are met, a randomly fluoresceinated antibody should carry amounts of fluorescein into the precipitated complex which are proportional to potency. This has been accomplished in connection with antibody-dye complexes on a colorimetric basis (8).

EXPERIMENTAL

Materials.—Diphtheria antitoxin concentrate (Lilly lot No. 729077) was used for the preparation of all fluorescent antitoxins. This material had a potency of 3,800 guinea pig protective units per ml., 3,700 *Lf* (flocculating) units per ml., and a *Kf* value of 4 minutes. The total protein content, chiefly gamma globulin, was 20%. The antitoxin was of equine origin. Various diphtheria toxins and toxoids were also supplied by Eli Lilly and Co.

Fluorescein amine and fluorescein isothiocyanate were obtained from Nutritional Biochemicals Corp. Reagent grade formamide was purchased from Matheson, Coleman, and Bell.

Preparation of Fluorescent Antitoxins.—The initial preparation was made by coupling fluorescein isocyanate to the antibody molecule as previously described (1). The dialysis purification was carried out at room temperature until no appreciable fluorescence was observed in the external fluid (17 to 20 days). The product was adjusted to a convenient volume with 0.9% saline and made 1:10,000 in thimerosal. All subsequent preparations were made by essentially the same process with the exception of using fluorescein isothiocyanate and maintaining a cold temperature, 4 to 6°, throughout the process, including the dialysis.

Titration of Diphtheria Toxin or Toxoid with Homologous Fluorescent Antitoxin.—A classical Ramon titration was employed wherein small increments of antitoxin were added to constant amounts (0.5 ml.) of toxin or toxoid in several tubes. The tubes were brought to equal volume, 2 to 4 ml. depending on antitoxin potency, with phosphate buffer, pH 7.4, 0.066 *M*, then either incubated at 50° for 2 hours and stored overnight under refrigeration or incubated overnight at 50° and allowed to stand an additional day at room temperature to reach equilibrium. These procedures were used to insure quantitative precipitation of floc. Usually, toxin flocs were refrigerated and toxoid flocs were developed at room temperature. It was found that toxoid flocs with fluorescent antitoxin were partially redissolved when cold, and additional floc would form as the tubes warmed to room temperature. The precipitates were separated by centrifuging and were resuspended and washed once with 1 to 3 ml. of 0.9% saline to remove residual unreacted fluorescent material.

Measurement of Floc Fluorescence.—In order to measure the fluorescein, the flocs had to be redissolved after washing. A study was undertaken to find the appropriate solvent. Of 25 solvents tested, formamide proved to be the most acceptable, dissolving large quantities of floc and simultaneously enhancing fluorescence because of its basic qualities.

The flocs were dissolved with either 3 or 4 ml. of formamide. Fluorescent intensity measurements

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† Present address: School of Pharmacy, Auburn University, Auburn, Ala.
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were taken with an Aminco-Bowman spectrophotofluorometer using an activation wavelength of 490 mμ. The emission intensity at 525 mμ was recorded with the appropriate sensitivity setting. To standardize the instrument, a formamide solution of fluorescein, containing 0.333 mcg./ml., was used. A typical fluorescent floc emission spectrum is shown in Fig. 1.

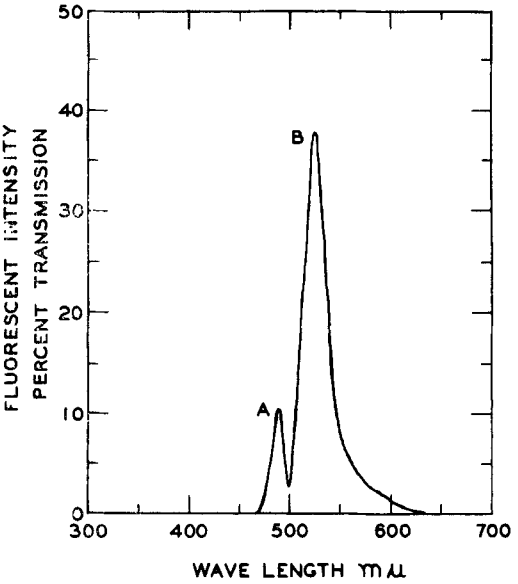

Fig. 1.—Emission spectrum of a fluorescent diphtheria floc. Activation 490 mμ. A, Activation crossover peak; B, fluorescent emission peak.

