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Ian R. Mackay, … , Nils Eriksen, Patricia Ann Wood

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PAPER ELECTROPHORESIS OF SERUM PROTEINS: PHOTOMETRIC QUANTITATION AND COMPARISON WITH FREE ELECTROPHORESIS [1]

By IAN R. MACKAY,[2] WADE VOLWILER, AND PATRICK D. GOLDSWORTHY WITH THE TECHNICAL ASSISTANCE OF NILS ERIKSEN [3] AND PATRICIA ANN WOOD

(From the Department of Medicine, University of Washington, Seattle, Wash.)

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Methods for electrophoretic separation of amino acids, peptides and proteins on filter paper were developed independently by several investigators (1–5) during the years 1948–50. The major protein components of serum could be separated satisfactorily by this principle. The serum proteins on the paper were stained with dye; by cutting the paper into segments and eluting the dye, the amount bound in each segment could be estimated and an electrophoretic pattern constructed. The quicker procedure of photometric quantitation of the resolved serum components directly from the paper strip was introduced by Grassmann, Hannig, and Knedel (6, 7). Variables which affect electrophoretic movement of protein and peptide in a paper supported buffer medium include variations in potential gradient, temperature, pH, ionic strength and nature of the buffer used, thickness of the filter paper, evaporation, time of run, and nature of the protein itself. Paper electrophoresis is well established as a research technique, having been successfully used in serum protein and lipid analysis (2–20), identification of the iron-binding globulin (21), study of gastric mucins (22), serum transport of thyroglobulin (23) and identification of abnormal hemoglobins (24, 25). This report relates experiences with the Grassmann-Hannig and Durrum methods of paper electrophoresis of serum proteins and photometric measurement of the amount of dye bound by the serum protein fractions. The significance of results obtained, their relationship to those obtained by free electrophoresis, and the accuracy and value of paper electrophoresis as a clinical laboratory procedure are discussed.

MATERIAL AND METHODS

Collection of samples. Sera were obtained from fasting subjects and stored at 4° C. Electrophoresis was usually performed on the following day, and not longer than seven days after obtaining the specimen. The results of analyses on 55 subjects are presented.[4]

Paper electrophoresis apparatus and technique. The apparatus described by Grassmann and Hannig (7), and a minor modification of that described by Durrum (26) were used. Both employ similar principles.

Each Grassmann-Hannig box holds two papers in a horizontal position, and three boxes were usually run simultaneously. Strips of Whatman No. 1 filter paper, 4 cm. × 30 cm. were used. After the paper strip had been moistened in buffer, gently blotted and placed in position in the box,[5] approximately 0.01 ml. of serum was carefully applied [6] as a narrow band across the center of the strip to within 0.5 cm. of either edge. Even distribution and linear application of protein were important in determining the quality of the resolution. A diethylbarbituric acid-sodium barbital buffer, pH 8.6, ionic strength 0.1 was used. Several runs could be made with one lot of buffer without change in pH, but the polarity was changed after each run; after every second run, the electrodes were washed free of deposited barbiturate. A power supply and voltage regulator were used to supply constant d.c. voltage from a 110 a.c. volt supply. For six paper strips connected in parallel (three boxes), optimal separations were obtained with 150 v. and 9 m.a. for 14 hours. Although separation could be achieved in 6 to 7 hours with currents of 15 m.a., longer runs with lower currents gave better results. During the run, there was a slight drop in voltage and rise in amperage, due to the properties of the power supply used. No gross fluctuations in cur-

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[2] A. H. Robins Research Fellow in Gastroenterology.

[3] Department of Pathology.

[4] Samples of abnormal sera were obtained from patients in the King County and Veterans Administration hospitals, Seattle.

[5] The paper was kept under tension by use of counterweights.

[6] "Misco" micro pipettes from the Microchemical Specialties Corp., Berkeley, California.

rent strength occurred, and papers could thus be run conveniently overnight.

A similar technique was employed with the Durrum apparatus. The model used in this laboratory has two buffer compartments 34 cm. by 7 cm. into which the paper dips, and two electrode compartments 34 cm. by 2 cm.; each inner buffer compartment is connected to its corresponding outer electrode compartment by three small openings plugged with glass wool. Up to seven strips of paper, 35 cm. by 4 cm., are supported 20 degrees from the vertical by a plastic rack which has an apex height of 18.5 cm. A diethylbarbituric acid-sodium barbital buffer of pH 8.6, ionic strength 0.1 was employed, with a current of 1.5 m.a. per strip (180–220 volts) for 14 hours. Initially, the dry papers were suspended over the rack and buffer was allowed to gradually soak upwards from the buffer chamber; the serum was applied as a band across the dry section of the paper just before the buffer rising up the two halves of the paper met at the top. It was later found more satisfactory to moisten and blot the entire paper, suspend it horizontally, apply the serum across the center, and then suspend the paper strip over the rack. Movement of albumin during electrophoresis could be followed by adding a small amount of dye (azocarmine or bromphenol blue) to the serum specimen to be used.

Electrophoresis was carried out routinely in the refrigerator at 4° C., although temperature control may not actually be important (7, 27). No studies on the effect of temperature on quality of separation were made in this laboratory.

With either apparatus, best results were obtained with a diethylbarbituric acid-sodium barbital buffer pH 8.6, ionic strength 0.1. Other types of buffers appraised included sodium barbital-acetate, pH 7–9, sodium barbital-citrate pH 8.6, phosphate pH 7–9, phosphate-citrate pH 6–8, and phosphate-citrate-borate pH 7–9, all of ionic strength 0.1. Phosphate buffers are prepared in a simpler fashion and less expensively than barbiturate buffers, permit more rapid movement of serum components, and give a satisfactory separation of β and γ globulins. However, of the buffers evaluated, only barbiturate solutions resolved α_1 globulin from albumin. Protein movement was faster using buffers of ionic strength 0.05, and a run could be completed in 6 to 7 hours; good patterns were obtained but protein "trailing" seemed more pronounced.

