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## Purification and immunochemical characterization of alpha-fetoprotein from rat fetal serum and liver

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# Purification and immunochemical characterization of alpha-fetoprotein from rat fetal serum and liver\*

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## Abstract

Two alpha<sub>1</sub>-globulin bands of fetal serum with relative mobilities against bromophenol blue of 0.55 and 0.58 on 7% polyacrylamide gel electrophoresis reacted with a monospecific rabbit antiserum to alpha-fetoprotein (AFP). The former globulin band was clearly detected in the fetal liver supernatant. AFP was immunochemically purified from both the fetal serum and liver, and their electrophoretic and immunochemical properties were compared. Liver AFP purified by immunoabsorbent column yielded electrophoretic mobilities and relative amounts of the two electrophoretically distinct components identical with the purified serum AFP. The immunological reactivity of the two components of the purified preparations from serum and liver against the monospecific anti-AFP serum was also indistinguishable. After the removal of the sialic acid residues from purified serum and liver AFP by treatment with neuraminidase for 6 to 12 hr, disc electrophoretic patterns on 5% polyacrylamide gel and immunoelectrophoretic patterns of the treated AFP were found to be closely similar in both preparations. It may be possible to conclude that serum and liver AFP are structurally indistinguishable and probably identical.

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**PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION OF ALPHA-FETOPROTEIN FROM RAT FETAL SERUM AND LIVER**

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*Abstract:* Two alpha<sub>1</sub>-globulin bands of fetal serum with relative mobilities against bromophenol blue of 0.55 and 0.58 on 7% polyacrylamide gel electrophoresis reacted with a monospecific rabbit anti-serum to alpha-fetoprotein (AFP). The former globulin band was clearly detected in the fetal liver supernatant. AFP was immunochemically purified from both the fetal serum and liver, and their electrophoretic and immunochemical properties were compared. Liver AFP purified by immunoabsorbent column yielded electrophoretic mobilities and relative amounts of the two electrophoretically distinct components identical with the purified serum AFP. The immunological reactivity of the two components of the purified preparations from serum and liver against the monospecific anti-AFP serum was also indistinguishable. After the removal of the sialic acid residues from purified serum and liver AFP by treatment with neuraminidase for 6 to 12 hr, disc electrophoretic patterns on 5% polyacrylamide gel and immunoelectrophoretic patterns of the treated AFP were found to be closely similar in both preparations. It may be possible to conclude that serum and liver AFP are structurally indistinguishable and probably identical.

Rat alpha-fetoprotein (AFP) purified from fetal serum (1) or adult serum with hepatoma (2) was separated into two immunologically identical components with different charges by conventional polyacrylamide gel electrophoresis. Our previous studies of their physicochemical and immunological properties (3) have revealed that AFP<sub>a</sub> and AFP<sub>b</sub>, the two AFP components of rat, contain at least 2.5 and 4.5 molecules of sialic acid per mole of fetoprotein, respectively, and that negative charges other than the carboxyl groups of sialic acid are required for AFP<sub>a</sub> to move faster than AFP<sub>b</sub> at an alkaline pH by disc electrophoresis.

It has been established that AFP is synthesized mainly in the fetal liver with a trace production in the yolk sac of rats (4). However, whether the two components of rat AFP are synthesized *de novo* in the fetal liver under a genetically controlled mechanism or modified in charge after synthesis is

unknown at present. The binding of sialic acid, hexoseamine and other hexoses to serum glycoproteins occurs after the synthesis of proteins and may affect their metabolic function and degradation (5, 6). These considerations led us to investigate the electrophoretic and immunological properties of AFP circulating in the blood and the newly synthesized AFP in the liver. In this report, the results of comparative studies on the immunochemical properties of serum and liver AFP prepared by an immunoadsorbent column are described.

#### MATERIALS AND METHODS

*Animals:* Sprague-Dawley rats were used in the present experiment unless otherwise stated. Rat fetuses were obtained by cesarean section of female rats of the same strain at 15 20th days of pregnancy. The serum and liver samples were collected after decapitation. The preparation of liver supernatant was carried out as described in our previous paper (7).

