PHOTOMETRIC MICRODETERMINATION OF HUMAN GAMMA GLOBULIN. II. COMPARISON OF QUANTITATIVE FLOCCULATION-NINHYDRIN METHOD WITH ELECTROPHORETIC METHOD [1]

By ABRAHAM SAIFER, MICHAEL C. ZYMARIS, and HENRY BERGER

(From the Biochemistry Department of the Division of Laboratories, Jewish Sanitarium and Hospital for Chronic Diseases, Brooklyn, N. Y.)

(Submitted for publication August 9, 1951; accepted October 1, 1951)

The main purpose in performing the detailed experimental studies in Part I is the application of the photometric method to the determination of the gamma globulin content of human sera. These experimental studies show that protein fractions such as albumin or alpha globulins, whose electrophoretic mobilities are far removed from gamma globulin, cause little interference with its determination. Whereas those fractions, such as beta globulins or fibrinogen, whose electrophoretic mobilities are close to that of gamma globulin, are apt to cause positive errors, unless their concentrations are reduced below a certain *critical ratio,* as compared to gamma globulin. This furnishes experimental evidence that there is a relationship between mobility and flocculation of protein fractions which is to be expected from theoretical considerations (1).

A similar close parallelism between mobility and solubility of proteins in concentrated salt solution, *e.g.,* ammonium sulfate, has been pointed out by Svensson (2) and Pedersen (3). Svensson concludes that it is not a simple matter to isolate pure electrophoretic components by salt fractionation. His results were confirmed by the careful experimental studies of Majoor (4) on the solubility of protein fractions in various concentrations of sodium sulfate in comparison with results of electrophoresis experiments. His results show that particularly in the case of disease sera, a wide range of concentrations of sodium sulfate is required for complete precipitation of gamma globulin. He states that at any single salt concentration there is undeniable overlapping of protein fractions and no absolute separation takes place. The experimental technique in this paper conforms closely to that of Majoor in that a salt fractionation procedure is used which will precipitate quantitatively gamma globulin under *all* conditions with minimum contamination from the other protein fractions. Since the salt fractionation procedure is performed prior to the flocculation method, it results in a sufficient removal of the interfering fractions so as to make the method quantitative solely for gamma globulin.

In spite of certain difficulties with electrophoretic separation of protein fractions, particularly in disease sera, *e.g.,* nephrosis, in which lipids are bound to the globulin fractions (5), it still remains the standard procedure against which other quantitative methods are compared. A large series of normal and disease sera were run comparatively with both the photometric flocculation-ninhydrin procedure and the electrophoretic method. The results obtained prove conclusively that the photometric flocculation-ninhydrin method provides a simple, accurate micro-procedure for the quantitative estimation of the gamma globulin content of human sera in both health and disease.

In addition, the flocculation-ninhydrin method furnishes a simple technique for the quantitative estimation of another protein fraction present in the whole sera which is probably a slower migrating beta globulin or beta lipoprotein. The ratio of this component to the gamma globulin, as estimated with the flocculation-ninhydrin method, furnishes a *new index* of *disease* which remains relatively constant for normal sera but which rises in cases of nephrosis and falls in cases of hepatitis or multiple myeloma. Unlike more complicated methods, the quantitative flocculation-ninhydrin procedures described in this paper can be used for large scale clinical evaluation of gamma globulin changes in disease, *e.g.,* tuberculosis sera. It should also prove useful in supplementing the elec-

[1] Presented before the Biological Chemistry Section of the International Congress of Pure and Applied Chemistry, 12th meeting, New York, N. Y., September 10, 1951.

trophoretic procedure in obtaining quantitative results where electrophoretic patterns are difficult to interpret as in cases of nephrosis, and multiple myeloma, and with certain animal sera. The described procedures should prove especially applicable to clinical studies in laboratories where electrophoretic or immunochemical methods are presently unavailable.

EXPERIMENTAL

A. Reagents and apparatus

In addition to those described in the previous paper, the following are required:

1. *Sodium sulfite*—20% solution. Dissolve 200 g. of anhydrous sodium sulfite, reagent grade, in distilled water by vigorous shaking at room temperature and dilute to a liter in a graduated cylinder.

2. *Sodium chloride solution*—11% solution.

3. *Cellophane tubing,* 1 inch wide, obtained from Visking Corporation, Chicago, Illinois.

B. Method for determination of gamma globulin content of human serum

1. *Photometric flocculation-ninhydrin procedure for dialyzed globulin clot as gamma globulin.* To 7.5 ml. of 20% sodium sulfite in a 15 ml. graduated centrifuge tube, add 0.5 ml. of clear serum. Mix contents well and let stand 20 minutes or longer at room temperature. Add approximately 5 ml. of anhydrous ether, stopper with a clean rubber stopper and shake vigorously for 30 seconds. Centrifuge at about 2,500 r.p.m. for about 10 minutes. Remove tube from centrifuge and tilt gently to an almost horizontal position so that the globulin button at

TABLE I

Normal sera: Comparison of electrophoretic data with the flocculation-ninhydrin method

