Use of Optical Density of Fluorescent Conjugates for Analysis of Co-Precipitating Antibody.* (26013)

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The possibility that concentration of fluorescein in fluorescein-conjugated protein solutions might be used as an index of their protein content was suggested by the work of Schiller *et al.*(1), who demonstrated that fluorescein-protein conjugates, like fluorescein dye, have a light absorption peak at a wave length around 490 mμ. Optical density (O.D.) at 490 mμ of a fluorescein-protein conjugate is an index of its dye content and, for that conjugate, of its protein concentration. For any preparation this direct relationship exists over a wide enough range to suggest the spectrophotometric determination of fluorescein content as a means of determining protein content.

A procedure for quantitative determination of non-precipitating antibodies has been described(2). Total protein precipitated in the equivalence zone from a mixture of precipitating and non-precipitating antibodies and homologous antigen is compared to amount of precipitate obtained by using precipitating antibody and antigen alone. The difference is taken as the amount of non-precipitating antibody [(precipitins + co-precipitating antibodies + antigen)—(precipitins + antigen) equals co-precipitating antibodies]. The validity of the results depends on the assumption that addition of the co-precipitating factor does not alter amount of protein precipitated from the precipitating serum.

Since concentration of the fluorescein-protein conjugate can be estimated directly on the basis of the O.D. at 490 mμ, use of conjugated antibodies (precipitins in the system described above) should obviate the need of the double sample. We are reporting preliminary experiments for titrating conjugated added to unconjugated precipitating antibodies.

Procedure. Anti-egg albumin and anti-human globulin rabbit antisera were obtained from animals inoculated weekly with 5 subcutaneous injections of antigen† in Freund's complete adjuvant. One portion of each serum was precipitated at 33% saturation with ammonium sulphate to obtain the crude gammaglobulins that were conjugated with fluorescein isothiocyanate following the method of Riggs *et al.*(3).

After 7 days' dialysis against phosphate buffered saline at pH 7.4, variable amounts of the conjugates were dissolved in N/1 NaOH and the solutions were examined for the spectrum absorption between 300 and 1000 mμ with a Beckman DU Spectrophotometer, against a blank of N/1 NaOH. Fig. 1 shows that in this alkaline medium conjugates have a sharp absorption peak between 420 and 540 mμ with a maximum near 500 mμ.

The absorption characteristics of the various concentrations of the fluorescent protein preparations were determined by their O.D. at 490 mμ. Results were proportional to their protein concentration for a range of 5-200 γ/ml.

Because the antibodies, specifically precipitated from the conjugated gamma globulins and then redissolved in N/1 NaOH, had the same fluorescein-protein ratio as total conjugated gamma globulins from the same batch the standard was always prepared with crude conjugated gamma globulins.

Protein content of the standard was determined by the Lowry method(4) at 750 mμ and the O.D. of known dilutions of the same preparation, determined at 490 mμ was plotted against their protein content (Fig. 2).

The following antigen/antibody systems were prepared:

a) unconjugated rabbit anti-egg albumin serum + conjugated rabbit anti-egg albumin globulins + egg albumin.

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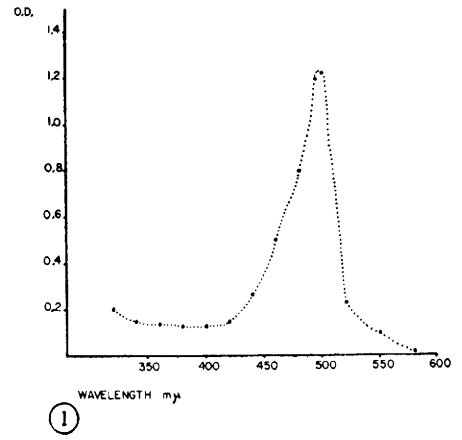
† Thrice crystallized egg albumin (Armour) poliomyelitis immune human globulin (Lederle).



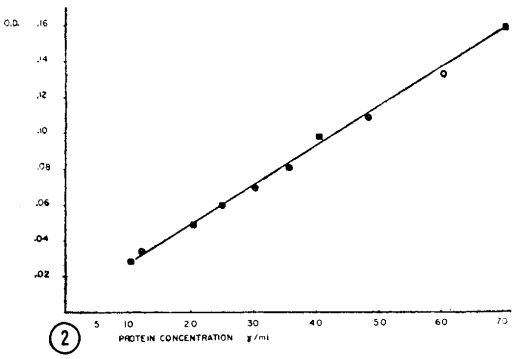


FIG. 1. Absorption spectrum of a fluorescein isothiocyanate conjugated rabbit anti-human globulin solution in N/1 NaOH. Concentration of protein 200 γ/ml.

