THE EFFECTS OF AMNIOTIC FLUID ON THE DIRECT ACTIVATION OF THE FIBRINOLYTIC SYSTEM

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By

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I wish to dedicate this thesis to my wife, Sande, who has somehow weathered it all.

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INTRODUCTION

Recent emphasis has been placed upon ellucidating the relationship between amniotic fluid and its coagulant and fibrinolytic properties with respect to mammalian blood. Specifically, one main area of concern is the response of a maternal blood vascular system to infusions of amniotic fluid during pregnancy and parturition.

The fact that amniotic fluid possesses coagulant properties is well documented. In vitro studies (6, 31) indicate that amniotic fluid, when mixed with whole blood, shortens blood clotting times. In addition, infusions of amniotic fluid into the mammalian blood vascular system produce intravascular clotting, specifically in the pulmonary regions, and a lowered plasma fibrinogen content (14). It has been suggested (6, 7, 31) that amniotic fluid acts as a potent thromboplastic substance when introduced into the bloodstream. Two necessary factors qualify amniotic fluid as such a substance: first, amniotic fluid is a type of body tissue (15); second, this fluid is a particulate matter containing cells, cellular debris and organic and inorganic solids (15). It is well known that infusions of

body tissue substances or any particulate matter into the blood will produce coagulant activity (14).

Paradoxically, or seemingly so, the entrance of amniotic fluid into the mammalian blood vascular system may cause subsequent spontaneous bleeding or hemorrhagic conditions (7, 14). This type of action suggests that amniotic fluid has certain fibrinolytic properties (14). Conditions or substances which result in intravascular clotting (i.e., the conversion of fibrinogen to fibrin within the blood vessels) spontaneously activate the conversion of plasma plasminogen to plasmin, a substance which acts to dissolve any vascular clots. Since amniotic fluid is thromboplastic in nature and therefore can cause intravascular clot formation, it follows that amniotic fluid should spontaneously, though indirectly, produce fibrinolysis. In addition, amniotic fluid may possess significant quantities of plasminogen activators which effect the conversion of plasminogen to plasmin and produce fibrinolytic properties (1). Activators known to be specific for the conversion of plasminogen to plasmin include those of bacterial origin, staphylokinase and streptokinase, as well as urokinase, trypsin and a variety of tissue activators (1). Amniotic fluid has the potential for possessing from one to several of these activator substances (15). However, the total effect of these

activator substances, if present, in determining the fibrinolytic properties of amniotic fluid has not been demonstrated. Nor has the direct conversion of plasminogen to plasmin by activators present in amniotic fluid (disregarding thromboplastic effects) been demonstrated experimentally or quantitatively measured.

The research described in this paper involves the investigation, by means of two separate assay procedures, of the ability of amniotic fluid to initiate directly the conversion of plasminogen to plasmin. It is hoped that the results of this work will help clarify the nature of the fibrinolytic properties of amniotic fluid.

EXPERIMENTAL PROCEDURE

Two assay techniques were employed in this study, both of which were dependent on the conversion of plasminogen to plasmin by amniotic fluid and the measurement of the plasmin generated. Agreement between the results obtained by fluorometric and caseinolytic analyses was expected (4). Therefore, experimental results and conclusions could be verified by two separate assays.

One technique used was a highly sensitive fluorometric assay for plasmin (4). This method is based upon the enzymatic liberation of 2-napthol from the synthetic substrate α-N-methyl α-N-tosyl-L-lysine 2-napthol ester (MTLNE) by plasmin. The 2-naptholate ion emits a strong fluorescence at 420 nm (4). The fluorometric assay used in these experiments followed the procedures set down by Bell, et al., (4).

A second assay procedure is a modification of a casein assay procedure originally described by Remmert and Cohen as refined by Robbins and Summaria (24). This assay is based on the enzymatic liberation of trichloroacetic acid-soluble tyrosine from casein by plasmin and the subsequent measurement of absorbance at 280 nm.

Both the plasminogen and the amniotic fluid were obtained from rabbits and were kept frozen prior to use.