Standard Curves.—The fluorescent intensities of all flocs, adjusted to the same instrumental sensitivity setting, were plotted *vs.* the corresponding flocculating units of antitoxin added. These curves, as seen in Fig. 2, have the general shape of the diphtheria precipitin curve obtained by weight or nitrogen analysis. If toxins or toxoids of different potencies were used, a plot of the various maximum fluorescent intensities at the equivalence points in the individual titration curves *vs.* potency, either in *Lf* units or guinea pig protective units, would be linear with zero intercept. The slope would be determined by the degree of fluorescein labeling of the antitoxin. This concept is shown in Fig. 3. Construction of these standard curves with each fluorescent antitoxin was not necessary for quantitative potency determinations; it was even more accurate to test a standard toxin along with an unknown and compare equivalence point floc fluorescence and potency on a proportional basis.

Comparison Analytical Method.—The standard *Lf* test was used as a reference method with which to compare the fluorescent antibody method and to determine flocculating power of various antigens and antitoxins. This method is a titration similar to that already described using small increments of standard antitoxin or toxin. The tubes were incubated at 50° and constant observation maintained until the first floccules appeared. The tube showing the first trace of floc is the equivalence point tube and the time required for flocculation

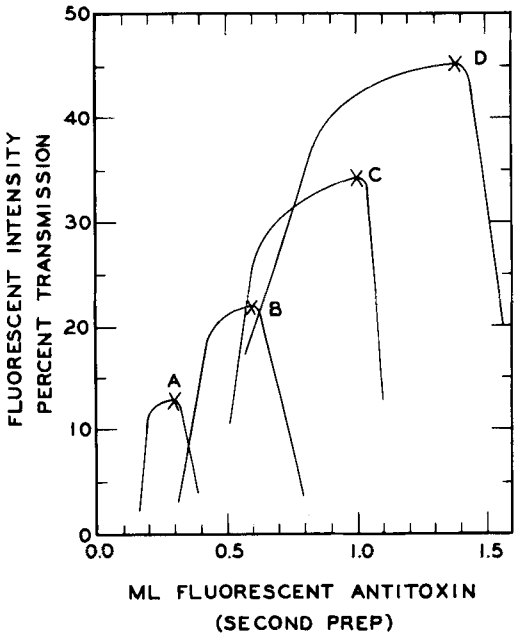

Fig. 2.—Precipitin curves obtained by measurement of floc fluorescence. Curve A, diphtheria toxin potency, 27.5 *Lf*/ml.; curve B, diphtheria toxin potency, 55 *Lf*/ml.; curve C, diphtheria toxin-potency, 82.5 *Lf*/ml.; curve D, diphtheria toxin potency, 110 *Lf*/ml.; × indicates the equivalence points.

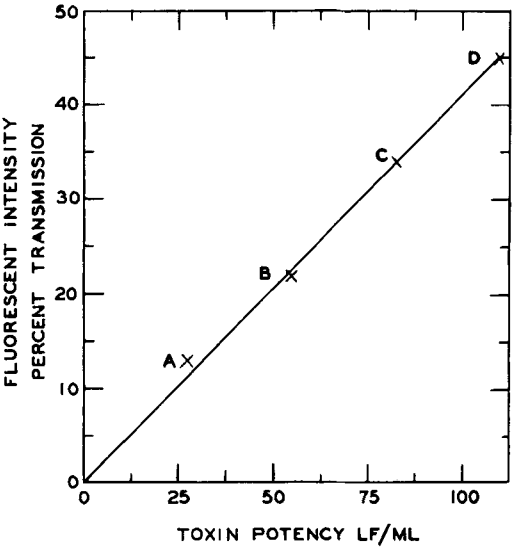

Fig. 3.—Equivalence point fluorescence (Fig. 2) *vs.* toxin potency. A, B, C, D, toxin potencies (same as Fig. 2).