After removal from the apparatus, excess moisture was immediately blotted from the buffer-soaked ends of the papers; otherwise this moisture moved towards the center of the strip, displacing the albumin band back towards the point of application and causing some smearing of the pattern. Paper strips were partially dried in air, then transferred to an oven at 100° C. for 30 minutes.

Staining of paper strips. Both amido black 10 B (Grassmann and Hannig [7]) and azocarmine (Turba and Enenkel [4]) stains have been used. Papers were stained for exactly 10 minutes, as it had been emphasized that dye uptake by globulins increases slowly with time (8). For amido black, removal of background stain of the protein-free filter paper required repeated washings with 10 per cent acetic acid in methanol.[7] Decolorization was never quite complete; however the faint blue background did not interfere with photometric registration of protein-bound dye. Azocarmine gave a less intense stain. Prior to its application, protein was "fixed" by immersion of the strip in methanol for 5 minutes. After staining, papers were easily and quickly decolorized with 10 per cent acetic acid. The azocarmine stain faded appreciably in 5 to 6 weeks, whereas amido black did not appear to do so. With either dye, easy decolorization of the protein-free paper with dioxane was not possible, as reported by Griffiths (15) for bromphenol blue. Although azocarmine was easier to handle, and results appeared quantitatively similar to those obtained with amido black, the latter dye was found preferable, particularly for bringing out bands of low protein concentration.

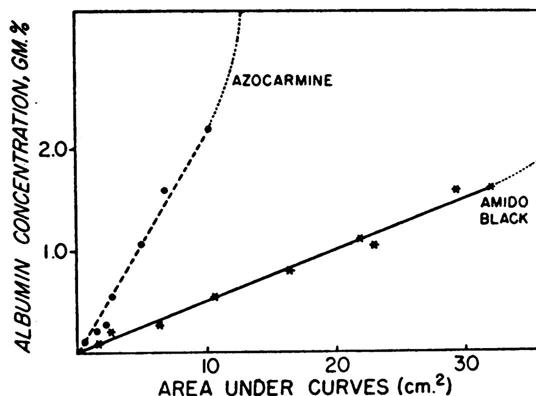


FIG. 1. RELATIONSHIP BETWEEN PLANIMETER AREAS CALCULATED FROM PHOTOMETER READINGS AND QUANTITY OF DYED PROTEIN

Photometric measure of protein concentration. Papers were rendered translucent by immersion in a solution of α-bromnaphthalene and mineral oil (7) (10 parts of mineral oil to one part of α-bromnaphthalene) before being subjected to photometry.

The Grassmann-Hannig photometer[8] was employed. The oil-soaked paper was passed millimeter by millimeter across a 4 cm. long and 1 mm. wide slit over a white light source, the light absorption being measured by a photocell and registered in optical density units. Errors associated with irregular protein movement at the edges of the paper ("edge effects") were eliminated by shortening the light slit by 1 cm. with black adhesive tape. When consecutive photometer readings (ordinate) were plotted against millimeter distance along the paper strip (abscissa), a smooth electrophoretic curve was obtained showing the characteristic peaks for whole serum components. The percentages of the individual serum protein components present were calculated from planimeter measurements of areas under the peaks, after dropping perpendiculars to

[7] Water washes may be alternated with methanol washes.

[8] Obtained from Bender and Hobein, Munich, Germany.

TABLE I

Results of multiple analyses of a single abnormal serum (Case 52, tuberculous adenitis)

Experiment number	Serum components (per cent total planimeter area)				
		Globulins			
	Albumin	α_1	α_2	β	γ
1	37.0	5.7	14.3	11.9	31.1
2	36.7	5.2	11.9	11.2	35.0
3	39.1	5.7	12.4	11.2	31.6
4	37.5	5.1	13.7	11.0	32.7
5	36.0	5.5	13.2	10.4	34.9
6	35.7	6.4	14.2	11.2	32.5
7	39.2	5.2	13.7	11.6	30.3
8	37.3	6.3	13.4	12.6	30.4
Means	37.3	5.6	13.4	11.4	32.3
Maximum differences	3.5	1.3	2.4	2.2	4.7
Maximum differences from mean	1.8	0.8	1.5	1.2	2.7
Standard deviations from mean	1.20	0.46	0.77	0.61	1.72

the baseline from the lowest points between peaks (28).

Free electrophoretic methods. Free electrophoretic analysis of sera was performed with two different types of apparatus, the Pearson model of the Tiselius apparatus with a slit replacing the knife edge for recording,[9] and the Perkin-Elmer model 38. Sera were diluted 1:4 or 1:4.5 with barbital buffer and dialyzed against buffer for 18 hours at 5° C. Runs were made in a diethylbarbituric acid-sodium barbital buffer, pH 8.6, ionic strength 0.1 at 0–1° C. Magnified schlieren diagrams were traced onto paper and the areas under peaks determined by planimetry. The tracings were divided into areas by lines dropped perpendicularly to the base from the lowest point between the peaks.

[9] Analyses performed by Dr. N. Eriksen.

RESULTS

Readings on the photometer were demonstrated to be directly proportional to amounts of albumin protein on the paper strip, within certain limits of protein concentration (Figure 1): Known quantities of human albumin in 0.01 ml. of solution were applied as a band to the paper, stained, and subjected to photometry. The planimeter areas obtained were then plotted against protein concentration; the relationship of planimeter area to protein concentration was found to be linear only up to 1.6 Gm. per cent using amido black stain, and 2.2 Gm. per cent using azocarmine. These concentrations corresponded, respectively, to protein densities produced by 2.9×10^{-4} and 4.6×10^{-4} mg. albumin per square millimeter averaged over the area scanned by the photometer (1 × 39 mm.). The density of the albumin band in patterns of normal serum often exceeded these values, being of the order of 9×10^{-4} mg. albumin per square millimeter.

