STUDIES ON FLUORESCENT ANTIBODY STAINING

I. Non-Specific Fluorescence with Fluorescein-Coupled Sheep Anti-Rabbit Globulins*

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Plate 18

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When tissues or smears are treated with the usual preparations of fluorescent antibodies, the accompanying non-specific fluorescence found in the preparations (1–19) has imposed limitations upon the use of the fluorescent antibody technique. Dilution, absorption of the antibody preparation with tissue powders or fresh or lyophilized tissues or ion-exchange resins, and masking of sites by treatment with "normal" globulins coupled with fluorescent dyes of contrasting color, have all been practiced. The need for such devices seems to depend upon the discreteness of the antigens sought within the preparations and the relative thicknesses of the latter. With bacteria and certain intracellular viral particles, one may establish localization with relative ease. In contrast, precise localization of soluble antigens that are present in low and variable concentrations, and of some other types of viral particles, is rendered unsure by the accompanying non-specific fluorescence.

The present study was undertaken for the purpose of learning something of the reasons for non-specific fluorescence with the aim that this information would aid in the preparation of fluorescent antibody reagents that stain only at sites of specific antigen deposits.

Absorption methods using tissue powders were employed only on a limited scale, and the major effort was directed to the coupled globulins themselves.

The ultimate criterion for the specificity of fluorescence was based on determining the inhibition of fluorescence by pretreatment with non-fluorescent antibody. Reliable information as to non-specific fluorescence was obtained also by staining tissues where no antigen-antibody reaction was possible.

Individual preparations of fluorescent antibody were found to be hetero-

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geneous with respect to the number of fluorescein isothiocyanate (FITC) molecules attaching per antibody molecule, and the degree of non-specific fluorescence was demonstrated to be directly related to the average number of fluorescein linkages effected per molecule of antibody.

It was possible to obtain fluorescent antibody preparations with adequately bright specific staining and negligible or no non-specific staining, by means of gradient elution chromatography on DEAE[1]-cellulose.

A technique was developed for rapid removal of residual fluorescent products not coupled to protein (16).

Materials and Methods

Fluorescein Isothiocyanate.—One amorphous acetone-soluble preparation (Lot 12331) and one crystalline preparation (Lot 912606) of fluorescein isothiocyanate, from Baltimore Biological Laboratories, were used in the present study to prepare fluorescent antibodies. The crystalline preparation, viewed with polarized light, showed that only a small amount of amorphous material could be present.

Antisera Used.—Sheep immune sera prepared against rabbit serum fractions (Sylvana Chemical Company, Orange, New Jersey, Lot 637-259, and Surburban Serum Laboratories, Silver Spring, Maryland, Lot 43059) were secured in approximately 100 ml amounts. Both specimens came from the same sheep, drawn at two times in the course of immunization. Normal sheep serum from Cappel Laboratories was used also. Of these sera two were without preservative and Lot 637-259 had been preserved with 1:10,000 merthiolate. All sera were fractionated at 6°C and 40 per cent ammonium sulfate saturation by adding 1 volume of saline and 1.33 volumes of cold saturated ammonium sulfate. The resulting precipitate separated in the cold and washed once with 50 per cent saturated ammonium sulfate was dispensed in portions of approximately 180 mg protein (dry weight basis). The precipitates were packed by centrifugation, stripped, treated with 1 drop of toluol per tube, and stored in the cold. For use, the moist ammonium sulfate precipitates of crude globulin were suspended in saline, dialyzed for 8 hours against 0.45 per cent sodium chloride containing 1 drop N NaOH per 100 ml, and clarified from any denatured protein by centrifugation. The concentration of the final solution was determined by its absorption at 280 mμ, with 1.24 O.D. being taken as 1 mg/ml.

For selected purposes, the gamma globulin component was obtained by chromatography on DEAE-cellulose (20) using 0.01 M sodium phosphate, pH 7.5. The protein peak, eluted from the DEAE-cellulose with the same buffer, was concentrated by dialysis (21) under nitrogen at 14 pounds pressure. This material represented 45 to 60 per cent of the dialyzed ammonium sulfate globulin fraction applied to the column.

Conjugation of Serum Globulins with Fluorescein Isothiocyanate.—Globulin was coupled in concentration not less than 2.5 per cent, such that volumes of 2 to 10 ml were coupled usefully in flat-bottomed glass stoppers of 24/40 standard taper, held in a cooling bath, and provided with magnetic stirrer and probe-type glass electrodes for constant observation of the reaction. At 0°–2°C, 0.10 volume of cold M carbonate-bicarbonate pH 9.0 was added, and then, in a few increments, the desired amount of fluorescein isothiocyanate contained in dry acetone. The acetone, not exceeding 10 per cent of the volume, had no detectable effect upon the globulins. The reaction was maintained between pH 9.0 and 9.5 by adjusting as necessary with sodium carbonate or bicarbonate. The chemical reaction was allowed to con-

[1] DEAE, diethylaminoethyl.

tinue for a period of 2 to 24 hours. For couplings longer than 2 hours, the reaction vessel was sealed and transferred to a cold room at 6°C. At the end of the coupling period, the pH of the solution was adjusted with N HCl to 7.2–7.5.

Factors that were varied at the coupling step were the fluorescein isothiocyanate preparation, the amount added relative to protein, and the length of the coupling reaction. The couplings appeared to proceed best with the relatively high concentrations of globulin that were adopted. No precipitation occurred during the couplings.

Removal of Fluorescent Dialyzable Products.—At first, unreacted fluorescein isothiocyanate and its degradation products were separated from fluorescent globulins by the usual dialysis through Visking tubing against 30 to 60 volumes of cold toluized 0.15 M NaCl, having 1 drop N NaOH per 100 ml. The outer fluid was changed every few hours during the 1st day, and then daily. This step required 4 to 7 days before the dialysate was clear and colorless.

A technique superior to the above, which provided complete and rapid separation of the low molecular weight fluorescent compounds, was accomplished by passing the reaction mixture through a column of sephadex G-50 to utilize its property of molecular sieving (16, 22). The smaller molecules diffuse into the swollen hydrophilic particles and are retained temporarily while the protein molecules pass through directly. The procedure is described below.

Fluorescein-Protein Ratios of Fluorescein-Antibody Preparations.—The methods employed in reference 9 were followed in the main. Protein concentration was determined by the biuret reaction (23). For the concentration of coupled fluorescein, the absorption value was read in a Beckman DU spectrophotometer at 495 mμ, the point of maximal absorption. Two standard curves for the absorption at 495 mμ of amorphous and of crystalline fluorescein isothiocyanate, with the respective actual weights reduced by the mole equivalent of the isothiocyanate grouping, were used to convert absorption values into micrograms of "fluorescein equivalents" for each preparation. It should be remarked that the "fluorescein equivalent" values were, in the case of the crystalline preparation, close to those of recrystallized fluorescein itself, and much less so with the amorphous one.

Column Electrophoresis.—Electrophoresis through ethyl-esterified cellulose was carried out as in references 24 and 25, using vertical water-cooled jacketed columns with column length 50 cm and internal diameter 2 cm, containing 50 gm of the cellulose preparation (Munktell). Details are given below.