Reagents

Two-napthol was purchased from Eastman Kodak Company and was purified by sublimation prior to use in the preparation of standard curves. MTLNE and p-nitrophenyl-p'-guanidinobenzoate (NPGB) were purchased from Nutritional Biochemical Corporation. MTLNE was prepared daily to a final concentration of 2×10^{-4} M (reaction volume = 5 ml) in 10% dimethylformamide (DMF) and was filtered before use through Whatman No. 2 filter paper. NPGB was prepared daily in DMF to a final concentration of 3×10^{-5} M and was used without further treatment. Amniotic fluid from mature rabbits (Pel-Freez, Inc.) was pooled and kept frozen at -10° C until use. All amniotic fluid samples were free of blood contamination. Casein, purchased from Nutritional Biochemicals Corporation, was used in the caseinolytic assay. A 4% suspension of casein in 0.067 M phosphate buffer, pH 7.4, was prepared and clarified by centrifugation, and stored at 4° C until use. Trypsin, used for the measurement of plasminogen viability, was purchased from the Sigma Chemical Corporation.

Plasminogen Purification

Plasminogen was isolated from frozen rabbit plasma by affinity chromatography. This procedure is a modification of the method for plasminogen isolation of Sodetz, et al., (26), and Walther, et al., (28). The starting material was citrated (4% sodium citrate) whole rabbit blood. From this material, citrated plasma was obtained by refrigerated centrifugation at 4^{o} C at 3000 X g for 5 minutes. The plasma was frozen and stored at -10^{o} C.

The L-lysine coupled agarose gel column (Affi-Gel 10, Bio-rad Laboratories) was prepared by mixing 2 g of Affi-Gel 10 and 4 g of L-lysine with 40 ml of 0.1M sodium bicarbonate buffer, pH 8.5, at $3-5^{o}$ C. The resulting mixture was stirred gently for 2 hours at chromatography column (1.5 X 16 cm) and was washed overnight with 0.1M sodium phosphate buffer (approximately 600 ml), pH 7.4 at $3-5^{o}$ C until the effluent exhibited no further change in absorbance at 260 nm. After addition of the citrated plasma solution to the column, all subsequent chromatography procedures were performed at room temperature. While not in use, the column was stored at $3-5^{o}$ C.

The frozen rabbit plasma was thawed in an equal volume of 0.1M sodium phosphate buffer, pH 7.4, 0.001 M EDTA. The

resulting solution was placed over the L-lysine coupled agarose gel column, equilibrated, and washed with 0.3M sodium phosphate buffer, pH 8.2, until the eluate had a constant absorbance of 0.005 or less at 280 nm. The plasminogen was eluted with 0.1M sodium phosphate buffer, pH 7.4, containing 0.001 EDTA and 0.2M L-lysine. The plasminogen solution was then frozen at -10° C. The concentration of plasminogen obtained by this procedure was determined according to the method of Warburg and Christian (29) by reading the absorbances of the plasminogen at 260 and 280 nm with a Beckman DU-2 Ultraviolet Spectrophotometer.

Reliability of this affinity chromatography procedure for isolating plasminogen in a relatively pure form was determined by using sodium dodecyl sulfate (SDS) disk gel electrophoresis with 5% polyacrylamide-8M urea gels according to the method of Weber and Osborn (30). SDS electrophoresis was carried out for 30 minutes at 48 ma, 42 mv and for 3 1/2 hours at 96 ma, 80 mv. Gels were stained with Coomassie Brilliant blue (454 ml of 50% ethanol, 46 ml of glacial acetic acid) for 3 hours and were destained in 25 ml 10% acetic acid, 10% methanol solution, 80% distilled water (changed once daily), for three days. The length of the gel, the distance moved by each dye marker and the color intensity of each band were measured.

Three standard solutions, bovine serum albumin (molecular weight 68,000), glyceraldehyde-3-phosphate (molecular weight 36,000), and cytochrome C (molecular weight 11,700) were included as standards in the electrophoresis for reference points. The molecular weight of the plasminogen was obtained according to the method indicated by Weber and Osborn (30).

Fluorometric Assay

A Beckman DU-2 Ultraviolet Spectrophotometer with a fluorometric attachment (catalog no. 73500) was used for all fluorometric measurements. The light source used was a mercury phosphor lamp which emitted light in the range of 300-400 nm.

