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Research Article

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Sedimentation velocity experiments indicated that the $S^{o}_{20,\,[unk]}$ of fibrinogen Baltimore was slightly greater (8.13S vs. 7.85S) than that of normal fraction I-4. Differences in concentration dependence (- 0.65 c vs. - 1.30 c for normal) of the sedimentation coefficient could be attributable […]

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Chromatographic, Ultracentrifugal, and Related Studies of Fibrinogen "Baltimore"

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ABSTRACT Chromatographic, ultracentrifugal, and related studies of the fibrinogen of a patient with a congenital disorder of fibrinogen (fibrinogen "Baltimore") have provided evidence of structural differences from normal.

Diethylaminoethyl-cellulose (DEAE-cellulose) gradient elution chromatography demonstrated two major peaks in the elution pattern of fibrinogen Baltimore as was the case for normal fibrinogen. However, the first peak of fibrinogen Baltimore was somewhat broader and more symmetrical and was eluted significantly later in the chromatogram than the corresponding peak of normal fibrinogen. Additionally, in some elution patterns, a shoulder on the ascending limb of peak 1 was present, suggesting the presence of chromatographically "normal" fibrinogen. Thrombin time determinations of eluted column fractions from a chromatogram of propositus fibrinogen supported this conclusion by demonstrating that fibrinogen from the ascending portion of peak 1 behaved functionally more like normal than that later in the chromatogram. Chromatograms of mixtures of propositus and normal fibrinogen confirmed the ability of this technique to distinguish normal from Baltimore fibrinogen. Chromatograms of fibrinogen isolated from two affected daughters displayed the characteristic increased anionic binding of peak 1 fibrinogen.

Sedimentation velocity experiments indicated that the $s^{\circ}_{20,w}$ of fibrinogen Baltimore was slightly greater (8.13S vs. 7.85S) than that of normal fraction I–4. Differences in concentration dependence (− 0.65 c vs. − 1.30 c for normal) of the sedimentation coefficient could be attributable in part to spatial conformational differences. Molecular sieving experiments in acrylamide gels indicated that the molecular weight of propositus fraction I-2 was about the same as that of normal fibrinogen of comparable solubility (i.e. I–4, mol wt 325,000).

Studies of the UV spectra, tyrosine/tryptophan ratios, sialic acid and hexose content, and *N*-terminal amino acids demonstrated no consistent significant differences from normal fraction I–4.

The results of these studies were presented in part at the National Hemophilia Foundation Symposium on "Recent Advances in Hemophilia and Hemophilioid Disease," 30 August 1968, New York City.

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INTRODUCTION

In 1964 Beck (1) reported the results of studies on the blood of a young woman with a history of recurrent thrombosis, pulmonary embolism, and mild bleeding tendency. The rate of coagulation of her plasma by thrombin or reptilase was delayed markedly although the amount of fibrinogen detectable by immunochemical techniques was normal (2). Thrombin clots formed from recalcified plasma were insoluble in 5 M urea. There was no evidence of increased fibrinolysis or fibrinogen turnover. Immunoelectrophoretic studies suggested slightly greater anodal mobility of patient plasma fibrinogen compared with that of normal (3). On the basis of this evidence and associated family studies demonstrating the abnormality in three successive generations, it was proposed that this kindred was another example of a functionally defective fibrinogen such as had already been described by Imperato and Dettori (4) and Ménaché (5). Employing a nomenclature similar to that for hemoglobinopathies the abnormal fibrinogen was designated fibrinogen "Baltimore."

Since that time several other kindred have been recognized with abnormally slowly clotting fibrinogen (6–12); evidence of amino acid substitution (serine for arginine) has been demonstrated in the *N*-terminal portion of the α(A)-chain of fibrinogen Detroit (13). Similar studies of fibrinogen Baltimore have failed to demonstrate any abnormality of that portion of the molecule.[1]

[1] Blombäck, M., and B. Blombäck. Unpublished data.