Chromatography.—Selectacel-DEAE (Brown and Company, Berlin, New Hampshire) was equilibrated with 0.01 M sodium phosphate buffer pH 7.5. (In later experiments, a preliminary washing was conducted with 0.5 N NaOH, followed by distilled water.) The product was employed in columns packed to 20 × 2 cm under a head of 130 to 150 cm water. To establish a gradient, two 250 ml mixing chambers were employed between the column and its 1 liter reservoir constituting a closed system. Column effluents were collected at 6°C in fractions of 3 to 6 ml at selected rates, varying in individual experiments between 20 and 200 ml/hour.

Pressure Dialysis.—Proteins were concentrated from dilute solution by adapting the method of Porter (21). Visking dialysis grade tubing ($2\frac{9}{32}$ inches) was tied at one end and affixed at the other by cord to a rubber stopper through which passed an 8 mm O.D. glass tube. For this purpose, English one-holed rubber stoppers, size 15, BLWA Standard TCD 161, offer a better taper than American molds. Over the cellophane was drawn a close-fitting net of Dufour bolting cloth (Turtox Company, Chicago) sewn to form an open tube 38 cm long, of a size fitting snugly around a culture tube of 20 mm O.D. The netting was secured likewise to the rubber stopper by cord. After the sac had received its charge, the 8 mm glass tube was fitted with pressure tubing to a thick-walled glass bulb provided at the top and bottom with tapered side arms; the other side arm was joined via pressure tubing and clamping device to a tank of nitrogen bearing a pressure gauge. Nitrogen was introduced slowly to

14 pounds pressure, the clamp tightened, and sac and pressure bulb were suspended in a 2000 ml graduate so that the full length of sac and netting was immersed in cold outer fluid of the desired composition. The outer fluid was stirred by means of a magnetic stirrer. Concentration of proteins and equilibration with outer electrolytes would occur simultaneously at 6°C in the course of 12 to 30 hours according to the volume to be reduced.

Splenic Imprints.—The preparations used for fluorescent antibody studies consisted of imprints made from the spleens of exsanguinated rabbits and guinea pigs. Sections of spleen *ca.* 2 mm thick were blotted on filter paper to remove excess fluid and then were used to make impression imprints on microscope slides, close to a level surface of pulverized dry ice. From two to four imprints were made on a single slide, and then the slide was placed at once on dry ice to freeze the imprints rapidly. Slides were stored at −16°C until used for staining. The preservation of cellular morphology was not uniform, but each imprint contained many areas that were representative and were only one or two cell layers thick. Examples are given in Figs. 1 *a*, 1 *b*, and 2, from spleens of antigen-stimulated animals (see below for details).

Not more than 2 hours before staining, the slides were thawed by flooding with 95 per cent ethanol, and then were fixed by immersion in ethanol for 30 minutes and air-dried.

Staining of Imprints.[2]—The desired individual imprints were encircled separately by application of rings of molten Vaspar (equal parts of paraffin wax and vaseline) to provide separation wells. The imprints were moistened in buffered saline pH 7.2, $\mu = 0.15$ (4.09 gm Na_2HPO_4, 1.55 gm $NaH_2PO_4 \cdot H_2O$, and 3.04 gm NaCl, per liter of distilled water) and drained of excess saline. Then 2 to 3 drops of fluorescent globulin diluted as desired in buffered saline were placed within the Vaspar rings. The slides were covered with Petri dish lids lined with moistened filter paper and were rotated at room temperature for 30 minutes on a Fisher rotator at 100 excursions per minute. The fluids were then drained off, rinsed, and immersed in two further changes of buffered saline for 15 minutes each. The Vaspar rings were scraped away, and the imprints were mounted for examination in buffered glycerine under coverslips.

Inhibition Tests.—In order to determine whether fluorescence was specific, an initial coating of non-fluorescent antibody was applied to coat specific sites and specifically to inhibit subsequent fluorescent staining.

On a slide containing three satisfactory imprints, one would be overlaid with a 1 to 2 per cent solution of normal sheep globulin, another with buffered saline, and the third with 1 to 2 per cent of specific but non-fluorescent antibody solution. After being kept on the rotary shaker for 1 hour, and being washed in buffered saline, all imprints would be overlaid with a 0.05 to 0.1 per cent solution of fluorescent antibody for 30 minutes. The slide was then washed as usual and mounted. Specific fluorescence would be inhibited completely by pretreatment with non-fluorescent antibody. When inhibition of fluorescence was found to be only partial as compared to the saline-, or globulin-pretreated imprints, the fluorescence was considered to be partly specific and partly non-specific. When no inhibition of fluorescence occurred, the fluorescence was considered to be non-specific.

As an additional control of the specificity of fluorescent sheep anti-rabbit globulins, guinea pig spleen imprints were used as a test substrate (Figs. 1 *a* and 1 *b*).

Fluorescence Microscopy.—A Zeiss power supply powered with constant line voltage of 122 v and an osram HBO-200 light source were used with a Zeiss UG-2 exciter filter and a Wratten-2B barrier filter. Besides the initial heat-absorbing filter, a similar heat-absorbing filter was mounted directly against each exciter filter to prevent cracking from uneven heating. An A.O. microscope was equipped with a wide angle Abbé condenser N. A. 1.40 and a glass "dark field disc," set in a centerable mount, with a first-surface mirror, and a trinocular head with "T" pull-out prism. For photography, exposures were made on 35 mm super ansco-

[2] The technique was worked out by C. D. Dukes and M. W. Chase.

chrome daylight film, using two exciter filters (one Zeiss BG-12 and one Corning 5840, the latter 2.25 mm thick and having color specification 7-60) and a Wratten K2 barrier filter. The immersion oil was Cargille's type B low fluorescence non-drying immersion oil. For illumination on a writing surface in the dark room, we employed a 15 w red fluorescent bulb (26).

RESULTS

To test the relative effectiveness of different fluorescent preparations made from antisera of the same sheep for their properties of specific and non-specific fluorescence staining, rather thin tissue imprints were made from the spleens of rabbits immunized with fluid bovine serum albumin and sacrificed 3 to 5 days after restimulation, and from the spleens of guinea pigs sacrificed at varying intervals after sensitization with ovalbumin administered in Freund's complete adjuvant. The serum of all of these animals contained antibody, and the splenic imprints gave full evidence of the associated cellular changes. The spleens of normal guinea pigs were employed at times, but the absence of cellular changes rendered them less useful.

In addition to the fluorescent globulins of sheep immunized with rabbit serum protein, fluorescent serum globulins of non-immunized sheep were used, here only for their non-specific staining.

