[CANCER RESEARCH 35, 1991-1995, August 1975]

Use of Cationic Disc Electrophoresis Near Neutral pH in the Evaluation of Trace Proteins in Human Plasma[1]

Charles W. Young, Wilner Dessources, Sadie Hodas, and Edward S. Bittar

Memorial Sloan-Kettering Cancer Center, New York, New York 10021

SUMMARY

Cationic discontinuous electrophoresis can be carried out at pH 6.0 with excellent resolution in urea-containing acrylamide gels with potassium as the leading ion, 3-picoline as the trailing ion, and cacodylic acid as the buffer. This analytic technique has consistently demonstrated trace protein abnormalities in plasma or serum from febrile patients. It has made possible the detection of three protein bands that were obscured in similar electrophoretic assays at pH 3.8. A rough parallelism is found in the plasma (or serum) content of the five protein bands regardless of the apparent clinical cause of the febrile state.

INTRODUCTION

Young *et al.* (8, 9) have demonstrated the presence of abnormal trace proteins in plasma, serum, and urine obtained from febrile patients. The trace proteins were detected and quantitated by cationic electrophoresis in acrylamide gel. Their initial studies focused on a cationic protein of low molecular weight in urine and CSF[2] and on a more complex protein in plasma. The electrophoretic migration of the plasma protein was identical to that of the urine and CSF protein(s) but displayed a doublet configuration. The presence of these proteins in plasma and urine correlated well with the overall intensity of pyrexia but less closely with hr to hr fluctuations in the actual body temperature (9). These studies were carried out by a modification of the electrophoretic technique of Reisfeld *et al.* (4), wherein proteins undergo stacking at pH 5 and run in the separation gel at pH 3.8. Because of the discordance between the observations on urine and CSF and those on plasma, it seemed possible that multiple species of protein might be involved that were not distinguishable in an electrophoretic assay run at low pH. Accordingly, we have designed a cationic disc electrophoresis system with stacking and separation closer to neutrality. It has permitted detection and quantitation in plasma from febrile patients of 3 additional trace protein bands that were previously obscured by the much more prominent plasma DP. This communication will provide details and comparison of methodology at the 2 pH ranges and initial support for the pertinence of these newly detected proteins to the febrile state.

[1] This work was supported in part by a grant from the Bodman Foundation and by Grants CA 08748 and CA 15928 from the National Cancer Institute.

[2] The abbreviations used are: CSF, cerebrospinal fluid; DP, doublet proteins.

Received November 19, 1974; accepted April 22, 1975.

MATERIALS AND METHODS

The 2 techniques are based upon the formulation of leading ion and trailing ion initially enunciated by Ornstein (3) and lean heavily on the suggestions for buffer strategy made by Williams and Reisfeld (6). The leading ion is potassium, the trailing ion is β-alanine in the pH 3.8 system or 3-picoline, (3-methylpyridine) (pK 6.29) in the pH 6.0 system. Acetic acid and cacodylic acid (pK 6.4) provide suitable buffering respectively, for the 2 systems. Precise formulation and comparison of the 2 electrophoretic systems are given in Table 1.

Chemicals. The reagents used and their commercial sources were: acrylamide, Canalco, Inc., Rockville, Md.; *N,N'*-methylenebisacrylamide, *N,N,N',N,'*-tetramethylethylenediamine, and 3-picoline (practical), Eastman Organic Chemicals, Rochester, N. Y.; ammonium persulfate, E. C. Apparatus Corp., Philadelphia, Pa.; ultrapurified urea, Research Plus Labs., Denville, N. J.; sucrose, Sigma Chemical Co., St. Louis, Mo.; β-alanine and Coomassie Brilliant blue-R-250, Schwarz/Mann, Orangeburg, N. Y.; Amido schwarz (naphthol blue black), Roboz Surgical Instruments Co., Washington, D. C., or Allied Chemical, Morristown, N. J.; Sephadex G-75 and QAE Sephadex A-50, Pharmacia Fine Chemicals, Piscataway, N. J.; cacodylic acid, ethylenediamine, and Pyronine Y, Fisher Chemicals, New York, N. Y.