Using amido black, multiple (8) analyses of a single abnormal serum gave a range of variation within satisfactory limits for each component (Table I). The maximum deviation from the mean, in terms of percentage of concentration of total protein, was for albumin 1.8 per cent, α_1 globulin 0.8 per cent, α_2 globulin 1.5 per cent, β globulin 1.2 per cent and γ globulin 2.7 per cent. No difference in electrophoretic separation between the Durrum and Grassmann-Hannig types

TABLE II

Comparison of serum protein staining by amido black and azocarmine dyes

Case No.	Diagnosis	Serum components (per cent total planimeter area)									
		Amido-Black					Azocarmine				
			Globulins					Globulins			
		Albumin	α_1	α_2	β	γ	Albumin	α_1	α_2	β	γ
5	Normal	54.6	4.3	8.9	11.6	20.6	54.6	3.4	9.1	11.3	21.6
10	Normal	60.5	2.4	7.8	13.7	15.6	63.4		7.3	12.7	16.5
18	Portal cirrhosis	44.6	2.7	6.4	10.8	35.5	40.0	4.5	6.9	10.0	38.5
26	Biliary cirrhosis	37.8	13.4		19.5	29.3	36.0	13.6		18.9	31.5
33	Myeloma	36.8	4.2	8.0	9.6	41.4	39.3	5.2	6.8	9.4	39.3
34	Myeloma	24.7	3.4		65.1	6.8	27.8	4.8		62.8	4.6
38	Nephrotic syndrome	38.7	13.3	17.3	15.9	14.8	34.3	11.6	20.1	19.0	15.0
40	Hypercholesterolemic xanthomatosis	60.4	2.7	7.4	14.3	15.1	56.9	2.6	8.3	15.3	16.9
42	Lupus erythematosis	26.7	3.1	8.3	11.0	50.9	24.0	3.5	6.5	10.5	55.5
43	Lupus erythematosis	40.0	7.4	14.1	11.7	24.8	42.4	6.3	13.3	10.5	27.5
45	Myeloid leukemia	45.1	8.2	11.9	9.0	25.8	45.7	7.3	13.7	9.4	23.9
49	Pulmonary abscess	40.4	5.5	11.6	14.2	28.3	40.6	4.9	12.2	15.0	27.3

TABLE III

Comparison of amido black stain and photometric analysis with bromphenol blue staining, elution and colorimetry, and with free electrophoresis

Analytical procedure	Serum components (per cent total planimeter area)				
		Globulins			
	Albumin	α_1	α_2	β	γ
Case No. 9					
Photometric method	55.8	6.0	9.4	13.4	15.4
Elution and colorimetry*	57.4	4.8	9.8	15.1	12.8
Free electrophoresis	58.7	4.8	10.6	12.2	13.7
Case No. 47					
Photometric method	31.1	6.0	7.8	9.0	46.1
Elution and colorimetry*	30.5	3.7	7.6	9.1	49.1
Free electrophoresis	32.4	4.7	6.9	13.8	42.1
Case No. 54					
Photometric method	47.5	5.5	9.5	13.3	24.1
Elution and colorimetry*	47.2	3.5	9.7	15.6	23.8
Free electrophoresis	55.2	4.0	9.6	15.6	15.6

* Data obtained by Drs. B. C. Houghton and C. E. Bender.

of apparatus [10] was observed. Azocarmine and amido black stains yielded almost identical results with a given serum (Table II).

The dye-binding properties of albumin [11] and γ globulin [12] for azocarmine and amido black were studied individually by applying known amounts of protein to paper strips, staining, and measuring by planimeter the areas obtained by photometric measure of dye concentration. When bands representing equal amounts of protein (micro-Kjeldahl nitrogen determination) were compared, the albumin area was found to be greater than that for γ globulin by a factor of 1.3 for amido black and 1.35 for azocarmine.[13] However, this factor did not appear to apply in the following analysis of a normal whole serum: A known quantity of γ globulin was added to the serum of the same subject from which it was obtained. Measurement by both free and paper electrophoresis, employing no factor of correction, almost completely accounted for the γ globulin increment (Table IV). In electrophoretic analysis of whole serum, gamma globulin values were actually slightly greater by the paper method than by free electrophoresis (Table V); this was presumably due to "trailing" of albumin on the paper.

Analyses by paper electrophoresis of 55 different normal and abnormal sera are presented in Table V and Figure 2. In most cases, a corresponding free electrophoretic pattern was obtained, and the

[10] In a limited series, electrophoresis by the above methods, with photometric analysis of the paper strips, gave results which closely corresponded with those obtained by paper electrophoresis between sealed glass plates followed by bromphenol blue staining and elution (Kunkel and Tiselius [5]) (Table III).

[11] Red Cross normal human serum albumin.

[12] Ninety per cent electrophoretically homogeneous normal human γ globulin prepared in this laboratory by Cohn's Method 10 (29).

[13] Bromphenol blue gave erratic results with direct photometry due to instability of color intensity and shade, as was also noted by Köiw (30).

