Factor IX and Prothrombin in Amniotic Fluid and Fetal Plasma: Constraints on Prenatal Diagnosis of Hemophilia B and Evidence of Proteolysis

By Arthur R. Thompson

Potential limitations of prenatal diagnosis of hemophilia B, as compared to hemophilia A, include (1) occurrence of far more frequent defects with abnormal circulating antigen, (2) lower levels of factor IX in fetal plasma at 16 to 20 weeks gestation, and (3) the presence of factor IX antigen in amniotic fluid. In addition, proteolysis could occur, especially with amniotic fluid contamination of fetal plasma. A sensitive polyclonal immunoradiometric assay for factor IX antigen was used to characterize the range of levels in amniotic fluids and fetal plasma samples. To assess for altered forms, factor IX species were compared to those of a homologous clotting factor, prothrombin. Fourteen postmortem abortus blood samples from fetuses of 14 to 23 weeks gestation had factor IX antigen levels that averaged 5.1 U/dL and ranged from 1.7 to 15 U/dL. Amniotic fluid factor IX antigen averaged 2.9 U/dL, with a range from 1.4 to 8.5 U/dL in 19 separate amniocentesis samples. Thus, in a male fetus at risk of hemophilia B and with a low circulating level of gene product, mixture of fetal plasma with amniotic fluid could severely limit prenatal diagnosis, assuming that the amniotic fluid factor IX is of maternal origin. Despite rapid processing of amniotic fluid samples, the prothrombin was extensively cleaved, suggesting that it had been activated in vivo. On gel electrophoresis of amniotic fluid samples, however, factor IX was only minimally cleaved. In the postmortem fetal blood specimens, prothrombin was partially cleaved. On crossed-immuno- electrophoresis, fetal plasma prothrombin showed decreased migration in calcium, compared to EDTA, indicative of mature γ-glutamyl carboxylation. The latter presumably resulted from fetal hepatic synthesis.

Accurate Prenatal Diagnosis of hemophilia A has been an important clinical outgrowth of factor VIII coagulant antigen assays. Although several methods have been established to examine factor IX antigen and much more is known about the structure of factor IX as compared to factor VIII, prenatal diagnosis of hemophilia B has been limited by three major differences between the two types of hemophilia. First, over half of the hemophilia B patient pedigrees (including only those with clinically severe disease) in one series had at least 10 U/dL circulating abnormal factor IX antigen, whereas the vast majority of hemophilia A patients lack factor VIII coagulant antigen. Secondly, the level of factor IX antigen, as shown in midtrimester fetal blood samples from abortus specimens or from fetoscopy, is considerably lower than the factor VIII coagulant antigen at that stage of development. Finally, factor VIII coagulant antigen is undetectable in amniotic fluid, whereas factor IX is readily detected by sensitive immunooassays. Upon obtaining fetal specimens, gross contamination of blood with amniotic fluid occurs with the simpler technique of vessel puncturing, as opposed to withdrawing a blood sample from a cannulated fetal vessel. The latter approach has been successful in at least one series, although gross levels of amniotic fluid contamination frequently occurred in other series and trace contamination would be difficult to avoid.

The problem of amniotic fluid interference in prenatal diagnosis of hemophilia B is potentially greater than that of the mere presence of factor IX antigen. Amniotic fluid is a complex mixture of proteins and lipids that is capable of activating coagulation factors, especially through the extrinsic system. This presumably accounts for the fact that apparent coagulant activities of even fetoscopy-obtained fetal plasma samples are in from twofold to fourfold excess over simultaneously determined factor IX antigen levels.

The degree to which these limitations apply to the prenatal diagnosis of hemophilia B has been investigated in two ways. Factor IX antigen levels were determined by sensitive immunoradiometric assays, in both fetal blood and amniotic fluid samples, to explore the range of values observed. In addition, factor IX and a homologous vitamin K-dependent clotting factor, prothrombin, were studied by crossed-immuno-electrophoresis and polyacrylamide gel electrophoresis, followed by immunologic detection to screen for altered species of these proteins.