In this report the results of investigations relating to the characterization of fibrinogen Baltimore by gradient elution chromatography, and ultracentrifugal and related techniques are presented.

METHODS

Single donor and propositus fibrinogen was prepared from citrated or acid, citrate, dextrose (ACD) plasma. It is noteworthy that of the total plasma fibrinogen measured in five samples of propositus plasma by the method of Ratnoff and Menzie (14) (mean = 141 mg/100 ml; range = 113–159) approximately 22% (range = 20–25) remained in the supernatant of fraction I, a finding similar to that for normal plasma fibrinogen (15). Patient or normal material equivalent in solubility to fraction I-2 was prepared by the method of Blombäck and Blombäck (16) or by the procedure of Laki (17) from fraction I. Certain preparations were reprecipitated with 2.1 M glycine (18) to increase clottability (usually to 94% or more). Alternatively, the procedure described by Mosesson and Sherry (15) was employed. Fibrinogen for chromatography from two affected daughters of the propositus was prepared from relatively small amounts (9–10 ml) of plasma by 2.1 M glycine reprecipitation of Cohn fraction I. The clottability of each sample was about 70%. Normal human fraction I-4 (more than 97% clottable) was prepared from pooled outdated ACD plasma (15, 16). Only material of 94% or more clottability was used for ultracentrifugal and other quantitative analytical procedures performed on propositus fibrinogen.

Clottability was determined by the spectrophotometric procedure of Laki (17) modified in that the clots were incubated at 2°–5°C for at least 18 hr before analysis.

Thrombin time determinations were carried out at 37°C by adding 0.05 ml of thrombin solution (approximately 5 U. S. Standard U/ml) in phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.0) to a 0.05-ml sample which had been dialyzed against phosphate-buffered saline. The thrombin was bovine thrombin (Parke, Davis & Co.) stored as a stock solution of 200 U. S. Standard U/ml in 50% glycerol at 5°C and diluted for use. End points were determined with the aid of a Nichrome wire loop. Timing of end points was not carried beyond 120 sec. For normal fibrinogen the earliest evidence of clot formation (i.e. a single small fibrin strand) was followed very closely by evidence of definite fibrin formation (i.e. firm clot or thick strand adhering to wire loop). In the case of fibrinogen Baltimore samples, particularly at the concentrations studied (vide infra), evidence of definite fibrin formation often lagged far behind the earliest evidence of clot formation. Because of the possibility that early evidence of clot formation indicated the presence of "normal" fibrinogen in fibrinogen Baltimore samples, whereas delayed formation of a firm fibrin clot reflected the presence of fibrinogen Baltimore itself, two end points were recorded for all samples analyzed in this manner (e.g. Table I).

Column chromatography on DEAE-cellulose was performed at 3°–5°C using a continuous concave Tris–phosphate salt and pH gradient from 0.005 M phosphate (pH 8.6) to 0.50 M phosphate (pH 4.1–4.3) as described for the chromatography of fibrinogen (19) modified for examination of small samples (20). Acrylamide-gel electrophoresis was carried out as previously outlined (20).

Eluted column fractions for thrombin time determinations were pooled, precipitated with 1/3 saturated ammonium sulfate, and redissolved in saline; further reduction in volume was accomplished by pervaporation before dialysis against phosphate-buffered saline, pH 7.0. Protein concentration of individual samples was estimated spectrophotometrically assuming $A^{1\%}_{1\ cm}$ at 280 $m\mu = 15$. Owing to the small amounts