Case No.	Electrophoretic data							Photometric data*			Electrophoretic
	T. P. g. %	Alb. g. %	Glob. g. %	$Alpha_1$ g. %	$Alpha_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. serum g. %	Total serum / Glob. clot	Beta + gamma / Gamma
1	7.6	4.6	3.0	0.4	0.7	1.0	0.9	0.96	—	—	2.1
2	7.7	4.7	3.0	0.3	0.6	1.0	1.1	1.25	—	—	2.0
3	6.7	3.6	3.1	0.4	0.8	0.8	1.0	0.94	—	—	1.8
4	6.6	3.5	3.1	0.4	0.7	0.7	1.2	0.96	1.43	1.49	1.6
5	7.5	4.0	3.5	0.4	0.7	1.1	1.2	1.25	1.82	1.46	1.9
6	7.0	4.6	2.4	0.2	0.6	0.8	0.9	1.05	1.59	1.51	1.9
7	6.8	3.6	3.2	0.4	0.7	1.2	0.9	1.09	1.55	1.42	2.3
8	7.9	4.3	3.6	0.5	0.7	1.2	1.2	1.14	1.64	1.42	2.0
Mean	7.2	4.1	3.1	0.4	0.7	1.0	1.1	1.08	1.61	1.46	2.0

Summary of data for normal sera run with the flocculation-ninhydrin method

No. of sera	Gamma glob. clot g. %		Gamma glob. tot. serum g. %		Gamma glob. (tot. serum) / Gamma glob. (glob. clot)	
	Range	Mean	Range	Mean	Range	Mean
32	0.72–1.25	1.00	1.09–2.00	1.54	1.40–1.83	1.55

Typical results for the flocculation-ninhydrin method

Case no.	Glob. clot (1:50 diltn.) ml.	Gamma glob. clot g. %	Standard deviation from mean
100	0.50 1.00	1.34; 1.34; 1.27. 1.28; 1.34; 1.27. Aver.: 1.30	±.040
101	0.50 1.00	0.78; 0.85; 0.79. 0.86; 0.84; 0.85. Aver. = 0.83	±.031

Recovery of added gamma globulin (Fraction II)

No. of sera	Gamma glob. added μg.	Average gamma glob. recovered* μg.	Average error %
6	173	188	+8.7
6	288	299	+3.5
8	310	312	+0.6

* Each value given for the flocculation-ninhydrin method is the average of six determinations at two concentrations.

the bottom of the ether layer floats away from the side of the tube and permits access to a capillary-tipped pipette to the bottom of the tube. Remove all the liquid with gentle suction taking care not to disrupt the globulin clot. Add about 3 ml. of distilled water and suspend the protein by vigorous shaking of the tube.

Prepare cellophane bags by cutting 6 inch strips of cellophane tubing and soaking in water for about five minutes. Open one end of the tubing, then twist this end several times and tie off this end with a double knot. Separate the other end of the tubing and open wide by blowing into it. Transfer the 3 ml. of the protein suspension to the cellophane bag and rinse out centrifuge tube with three 2 ml. portions of distilled water, and transfer the washings quantitatively to the cellophane bag. Wash down inside of cellophane bag with another 2 ml. portion of distilled water and tie off the open end with a double knot leaving only a small amount of air space. Label bags by stapling on tag and dialyze out all soluble material from the protein solution by placing bag in a container and running cold tap water over it for 12 hours or longer. Remove bag from water bath, cut open one end of the bag and transfer contents quantitatively with distilled water to 25 ml. volumetric flasks keeping total volume of solution and washings below 20 ml. Add 2 ml. of 11% sodium chloride solution, dilute to 25 ml. mark with distilled water and mix.

Pipette three 0.50 ml. aliquots and three 1.00 ml. aliquots into selected test tubes (75 × 12 mm.) and then add 0.85% NaCl where necessary, so that the volume in each tube is exactly 1.00 ml.

2. *Photometric flocculation-ninhydrin procedure for total serum as gamma globulin.* Prepare a 1:50 dilution of same serum with 0.85% NaCl in a volumetric flask. Pipette three 0.5 ml. aliquots and three 1.00 ml. aliquots of the diluted serum into selected test tubes (75 × 12 mm.) and then add 0.85% NaCl, where necessary, so that the volume in each tube is exactly 1.00 ml.

3. *Standards and blanks.* Pipette in triplicate 0.5 ml. and 1.00 ml. aliquots of pure gamma globulin standard solution, prepared as described in Section A-5 of the preceding paper, into selected test tubes and then add 0.85%

TABLE II

Disease sera: Effect of increased alpha globulins (electrophoretic) on the flocculation-ninhydrin method

