gain important information in an artificial, *in vitro* system, which can later be applied to the physiological process.

Summary. Electrophoretic mobilities and thromboplastic activities of emulsions of mixtures of phospholipids derived from beef brain and egg yolk were determined. The studies included mixtures of varying amounts of phosphatidyl serine in lecithin, phosphatidyl serine in phosphatidyl ethanolamine, and phosphatidic acid in lecithin. It was concluded that the manifestation of "thromboplastic activity" by a particular phospholipid emulsion will depend largely upon the surface charge of the particles. The possibility of other factors involved was not excluded.

1. Rouser, G., White, S. G., Schloredt, D., *Biochim. Biophys. Acta,* 1958, v28, 71.

2. Wallach, D. F. H., Maurice, P. A., Steele, B. B., Surgenor, D. M., *J. Biol. Chem.,* 1959, v234, 2829.

3. Marcus, A. J., Spaet, T. H., *J. Clin. Invest.,* 1958, v37, 1836.

4. Troup, S. B., Reed, C. F., Marinetti, G. V., Swisher, S. N., *ibid.,* 1960, v39, 342.

5. Rouser, G., White, S. G., Schloredt, D., *Biochim. Biophys. Acta,* 1958, v28, 81.

6. Rapport, M. M., *Nature,* 1956, v178, 591.

7. Theriault, D., Nichols, T., Jensen, H., *J. Biol. Chem.,* 1958, v233, 1061.

8. Bangham, A. D., *Nature,* 1961, v192, 1197.

9. Rouser, G., Bauman, A. J., Kritchevsky, G., Heller, D., O'Brien, J. S., *J. Am. Oil Chem. Soc.,* 1961, v38, 544.

10. Hanahan, D. J., Dittmer, J. C., Warashina, E., *J. Biol. Chem.,* 1957, v228, 685.

11. Kates, M., *Can. J. Biochem. and Physiol.,* 1954, v32, 571.

12. Bergsagel, D. E., Hougie, C., *Brit. J. Haematol.,* 1956, v2, 113.

13. Horowitz, H. I., Spaet, T. H., *J. Applied Physiol.,* 1961, v16, 112.

14. Bangham, A. D., Heard, D. H., Flemans, R., Seaman, G. V. F., *Nature,* 1958, v182, 642.

Received June 18, 1962. P.S.E.B.M., 1962, v111.

Studies on Fluorescent Antibody Staining II. Inhibition by Sub-Optimally Conjugated Antibody Globulins.* (27810)

GERALD GOLDSTEIN,† BRANCH H. SPALDING,‡ WILLIAM B. HUNT, JR.§
(Introduced by M. W. Chase)
Department of Microbiology, University of Virginia School of Medicine, Charlottesville

The principle followed until recently for preparing fluorescent antibody reagents has been to attach to antibody globulins as many fluorescent groupings as possible without precipitating the antibody. Then, to reduce the large amount of resulting non-specific fluorescence, it has been customary to subject the fluorescent antibody to multiple absorptions with tissue powders, charcoal, or resins.

Recent reports(1-3) have shown that the attachment of fluorescein isothiocyanate (FITC) to protein is not a uniform process, and that within each fluorescent antibody preparation there may be a marked variation in the number of fluorescent groupings per protein molecule. Furthermore, it has been shown that the specific and the non-specific staining properties of fluorescent antibodies are related to the number of fluorescent groupings conjugated with antibody. Thus, homogeneous fractions of fluorescent antibodies with fluorescein:protein (F:P) ratios of 2 to 3.5×10^{-3} have satisfactorily bright specific staining and negligible non-specific staining (*cf* 1).‖ Fractions with higher F:P ratios stain both non-specifically and specifically. These findings have led to the realization that it is not desirable to couple more than a certain chosen number of fluorescent groupings.

* This work was supported by USPHS research grants.

† Recipient USPHS Research Career Development Award.

‡ Deceased.

§ Post Doctoral Fellow USPHS Training Grant.