To each sample tube was added 60 µg (0.2 ml) rabbit plasminogen. Rabbit amniotic fluid was added in varying amounts to each tube and was followed by the addition of 0.5 ml MTLNE and 0.5 ml NPGB. MTLNE final concentration was 2×10^{-4} M; NPGB concentration was 3×10^{-5} M. Each sample tube was adjusted to a final volume of 5 ml by the addition of 0.05 M sodium phosphate buffer, pH 7.0.

The plasminogen and amniotic fluid were allowed to incubate for 10 minutes at 25° C. At the end of this incubation period, the substrate was added. Any plasmin produced by activation

of plasminogen hydrolyzes MTLNE with a subsequent release of 2-napthol (4). Thus, any turnover of MTLNE by plasmin could be measured by the fluorescence of 2-napthol at 420 nm (4). Because MTLNE has an inhibitory effect on tryptic-like enzymes (4), addition of the substrate effectively inhibits any subsequent plasminogen to plasmin conversion during the remainder of the assay. NPGB was then added to each sample tube. NPGB, when added to plasmin, acylates the enzyme site rapidly and irreversibly (4), thus stopping any further action by plasmin on the substrate MTLNE. Final DMF concentration per sample was approximately 8%. A zero-time blank standard for reference purposes was determined for each assay.

After completion of the reactions, each sample was transferred to a Vycor tube which was placed in the fluorometric attachment of the Beckman DU-2 Ultraviolet Spectrophotometer for fluorescent measurement of the 2-naptholate ion. Per cent transmittance was recorded for each sample. Per cent transmittance was compared to a standard fluorometric curve for 2-napthol, run in accordance with each sample measured, and 2-napthol concentration was determined for each sample.

Plasminogen activation was determined also by the fluorometric assay procedures, substituting trypsin in varying

quantities for the amniotic fluid. With the exception of substituting trypsin for anmiotic fluid, the fluorometric assay procedure was identical to the method described previously. Since trypsin has the potential for converting plasminogen to plasmin (as measured fluorometrically by the release of 2-napthol), this assay was used to offer evidence for a starting zymogen capable of being converted to plasmin.

Casein Assay

Cold plasminogen solutions (stored at 4° C) were pipetted into test tubes situated in a 4° C water bath. Each sample tube contained 0.2 ml plasminogen (60 µg). The activator, rabbit amniotic fluid, was added to each sample tube in varying amounts and the volume was adjusted to 2.0 ml with 0.067 M sodium phosphate buffer, pH 7.4. Addition of 2 ml cold (4° C) casein solution per sample tube followed and the resulting solutions per sample tube followed and the resulting solutions were mixed thoroughly. After mixing, the tubes were transferred to a 37° C water bath and remained there for 30 minutes. At the end of this incubation period, the samples were again placed in the 4° C water bath and 6.0 ml of 15% trichloroacetic acid were added to each tube. The samples remained in the 4° C

water bath for 18 hours, after which they were allowed to warm up to room temperature. The precipitate which formed was removed by filtration through Whatman No. 2 filter paper. The absorbance of each solution was read at 280 nm using the Beckman DU-2 Ultraviolet Spectrophotometer.

The plasmin proteolytic unit was calculated as the amount of enzyme which liberated 450 μg of trichloroacetic acid soluble tyrosine in one hour (24). This proteolytic unit, measured in Casein Units, was calculated as follows: (24)

$$1 \text{ Casein Unit per 1 ml} = \frac{\text{Absorbance at 280 nm}}{\text{Sample Volume}} \times 6.67.$$

As with the fluorometric assay, a zero-time blank for reference purposes was prepared in conjunction with the sample assays.

Statistical Analysis

After collection of data, standard deviations were compiled for both the fluorometric and caseinolytic assays. A standard T test was used to determine any significant statistical differences. All testing for statistical significance was at the 0.01 α-level (11).

RESULTS

Plasminogen Concentration

The absorbance at 280 nm of the plasminogen isolated by affinity chromatography procedures was 0.04/ml plasminogen (Table I). This value corresponds to a plasminogen concentration of 0.34 mg protein/ml (29) using an extinction coefficient ($E_{280}^{1\%}$) of 17 (28). Total plasminogen obtained by this procedure was 5.12 mg. The plasminogen sample was adjusted to a final volume of 17.05 ml, to yield a plasminogen concentration of 0.300 mg protein/ml, making measurement of protein samples used in each assay more precise and convenient.