to occur is the *Kf* value. This value is indicative of the degree of denaturation, a longer time being required for flocculation by a more highly denatured toxin or antitoxin. The standard antitoxins or toxins were National Institutes of Health primary standards or secondary standards derived from them.

TABLE I.—CHARACTERISTICS OF VARIOUS FLUORESCENT ANTITOXINS

Prepn.	Potency Loss (from Original) Toxin, %	Potency Loss (from Original) Toxoid, %	Reactants: Fluorescein Derivative, mg.	Reactants: D.A.T.C.,[a] ml.	*Kf*, min.	Inert Adsorbed Fluorescein	S.P.N.,[b] %	Fluorescein Ratio[c]
No. 1	58	100	50	5	25	[d]	[d]	[d]
No. 2	28.5	100	50	5	15	Yes	9.36[e]	5.35
No. 3	20.5	100	50	10	10	Yes	10.44[e]	5.4
No. 4	44	44	[f]	[f]	8	No	5.14	5.25
No. 5	0	0	4	10	5	Very slight	5.54[g]	1.5
No. 6	0	0	20	10	5	Yes	5.76	4.25

[a] Diphtheria antitoxin concentrate. [b] Specifically precipitable nitrogen. [c] Number of fluorescein molecules per antitoxin molecule. [d] Not determined. [e] These values determined with use of toxin, giving greater precipitability than the last three, which were determined with the use of toxoid. [f] Preparation made from No. 3. [g] Nitrogen content per *Lf* toxoid, used in calculating this value, was calculated from data obtained from the reaction of No. 4 and No. 6 antitoxins with the same toxoid.

Determination of Specifically Precipitible Nitrogen and Fluorescein to Antitoxin Ratios.—Since diphtheria antitoxin concentrate contains a considerable amount of inert globulin, gross fluorescence and total protein measurements will not give much information concerning that fluorescein bound to antitoxic globulin molecules. To obtain these data, a method described by Kabat and Mayer (9) was used. This technique enables the determination of the amount of antitoxin precipitated. Fluorescence measurements of the dissolved flocs reveal the amount of fluorescein present. From these figures, the degree of labeling is easily calculated. The molecular weight value of the antitoxin molecule is 184,000 (10).

RESULTS AND DISCUSSION

First Fluorescent Antitoxin.—By means of *Lf* tests, this material was found to have lost 58% of its potency against toxin and 100% of its antitoxoid activity. The *Kf* value was 25 minutes. In addition to potency loss, only a slight similarity to typical precipitin curves could be obtained. The material, after standing for a few weeks under refrigeration, deposited an appreciable amount of granular fluorescent material and was considered to be an altogether unsatisfactory preparation. A summary of the proportion of reactants and the characteristics of this and all other fluorescent antitoxins prepared is given in Table I.

Second Fluorescent Antitoxin.—Toxoid flocculating power was again destroyed, but 72.5% of the toxin flocculating ability was retained. The *Kf*, being 15 minutes, was still 11 minutes longer than the original unconjugated antitoxin. A standard curve was prepared using this antitoxin with good linearity and zero intercept. Twenty-five *Lf*, corresponding to approximately 0.25 mg. toxin protein, was the smallest amount of toxin that could be accurately assayed. Since the *Kf* of this fluorescent antitoxin was still comparatively long, it was possible that excess fluorescein had been used and was occupying specific reactive sites in the molecule without contributing materially to the fluorescence of the flocs.