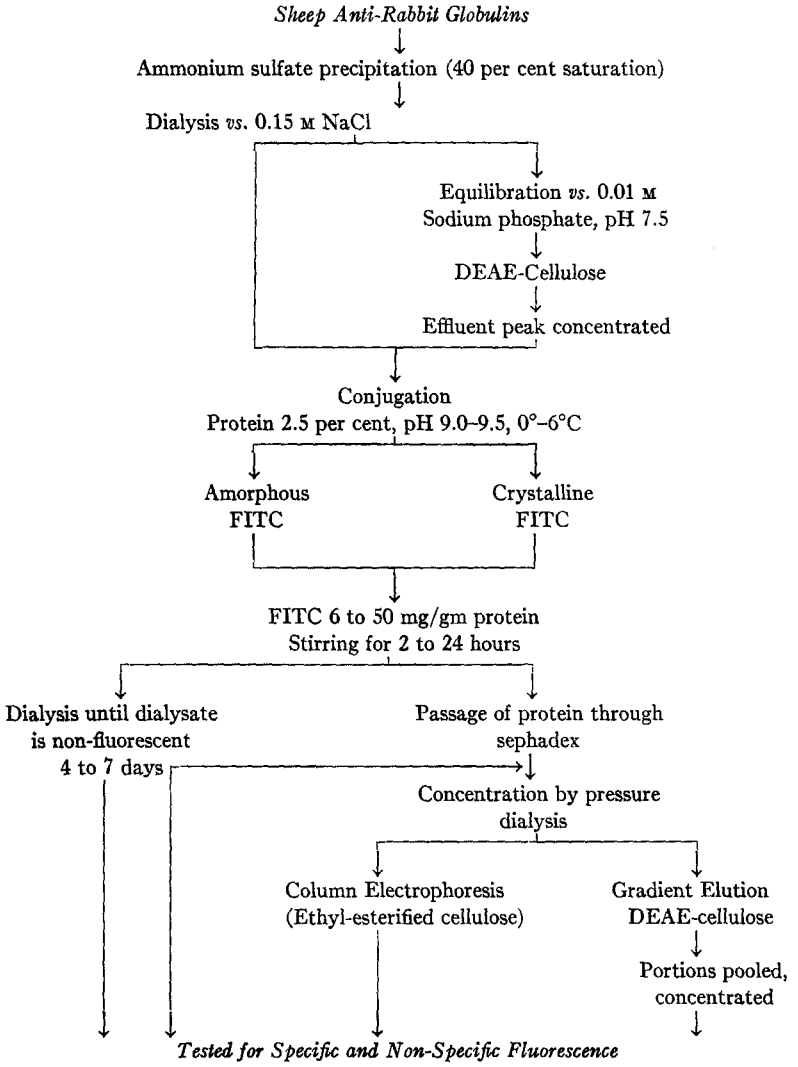
The three or four consecutive splenic imprints on each slide allowed direct comparison between the degree of fluorescent staining secured by testing different reagents and concentrations, and also facilitated observing the outcome of attempts to inhibit fluorescent staining by pretreatment with non-coupled antibody globulins, as described above.

The latter test was used for the final decision as to whether fluorescence staining was wholly non-specific, wholly specific, or a summation of specific and non-specific staining. It may be stated at the outset that staining of guinea pig tissue imprints by sheep globulin was found always to be non-specific, as expected, as was the staining of antibody-containing rabbit imprints by fluorescent globulins of normal sheep. The proper concentration of fluorescent globulins to apply to an imprint was found to fall between 0.5 and 1.5 mg/ml, as is discussed below.

Outline of the Investigation.—The general plan of this study is outlined in Text-fig. 1. An estimate of the factors contributing to non-specific fluorescence was obtained by using alternate procedures at many stages in the preparation of fluorescent antibodies.

Besides investigating differences owing to purity of the coupler, its initial ratio to protein, and the time of coupling, non-specific staining attributable to fluorescent protein fractions other than the electrically neutral globulins was examined. Finally the reaction products, separated by application of column electrophoresis and column chromatography, were studied individually. All of the steps shown were performed at 0°–6°C, but the staining and examination of

imprints was done at room temperature. The presentation of results follows the order indicated in Text-fig. 1.



TEXT-FIG. 1. Operative pathways under investigation. FITC signifies fluorescein isothiocyanate.

Effect of Purity of Globulin Preparation.—Besides excluding the soluble albumins and some other serum components by means of ammonium sulfate at 40 per cent saturation, gamma globulins were then separated by chromatography on DEAE-cellulose. Tests of specific and non-specific fluorescence were

made both on crude globulins and the purified products after being coupled with FITC.

When guinea pig spleen imprints were stained at a concentration of 1 mg/ml of fluorescent crude antibody globulin and 1 mg/ml of fluorescent purified gamma globulin at equal fluorescein:protein ratios, the amount of non-specific fluorescence was equivalent. Accordingly, there was no selective elimination of the cause of non-specific fluorescent staining by further purification of the globulins.

Even though chromatographic separation of gamma globulin on DEAE-cellulose did not eliminate or reduce non-specific staining, the step was adopted to allow subsequent studies on the relative homogeneity of coupling.

It may be pertinent to add that in preliminary experiments employing the methods of Porter (27) for splitting antibody by papain digestion (using in this instance rabbit antibody) non-specific staining was found to remain a property even of fluorescein-coupled fraction I.

Effect of Relative Purity of Fluorescein Isothiocyanate.—Two preparations of fluorescein isothiocyanate were used for preparing fluorescent antibodies and/or fluorescent normal sheep globulins, namely, a brownish yellow amorphous powder and a yellow crystalline preparation. Both exhibit maximal absorption at 495 mμ, but at equal concentrations the crystalline product shows approximately 20 per cent greater absorption.

Table I lists various fluorescent preparations of globulins from sheep anti-rabbit serum and from normal sheep. Some are coupled with the amorphous preparation, others with the crystalline fluorescein isothiocyanate. By comparing the amount of fluorescein isothiocyanate offered per gram of protein and the resultant fluorescein:protein ratio of the coupled product, it will be noted that crystalline fluorescein isothiocyanate is about 2 times as reactive with globulins as the amorphous product. One may infer that only part of the latter product is competent fluorescein isothiocyanate. While preparations 1, 2, 3, and 7 were made from DEAE-separated globulins of merthiolate-preserved serum, there was no evidence of any difference in result from similar products made from serum containing no additive.

The use of crystalline fluorescein isothiocyanate for preparing fluorescent antibodies did not decrease non-specific fluorescence. When the fluorescent reagents listed in Table I were used at equal protein concentrations (1 mg/ml) for staining spleen imprints, the degree of non-specific fluorescence increased as the fluorescent-protein ratio of the antibody increased. The intensity of specific staining also increased as the fluorescent-protein ratio increased. Indeed, the usual coupling ratio found in the literature (50 mg per gm of protein) results in a product that offers great hazard in interpreting specific fluorescence when the new crystalline FITC is used.

The extent to which fluorescent groupings attached to protein under the conditions of coupling for 24 hours usually varied between 67 and 86 per cent

of the amount of crystalline FITC offered. Within 2 hours the coupling was about two-thirds as great as at 24 hours. The lesser time for coupling represents an unsuccessful endeavor to reduce the amount of non-specific fluorescence. In contrast, the use of amorphous FITC under similar conditions led to the coupling of only 28 to 36 per cent of the amount offered.

Rapid Recovery of Coupled Globulins through Sephadex.—For investigating the effect of multiple variations in coupling ratios, particularly with a view to

TABLE I

Preparation of Fluorescent Antibodies

Prep. No.	Product coupled	FITC offered for reaction‡ (mg/gm of protein)	F/P × 10^3	
			Amorphous	Crystalline
1	Sheep anti-rabbit globulin	6		4.4*
2	"	7		4.9* (2 hrs.)
3	"	7		6.8*
4	"	7.5	2.3	
5	"	8		5.0*
6	"	15	5.4	
7	"	20		10.6* (2 hrs.)
8	"	20		17.2*
9	"	30 (2 × 15)	9.6	
10	"	45 (3 × 15)	13.2	
11	"	50	14.0	
12	"	50		33.4*
13	Normal Sheep globulin	50	12.1	
14	"	50	17.5*	

* Asterisk indicates that the gamma globulins were obtained by chromatography on DEAE-cellulose. In other instances, globulins were used after precipitation at 40 per cent saturation with ammonium sulfate and dialysis.