TABLE I
Thrombin Times of Various Fibrinogen Fractions

Sample	First end point*	Second end point‡	No. of determinations	Protein concentration§
	sec, mean (range)	*sec, mean (range)*		*mg/ml*
Fibrinogen Baltimore				
Plasma	>120 (>120)	>120 (>120)	2	1.42 (fibrinogen‖)
I-2	12.7 (10–15.4)	>120 (>120)	2	0.85
Chromatographic peak 1 fractions:				
Ascending limb	18.9 (13–23)	26.1 (24.4–30.2)	4	0.61
Peak	25.9 (23.6–31.7)	52.9 (40–86)	4	0.57
Descending limb	>120 (>120)	>120 (>120)	2	0.68
Normal Fibrinogen				
Plasma	11.1 (10.6–12.0)	11.7 (10.6–12.7)	3	2.26 (fibrinogen‖)
I-2	11.1 (10.6–11.6)	12.1 (11.6–12.6)	2	0.69
Chromatographic peak 1	11.3 (10.0–12.7)	14.2 (14.0–14.4)	2	0.53

* i.e. first trace of fibrin (see Methods).
‡ i.e. definite fibrin formation (see Methods).
§ Before thrombin addition.
‖ Estimated by method of Ratnoff and Menzie (14).

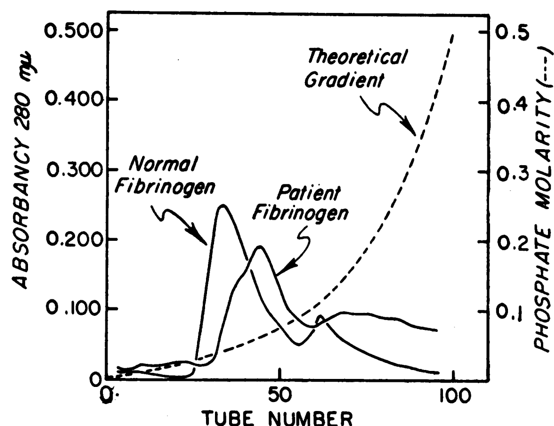

FIGURE 1 DEAE-cellulose gradient chromatographic pattern of 22 mg of fibrinogen Baltimore (I-2) compared with 28 mg of normal fibrinogen (I-4). Each was chromatographed separately under identical conditions. The molarity of the theoretical phosphate gradient is illustrated by the dashed line.

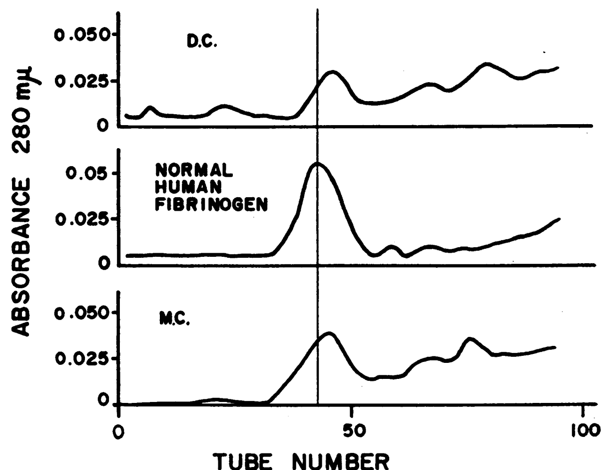

FIGURE 2 DEAE-cellulose gradient chromatographic patterns of partially purified fibrinogen from two affected daughters (D. C. and M. C.) of the propositus compared with normal fibrinogen (I-4). The total amount of protein chromatographed in each instance was: D. C., 3.5 mg; M. C., 3.5 mg; I-4, 2.9 mg. Clottability before chromatography was, respectively: D. C., 71%, M. C., 69%, I-4, 98%.

of material recovered in the precipitated column fractions the thrombin time assay was carried out within the range 0.53–0.87 mg/ml protein. Thrombin time determination of normal fibrinogen within this concentration range indicated that the end point was relatively independent of concentration (about 1 sec difference at the extremes of concentration).

Ultracentrifugation of purified, dialyzed fibrinogen fractions was performed in a Spinco Model E analytical ultracentrifuge using schlieren optics. Concentration was determined with a Brice-Phoenix differential refractometer, assuming a specific refractive increment of 0.188 ml/g at 546 mμ (21). Tyrosine/tryptophan ratios were calculated by the method of Bencze and Schmid (22).