*Preparation of antiserum:* Male rabbits were immunized by subcutaneous injections of pooled fetal serum, and a monospecific anti-AFP serum was obtained by absorbing 30 ml of whole antifetal-serum-immune serum with 10 ml of pooled adult serum and further with 13 ml of cadmium chloride-poisoned rat serum, as described previously in detail (8). Immunological properties of anti-AFP serum thus obtained have already been reported (8).

*Analytical techniques:* The following techniques were employed as reported originally—vertical disc electrophoresis on 5 or 7% polyacrylamide gels at 4°C for 60 70 min with a constant current of 4 mA per tube (5×90 mm) (9), relative mobilities against bromophenol blue ( $R_{FPB}$ ) as a marker being determined for identification of AFP components; immunoelectrophoresis (10); agar double-diffusion test (11); and determination of AFP concentration by a double-antibody technique with  $^{131}\text{I}$  or  $^{125}\text{I}$ -labeled AFP (12). The protein was stained with Amido Schwarz. Densitometric profiles were recorded by a Fujiox densitometer.

*Neuraminidase treatment of AFP:* In incubation of AFP with neuraminidase (type VI, chromatographically purified from *Clostridium perfringens*) at 37°C for 6 hr, 0.64 unit of the enzyme per mg AFP at a final concentration of 0.5 mg per ml was used. However, for 12 hr-treatment, 20 milliunits of fresh enzyme were further added after 6 hr of incubation to achieve complete hydrolysis.

*Materials:* Freund's complete adjuvant and Noble agar were purchased from Difco Laboratories Inc. and neuraminidase from Sigma Chemical Co. Cyanogen bromide-activated Sepharose 4B and Sephadex G-200 were purchased from Pharmacia Fine Chemicals AB. Other reagents used were purchased from sources reported previously (1, 3, 7, 8).

#### RESULTS

*Electrophoretic and immunochemical properties of fetal serum and liver supernatant proteins:* Disc electrophoretic patterns of fetal serum and liver

supernatant proteins were compared to those of the respective adult proteins. The presence of two bands with  $R_{BPB}$  of 0.55 and 0.58, which were located between albumin and  $\alpha_1$ -globulin, was the most outstanding feature in fetal serum (Fig. 1). The fetal liver proteins yielded only the former protein

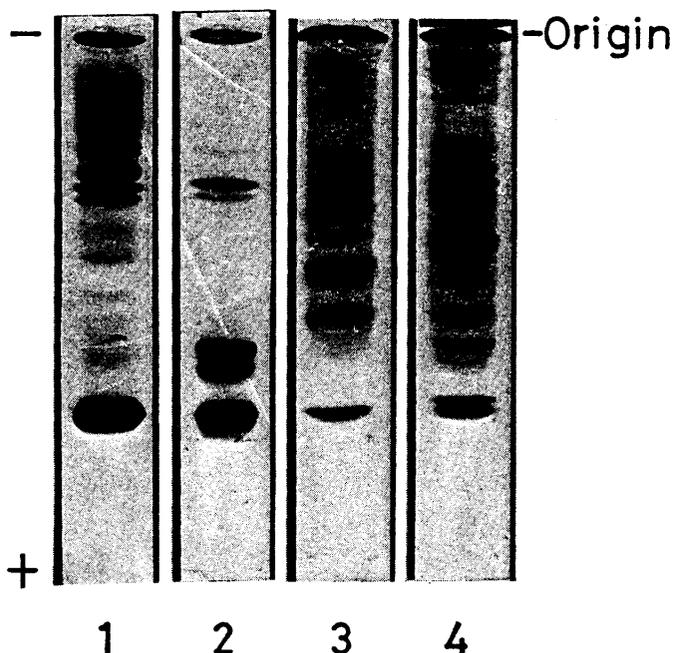


Fig. 1. Disc electrophoretic patterns of serum and liver supernatant proteins from adult and fetal rats. Three  $\mu$ l of serum and 10  $\mu$ l of liver supernatant were put on 7% polyacrylamide gel columns and electrophoresed for 60 min at 4°C and 4 mA/tube. Samples applied on columns were as follows: 1, adult serum; 2, fetal serum; 3, liver supernatant of adult rat; and 4, liver supernatant of fetal rat. Proteins were stained with Amido Schwarz.

band in the  $\alpha_1$ -globulin region. These protein bands absent in adult serum and liver supernatant were more clearly demonstrated in densitometric profiles (Fig. 2). Disc electrophoretic location of AFP in fetal serum and liver supernatant was demonstrated by acrylamide gel immunoelectrophoresis with a monospecific anti-AFP serum (Fig. 3). The single precipitin arcs of AFP were formed behind the albumin band, corresponding to the bands of protein absent in the adult serum and liver supernatant. These protein bands which were seen on the conventional polyacrylamide gel are thus at least partly AFP.