Electrophoretic data								Photometric data			Electrophoretic	Diagnosis
Case no.	T. P. g. %	Alb. g. %	Glob. g. %	$Alpha_1$ g. %	$Alpha_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. ser. g. %	Tot. ser. / Glob. clot	(Beta+gamma) / Gamma	
34	7.3	2.1	5.2	1.3	1.7	1.1	1.0	1.13	—	—	2.1	Reticulocell sarcoma (4/12)
35	7.9	3.3	4.6	1.0	1.7	0.9	1.0	1.14	—	—	1.9	Same case (4/25)
36	7.2	2.5	4.7	1.0	1.7	1.1	1.0	1.13	1.78	1.48	2.1	Same case (4/29)
37	6.8	3.4	3.4	0.4	1.0	0.9	1.0	1.10	1.59	1.45	1.9	Possible T.B.
38	6.8	3.0	3.8	0.6	1.2	1.2	0.9	1.11	1.69	1.52	2.3	Collagen disease
39	7.5	3.6	3.9	0.6	1.1	1.1	1.1	1.27	1.87	1.47	2.0	Rheumatic fever, acute
40	7.8	4.0	3.8	0.6	0.9	1.2	1.1	0.98	1.75	1.79	2.1	Rheumatoid arthritis
41	7.2	3.3	3.9	0.6	1.1	1.2	1.0	1.02	1.62	1.58	2.2	Unknown
Mean	7.3	3.2	4.2	0.86	1.3	1.1	1.0	1.11	1.70	1.55	2.1	
Increased alpha + gamma globulins												
42	7.4	2.5	4.9	0.9	1.6	1.2	1.3	1.36	1.84	1.35	1.9	Lupus erythematosus, pneumon.
43	7.5	3.0	4.5	0.5	1.2	1.2	1.5	1.51	1.78	1.18	1.8	Lupus erythematosus
44	7.9	3.0	4.9	0.8	1.5	1.1	1.4	1.50	2.04	1.36	1.8	Unknown
Increased alpha and decreased gamma globulins												
45	6.8	4.1	2.7	0.3	0.9	0.8	0.7	0.79	1.69	2.14	2.2	Baby—21 days old
Increased alpha and decreased beta + gamma globulins												
46	4.8	1.3	3.5	0.4	2.0	0.6	0.5	0.65	1.25	1.92	2.2	Nephrotic syndrome
Increased alpha + beta + gamma globulins												
47	7.9	3.5	4.4	0.4	1.1	1.4	1.5	1.45	2.17	1.49	1.9	Lupus erythematosus
48	7.5	2.6	4.9	0.7	1.1	1.2	2.0	2.06	2.52	1.22	1.1	Unknown

TABLE III

Disease sera: Effect of increased beta globulins (electrophoretic) on the flocculation-ninhydrin method

Case no.	Electrophoretic data: T. P. g. %	Alb. g. %	Glob. g. %	Alpha1 g. %	Alpha2 g. %	Beta g. %	Gamma g. %	Photometric data: Gamma glob. clot g. %	Gamma glob. tot. ser. g. %	Tot. ser. / Glob. clot	Electrophoretic: (Beta+gamma) / Gamma	Diagnosis
49	7.3	3.6	3.7	0.5	0.8	1.3	1.1	0.93	1.61	1.73	2.2	Unknown
50	7.2	3.9	3.3	0.3	0.6	1.4	1.1	1.26	1.85	1.47	2.3	Lupus erythematosus
51	7.8	4.5	3.3	0.2	0.8	1.5	0.9	0.98	1.44	1.47	2.6	Unknown
52	7.5	3.4	4.1	0.4	0.8	2.0	0.9	0.92	1.46	1.59	3.2	Multiple myeloma?
Mean	7.5	3.9	3.6	0.4	0.8	1.6	1.0	1.02	1.59	1.57	2.6	
Increased alpha + beta globulins												
53	7.2	3.2	4.0	0.4	0.8	1.4	1.4	1.30	1.98	1.52	2.0	Pregnancy 9 months
54	7.0	3.0	4.0	0.4	0.9	1.3	1.4	1.34	1.79	1.34	1.9	Diabetes
55	7.0	2.9	4.1	0.5	0.9	1.3	1.4	1.33	1.75	1.32	1.9	Same case—serum drawn 1 hour later
Increased gamma + beta globulins												
56	7.6	3.7	4.1	0.4	0.7	1.3	1.6	1.58	2.20	1.40	1.8	Sarcoidosis
57	8.2	1.9	6.3	0.6	0.5	2.1	3.1	2.96	3.33	1.13	1.7	Hodgkin's disease
Increased beta and decreased gamma globulins												
58	7.0	4.3	2.7	0.2	0.7	1.2	0.6	0.72	1.45	2.02	3.0	Slight polyerythemia
59	No results obtainable from electrophoretic pattern							0.52	1.99	3.82	—	Lipoid nephrosis Total cholesterol = 772 mg.%
60	4.2	0.5	3.7	3.2			0.5?	0.18	1.10	6.12	—	Lipoid nephrosis Total cholesterol = 499 mg. %

NaCl where necessary so that the volume in each tube is exactly 1.00 ml. For blank tubes, pipette in triplicate 1.00 ml. aliquots of 0.85% NaCl into the selected test tubes. The remainder of the procedure, including the flocculation and washing of the protein precipitate and the development of the color with the ninhydrin reaction, is exactly as described under Section B of the preceding paper. Blanks, standard and unknowns, are run simultaneously under identical conditions and the spectrophotometric readings and calculations performed in the usual manner according to the Bouger-Lambert-Beer Law.

C. Comparison of the flocculation-ninhydrin procedure with the electrophoretic method for gamma globulin

Fifty sera, from both normal and disease cases were analyzed with the photometric flocculation-ninhydrin method and the electrophoretic method independently in two different laboratories.[2] The instrument used for the electrophoretic determinations is the Tiselius electrophoresis apparatus. Patterns were obtained by a modification of the scanning method of Longsworth (6). Each result is the mean of two measurements. Each pattern was enlarged to three times its actual size, was traced and the areas measured with a planimeter. Areas were defined by the method of Longsworth (7). In all cases the data were computed from the ascending pattern as it gives better resolution and has no beta anomaly. Barbiturate buffer at pH 8.6 and 0.1 ionic strength was used for these runs and the power was adjusted so that it was always equal to 2 watts.

To prepare serum for electrophoretic analysis, 1.0 ml. of serum was added to 3.0 ml. of buffer and dialyzed against 300 ml. of buffer for 17 hours at 4° C. All analyses were made in the Tiselius microcell of 2 ml. capacity. The various protein components expressed as percentages are converted into absolute values, *i.e.*, grams %, from the total protein value as determined by a micro-Kjeldahl digestion-steam distillation procedure.