Materials and Methods

Preparations and Materials

The following proteins and antibodies were prepared by methods previously described, as indicated: isolated human factor IX and rabbit anti-factor IX serum, purified polyclonal goat and mono-

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clonal mouse anti-human factor IX antibodies, human prothrombin and rabbit antiprothrombin serum,\textsuperscript{11} and human thrombin.\textsuperscript{12} Staphylococcal protein A was from Pharmacia, Piscataway, NJ; sodium \[^{125}\text{I}]\text{iodide was from New England Nuclear, Boston, MA; and lactoperoxidase-glucose oxidase reagent (Enzymobeads) was from Bio-Rad, Richmond, CA. Electrophoretic materials and chemicals used were as previously described.\textsuperscript{9,13,14}

Fetal plasma samples were provided by Dr Thomas Shepard, University of Washington, as previously described.\textsuperscript{5} In brief, they were obtained by direct cardiac puncture, heparinized, and centrifuged; however, up to several hours had elapsed from the time of the abortion to the sampling. Gestational ages were determined by size criteria, as previously presented.\textsuperscript{5} Blood from one fetus was only available from the plural space at necropsy and was grossly hemo
dyzed. An additional fetus had blood drawn both by cardiac puncture and as a hemolyzed specimen from the plural space for comparison. Five of the samples were from spontaneous abortions without grossly detectable abnormalities in three (one of which had been exposed to excessive phenytoin ingestion during the first trimester) and tetralogy of Fallot and osteogenesis imperfecta, respectively, in the other two; the other nine were from progestaglandin-induced abortions, with Down’s syndrome in three, anencephaly in two, trisomy 18 in another, mosaic Turner’s syndrome in a seventh, a neural tube defect in an eighth, and no detectable defect in the ninth.

Amniotic fluid samples were obtained from consenting women undergoing diagnostic, midtrimester amniocentesis by the University’s Department of Obstetrics and following a protocol approved by the University’s Human Subjects Review Committee. Gestational ages were based on clinical estimation of fetal size by Doppler ultrasound assessment. Approximately 1 mL of the fluid was transferred from the syringe to a polypropylene tube containing 10 μL 0.15 mol/L NaCl with 10 U heparin within five to ten minutes after sampling; after storage for up to two hours on ice, samples were centrifuged at 40,000 g for 20 minutes to remove most of the cellular elements prior to freezing. Samples were then frozen at \(-80 \degree C \) and thawed prior to assay. For the initial three samples obtained, heparin was not added for two hours, and the samples were not centrifuged or frozen for 24 hours; results from these three samples were treated as a pilot study and were separate from results from the subsequent 19 samples. For electrophoreses, samples of up to 500 μL were concentrated tenfold in Amicon concentrators (Minicon A-25, Lexington, Mass), where indicated.

Because volumes of samples varied, as did the amount of dilution with anticoagulant, samples were assumed to be comparable to the citrated normal plasma pool. This assumption led to an overestimation of the amounts of any protein by up to 9% in some of the sample. Similarly, the number of tests that could be run on any given sample was limited by sample size, although two sets of determinations were run for immunoradiometric assays and for most of the other results reported.

Antithrombin III levels were determined by Dr Gottfried Schmer (Laboratory Medicine, University of Washington) by a fluorometric (Protopath) method (Dade, Miami, Fla), according to manufacturer’s directions, on four amniotic fluid samples (AF-4, 13, 15, 18) using a tenfold increase in sample volume (50 μL) and less buffer for the same final volume. Values of functional antithrombin III (corrected for volume) were 4%, 8%, 7%, and 4% of normal adult levels, respectively.

