Developmental Microheterogeneity of Mouse α-Fetoproteins: Purification and Partial Characterization†

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ABSTRACT: Mouse α-fetoprotein shows reproducible changes in concentration of its electrophoretic variants with time of development. Mixtures of the α-fetoprotein containing Fp1-3, Fp1-5, and Fp5 were purified from day 12.5 amniotic fluid, day 15.5 amniotic fluid, and day 18.5 plasma, respectively. The molecular weights of these purified protein mixtures, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were similar (about 70 000). Antibody produced against Fp1-3 immunologically reacted with Fp1-5 and Fp5. Analyses of amino acids and carbohydrates indicated that they were similar in these α-fetoprotein mixtures except in the number of sialic acid residues. Neuraminidase treatment of the α-fetoproteins caused a disappearance of the faster moving electrophoretic variants with a corresponding increase in the concentration of the less acidic components. Purification of each electrophoretic component (Fp1, Fp2, Fp3, Fp4, and Fp5) from day 14.5-15.5 amniotic fluid was carried out in long preparative polyacrylamide gels. *V. cholerae* neuraminidase treatment of each α-fetoprotein component gave rise to proportionally slower moving electrophoretic components. However, since Fp3 was partially resistant to the enzyme, Fp3 and Fp1 were the final products. The increasing mobility of each α-fetoprotein was shown to be proportional to an increase in sialic acid content and a decrease in isoelectric point. Fp5 was separated into two components Fp5a and Fp5b by isoelectric focusing in polyacrylamide gels.

Fp5 was labeled with [^{3}H]leucine in day 14.5 embryos, isolated from amniotic fluid, and purified by preparative acrylamide gel electrophoresis. Labeled Fp5a and Fp5b were separated by QAE-Sephadex chromatography as determined by isoelectric focusing in polyacrylamide gels. Neuraminidase converted Fp5a to Fp3 and Fp5b to Fp1. It is concluded that with time of development the α-fetoproteins synthesized contain increasing amounts of sialic acid.

Many glycoproteins show microheterogeneity due to variations in the type and amount of carbohydrate covalently attached to their polypeptide chains. In several cases electrophoretic variants of these glycoproteins, which appear homogeneous by many other criteria, have resulted from variations of the terminal sialic acid residues of the glycoprotein. Sialic acid is responsible, at least in part, for the microheterogeneity of transferrin (Chen and Sutton, 1967; Gustine and Zimmerman, 1973), fetuin (Oshira and Eylar, 1968), mouse myeloma immunoglobulin (Melchers et al., 1966), and α-fetoprotein of human (Ruoslahti and Sepällä, 1971; Alpert et al., 1972) and mouse (Gustine and Zimmerman, 1973).

During gestation, marked changes in plasma proteins in various species have been noted. For example, the concentration of α-fetoprotein at first increases during development and then decreases during the later fetal period (Bergstrand and Czar, 1957; Weller and Schectman, 1962). Some of these plasma proteins also show electrophoretic variations during development (Pantelouris and Hale, 1962; Parker and Bearn, 1962; Wise et al., 1963). While exploring the developmental changes in fetal plasma proteins of mice, we observed three electrophoretically separable transferrins (Tr1-3) and five α-fetoproteins (Fp1-5)[1] at day 14.5 of development. By day 15.5, the more acidic proteins Tr3, Fp4, and Fp5 had reproducibly increased in concentration, while the less acidic ones decreased. Neuraminidase treatment of fetal plasma at this time of development converted the acidic components (Tr3, Fp4, Fp5) to Tr1, Tr2, and Fp1, Fp2, and Fp3. Furthermore, at day 12.5 of gestation, the less acidic glycoproteins were synthesized, while at day 14.5 only the more acidic ones were being synthesized. It was postulated that the glycoproteins which are synthesized later in development contain increased sialic acid and that the observed changes in microheterogeneity of these proteins represent regulation of glycoprotein synthesis at the level of carbohydrate attachment (Gustine and Zimmerman, 1973).

In this report, the putative electrophoretic variants of the α-fetoproteins observed at different times in development were purified and partially characterized. Evidence is presented that these proteins are, in fact, electrophoretic variants of α-fetoprotein and that the differences can be attributed, at least in part, to differences in sialic acid content of these proteins.

Experimental Procedure

Materials. Neuraminidase[2] (EC 3.2.1.18) from *Clostridium perfringens* (1.1 units/mg) and from *Vibrio cholerae* (500 units/mg) was purchased from the Sigma Chemical Co. and Calbiochem, respectively.

Breeding of Mice. Inbred strains C3H/An (Cumberland View Farms) and A/J (Jackson Laboratories) were used as the source of fetal plasma and amniotic fluid. There were no developmental differences in the electrophoretic components of α-fetoproteins (as well as transferrins) in amniotic fluid or fetal plasma between these two strains. Evidence of a vaginal plug the morning after mating was used as the criterion for

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[1] In previous publications (Gustine and Zimmerman, 1972a,b), we have referred to the mouse α-fetoproteins as mouse β proteins and numbered them β1, β2, etc. In order to conform to accepted practice, we now refer to these fetal specific proteins as α-fetoproteins and number them Fp1, Fp2, etc.

[2] Units (500) of *Vibrio cholerae* enzyme are roughly equivalent to 0.1 unit of *Clostridium perfringens* enzyme (Pricer and Ashwell, 1971).

conception, and this time was considered to be day 0.5 of gestation.

Purification of α-Fetoprotein Mixtures. At the appropriate gestational age, amniotic fluid or fetal plasma was collected (Gustine and Zimmerman, 1972a) and protein concentration was determined (Lowry et al., 1951). Proteins from amniotic fluid or fetal plasma were separated by polyacrylamide gel electrophoresis (Ornstein, 1964; Davis, 1964). Basic gels (pH 8.3) containing 7% acrylamide were run in the cold room at 3.5 mA per gel for about 2 h until the tracking dye was about 1 cm from the end of the gel (6 × 90 mm). Approximately 300–500 μg of total protein was applied per gel. The absorbance of extruded gels was recorded at 280 nm with a Gilford Model 2400 spectrophotometer, equipped with a gel scanner. The portions of the gels corresponding to α-fetoproteins were cut out and slices from 24 gels were homogenized with 8 ml of 0.05 M Tris (pH 7.0) in a Dounce homogenizer. The homogenized acrylamide particles were extracted in the buffer overnight at 4 °C and then centrifuged at 800*g* for 10 min. The acrylamide particles were reextracted four times with a 4-ml buffer and the washes were combined with the original supernatant. α-Fetoprotein was precipitated by addition of 4 volumes of cold 100% ethanol. After standing overnight at −20 °C, the precipitated protein was centrifuged and the protein pellet was dissolved in 1–2 ml of 0.05 M sodium acetate (pH 5.0), yielding a protein concentration of about 1 mg/ml.

In other experiments gels were run in duplicate and proteins were stained in 1% Aniline Blue-Black in 7% acetic acid. The unbound dye was removed by diffusion destaining overnight in 7% acetic acid. Absorbance patterns were then recorded at 600 nm.

Molecular Weight Determination. The apparent molecular weights of the α-fetoprotein mixtures were determined by the method of Weber and Osborn (1969) in sodium dodecyl sulfate–polyacrylamide gels. Gels were stained with Coomassie brilliant blue.

Immunoelectrophoresis Assay. Immunoelectrophoreses of amniotic fluid, sera, and purified α-fetoprotein mixtures were carried out by adding 5 μl of antigen to the appropriate well of a Meloy immunoelectrophoresis plate. Electrophoresis at 100 V for 105 min was performed and the appropriate antibody was added to each trough. Anti-Fp1–3 antibody was prepared against purified Fp1–3 in a goat as previously described (Gustine and Zimmerman, 1972).

Carbohydrate Analyses. Sialic Acid. The purified α-fetoprotein preparations were precipitated by ethanol (80%) and dissolved in distilled water. Portions were assayed for protein concentration or made to 0.1 N H_2SO_4 and hydrolyzed at 80 °C for 1 h. Aliquots (50 μl) were assayed for sialic acid by the thiobarbituric acid method of Warren (1959) employing a fivefold less volume of reagents and 50–150 μg of protein.