‡ The time for chemical reactions was 24 hours excepting as indicated.

making successive couplings of a single product, it seemed advisable to avoid the usual procedure of lengthy dialysis and to minimize the accompanying surface denaturation, even if the latter is minor. The introduction of cross-linked dextran gel of molecular weight great enough to provide a large inner volume of water with the exclusion of entry of large protein molecules into the gel appeared to offer the needed technical advantage (22). This proved to be the case. Sephadex G-50 (10 gm) was suspended in water and freed of finer particles; a column was poured and equilibrated with phosphate-buffered saline, pH 7.2. To such a column, amounts up to 250 mg of fluorescent globulin (10 to 12 ml) were applied, the fluorescent protein passing through the column in the initial 15 to 35 ml of eluate. The dialyzable fluorescent compounds emerged later, after

at least 60 ml of eluate had been collected. The recovery of fluorescent antibody protein was 90 per cent or more and was contained in a volume only 2 to 3 times the initial volume. The recovered fluorescent antibody was usually concentrated by means of pressure dialysis preparatory to further procedures. In this way, for example, a second coupling of a product would be possible 24 hours after a primary coupling (Table I, preparations 9 and 10).

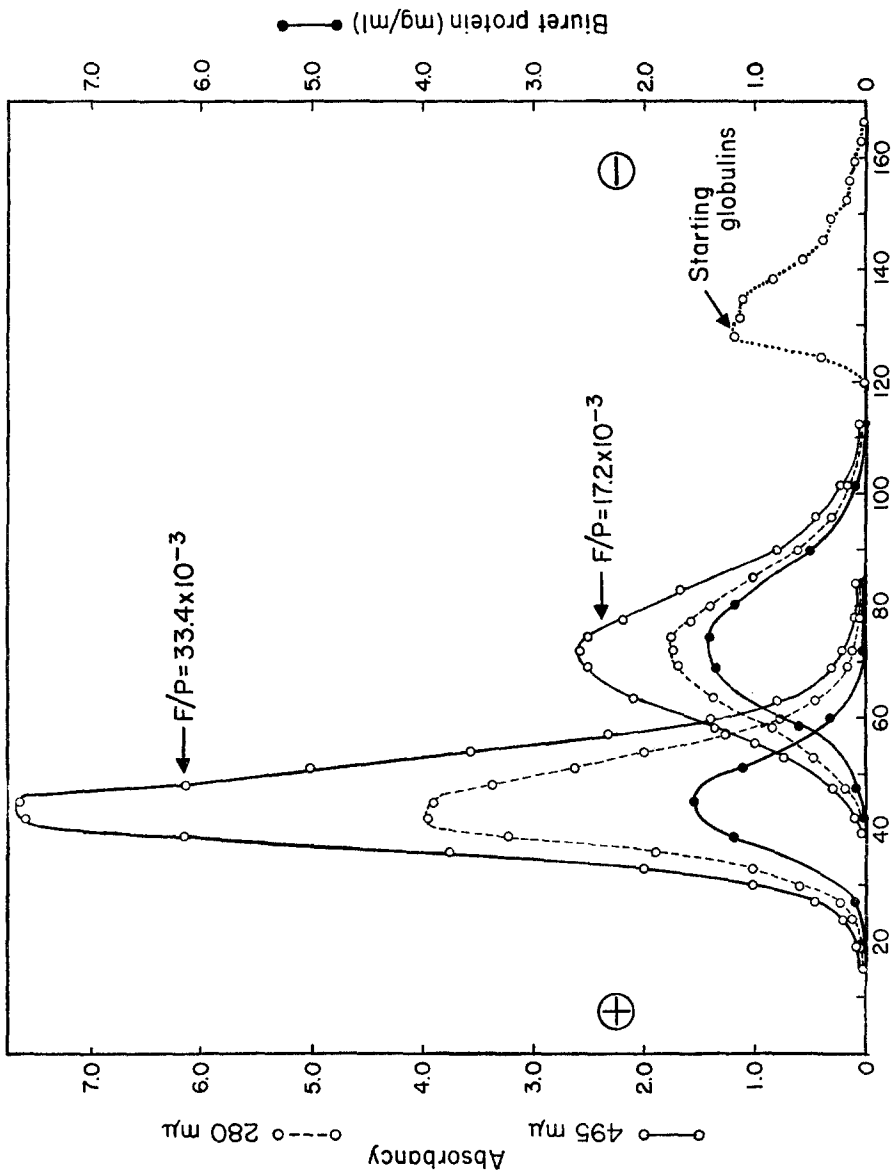
The fluorescent antibody preparations so obtained possessed no fluorescent constituents dialyzable through Visking dialysis membranes 18/32. Even with the very effective separation of dialyzable fluorescent products so obtained, the preparations had no less non-specific fluorescence than similarly coupled antibody globulin dialyzed through Visking membranes for 4 to 7 days.

The Relation of Fluorescein:Protein Ratio and Electrophoretic Mobility.— Column electrophoresis of sheep anti-rabbit serum proteins before and after coupling was carried out on columns of ethyl-esterified cellulose (24, 25), in an attempt to determine whether the more highly coupled molecules contributed importantly to non-specific fluorescence (11).

The ethyl-esterified cellulose was equilibrated with a sodium chloride, borate, phosphate buffer, pH 8.6, ionic strength of 0.1, and packed in the water-jacketed column already described. Approximately 25 mg of antibody in 0.5 ml buffer was placed on the column and allowed to enter the column to a known position. The lower end of the column was established as the anode. Electrophoretic runs were allowed to proceed for 16 to 23 hours at 800 volts and 72 milliamperes. At the end of the electrophoretic run, the column was placed on a fraction collector, and the globulin was eluted with the same borate buffer.

The alteration in electrophoretic mobility that results from conjugation with fluorescein isothiocyanate is illustrated in Text-fig. 2. Sheep antiserum to rabbit serum proteins, precipitated with ammonium sulfate and chromatographed on DEAE-cellulose as described above, is seen to move slightly towards the cathode. Contrary to a recent report (19), the same gamma globulin preparation after reaction with two different ratios of crystalline fluorescein isothiocyanate migrated as expected towards the anode. Both products had been freed of dialyzable constituents by passage through sephadex and then concentrated to 5 per cent protein. The product resulting from an offering of 50 mg per gm of protein, having an F:P ratio of 33.4×10^{-3}, migrated faster towards the positive pole than the product coupled at 20 mg/gm protein (F:P ratio 17.2×10^{-3}).

Fractions obtained by column electrophoresis were evaluated for protein (biuret method) and fluorescein concentration, and for specific and non-specific staining. As is indicated on the graph, readings of optical density were made at 280 mμ as well. Whereas this reading on the starting globulins is useful in determining concentration of protein, it is seen to be progressively less useful for this purpose as fluorescein groupings become conjugated with the protein. Fluorescein contributes significantly to the reading at 280 mμ, as is evident



TEXT-FIG. 2. Column electrophoresis of sheep anti-rabbit globulin on ethyl-esterified cellulose.

since the sample size of protein was the same in all three cases. Readings made at 280 mμ are, accordingly, informative only for special purposes unless one is in a position to apply correction factors appropriate to the extent of coupling.