Methods

Immunoassays for factor IX antigen were performed as previously described.\textsuperscript{5} In brief, dilutions of goat antibody were adsorbed at pH 9.5 to polystyrene microtiter wells, which were washed with albumin-containing buffers. Samples were diluted in phosphate-buffered saline with albumin, and duplicate 100-μL aliquots of each dilution were incubated overnight in the antibody-coated wells. After washing with the same buffer used for dilutions, \[^{125}\text{I}]\text{-anti-human factor IX (around 20,000 cpm and 2 μg/dL) was incubated for six hours at 4 \degree C \) and, after a final washing, the wells were counted. An immunoelectrophoretic procedure was used to assess prothrombin antigen levels, and rabbit antiprothrombin serum (50 μL in 3 mL agarose) was used for the second dimension of crossed-immunelectrophoresis.\textsuperscript{11} Prothrombin \(^{11}\) was radiolabeled by the chloramine T method\textsuperscript{5} and contained 100 cpm/μg; 90% of the radioactivity was confined to the prothrombin migration peak position on electrophoresis in polyacrylamide gels with sodium dodecyl sulfate. For crossed-immunelectrophoresis of human factor IX, the purified labeled goat antibody alone was limited in its ability to form precipitin arcs with dilute solutions of factor IX. Therefore, 10 μL rabbit anti-factor IX serum\textsuperscript{9} was added with 3 × 10\(^{10}\) cpm of \[^{125}\text{I}]\text{-anti-factor IX to the 3 mL agarose for the second dimension; electrophoresis was otherwise run as previously described.\textsuperscript{121} Electro
radiophoretic blotting to nitrocellulose was as previously described,\textsuperscript{4} except that the blotted strips from up to five lanes were incubated in either \[^{125}\text{I}\text{-anti-factor IX (100 μg/cass/10 mL) or with rabbit antiprothrombin sera followed by \[^{125}\text{I}\text{-Staphylococcal protein A (2 × 10\(^{10}\) cpm/10 mL). The latter was prepared by radiolabeling with lactoperoxidase reagent according to the manufacturer’s direction, with a 20-minute incubation of 10 μg protein in 1 mCi of sodium \[^{125}\text{I}\text{iodide. Radioautography of the blotted gel or dried agarose plates, the latter from the crossed-immunelectrophoresis studies, was also as described.\textsuperscript{9}}

![Fig 1. Standard curve, immunoradiometric assay. A typical plot of log factor IX antigen concentration vs dilution (one to 20 through one to 1200) of normal plasma (circles) superimposed upon the curve of dilutions of isolated factor IX (squares), log of percent \[^{125}\text{I}\text{-anti-factor IX bound is shown. With subsequent preparations of \[^{125}\text{I}\text{-anti-factor IX of higher specific activity, a 10,000-fold dilution of normal plasma (0.5 ng/mL factor IX) was routinely fit to the curve, giving 3% to 4% bound (not shown).}](image-url)
the plasma from patients with hemophilia B; sensitivity to dilutions of normal plasma was comparable (Fig 1). For goat anti-factor IX preparations of higher specific radioactivity, sensitivity was tenfold greater. Coefficients of variation for duplicate determinations were less than 10%; nonspecific binding was less than 1%.

Factor IX antigen levels in ten samples from fetal plasmas averaged 5.1 U/dL, with a range from 1.7 to 15 U/dL (Table 1) over a gestational age range from 14 to 23 weeks. Within this series, gestational age or size and factor IX were not significantly correlated ($r = .32$ and $r = .43$, respectively). When these data were combined with previously published antigen levels from six fetal plasmas, the gestational age range was extended to 29 weeks and there was significant positive correlation between factor IX antigen and age ($r = .70, P < .01$). It should be noted that essentially the same results were obtained from the radioimmunoassays with rabbit antisera and the current immunoradiometric assay with goat anti-factor IX ($r = .93$ for 40 samples from different pedigrees with hemophilia B).

The initial three amniotic fluid samples had factor IX antigen levels of 1.6, 2.2, and 2.6 U/dL. For the subsequent 19 amniotic fluid samples, which were more rapidly processed, factor IX antigen averaged 2.9 U/dL, with a range from 1.4 to 8.5 U/dL (Table 1). The distribution of these results was wide, and there was no correlation with gestational age ($r = -.16$) over the narrow age range sampled (15 to 20 weeks). Although four of the 19 samples were contaminated with a visible trace of red blood cells, three of these were within 1 SD of the mean, but the fourth had the highest level of any.