FIG. 2. Relation between optical density and protein concentration in a conjugated anti-egg albumin (○) and in a conjugated anti-human globulins (■) rabbit globulin solutions.

b) unconjugated rabbit anti-egg albumin serum + conjugated anti-human rabbit globulins + egg albumin.

c) unconjugated rabbit anti-human serum + conjugated rabbit antihuman globulins + human globulins.

d) unconjugated rabbit anti-human serum + conjugated rabbit anti-egg globulins + human globulins.

The conjugated globulins were in the concentration of 1% protein, the heterologous conjugated antibody in b) and in d) served as controls.

The various mixtures were placed at 37°C for 2 hr, then overnight at 4°C. Resulting precipitates were washed 3 times with saline buffered at pH 7.4 at 4°C, then dissolved in N/1 NaOH.

One part of the dissolved precipitate was used for spectrophotometric determination of the conjugated protein content by means of the O.D. at 490 mμ using the standard curve of O.D. *vs.* protein concentration prepared from the same batch. The other part of the dissolved precipitate was used to estimate protein content by the Lowry method. Volumes used for the latter were calculated on the basis of the expected amount of specific precipitate so that the values were in the workable range of the Lowry method either at 750 or 560 mμ. The 490 mμ more suitable for higher protein concentrations was not used because of interference of the fluorescent dye.

Results. The results of a typical determination are shown in Table I. Only when homologous conjugated antibody was added did an appreciable amount of fluorescent dye appear in the dissolved precipitate. No significant O.D. readings could be obtained at 490 mμ in the redissolved precipitate from the unconjugated antibody to which heterologous conjugated antibody and specific antigen had been added.

Amount of protein precipitated from the conjugated globulins was calculated by 2 methods: (1) using the Lowry technic and subtracting the results of b) from a) and of d) from c) respectively; (2) by direct estimation of O.D. of the precipitate at 490 mμ, interpolating this result on the standard. It should be emphasized that the results obtained by the two methods agree only when the data in the equivalence zone are compared; at other antigen/antibody ratios the data, calculated by difference, do not agree with the direct spectrophotometric determination. In Table I the results obtained in equivalence zone are italicized.

The experiment has been repeated using unconjugated rabbit antiserum heated at 75°C to destroy its precipitating activity without eliminating its capacity of co-precipitating in presence of homologous precipitating antibody and antigen(5). Table II shows that, in this condition too, use of fluorescein conjugated precipitins permits us to distinguish in

TABLE I. Analysis of Specific Precipitate Obtained from Mixtures of Unconjugated Precipitins and Conjugated Precipitins.

Antibodies	Antigen (γ)	Total pptd. proteins (ag + ab) (γ)	O.D. at 490 mμ	Anti-bodies pptd. (γ)	Conjugated proteins in precipitate (γ)	Unconjugated proteins in precipitate (γ)
.1 ml anti-egg serum	200 EA	600	.045		72	
+ .3 ml conjug.	150	820	.061		104	
anti-egg glob.	100	*960*	.080	*860*	*134*	*726*
(1% proteins)	75	860	.075	785	120	665
	50	800	.065	750	110	640
.1 ml anti-egg serum	200 EA	400	.005		traces	
+ .3 ml conjug.	150	650	—		—	
anti-hum. glob.	100	820	—	(720)	—	
(1% proteins)	75	790	.005	(715)	traces	
	50	*780*	—	*730*	—	
.1 ml anti-hum. serum	100 HG	390	.078		128	
+ .3 ml conjug.	75	*510*	.096	*435*	*152*	*283*
anti-hum. glob.	50	420	.074	370	124	246
(1% proteins)	25	370	.071	345	116	229
.1 ml anti-hum. serum	100 HG	230	—		—	
+ .3 ml conjug.	75	260	.002	(185)	traces	
anti-egg glob.	50	*340*	.006	*290*	"	
(1% proteins)	25	250	.005	225	"	

Washed precipitates were dissolved in 4 ml of N/1 NaOH; the Lowry method was performed on .2 ml of these solutions and results multiplied by 20; O.D. were interpolated on standard curve to obtain conjugated protein concentration and results multiplied by 4. Proteins precipitated from unconjugated serum were given by the difference: Antibodies pptd. – conjugated proteins pptd.