The various fractions were found to be unlike with respect to the number of molecules of fluorescein conjugated to antibody, indicative of heterogeneity of coupling as stressed by Curtain (11). For example, the product exhibiting the over-all F:P ratio $\times 10^3$ of 33.4 contained fractions varying from 35.0 to 29.2, and the preparation showing the F:P ratio $\times 10^3$ of 17.2 had fractions varying from 20.8 to 15.9. The faster the migration of a fraction, the greater was its F:P ratio.

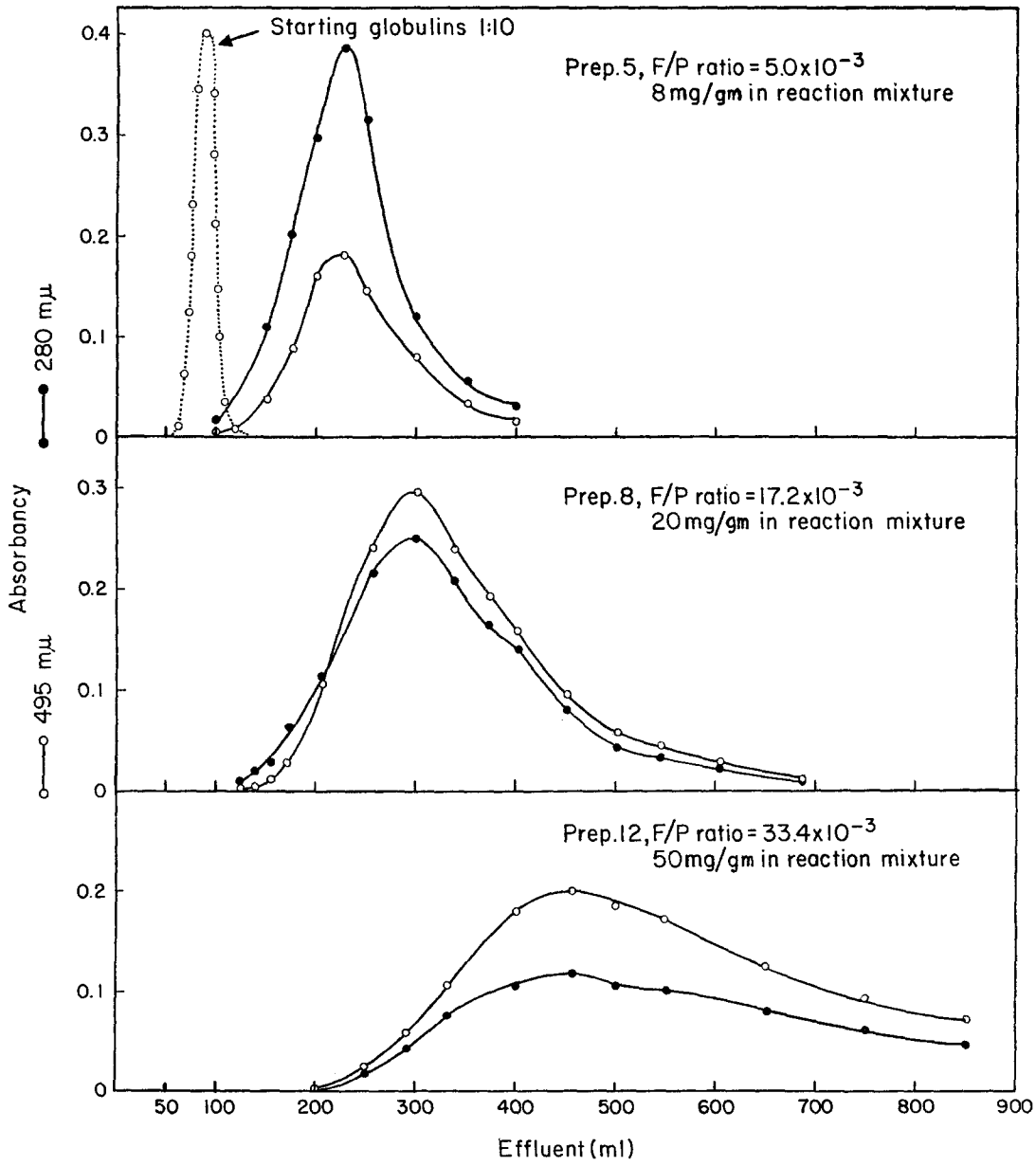
When compared for the property of non-specific staining on splenic imprints, both these preparations and all their individual fractions exhibited non-specific fluorescence in high degree (Figs. 1 *a* and 1 *b*).

Gradient Elution Chromatography on DEAE-Cellulose.—Individual fluorescent antibody preparations could be separated into fractions differing in the average number of fluorescent groupings per antibody molecule. This was achieved satisfactorily by gradient elution from DEAE-cellulose by means of sodium chloride.

The fluorescein-globulin preparations, freed of fluorescent dialyzable materials, were placed on the column (described above) in amounts of 25 to 70 mg in 2 ml volume that had been equilibrated with 0.01 M sodium phosphate, pH 7.5. The packing of selectacel-DEAE was roughly 12 gm. This buffer was passed through the column as long as fluorescent material emerged, which event occurred chiefly in the case of the lower coupled products. A narrow, faintly colored band of protein would separate from the bulk of the bound fluorescent globulins and start to emerge at 1 column volume. The gradient was next applied. In the apparatus described earlier, the final mixing chamber contained the same phosphate buffer as the column. The intermediate mixing chamber contained 0.5 M sodium chloride in the same buffer (pH 7.4–7.5), while the reservoir contained 1.0 M sodium chloride in the same buffer (pH 7.3-7.5). Under these conditions, the fluorescent preparation bound to the column packing was seen to remain in its upper portion until, after the passage of at least 1 further column volume, an advancing front of fluorescent globulins moved through the column. The concentration gradient of sodium chloride could be determined by measuring the conductivity of the eluate (28). With elutions from this apparatus involving 400 ml volumes, the sodium chloride would increase almost linearly to about 0.2 M (Table II).

Eluates were pooled as desired (100 to 150 ml amounts) and concentrated by pressure dialysis to less than 8 ml. Then the protein concentration, F:P ratio, and specific and non-specific fluorescence were determined on the pools.

Text-fig. 3 illustrates chromatographic separation of the same two preparations (Nos. 8 and 12) that were used for column electrophoresis, and a third preparation (No. 5), all compared with the original antibody globulin before coupling. Uncoupled globulin is not retained by the column and it is evident that the strength of binding to the column increases as the number of fluorescing radicals increases. In the case of preparation 5, discharge is almost symmetrical and is essentially completed within 4 column volumes. With



TEXT-FIG. 3. Gradient elution of various conjugates (25 mg portions) chromatographed on DEAE-cellulose. Preparations made with DEAE-cellulose–separated globulins of sheep anti-rabbit serum proteins and crystalline FITC.

the highly coupled preparation 12, having about 15 fluorescein residues per molecule, a higher initial concentration of salt was required for emergence and the process of elution was only 5/6 complete even after the passage of a further 16.5 column volumes with ascending salt concentrations. Only the portions emerging through the initial 7.5 column volumes are shown in Text-fig. 3. Owing to a need to conserve the samples as secured, it was not expedient to determine protein accurately by the biuret method; instead, the optical density at 280 mμ was read as a means to acquiring relative information about concentrations. The heterogeneity of the coupling is illustrated well by preparation 8 (F:P $\times$ 10^3, 17.2). Here the absorption at 280 mμ was higher than at 495 mμ in the fractions emerging first, whereas portions eluting at the higher salt concentrations had higher absorption at 495 mμ than at 280 mμ. Preparation 5, more lightly coupled, showed higher readings at 280 than at 495 throughout the range of eluates, whereas the reverse was true of heavily coupled preparation 12.