Plasminogen Molecular Weight Determination

The molecular weight value for the isolated plasminogen was determined by SDS disk gel electrophoresis in Table II and Figure I to be approximately 87,000. Results indicate that affinity chromatography procedures used in preparing the plasminogen for this study are reliable for producing adequately purified plasminogen for the following three reasons:

(1) a single band present on the sample plasminogen gel after electrophoresis indicated isolation of a single protein component;

TABLE I. PLASMINOGEN CONCENTRATION

SUBSTANCE	A 280	A 260	RATIO	FACTOR	EXTINCTION COEFFICIENT	PROTEIN CONC. mg/ml	TOTAL VOLUME ml	TOTAL PROTEIN mg
Plasmin-ogen	0.58	0.40	1.45	1.0	17.0	0.34	15.0	5.12

TABLE II. SUMMARY OF DATA OBTAINED BY SDS GEL ELECTROPHORESIS

SUBSTANCE	LENGTH OF GEL (cm)	DISTANCE MOVED BY DYE MARKER (cm)	DISTANCE MOVED BY EACH BAND (cm)
Plasminogen	10.8	10.3	3.6 (one band)
Plasminogen	10.8	9.3	3.5 (one band)
Plasminogen Free Plasma	11.2	9.0	.4, .7, .95, 1.2, 1.6, 1.9, 2.3, 2.7, 3.7, 4.5, 5.2, 5.9, 6.5, 7.2, 7.5, 7.9 (twelve bands)
Glyceralde-hyde-3-Phos-phate	10.9	9.4	6.8 (one band)
Bovine Serum Albumin	10.9	9.4	4.7 (one band)
Cytochrome C	10.9	9.4	9.5 (one band)