Neutral Sugars. The α-fetoproteins were assayed for neutral sugars by the anthrone method (Hewitt, 1958) employing about 200 μg of protein and 1 ml of anthrone reagent. A mannose–galactose (1:1) mixture was used as a standard.

Amino Sugars. α-Fetoproteins were assayed for amino sugars by the Elson–Morgan reaction as modified by Gatt and Berman (1966) employing a fourfold less volume of reagents. Glucosamine was employed as the standard and 100 μg of α-fetoprotein was used for each assay.

Amino Acid Composition. One milligram samples of purified Fp1–3, Fp1–5, and Fp5 preparations were hydrolyzed in 6 N HCl at 110 °C in vacuo for 22 and 72 h. The amino acid mixtures were analyzed in a Beckman Spinco 120C amino acid analyzer according to the procedure of Moore et al. (1958). Values represent averages of the 22- and 72-h hydrolyses. The amounts of threonine and serine were calculated by extrapolation to zero time. Tryptophan was separately determined according to the spectrophotometric procedure of Edelhoch (1967).

Purification of Each Electrophoretic Component of α-Fetoprotein. Each individual electrophoretic component of α-fetoprotein (Fp1, Fp2, Fp3, Fp4, Fp5) was purified from day 14.5–15.5 amniotic fluid (equal volume of each) employing preparative polyacrylamide gels (Zimmerman and Bowen, manuscript in preparation). Samples were concentrated either by dialysis against 30% poly(ethylene glycol) or with an Amicon filtration apparatus (UM-10 filter) in the cold room and were layered directly over a 2-ml stacking gel. Approximately 1 ml volume of concentrated amniotic fluid containing 2–10 mg of protein was employed for each 1.8 × 44 cm polyacrylamide separating gel. Electrophoresis was for 65 h at 9.1 mA/gel in the cold room. After electrophoresis, gels were extruded and a longitudinal wedge in the approximate region of the α-fetoprotein bands was cut out of the gel and incubated with a 0.03 mg/ml solution of anilinonaphthalenesulfonic acid, magnesium salt for 5 min, and then transferred to 3 N HCl for 2 min (Hartman and Udenfriend, 1969). The wedge was then aligned with the untreated gel and protein fluorescence in the wedge visualized using a hand-held UV[3]-light source. The portions of the untreated gel corresponding to the Fp1, Fp2, Fp3, Fp4, and Fp5 bands were cut out and homogenized with 3 volumes of 0.05 M Tris–0.1 M NaCl, pH 7.0, and each α-fetoprotein was extracted by incubation overnight at 4 °C. The extracted α-fetoproteins were isolated by centrifugation at 300*g* for 20 min and the acrylamide debris was washed an additional four times with 3 ml of the same buffer. The supernatants and washes were combined, again centrifuged at 300*g* for 10 min, and filtered through a Millipore prefilter. The solutions were concentrated again with the Amicon filtration unit, dialyzed against the Tris-NaCl buffer, and ethanol-precipitated as previously described.

Isoelectric Focusing. Purified electrophoretic components of α-fetoprotein were separated by isoelectric focusing in polyacrylamide gels by a modification of the method of Wrigley (1968). Differences in isoelectric points of Fp1, Fp2, Fp3, Fp4, and Fp5 could not be reproducibly measured using a gradient of pH 4–6 ampholyte (LKB). Therefore the pH 4–6 ampholyte solution was first subjected to isoelectric focusing with a LKB column apparatus for 38 h and a mixture of separated ampholytes (about pH 4.4 to 5.4) was pooled. To prevent bacterial contamination due to the presence of sucrose, the mixture was sterilized by filtration through a Millipore nitrocellulose filter (0.45-μm pore size). The ethanol-precipitated α-fetoprotein components were dissolved in the ampholyte solution and catalyst and acrylamide solutions were added. Final volume was 1.8 ml and ampholyte concentration was 2.5%. Polymerized gels (7.5 cm) were run in the cold room overnight, at a constant voltage of 350.

Gels were sliced into 2.2-mm pieces and eluted with 0.2 ml of glass-distilled water for 2 h at room temperature. The pH in each sample was measured with a microelectrode. α-Fetoprotein in 5–10 μl of each sample was determined by radial immunodiffusion with sera of anti-Fp1–3 (1:25 dilution). Purified Fp5 served as the reference standard.

Synthesis of Labeled Fp5. Labeled Fp5 derived from amniotic fluid was prepared essentially as described by Gustine

[3] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid.

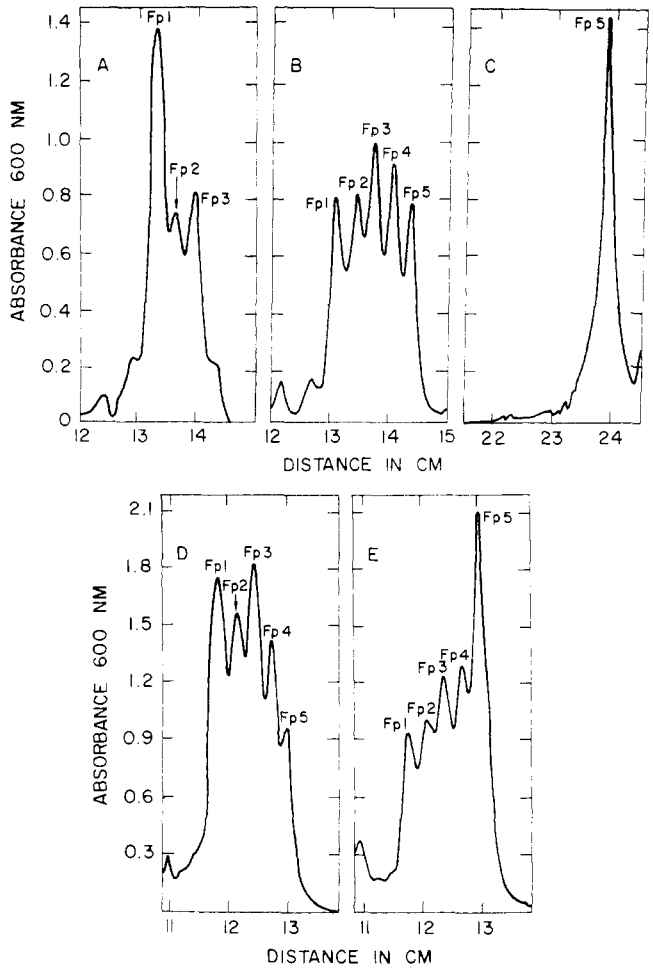

FIGURE 1: Polyacrylamide electrophoresis of day 12.5 amniotic fluid, day 15.5 amniotic fluid, and day 18.5 fetal plasma on long gels. Samples (100 μg of protein each) were subjected to electrophoresis at 4 °C: day 12.5 amniotic fluid (A) and day 15.5 amniotic fluid (B) were separated on 15-cm gels at 3.5 mA/gel for 4 h and day 18.5 fetal plasma (C) was separated on a 24.5-cm gel at 1.5 mA/gel for 16 h. In a separate experiment, day 12.5 (95 μg) and day 15.5 (108 μg) amniotic fluid were mixed (D), as well as day 15.5 amniotic fluid (108 μg) and day 18.5 fetal plasma (84 μg) (E), and separated on 15-cm gels. The gels were run for 2 h at 3.5 mA/gel and then an additional 2 h at 2.6 mA/gel. Areas of gels containing α-fetoproteins are presented.

and Zimmerman (1973). Day 14.5 embryos with placentas and membranes intact were incubated on a wire grid in a Falcon petri dish (3 embryos/dish) containing 0.5 ml of sterile Triple Eagle's Medium (Gibco), 10% fetal calf serum, 10% chick embryo extract, and 5 mM glutamine. A total of 27 embryos were preincubated at 37 °C. Neutralized and sterilized [4,5-^{3}H]-L-leucine (10 μl, 5 μCi, 46 Ci/mmol) was then injected into the amniotic sac of each embryo and incubation continued for another 22 h. Embryos with their sacs intact were washed with phosphate-buffered saline (0.01 M sodium phosphate–0.15 M NaCl, pH 7.5). The membranes were torn and amniotic fluid was collected. Each embryo and its membranes were then washed with 3 drops of a buffer containing 0.02 M Tris, pH 7.6, 0.001 M $MgCl_2$, 0.01 M NaCl, 0.25 M sucrose, and 0.01 M leucine and the wash was combined with the collected amniotic fluid. Labeled Fp5 was then purified on a preparative acrylamide gel, as described above.