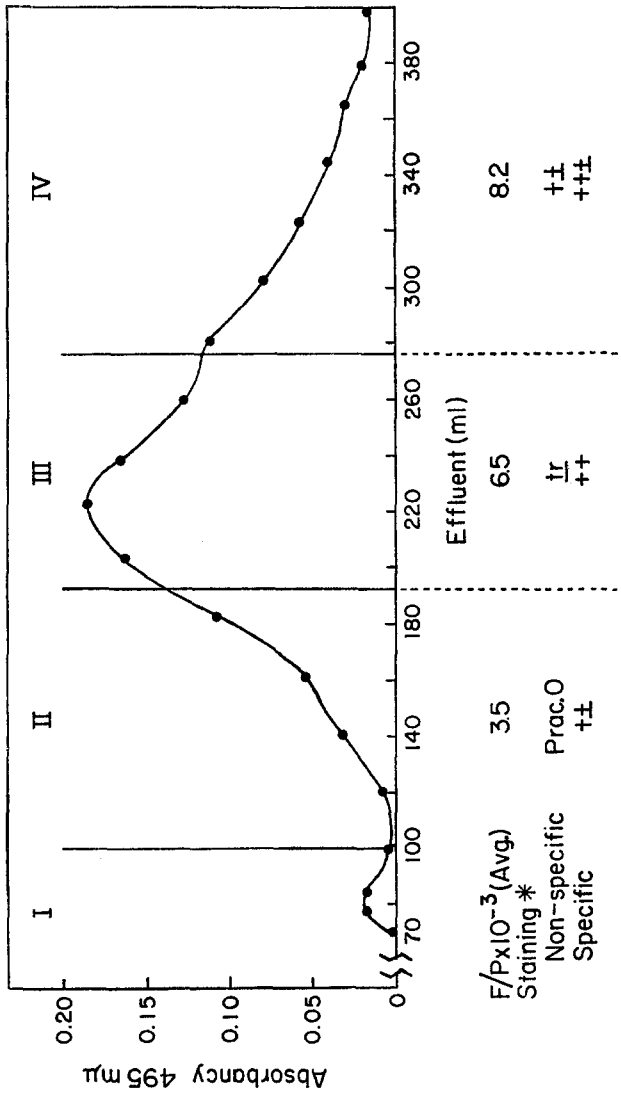
All the fractions from fluorescent antibody preparations 8 and 12 exhibited non-specific fluorescence, and were not useful. Preparation 5 will be discussed further below. To reduce the degree of non-specific fluorescence, the less coupled products evidently held more promise.

Concentration of Fluorescent Globulins.—The concentration of fluorescent antibody globulins adhering to a tissue imprint, and the intensity of the emitted fluorescence are intimately related to the concentration of fluorescent antibody globulins applied to the slide under constant conditions of contact.

Individual preparations in Table I were serially diluted with buffered saline and the non-specific staining of the respective dilutions was determined. The concentration that could be applied to an imprint without producing non-specific fluorescence varied with the particular fluorescent antibody tested. For example preparation 6 (F/P = 5.4×10^{-3}) at a concentration of 0.125 mg/ml was acceptable, while at the same concentration preparation 12 (F/P = 33.4×10^{-3}) saturated the imprint with non-specific staining. At a concentration of 0.002 mg/ml this preparation still had a high degree of non-specific staining. Specific and non-specific staining were extinguished at about the same concentration.

It was observed that as the F/P ratio increased, less protein must be applied to avoid producing non-specific staining. Although titrating each fluorescent preparation for the extinction of non-specific staining provided useful comparisons, better comparisons of products were obtained by using concentrations of 0.5 to 1.5 mg/ml. In this way, staining varied from no staining to saturation of imprints with a brilliant yellow-green fluorescence.

Comparison of the concentrations used in this study for staining with concentrations used by others is difficult as in other studies the concentration applied to the tissues is mentioned only rarely.



TEXT-FIG. 4. Gradient elution of preparation 5 from DEAE-cellulose.

Practical Dissociation of Specific from Non-Specific Fluorescence.—As mentioned, attention was turned to lighter couplings of fluorescent antibodies prepared with crystalline fluorescein isothiocyanate. Text-fig. 4 illustrates the

TABLE II

Characteristics of Fractions of Fluorescent Antibody Obtained by Chromatography on DEAE-Cellulose

Prep. No.	Offering *FITC* crystalline, mg/gm of protein	Eluate	Sodium chloride concentration	F/P × 10^3	Specific staining at 1 mg/ml	Non-specific staining at 1 mg/ml
		ml	*molality*			
1	6	120–180	0 –0.05	2.2	+	0
		181–240	0.05–0.10	4.6	+	±
		241–310	0.10–0.16	6.8	++	+ st
2	7*	108–170	0 –0.05	2.3	+ st	0?
		171–240	0.05–0.10	5.6	+±	tr
		241–390	0.10–0.20	8.2	++	+ st
3	7	112–200	0 –0.08	2.0	+	fffftr
		201–294	0.08–0.15	6.9	++	+w
		295–451	0.15–0.24	9.5	—	—
5	8	100–190	0 –0.05	3.5	+±	0?
		191–280	0.05–0.15	6.5	++	tr
		281–400	0.15–0.20	8.2	++±	+±
8	20	105–238	0 –0.10	5.2	+±	±
		239–364	0.10–0.19	9.9	—	++
		365–577	0.19–	12.8	—	++
12	50	400–520	0.22–	27	—	+++
		521–640		29	—	+++
		641–760		34	—	+++

tr, trace; tr, strong trace; st, strong; w, weak; fff, exceedingly faint; ±, one-half on scale of +++.

* Coupling time, 2 hours. All other preparations were coupled for 24 hours.

absorption at 495 mμ of a coupling made at 8 mg/gm for 24 hours and fractionated by gradient elution on DEAE-cellulose. At 70 to 90 ml effluent volume, a small peak of scarcely fluorescent material emerged. The remainder of the eluate was pooled into three fractions as shown. The heterogeneity is quite evident. The average F:P ratios of fractions II, III, and IV were respectively 3.5×10^{-3}, 6.5×10^{-3}, and 8.2×10^{-3}. Fraction IV exhibited marked non-specific fluorescence, while fraction III showed it but only slightly

when both were tested at equal concentrations of protein. A striking separation of specific from non-specific fluorescence was shown by fraction II. This fraction had bright, but not brilliant, specific fluorescence and no sure evidence of non-specific fluorescence.