Hexose analysis was performed by the orcinol method as described by Winzler (23) using a 1:1 galactose-mannose standard. Sialic acid content was determined by the thiobarbituric acid method (24) and calculated as *N*-acetyl neuraminic acid. *N*-terminal amino acid analysis was performed by the dinitrofluorobenzene (DNFB) method as described by Fraenkel-Conrat, Harris, and Levy (25). Thin-layer chromatography of dinitrophenyl (DNP) amino acids was performed on 20 × 20 cm glass plates coated with Silica Gel G[2] using the toluene: 2-chlorethanol: pyridine: 0.8 N ammonium hydroxide (30:18:9:18) solvent for the first dimension and chloroform: methanol: acetic acid (95:5:1) for the second (26).

RESULTS

Gradient chromatography of propositus fibrinogen (8 chromatograms of five preparations) on DEAE-cellulose (including one chromatogram each of propositus fraction I and I-1) demonstrated small but consistent differences between patient and normal fibrinogen (Fig. 1). As is the case with normal fibrinogen (19, 20) two major chromatographic peaks were discernible for propositus fibrinogen. The first chromatographic type of Baltimore fibrinogen was somewhat broader and more symmetrical and peaked later (mean = tube 42; range = tube 38–45) than its presumed normal counterpart.[3] The difference in the position of the peak from normal was statistically significant at $P < 0.001$. The onset of elution of the first peak of fibrinogen Baltimore samples tended to occur up to five tubes later than that of normal fibrinogen samples but this phenomenon was more variable than the position of the peak tube itself. In some preparations (three of five), as illustrated by Fig. 1, the ascending limb of peak 1 had a shoulder, suggesting that there were at least two components to the peak. The second peak was eluted in approximately the same position as peak 2 of normal fibrinogen but was usually broader and less well defined, making it difficult to determine its precise location. Chromatographic analyses of the partially purified fibrinogen of two affected daughters of the propositus demonstrated (Fig. 2) the increased anionic binding of peak 1 characteristic of the propositus' fibrinogen. However, the small amount of material studied did not permit any additional meaningful conclusions from these data.

To further explore the nature of the ascending limb of propositus peak 1, mixtures of propositus (P) and normal (N) fibrinogen were chromatographed (Fig. 3).

[2] Brinkmann Instruments Inc., Long Island, N. Y.

[3] Single donor fibrinogen prepared from plasma obtained from 21 normal individuals, two patients with hemophilia A, and two patients with von Willebrand's disease were used for comparison. Chromatograms of these 25 preparations of normal fibrinogen (8–36 mg) revealed the peak of the first chromatographic type between tubes 32 and 37 (mean = 34 ±2), and that of the second chromatographic type between tubes 53 and 65 (mean = 60 ±3).

The elution profile of propositus fibrinogen (100%, P) again demonstrated the increased anionic binding capacity characteristic of peak 1. The ascending limb of peak 1 in this instance appeared smooth, and peak 2 was well defined. The elution profile of a mixture consisting predominantly of propositus fibrinogen (87%, P; 13%, N) was somewhat different, most notable being the appearance of a small 'shoulder" on the ascending limb of peak 1. The elution profile of a mixture which was predominantly normal fibrinogen (7%, P; 93%, N) was virtually indistinguishable from the normal (100% N). A chromatogram of a mixture of approximately equal amounts of propositus and normal fibrinogen (55%, P; 45% N) displayed an elution profile with features of both fibrinogens, particularly in the appearance of peak 1 which was broader than either the normal or abnormal and which had a "shoulder" at the peak. Thus, these results demonstrated that peak 1 of normal fibrinogen could be distinguished in the presence of Baltimore fibrinogen and further suggested that the shoulder present on the ascending limb of peak 1 in certain fibrinogen Baltimore preparations might represent normal fibrinogen.