*AFP contents in fetal serum and liver:* The AFP concentration in the

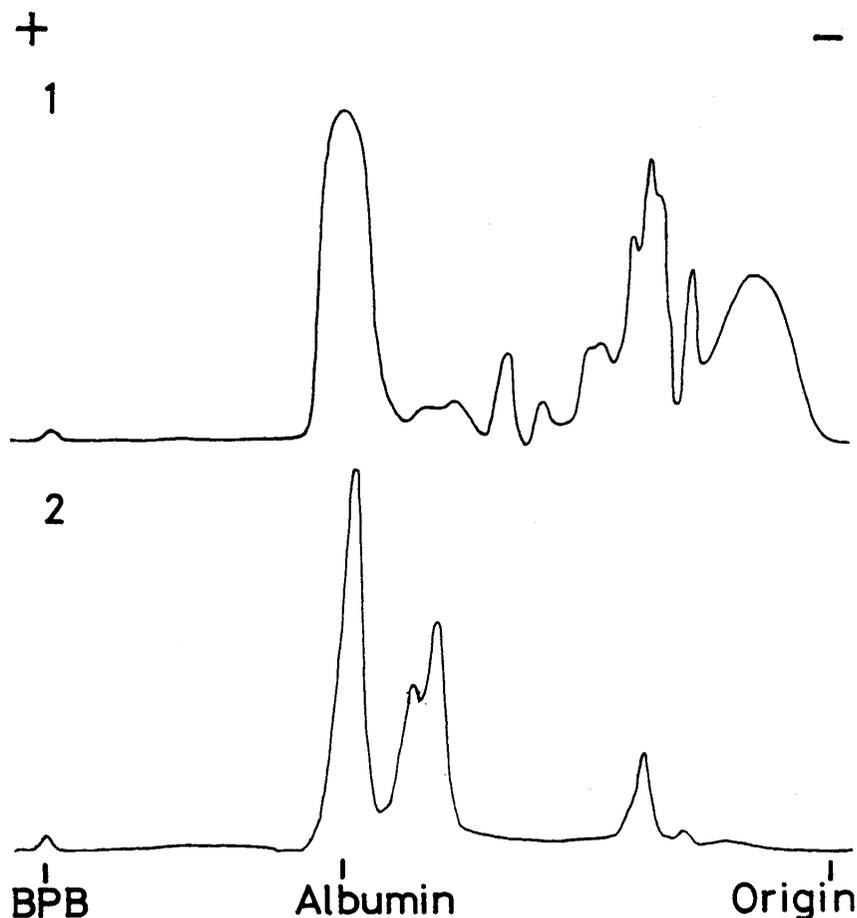
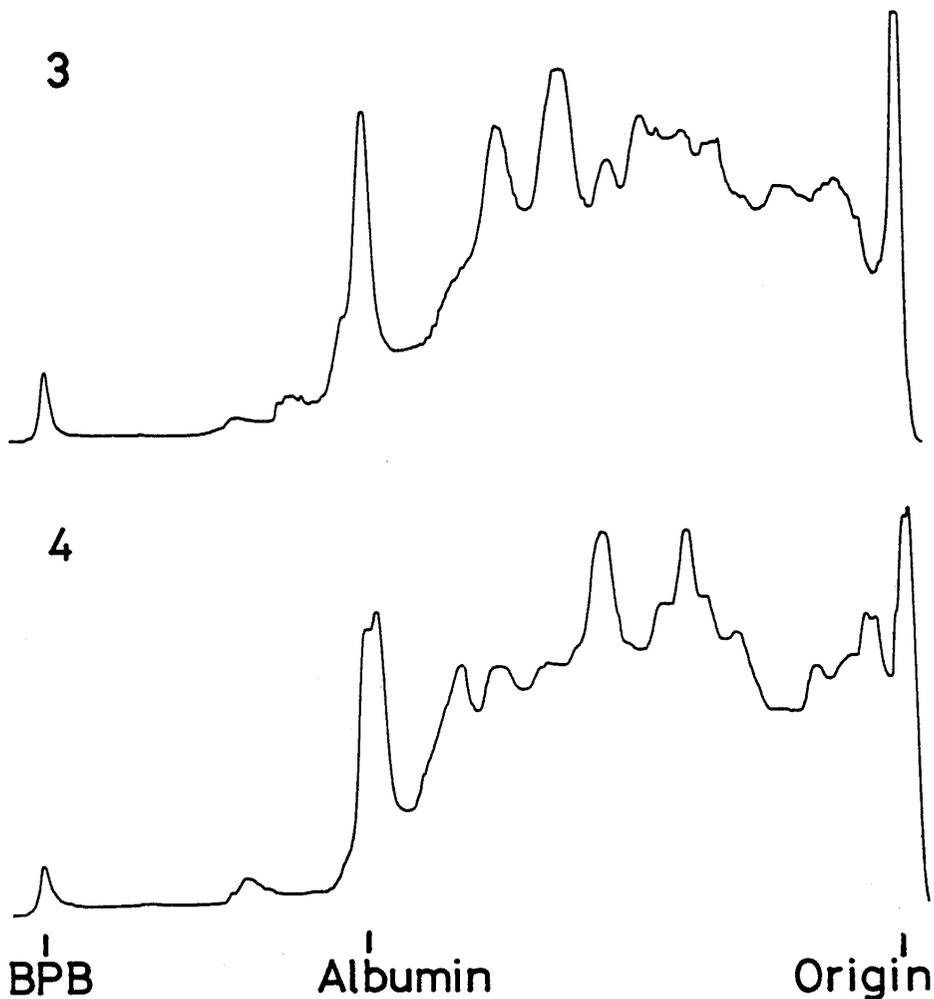