Table II lists the properties of several fluorescent sheep antibodies eluted from DEAE-cellulose columns. A satisfactory fluorescent antibody fraction was obtained from each of the products that resulted from offering 6 to 8 mg crystalline fluorescein isothiocyanate per gm of globulins. In our system, the products exhibiting a satisfactory absence of non-specific fluorescence emerged between 100 and 200 ml effluent volume. The fluorescein:protein ratio of these satisfactory fractions ranged from 2.0 to 3.5 $\times$ 10^{-3}, (Figs. 2 and 3). By staining with high concentrations (greater than 2.0 mg/ml) of each of these fractions, however, non-specific fluorescence was observed. The initial small peak emerging in each of these preparations in about 1 column volume (70 to 85 ml) exhibited low 495 mμ and high 280 mμ absorptions. The material represented by this peak was recovered and concentrated. It was found not to impart fluorescent staining to specific imprints.

Accordingly, it is seen that satisfactorily bright, although not brilliant, staining can be secured in the practical absence of non-specific fluorescent staining, by means of excluding the more highly coupled molecules of globulin.

DISCUSSION

This study was designed to determine factors that lead to the production of non-specific fluorescent staining by fluorescein-globulin conjugates, with the intention of making preparations that would be essentially free from non-specific staining.

As test objects for investigating specific and non-specific staining, tissue imprints were used from the spleens of antigen-stimulated animals such that abundant cellular changes were secured. These sections were subjected to coating with the fluorescent globulins of sheep anti-rabbit proteins and with fluorescent globulins of normal sheep; moreover, the imprints of antigen-stimulated rabbits were stained in parallel with those from antigen-stimulated guinea pigs.

The two measures employed for determining non-specific fluorescence showed excellent correlation: (*a*) the fluorescent staining that appeared in guinea pig spleens treated with fluorescent globulins of sheep anti-rabbit serum proteins—staining which could not be inhibited by a preceding contact with non-fluorescent sheep anti-rabbit globulins used in tenfold higher concentration than the fluorescent preparation, and (*b*) fluorescent staining that would occur in rabbit splenic imprints after the antigenic sites on the rabbit antibody had been coated with non-fluorescent specific sheep antibody. It should be borne in mind that many cells exhibiting non-specific fluorescence were not dis-

tinctively different from those associated with antibody production, and decision had to be reached by means of inhibition tests (Figs. 1 *a* and 1 *b*).

Several types of experiments were undertaken to ascertain possible causes for non-specific fluorescent staining. A practical exclusion of serum proteins other than gamma globulins, secured by chromatographing on DEAE-cellulose those serum proteins that precipitate at 40 per cent saturated ammonium sulfate, did not diminish the non-specific staining of the final fluorescent globulin preparation in comparison with conventional fluorescent preparations made from crude globulins. Similarly, it appeared unlikely that the possible presence of low molecular weight fluorescent compounds (unreacted fluorescein isothiocyanate, fluorescein amine, and other products) contributes importantly to non-specific fluorescence (7, 17). For example, neither the sharp and rapid separation of soluble low weight fluorescent compounds on sephadex G-50 or the use of a more purified fluorescein isothiocyanate yielded conjugates with less non-specific fluorescence than preparations resulting from impure FITC and conventional dialysis.

These findings suggested that fluorescein-coupled gamma globulin molecules might be a major cause of non-specific fluorescent staining. Two problems could arise: a uniform addition of too many fluorescein groups per molecule, leading to marked lowering of the isoelectric point of the globulins and ready precipitation as well as changes in the secondary or tertiary structure, or a lack of uniformity in the coupling of fluorescein groupings to globulin molecules so that only certain molecules are altered markedly.

As regards the former, it is well known that coupling small radicals to proteins alters the isoelectric point and the solubility, as by coupling tyrosine and histidine residues of proteins with diazonium compounds (29, 30) and also by coupling the ϵ-amino groupings of lysine residues with chloro- and nitro-substituted benzenes (see reference 31). Similar in principle to the latter case is the coupling of proteins with aromatic isocyanates (1, 32–34). Studies on the reaction site between aromatic isocyanates and proteins have been reviewed by Coons and Kaplan (1). They point out that the most likely reaction site is at the epsilon amino group of lysine with the formation of a disubstituted urea. The net loss of three positive charges on the protein molecule then results in a lowering of the isoelectric point and in alterations of electrophoretic mobility. More recently, Schiller *et al.* (33) have studied the shift in isoelectric point when fluorescein isocyanate conjugates with bovine serum albumin.

Heterogeneity as exhibited in unlike coupling with protein molecules was indicated in early studies by Creech and Jones (34), for serum albumin conjugated with 1,2-benzanthryl isocyanate. More recently, Curtain (11), employing convection electrophoresis, demonstrated that the coupling of fluorescein isocyanate with gamma globulin was heterogeneous. His studies led him to associate a rapidly moving peak with non-specific fluorescence.

By employing column electrophoresis in the present study (Text-fig. 2), we have confirmed (*a*) the alteration in mobility produced by conjugation with fluorescein isothiocyanate, (*b*) the heterogeneity in the coupled globulin molecules, and (*c*) the fact that the factor producing non-specific fluorescence migrated with the fluorescent antibody (11).

Gradient elution chromatography on diethylaminoethyl cellulose was then undertaken. This procedure yielded fractions that differed in the number of fluorescent groups conjugated to globulin molecules. Non-specific fluorescence was found clearly to be associated with antibody molecules that contained too many fluorescent groups, *i.e.*, fluorescein:protein ratios greater than 3.5×10^{-3}. Relatively homogeneous fractions showing F:P ratios between 2.0 and 3.5×10^{-3} gave bright specific staining and no or negligible non-specific staining. The fluorescein:protein ratio of these particular fractions is about the same as the average ratio of the entire conjugate made by Coons and Kaplan (1) with the use of fluorescein isocyanate and found by them to yield satisfactory staining.

These results implicating fluorescent gamma globulins in the production of non-specific staining differ from the interpretation by Riggs, Loh, and Eveland (19) of their results using chromatography of fluorescent whole antiserum. They observed a decrease in non-specific staining in some of the material eluted from a DEAE-cellulose column charged with fluorescent serum, and they attribute the marked reduction of non-specific fluorescence to elimination of fluorescent non-antibody–containing serum components. Our findings indicate that fluorescein-labeled gamma globulins are retained by DEAE-cellulose and that fractions binding most strongly are to be associated with the production of non-specific staining.

Methods designed to "remove" non-specific fluorescent staining from fluorescent antibody preparations by absorptions with tissue powders or activated charcoal, or extraction with ethyl acetate, were wholly disappointing when, as is usually practiced currently, the coupled globulins exhibit relatively high fluorescein:protein ratios. In our hands, indeed, the main result of tissue powder absorption or the other procedures was to decrease the protein concentration. When a fluorescent antibody globulin with a low fluorescein:protein ratio was absorbed with tissue powders, however, there was an indication that non-specific fluorescence was reduced, albeit with high losses of fluorescent antibody.

The use of columns of dextran gel (sephadex G-50) is found to be far superior to ordinary dialysis in freeing conjugates of diffusible fluorescent compounds. In contrast to the slow and incomplete process of dialysis, one can recover the coupled antibody within 1 to 4 hours after conjugation. Recovery of the coupled antibody is greater than 90 per cent. Further, there remain no diffusible small molecules, so that the F:P ratio becomes rather more meaningful.