Plasma Samples. Venous blood was obtained in Versene-containing Vacutainer tubes (No. 3300Q; Becton-Dickenson, Rutherford, N. J.) and was placed on ice. The red blood cells were removed by centrifugation in the cold at $800 \times g$ for 20 min. The plasma supernatant was recentrifuged at $1250 \times g$ for 30 min to remove residual cellular elements. Serum was obtained from clotted blood in a similar manner except that cooling was omitted to permit clot formation and retraction. Since trace quantities of hemoglobin interfered with later assays, all samples were tested with orthotolidine peroxide reagent (Bili-Labstix; Ames Co., Elkhart, Ind.), and positive samples were discarded. Plasmas were stored at $-20°$ until used. The trace protein content of repeatedly studied samples did not change with storage or with repeated freezing and thawing.

Acrylamide Gel Electrophoresis. Cationic electrophoresis was carried out in 6- x 0.5-cm glass tubes with the

Downloaded from http://aacrjournals.org/cancerres/article-pdf/35/8/1991/2395467/cr0350081991.pdf by guest on 07 August 2024

Table 1

Comparison of stock and working solutions for cationic electrophoresis at pH 3.8 [modified from the method of Reisfeld et al. (4)] and pH 6.0 (this paper)

pH 3.8	pH 6.0
Stock A	
Urea, 36 g	Urea, 36 g
KOH, 2.6 g	KOH, 13.4 g
TEMED, 4 ml	TEMED, 4 ml
Glacial acetic acid, q.s. to pH 4.3	Cacodylic acid, 68 g (pH to 6.4)
H_2O q.s. to 100	H_2O q.s. to 100 ml
Stock B	
Urea, 36 g	Urea, 36 g
KOH, 2.6 g	KOH, 13.4 g
TEMED, 0.46 ml	TEMED, 0.4 ml
Glacial acetic acid, q.s. to pH 6.7	Cacodylic acid, 3.2 g (pH to 8.2)
H_2O q.s. to 100 ml	H_2O q.s. to 100 ml
Stock C	
(7% gel)	*(6% gel)*
Acrylamide, 28 g	Acrylamide, 24 g
Bis 0.735 g	Bis 1.2 g
H_2O q.s. to 100 ml	H_2O q.s. to 100 ml
Stock D	
Acrylamide, 20 g	
Bis, 5.0 g	Unchanged
H_2O q.s. to 100 ml	
Stock E	
Riboflavin, 4 mg	
Urea, 36 g	Unchanged
H_2O q.s. to 100 ml	
Stock G	
Ammonium persulfate, 0.056 g	Ammonium persulfate, 0.028 g
10 M urea, 16.0 ml	10 M urea, 16.0 ml
H_2O q.s. to 20 ml	H_2O q.s. to 20 ml
Working solutions	
Separating gel: Stock A, 1 vol; Stock C, 2 vol; 10 M urea, 1 vol; Stock G, 4 vol.	
Stack gel: Stock B, 1 vol; Stock D, 1 vol; Stock E, 1 vol; 10 M urea, 4 vol; H_2O, 1 vol.	
Sample gel: Stock B, 1 vol; Stock D, 1 vol; 10 M urea, 1 vol; Stock E, 1 vol.	
Tray buffer	
Anode and cathode	*Anode*
β-Alanine, 34 g	KOH, 0.017 g
Glacial acetic acid, 7.0 ml	3-Picoline, 4.7 g
H_2O q.s. to 1000 ml	Cacodylic acid, 0.7 g
	H_2O q.s. to 1000 ml
	Cathode
	KOH, 1.7 g
	Cacodylic acid, 6.0 g
	H_2O q.s. to 1000 ml

[a] TEMED, *N,N,N',N'*-tetramethylethylenediamene; q.s., sufficient quantities; Bis, *N,N'*-methylenebisacrylamide; vol, volume.

conventional Canalco analytical disc electrophoresis apparatus (Canalco). All stock solutions used were made up in deionized-double distilled water. The sample gel was mixed with an equal volume of 6 M urea containing the plasma sample. The gels were polymerized in the sequence listed in Table 1. They measured 4, 1, and 1 cm, respectively, in the glass electrophoresis tubes. The conventional sample was 10 μl plasma or serum (500 to 800 μg protein), except in pH 6.0 runs for spectrophotometric scans where 20 μl were used. Pyronine Y served to mark the electrophoretic front. Electrophoretic voltage was adjusted to provide a constant current of 4.5 ma/gel. The conventional run was 2 hr for visual inspection and 2.5 to 3 hr for spectrophotometric scanning.