The results obtained in the comparative study with the two methods are given in Tables I through IV. The flocculation-ninhydrin values given for both the dialyzed globulin clot and diluted whole sera represent triplicate determinations at two different concentrations, *i.e.*, 0.50 and 1.00 ml. aliquots of a 1 : 50 dilution of the original se-

[2] The electrophoretic determinations for this paper were performed by Mr. Henry Berger of the Mattie Billings Lowe Laboratory of the Jewish Hospital of Brooklyn.

rum. These values were obtained without prior knowledge of the electrophoretic values for gamma globulin. Some typical results as obtained for two sera, one low and the other high in gamma globulin, are included in Table I so as to indicate the reproducibility of the procedure. Some data on the recovery of pure gamma globulin added to sera are also included in Table I to show the accuracy of the method.

Thirty-two normal sera obtained from the hospital staff were run with the flocculation-ninhydrin method alone. The subjects used were adults in good health, from 20 to 50 years of age and about equally distributed as to sex. The summary of the results obtained for these normal sera is given in Table I. These results are compared with those obtained electrophoretically by Reiner, Fenichel, and Stern (8) in Table V for a similar series.

Twenty-five sera from hospitalized tuberculosis patients were run by the flocculation-ninhydrin method. These results were compared with those obtained electrophoretically by Seibert, Pfaff, and Seibert (9) for a similar group of tuberculosis patients.

The rest of the comparison experiments between the photometric flocculation-ninhydrin procedure and the electrophoretic method are divided into tables based upon the increase in a particular electrophoretic globulin component, *e.g.*, Table II (increased alpha globulins), Table III (increased beta globulins) and Table IV (increased gamma globulins). Table V summarizes all the preceding data and gives a statistical analysis of the results obtained with the electrophoretic and flocculation-ninhydrin methods for both normal and disease cases.

DISCUSSION

The comparative data between the electrophoretic method and the photometric flocculation-ninhydrin methods are shown in Tables I through V inclusive. These data prove conclusively that the globulin clot-gamma globulin values are equivalent to electrophoretic gamma globulin values over a wide range of values for both normal and

TABLE IV

Disease sera: Effect of increased gamma globulins (electrophoretic) on the flocculation-ninhydrin method

Electrophoretic data								Photometric data			Electrophoretic	Diagnosis
Case no.	T. P. g. %	Alb. g. %	Glob. g. %	$Alpha_1$ g. %	$Alpha_2$ g. %	Beta g. %	Gamma g. %	Gamma glob. clot g. %	Gamma glob. tot. ser. g. %	Tot. ser. / Glob. clot	(Beta+gamma) / Gamma	
61	7.3	4.0	3.3	0.3	0.7	1.1	1.3	1.32	1.93	1.46	1.8	Rheumatoid arthritis
62	7.0	3.7	3.3	0.3	0.5	1.1	1.3	1.47	2.06	1.40	1.9	Idiopathic uveitis
63	6.8	3.2	3.6	0.4	0.6	1.1	1.5	1.72	2.38	1.38	1.7	Psoriasis; arthritis of cervical spine
64	7.5	4.2	3.3	0.3	0.7	0.9	1.5	1.51	2.00	1.32	1.6	Paroxymal hemaglobinuria
65	6.2	2.5	3.7	0.5	0.7	1.0	1.6	1.62	1.95	1.20	1.6	Homologous serum jaundice
66	6.6	3.0	3.6	0.4	0.7	1.0	1.6	1.81	2.14	1.17	1.6	Lupus erythematosus
67	7.9	4.3	3.7	0.3	0.6	0.7	2.1	2.00	2.37	1.18	1.3	Hemolytic jaundice
Mean	7.0	3.6	3.5	0.4	0.6	1.0	1.6	1.64	2.12	1.30	1.6	
68	11.2	2.7	8.5	0.5	0.8	0.8	6.4	6.70	6.45	0.97	1.1	Multiple myeloma
Increased alpha + gamma globulins												
69	7.6	3.3	4.3	0.4	0.9	1.1	1.8	1.87	2.35	1.25	1.6	Rheumatoid arthritis
70	7.5	3.2	4.3	0.6	1.0	1.1	1.7	1.57	1.84	1.17	1.6	Pregnancy 9 months
Increased beta + gamma globulins												
71	7.4	2.4	5.0	0.5	0.6	1.3	2.6	2.77	3.09	1.11	1.5	Infectious hepatitis with jaundice
Increased gamma and decreased beta globulins												
72	6.3	1.6	4.7	0.3	0.6	0.5	3.3	3.03	3.47	1.15	1.2	Multiple myeloma
73	—	—	—	—	—	—	1.4	1.44	1.60	1.11	—	Same case after remission
Plasma sample containing fibrinogen												
74	7.8	3.9	3.9	0.5 (Fibrinogen = 0.6 g. %)	0.8	1.0	1.0	1.62	2.23	1.37	1.6 (plus fibrinogen)	Possible pseudohemophilia

TABLE V

Summary of data for flocculation-ninhydrin method for normal and disease cases and comparison with electrophoretic data