It was possible that a trace plasma protein other than factor IX was recognized by a minor component in the purified polyclonal antibody preparation, especially if it were of a disproportionately high level in fetal fluids. To exclude such a spurious influence on the factor IX antigen level results, samples were selected for comparison of factor IX antigen levels by a monoclonal immunoradiometric assay with those from the polyclonal antibody system. Four fetal plasmas (FP-7, 8, 13, and 14; Table 1) and three amniotic fluids (AF-3, 4, 15) were simultaneously run in both assay systems, and the factor IX antigen levels by the two methods were in excellent agreement ($r = .96; P < .01$).

**Immunoelectrophoretic Studies**

*Fetal plasma prothrombin.* On crossed-immunoelectrophoresis against rabbit antiprothrombin, samples from the 14 fetal plasmas (Table 1) and from plasmas D, E, and F (Table 2)$^5$ all gave peaks that had identical migration to that of normal plasma. When EDTA was added in the first dimension, migration was more anodal (prealbumin) than when calcium was used, again as with normal plasma prothrombin. Six of the 13 samples showed an additional, more anodally migrating species present when run in either EDTA or calcium, and in three of these (FP-2, 8, and 13), the more rapidly migrating species gave a peak that was at least as high as that corresponding to the normally migrating antigen. The more rapidly migrating peak

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**Table 1. Factor IX Antigen Levels in Fetal Plasma and Amniotic Fluid Samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gestational Age (Weeks)</th>
<th>Factor IX Antigen (U/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal plasmas</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP-1</td>
<td>14.0</td>
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</tr>
<tr>
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</tr>
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<td><strong>Mean ± SD</strong></td>
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</tr>
<tr>
<td>AF-19</td>
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<td>2.9</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>16.7 ± 1.3</td>
<td>2.9 ± 1.8</td>
</tr>
</tbody>
</table>

*Gestational ages were determined by direct fetal measurements on abortus specimens (crown–rump and foot lengths; weight) or by estimated size from clinical Doppler ultrasound examination prior to amniocentesis.

*Unclosed, hemolyzed blood from thoracic cavity.

†The same as footnote , except an additional, unhemolyzed sample was also obtained by cardiac puncture.

‡Reddish cast was present in sample and RBCs were visibly present in pellet after centrifugation.
corresponded in migration to a major peak observed in glass-clotted normal human serum.

**Amniotic fluid prothrombin.** Quantitative immunoelectrophoresis\(^{11}\) of seven of the amniotic fluids showed prothrombin antigens present in twofold to fourfold greater concentration (relative to normal adult levels) than the corresponding factor IX antigen level ratios. In terms of protein concentrations, these reflect at least a 50-fold greater concentration of prothrombin compared to factor IX. Prothrombin was further characterized on crossed-immunoelectrophoresis for all 22 amniotic fluid samples (the 19 in Table I and the three that were processed more slowly). Although the prothrombin concentration from the peak height was semiquantitative, it again clearly showed an excess of prothrombin antigen ratio (amniotic fluid to normal plasma) over the corresponding ratios of factor IX antigen levels in all cases.

For crossed-immunoelectrophoresis, prothrombin antigen consistently had faster migration than that of normal plasma. The \(R_f\) value for samples run in EDTA averaged 1.25-fold (SD ± 0.06) greater anodal migration for the amniotic fluid species than that obtained with the concurrent normal human plasma control. Four amniotic fluid samples were run in tandem with normal plasma dilutions, and the two peaks obtained shared complete identity. Figure 2A shows the one amniotic fluid sample where a second peak of equal size and normal migration was observed; for six additional amniotic fluid samples, a smaller peak, representing 10% to 20% height of the major one and with normal migration, was also present. For the remaining 15 samples, the more rapidly migrating peak was the only one observed. When crossed-immunoelectrophoresis was performed with calcium in the first dimension, the same relationships held, ie, the major rapid prothrombin peak from amniotic fluid consistently ran 2 to 4 mm more anodal than the migration of simultaneously run normal plasma controls (right panel of Fig 2A). The more anodal migrating peak had the same migration position as the minor peak seen in some of the fetal plasma samples (above) in either system, being identical to the normal serum peak. One concentrated amniotic fluid was adsorbed with \(\text{Al(OH)}_3\), and its reaction with antiprothrombin was lost. Upon elution with 0.15 mol/L trisodium citrate, the more rapidly migrating peak was again observed, suggesting that it contains the calcium-binding (\(\gamma\)-carboxyglutamic acid-rich), amino-terminal region. In two samples (AF-3 and 8), a small arc migrating ahead of the more anodal of the two peaks was observed when electrophoresed in calcium only. This trace additional peak was similar to that seen on crossed-immunoelectrophoresis of plasma of patients on warfarin, but it was not apparent in other amniotic fluid or fetal plasma specimens tested.