Separation of Labeled Fp5a and Fp5b by QAE-Sephadex Chromatography. ^{3}H-Labeled Fp5 was chromatographed on a column of QAE-Sephadex A-50 (1.7 × 21 cm) employing a linear pH gradient of 4.8 to 6.0 with 0.1 M sodium cacodylate. Ethanol-precipitated ^{3}H-labeled Fp5 (0.54 mg) was dissolved in 2 ml of pH 6.0 sodium cacodylate buffer and layered over the column. Flow rate was 1 ml/4 min and 4-ml fractions were collected. pH and radioactivity in each fraction were monitored; 0.2 ml was added to 2.5 ml Multisol and radioactivity was assayed.

Results

Developmental Pattern of α-Fetoproteins from Fetal Plasma and Amniotic Fluid. Figure 1 shows the changes in electrophoretic mobilities of mouse α-fetoprotein observed during development. With day 12.5 amniotic fluid, Fp1 was found in the greatest concentration (Figure 1A) followed by Fp3 which was only slightly greater than Fp2 in concentration. Figure 1B shows that there were five discernible protein peaks in the α-fetoprotein region of day 15.5 amniotic fluid. In contrast, only a single protein peak (Fp5) was found in day 18.5 fetal plasma (Figure 1C). In general, qualitative changes in these proteins during development were quite reproducible. However, quantitative changes in the proportion of each protein from an individual fetus at a particular time in development were observed. To test whether the α-fetoproteins observed at different times of development in amniotic fluid and plasma had identical mobilities in polyacrylamide gels, mixing experiments were performed. When day 12.5 and 15.5 amniotic fluids were mixed and subjected to polyacrylamide gel electrophoresis, all α-fetoproteins superimposed on each other (Figure 1D). Similarly when day 15.5 amniotic fluid and day 18.5 plasma were mixed, electrophoresis revealed five α-fetoprotein peaks with Fp5 in greatest concentration, as would be expected (Figure 1E).

Purification of α-Fetoprotein Mixtures. Since α-fetoproteins form nearly 60% of the total protein in day 15.5 amniotic fluid (Gustine and Zimmerman, 1973) and plasma (Gustine and Zimmerman, 1972b), a one-step isolation of α-fetoprotein from day 12.5 and 15.5 amniotic fluid and day 18.5 fetal plasma by separation on polyacrylamide gels and elution from the gels was attempted. When aliquots of the purified α-fetoprotein preparations were reexamined by polyacrylamide gel electrophoresis, it can be seen that the only major peaks observed were the α-fetoproteins showing their putative microheterogeneity (Figures 2A–C, see also Figure 5 for greater resolution of the α-fetoproteins). However, small protein peaks also appeared that were slower moving than the major α-fetoprotein peaks, designated X. Visual inspection of these peaks from many different preparations indicated multiple peaks in the X region (e.g., Figure 2C). Treatment of the purified α-fetoprotein preparations with clostridial neuraminidase showed a decrease in mobility and number of bands in the α-fetoprotein area of the gels (e.g., see Figure 4). This result suggests that electrophoretic variants of these glycoproteins exist as a result of differences in the amount of sialic acid.

When all the α-fetoprotein preparations were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, they moved as single bands in the gels (Figures 2D–F). The slower moving peaks (X), observed in Figures 2A–C, were now not seen. In addition each α-fetoprotein taken from different times of development had the same mobility.

Molecular Weight Measurements. The apparent molecular weights of Fp1–3, Fp1–5, and Fp5 were calculated based upon their mobilities in sodium dodecyl sulfate–polyacrylamide gels and were found to be 71 000, 70 000, and 71 000, respectively (data not presented). Therefore mouse α-fetoproteins taken from different times in development appear to have similar molecular weights of about 70 000.

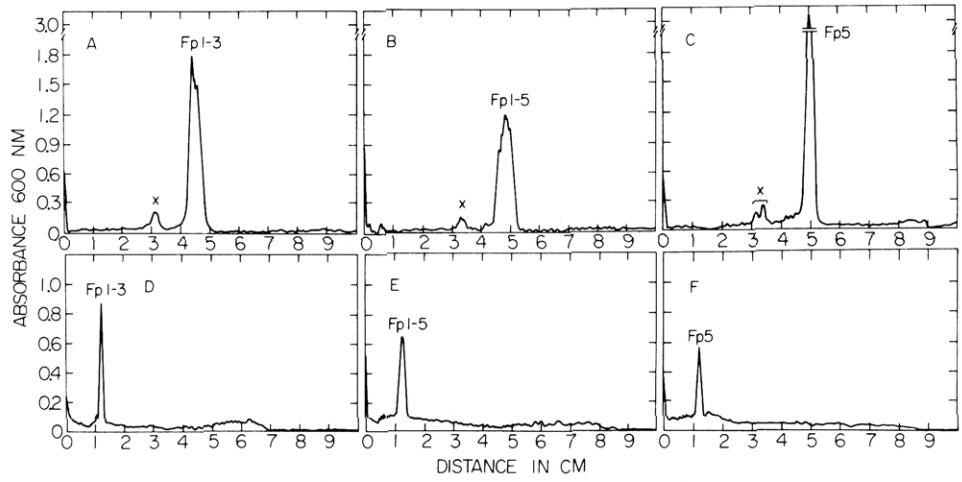

FIGURE 2: Purification of Fp1-3, Fp1-5, and Fp5 mixtures. The appropriate α-fetoprotein mixtures were purified as described under Experimental Procedure. (A-C) Polyacrylamide gel electrophoresis of purified Fp1-3, Fp1-5, and Fp5. Day 12.5 amniotic fluid was the source for Fp1-3 (A), day 15.5 amniotic fluid for Fp1-5 (B), and day 18.5 fetal plasma for Fp5 (C). Aliquots of the purified α-fetoprotein mixtures (43 μg of each protein) were subjected to polyacrylamide gel electrophoresis on 9-cm gels to analyze purity. Gels were stained with Aniline Blue-Black. (D-F) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified Fp1-3, Fp1-5, and Fp5. The α-fetoprotein preparations (25 μg of protein each) were precipitated with ethanol (80%) and the pellets dissolved in 50 μl of 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 0.01 M sodium phosphate (pH 7.0). After incubation for 2 h at 37 °C, proteins were subjected to electrophoresis for 4 h and stained with Coomassie brilliant blue. Densitometric scans of whole gels are presented.

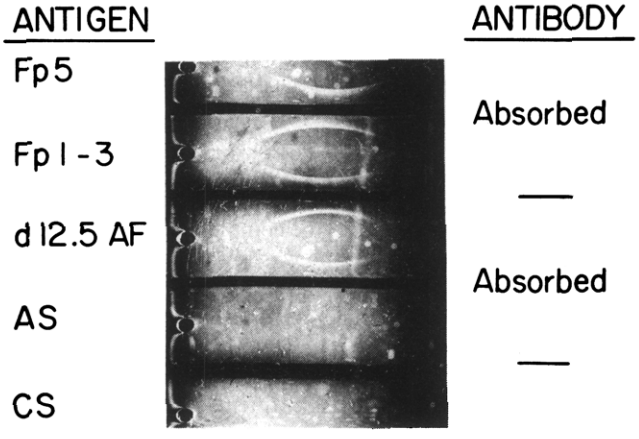

FIGURE 3: Immunoelectrophoresis of sera and purified α-fetoprotein. Antigens include purified Fp5, purified Fp1-3, day 12.5 amniotic fluid, adult mouse sera from strain C3H (AS), and control sera from nonimmune goat (CS). Migration of protein is from left to right. After the run, each antigen was tested against either undiluted anti-Fp1-3 (—) or anti-Fp1-3 immunoabsorbed with C3H adult serum (100 μg/ml adult protein) (Absorbed).

