Paper chromatographic analysis and scanning with ultraviolet light showed trace contamination of the FMN with riboflavin and trace contamination of the FAD with FMN and riboflavin. The amount of the contaminants was so low as not to be of the order that would statistically alter the observed results.

Based upon the observations described herein, it is suggested that in the turbidimetric microbiological determination of tissue flavin, where the flavins are extracted by acid hydrolytic means, FMN should be used as the primary standard.

Summary. The growth responses, measured turbidimetrically, of *Lactobacillus casei* (ATCC 7469) to riboflavin, FMN, and FAD differ significantly from each other. In the assay of acid extracts of tissues for flavin content, the use of FMN, as the standard, is recommended.

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Isolation of a Reaginic Antibody Fraction with Properties of γ_G-Globulin.* (31078)

A. MALLEY AND FRANK PERLMAN (Introduced by George Kittinger)
Oregon Regional Primate Research Center, Beaverton, and University of Oregon Medical School, Portland

Attempts to isolate highly purified and highly active reaginic antibodies have, in general, achieved limited success. Previous attempts to apply DEAE-cellulose ion-exchange chromatography(1-4) to the isolation of reagin from grass and ragweed pollen sensitive patients resulted in a distribution of the reaginic activity into several fractions containing both γ_G- and γ_A-globulins. With the availability of Sephadex G-200, attempts to apply the gel-filtration method in conjunction with ion-exchange chromatography(4-6) have yielded much the same results. This report describes the isolation of a highly active reaginic antibody fraction which has a sedimentation coefficient of $6.7S_{20,w}$, is heat unstable at 56°C, and gives a single line of precipitation against rabbit anti-total human globulins and specific rabbit anti-human γ_G-globulin.

Materials and methods. Allergens and reagin. Reaginic serum was obtained from an untreated timothy sensitive patient (E.F.). This serum was collected during the pollen season, and was kept at —20°C until used. Preliminary to the isolation of the reaginic antibody fraction described below, this serum gave positive Prausnitz-Kustner (P-K) reactions at a dilution of 1:10,000 in 4 non-allergic human volunteers. The allergens used in passive transfer experiments in both man and rhesus monkeys were a crude water soluble timothy extract (WST) and a partially purified fraction of timothy pollen (>40% ETOH). Methods for preparation of both WST and >40% ETOH fraction have been described(7).

Antisera to human serum proteins. Rabbit

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anti-human γ_M-, γ_A- and γ_G-globulins were purchased from Behringwerke Ag., distributed by Lloyd Brothers, Inc., Cincinnati, Ohio. Human γ_G-globulin was purified on DEAE-cellulose by the method of Stanworth(8), and rechromatographed on DEAE cellulose by the method of Fahey and McCloughlin(9). The purified γ_G-globulin was utilized to check the specificity of the commercially obtained rabbit anti-human γ_G-globulin both by immunodiffusion and immunoelectrophoresis. Rabbit anti-total human globulin was prepared by immunizing 3 rabbits with 10 mg protein/ml of a solution of serum proteins (precipitated by 50% saturated ammonium sulfate and resuspended in saline) in complete Freund's adjuvant. The adjuvant was administered at weekly intervals for 3 weeks, and the animals were bled 4 weeks after the last injection.

Passive transfer reactions. Prausnitz-Kustner (P-K) reactions were carried out on 4 non-allergic human volunteers having no clinical history of allergy, and having negative skin reactions to both WST and the >40% alcohol fraction of timothy pollen. Each individual received 0.1 ml intradermal injections of a 1:6 dilution of a reagin containing serum and various chromatographically purified (gel-filtration and DEAE cellulose) fractions of this serum. Forty-eight hours later, the sites were challenged with 0.05 ml of a 1:100,000 (W/V) dilution of the >40% ETOH fraction, and reactions were graded 0-4+ twenty minutes after injection of the allergens.

PCA reactions in 3 kg rhesus monkeys were accomplished by the method described by Layton *et al*(10). Each animal received 0.1 ml intradermal injections of a 1:6 dilution of a reagin containing serum and the same chromatographically purified fractions of this serum used previously in the P-K tests in man. Twenty-four hours later each animal received a 5 cc intravenous injection of a 1% Evans Blue dye. Fifteen minutes later each animal was given a second intravenous injection of 5 mg protein of WST, and the reactions were graded 0-4+ twenty minutes later.