![Fig 2. Crossed-immunoelectrophoresis of amniotic fluid samples. Samples (20 \(\mu\)L) on upper slides were 20-fold dilutions of normal human plasma (NP) and, on lower slides, of concentrated amniotic fluids (AF). In the first dimension, samples were electrophoresed with the anodal migration of the marker dye to the vertical bar indicated to the lower left of each slide; the anode was to the top in the second dimension. Further details are presented in Materials and Methods and in Thompson et al.\(^{11}\) (A) Prothrombin. Samples were run with 2 mmol/L EDTA (left) and 2 mmol/L calcium (right) in the first dimension and against agarose containing antiprothrombin serum (in 2 mmol/L EDTA) in the second dimension. The amniotic fluid sample shown (lower slides) was concentrated AF-12 (as numbered in Table 1). All other amniotic fluid samples had the prothrombin peak that migrated more rapidly than that of normal plasma in both EDTA and calcium; in about half, a smaller peak, corresponding to that in normal plasma, was seen (see Results). (B) Factor IX. Electrophoresis was in 2 mmol/L EDTA with \(^{125}\text{I}-\text{anti-factor IX} in the second dimension, followed by washing and autoradiography of the dried gel. The lower sample was concentrated amniotic fluid sample AF-9. Samples were applied in rectangular wells, as indicated by the darkened bars on the lower right of the slides. Of six other amniotic fluid samples run (AF-3, 8, 11, 17, and two of the first three drawn without rapid processing), the factor IX peak had the same migration as that of normal plasma, whereas in the sample shown, amniotic fluid factor IX migrated 1.1 times further than that of normal plasma.

**Amniotic fluid factor IX.** Results from crossed-immunoelectrophoresis of one amniotic fluid sample run in a system in which radiolabeled anti-factor IX antibody was included in the second dimension are shown in Fig 2B. For this one sample, the amniotic fluid factor IX migrated slightly ahead of that of normal plasma (by a factor of 1.1), but for the four additional amniotic fluid samples tested, the migration peak was within 0.5 mm that of the normal plasma factor, IX. Controls with isolated factor IX showed that these species had essentially the same migration as that of factor IX in normal plasma (not shown). Crossed-immunoelectrophoresis of isolated factor IX\(^a\) produced a distinct precipitin peak that had decreased anodal migration, as would be expected from a dissociable, sialic acid-rich, activation peptide (not shown).