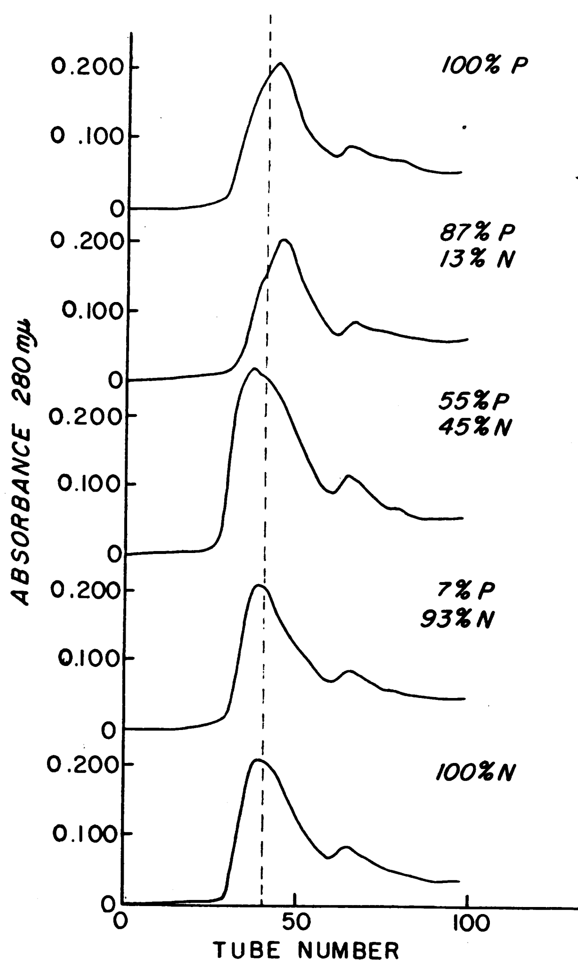

FIGURE 3 DEAE-cellulose gradient chromatographic patterns of various mixtures of propositus (P) and normal (N) fibrinogen. The percentage of "P" and "N" in the mixture is indicated at the right of each pattern. The total amount of material chromatographed in each instance was, from top to bottom: 19, 17, 22, 17, and 16 mg.

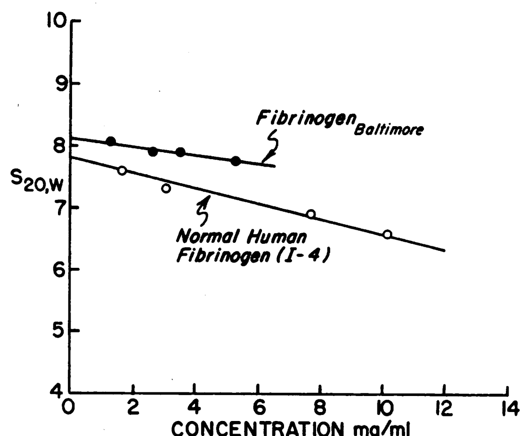

FIGURE 4 Sedimentation coefficient ($s_{20,w}$) vs. concentration (mg/ml). Propositus fibrinogen (I-2) was in 0.15 M NaCl, 0.01 M phosphate, pH 6.4 solution, whereas normal human fibrinogen (I-4) was in 0.29 M NaCl, 0.01 M phosphate, pH 6.4 solution.

To test whether the ascending limb of propositus fibrinogen was functionally as well as chromatographically normal, the following additional experiment was performed. Only enough patient material for a single experiment was available. The eluted peak 1 fractions from a single chromatogram of 18 mg of propositus fraction I-2 were pooled into three fractions: ascending limb, peak, and descending limb (Table I). (The elution profile in this instance did demonstrate a shoulder on the ascending limb of peak 1). A comparable amount of normal single donor fraction I-2 was carried through the same procedures.[4]

The results of thrombin time determinations on each of the concentrated, dialyzed fractions and related sam-