Isoelectric Focusing. Plasma proteins were focused in urea-containing acrylamide gels according to a method published previously (7). The conventional onlay was 10 μl plasma. All isofocus and pH 6.0 electrophoresis gels were stained overnight by Coomassie blue according to the formulation of Spencer *et al.* (5); they were destained by diffusion in 7% acetic acid. The pH 3.8 electrophoresis gels were stained for 30 min in 1% Amido schwarz and 7% acetic acid; they were placed in dye-free acetic acid overnight to permit extensive leaching of the urea. Final destaining was accomplished by lateral electrophoresis (Canalco lateral destaining apparatus).

Densitometric tracings of the gels were carried out at 615 nm (Amido schwarz-stained gels) or 540 nm (Coomassie blue-stained gels) with a Gilford recording spectrophotometer and a linear gel transport. Tracings were made with a calibration of 1.000 A for the full-scale recorder deflection with a scanning rate of 1 cm/min and a chart speed of 10 sec/inch. The area under the curve was calculated by cutting out the tracing and weighing it. Within the range of observations on any given run, there was a linear relationship between the quantity of trace protein applied, *i.e.*, in crude plasma, and the area under the curve. Because there was modest variation in staining intensities from day to day, determinations were corrected to a standard value for a single standard sample run repeatedly. In the absence of adequately purified trace protein to afford a gravimetric or Kjeldahl standard, the relative plasma levels are expressed in arbitrary density units, *i.e.*, the weight (in mg) of tracing "cut out."

RESULTS

Comparisons of Electrophoretograms Run at pH 3.8 and 6.0. The potassium-β-alanine-acetate system of Reisfeld *et al.* (4) provides excellent cationic mobility of such proteins as muramidase and RNase and retains acidic molecules such as α_1-acid glycoprotein or haptoglobin within the sample gel. However, in this technique some relatively acidic molecules such as serum albumin (pI 4.3) also move wholly, or in part, into the separating gel (Fig. 1). Because it is retarded by its size, albumin does not obscure low-molecular-weight neutral or -cationic proteins. However, lower-molecular-weight proteins with pI's between 5 and 6 can and do migrate well into separating gel. Under the newly derived electrophoretic conditions where proteins stack at 6.5 and run at 6.0, albumin is retained completely within the sample gel (Fig. 2). Muramidase runs with approximately the same mobility seen in the Williams and Reisfeld method (6), but RNase has a considerably lower mobility (Figs. 1 and 2); γ-globulin still moves cationically, but by reason of its size it is retained within the

Downloaded from http://aacrjournals.org/cancerres/article-pdf/35/8/1991/2395467/cr0350081991.pdf by guest on 07 August 2024

proximal portion of the separating gel.

A distinct difference is obvious between plasma or serum from febrile patients and afebrile individuals (Figs. 2 to 4). Furthermore, the new technique makes evident the presence of trace components that were previously obscured in the more abundant DP. Both the electrophoretic modification and increased protein staining sensitivity by means of Coomassie blue (1) are necessary; the pH 6.0 cationic trace proteins are not detectable when gels are stained with Amido schwarz. When plasma or serum samples from febrile and afebrile patients are subjected to electrophoresis by 2.5 to 3 hr, the trace proteins are sufficiently well separated from each other and from the bulk of plasma protein to permit densitometric analysis (Chart 1). Pending more meaningful identification, the 3 protein bands can conveniently be called (from the cathodal) *a*, *b*, and *c*. The intensity staining of afebrile plasma in these areas is markedly below that of the plasma from febrile patients. Furthermore, the configuration of staining in the plasmas from febrile and afebrile individuals is sufficiently different that an identity between bands present in the 2 circumstances cannot be firmly asserted.