Immunochemical Studies. First, anti-Fp1-3 was used to attempt to immunoprecipitate Fp1-3, Fp1-5, and Fp5 and the immunoprecipitates were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. All three α-fetoprotein preparations were immunoprecipitated and the presence of the α-fetoprotein band was confirmed (data not presented). Furthermore day 12.5 amniotic fluid and purified Fp1-3 gave single precipitin arcs with anti-Fp1-3 when immunoelectrophoresis was carried out (Figure 3). In addition, anti-Fp1-3 was mixed with adult sera to immunoabsorb any possible contaminating antibodies. Employing the immunoabsorbed antibody, single precipitin arcs were seen with purified Fp5 and Fp1-3 and day 12.5 amniotic fluid. As a negative control, adult sera were reacted with immunoabsorbed antibody and no precipitin arc was found, as expected. In summary, it is concluded that Fp5 found late in development is immunoprecipitated by anti-Fp1-3.

TABLE I: Amino Acid Composition of Fp1-3, Fp1-5, and Fp5 Mixtures.

	Fp1-3[a]	Fp1-5	Fp5	Human[b] α-Fetoprotein
Lys	51	45	48	49
His	17	16	17	17
Arg	23	22	23	20
Asp	38	41	41	43
Thr	46	47	49	38
Ser	34	34	33	34
Glu	81	84	83	92
Pro	24	23	24	25
Gly	35	51	43	35
Ala	40	42	42	50
½-Cys	21	23	23	28
Val	24	26	26	31
Met	9.7	10	9.4	6.4
Ile	32	31	30	33
Leu	63	61	62	60
Tyr	10	11	10	18
Phe	26	26	26	28
Trp	2.1	0.90	2.1	ND

[a] Number of residues per molecule. Average values calculated on a molecular weight of 66 850 for the peptide portions since carbohydrate contents and molecular weights were assumed to be 4.5% and 70 000, respectively. [b] Data from Ruoslahti and Sepällä (1971).

Amino Acid Analyses. Amino acid analyses are presented in Table I. The values are given in residues of amino acid per molecule of protein. Within the limits of the analyses, the compositions of the three α-fetoprotein mixtures were similar except for glycine. The reason for this variation with glycine is unknown but may be due to the amino acid absorbed to the proteins from the glycine-containing electrophoresis buffer in which the α-fetoproteins were originally separated. It is interesting to note that these three α-fetoprotein preparations show a striking similarity to human α-fetoprotein. It is not known whether the differences in threonine, methionine, and tyrosine from the two species are significant.

TABLE II: Carbohydrate Analyses for α-Fetoproteins.[a]

Sample	Sialic Acid Content (%)	Sialic Acid Residues/mol[b]	p[c]	Neutral Sugar Content (%)	Neutral Sugar Residues/mol	Amino Sugar Content (%)	Amino Sugar Residues/mol	p	Total Content (%)
Fp1-3	0.27 ± 0.039	0.61 ± 0.088		1.6 ± 0.02	6.2 ± 0.08	2.2 ± 0.05	8.5 ± 0.21		4.1
Fp1-5	0.54 ± 0.068	1.2 ± 0.15	<0.01	1.6 ± 0.05	6.2 ± 0.19	2.5 ± 0.10	9.8 ± 0.34	<0.01	4.6
Fp5	0.96 ± 0.078	2.2 ± 0.18	<0.01	1.5 ± 0.06	5.8 ± 0.15	2.2 ± 0.04	8.5 ± 0.17	<0.01	4.7
Fp1&3[d]	0.076 ± 0.009	0.17 ± 0.02							

[a] The results are the mean ± SE of four determinations for sialic acid, mean ± range of 2 for neutral sugars and mean ± SE of 6 for amino sugars in the α-fetoproteins. In addition duplicate determinations for sialic acid were performed on Fp1&3; the mean ± range are presented. [b] Calculated on a basis of a molecular weight of 70 000 for Fp1-3, Fp1-5, Fp5, and Fp1-3. [c] p value for Student's t test comparing Fp1-3 with Fp1-5, and Fp1-5 with Fp5. [d] Fp1&3 were produced by treatment of 1 mg of Fp1-5 with 500 units of neuraminidase (*V. cholerae*) as described in Figure 5. After incubation a portion of the reaction mixture (32 μg of Fp) was subjected to electrophoresis on a 15-cm polyacrylamide gel. Analysis of the stained gel revealed that Fp1 and Fp3 were present in the proportion of 81 and 19%, respectively. The remainder of the reaction mixture was passed through a Bio-Gel P-30 column (1.4 × 6 cm, 0.05 M sodium acetate, pH 5.6) and Fp1&3 were collected in the void volume to separate them from the enzyme. The α-fetoproteins were ethanol precipitated.

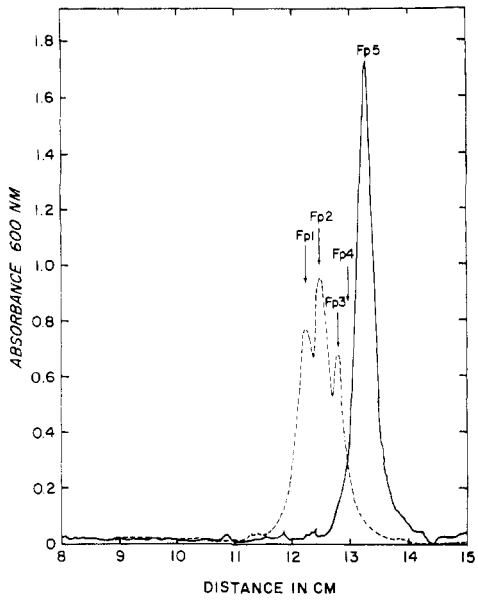

FIGURE 4: Effect of *Clostridium perfringens* neuraminidase on mobility of purified Fp5 in polyacrylamide gels. The reaction mixtures contained 66 μg of Fp5, 6 μg of enzyme, and 12.5 μmol of sodium acetate (pH 5.0) in a final volume of 0.25 ml. Samples were incubated for 30 min at 37 °C, and control and enzyme-treated samples each containing 26 μg of α-fetoprotein were subjected to electrophoresis on 15-cm polyacrylamide gels. Areas of gels containing α-fetoproteins are presented and assignments of mobilities of Fp1, 2, 3, 4, and 5 were derived from Fp1-5 mixtures also run. (—) Control; (- - -) neuraminidase treated.

Carbohydrate Analyses. The distribution of sialic acid, neutral sugars, and amino sugars in Fp1-3, Fp1-5, and Fp5 is shown in Table II. There were no apparent differences in the content of neutral sugars between the α-fetoproteins. Fp1-5 showed 9.8 residues of amino sugar per mole compared with 8.5 for Fp1-3. However, Fp5, which moved as a single band electrophoretically, has a lower value of 8.5 residues per mole. Sialic acid content was significantly different ($p < 0.01$) in the preparations isolated at the three different times in development. Fp1-3 contained 0.61 residue per mole while Fp5 contained about fourfold more: 2.2 residues. This latter value agrees with that reported for human α-fetoprotein (Ruoslahti and Sepällä, 1971). Fp1-5 contained an intermediate content of 1.2 residues. These results support the concept that the electrophoretic variation of the α-fetoproteins is due to differences in sialic acid content. Finally, all the α-fetoproteins contained between 4.1 and 4.7% carbohydrate, which is in agreement with the values reported for human α-fetoprotein (Ruoslahti and Sepällä, 1971).