Fig. 2. Densitometric profiles of serum and liver supernatant proteins from adult and fetal rats on polyacrylamide gel electrophoresis. The stained gels shown in Fig. 1 were scanned from left (bottom) to right (top) by a densitometer. Electrophoretic locations of BPB and albumin are shown at the bottom of the figure. The numbers on the densitometric profiles correspond to those of gels shown in Fig. 1.

serum of fetal rats used in the experiment was estimated to be 6.8 mg/ml and 9.2 mg/ml by disc electrophoresis and radioimmunoassay techniques, respectively. On the other hand, the liver AFP level of the same fetal rats was determined to be approximately 2.1 mg and 1.2 mg/g wet liver weight, respectively, by the same two techniques.

*Purification of AFP*: Rat AFP was purified from pooled fetal serum and liver by using an immunoadsorbent column of Sepharose 4B coupled with



rabbit antibody to rat serum AFP by means of cyanogen bromide. For this purpose, an antibody-rich gamma-globulin fraction was isolated from specific anti-AFP serum by conventional ammonium sulfate fractionation (see Fig. 4, Column 1) and lyophilized. The lyophilized gamma-globulin fraction (1.3 g protein) was dissolved in 30 ml of 0.1 M  $\text{NaHCO}_3$ , added to Sepharose 4B (7.5 g) activated previously with cyanogen bromide and mixed mechanically for 6 hr at  $4^\circ\text{C}$ . The Sepharose-antibody conjugate was then washed extensively with saline and packed in a column ( $2 \times 50$  cm). Nearly complete adsorption of gamma-globulin on Sepharose 4B in this step was confirmed by analysis of unadsorbed proteins (Fig. 4, Column 2). The AFP fraction

obtained from fetal serum (Fig. 4, Column 3) or liver supernatant by 43% saturation of ammonium sulfate was applied to the column coupled with the antibody-rich globulin. The column was washed exhaustively with saline until no protein was eluted. The protein washed out in this step was mostly albumin, and AFP was specifically bound to anti-AFP-Sepharose 4B (Fig. 4, Column 4). AFP was then eluted with 0.1 M glycine-HCl buffer, pH 2.8, at a flow rate of 150 ml/hr and immediately neutralized with 0.5 M phosphate buffer, pH 7.3. The AFP fraction was concentrated by ultrafiltration and further purified by gel filtration on a Sephadex G-200 column, which was equilibrated with saline, to remove small amounts of contaminating proteins