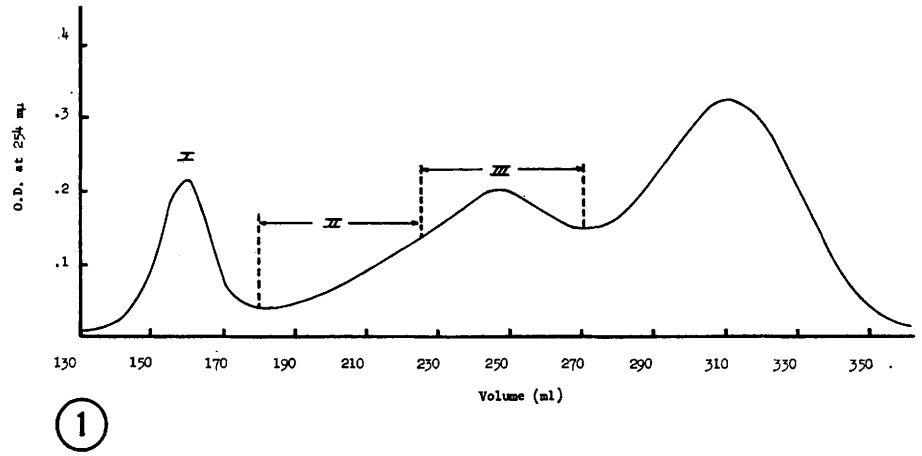
Immunodiffusion and immunoelectrophoresis. Double diffusion in agar plates was carried out by the method of Ouchterlony(11). Immunoelectrophoresis was performed by the method described by Campbell *et al*(12).

Gel-filtration and ion-exchange chromatography. Gel filtration of a reagin containing serum from an untreated timothy sensitive patient (E.F.) was carried out on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). Five milliliters of the serum was adjusted to 1 M NaCl by addition of solid NaCl. This sample was then applied to a Sephadex chromatographic column (2.5 × 100 cm) which had been packed with Sephadex G-200 prepared by the method of Borsos and Rapp(13) and equilibrated with 0.1 M Tris-HCl buffer, pH 8, containing 1 M NaCl, and the optical density of the eluted fractions was determined by passing the effluent through an LKB Absorptionmeter. Fractions I, II, and III were concentrated by centrifugation in a Spinco Model L preparative ultracentrifuge, 50 Rotor, 90 minutes for Fraction I, and 10 hours for Fractions II and III at a speed of 49,000 rpm at 4°C. The protein-free supernatant was carefully removed and the resulting pellet was resuspended in 0.15 M NaCl. Concentration of the resuspended material by centrifugation as described above was repeated one more time, and resuspended fractions were tested for their skin test activity in both rhesus monkeys and non-allergic human volunteers. Fraction II was dialyzed against 1 liter of 0.01 M phosphate buffer, pH 8.0, for 48 hours. During the 48 hours the dialysis bath was changed 6 times. The dialyzed Fraction II was further fractionated by ion-exchange chromatography on DEAE-cellulose (Sigma Chemical Co., St. Louis, Mo.) by the method of Fahey and McCloughlin(9). Fractions eluted from the DEAE-cellulose column were concentrated by the same centrifugation procedure described above, and tested for their skin-test activity in both rhesus monkeys and non-allergic human volunteers.

Ultracentrifugation. Ultracentrifugation of the γ_G-globulin fraction obtained from DEAE-cellulose chromatography was carried out in the Model E Analytical Ultracentrifuge (Beckman Instrument Co., Spinco Division) in an AN-D rotor at a speed of 59,780 rpm at 20°C.

Results. The results of gel-filtration on Sephadex G-200 of human serum from an untreated timothy sensitive patient are shown in Fig. 1. The concentrated fractions (G.F.-I, G.F.-II, G.F.-III) were tested for their skin test activity by passive transfer in rhesus monkeys and man (Table I). The reaginic antibody activity of this serum was entirely in Fraction-II, and this fraction was shown to contain both γ_G- and γ_A-globulins by immunoelectrophoresis.