Effect of Neuraminidase. Previously it was demonstrated that neuraminidase from *Clostridium perfringens* when incubated with day 15.5 amniotic fluid (Gustine and Zimmerman, 1973) and plasma (Gustine and Zimmerman, 1972a), both containing Fp1-5, caused the disappearance of Fp4 and Fp5 and an increase in the concentration of Fp1-3. To extend these studies, purified Fp5 was incubated with clostridial neuraminidase (fetoprotein-enzyme ratio of 10:1) and the products were separated by polyacrylamide gel electrophoresis. Figure 4 reveals that the enzyme completely converts Fp5 to Fp1, Fp2, and Fp3, and no Fp4 was found (see arrow).

When the effects of *Vibrio cholerae* neuraminidase on the mobility of the α-fetoproteins were tested, surprisingly Fp2 showed a sensitivity to the enzyme. Fp1-3 after enzyme treatment showed an increase in Fp1 and a disappearance of Fp2 (Figure 5A). Similarly, treatment of Fp1-5 produced predominantly Fp1 and Fp3 after incubation with the lower amount of enzyme. Fp2 was present only as a shoulder. Incubation in the presence of tenfold greater enzyme concentration shows that no Fp2 remained. Finally when Fp5 was incubated with the low concentration of *V. cholerae* enzyme, four peaks appeared in the polyacrylamide gels: Fp1, Fp2, Fp3, and Fp4. Incubation with the high concentration of enzyme caused the disappearance of Fp2 and Fp4 and left only Fp1 present in the highest concentration and Fp3 in a much lower concentration.

In summary, both sources of neuraminidase converted the faster moving Fp4 and Fp5 to the slower moving fetoproteins, while *V. cholerae* neuraminidase, under these conditions, alone converted Fp2 to Fp1.

In addition, the sialic acid content of a Fp1 and Fp3 mixture produced by the treatment of Fp1-5 with *Vibrio cholerae* neuraminidase was determined. This mixture was composed of 81% Fp1 and 19% Fp3 and contained 0.17 sialic acid residue per mole (Table II). Thus it is concluded that Fp1 lacks sialic acid while Fp3 contains sialic acid resistant to the action of *V. cholerae* neuraminidase.

Purification of Each Electrophoretic Component of α-Fetoprotein. The results presented so far would indicate that the electrophoretic components found in the region of α-fetoprotein throughout development indeed represent α-fetoproteins differing, at least in part, in their sialic acid content. To

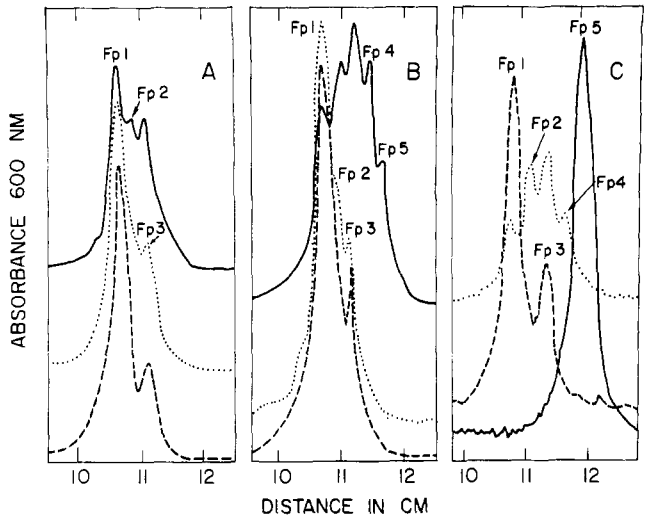

FIGURE 5: Effect of *Vibrio cholerae* neuraminidase on mobility of purified Fp1-3, Fp1-5 and Fp5 in polyacrylamide gels. Fp1-3 (80 μg), 115 μg of Fp1-5, and 58 μg of Fp5 were incubated with neuraminidase in the following proportions (protein (μg):enzyme units) 50:0, 50:3, 50:30. The incubation mixture (total volume 0.44 ml) contained, in addition to fetal proteins and enzyme, 0.44 μmol of $CaCl_2$ and 44 μmol of sodium acetate (pH 5.6). The enzyme was allowed to react at 37 °C for 24 h, and one-half of the fetal proteins were separated by electrophoresis on 15-cm polyacrylamide gels. The gels were scanned at full scale of 2.0 absorbance units. A, B, and C show the effect of neuraminidase on Fp1-3, Fp1-5, and Fp5, respectively. (- - -) High concentration of enzyme; (. . .) low concentration of enzyme; and (—) no enzyme.

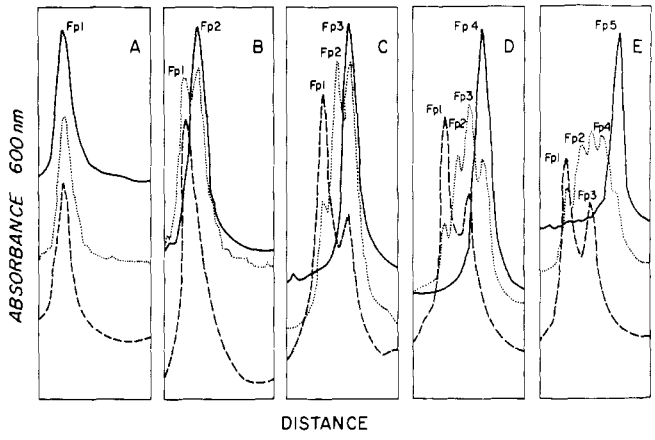

FIGURE 6: Purification of Fp1, Fp2, Fp3, Fp4, and Fp5 and the effect of *V. cholerae* neuraminidase on the mobility of each in polyacrylamide gels. Each electrophoretic component of α-fetoprotein from day 14.5-15.5 amniotic fluid was purified from large preparative acrylamide gels (44 cm) as described in Experimental Procedure. The purity of each α-fetoprotein component (approximately 10 μg) was analyzed by polyacrylamide gel electrophoresis on 15-cm polyacrylamide gels (—). In addition, each α-fetoprotein component was incubated with 0.5 unit (. . .) or 5 units (- - -) of neuraminidase, as described in Figure 5 except that the final volume was 0.12 ml. In addition, activity of the enzyme was inhibited by binding Ca^{2+} ion with twice its concentration of sodium EDTA and the pH of the reaction mixture was brought to 7.0 with 0.2 M unneutralized Tris buffer. Mobility of these electrophoretic components could then be ascertained by adding 50 μg of carrier day 15.5 amniotic fluid to all replicate samples, as described in Figure 1. Tracings of these samples containing amniotic fluid are not presented. Comparison of peak areas can not be made since gels were scanned at either 1.0 or 2.0 absorbance units full scale in order to optimize location of peaks. Sensitivity of detection of each electrophoretic band was enhanced by staining with Coomassie brilliant blue. (A) Fp1; (B) Fp2; (C) Fp3; (D) Fp4; (E) Fp5.

further characterize these electrophoretic components, Fp1, Fp2, Fp3, Fp4, and Fp5 from day 14.5-15.5 amniotic fluid were separated from each other on large diameter, long gels. Figure 6 indicates that, when each of these electrophoretic components was reapplied to 15-cm analytical polyacrylamide gels, only single peaks were now observed. We could determine that each peak represented the intended α-fetoprotein by mixing it with day 15.5 amniotic fluid and observing its mobility, as in Figure 1 (data not presented).

Effect of Neuraminidase. If each of these five electrophoretic components represented the same α-fetoprotein differing in sialic acid content, then *V. cholerae* neuraminidase should allow a progressive shift in mobility from the more acidic components to the less acidic ones. Indeed this was found. With low concentrations of enzyme, Fp5 was converted to Fp4, Fp3, Fp2, and Fp1; Fp4 was converted to Fp3, Fp2, and Fp1; Fp3 was converted to Fp2 and Fp1. As would be expected from the results with the α-fetoprotein mixtures isolated from different times in development, Fp2 was converted to Fp1 and Fp1 was not altered in its mobility. Similarly, treatment of Fp5, Fp4, or Fp3 with high concentrations of neuraminidase gave rise to Fp3 and Fp1. Fp1 was the only product of treatment of Fp2 or Fp1.