Kind of sera used	Electrophoretic data				Flocculation-ninhydrin procedure					
	Gamma glob. g. %		Beta+gamma / Gamma		Gamma glob. clot g. %		Gamma glob. tot. serum g. %		Total serum / Glob. clot	
	Mean, S.D.,	Range	Mean, S.D.,	Range	Mean, S D.,	Range	Mean, S.D.,	Range	Mean, S.D.,	Range
*Normal sera** (80E; 32F-N)	1.0±.21	0.8–1.2	1.9	1.5–2.2	1.00±.13	.72–1.25	1.54±.23	1.09–2.00	1.55±.12	1.40–1.83
Disease sera Increased alpha glob. (8 cases)	1.0±.06	.9–1.1	2.1±.13	1.9–2.3	1.11±.08	.98–1.27	1.70±.10	1.59–1.87	1.55±.12	1.45–1.79
Increased beta glob. (4 cases)	1.0±.10	.9–1.1	2.6±.39	2.2–3.2	1.02±.14	.92–1.26	1.59±.16	1.44–1.85	1.57±.11	1.47–1.73
Increased gamma glob. (8 cases)	1.6±.25	1.3–2.1	1.6±.18	1.3–1.9	1.64±.21	1.32–2.00	2.12±.17	1.93–2.38	1.30±.11	1.17–1.46
Tuberculosis sera† Minimal cases (22E; 4F-N)	1.25±.29	.73–1.94	1.8		1.35±.17	1.07–1.50	2.49±.39	1.94–3.06	1.85±.12	1.70–2.04
Mod. adv. cases (16E; 10F-N)	1.59±.56	.98–2.87	1.7		1.69±.31	1.18–2.42	2.30±.46	1.77–3.56	1.36±.19	1.03–1.74
Far adv. cases (21E; 8F-N)	1.81±.61	.96–3.27	1.7		1.70±.28	1.07–2.23	2.33±.56	1.59–3.45	1.38±.14	1.06–1.55

* Electrophoretic data for 80 normal sera taken from paper by Reiner, Fenichel and Stern (8).
† Electrophoretic data for 59 tuberculosis sera taken from paper by Seibert, Pfaff and Seibert (9).
E = Electrophoretic determinations.
F-N = Flocculation-ninhydrin determinations.

disease cases. The total serum-gamma globulin values are presumed to be equivalent to the electrophoretic gamma globulin plus a beta fraction which corresponds to about 50% of the normal beta globulins. This beta fraction is believed to be composed mainly of $beta_2$ globulins and beta lipoproteins. Experimental evidence to support this viewpoint is furnished by the data obtained with Cohn's fractions in Part I of these papers. These interfering fractions, *i.e.*, III and III–0, have been characterized electrophoretically by Cohn and his associates (10) as containing increased amounts of $beta_2$ globulins and beta lipoproteins. Further experimental support is furnished by the results obtained with lipoid nephrosis sera which have been found by numerous investigators (5, 11–14) to be markedly increased in beta globulins and beta lipoproteins as given in Table III. If these assumptions are correct then changes in the total serum/globulin clot ratio should roughly approximate those in the electrophoretic (beta + gamma/gamma) ratio. The values included in the various tables show this to be true except for some cases of increased beta globulins which will be discussed later.

Campbell and Hanna (15) have shown that 20% sodium sulfite is approximately equal to 20% sodium sulfate for the precipitation of globulins. This concentration has been shown by Majoor (4) to precipitate all the gamma globulin with minimum contamination from other globulins for both normal and disease sera. It is this prior concentration of the gamma globulin by means of salt fractionation that permits the *quantitative* flocculation of gamma globulin from solution over a wide range of concentrations. That this is not possible with salt fractionation alone is shown by the electrophoretic studies of Majoor (4) and Petermann, Young, and Hogness (16) with salt fractionation of both normal and disease sera.

It is clear from the data in this paper that, while salt fractionation followed by the flocculation-ninhydrin procedure effectively separates the gamma globulin fraction from albumin and alpha and beta globulins, no such separation occurs with fibrinogen as can be seen from the plasma run in Table IV, Case No. 74. Since the electrophoretic mobility of fibrinogen lies between the beta and gamma globulins, its interference with the gamma globulin procedure is not unexpected.

From an analytical viewpoint the accuracy of the electrophoretic method is bound to be low because

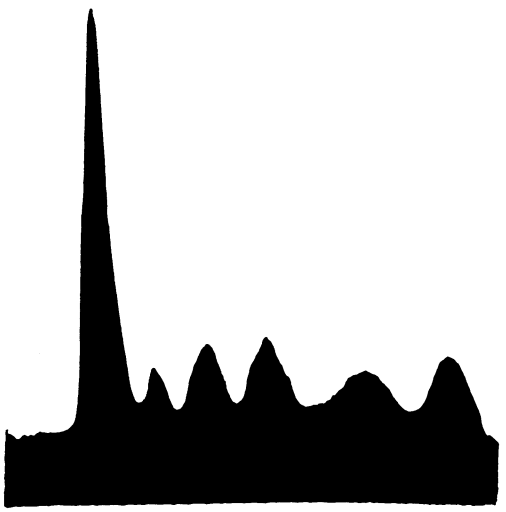

FIG. 1. ELECTROPHORETIC PATTERN FOR NORMAL SERUM

only a single determination is performed on each sample and all subsequent measurements and calculations are based on this *one* determination. Moore and his coworkers (17) have shown that variations in buffer used, hemolysis, and method of dialysis and in period of storage and transportation of sample cause alterations in the electrophoretic pattern obtained. Even measurement of the *same* pattern by different individuals may introduce considerable errors in the final result. When absolute rather than relative concentrations are desired, the error in the micro-Kjeldahl determination may be additive with those of the electrophoretic method. For these reasons, in spite of the much smaller amounts of serum used in the photometric method, *e.g.*, 0.01 to 0.02 ml., the variability of results as measured in a large series of normal and of tuberculosis sera is about one-half that obtained with the electrophoretic method as is shown in Table V. In addition, the data in Table I show the excellent reproducibility of the method and that gamma globulin added to sera may be recovered with an average value of + 105% of the protein added. Repeat values on the same patients' sera, as seen in Tables II and III, gave highly reproducible results with the photometric procedure.