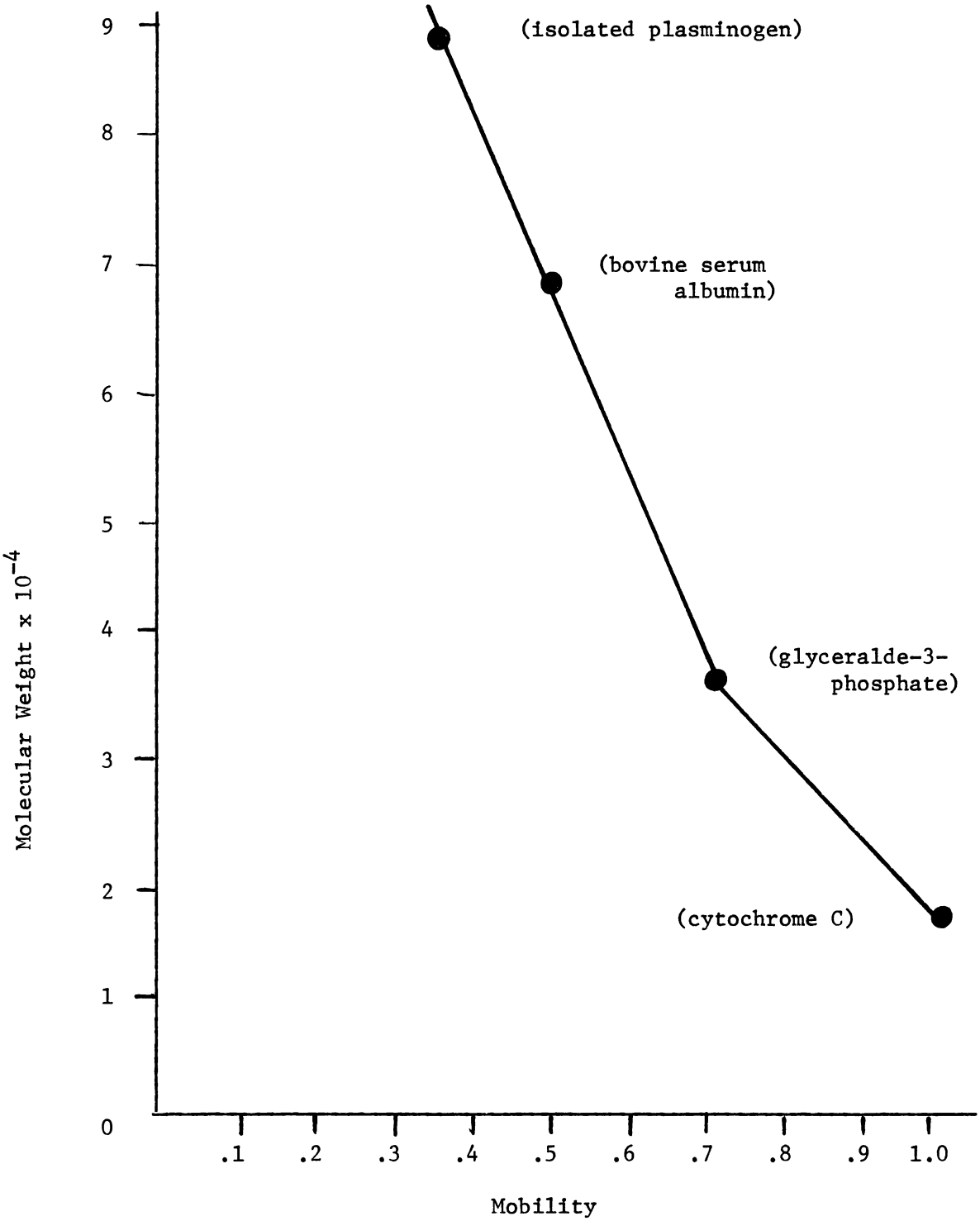

FIGURE 1. PLASMINOGEN MOLECULAR WEIGHT

(2) corresponding gels run with plasma (minus plasminogen) eluted from the column showed a complete absence of the plasminogen band found on the sample gel containing only plasminogen, suggesting effective and entire removal of plasminogen from raw citrated plasma;

(3) molecular weight values for the isolated plasminogen correspond to the expected molecular weight values for that particular zymogen (28).

Plasminogen Viability

The ability of the isolated plasminogen to be activated enzymatically was verified by fluorometric procedures using trypsin as the activator substance. Data for this assay is summarized in Table III. Corresponding 2-napthol concentrations were determined by comparing per cent transmittance readings to the standard curve illustrated in Figure 2.

Each sample containing trypsin as the activator exhibited a marked increase in per cent transmittance (thus 2-napthol production) as compared to a zero-time blank (see Table III). Increased concentrations of trypsin produced increased per cent transmittance and increased 2-napthol production. The maximum per cent transmittance produced represented 46.3 μM of 2-napthol per 10 minute assay.

TABLE III. SUMMARY OF FLUOROMETRIC ASSAY DATA UTILIZING TRYPSIN ACTIVATOR

TUBE NO.	TRYPSIN CONC. (μg)	CORRECTED PER CENT TRANSMIT-TANCE	CORRECTED 2-NAPTHOL CONC. X 10^{-5} (M)	AMOUNT 2-NAPTHOL RELEASED PER MINUTE (μM)
1	10	8.4	1.80	1.80
2	20	12.5	2.58	2.58
3	30	19.9	3.80	3.80
4	40	24.4	4.50	4.50
5	50	25.0	4.58	4.58
6	100	25.8	4.63	4.63