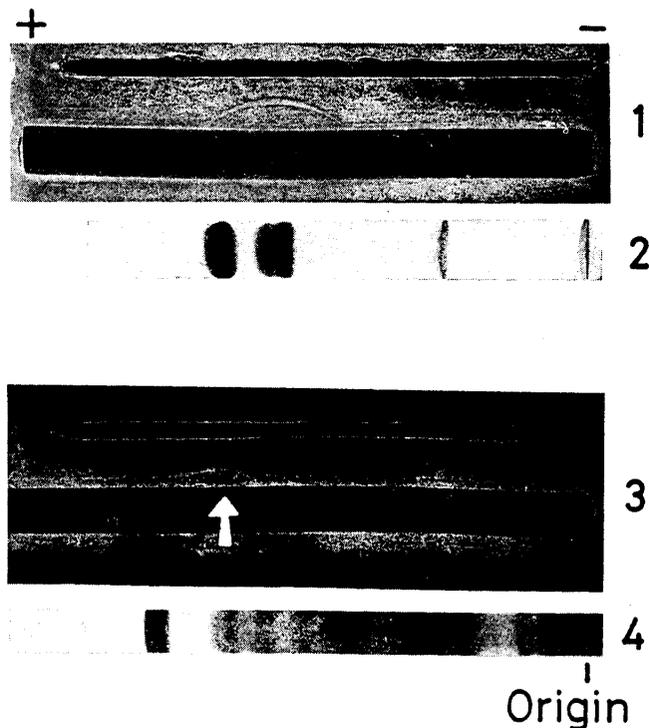


Fig. 3. Disc electrophoretic location of AFP in fetal serum and liver supernatant on polyacrylamide gels. Three  $\mu$ l of fetal serum (Plates 1 and 2) and 10  $\mu$ l fetal liver supernatant (Plates 3 and 4) were applied on 7% polyacrylamide gels and electrophoresed, as described in the legend to Fig. 1. Electrophoresed gels were stained for protein with Amido Schwarz (Plates 2 and 4). Identical gels were placed on glass plates and covered with 1.5% Difco-Noble agar at 50°C (Plates 1 and 3). Double-diffusion test was performed after the specific anti-AFP serum was put into troughs of Plates 1 and 3. A faint precipitin line for liver supernatant is indicated by the arrow (Plate 3).

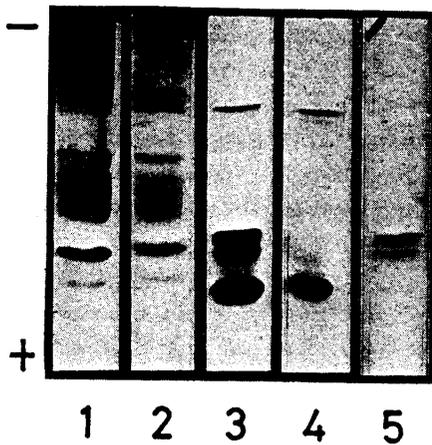


Fig. 4. Disc electrophoretic patterns of protein preparations from anti-AFP serum and fetal serum obtained during purification of rat AFP. Each 50-120  $\mu\text{g}$  of protein sample was put on 7% polyacrylamide gel column and electrophoresed. Samples applied were as follows: 1, antibody-rich gamma-globulin fraction isolated from monospecific anti-AFP serum by conventional ammonium sulfate fractionation; 2, unadsorbed proteins on Sepharose 4B from antibody-rich gamma-globulin fraction; 3, AFP-rich fraction of fetal serum isolated by 43% saturation of ammonium sulfate; 4, unadsorbed fraction from AFP-rich fraction of fetal serum on anti-AFP antibody-bound Sepharose 4B; and 5, a purified AFP preparation eluted with 0.1 M glycine buffer, pH 2.8, from a column containing antigen and antibody-bound Sepharose 4B.

respectively (Fig. 4, Column 5). Identical results were obtained with similarly purified liver AFP, although the crude liver supernatant revealed only one protein band lacking in adult liver supernatant. The protein band corresponding to  $R_{\text{BPB}}$  of 0.58 found in fetal serum would also be present in the fetal liver supernatant but was not distinctly observed on stained gels because of the much lower AFP content as compared with other liver proteins. The fast and slow migrating components are referred to as AFP<sub>a</sub> and AFP<sub>b</sub>, as previously reported (1, 3). AFP<sub>a</sub> and AFP<sub>b</sub> which were electrophoretically separated from the purified AFP reacted identically with the antiserum in the Ouchterlony test, indicating no immunological difference between the two components.