Resolution of Cationic Proteins from DP. Definitive differentiation has been made between the prominent DP of the pH 3.8 electrophoretograms and the 3 newly detected trace protein molecules by parallel analyses on crude plasma and chromatographically separated fractions thereof. Plasma (10 ml) was diluted and equilibrated with ethylenediamine-acetic acid buffer *I* 0.1, pH 7.0 and placed upon a QAE Sephadex A-50 column (40 x 2.5 cm) equilibrated with the same buffer according to the method of Joustra and Lundgren (2). The IgG fraction was eluted with the initial buffer, but the vast majority of both the DP and *a*, *b*, and *c* bands adhered to the column. These proteins were eluted when the column was developed with the initial buffer made 6 M with respect to urea. The urea eluate was pooled into 2 fractions, the 1st containing the initial bed volume eluent; 2nd was a pool of the 2nd and 3rd bed volumes. When subjected to gel diffusion chromatography in acetate-buffered NaCl solution at pH 3.5, the early (urea-released) cationic proteins enter a Sephadex G-75 column to some degree (Chart 2) while the DP form aggregates that are eluted in void volume.

When the crude serum and the QAE-Sephadex fractions are compared by the low-pH electrophoretic technique, the trace proteins overlap (Fig. 5). However, in the new technique the DP fail to enter the separating gel in significant quantities, while the small, more cationic, trace proteins give the configuration typical of febrile plasma (Fig. 6). When analyzed in isoelectrical focusing (in urea-containing acrylamide gels) 1 of the doublet proteins is readily visible between transferrin and the bulk of plasma proteins (Fig. 7); the other is obscured by other proteins. The more cathodal trace proteins can be detected within the upper reaches of the pH range, but they are difficult to quantitate in isoelectric focusing because they overlap with diffuse staining produced by the immunoglobulins.

Comparison of the Individual Trace Protein Abnormalities According to Diagnosis. Although the DP, *a*, *b*, and *c* bands are physically distinct and separable from one another, they increase in blood of febrile individuals more or less in parallel. Charts 3 to 6 provide scattergram comparison of *a* and *b*, *a* and DP, *b* and DP, and *b* and *c*, respectively, in samples obtained from febrile patients with Hodgkin's

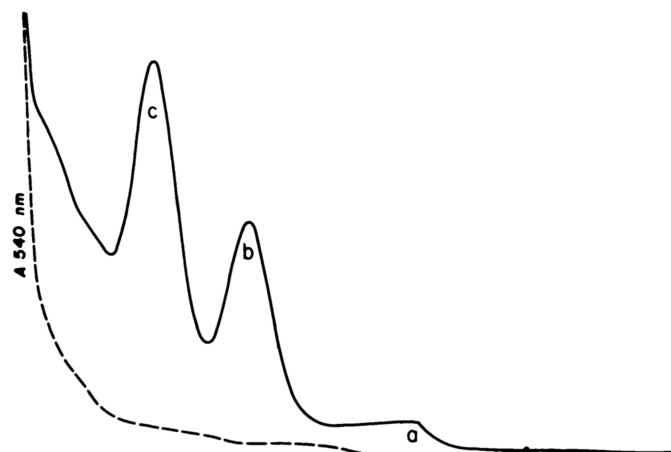


Chart 1. Densitometric comparison of electrophoretograms of plasma from a normal individual (- - -) and a febrile patient with Stage IV Hodgkin's disease (——) following cationic electrophoresis at pH 6.0.