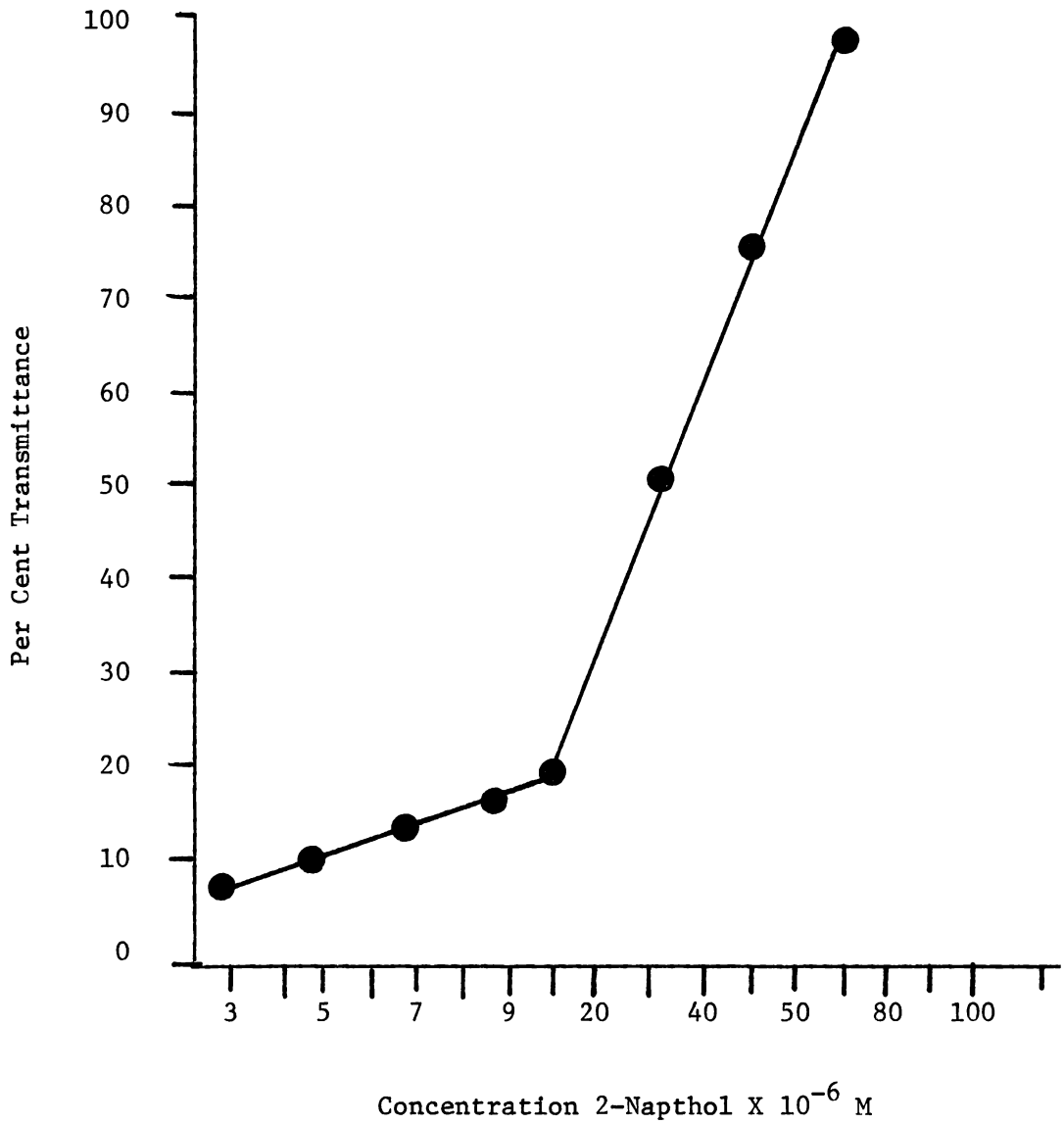

FIGURE 2. 2-NAPTHOL STANDARD CURVE FOR TRYPSIN ASSAY

Fluorometric Assay: Amniotic Fluid

Data for the fluorometric measurement of samples incubated with varying concentrations of amniotic fluid is summarized in Table IV. The 2-napthol standard curve used in conjunction with the assay using amniotic fluid as the activator is shown in Figure 3. With the possible exception of Tube 1, each sample containing amniotic fluid exhibited a slight increase in per cent transmittance and 2-napthol liberation as compared to a zero-time blank. Samples using 1.5 ml of amniotic fluid (Tubes 3 & 9) produced the highest amount of activity, a net produced the highest amount of activity, a net production of 1.9 μM of 2-napthol per 10 minute assay.

Statistical analysis of per cent transmittance readings indicated that only 3 of the 12 sample tubes produced statistically significant increases in transmittance. Tubes 3 and 9 (both containing 1.5 ml amniotic fluid) and Tube 8 (containing 1.0 ml amniotic fluid) produced the only statistically significant increases in per cent transmittance, i.e., plasminogen activation.