The flocculation-ninhydrin method has other advantages over the electrophoretic method in that it requires no equipment not presently available in most clinical laboratories and in that as many as 100 determinations may be performed each day by one technician as compared to about four per day for the electrophoretic method. This makes the method especially suitable for clinical research involving large numbers of determinations. It is presently being employed for a mass survey of tuberculosis patients, part of the data from such a study being shown in Table V.

The experimental data obtained for human sera in the comparative studies with the electrophoretic and flocculation-ninhydrin methods have been divided into normal cases (Table I) and into disease cases (Tables II–IV) based upon the increase in a particular electrophoretic component. Since extensive data have been compiled for normal sera with the electrophoretic method by numerous investigators as summarized in the paper by Reiner, Fenichel, and Stern (8) there seemed little need to repeat such a study. A small series of normals was therefore run with both procedures and a large series (32 cases) with the flocculation-ninhydrin method alone. Figure 1 shows a typical electrophoretic pattern for a normal serum. A summary of the data obtained for these normal cases is given in Table I and the results compared statistically with the electrophoretic method in Table V.

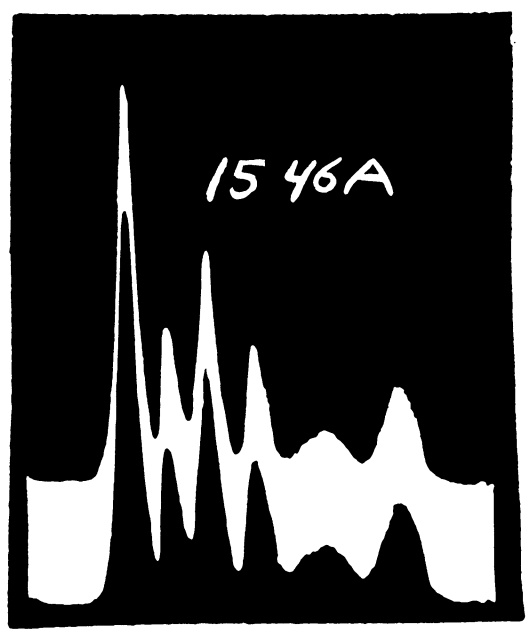

FIG. 2. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF RETICULOCELL SARCOMA (INCREASED ALPHA GLOBULINS)

The method of classification for disease sera based on an increase in a particular electrophoretic component serves to emphasize their relationship with the data obtained with Cohn's fractions in the preceding paper.

The data in Table II lists those cases (Nos. 34 through 41) in which there is an increase in either the $alpha_1$ or $alpha_2$ globulin component. The average increase in each case is about twice that for normal sera. These sera correspond to the runs in Part I dealing with Fractions IV–1 and IV–4 which were non-interfering fractions. Table II shows the excellent agreement between the electrophoretic gamma globulin values and the flocculation-ninhydrin values. The mean value of the total serum/globulin clot ratio for this series of cases is 1.55 as compared to a mean value of 1.55 for normal sera. The electrophoretic (beta + gamma/gamma) ratio is 2.1 for this series as compared to a mean value for normal sera of 1.9. The fact that increases in the alpha globulins have no effect on the flocculation-ninhydrin method for gamma globulin is shown by the data for Case Nos. 42, 43, and 44 where an increase in both alpha and gamma globulin causes a decrease in the ratio *below* normal values, whereas increased alpha globulin and decreased gamma globulin cause a rise in the ratio above normal values. These are the results that would be expected from changes in the gamma globulin alone.

In disease cases reversal of the electrophoretic (albumin/globulin) ratio is of rather frequent occurrence. Because experimental work with the albumin fraction in Part I has shown it to be a non-interfering fraction for the gamma globulin method, changes in this fraction were ignored in classifying the electrophoretic data in this paper. However, in certain cases, *e.g.*, reticulocell sarcoma (Nos. 34, 35, and 36), there is such a marked reversal of the A/G ratio, that such changes can be readily detected with the more insensitive salt fractionation methods. Therefore, where a patient's serum has a normal gamma globulin value, a normal total serum/globulin clot ratio (indicating a normal or slightly increased beta component) and a marked increase in the total globulin over the albumin component (as determined by the usual chemical methods for the A/G ratio), it can be generally assumed that there is an increase in the alpha globulin content of the patient's serum.

FIG. 3. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF LIPOID NEPHROSIS (INCREASED BETA GLOBULINS AND BETA LIPOPROTEINS)

The flocculation-ninhydrin procedure described in this paper can therefore be used in conjunction with A/G ratio determinations as a gross measure of increases in the electrophoretic alpha globulin values. Figure 2 shows the electrophoretic pattern obtained for a case of reticulocell sarcoma.