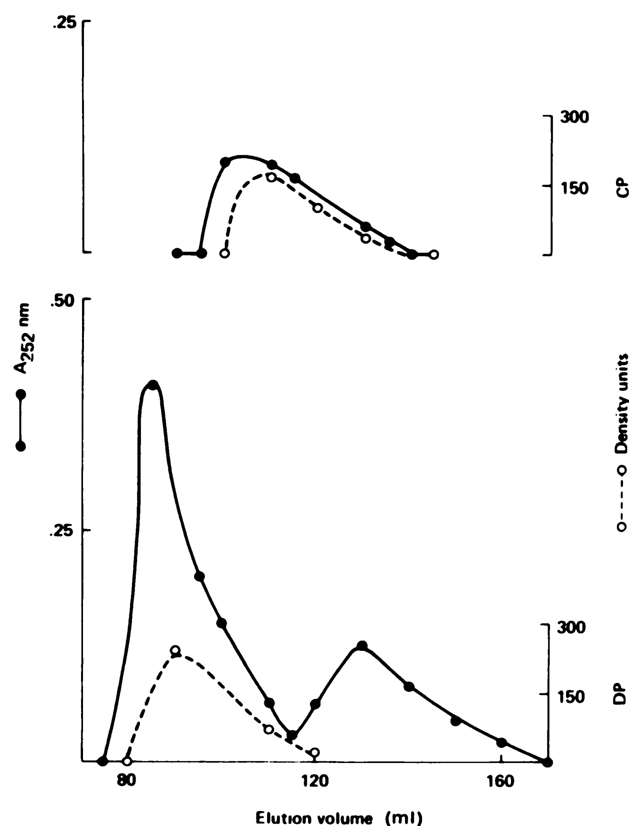
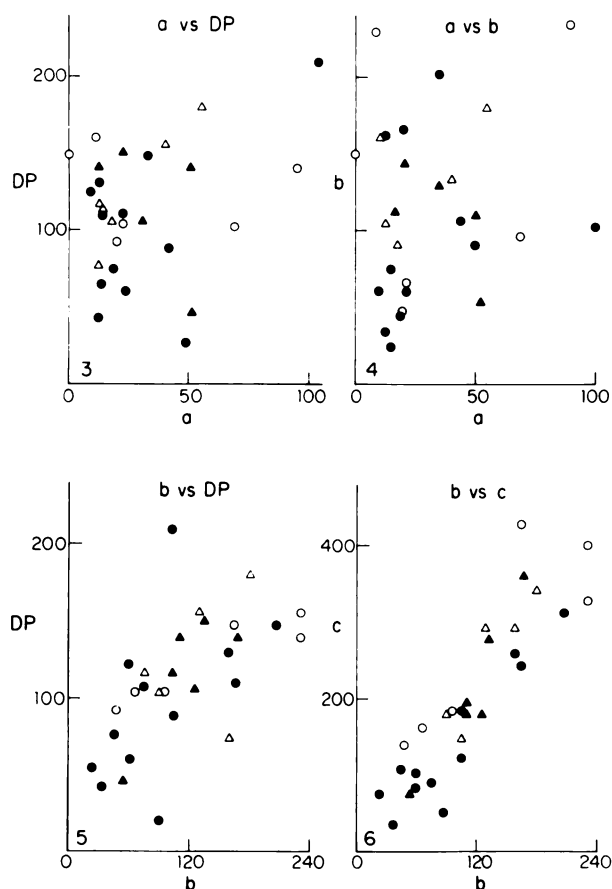


Chart 2. UV and electrophoretic-densitometric elution profiles of fractions corresponding to *c* and *d* from Figs. 5 to 7 rechromatographed on a 45- x 2.5-cm Sephadex G-75 column in 0.1 M NaCl titrated to pH 3.5 with acetic acid. The DP and CP units are arbitrary, representing the weight of the area under the curve in densitometric analysis of Coomassie blue-stained gels. Electrophoresis was carried out at pH 3.8 and 6.0, respectively, for the 2 trace protein fractions.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/35/8/1991/2395467/cr0350081991.pdf by guest on 07 August 2024



Charts 3 to 6. Scattergram comparison of plasma content of the trace proteins *a*, *b*, *c*, and DP in patients manifesting fever of unknown origin in Hodgkin's disease (●), non-Hodgkin's lymphoma or leukemia (○), and solid tumors (▲) and in patients with fever of bacterial infection (△). The values expressed are arbitrary density units based upon densitometric scanning of stained gels: *a*, *b*, and *c*, Coomassie blue; DP, Amido schwarz. Chart 3, ***a versus*** DP; Chart 4, ***a versus b***; Chart 5, ***b versus*** DP; Chart 6, ***b versus c***.

disease, non-Hodgkin's lymphoma or leukemia, solid tumors, and bacterial infections. There is extensive intermingling of points from patients with different diagnoses, suggesting the existence of a general relationship between these blood proteins and the febrile state rather than with any specific diagnostic entity. With rare exception, the content of DP $> c > b >> a$. The closest quantitative parallelism occurs between bands *c* and *b*.

DISCUSSION

This report indicates that the potential utility of cationic disc electrophoresis in acrylamide gels can be increased by the use of pH conditions near to neutrality. The method described here provides excellent reproducibility and gels that are suitable for densitometric quantitation. For optimal buffering, the concentration of 3-picoline in the upper bath should be greater; however, the strong odor of this chemical makes it difficult to work with at concentrations greater than those utilized. A variety of alternative trailing ions were tested. Pyridine itself and 2,6-lutidine have odors that are fully as strong as that of 3-picoline and did not give comparable resolution. Although the pK of 2-aminothiazole was in the proper range, its very low solubility in aqueous solutions and its tendency to damage the integrity of the acrylamide gel made it an unsuitable choice. Similarly, maleic acid was descarded as a candidate buffer because it injured the gels.