Casein Assay: Amniotic Fluid

Data summarizing possible activation of plasminogen by

TABLE IV. SUMMARY OF FLUOROMETRIC ASSAY DATA UTILIZING AMNIOTIC FLUID

TUBE NO.	AMNIOTIC FLUID CONC. (ml)	CORRECTED PER CENT TRANSMIT-TANCE	CORRECTED 2-NAPTHOL CONC.x 10^{-5} (M)	AMOUNT 2-NAPTHOL RELEASED PER MINUTE (μM)
1	0.5	-1.9	-	-
2	1.0	3.5	0.06	0.06
3	1.5	7.5	0.19	0.19
4	2.0	4.5	0.09	0.09
5	2.5	2.6	0.06	0.06
6	3.0	2.1	0.06	0.06
7	0.5	3.9	0.10	0.10
8	1.0	6.1	0.15	0.15
9	1.5	7.5	0.19	0.19
10	2.0	5.2	0.12	0.12
11	2.5	2.5	0.06	0.06
12	3.0	1.8	0.05	0.05

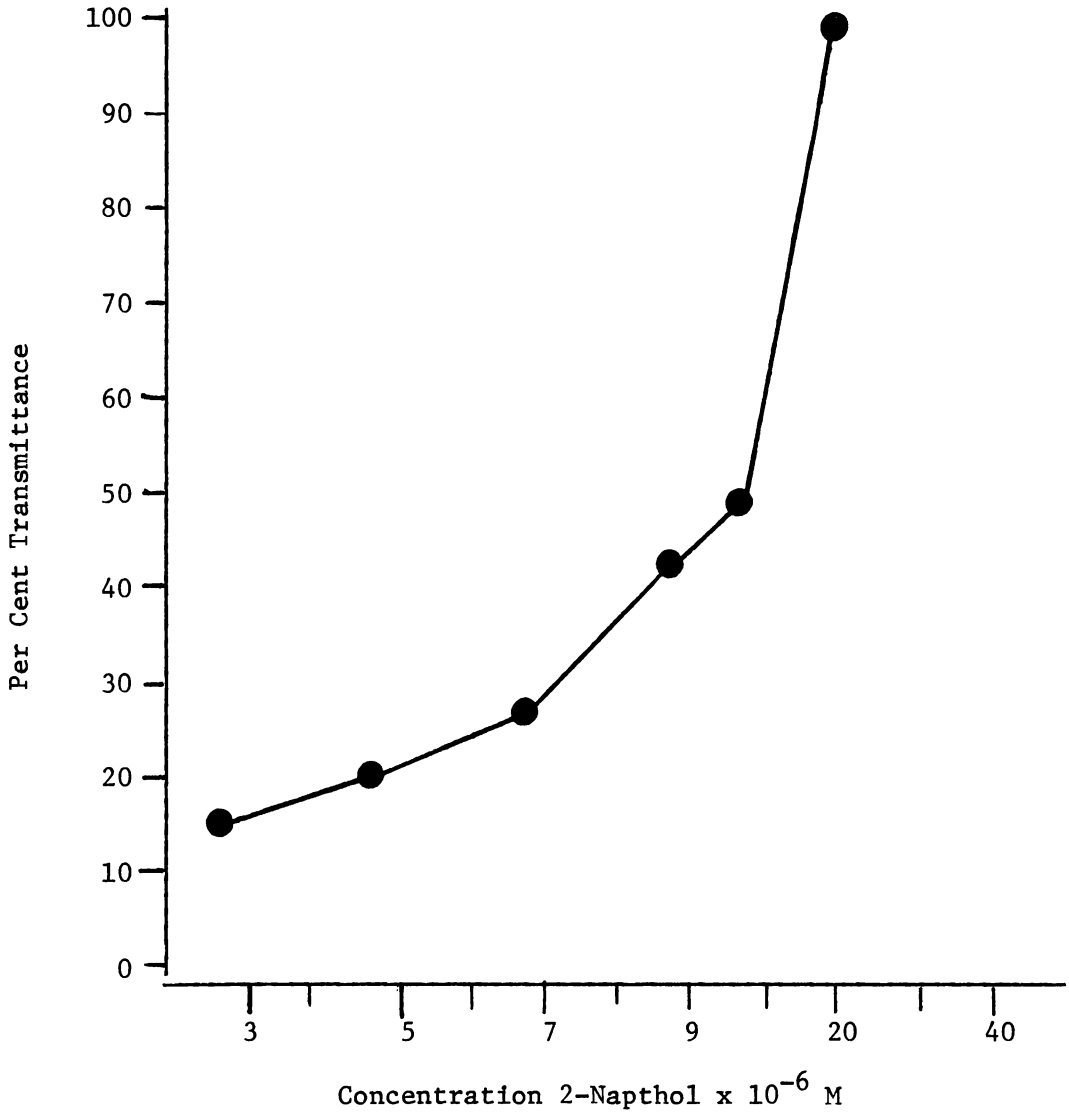

FIGURE 3. 2-NAPTHOL STANDARD CURVE FOR AMNIOTIC FLUID ASSAY

varying concentrations of amniotic fluid is shown in Table V. Statistical analysis performed on the changes in absorbances indicated no significant changes in absorbances for any of the sample tubes; i.e., no significant activation could be detected. The increase in absorbance observed can be explained by the additional amounts of protein inherently contained in the amniotic fluid added in increasing amounts to each sample.