The data for the effect of increased beta globulins on the flocculation-ninhydrin method for gamma globulin, as given in Table III, show that the values obtained for gamma globulin with the two procedures are in excellent agreement. This furnishes experimental proof that the interfering beta globulin fractions described in Part I are effectively removed during the procedure. From the increase in the electrophoretic (beta + gamma/gamma ratio), a rise in the total serum/globulin clot ratio would be expected. However, the mean value of this ratio for Nos. 49 through 52 is only 1.57 as compared to a value of 1.55 for normal sera. It is quite possible that the rise in beta globulins in these cases is due solely to an increase in $beta_1$ globulins which are not flocculated out of the diluted serum by this procedure. Experimental proof of this contention can only be furnished by the use of an instrument which can separate the beta globulin into $beta_1$ and $beta_2$ fractions and which is not presently available to the authors. As would be expected an increase in beta globulin accompanied by an increase in gamma globulin causes a fall in the total serum/globulin clot ratio as can be seen in Nos. 54 through 57. Whereas in cases where the increase in beta globulins is accompanied by a decrease in gamma globulin the total serum/globulin clot ratio rises. The flocculation-ninhydrin method should be especially useful in following the prog-

nosis in cases of lipoid nephrosis, Nos. 59 and 60, where the ratio rises to four times the normal value and where the electrophoretic patterns, as seen in Figure 3, are difficult to evaluate quantitatively.

The data for sera containing increased gamma globulin are given in Table IV. In this series both the mean value of the electrophoretic (beta + gamma/gamma) ratio and the total serum/globulin clot ratio show significant decreases from the corresponding values for normal sera. In fact, as the gamma globulin increases from 1.32 g. % to 2.00 g. %, there is a corresponding continuous decrease in the ratio from 1.46 to 1.18. This decrease in the ratio is especially marked in cases of multiple myeloma. A typical electrophoretic pattern obtained with a multiple myeloma serum is shown in Figure 4. It is interesting to note that all three liver disease cases (Nos. 65, 67, and 71) fall into the same series and that all three have ratios below 1.20. Figure 5 shows an electrophoretic pattern for a case of homologous serum jaundice.

In view of the large number of semi-quantitative protein flocculation tests which have appeared in the literature, it should perhaps be emphasized that it is not the intention of the authors to add another such flocculation test to the medical literature. Rather it is the purpose of the authors to conduct investigations on the physico-chemical concepts upon which all flocculation reactions are based and to employ these basic concepts in conjunction with quantitative reactions for the direct quantitative microdetermination of various protein fractions which may or may not correspond to those obtained electrophoretically. Work is presently in progress on the application of these quantitative flocculation methods to the determination of fibrinogen and albumin in plasma, and on the application of these methods to other biological fluids, *e.g.*, cerebrospinal fluid.

FIG. 5. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF HOMOLOGOUS SERUM JAUNDICE (INCREASED GAMMA GLOBULIN)

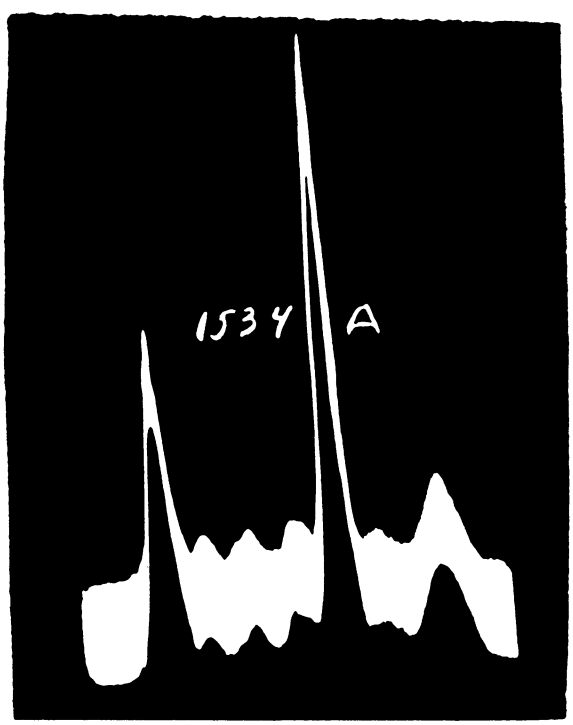

FIG. 4. ELECTROPHORETIC PATTERN FOR SERUM FROM A CASE OF MULTIPLE MYELOMA (INCREASED GAMMA GLOBULIN)

SUMMARY

1. The application to human sera of a previously described photometric flocculation-ninhydrin method for the quantitative microdetermination of gamma globulin is described.

2. The principle of the method is based upon precipitation of gamma globulin quantitatively, with a minimum contamination from the other globulin fractions, by means of 20% sodium sul-

fite prior to the application of the flocculation reaction to the dialyzed globulin clot. Results obtained with this procedure are called the globulin clot-gamma globulin values.

3. When the flocculation-ninhydrin method is applied directly to diluted whole sera, the values obtained are usually about 50% higher than the corresponding globulin clot values and are called total serum-gamma globulin values.

4. Fifty sera from both normal and disease cases were run comparatively with the two procedures described above and with the Tiselius electrophoresis apparatus. From these data it was determined that the globulin clot-gamma globulin value corresponds to the electrophoretic gamma globulin value. Also that the total serum-gamma globulin value corresponds to gamma globulin plus another fraction which is believed to be composed of $beta_2$ globulins and beta lipoproteins.

5. Thirty-two normal sera and 25 tuberculosis sera were run with the photometric flocculation-ninhydrin method. The gamma globulin values obtained for each series checked closely with the electrophoretic data reported in the literature by other investigators for a similar series of cases.