TABLE IV

Determinations of γ globulin before and after adding known amount of γ globulin to normal serum*

Serum components	Free electrophoresis				Paper electrophoresis			
	Before addition of γ globulin		After addition of γ globulin		Before addition of γ globulin		After addition of γ globulin	
	Per cent of total area	Protein conc. *Gm. %*	Per cent of total area	Protein conc. *Gm. %*	Per cent of total area	Protein conc. *Gm. %*	Per cent of total area	Protein conc. *Gm. %*
Alb	55.7	4.21	47.8	4.23	55.9	4.23	49.1	4.41
α_1	4.7	0.36	4.1	0.36	4.4	0.33	4.4	0.37
α_2	7.6	0.57	7.7	0.68	9.1	0.69	9.3	0.78
β	16.1	1.22	15.2	1.34	11.8	0.89	10.9	0.82
γ	15.9	1.20	25.2	2.23	18.8	1.42	26.3	2.46
Total protein†		7.56		8.84		7.56		8.84
γ globulin increment	2.23 − 1.20 = 1.03 Gm. %				2.46 − 1.42 = 1.04 Gm. %			

* 1.28 Gm., 85 per cent electrophoretically homogeneous (1.09 Gm. γ globulin).
† Biuret.

results for the two techniques were compared. For normal subjects, values for albumin and for the individual globulins fell within the expected ranges, and divergence from free electrophoretic values was small. It is evident from Figure 2 that there is a lack of correspondence in position of the peaks in the paper and free electrophoretic patterns, due to differing protein mobilities between the two techniques. On paper, albumin and α_1 globulin separate well, whereas the other globulins tend to be compressed. This was particularly noticeable in the sera of portal cirrhosis. Without knowing mobilities, precise identification of the various globulin fractions on the paper strip may occasionally be uncertain.

Poor correlation between paper and free electrophoretic analysis was observed in two cases of hypercholesterolemic xanthomatosis tuberosum (Cases 40 and 41). These sera, together with a highly lipemic serum from a patient with the nephrotic syndrome (Case 37), were studied as follows: Total nondialyzable solids (TNDS) were determined by dialyzing the serum against distilled water for 7 to 10 days, then drying the dia-

TABLE V

Comparison of paper and free electrophoresis for normal and pathological sera
(Components expressed as percentages of total planimeter area)

Case No.	Diagnosis	Paper electrophoresis					Free electrophoresis					
			Globulins					Globulins				
		Albumin	α_1	α_2	β	γ	Albumin	α_1	α_2	β	γ	
	Normals											
1		57.8	3.6	8.1	17.8	12.7	60.8	4.0	8.9	15.6	10.7	a
2		65.9	3.9	6.8	12.4	11.0	63.8	5.2	8.1	14.9	7.9	a
3		54.9	5.2	6.9	12.9	20.1	56.7	4.6	7.8	14.8	16.1	a
4		51.5	3.8	9.4	13.8	21.5	57.1	5.3	7.5	14.9	15.2	b
5		54.6	4.3	8.9	11.6	20.6	55.1	4.6	7.5	15.5	16.1	a
6		60.3	4.5	8.9	14.0	12.3	59.2	4.9	8.9	15.1	11.9	a
7		58.7	5.3	9.2	12.1	14.7	54.7	4.7	8.8	18.0	13.8	a
8		57.4	3.6	8.0	14.5	16.5	53.2	4.7	9.0	16.7	16.4	a
9		55.8	6.0	9.4	13.4	15.4	58.7	4.8	10.6	12.2	13.7	b
10		60.5	2.4	7.8	13.7	15.6	56.1	5.9	7.6	14.8	15.6	a
11		59.5	2.4	7.7	15.2	15.2	56.6	5.2	7.6	15.3	15.3	a
12		52.1	4.9	11.2	16.1	15.7	56.6	5.8	10.1	14.3	13.2	a
Means:		57.4	4.2	8.5	14.0	15.9	57.4	5.0	8.5	15.2	13.9	
Mean differences from free electrophoretic values:		−0.	−0.8	−0.	−1.2	+2.0						
Maximum differences from free electrophoresis:		5.6	3.5	1.9	5.9	6.3						
	Portal Cirrhosis											
13		26.4	3.2	6.0	15.3	49.1						
14		36.1	3.1	9.2	15.6	35.9						
15		37.2	4.9	9.9	21.0	27.0						
16		39.6	8.2	15.0	12.8	24.4						
17		40.0	6.8	7.7	13.1	32.4						
18		44.6	2.7	6.4	10.8	35.5						
19		34.6	3.0	9.2	10.0	43.1	34.1	2.9	10.9	11.0	40.9	b
20		38.7	7.2	9.8	11.0	33.3	37.1	5.3	7.8	19.5	30.4	a
21		34.9	4.3	7.6	7.6	45.6	34.0	2.1	5.3	14.5	44.1	a
22		30.6	5.0	8.7	17.2	38.5	28.6	5.2	6.9	17.9	41.4	b
23		26.3	3.2	5.3	9.4	55.8	22.6	3.7	5.6	11.3	56.8	b
24		31.7	5.0	7.3	12.0	44.0	33.7	4.9	6.6	13.8	41.0	b
25		32.4	11.5	10.5	10.4	35.2	33.0	9.1	13.1	10.5	34.3	a
Means:		34.9	5.2	8.7	12.8	38.4	31.9	4.7	8.0	14.1	41.3	
Mean differences from free electrophoretic values (for cases 19–25)		+0.9	+0.9	+0.3	−3.0	+0.9						
Maximum differences from free electrophoretic values (for cases 19–25)		3.7	2.4	2.6	8.4	3.0						

a = Electrophoretic analysis on Perkin-Elmer apparatus model 38 (Ascending limb).
b = Electrophoretic analysis on Pearson model of Tiselius apparatus (Descending limb).