TABLE V. SUMMARY OF CASEIN ASSAY DATA

TUBE NO.	AMNIOTIC FLUID CONTENT (ml)	CORRECTED A_{280}	CASEIN UNITS / ML
1	0.5	0.03	0.02
2	1.0	0.10	0.06
3	1.5	0.15	0.10
4	2.0	0.19	0.12

DISCUSSION

Amniotic fluid contains plasminogen activator substances as well as inhibitors of these activators (27). Specifically, the ratio of these activator substances to inhibitors is 1:13.9, suggesting that amniotic fluid has the ability to activate plasminogen by converting it to plasmin. This ability is masked to a certain extent by inhibitory action (27). Data obtained by the fluorometric assay techniques in this study apparently implied to a certain extent this activation-inhibition property of amniotic fluid.

Slight activation of plasminogen was made evident by the addition of small amounts of amniotic fluid per sample. As the level of amniotic fluid increased (up to a level of 1.5 ml), activation response increased. Peak or maximum activation response (as measured by 2-napthol liberation) was observed with samples containing 1.5 ml amniotic fluid. This maximum response, however, corresponded to a 2-napthol production of only 0.19 μM of 2-napthol per minute, a small amount of activation as compared to trypsin activated plasminogen. Maximum activation of plasminogen by amniotic fluid produced only 10.5% of the plasmin activity produced by the minimum amount

of trypsin used in the assay procedures described and only 4.1% of the activity by 100 μg of trypsin, the maximum amount of trypsin used in the assay.

Addition of amniotic fluid (in excess of 1.5 ml per sample) decreased the amount of plasmin activation. These results suggest a proportional increase in the inhibitory effect of amniotic fluid on any activator substances as the amount of amniotic fluid increases.

Results from the casein assay indicate similar results, though the ability of amniotic fluid to activate plasminogen in this instance is not as clearly evident. Increased amounts of amniotic fluid per casein assay produced an increase in casein units per 1 ml; however, this increase amounted to only a small change in activation response, and none of these changes was statistically significant. Addition of increasing amounts of amniotic fluid should, by the nature of the fluid itself, produce an increase in absorbance readings at 280 nm, regardless of any changes produced by any activation of plasminogen by activators within the fluid.

In reality, then, it remains to be indisputably proven that amniotic fluid does actually have activation effects upon plasminogen. In fact, the small increases in 2-napthol response

exhibited by the results of the fluorometric assay could possibly be a result of experimental technique, involving an unidentified effect on the MTLNE substrate by one or more undefined factors. Perhaps some of these additional factors are contained within the amniotic fluid itself. Casein assay response to increases in amounts of amniotic fluid leave little doubt that any significant activation effects on plasminogen by amniotic fluid occur. Moreover, the results demonstrated by the two assays clearly imply that upon infusion of amniotic fluid into a maternal blood vascular system, the production of plasmin, due to direct activation or conversion of plasminogen by activator substances within the fluid, should be minimal, if not insignificant. Assuming this, hemorrhagic conditions experienced upon amniotic fluid's infusion into a blood vascular system then would be due almost entirely to the activation of plasminogen by the presence of an intravascular fibrin clot. This possibility remains to be tested directly. Further investigation involving <u>in vivo</u> studies comparing the amount of plasmin produced indirectly by amniotic fluid's thromboplastic properties versus any plasmin produced by direct activation is suggested.

SUMMARY

The ability of amniotic fluid to catalyze directly the conversion of plasminogen to plasmin was measured by two separate *in vitro* assays. Results from fluorometric and caseinolytic assays indicate little or no direct activation of plasminogen by amniotic fluid. This implies that hemorrhagic conditions occuring *in vivo*, due to amniotic fluid infusions, are caused by indirect activation of plasminogen by thromboplastic substances within the fluid itself.

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