**Polyacrylamide Gel Electrophoresis Studies**

**Prothrombin.** The prothrombin species were further evaluated by gel electrophoresis of concentrated amniotic fluid samples. As shown in Fig 3A, antipro-
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Fig 3. Polyacrylamide gel electrophoresis of amniotic fluid samples. Slab gels were electrophoretically blotted to nitrocellulose, incubated with antibodies, dried, and autoradiographed. The origin of the stacking gel is indicated (O), as is the anodal migration of the marker dye (+), the latter at around 8 cm. (A) Prothrombin. Nonreduced, concentrated amniotic fluids (10 μL) were electrophoresed in 7.5% polyacrylamide gels, blotted, and portions of the blot were incubated with rabbit antiprothrombin sera, washed, and then incubated with 125I-Staphylococcal protein A (see Materials and Methods). Lane 1 contained 1 μL normal plasma that had been adsorbed with 0.1 vol of Al(OH)3; lane 2, 1 μL normal plasma; lanes 3 and 4, 210 and 70 ng purified prothrombin, respectively; and lanes 5 through 10, amniotic fluid samples numbered (as in Table 1) AF-12, 14, 5, 3, 1, and 11, respectively. Migration of native prothrombin is indicated (P). The higher molecular weight radioactivity zone represents human IgG in amniotic fluid samples, and the lower molecular weight band has properties of fragment 1 - 2, the amino terminal cleavage product of prothrombin (see Results). Similar patterns, including the prominent low molecular weight form, were observed on each of the four other samples tested (AF-10, 17, 18, 19, not shown). (B) Factor IX. Nonreduced, concentrated amniotic fluids (10 μL) were electrophoresed on 7.5% polyacrylamide gels, blotted, and incubated directly with 125I-goat anti-factor IX. Lanes 1 and 2 contained 20 ng each of isolated factor IX and factor IX partially activated by factor IXa, respectively; lanes 3 through 5, 10-μL samples of concentrated amniotic fluids numbered (as in Table 1) AF-8, 7, and 10; the x-ray exposure on the latter three lanes was four times longer than that on lanes 1 and 2. The lower molecular weight factor IX band essentially corresponded to that seen with unreduced factor IXa, or inactivated factor IX as cleaved by elastase. Ten other samples run gave similar results (AF samples, 1, 3, 5, 9, 11, 12, 14, 15, 17, 19, not shown), with all but trace amounts of factor IX migrating to the same position as intact native species.

thrombin reacted with the blotted gel to give two distinct bands; one of normal molecular weight and one of low molecular weight. This pattern was seen in each of the ten samples so tested, although the relative intensity of the radioactivity in each peak varied among samples. On other gels (not shown), the low molecular weight peptide was consistently of higher molecular weight than purified thrombin, which also reacted with antiprothrombin serum. In one amniotic fluid sample (AF-14), a control was run in which the antiprothrombin antibody was omitted. Only the broad zone of higher molecular weight proteins bound radioactivity. Likewise, when samples AF-10 and AF-18 were adsorbed with 0.1 vol of Al(OH)3, both the normal prothrombin and the lower molecular weight bands disappeared, but the higher molecular weight zone binding 125I-Staphylococcal protein A remained.

In another sample, AF-12, the concentrated fluid was preadsorbed with agarose substituted with an insolubilized inhibitor of thrombin (p-chlorobenzylaminophenyl-aminocaproyl-agarose); the two specific prothrombin bands were unchanged. These data are consistent with the more anodal band representing the smaller, amino-terminal fragment 1 - 2 ("pro-piece") of prothrombin. There was no radioactivity corresponding to the thrombin migration position in any of the amniotic fluid samples tested, although thrombin complexed with inhibitor(s) could have been masked within the higher molecular weight, dark background, radioactivity zone. When 125I-prothrombin was added to each of three amniotic fluid samples (AF-6, 10, and 16) and incubated for two hours at 4 °C or 37 °C, frozen and thawed, there were no degradative cleavages seen on gels from samples at either temperature. When added to one of the first three fluids, which were more slowly processed, however, cleavage was observed after two hours (51% at 37 °C and 13% at 4 °C). With control, 125I-prothrombin incubated with normal human serum (from recalcified plasma clotted with kaolin and lipid), 87% was cleaved after ten minutes at 37 °C.

Five fetal plasma samples were analyzed in a similar fashion as polyacrylamide gel electrophoresis for prothrombin antigen. As with normal plasma or serum samples, there was a higher molecular weight radioactivity zone that extended to the migration of the prothrombin peak, and it was more intense than that of the amniotic fluids. It was present even when specific rabbit antibody to prothrombin was omitted. Nevertheless, in each sample (FP-4, 7, 8, and 14 and E, Suzuki and Thompson, a faint band of radioactivity, corresponding to the more anodal lower molecular weight prothrombin, was observed (as in Fig 3A), suggesting at least partial cleavage of fetal plasma prothrombin.