TABLE V—*Continued*

Case No.	Diagnosis	Paper electrophoresis					Free electrophoresis					
			Globulins					Globulins				
		Albumin	α_1	α_2	β	γ	Albumin	α_1	α_2	β	γ	
	Biliary Cirrhosis											
26	Obstructive biliary cirrhosis	36.0	5.4	8.2	18.9	31.5	28.2	5.3	10.0	28.0	28.5	a
27	Obstructive biliary cirrhosis	41.8	6.0	8.2	10.5	33.5	36.6	4.7	8.2	16.3	34.2	a
28	Primary biliary cirrhosis	33.7	7.3	11.6	18.2	29.2	26.4	6.3	9.7	29.9	27.7	b
29	Primary biliary cirrhosis	46.4	4.9	12.2	15.5	21.0	38.0	6.0	12.1	24.1	19.8	a
	Multiple Myeloma											
30		26.9	7.6	11.8	10.0	43.7						
31		46.4	7.7	12.6	16.9	16.4	45.4	9.6	14.9	18.5	11.6	b
32		26.1	2.4	6.4	57.9	7.2	24.4	2.9	7.1	60.9	4.7	b
33		36.8	4.2	8.0	9.6	41.4	26.9	3.5	7.6	7.1	54.8	b
34		24.7	3.4		65.1	6.8	20.0	2.4		75.1	2.5	b
35		28.4	5.8	8.6	7.0	50.2	20.1	5.0	7.0	6.7	61.2	b
	Nephrotic Syndrome											
36	Glomerulonephritis	32.1	6.6	18.9	17.3	25.0	30.6	9.8	20.8	19.3	19.5	a
37	Diabetic nephropathy	37.6	7.4	17.9	22.8	14.3	30.7	7.8	22.6	28.8	10.1	b
38	Diabetic nephropathy	38.7	13.3	17.3	15.9	14.8	30.0	16.3	19.0	18.4	14.3	b
39	Toxemia of pregnancy	22.8	8.3	22.0	21.5	24.5	22.9	15.9	25.8	19.9	15.5	b
	Miscellaneous											
40	Essential hyper-cholesterolemic xanthomatosis	60.4	2.7	7.4	14.3	15.1	46.0	5.5	10.2	27.7	10.6	b
41	Essential hyper-cholesterolemic xanthomatosis	53.4	2.3	9.9	16.6	17.8	40.0	4.5	10.5	33.3	11.7	b
42	Lupus erythematosis	26.7	3.1	8.3	11.0	50.9						
43	Lupus erythematosis	42.4	6.3	13.3	10.5	27.5						
44	Lymphosarcoma	57.1	9.1	11.8	11.9	10.1						
45	Myeloid leukemia	45.1	8.2	11.9	9.0	25.8						
46	Neutropenia, recurrent infections	47.1	5.9	11.8	11.4	23.8	50.2	6.2	10.1	14.9	18.6	b
47	Lymphoma	31.1	6.0	7.8	9.0	46.1	32.4	4.7	6.9	13.8	42.1	b
48	Hypopituitarism	24.7	10.9	21.3	12.3	30.8						
49	Pulmonary abscess	40.4	5.5	11.6	14.2	28.3	33.0	6.6	12.2	18.7	29.5	a
50	Recurrent pulmonary infection	51.3	5.8	15.0	11.8	16.1	53.6	7.6	13.5	13.9	11.4	b
51	Staphylococcal infections	53.0	7.5	11.4	12.3	15.8	53.7	6.4	12.1	14.9	12.9	b
52	Tuberculous adenitis	37.0	5.2	14.5	12.7	30.6	36.7	5.1	10.2	14.6	33.4	a
53	Infectious hepatitis	54.9	4.4	6.9	14.1	19.7						
54	Bronchial asthma	47.5	5.5	9.5	13.3	24.1	55.2	4.0	9.6	15.6	15.6	b
55	Agammaglobulinemia	64.4	4.4	10.4	17.8	3.0	66.3	5.5	11.5	14.7	2.0	b

lyzed protein for 72 hours at 85° C., with repeated weighings over this period. The minimum weight was taken as the best estimate of the TNDS of the serum (31). The serum concentration in grams per cent of each component was calculated by multiplying the corresponding per cent of the total planimeter area of the free electrophoretic pattern by the weight of the TNDS.[14] These values were compared with results obtained by paper electrophoresis (amido black) wherein the concentration in grams per cent of each component was determined by multiplying the corresponding per cent of the total planimeter area of the paper electrophoretic pattern by the biuret total protein (Table VI). Values for albumin and γ globulin cor-

[14] The calculation of individual serum components from free electrophoretic patterns as per cent of TNDS would seem more valid than values based upon biuret reaction of the whole serum (32); obviously more substances contribute to refractive change than are determined by dye-staining or biuret-reacting groups, the lipid portions of α and β lipoprotein being good examples.

responded, whereas values for α and β globulins, which contain the lipoproteins, were considerably lower by paper electrophoretic analysis.

In advanced portal cirrhosis, the characteristic albumin depression and γ globulin elevation were consistent findings (Table V). The γ globulin peak of the pattern was usually tall and broad, but certain other abnormalities in this area were also observed. A well-marked and definite band was frequently observed between the β and γ globulin areas corresponding to the previously described γ_1 fraction (33, 34), or H component of Viollier (35). This band was more often apparent in terminal cases, where it occasionally amounted to as much as 12 per cent of the total serum protein