We have only begun detailed studies on the nature of the *a*, *b*, and *c* proteins and the physiological circumstances associated with their occurrence in blood and urine. Under physiological conditions these trace proteins are, in major part, bound to other, larger serum proteins. They are released from these proteins by urea both in the electrphoretic assay and in the ion-exchange chromatographic separation described above. Their interaction with blood proteins is not random. Although the nature of the "binding" proteins is not established, immunoglobulins (Figs. 5 to 7) and, on the basis of work currently in progress, albumin can be excluded. The results of these investigations will appear in later communications.

REFERENCES

1. Fazekas de St. Groth, S., Webster, R. G., and Datyner, A. Two New Staining Procedures for Quantitative Estimation of Proteins on Electrophoretic Strips. Biochim. Biophys. Acta. *71:* 377-391, 1963.
2. Joustra, M., and Lundgren, H. Preparation of a Freeze-dried Monomeric and Immunochemically Pure IgG by a Rapid and Reproducible Chromatographic Technique. *In:* H. Peeters (ed.), Protides of the Biological Fluids, pp. 511 515. New York: Pergamon Press, 1970.
3. Ornstein, L. Disc Electrophoresis-I Background and Theory. Ann. N. Y. Acad. Sci., *121:* 321 349, 1964.
4. Reisfeld, R. A., Lewis, W. J., and Williams, D. E. Disk Electrophoresis of Basic Proteins and Peptides on Polyacrylamide Gels. Nature, *195:* 281-283, 1962.
5. Spencer, E. M., and King, T. P. Isoelectric Heterogeneity of Bovine Plasma Albumin. J. Biol. Chem., *246:* 201-208, 1970.
6. Williams, D. E., and Reisfeld, R. A. Disc Electrophoresis in Polyacrylamide Gels. Extension to New Conditions of pH and Buffer. Ann. N. Y. Acad. Sci., *121:* 373 381, 1964.
7. Young, C. W., and Bitter, E. S. Analysis of Tissue Esterases from Patients with Hodgkin's Disease and Other Types of Advanced Cancer by Isoelectric Focusing in Acrylamide Gel. Cancer Res. *33:* 2692-2700, 1973.
8. Young, C. W., and Hodas, S. Demonstration of a Characteristic Protein in Plasma of Febrile Patients with Hodgkin's Disease. J. Clin. Invest., *50:* 101a, 1971.
9. Young, C. W., Hodas, S., Dessources. W., and Korngold, L. Observations on Trace Proteins in Plasma of Febrile Patients by Cationic Disc Electrophoresis in Acrylamide Gel at pH 3.8. Cancer Res., *35:* 1985-1990, 1975.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/35/8/1991/2395467/cr0350081991.pdf by guest on 07 August 2024