‖ The F:P ratio is a relative measure of the weight of fluorescein and protein in a fluorescent protein conjugate (Table I).

Griffin and coworkers(4) suggest that "a logical procedure for the preparation of satisfactory conjugates would seem to lie in the labeling of high titer antisera or fractions thereof at low dye-to-protein ratios." Goldstein, Slizys, and Chase(1) in addition to lowering the dye-to-protein ratio, fractionated their coupled sheep globulins by salt gradient elution from DEAE-cellulose. In this way the globulins with bright specific and no non-specific staining were separated from the excessively coupled globulins. Each of their preparations had a fraction with a low fluorescein content that did not impart fluorescence to specific tissue imprints. The portion of such under-coupled globulins was too small to influence the brightness of staining of the whole fluorescent antibody preparation.

The purpose of this paper is to report observations on a series of fluorescent rabbit antibody globulins prepared by adding different amounts of FITC to protein to provide various low dye-to-protein ratios. In each of these preparations, a significant proportion of the whole preparation contained less than the optimal range of fluorescent groupings. Inclusion of such material with optimally coupled globulins inhibited the brightness of specific staining, and thereby the sensitivity of the fluorescent antibody technic.

Elimination of sub-optimally coupled globulins from the fraction with bright specific staining was obtained in the same step used to separate the excessively coupled globulins, *i.e.*, chromatography on DEAE-cellulose.

Materials and methods. A detailed description of the procedures appears in(1). Slight modifications and a description of new materials follow:

Fluorescein isothiocyanate. A crystalline preparation obtained from Baltimore Biological Laboratories (Lot #006652) was used for conjugation with antibody globulins.

Antisera. Anti-Staphylococcus aureus *serum.* A coagulase-producing hemolytic strain of *S. aureus* was used to immunize 2 groups of New Zealand albino rabbits. Preparation 1 was obtained from a rabbit that received intradermal injections of killed *S. aureus,* and was bled 7 days after the last injection. Preparation 2 was a pool of sera with agglutination titers of 1:4000 to 1:65,000 obtained from 2 rabbits immunized by the method of Stern and Elek(5).

Anti-ragweed serum. Dwarf ragweed pollen extract was prepared by defatting pollen in ethyl ether, and extracting the defatted pollen in glass distilled water. New Zealand albino rabbits were injected subcutaneously with the extract in a stable water-in-oil emulsion containing heat killed *Mycobacterium bovis.* Three weeks after the initial injection, the animals were restimulated with aqueous ragweed extract injected intravenously. The animals were bled 5 and 6 days after the last injection. Each serum gave a rapid flocculent precipitin reaction with ragweed extract.

All sera were stored without preservatives at −22°C.

Preparation of globulins. Serum was diluted with an equal volume of 0.15 *M* sodium chloride and solid ammonium sulfate was added to a final concentration of 1.6 *M*. The precipitate was washed with 2.0 *M* ammonium sulfate in distilled water, redissolved, dialyzed, and applied to a DEAE-cellulose column to secure a chromatographically homogeneous fraction.

Biuret protein determinations were made using the biuret reagent described in(6).

Conjugation of serum globulins and FITC. Two modifications were arbitrarily introduced. To minimize brief local increases in FITC and acetone concentrations, the FITC dissolved in acetone was delivered slowly (15 minutes to 2 hours) from a tuberculin syringe through a fine polyethylene tubing (Clay Adams P.E. 50) to the bottom of the rapidly stirring reaction mixture.

In Preparations 1 and 2, the pH was maintained between 9.0 and 9.5 during the initial 2 hours of coupling by addition of 0.1 *N* NaOH rather than of sodium carbonate.

Staining of imprints. Suspensions of *S. aureus* in 0.15 *M* sodium chloride were smeared onto albumin coated microscope slides. The smears were fixed for 3 minutes in 95% ethyl alcohol prior to staining.