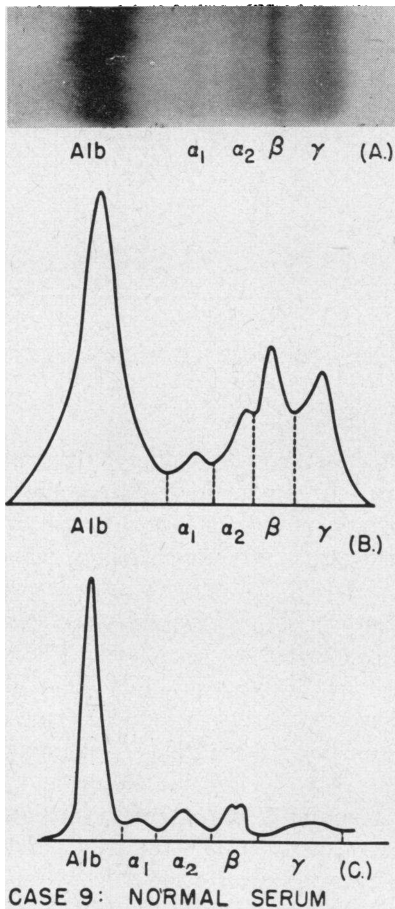


FIG. 2. COMPARISON OF DYED PAPER ELECTROPHORETIC STRIPS AND CORRESPONDING PHOTOMETER PATTERNS WITH TISELIUS SCHLIEREN DIAGRAMS

A = Stained paper strip.
B = Photometer pattern of A.
C = Free electrophoretic pattern.

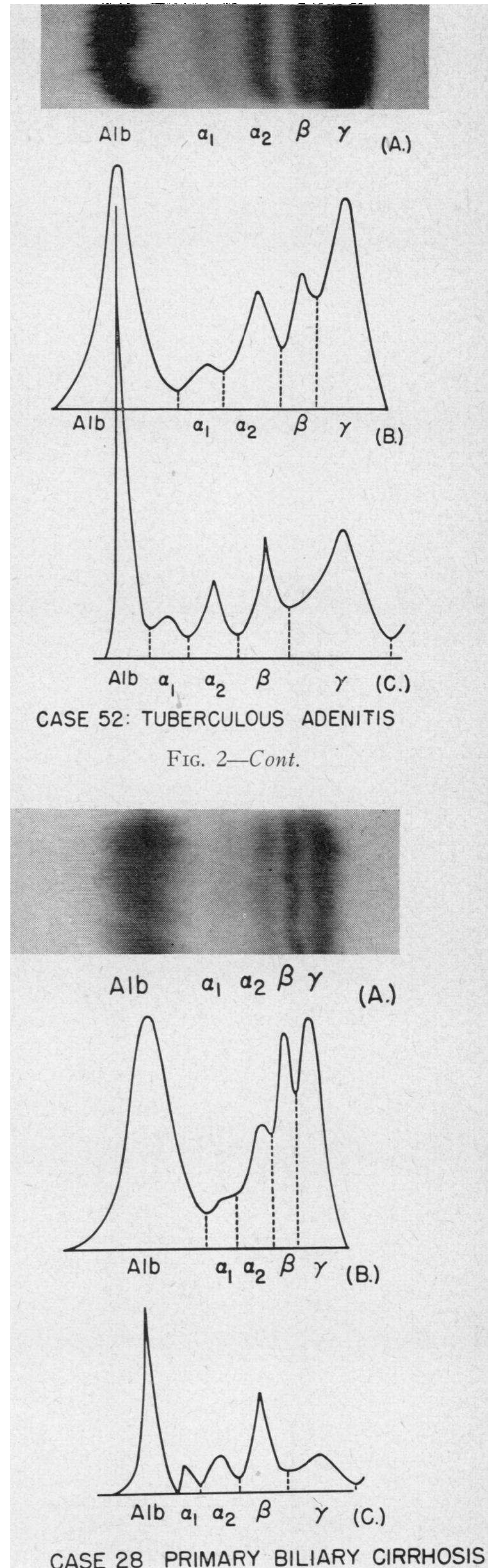
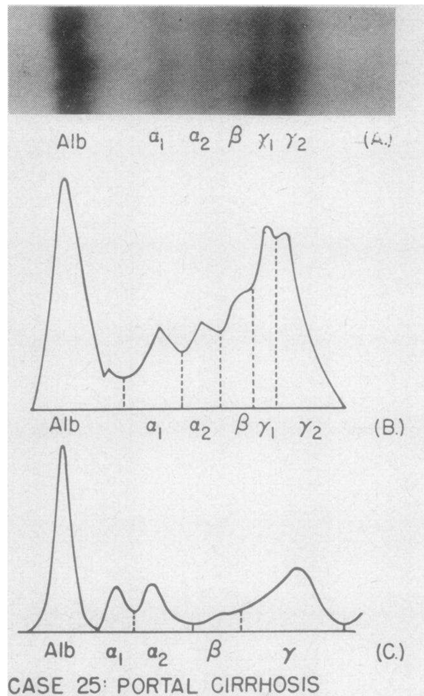