EA = egg albumin; HG = human globulins.

TABLE II. Analysis of Specific Precipitate Obtained from Mixtures of Conjugated Precipitins and Heated Rabbit Antisera.

Antibodies	Antigen (γ)	Total pptd. proteins (ag + ab) (γ)	O.D. at 490 mμ	Anti-bodies pptd. (γ)	Conjugated proteins in precipitate (γ)	Unconjugated proteins in precipitate (γ)
.5 ml anti-human	100 HG	310	.071		130	
heated serum +	75	*340*	.093	*265*	*148*	*117*
.3 ml conjugated	50	320	.070	270	114	156
anti-human glob.	25	210	.066	185	108	77
(1% proteins)						
.5 ml anti-egg heated	100 HG	160	.075		120	
serum + .3 ml	75	190	.069		114	
conjugated anti-	50	220	.090	(170)	144	(16)
human glob.	25	*190*	.089	*165*	*142*	*23*
(1% proteins)						

Anti-human globulins and anti-egg albumin rabbit sera, diluted 1 to 5, were heated at 75°C for 15', kept in cold for 2 wk and centrifuged before using; no precipitins were present in these sera. The mixtures unconjugated heated antisera, conjugated anti-human globulins, human globulins were kept for 3 days at 4°C before titration of precipitates.

the specific precipitate the proteins of the two sera.

The optical characteristics of the fluorescein isothiocyanate might be applicable for determination of protein content of non-precipitating antibodies when they are co-precipitated by fluorescein-conjugated precipitins. We are at present trying to apply this method to quantitative determination of the reagins.

Summary. The O.D. of fluorescein isothiocyanate permits determination of quantity of protein derived from a conjugated precipitin serum when this is added to an unconjugated precipitin serum. This permits one to distinguish in a specific precipitate the antibodies of

the 2 sera. This finding could have practical application in co-precipitation technics.

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Experimental Histoplasmosis in Large Domestic Animals.* (26014)

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Previous studies from these laboratories have been concerned with pathogenesis of histoplasmosis in mice(1-4), dogs(5,6), and monkeys(7). The present report describes experimental disease in horses, cattle, sheep, and swine. Although naturally occurring clinical histoplasmosis is rare in these species(8), skin-test and other evidence(9) suggest that, as in man, asymptomatic infection may be more common. To our knowledge, experimental infection of these species with *H. capsulatum* has not been reported.

Materials and methods. Eight healthy young adult animals, 2 of each species and one of each sex, were used. After completion of base line studies, each was inoculated intratracheally(10) with 5.0 ml of saline suspension of yeast phase *H. capsulatum*† containing 2.0 ml of packed cells from 3-day cultures grown on brain heart infusion (Difco) blood agar. Pontocaine (Winthrop), 2.0 ml, was administered intratracheally 5 minutes prior to inoculation of yeast cells to depress reflex coughing. Animals were observed daily for clinical signs of illness, and blood samples for cultures, complete blood counts, and serologic studies were taken 7, 10, 17 and 24 days after inoculation. Serologic response was studied by collodion agglutination(11) and complement fixation(12) tests at these intervals, then weekly for about 3 months. Skin tests were performed 31 and 73 days after inoculation, using histoplasmin prepared and standardized in infected dogs by previously described methods(13-14). Seven of 8 animals were sacrificed about 3 months post-inoculation, and complete autopsies performed. Tissues taken at autopsy and all blood samples were treated and cultured by the method of Conant(15).

Results. Table I summarizes findings in 8 large domestic animals inoculated intratracheally with *H. capsulatum*. All except the female sheep exhibited clinical evidence of infection and all produced antibodies and developed positive skin tests as compared to pre-inoculation antibody-negative sera and negative skin tests. Fever was first observed in 7 of 8 animals 3-5 days after inoculation, and persisted for about 1 week except in the male sheep and swine, in which 3 weeks of fever was observed. Symptoms varied from slight cough in the 2 swine to definite respiratory symptoms in the male sheep characterized by frequent cough, dyspnea, dry rales, tachypnea and hyperpnea; rales and hyperpnea persisted in this animal up to time of sacrifice at 88 days. A positive blood culture was obtained from the male sheep 7 days after inoculation, and *H. capsulatum* was seen in lung lesions at autopsy. In other animals, symptoms subsided within a few days after the end of the febrile period. A positive blood culture was also obtained from the female swine 7 days after inoculation. No significant

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† Strain G 13—an isolate from spontaneous histoplasmosis in a dog.