[4] There was insufficient fibrinogen recovered from the ascending limb of peak 1 to permit thrombin time determination of this fraction; therefore, peak 1 fractions were pooled (i.e. "chromatographic peak 1," Table I). However, in a subsequent chromatographic experiment on normal fraction I-2, sufficient material was recovered from pooled subfractions of peak 1 to allow comparative thrombin time determinations to be carried out. The mean (first end point) of replicate thrombin time determinations (protein concentration in parentheses) of starting fraction I-2 (0.73 mg/ml), ascending limb (0.54 mg/ml), peak (0.58 mg/ml), and descending limb (0.87 mg/ml) of chromatographic peak 1 were 12.2, 13.6, 13.6, and 12.3 sec, respectively. These values were not significantly different from one another nor were the second end points, which occurred within 1 sec of the first in every instance.

ples (Table I) indicated that the thrombin time of the fraction obtained from the ascending limb of propositus peak 1 was shorter than that of the peak or of the descending limb, and approached that of normal fibrinogen.

Analytical ultracentrifugation of the propositus' fibrinogen (94% clottable) demonstrated a single symmetrical peak sedimenting at a slightly higher $s^{\circ}_{20,w}$ (8.13 vs. 7.85 s) than that of normal fibrinogen (Fig. 4). There were differences in the concentration dependence ($s_{20,w}$ vs. concentration) of propositus fibrinogen (-0.65 c) compared with that of normal (-1.30 c).

Under appropriate conditions, human fibrinogen fractions of differing molecular weights can be distinguished by the molecular sieving properties of acrylamide gels (20). The patterns of propositus fibrinogen and normal fraction I-4 (mol wt = 325,000) electrophoresed at two gel concentrations (8 and 10%) were virtually indistinguishable.[5] In contrast, low molecular weight human fibrinogen (fraction I-8, mol wt = 270,000) was easily distinguished from fraction I-4 under these conditions; these observations supported the notion that the molecular weight of fibrinogen Baltimore does not differ significantly from that of classically prepared normal fibrinogen (i.e. fraction I-4).

The UV spectrum (245–360 mμ) of propositus fibrinogen in 5 M urea and 0.1 M NaOH was indistinguishable from normal. Further analysis of these curves (22) indicated that there were no significant differences in tyrosine/tryptophan ratios (1.34:1 vs. 1.32:1 for normal). Differences in the absorbancy coefficient ($A_{1\,cm}^{1\%}$) at 282 mμ in alkaline urea were marginal (16.2 vs. 16.7 for normal). There were no consistent significant differences in sialic acid and hexose content.

N-terminal amino acid analysis was undertaken on an amount of material sufficient only for qualitative analysis (approximately 8 mg). The results of this analysis demonstrated the presence of alanine and tyrosine, which are known to be the major amino terminals of normal human fibrinogen. Smaller amounts of other amino acids (e.g. aspartic acid) could not have been detected with the amount of material analyzed.

DISCUSSION

Ultracentrifugal studies indicated a small difference in the $s^{\circ}_{20,w}$ of propositus fibrinogen as compared with normal. The differences in the concentration dependence of the sedimentation coefficient might be partially explicable by the somewhat different ionic strength (see legend Fig. 4) of the solutions in which the two fibrinogens were studied (27). However, the difference was greater than could be accounted for on the basis of salt concentration alone; the possibility of a spatial conformational difference (28) was also raised by these data. A positive slope for fibrinogen prepared from certain patients with hemophilia A has been described and attributed to a tendency for molecular aggregation at higher concentrations (29).

Results of molecular sieving experiments in acrylamide gels suggested that the molecular weight of Baltimore fibrinogen was the same as that of normal fibrinogen (i.e. fraction I-4, mol wt 325,000) of similar solubility. Estimation[6] of the $D_{20,w}$ of propositus fibrinogen by the method of Allison and Humphrey (30) has demonstrated no difference from normal fraction I-4. Assuming that the partial specific volume of Baltimore fibrinogen is the same as that of normal fraction I-4, it can be estimated from the Svedberg equation that the molecular weight of Baltimore fibrinogen is no more than 4% greater than that of normal, a marginal difference.