Isoelectric Point and Sialic Acid Content. Polyacrylamide gel electrophoresis separates according to both size and charge. Therefore, to further determine whether these components differ from each other with respect to charge and hence sialic acid content, the isoelectric points and sialic acid contents of these electrophoretic components were measured. To determine their respective p*I* values, isoelectric focusing in polyacrylamide gels was carried out. It soon became obvious that the p*I* values of each α-fetoprotein were so close to each other that using a pH 4-6 gradient would not allow sufficient resolution. Figure 7 shows the results of a representative experiment for each electrophoretic component of α-fetoprotein employing a narrow pH gradient. As can be seen, there is a progressive decrease in the p*I* from Fp1 toward Fp5. Table III indicates that each α-fetoprotein differs by about 0.1 p*I* unit from each other in the series. With such small differences to show this trend it was necessary to use many replicate determinations. Employing a one-tail Student's *t* test, the α-fetoproteins in the series showed statistically significant differences ($p < 0.05$) except for Fp3 when compared with Fp2.

These results indicate that there is a positive correlation with respect to an increase of electrophoretic mobility in polyacrylamide gels and a decrease in isoelectric point by isoelectric focusing. Similarly, as would be expected, the sialic acid content increased progressively from Fp1 to Fp5; the differences were statistically significant with *p* values indicated in Table III.

The sialic acid contents were not integer values using the thiobarbiturate assay, as would have been expected. Since the color test depends on release of the sialic acid moiety from the glycoprotein and it has been shown that O-acetylated derivatives of sialic acid are not released from glycoprotein under these mild acid hydrolyses (Schaurer et al., 1974), mild alkaline hydrolysis of Fp5 was carried out prior to the thiobarbiturate assay (Neuberger and Ratcliffe, 1972). No increase in sialic acid content was observed (see Table III). Furthermore, hydrolysis in 0.1 N H_2SO_4 at 80 °C for up to 6 h also did not increase the value of sialic acid in Fp5 (data not presented).

Examination of the patterns of these α-fetoprotein components on isofocusing gels suggested further microheterogeneity. Fp5 consistently showed two peaks which were termed Fp5a and Fp5b (Figure 7E). The profile of Fp3 and Fp4 frequently

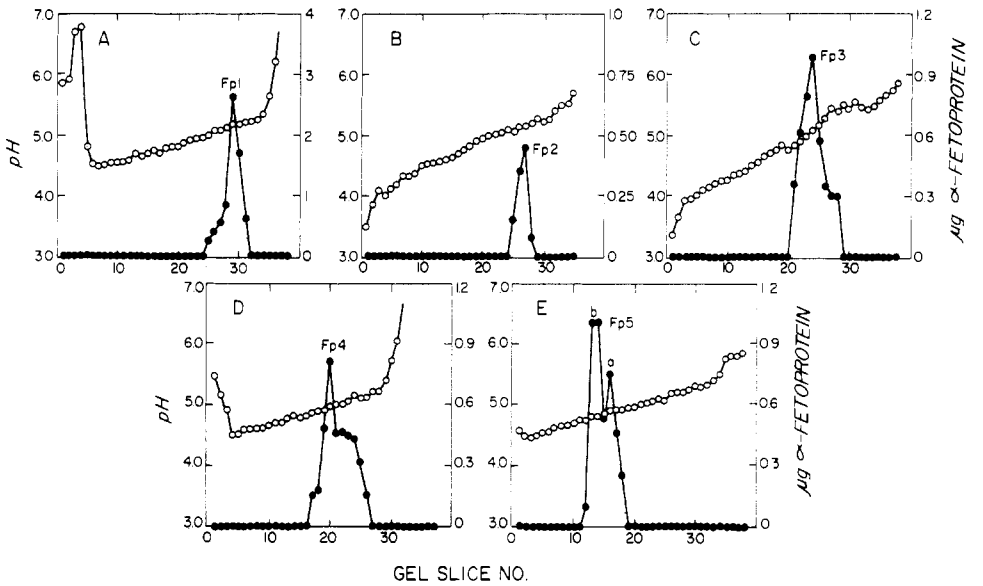

FIGURE 7: Isoelectric focusing of purified Fp1, Fp2, Fp3, Fp4, and Fp5. Representative experiments for each electrophoretic component are shown. (A) Fp1; (B) Fp2; (C) Fp3; (D) Fp4; (E) Fp5. In all experiments performed two peaks of Fp5 were observed and hence termed Fp5a and Fp5b. Similar dual peaks were sometimes observed with Fp3 and Fp4. In other experiments, the more basic component was only observed as a shoulder (e.g., C, D). pH (○) and amount of α-fetoprotein (●) are indicated.

TABLE III: Isoelectric Point and Sialic Acid Content of Fp1, Fp2, Fp3, Fp4, and Fp5.[a]

Sample	p*I*			Sialic Acid[c] (Residues/mol)		
	Mean ± SE	*n*	*p*[b]	Mean ± SE	*n*	*p*
Fp1	5.21 ± 0.032			0.32 ± 0.037	4	
Fp2	5.13 ± 0.028	8	<0.05	1.02 ± 0.048	4	<0.01
			NS			
Fp3	5.05 ± 0.03	8	>0.05	1.37 ± 0.068	4	<0.01
Fp4	4.96 ± 0.04	5	<0.05	1.72 ± 0.14	4	<0.05
Fp5a	4.86 ± 0.021		<0.05			
		8		2.72 ± 0.11	8	<0.01
Fp5b	4.80 ± 0.018		<0.05			

[a] Isoelectric point of each α-fetoprotein component was determined from replicate experiments as described in Figure 7. p*I* values for both Fp5a and Fp5b are presented, whereas those for only the more acidic components of Fp3 and Fp4 are shown. [b] *p* value for one-tail Student's *t* test of two consecutive α-fetoproteins in the series. [c] Four determinations of sialic acid content of Fp5 were carried out by the thiobarbiturate assay after a prior mild alkaline hydrolysis. The ethanol-precipitated Fp5 was dissolved in 0.5 N NaOH and incubated at 4 °C for 2 days. The samples were then neutralized with H_2SO_4 and made to 0.1 N with additional acid. The sialic acid content after alkaline pretreatment was not significantly different from Fp5 samples directly assayed for sialic acid. Therefore all determinations were pooled and a mean value is presented.

showed dual peaks and in other experiments revealed a less acidic peak present as a shoulder (Figures 7C and 7D). The observed p*I* values for Fp5a and Fp5b were 4.86 and 4.80 ($p < 0.05$), respectively (Table III).

Synthesis and Separation of Fp5a and Fp5b. Analyses of human and rat α-fetoprotein also suggest a microheterogeneity (Ruoslahti and Sepällä, 1971; Alpert et al., 1972; Belanger and Dufour, 1974), which appears analogous to that of mouse Fp5a and Fp5b. Therefore, a further analysis of these two components was carried out to determine whether they differ from each other. Due to the limiting quantity of these components normally available, labeled Fp5 was employed as the starting material. In order to separate Fp5a and Fp5b, the labeled α-fetoprotein was chromatographed on a QAE-Sephadex column with a pH gradient of 6.0 to 4.8 (Belanger and Dufour, 1974). Since only a single broad peak of radioactivity was found, aliquots from the leading (pH 5.24) and trailing (pH 5.0) edges of the peak were tested for purity of Fp5a and Fp5b by isoelectric focusing. Figure 8A reveals that in the leading edge there is a predominant peak in the position of Fp5a and only a small amount of radioactivity in the region of Fp5b. Conversely, the trailing edge contained a peak of radioactivity that comigrated with Fp5b (Figure 8B). Little or no radioactivity was associated with Fp5a. These results suggest that at the two edges of the radioactive peak fractionated on QAE-Sephadex, Fp5a and Fp5b were adequately separated from each other.

Next, fractions from each of these areas of the column eluates were pooled, carrier Fp5 was added, and α-fetoprotein was ethanol precipitated. Each fraction was then split into two, incubated in the presence and absence of very high concentrations of *V. cholerae* neuraminidase, and subjected to polyacrylamide gel electrophoresis. Figure 9 shows the results of this experiment. The radioactivity found in each gel was very low. It was observed that both Fp5a and Fp5b migrated with carrier Fp5, as expected. However, after neuraminidase digestion, Fp5a was converted to Fp3. In contrast, after neuraminidase treatment of Fp5b, practically all the radioactivity was now associated with Fp1.