Third Fluorescent Antitoxin.—The method of preparation was the same as the method employed for the second fluorescent antitoxin except that twice as much diphtheria antitoxin concentrate was used. Again, 100% toxoid activity was lost, but only 20.5% toxin activity was lost. The *Kf* decreased to 10 minutes. A standard curve was prepared using dilutions of toxin as before. Surprisingly, the slope of this curve was almost identical with the slope of the curve obtained with the second preparation instead of being approximately one-half as great. When the overall fluorescence of this third antitoxin was compared to the fluorescence of the second preparation, the latter showed appreciably more gross fluorescence by a ratio of 82 to 49. This greater fluorescence must be accounted for in some immunochemically inactive manner. Either this fluorescein is bound to the inert globulin present or is physically adsorbed on the globulin and antitoxin and is released upon reaction. The latter seems much more probable since the two proteins have no apparent chemical differences other than the spatial arrangement of polypeptide chains. Verification of this was obtained by setting up tests using identical quantities of toxin and titrating with the second and third antitoxin. The floc fluorescence and the fluorescence of the supernatants were measured. It was found that floc fluorescence was the same for both materials and the fluorescence of the supernatants was in the same approximate ratio as the unreacted antitoxins. Control tubes of toxin, antitoxin, and buffer were mixed in the same proportion as in the floc tests immediately preceding the fluorescence measurement. Aliquots were taken from these tubes before flocculation could occur and in this manner served to compensate for any buffer effects, slight toxin fluorescence, etc., that might be misinterpreted. The ratios of fluorescence for the two antitoxins were 1.65 for unreacted or unflocculated controls and 1.58 for the supernatants after flocculation.

By using the control tests, a greater amount of fluorescence was consistently observed in the supernatant fluids, even after removal of some fluorescein in the flocs, than was present in the unflocculated control tubes. This could be explained by the adsorption of fluorescein to the antitoxin surface and in some manner not exhibiting fluorescence. During the reaction with toxin the fluorescein could be liberated into solution This bound fluorescein might also be located at or near reactive sites in the molecule since dilution alone does not cause an increase in fluorescence. The location of fluorescein near these sites would also explain the increase in *Kf* observed, since the antigen molecule would have to displace fluorescein before complex formation could occur.

To verify further that the degree of labeling of the two antitoxins was the same, the number of

fluorescein molecules per antitoxin molecule was experimentally determined. The second fluorescent antibody contained an average of 5.35 fluorescein molecules to 1 antitoxin molecule while the ratio was 5.4 to 1 for the third fluorescent antitoxin. This very similar labeling probably occurs because the amount of fluorescein used in both preparations was in excess of that required, the larger excess being adsorbed to a greater extent in the second preparation.

Fourth Fluorescent Antitoxin.—The presence of physically adsorbed fluorescein has been confirmed in the literature (11) for a different protein. By treatment with charcoal, a large percentage of this adsorbed material was removed. The fourth fluorescent antitoxin was made from the third preparation by shaking it with activated charcoal for 3 hours and filtering all traces of charcoal. This product showed a 50% decrease in overall fluorescence from the third preparation but only a 29.8% loss of *Lf*, which is comparable to the literature value. Immunochemically inert fluorescein had definitely been removed. Tests run on supernatants after flocculation showed no release of adsorbed fluorescein as had been noted previously. In addition, this material had good toxoid flocculating power, which had not been observed with the previous preparation, and typical precipitin curves could be obtained. The *Kf* with toxin had decreased to 8 minutes and toxoid *Kf* was in the range of 30 to 45 minutes. The toxoid reactivity loss from the original unlabeled antitoxin was 44% which is the same overall loss of toxin reactivity. An "unknown dilution" of toxin was assayed within 98.6% of its value as determined by *Lf* tests using a comparison standard toxin test run simultaneously. Toxoid could also be quantitated and tests on a series of dilutions gave a typical standard curve. Analysis showed an average of 5.25 molecules fluorescein to each molecule antitoxin. The charcoal adsorption evidently did not remove any significant quantity of chemically bound fluorescein. The activity and characteristics of this material show that it should be possible to prepare an antitoxin without the charcoal adsorption step provided excess adsorbed fluorescein can be avoided.