Splenic imprints were prepared from rabbits sacrificed 5 days after restimulation with

ragweed extract. The indirect technic with layering of antigen over the imprint was used(7). Ragweed extract (.52 mg nitrogen/ml) was applied to individual imprints for 20 minutes. The slides were washed with buffered saline (0.15 *M* sodium chloride, 0.01 *M* sodium phosphate, pH 7.2) and stained with fluorescent antibody reagents.

Specificity of staining. To determine the specificity of fluorescence, inhibition tests with non-fluorescent antibody globulins were performed in the *S. aureus* : rabbit anti-*S. aureus* system. For the ragweed : antiragweed system, the antigen layer was replaced by buffered saline. In addition, spleen imprints from rabbits sacrificed after restimulation with bovine serum albumin were layered with ragweed extract and fluorescent antibody reagents.

Fluorescence microscopy. A power supply with a constant input voltage of 120 volts, and an Osram HBO-200 light source were used. A Corning 5840 exciter filter and a Euphos 2.5 mm barrier filter were used with a Leitz Wetzlar darkfield microscope.

Results. All globulins were reacted with FITC for approximately 24 hours. Rapid separation of the fluorescent globulins from both the acetone and the fluorescent dialyzables was obtained by passing the neutralized reaction mixture through Sephadex G-50 columns equilibrated with buffered saline(1,2, 8-13). Fig. 1 illustrates the separation achieved in the Preparation 2 reaction mixture. The first peak was the emergence of fluorescent antibody which was subsequently fractionated on DEAE-cellulose. The second peak is identified with acetone, which emerged in the same position as added samples of ribose. Unreacted fluorescent materials emerged in a broad band after the acetone. When amorphous FITC was used(1), traces of dialyzable fluorescent materials remained on the Sephadex particles despite extensive washing with buffered saline.

Properties of fluorescent antibody globulins Preparations 1-3. Preparations 1 to 3 were coupled with FITC at low dye to protein ratios to prevent excessive coupling of the antibody globulin and thereby to avoid non-specific staining. Table I presents the

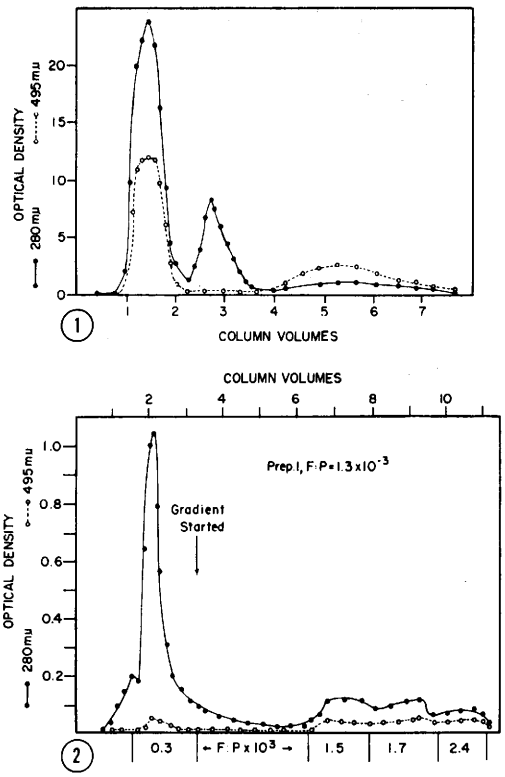

FIG. 1. Fractionation of fluorescent antibody reaction mixture (Preparation 2) on Sephadex G-50. Bed volume 100 cm³, 0.15 *M* NaCl, 0.01 *M* sodium phosphate, pH 7.2. Flow rate 30 ml/hr.

FIG. 2. Gradient elution of Preparation 1 on DEAE cellulose. Bed volume 110 cm³. Starting buffer 0.01 *M* sodium phosphate, pH 7.5 with chloride gradient. Flow rate 200 ml/hr.

coupling conditions and the F:P ratios of each preparation and of its fractions. The F:P ratios of Preparations 2 and 3 were in the optimal range ($2.0 - 3.5 \times 10^{-3}$) for satisfactorily bright specific staining and no non-specific staining. Preparation 1 was coupled at a 1% protein concentration and had an F:P ratio that was below the optimal range.