The increased anionic binding demonstrable on DEAE-cellulose chromatography of fibrinogen Baltimore compared with fibrinogen isolated from normal donors or from donors with no apparent abnormality of fibrinogen (*vide supra*) was consistent with the difference in mobility suggested by previous comparative studies employing immunoelectrophoresis (3). No procedure or manipulation employed in preparation of the various fibrinogen Baltimore fractions examined chromatographically can account for the observed differences from normal; normal human fibrinogen fractions of varying purity (19), prepared by a variety of techniques (19, 20), and (or) of differing solubility (20), have been shown to display the same or very nearly the same chromatographic behavior with respect to the clottable protein. Together with the ultracentrifugal data the chromatographic data seem to provide firm support that the previously demonstrated functional defect (1–3) reflects some structural alteration in the fibrinogen molecule itself, the nature of which remains to be determined.

Family studies of fibrinogen Baltimore suggested an autosomal inheritance pattern for the gene, expressed in three successive generations. It would be expected that each of the affected persons carries a normal fibrinogen allele and it is reasonable to suspect that some normal fibrinogen is being synthesized in addition to fibrinogen Baltimore. Indeed, some of the chromatographic patterns suggested that the shoulder on the ascending limb of peak 1 might represent chromatographically normal fibrinogen (Fig. 1). Determination of the thrombin time of propositus fibrinogen from different portions of a chromatogram (Table I) supported this notion by demonstrating that the thrombin time of fibrinogen from the ascending limb of peak 1 approached that of normal fibrinogen. Furthermore, the observation that there were

[5] Performed by Dr. B. Sweet. Present address: Austin Hospital, Melbourne, Australia.

[6] Performed by Dr. N. Alkjaersig, Washington University School of Medicine, St. Louis, Mo.

apparently two more or less widely separate thrombin time end points for propositus fibrinogen samples (i.e. first evidence of clot formation and evidence of definite fibrin formation) compared with normal fibrinogen raised the possibility that the early end point might indicate the presence of "normal" fibrinogen present in propositus samples. From chromatographic mixing experiments (Fig. 3) it was apparent that normal and propositus peak 1 fibrinogen were distinguishable although not separable from one another. It could be estimated that the apparent content of "normal" fibrinogen present in the propositus preparation represented by Fig. 1 was approximately 15–25%. The usefulness of the chromatographic method in these studies as a convenient means of recognition and semiquantitation of normal fibrinogen in the presence of abnormal was obvious. This technique has also proven of value[7] in differentiating fibrinogen Paris (5) from normal. On the other hand, Sherman, Gaston, and Spivak (12) could demonstrate no chromatographic abnormalities in the fibrinogen of their patient.

The presence of mixtures of normal and abnormal fibrinogen have been suggested by immunoelectrophoretic studies in the case of fibrinogen Cleveland (9) and fibrinogen Detroit (8) and are consistent with the autosomal inheritance pattern postulated for both kindreds. Paradoxically, however, Blombäck et al. (13) in their sequence studies of fibrinogen Detroit found a total absence of the normal α(A)-peptide chain. With regard to the results of recent similar studies on fibrinogen Baltimore in which no abnormalities of the α(A)-chain have yet been found,[1] the possibility that the presence of contaminating amounts of normal fibrinogen might complicate the discovery of an abnormal peptide sequence should be considered.

ADDENDUM

Since this manuscript was accepted for publication von Felten, Frick, and Straub (31) have provided evidence, based mainly upon the study of polymerization of fibrin derived from reptilase-treated plasma, for the copresence of functionally normal and abnormal fibrinogen in the plasma of their patient (fibrinogen Zurich) (7). This finding is consistent with the autosomal inheritance pattern suggested from previous studies (7).

ACKNOWLEDGMENTS

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