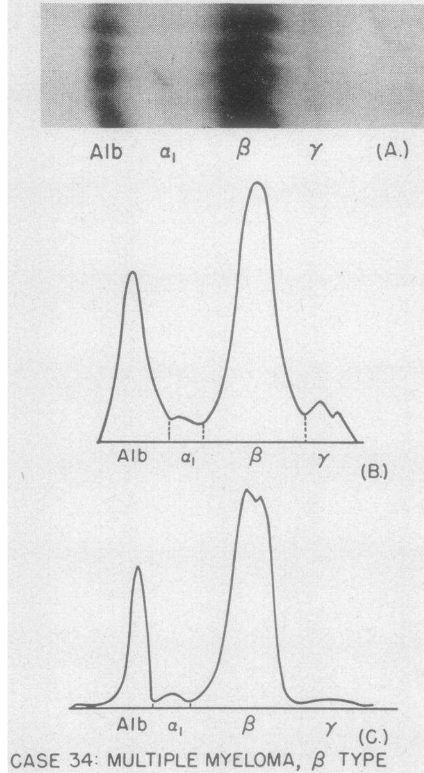
FIG. 2—*Cont.*

FIG. 2—*Cont.*



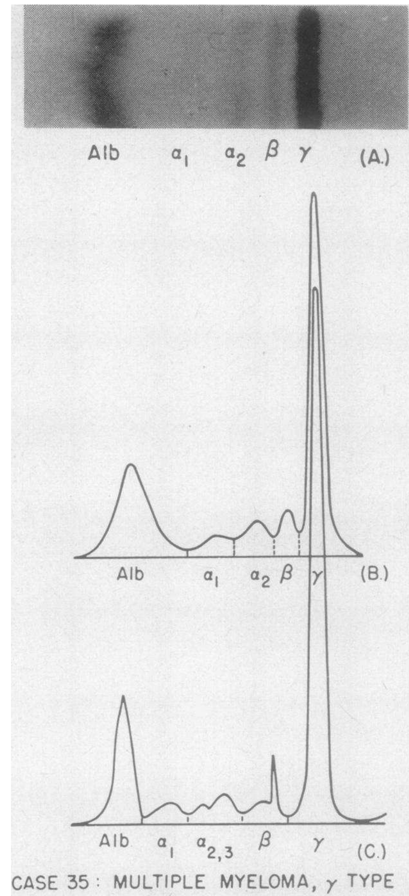
CASE 25: PORTAL CIRRHOSIS

FIG. 2—*Cont.*



CASE 34: MULTIPLE MYELOMA, β TYPE

FIG. 2—*Cont.*



CASE 35: MULTIPLE MYELOMA, γ TYPE

FIG. 2—*Cont.*

concentration, corresponding with Viollier's experience. In our methods of free electrophoresis, this component did not take the form of a discrete peak, but rather produced a plateau effect in the γ globulin area. In other cases of cirrhosis the paper pattern showed lack of clear separation, or smearing together, of the β and γ globulin bands, as noted by Brante (36). Slight α globulin increases were occasionally found, as have previously been noted in cirrhosis (37). Patients with biliary cirrhosis showed similar albumin and γ globulin changes, and also some increase of β globulin, but the discrepancy between the paper and free electrophoretic values for β globulin was marked; the paper method gave values nearly 50 per cent lower than obtained by free electrophoresis.

The seven cases of myeloma are representative of the various protein changes described in this disease (38). Five showed the characteristic narrow spiked peak in the γ globulin area. In Case

26 the abnormal γ globulin was a crystalline cryoglobulin. In Cases 32 and 34, the abnormal protein had a β globulin mobility on free electrophoresis, and accounted for nearly 60 per cent of the total protein present; Case 34 showed a double-spiked β globulin peak. Case 31 was of the unusual normo-proteinemic type. The abnormal myeloma proteins frequently gave lower values on paper electrophoresis as compared with free electrophoretic values. This is believed due to an error in photometric measurement, because the very narrow, deeply-stained myeloma band produced an optical density beyond the linear range of the instrument.

Low albumin and elevated α and β globulins (especially α_2) characterized the sera from the four patients with the nephrotic syndrome. The β globulin values were again found lower by paper than by free electrophoresis. Two cases showed a separate protein component appearing between the albumin and α_1 globulin areas.

In diseased states, measurement of fibrinogen in plasma is unsatisfactory by the paper electrophoretic technique, as has also been found with free electrophoresis (39). Fibrinogen usually appeared as a thin band adjacent to the γ globulin area on the paper, but seldom plotted out as a discrete peak. Particularly in the cases of cirrhosis showing a marked γ_1 globulin band, fibrinogen measurements by electrophoresis would seem impossible.

DISCUSSION

The precision of paper electrophoresis analysis, as evidenced by the reproducibility of results with serum, does not quite equal that of the Tiselius method. For free electrophoresis, Antweiler (40) reports a standard deviation of $\pm$ 0.5 per cent of total protein for each single component, and states that differences between duplicates should not exceed 1.5 per cent of total protein. Not all workers claim this degree of reproducibility (41). With paper electrophoresis, Grassmann and Hannig (7), from multiple determinations on different sera, give standard deviations (as per cent of total protein) of $\pm$ 1.2 per cent for albumin, $\pm$ 0.6 per cent for α_1 globulin, $\pm$ 0.7 per cent for α_2 globulin, $\pm$ 0.7 per cent for β globulin and $\pm$ 1.2 per cent for γ globulin. Sommerfelt (27), with repeated determinations on a single normal serum reports comparable figures. Our reproducibility has also been of this order (Tables I and II); for duplicate analyses, we regard differences within 3 per cent for albumin and γ globulin, and 2 per cent for the other globulin fractions as acceptable. However, when such deviations are related to the concentration in grams per cent of each component, the precision of the analysis is less impressive; large errors in the measurement of the smallest fractions, such as α_1 globulin, by paper electrophoresis are demonstrated.

Protein adsorption on the paper or "trailing," especially by the albumin fraction, is an important source of error, causing up to a 10 per cent reduction in the albumin value. The degree of adsorption has been demonstrated by electrophoresis and radioautography of I^{131} labeled albumin (16). "Trailing" may account for the occasional lower values obtained for albumin by paper as opposed to free electrophoresis. Gamma globulin behaves in a similar way, but no error is introduced with the technique described, owing to its limited mobility. "Trailing" of protein on the paper is largely responsible for the failure of the valleys between the peaks to approach the baseline of the electrophoretic pattern as closely as occurs in free electrophoresis. Thus, the area under the globulin peaks may not entirely represent single fractions, but will contain elements of the faster moving fractions, particularly albumin. When adsorption, or inadequate separation, results in the areas of individual globulin components appearing as "shoulders" rather than discrete peaks, any attempt to resolve the pattern into areas is quite inaccurate.