The staining properties at 1 mg/ml of Preparations 1 and 2 were satisfactory with regard to the absence of non-specific staining while Preparation 3 had traces of non-specific fluorescence. However, the specific staining of each preparation lacked the brightness seen in imprints stained with homogeneous fractions of fluorescent antibodies with F:P ratios of $2.0 - 3.5 \times 10^{-3}$. Each preparation was then fractionated on DEAE-cellulose to determine if a significant portion had less

TABLE I. Characterization of Fluorescent Antibody Globulins.

Preparation No.	Product coupled	Protein in reaction mixture	FITC added, mg/g protein	F:P × 10^3*		
				Whole	Low	High
1	Rabbit anti-*S. aureus*	1%	8	1.3	.3	2.4
2	*Idem*	2%	8	2.7	.6	3.2
3	Rabbit anti-ragweed extract	2%	10	2.4	.6	3.5

* Fluorescein is determined by reference to the absorption curve of crystalline FITC read at 495 mμ, corrected for the weight contribution of the isothiocyanate moiety. Protein is determined by the biuret method, referred to values obtained with a gamma globulin standard. The F:P ratio thus secured does not correct for difference in E_o at 495 mμ between free and conjugated fluorescein radicals.

than the optimal number of fluorescent groupings.

Fractionation of fluorescent antibody. Each fluorescent antibody preparation was dialyzed against 0.01 *M* sodium phosphate buffer, pH 7.5, and applied to a DEAE-cellulose column equilibrated with the same buffer. Globulins with very few fluorescent groupings (F:P = $0.3 - 0.6 \times 10^{-3}$) were not bound to DEAE-cellulose and were collected after a column volume of buffer passed through the column. After this fraction was collected, a gradient elution to 1 molar sodium chloride was started. The bound fluorescent globulins were thus eluted in order, starting with the lesser fluorescent and progressing towards the more fluorescent globulins. Successive portions of the eluate were pooled and concentrated by pressure dialysis. The protein concentration, relative fluorescein content, and the F:P ratio of each fraction was determined.

A measure of the separation obtained by DEAE-cellulose chromatography is shown under the Low and High columns of Table I. In each preparation there was a 6-8-fold difference in the fluorescein content of the low and the high fraction. The percentage of undercoupled antibody globulin in the whole preparation varied from 35% to 80%. These large quantities of sub-optimally conjugated antibody were responsible for the decreased brightness of specific staining of each preparation.

The elution curve in which 80% of the protein was poorly coupled (Preparation 1) is shown in Fig. 2. The 495 mμ absorption represents the absorption of FITC. The 280 mμ absorption represents the sum of the protein absorption and a secondary absorption peak of FITC. The F:P ratios of the pooled and concentrated fractions are shown below the curve. In this preparation, the major portion of the fluorescent globulins was not bound on DEAE-cellulose and was collected before the sodium chloride gradient was started.

There was a marked difference in the staining by the chromatographic fractions of fluorescent antibody preparations. Owing to the undercoupling, the whole products stained only dully. Table II records the intensity of specific staining by Preparation 1 and its high and low fractions. At equal protein concentrations, the staining by the whole product fell between the staining of the low and the high fractions. When the most highly coupled portion (F:P = 2.4×10^{-3}) was separated and used alone, the brightness of staining became acceptable. The staining by the low fraction was barely discernible.

Inhibition tests were performed to determine whether in the whole product, the low fraction merely diluted the high fraction, or whether it inhibited the staining by the high fraction. Smears of *S. aureus* were incubated first with the low fraction at a protein concentration of 7.5 mg/ml or with buffered saline. After washing the smears were incubated with the high fraction at a protein con-

TABLE II. Specific Staining by Preparation 1 and Its Highest and Lowest F:P Fractions.