The results of this study provide some understanding of non-specific fluorescence and some clues to the devising of a generally useful technique for preparing fluorescent antibodies. Of especial importance is the need to use appropriate simple quantitative techniques in applying fluorescent antibody staining. It is obvious that the aim is to find the most efficient means for coupling antibody in order to have a large percentage of the antibody conjugated uniformly with fluorescent groupings in the most suitable concentration.

The goal of securing satisfactory fluorescent staining with essential absence of non-specific staining may perhaps be attained more readily when thin splenic imprints constitute the test object. The studies should be extended to include the use of the usual, thicker tissue sections cut by microtome in a cryostat.

Whether the findings with sheep antibody will coincide exactly with antibody prepared in other species must also be learned.

SUMMARY

1. A study has been made of the non-specific fluorescent staining of splenic imprints treated with fluorescent sheep antibody globulins.

2. In tissue imprints made with the spleens of antigen-stimulated animals, no morphological distinction was evident between areas showing non-specific fluorescence and specific fluorescence.

3. Elimination of non-specific fluorescence was not achieved by any one, or any combination of the following: (*a*) conjugating only gamma globulins with fluorescein isothiocyanate; (*b*) removal of dialyzable fluorescent products on sephadex, followed by concentration through the use of pressure dialysis; (*c*) use of crystalline preparations of fluorescein isothiocyanate.

4. Individual preparations of fluorescent antibodies were separated by gradient elution chromatography on diethylaminoethyl (DEAE) cellulose into fractions possessing different numbers of fluorescein radicals per molecule of globulin.

5. The coupling ratio of 50 mg fluorescein isothiocyanate (FITC) per gm of protein, as commonly advocated, can not be recommended for the precise localization of antibody globulin in tissues owing to the capacity of the coupled products to give non-specific fluorescent staining. When crystalline preparations of FITC are used instead of the amorphous product at 50 mg/gm protein, far too high non-specific fluorescence results.

6. A fraction with bright specific fluorescence and no or negligible non-specific fluorescence was obtained from each fluorescent antibody that was prepared by using 6 to 8 mg of crystalline fluorescein isothiocyanate per gm of globulin and was then subjected to DEAE-cellulose chromatography and gradient elution to eliminate the most highly coupled molecules.

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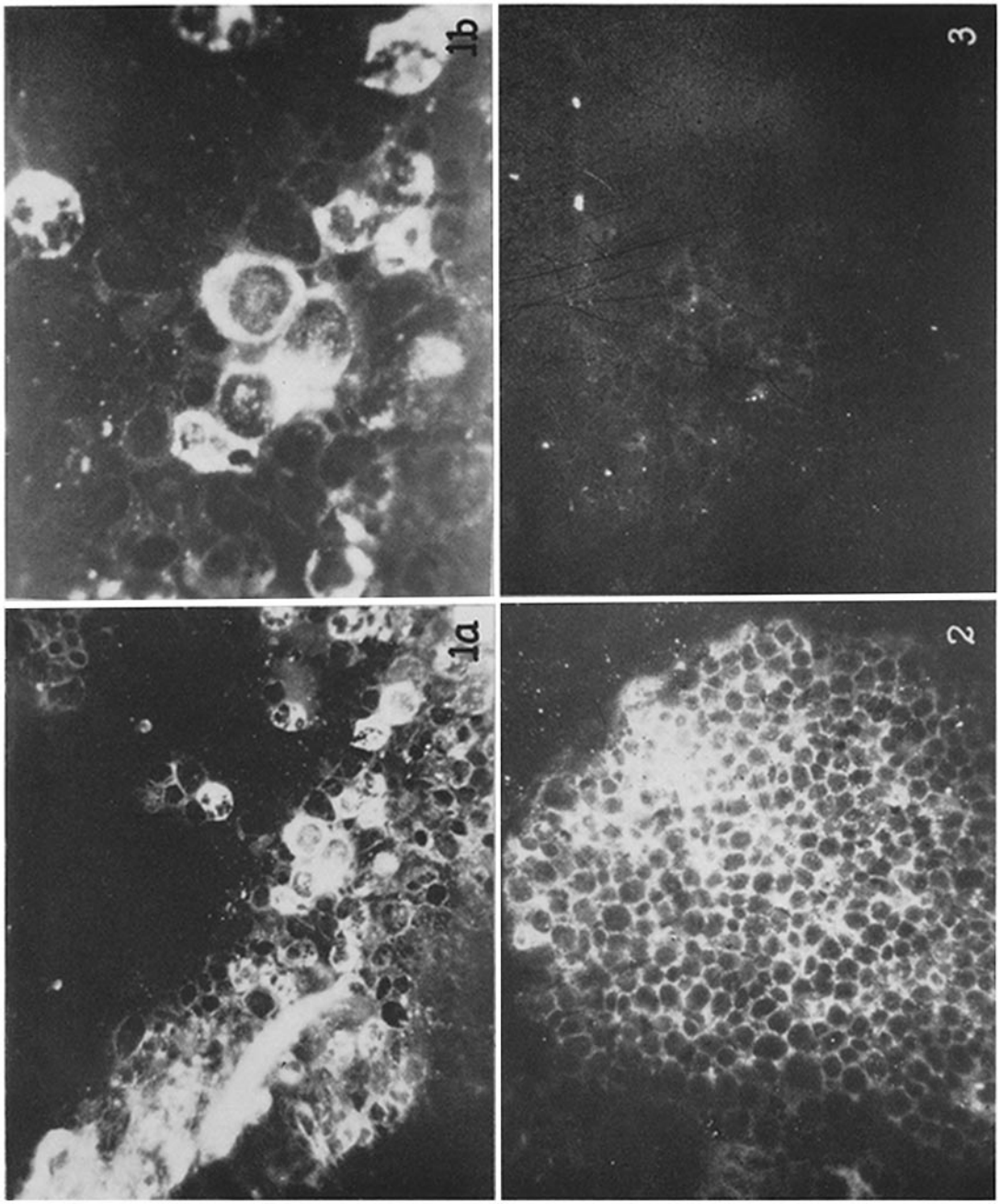
EXPLANATION OF PLATE

PLATE 18

FIG. 1 *a* and 1 *b*. Non-specific fluorescence shown by preparation 11 (Table I) of fluorescent sheep anti-rabbit serum proteins, twice "absorbed" with guinea pig liver powder, and applied at 1 mg/ml to a splenic imprint of an antigen-stimulated guinea pig. Note similarity of fluorescence in mononuclear cells and adjacent polymorphonuclear leukocytes. Super anscochrome, daylight type. Figure 1 *a*, × 400, 1 minute exposure; Fig. 1 *b*, × 880, 5 minute exposure.

FIG. 2. Specific fluorescence in absence of non-specific fluorescence (cf. Fig. 3), given by the first fraction (108 to 170) of preparation 2 (Table II) of fluorescent sheep anti-rabbit serum proteins, not absorbed, applied at 1 mg/ml to a splenic imprint of an antigen-stimulated rabbit. × 400, 1 minute exposure.

FIG. 3. Absence of non-specific fluorescence, same fluorescent antibody preparation as used in Fig. 2, but applied at twice the concentration (2 mg/ml) to a splenic imprint of an antigen-stimulated guinea pig. × 400, 1 minute exposure.



(Goldstein *et al.*: Non-specific fluorescent staining)