Noting a lack of agreement between the paper and free electrophoretic analysis of the same serum, previous investigators concluded that a "globulin factor" should be used to equate the results obtained by the two methods. However, there has been no unanimity of opinion as to the magnitude or desirability of such a factor, as evidenced by the following observations: Using bromphenol blue and elution, Cremer and Tiselius (3) observed that globulins appeared to bind less dye than albumin and derived a factor of 1.6 by which the globulin values should be multiplied to achieve comparability with free electrophoretic analysis. Similar factors have been used by subsequent workers employing the dye elution technic (8, 9, 12). Com-

TABLE VI

Comparison, methods of analysis of paper and free electrophoretic components, lipemic sera

Case No.	Biuret total protein *Gm. %*	Total non-dialyzable solids (TNDS)	Components	Paper electrophoresis % planimeter area	Paper electrophoresis Conc. from biuret *Gm. %*	Free electrophoresis % planimeter area	Free electrophoresis Conc. from biuret *Gm. %*	Free electrophoresis Conc. from TNDS *Gm. %*
37	6.23	8.23	Alb	37.6	2.34	30.7	1.91	2.53
Nephrotic			α_1	7.4	0.46	7.8	0.49	0.64
syndrome			α_2	17.9	1.12	22.6	1.41	1.86
Diabetic			β	22.8	1.42	28.8	1.79	2.37
nephropathy			γ	14.3	0.89	10.1	0.63	0.83
40	7.65	10.40	Alb	60.4	4.62	46.0	3.52	4.78
Hypercholes-			α_1	2.7	0.21	5.5	0.42	0.57
terolemic			α_2	7.4	0.57	10.2	0.78	1.06
xanthomatosis			β	14.3	1.09	27.7	2.12	2.88
			γ	15.1	1.16	10.6	0.81	1.10
41	7.40	10.70	Alb	53.4	3.95	40.0	2.96	4.28
Hypercholes-			α_1	2.3	0.17	4.5	0.33	0.48
terolemic			α_2	9.9	0.73	10.5	0.78	1.12
xanthomatosis			β	16.6	1.23	33.3	2.46	3.56
			γ	17.8	1.32	11.7	0.87	1.25

paring albumin and γ globulin fractions alone, Kunkel and Tiselius (5) found that, with a Folin tyrosine-reacting reagent, γ globulin gave a more intense color per unit of nitrogen than albumin; by bromphenol stain and elution, however, the reverse was true, giving a correction factor for globulin of approximately 1.3. Grassmann and Hannig (7) suggested a β globulin factor of 1.25 using their photometric method with amido black. Sommerfelt (42) reported that with amido black followed by elution, it was necessary to use a globulin factor of 1.3, but with the same dye and direct photometry, no factor was required. Griffiths (15), for bromphenol blue and direct photometry, stated that he found no evidence that any globulin factor was required. On the basis of many comparisons between paper and free electrophoresis, using pathological sera and bromphenol blue dye, Köiw, Wallenius, and Grönwall (11) derived separate factors for each individual globulin component ranging from 0.9 to 8.1; it was emphasized, however, that these factors varied greatly from one serum to another.

Our series of comparisons of paper with free electrophoresis indicates that results obtained with the two methods are in good general agreement. The major discrepancies occurred in the β globulin fraction, which often gave lower values with paper electrophoresis. The lipid components of these globulins presumably contribute to the refractive change in free electrophoresis, but do not stain with protein-binding dyes. As would be expected, values for lipemic sera and for sera with a high β globulin peak on free electrophoresis agreed least well.

We have shown that γ globulin also may have a slightly lower dye-binding capacity than albumin; however, values for albumin tend to be reduced, and γ globulin values increased by protein adsorption on the paper. All these considerations lead to the conclusion that paper electrophoresis measures serum protein complexes somewhat differently than does free electrophoresis; hence the use of an empirically derived globulin correction factor is not recommended, as no such factor can exactly equate the results obtained by the two methods.

The electrophoretic alterations in serum of diseased states which affect plasma protein metabolism have been fully reviewed (40, 43–45). The diagnostic value of any type of electrophoretic analysis of serum is limited by the fact that gross alterations of serum proteins in diseased states commonly follow general rather than specific patterns. In only a few situations, as in agammaglobulinemia, can electrophoretic patterns be considered of real diagnostic importance. The advantages of filter paper electrophoresis as compared with free electrophoresis are that it is simple, inexpensive, and requires very small amounts of protein. The technique is especially applicable to very opaque sera which often cannot be analyzed

by free electrophoresis. Our studies indicate that, for normal sera, results obtained with paper and free electrophoresis correspond well. The general protein disturbances in various pathologic conditions such as myeloma, portal and biliary cirrhosis, the nephrotic syndrome, and agammaglobulinemia are very satisfactorily revealed by the paper technique.

Photometric analysis of the paper pattern is much quicker than the more tedious elution method, but it does not seem more accurate than the latter. In most cases, simple inspection of the paper strip will demonstrate clearly the nature of any significant serum protein abnormality present, without any necessity for time-consuming quantitation of the pattern.

SUMMARY

1. Methods for paper electrophoresis using the Grassmann-Hannig and Durrum apparatus are described. Analyses of normal and abnormal sera were made by photometric measure of the serum protein components on the dyed paper strip.

2. Reproducibility of analysis is good, but does not quite meet the accuracy reported by others for free electrophoresis.

3. Results with paper electrophoresis compare well with analyses of duplicate samples by the Tiselius method, except with certain abnormal sera, such as those of high lipid content. Reasons for discrepancies between the two methods are investigated; it is concluded that a correction factor for globulin values should not be used.

4. Analyses of the serum protein changes in a wide variety of pathological states are presented, and the clinical usefulness of the procedure is discussed.

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