Product	Protein, mg/ml	F:P × 10^3	Brightness of staining
Whole	1	1.3	±
High	1	.3	+ str
Low	1	2.4	ffftr

ffftr, exceedingly faint; ±, one-half; + str, one-plus strong; on scale of +++.

centration of 1 mg/ml. At these protein concentrations, approximately equal concentrations of fluorescent groupings were applied by both fractions. Pre-treatment with the low fraction inhibited the subsequent staining by the high fraction. Such smears had a fluorescent staining intensity that was very weak (trace) compared to the saline pretreated smears which had acceptable fluorescent staining (one-plus strong).

Qualitatively similar results were obtained with Preparations 2 and 3 containing approximately 35% of sub-optimally coupled globulins. With these preparations the amount of inhibition of specific staining by the low fraction was much less than that seen in Preparation 1.

Discussion. These results are presented to emphasize the need to use fluorescent antibodies prepared at low dye to protein ratios, and to fractionate such fluorescent antibody preparations into portions with differing fluorescein content. Unless these procedures are performed, it will be difficult to secure a reagent that will possess maximal sensitivity and maximal specificity. It has already been demonstrated by several investigations that attaching too many fluorescent groupings to antibody globulins is associated with non-specific staining. To this we add the obvious finding that attaching too few fluorescent groupings may markedly decrease the sensitivity of specific fluorescent staining. If a large portion of the preparation is poorly coupled, the staining by the whole preparation may be so dull as to be unusable. Fortunately the use of column chromatography on DEAE-cellulose to separate excessively coupled globulins from optimally coupled globulins also functions to separate sub-optimally coupled globulins from the desired product. Although the meaning of the F:P ratio in terms of actual numbers of fluorescent groupings present is not established(14), these ratios provide a reliable relative measure of fluorescein content per unit concentration of protein. Homogeneous fractions with F:P ratios of 2–3.5 $\times$ 10^{-3} have bright specific and no non-specific staining at concentrations of 1 mg/ml.

Each of the fluorescent globulins described here was prepared from rabbit serum. The same purification and conjugation procedures applied to sheep serum have not produced fluorescent globulins with more than 5% of sub-optimally coupled globulins. A rabbit globulin prepared according to the procedures described in(1) at the laboratory of Dr. M. W. Chase of the Rockefeller Institute did not have a significant undercoupled fraction. Although the reasons for these differences in coupling have not been determined, these differences lend emphasis to the need for each laboratory to determine the range of heterogeneous coupling in its fluorescent products.

Summary. 1. Three rabbit immune globulin preparations were coupled at 1 or 2% protein concentration with 8 or 10 mg of crystalline FITC per gram of protein. 2. Each preparation was heterogeneous with respect to the number of fluorescent groupings coupled to individual globulin molecules. 3. In these preparations from 35 to 80% of the conjugated globulins contained less than an optimal number of fluorescent groupings. 4. Maximum brightness of specific staining was obtained after each preparation was fractionated on DEAE-cellulose to separate the optimally coupled from the under-coupled and the over-coupled globulins.

1. Goldstein, G., Slizys, I. S., Chase, M. W., *J., Exp. Med.* 1961, v114, 89.

2. Curtain, C. C., *J. Hist. and Cyto.,* 1961, v9, 484.

3. Peters, J. H., McDevitt, H. O., Pollard, L. W., Harter, J. G., Coons, A. H., *Fed. Proc.,* 1961, v20, 17.

4. Griffin, C. W., Carski, T. R., Warner, G. S., *J. Bact.,* 1961, v82, 534.

5. Stern, H., Elek, S. D., *J. Path. & Bact.,* 1957, v73, 473.

6. Mokrasch, L. W., McGilvery, R. W., *J. Biol. Chem.,* 1956, v221, 909.

7. Coons, A. H., Fluorescent Antibody Methods in *Gen. Cytochemical Methods,* Ed., C. F. Danielli, Academic Press, N. Y., 1958, p399.