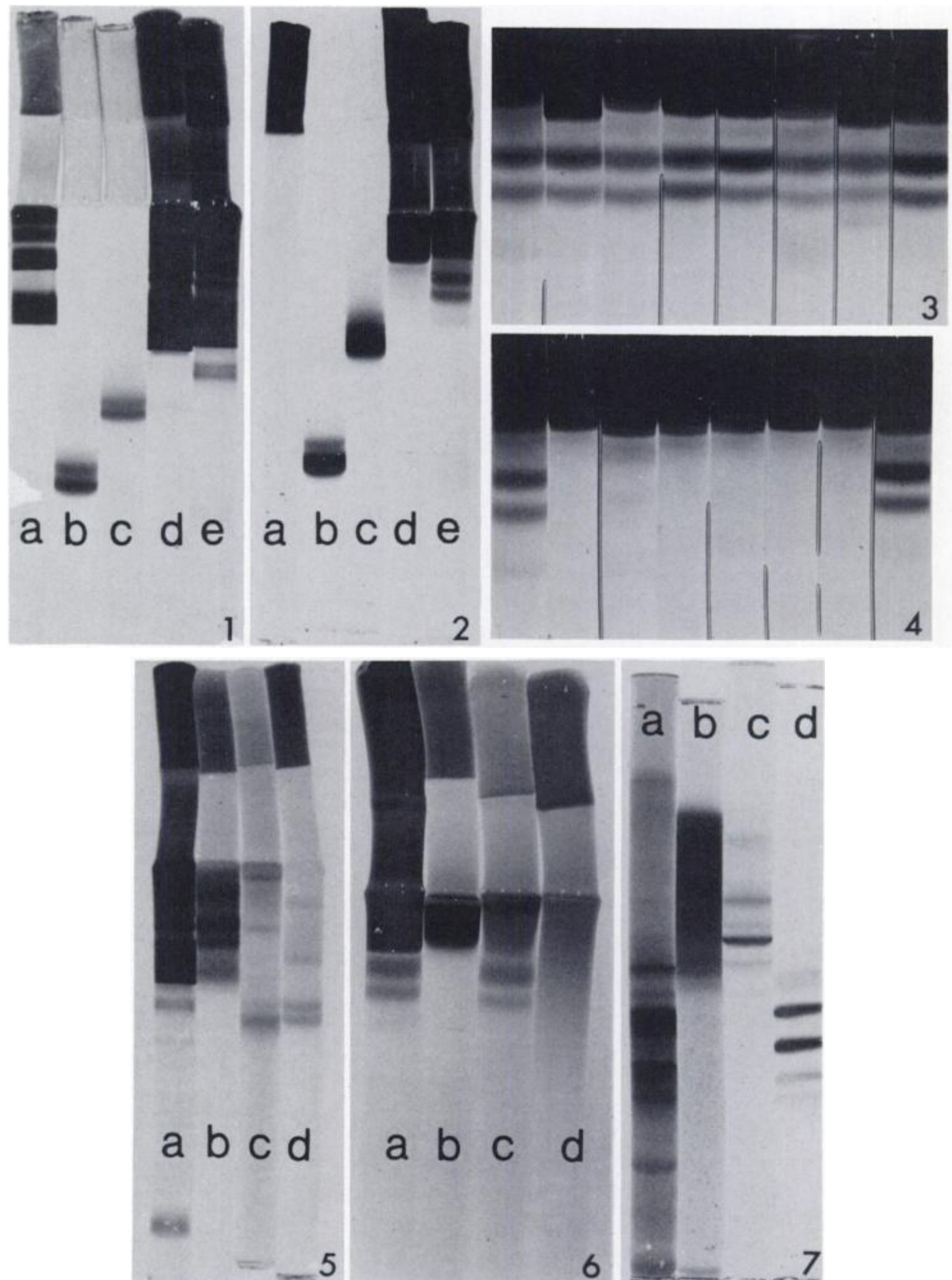


Fig. 1. Cationic disc electrophoretograms (at pH 3.8). *a*, human albumin; *b*, egg white muramidase; *c*, bovine pancreatic RNase; *d*, normal plasma; *e*, "febrile" plasma Stage IV Hodgkin's disease. Amido schwarz.

Fig. 2. Cationic disc electrophoretograms (at pH 6.0). *a* to *e*, see Fig. 1. Coomassie blue.

Fig. 3. Cationic disc electrophoretograms (pH 6.0). Plasma from 8 febrile individuals. Coomassie blue.

Fig. 4. Cationic disc electrophoretograms (at pH 6.0). Plasma from 6 normal individuals flanked by plasma from 2 febrile individuals. Coomassie blue.

Fig. 5. Cationic disc electrophoretograms (at pH 3.8) containing: (*a*) Febrile plasma Stage IV Hodgkin's disease; (*b*) γ-globulin fraction that failed to adhere to a QAE-Sephadex column, pH 7.0, *I* 0.1; (*c*) plasma proteins eluted from the QAE-Sephadex column within 1 bed volume of initial buffer made 6 M with respect to urea; (*d*) plasma proteins eluted from the QAE-Sephadex column in the 2nd and 3rd bed volumes of 6 M urea in initial buffer. Coomassie blue.

Fig. 6. Cationic disc electrophoretograms (pH 6.0), sequence as in Fig. 5. Coomassie blue.

Fig. 7. Plasma protein fractions following isoelectric focusing in urea-containing acrylamide gel. The sample sequence is the same as in Figs. 5 and 6. The cathode is at the top. Coomassie blue.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/35/8/1991/2395467/cr0350081991.pdf by guest on 07 August 2024