Twenty milliliters of the dialyzed Fraction-II was further fractionated by DEAE-cellulose chromatography (Fig. 2). The γ_G- and γ_A-globulin fractions designated were tested for their skin test activity by passive transfer in rhesus monkeys and man (Table I). Essentially all of the reaginic antibody activity was found in the γ_G-globulin fraction eluted with 0.01 M phosphate buffer, pH 8. Single lines of precipitation of the γ_G-globulin fraction against rabbit anti-human total globulins and rabbit anti-human γ_G-globulin were observed by immunoelectrophoresis (Fig. 3) and im-



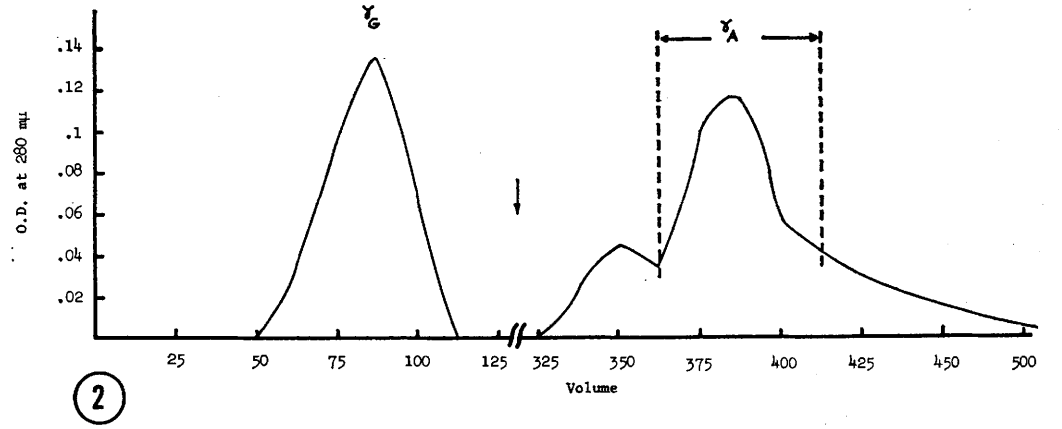


FIG. 1. Sephadex G-200 gel filtration of a reagin containing serum. Reagin activity was found in Fraction II (G.F.-II), which was shown by immunoelectrophoresis in Veronal buffer, pH8.2, μ-0.1, to contain both γ_G- and γ_A-globulins. Flow rate was 21 ml/hr, and column dimensions were 2.5 cm x 100 cm.

FIG. 2. DEAE chromatography of gel filtration Fraction-II. The γ_G-globulin was eluted with the starting buffer, 0.01M phosphate buffer, pH8.0. The ↓ indicates the point where a continuous gradient of phosphate buffer, pH8 was started. The mixing chamber (125 ml) contained 0.01M phosphate buffer, pH8.0, and the upper reservoir (500 ml) contained 0.3M phosphate buffer, pH8.0. Flow rate was 40 ml/hr, and column dimensions were 1.5 cm x 50 cm.