Fifth Fluorescent Antitoxin.—Reactants were used in proportions calculated to yield a product having a 1:1 ratio of fluorescein to protein. Examination showed that 100% of toxin and toxoid reactivity was retained in the process of synthesis and the *Kf* was 5 minutes. The molecular ratio of fluorescein to antitoxin was found to be 1.5 to 1. Supernatant examination after flocculation showed only a very slight liberation of bound fluorescein during reaction. The fast reaction and full retention of activity made this the best lot of antitoxin prepared at this point. The fluorescence level, however, was quite low, corresponding to the weak staining antibody discussed by Goldwasser (12).

Sixth Fluorescent Antitoxin.—The ratio of reactants was chosen to give a 5:1 fluorescein to protein product. One hundred per cent biological activity was retained for both toxin and toxoid. An examination of the supernatant after flocculation showed definite liberation of fluorescein during reaction, although this amount was much smaller than with the third antitoxin. The ratio of fluorescein to antitoxin in this preparation was 4.25 to 1. Flocculation fluorescence readings with this material read about 17% lower than those obtained with the fourth fluorescent antitoxin and the degree of labeling was 19% less, showing good correlation.

SUMMARY

Several fluorescent diphtheria antitoxins were prepared in order to determine the optimum conditions for obtaining a highly fluorescent antibody without denaturing the protein to the extent of losing significant flocculating power. The amount of fluorescein derivative used to prepare the fluorescent antitoxins was found to determine the *Kf* value and the amount of toxin or toxoid flocculating potency lost. There was an optimum amount of fluorescein which would couple chemically with the antitoxin, this value being an average of five molecules fluorescein to each antitoxin molecule. If amounts in excess of this were used, physical adsorption occurred which greatly decreased antitoxic potency. Antitoxoid potency was very easily destroyed by overlabeling. Some of the adsorbed fluorescein did not exhibit fluorescence until it was released by the reaction of the antitoxin with toxin or toxoid. It did not contribute to the fluorescence of the flocs and could be removed by competitive adsorption with activated charcoal.

The fluorescent antibody technique was shown to be applicable not only as a diagnostic or qualitative test but as a sensitive quantitative method of analysis as well. A comparison standard test, run simultaneously with the unknown, was more reliable than a standard curve procedure since varying conditions could be automatically controlled.

REFERENCES

(1) Coons, A. H., and Kaplan, M. H., *J. Exptl. Med.*, **91**, 1(1950).
(2) Deibel, R., and Hotchin, J. E., *Virology*, **8**, 367 (1959).
(3) Coons, A. H., Leduc, E. H., and Kaplan, M. H., *J. Exptl. Med.*, **93**, 173(1951).
(4) Kaplan, M. H., *J. Immunol.*, **80**, 254(1958).
(5) Marshall, J. M., *Exptl. Cell Research*, **6**, 240(1954).
(6) Coons, A. H., *et al.*, *J. Exptl. Med.*, **91**, 31(1950).
(7) Noyes, W. F., and Mellors, R. C., *ibid.*, **106**, 555 (1957).
(8) Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **62**, 467(1935).
(9) Kabat, E. A., and Mayer, M. M., "Experimental Immunochemistry," 2nd. ed., Charles C Thomas, Springfield, Ill., 1961, pp. 23–25.
(10) Petermann, M. L., and Pappenheimer, A. M., Jr., *J. Phys. Chem.*, **45**, 1(1941).
(11) Chadwick, C. S., *et al.*, *Biochem. J.*, **73**, No. 3, 41 (1959).
(12) Goldwasser, R. A., and Shepard, C. C., *J. Immunol.*, **80**, 122(1958).