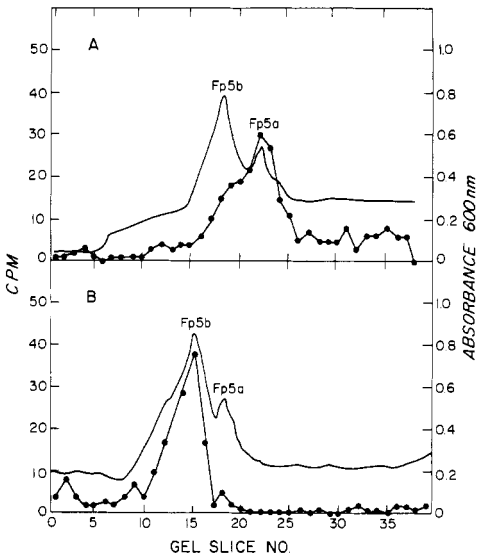

FIGURE 8: Isoelectric focusing of labeled Fp5 separated by QAE-Sephadex chromatography. (A) Aliquots of a fraction at pH 5.28 (5 μg, 300 cpm) and (B) that of a fraction at pH 5.0 (5 μg, 355 cpm) were taken from the chromatographic separation and precipitated with ethanol. The precipitates were subjected to isoelectric focusing as described in Experimental Procedure. The pH range of the ampholyte solution employed was 4.36 to 5.28. After completion of isoelectric focusing, the ampholyte in the gels was removed by placing them in tubes containing 10% trichloroacetic acid and rotating them overnight at 37 °C. The trichloroacetic acid was replaced four times every 2 h and incubation was continued overnight. Protein was stained with Coomassie brilliant blue. Gels were destained and densitometrically scanned. In spite of the extensive elution in trichloroacetic acid, removal of ampholyte was not complete and considerable background staining was encountered. Gels were sliced into 2.2-mm pieces which were placed in minivials and incubated in 0.1 ml of H_2O_2 for 2 h at 56 °C. Two milliliters of Multisol were added and radioactivity was measured. Cpm (●) and absorbance (—) are indicated.

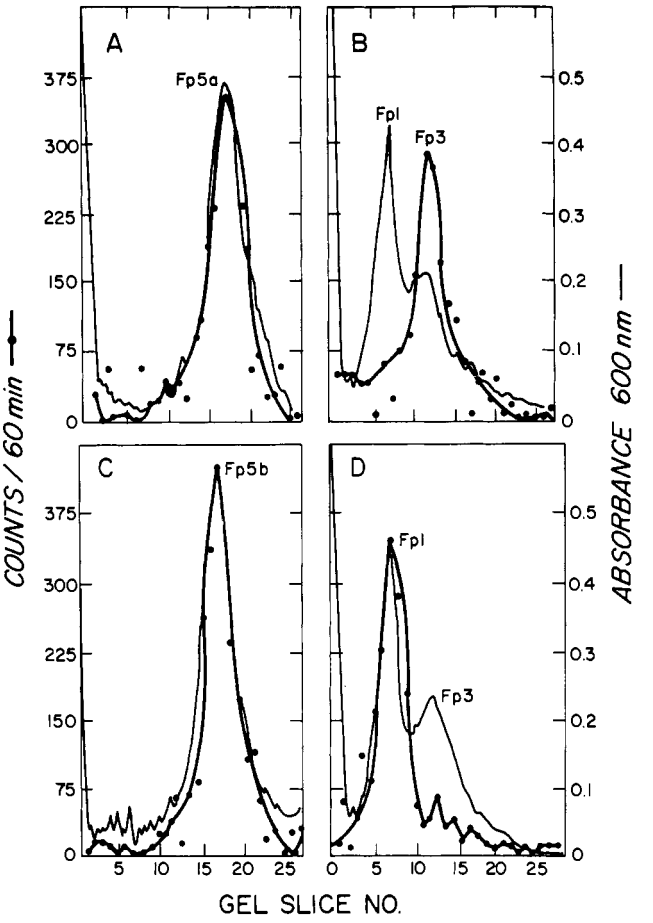

FIGURE 9: Polyacrylamide gel electrophoresis of labeled Fp5a and Fp5b and the effect of *V. cholerae* neuraminidase. Fractions from pH ranges of 5.19–5.24 and 5.00–5.05 from the chromatographic run on QAE-Sephadex were employed for the source of labeled Fp5a and Fp5b, respectively. Each pooled sample contained 25 μg of protein. An additional 75 μg of nonradioactive Fp5 was added to each fraction as carrier and the samples were ethanol precipitated. The pellets were dissolved in 350 μl of 0.2 M sodium acetate, pH 5.6. Aliquots were counted and from the remainder two 130-μl samples were incubated in the absence and presence of neuraminidase (60 units) as previously described. Samples were subjected to electrophoresis in 15-cm gels, which were then stained with Aniline Blue-Black and then diffusion destained. The gels were densitometrically scanned and radioactivity in the region of α-fetoprotein (1.1-mm slices) was measured as described in Figure 8. Since the radioactivity measured was so low (due to poor recovery of samples after ethanol precipitation and low amount of radioactivity as starting material), the radioactivity is expressed as counts/60 min after subtraction of background (455 counts/60 min). (A) Fp5a; (B) neuraminidase-treated Fp5a; (C) Fp5b; (D) neuraminidase-treated Fp5b.

Discussion

The α-fetoproteins from three times of development, which show characteristic electrophoretic variations (Figure 1), were purified by polyacrylamide gel electrophoresis. This single step of purification was sufficient to partially characterize these proteins on the basis of the following results. (a) Upon reelectrophoresis in polyacrylamide gels, the predominant bands were the α-fetoproteins; transferrins and albumin, the other major plasma and amniotic fluid proteins, were no longer present (Figures 2A–C). (b) Electrophoresis in sodium dodecyl sulfate–polyacrylamide gels revealed only a single molecular species of each α-fetoprotein. Ruoslahti and Sepällä (1971) found the same aggregation phenomenon with human α-fetoprotein.

The electrophoretic variation in the purified α-fetoproteins could be shown to be due to microheterogeneity by the following criteria. (a) Each α-fetoprotein mixture (Fpl–3, Fpl–5, Fp5) had an identical apparent molecular weight of about 70 000, which is in reasonable agreement with the value of total mouse α-fetoprotein of 74 000 previously described (Pihko and Ruoslahti, 1973). In addition, each protein in the mixtures (Fpl, Fp2, etc.) had the same molecular weight since only one band in sodium dodecyl sulfate–polyacrylamide gels could be discerned (Figures 2D–F). (b) Each α-fetoprotein mixture, including Fp5 which did not contain the other α-fetoproteins, immunologically cross-reacted with the monospecific Fpl–3 antibody. Positive reactions were found by immunodiffusion (Gustine and Zimmerman, 1972a), immunoelectrophoresis (Figure 3), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitated unlabeled α-fetoprotein mixtures as well as labeled Fp4 and Fp5 (Gustine and Zimmerman, 1973). (c) Each α-fetoprotein mixture exhibits a similar, if not identical, amino acid composition (Table I). (d) Each α-fetoprotein preparation has a similar carbohydrate composition, except for sialic acid (Table II). (e) Neuraminidase converted the faster moving α-fetoproteins to their slower moving counterparts in polyacrylamide gels (Gustine and Zimmerman, 1972a, 1973).

Further evidence that these α-fetoproteins differ by microheterogeneity with respect to sialic acids derives from the experiments with the purified Fpl, Fp2, Fp3, Fp4, and Fp5 isolated from day 14.5–15.5 amniotic fluid. (a) *V. cholerae* neuraminidase converted the faster moving α-fetoproteins to their respective slower moving counterparts (Figure 6). (b) The

faster moving α-fetoproteins showed proportionately lower isoelectric points than the slower moving ones in the series (Figure 7, Table III). The p*I* values of Fp5b (4.80) and Fp5a (4.86) are in close agreement with that of 4.7 for α-fetoprotein isolated from fetal mouse plasma during late development (Pihko and Ruoslahti, 1973). (c) The faster moving electrophoretic components were observed to have a proportionately greater sialic acid content (Table III).