Factor IX. Thirteen amniotic fluid samples were electrophoresed and studied for factor IX antigen migration after blotting and incubating with 125I-goat anti-factor IX antibody. Migration of the major immunoreactive peak was normal, as presented in Fig 3B. Trace amounts of a lower molecular weight species
were noted. This second band corresponded in size to the disulfide-bonded heavy-light chains of factor IX produced by factor Xla activation, although it has also been observed after inactivating proteolytic cleavage by granulocyte elastase.11

**DISCUSSION**

Fetal blood sampling for prenatal diagnosis of hemophilia is currently limited to gene product analysis during midtrimester, after sex determination. For hemophilia B, nine confirmed cases have been reported; five were accurately diagnosed as affected, two were unaffected, and two were unaffected but from pedigrees with defects in which circulating antigen was detectable.3,6,7 In all of these cases, an immunoradiometric assay with a high titer human alloantibody from a patient with hemophilia B (who was also hepatitis B surface antigen positive) has been used. As opposed to polyclonal,2 or even a monoclonal,8 antibodies prepared from immunization of animals, the human antibody is distinct in that it fails to cross-react with the plasmas of about half of the patients with hemophilia B.5,7 Also, in two of the diagnosed cases, the mother had an exceptionally low level of antigen (2 and 3 U/dL).3,7

Using the immunoassay with the human alloantibody, factor IX antigen was noted in amniotic fluid samples from hysterectomy abortuses and in a single fetoscopy control.3 However, because (1) the origin of factor IX in amniotic fluid (or even in fetal plasma) has not been established with certainty, (2) its species in this complex fluid has not been investigated, and (3) levels have not been determined in nonhuman antibody assays, the current study focused on a separate series of samples obtained by amniocentesis and processed rapidly. These were studied immunologically for both factor IX and a related vitamin K-dependent protein, prothrombin.

Using specific antigen assays, the levels of factor IX in fetal plasmas at midtrimester average about 5 U/dL.3,5,7 Although the opportunity for proteolysis was present in the postmortem fetal plasma samples, the mean level from this series (Table 1) is essentially the same as that reported in the literature. The similarity is also consistent with an observation that the only minor difference between factor IX antigen levels in paired samples of human plasma and serum can be accounted for by the 10% dilution of plasma with anticoagulant (A.R. Thompson, unpublished observations, 1977, 1980). The wide range of fetal plasma values (Table 1) is in part reflective of the threefold normal range in adults; however, additional factors, such as significant variability in the level of fetal hepatic protein synthesis, are probably operative.

It is of interest that fetal plasma prothrombin showed decreased mobility upon crossed-immunoelectrophoresis in calcium v EDTA. This pattern, as seen with normal adult prothrombin, suggests that the vitamin K-dependent carboxylation of these circulating clotting factors is essentially intact for the degree of liver maturity, assuming that these proteins are of fetal origin. Alternatively, maternal origin cannot be excluded.

From studies of protein polymorphisms, fetal sera can contain phenotypes from some of the major plasma proteins that are discordant from the corresponding maternal serum protein phenotypes.15,16 Although fetal liver synthesis of the vitamin K-dependent clotting factors would probably be similar, there is no data on polymorphisms to confirm fetal origin. This is particularly relevant to prenatal diagnosis of hemophilia B, because factor IX is a trace plasma protein of rather low molecular weight and a large volume of distribution.17

For several proteins, the exclusion from amniotic fluid or the presence of a large concentration gradient from amniotic fluid to fetal plasma is used as evidence for maternal or fetal origin.18 Thus, factor IX antigen in amniotic fluid5 presents an additional problem for prenatal diagnosis. The current range of levels overlap fetal plasma concentrations (see Table 1). Antigen levels were unlikely to have been underestimated in amniotic fluids, especially as there was only minimal cleavage. Therefore, antigen levels from samples in which there is gross contamination of fetal blood with amniotic fluid would be contaminated, assuming that amniotic fluid factor IX is of maternal origin. Phenotype testings of plasma proteins in amniotic fluids have shown concordance with maternal sera and, on some occasions, discordance with phenotypes in fetal sera for several different plasma proteins.18 Thus, if the major component of amniotic fluid factor IX is assumed to be similarly of maternal, as opposed to fetal, origin, amniotic fluid antigen levels could not be used for prenatal diagnosis.