8. Goldstein, G., Slizys, I. S., Chase, M. W., *Bact. Proc.,* 1960, 139.

9. Zwann, J., Van Dam, A. F., *Acta Histochem.,* 1961, v11, 306.

10. Fothergill, S. E., Nairn, R. C., *Nature,* 1961, v192, 1073.

11. George, W., Walton, K. W., *Nature,* 1961, v192, 1188.

12. Gordon, M. A., Edwards, M. R., Tompkins, V. N., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 96.

13. Rinderknecht, H., *Nature*, 1962, v193, 167.

14. Goldman, M., Carver, K. R., *Exp. Cell. Res.*, 1961, v23, 265.

Received June 28, 1962. P.S.E.B.M., 1962, v111.

Lipid Composition of Human Cerebrospinal Fluid.* (27811)

M. H. HACK AND F. M. HELMY

Departments of Medicine and Anatomy, Tulane University School of Medicine, New Orleans, La.

It has recently been reported(1) that human cerebrospinal fluid (CSF) contains cerebroside, sphingomyelin, cholesterol, cholesterol esters and triglycerides as well as a number of unidentified lipids. The present paper will present data confirming and extending some of these observations.

Materials and methods. The 14 samples of CSF examined in this study were obtained through the cooperation of Dr. R. C. Llewellyn during the course of pneumoencephalographic examination. The freshly collected samples (20-80 ml) were either centrifuged at 25,000 *g* for 20 minutes or they were filtered through coarse sintered glass filters, only water-clear samples were examined. The supernates or filtrates were promptly shell frozen in a dry-ice ethanol bath and freeze dried; the dry residue was extracted with 0.5 ml chloroform-methanol (2:1) per ml of CSF. Extracts obtained by both methods seemed to be identical and there were no obvious correlations to be made relating the resulting lipid pattern to the neurological complaint. Because of the large proportion of water soluble extractives, the chloroform : methanol extract, after filtering through a sintered glass filter (Corning porosity C), was fractionated according to Folch's technic(2) by addition of 0.73% NaCl. The resulting chloroform extract was dried over anhydrous Na_2SO_4, filtered again through sintered glass, and its volume reduced to 1/10th by means of a stream of nitrogen with the sample in a water bath at 40°C. Twenty-five to 50 μl of this concentrate was generally adequate for analysis by the paper chromatographic technic described elsewhere(3). Our experience has shown that when multiple spot-test analysis is applied to paper chromatograms their usefulness is greatly extended and serves to quite adequately identify various phosphatides.

Results and discussion. Fig. 1 clearly demonstrates the presence of both ethanolamine and choline plasmalogens; on hydrolysis with $HgCl_2$ (*cf.* 4) it was possible to show that both diester analogs were also present. As can be seen from the figure, ethanolamine plasmalogen was proportionately greater, whereas in plasma the reverse was true; this would suggest that CSF is not a simple plasma transudate. Choline spot-tests verified the presence of both lecithin (and choline plasmalogen) and sphingomyelin. When compared with authentic brain cerebroside and ganglioside (through the generosity of E. Klenk), no corresponding components, characterizable by the periodic acid-Schiff reaction, were observed in the CSF using the ketone, pyridine, water chromatographic system(5). There was, however, detectable by this system, a trace amount of a plasmalogen component which occupied a position on the chromatogram just above phosphatidyl ethanolamine. Due to insufficient material this component has not been characterized further, although it closely resembled cardiolipin when compared with a sample of this phosphatide (isolated from dog heart by column chromatography on silicic acid).

A second uncharacterized material could be seen between the phosphatidyl choline and phosphatidyl ethanolamine, stainable only by Rhodamin 6G and fairly rapidly leached by the subsequent SO_2 washings. This material did not give a positive phosphorus reaction.

* Supported by grants from Nat. Inst. Health, U.S.P.H.S.