6. The total serum/globulin clot ratio was found to be relatively constant for normal sera, *i.e.,* 1.55 $\pm$ .12, and to vary markedly in disease cases. It is not affected by changes in the alpha globulins, *e.g.,* reticulocell sarcoma. It is markedly elevated by increases in beta lipoprotein, especially when accompanied by decreases in the gamma globulin, *e.g.,* lipoid nephrosis. It decreases with increases in the gamma globulin, *e.g.,* multiple myeloma and liver disease, but increases with decreasing gamma globulin, *e.g.,* baby sera.

6. The many advantages of the photometric flocculation-ninhydrin method over the laborious single determination electrophoretic method for clinical work are discussed particularly with respect to the equipment needed and volume of work performed.

7. A statistical analysis of the data obtained with the two procedures shows that the photometric flocculation-ninhydrin method has about one-half the variation of the electrophoretic method in spite of the much smaller sample, *i.e.,* 0.01 to 0.02 ml. of serum used. The method is reproducible to about a $\pm$ 5% error and recovers gamma globulin from sera with an average value of about + 105% of the amount of protein added.

After these papers had been submitted for publication, a paper appeared by Ricketts, Sterling, and Levine (J. Lab. & Clin. Med., 1951, **38**, 153) dealing comparatively with the gamma globulin values obtained by turbidimetric methods (in Part I of these papers, Kunkel [24] and de la Huerga and Popper [8]), which presumably measure gamma globulin quantitatively, and those obtained by the electrophoretic method.

These authors found differences between the turbidimetric and electrophoretic methods of about 20% for normal sera, and as much as 100% for sera from disease cases, *e.g.,* portal or biliary cirrhosis. This furnishes additional experimental evidence to support our viewpoint that such procedures are at best *semi-quantitative* measurements of gamma globulin values.

ACKNOWLEDGMENTS

The authors wish to acknowledge the helpful advice and suggestions made during the course of this work by Dr. Bruno W. Volk, Director of Laboratories of the Jewish Sanitarium and Hospital for Chronic Diseases, and by Prof. Joseph Steigmann, Department of Chemistry of the Polytechnic Institute of Brooklyn. The authors also wish to express their gratitude to Dr. Sidney E. Lenke, Director of the Mattie Billings Lowe Laboratory of the Jewish Hospital of Brooklyn, for his approval in allowing the many electrophoretic determinations contained in this paper to be performed in his laboratory.

We also wish to express our appreciation to Mrs. Jennie Herbst for the editing and typing of the manuscript.

REFERENCES

1. Gouy, G., Constitution of the electric charge at the surface of an electrolyte. J. Physique, 1910, (4) **9**, 457.
2. Svensson, H., Fractionation of serum with ammonium sulfate and water dialysis, studied by electrophoresis. J. Biol. Chem., 1941, **139**, 805.
3. Pedersen, K. O., Ultracentrifugal studies on serum and serum fractions. Almquist & Wiksells, Uppsala, 1945.
4. Majoor, C. L. H., The possibility of detecting individual proteins in blood serum by differentiation of solubility curves in concentrated sodium sulfate solutions. II. Comparison of solubility curves with results of electrophoresis experiments. J. Biol. Chem., 1947, **169**, 583.
5. Thorn, G. W., Armstrong, S. H., Jr., Davenport, V. D., Woodruff, L. M., and Tyler, F. H., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXX. The use of salt-poor concentrated human serum albumin solution in the treatment of chronic Bright's disease. J. Clin. Invest., 1945, **24**, 802.

6. Longsworth, L. G., A modification of the schlieren method for use in electrophoretic analysis. J. Am. Chem. Soc., 1939, **61**, 529.
7. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev., 1942, **30**, 323.
8. Reiner, M., Fenichel, R. L., and Stern, K. G., Electrophoretic studies on the protein distribution in normal human serum. Acta haemat., 1950, **3**, 202.
9. Seibert, F. B., Pfaff, M. L., and Seibert, M. V., A serum polysaccharide in tuberculosis and carcinoma. Arch. Biochem., 1948, **18**, 279.
10. Cohn, E. J., History of plasma fractionation *in* Advances in Military Medicine. Little, Brown & Co., Boston, 1947, Vol. I, Chap. 28, p. 364.
11. Longsworth, L. G., and MacInnes, D. A., An electrophoretic study of nephrotic sera and urine. J. Exper. Med., 1940, **71**, 77.
12. Luetscher, J. A., Jr., The effect of a single injection of concentrated human serum albumin on circulating proteins and proteinuria in nephrosis. J. Clin. Invest., 1944, **23**, 365.
13. Routh, J. I., Knapp, E. L., and Kobayashi, C. K., Electrophoretic studies of plasma and urinary proteins in children with lipoid nephrosis. J. Pediat., 1948, **33**, 688.
14. Pearsall, H. R., and Chanutin, A., II. Electrophoretic, nitrogen and lipide analyses of plasma and plasma fractions in disease. Am. J. Med., 1949, **7**, 301.
15. Campbell, W. R., and Hanna, M. I., The albumins, globulins and fibrinogen of serum and plasma. J. Biol. Chem., 1937, **119**, 15.
16. Petermann, M. L., Young, N. F., and Hogness, K. R., A comparison of the Howe and the electrophoretic methods for the determination of plasma albumin. J. Biol. Chem., 1947, **169**, 379.
17. Moore, D. H., Roberts, J. B., Costello, M., and Schonberger, T. W., Factors influencing the electrophoretic analysis of human serum. J. Biol. Chem., 1949, **180**, 1147.