Amniotic fluid is a complex, dynamic liquid containing over 200 plasma proteins, most of which have apparent molecular weights under 100,000.19 Its total volume can change rapidly due to fetal swallowing and the contributions from fetal urine.20 Protease inhibitors are present in significant concentrations,21 and this may in part be due to the accumulation of inhibitor–protease complexes.22 The predominance of low molecular weight peptides19 and of procoagulant activity4,23 is consistent with active proteolysis having occurred within the fluid. These proteases could influence the species of various coagulation factors present; indeed, the cleaved forms of prothrombin observed in this current study provide physical evidence to support proteolytic activity in amniotic fluid. Prothrombin
clavage was less extensive in, and only observed in some of, the postmortem plasma samples, supporting the premise that proteolysis in the more rapidly processed amniotic fluid samples occurred in vivo. The failure to observe degradation of radiolabeled prothrombin, even at 37 °C, is further evidence that the cleavage occurred in vivo. Assuming that prothrombin cleavage was due to extrinsic system activation, this constitutes a situation in which prothrombin has been extensively cleaved with only minimal cleavage of factor IX by the same extrinsic system factors.

Amniotic fluid contamination, even at trace levels, could lead to in vitro proteolysis of fetal plasma samples. Proteolytic activation may well account for results from fetal plasmas where coagulant assays consistently show higher amounts of apparent factor IX than antigen levels. In one report, this coagulant activity was inhibited by specific antibodies to factor VII. Because factor IX can be activated by extrinsic system factors and is also susceptible to inactivation by a variety of proteases, the cleaved factor IX observed could represent factor IXa or an inactive species. The latter is seen following proteolysis by granulocyte elastase in calcium. Therefore, the factor IX antigen levels in the amniotic fluids primarily reflect native protein.

Antigen levels of the cleaved prothrombin species could have been altered by in vivo proteolysis. Possible mechanisms include decreased levels by accelerated clearance from the fluid space, or increased levels by accumulation of high molecular weight thrombin-inhibitor complexes, as seen with α1-antichymotrypsin. Decreased clearance is consistent with the current observation of prothrombin levels being disproportionately higher than factor IX, relative to their own normal adult levels.

Current results of amniotic fluid factor IX antigen levels were higher than the only other series, where five amniotic fluids ranged from 0.4 to 1.2 U/dL. The reason for this difference is unclear, but it may relate to populations, sample size, processing (which was not specified in the other study), or their use of a human monoclonal antibody assay system.

The limitations of prenatal diagnosis of hemophilia B are: (1) assay sensitivity and specificity, (2) variability in fetal production or destruction of the gene product, (3) the circulation of an abnormal gene product in affected members of many pedigrees, and (4) the broadly distributed normal range, which also applies to fetal fluids. At the very least, uncontaminated fetal blood samples are required. In addition, knowledge of a low reactivity to a given antibody preparation in an affected member of the pedigree would be necessary. Even under optimal conditions, and using sensitive assays, the limitations imposed make interpretations of results difficult. Testing would be most applicable to situations in which the affected fetus could be expected to have very low factor IX antigen levels, that is, where an affected family member has a level of less than 0.1 to 1 U/dL by the assay system employed. This situation is most commonly found with monoclonal antibodies, including half of the patients tested in one series with a human alloantibody and three of 55 cases in another with a mouse monoclonal antibody. Alternatively, as recently suggested, direct studies of the factor IX gene could be developed to circumvent the difficulties of gene product analysis for prenatal diagnosis of hemophilia B.

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Factor IX and prothrombin in amniotic fluid and fetal plasma: constraints on prenatal diagnosis of hemophilia B and evidence of proteolysis

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