α-Fetoprotein has been demonstrated in all species examined (Gitlin and Boesman, 1967). These present studies reveal additional similarities of mouse α-fetoprotein to the human protein. Not only does the mouse protein immunologically cross-react with the human one (Abelev, 1968), but it has about the same molecular weight of 70 000 to 72 000 as the human[4] (Ruoslahti and Sepällä, 1971; Alpert et al., 1972). Furthermore the two α-fetoproteins show similar amino acid and carbohydrate compositions, including that of sialic acid when mouse Fp5 is compared with the human species. The slower moving mouse proteins contain lesser amounts of sialic acid according to their "biological maturity". Another striking similarity of Fp5 and human α-fetoprotein is their own microheterogeneity; both can be separated into two main components by isoelectric focusing (Ruoslahti and Sepällä, 1971; Alpert et al., 1972) (Figure 7E). However, the mouse and human embryonic proteins are not identical since they show marked differences in their electrophoretic mobility; the human protein moves just behind the albumin in most electrophoretic systems (Uriel, 1969).

It would seem likely that the maximally sialylated mouse α-fetoprotein fits a four sialic acid model: Fp5, 4 sialic acid residues; Fp4, 3; Fp3, 2; Fp2, 1; Fp1, 0. These proposed structures are based on the sialic acid content found in each electrophoretic component (Table III) and the fact that *V. cholerae* neuraminidase converted Fp5 to Fp4, which was converted to Fp3, then to Fp2, then to Fp1. However, determination of the sialic acid content by the thiobarbituric acid method did not yield integer numbers. The values obtained for each α-fetoprotein were consistently less than expected; Fp5 contained 2.2–2.7 sialic acids. Sialic acid is known to contain various substituent groups: *N*- and *O*-acetyl, *N*- and *O*-glycolyl with one to three substituent groups on the neuraminic acid. Various modifications of the sialic acids on α-fetoprotein might explain the less than expected value by the color test. The presence of 4-*O*-acetyl group causes a decrease in the color yield with the thiobarbiturate reaction (Schaurer et al., 1974). However, the 4-*O*-acetyl group is readily hydrolyzed under alkaline conditions, yet, when Fp5 was alkali treated, no increased color yield was obtained. In any case, there appears to be a greater complexity in these sialylated derivatives, other than variation in number of sialic acid residues. First Fp5 could be further separated into two components Fp5a and Fp5b, and Fp4 and Fp3 showed varying amounts of these comparable acidic and basic components, which could not be attributed to contamination of the α-fetoproteins in the series. Fp2 was sensitive to *V. cholerae* but not to clostridial neuraminidase. Finally it was shown that Fp5b, after *V. cholerae* digestion, was converted to the asialoglycoprotein, Fp1, while Fp5a was incompletely desialylated (Figure 9). The explanations for these effects are as yet unknown. However, neuraminic acid derivatives with different substituent groups show different specificities to neuraminidase (Schaurer et al., 1974). In fact, preliminary experiments on Fp5 show the presence of both *N*-acetyl and *N*-glycolyl neuraminic acids (Zimmerman and Bowen, unpublished observations). In addition, these differences among the α-fetoproteins could be due to various linkages of sialic acid to the penultimate sugar (Hudgin and Schachter, 1972) (e.g., 2 → 3, 2 → 6), due to attachment to different sugars, and/or attachment to core and peripheral portions of the oligosaccharide (Carlson, 1968).

[4] By analogy with the human α-fetoprotein, it would appear that mouse α-fetoprotein is a monomer since human α-fetoprotein was shown to be monomeric. The same molecular weight was observed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Sephadex G-200 chromatography (Ruoslahti and Sepällä, 1971).

Acknowledgment

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Characterization and Isolation of Proteolytically Modified Nerve Growth Factor†

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ABSTRACT: The peptide chains of β nerve growth factor (βNGF) were shortened at their NH_2 termini by cleavage with a specific submaxillary gland endopeptidase(s) after histidine in position number 8 and the modified proteins characterized by sequence analysis, electrophoresis in the presence of sodium dodecyl sulfate, and by nonequilibrium isoelectric focusing. The NH_2-terminal octapeptide released by the endopeptidase was recovered in 11% yield. Removal of >97% of the NH_2-terminal octapeptide sequences from βNGF had no effect on its dimeric structure, its biological activity, or its ability to reform the 7S NGF complex with stoichiometric amounts of the α and γ subunits. Bisdes(1-8)-βNGF proteins lacking from 15 to 90% of their COOH-terminal arginine residues were prepared by altering the pH, temperature, or salt concentration at which βNGF was incubated with various fractions of the submaxillary gland homogenate. A rapid procedure for the isolation of NGF was devised which involved a two-column fractionation of crude extracts of adult male mouse submaxillary glands, taking advantage of the differing isoelectric points of the parent 7S NGF complex and its βNGF subunit. During isolation by this procedure the NGF dimer lost 17-20% of its COOH-terminal arginine residues and 35% of its NH_2-terminal octapeptide sequences. A method for determining the proportion of NGF chains which have lost COOH-terminal arginine residues or NH_2-terminal octapeptide sequences, or both, was developed. The method depended on the observation that intact chains, which contain two extra histidine residues compared with chains lacking the NH_2-terminal octapeptide, migrated more rapidly than the cleaved chains in the acidic region of the pH gradient established for isoelectric focusing.

Several different methods of isolating the nerve growth factor (NGF)[1] protein from the submaxillary glands of adult male mice have been described (Varon et al., 1968; Bocchini and Angeletti, 1969; Perez-Polo and Shooter, 1975; Jeng and Bradshaw, 1976). In one of them (Varon et al., 1968) the protein, βNGF, is isolated as one of the subunits of the purified 7S complex (Varon et al., 1967). In the other methods the NGF protein is isolated directly from the gland homogenate without prior separation of the complex. These proteins do not have exactly the same structure because the βNGF dimer undergoes two specific proteolytic cleavages when exposed to enzymes in the submaxillary gland extract. One of these cleavages results in the removal of either or both the COOH-terminal arginine residues from the two identical peptide chains in the NGF dimer (Angeletti et al., 1973; Moore et al., 1974). The second results in the production of chains which are shorter by eight amino acid residues at their NH_2 termini than the βNGF chains (Angeletti and Bradshaw, 1971; Angeletti et al., 1973; Mobley et al., 1974).

The properties of the βNGF dimers which lack one or both COOH-terminal arginine residues have been studied in detail (Moore et al., 1974). While complete removal of these residues does not affect the biological activity of βNGF (Moore et al., 1974), it does prevent the protein from reassociating with the other α and γ subunits of 7S NGF to reform the high-molecular-weight complex, a finding which argues in favor of the existence of a longer precursor to the βNGF chain (Angeletti and Bradshaw, 1971; Moore et al., 1974; Berger and Shooter, 1976). This report now describes a method for the complete

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[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; NGF, nerve growth factor; βNGF, the preparation made according to the procedure of Varon et al. (1968); βNGF dimer and βNGF chains, the intact βNGF dimer and βNGF chains, respectively; mono- and bisdes(1-8)-βNGF, the βNGF dimers lacking one and both, respectively, of the NH_2-terminal octapeptide sequences; mono- and bisdes-$Arg^{1\,8}$-βNGF, the βNGF dimers lacking one and both, respectively, of the COOH-terminal arginine residues; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Bis, *N,N'*-methylenebisacrylamide; Tris, tris(hydroxymethyl)aminomethane; CPB, carboxypeptidase B; endopeptidase, the endopeptidase which specifically cleaves the histidine-methionine peptide bond—the eighth peptide bond from the NH_2 terminus of the βNGF chain; CM, carboxymethyl.