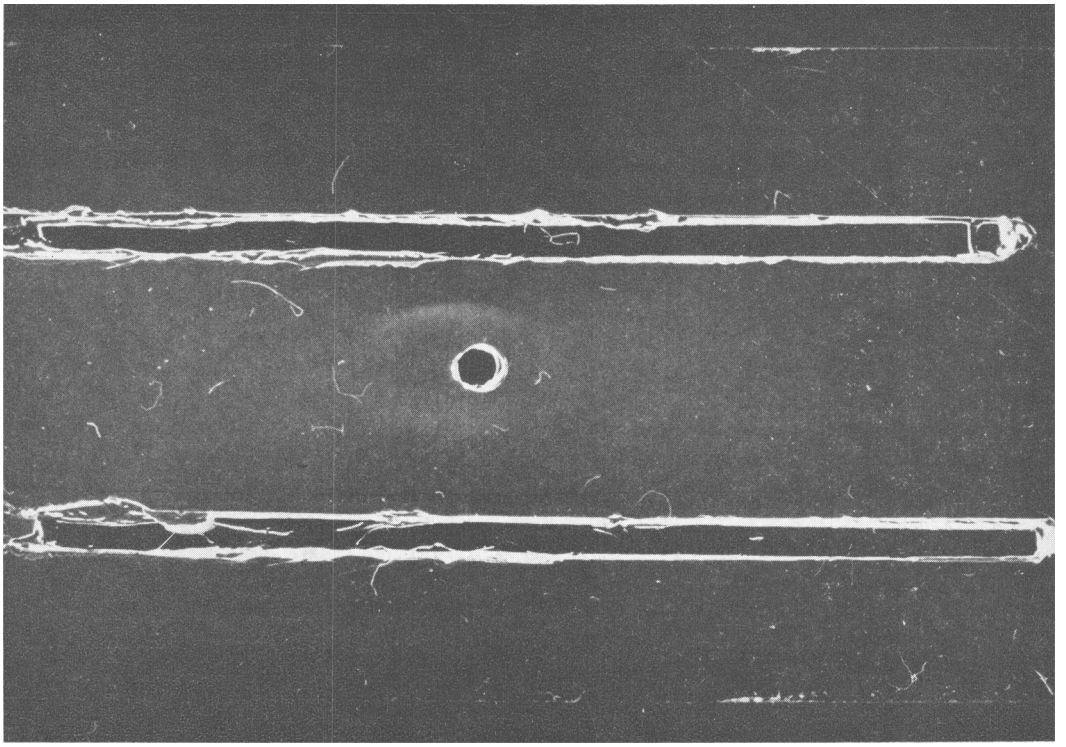


FIG. 3. Immunoelectrophoresis of γ_G-globulin fraction obtained from DEAE-cellulose chromatography in 0.05M Veronal buffer, pH8.2. Duration of the run was 2 hours at a constant current of 10 ma and 125 V at 4°C. Center well contained γ_G-globulin fraction, upper and lower trough contained anti-total human globulins and rabbit anti-human γ_G-globulin respectively. Left side of the slide represents the cathode.

munodiffusion. No line of precipitation of the γ_G-globulin fraction against rabbit anti-human γ_A-globulin was observed by either immunoelectrophoresis or immunodiffusion.

Discussion. Gel-filtration on Sephadex G-200 followed by ion-exchange chromatography on DEAE-cellulose resulted in isolation of a highly active fraction of reaginic antibody from the serum of an untreated timothy sensitive patient. The isolated reaginic antibody has chromatographic, immunochemical, and sedimentation characteristics of human γ_G-globulin. Previous reports by several investigators(3-5,14) demonstrated that reagin activity was chiefly present in γ_A-globulin enriched fractions. These authors concluded that reagin activity was distinctly associated with γ_A-globulin. However, Loveless (15) reported that a highly allergic individual, whose serum did not contain γ_A-globulin, had his reagin activity associated

TABLE I. Passive Transfer of Allergic Serum Fractions in Humans and Rhesus Monkeys.

Serum fraction	Protein conc, mg/ml	Recipients: No. tested	Rhesus‖	No. tested	Human¶
		4		4	
E.F.*	10		3+		4+
G.F.-I†	8.13		—		—
G.F.-II	8.62		4+		4+
G.F.-III	9.38		—		±
γ_G‡	1.33		3+		2+
γ_A‡	1.05		—		±
γ_G§	1.33		—		—

* Patients serum diluted 1:6 with 0.15M NaCl.

† Fractions obtained from Sephadex G-200 Gel-filtration (Fig. 1).

‡ Fractions obtained from DEAE chromatography (Fig. 2).

§ The γ_G-globulin fraction was heated in a water bath at 56°C for two hours.

‖ Animals challenged with 5 mg protein of WST extracts 15 min after a 5 cc intravenous injection of 1% Evans Blue dye.

¶ Each site was challenged with 0.05 cc of 1/100,000 (W/V) dilution of partially purified timothy allergens (>40% ETOH).

with γ_G-globulin. The present report demonstrates that an allergic individual, containing γ_M-, γ_G- and γ_A-globulins in his serum, has reaginic antibody activity entirely in a γ_G-globulin fraction. These observations would suggest that a certain degree of caution should be used in concluding that all reaginic antibodies are of a distinct molecular species. The methods for detection of reagin take advantage of the skin-fixing properties of this antibody. Although it has been demonstrated that γ_A-globulin will fix to the skin of human and various non-human primates, this report suggests that the property of skin fixation is not limited to γ_A-globulin. The presence of reaginic antibody actively associated with a γ_G-globulin fraction may be analogous to the skin fixing properties of guinea pig $7S\gamma_1$-globulin described by Ovary, Benacerraf and co-workers(16-18). Sera of several additional timothy sensitive patients are being investigated to determine the nature of the reaginic antibody activity.

Summary. 1. Serum from untreated timothy sensitive patient (E.F.) was fractionated by Sephadex G-200 gel-filtration and DEAE-cellulose ion-exchange chromatography, and resulted in the isolation of a single fraction with reagin activity. 2. The isolated reagin possesses chromatographic and immunochemical characteristics of γ_G-globulin.

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Production of Urea Nitrogen and Creatinine in Chronic Azotemia And Effect of Hemodialysis.* (31079)

N. J. BLONDEEL,† S. GOODMAN, N. M. SIMON AND F. DEL GRECO

Department of Medicine and Clinical Research Center, Northwestern University Medical School, Chicago, Ill.

The rate of urea nitrogen production in patients with chronic azotemia has not been adequately investigated. Since unlimited pro-

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