*Electrophoretic and immunochemical properties of purified AFP from fetal*

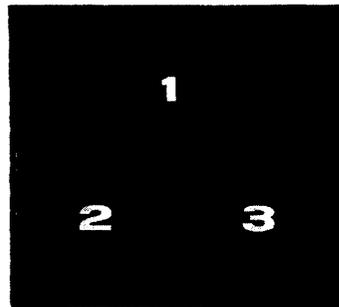


Fig. 5. Immunological identity of AFP purified from fetal serum and liver. Each 100  $\mu\text{g}$  of AFP purified from fetal serum and liver was electrophoresed on 7% polyacrylamide gel. Portions of electrophoresed gels behind BPB-bound albumin and corresponding to the position of AFP migration were cut in 5 mm thickness and placed into the outer larger wells (Wells 1 and 2). Portions of the gel at origin were cut out similarly and put into the remaining outer well (Well 3) as a control. The center well contained specific anti-AFP serum.

eluted in the void volume of the column.

Disc electrophoresis of the purified AFP from fetal serum on 7% polyacrylamide gels revealed two components with  $R_{\text{BPB}}$  of 0.58 and 0.55; the relative amounts of each component being 35% and 65%, respectively

*serum and liver*: The purified preparations of AFP from fetal serum and liver gave single fused precipitin lines against the antiserum in Ouchterlony double-diffusion analysis (Fig. 5).

Disc electrophoresis of the purified serum AFP treated with neuraminidase for 6 hr showed a clear separation into two bands with slower mobilities; each of these bands appeared to be further composed of subcomponents. We have previously demonstrated further heterogeneity of the neuraminidase-treated serum AFP by using disc electrophoresis with 5% acrylamide monomer (3). Therefore, the microheterogeneity of liver AFP treated with neuraminidase was also investigated by employing this analytical procedure. Electrophoresis of the purified liver AFP treated for 12 hr with neuraminidase also showed a clear separation into two bands with slower mobilities;  $R_{BPB}$  values of untreated AFPa and AFPb were 0.88 and 0.85, respectively, and those of treated components were 0.83 and 0.76, respectively (Fig. 6, Columns 3 and 4). The results were virtually identical to those with purified serum

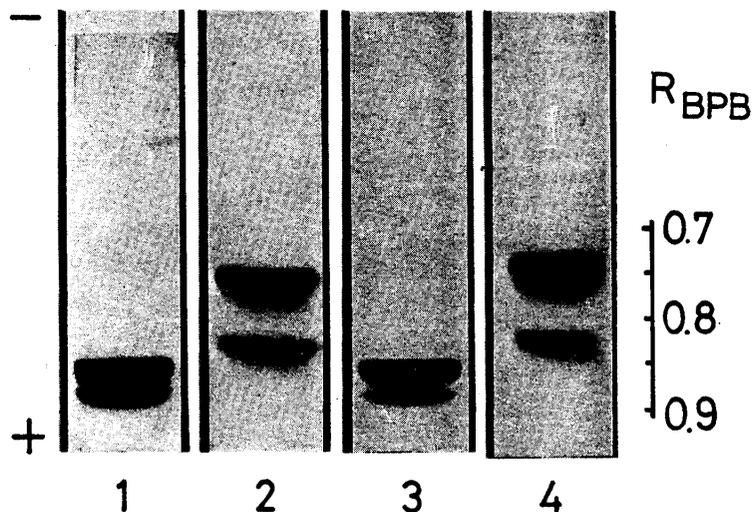


Fig. 6. Effect of neuraminidase treatment on disc electrophoretic mobilities of the two components of AFP purified from fetal serum and liver. Forty  $\mu$ g of AFP were electrophoresed on 5% polyacrylamide gels for 70 min. Neuraminidase treatment was carried out at 37°C for 12 hr as described under Materials and Methods. 1, untreated AFP purified from serum; 2, neuraminidase-treated AFP from serum; 3, untreated AFP purified from liver; and 4, neuraminidase-treated AFP from liver. Neuraminidase ran off the gels after electrophoresis under the tested condition.

AFP (Fig. 6, Columns 1 and 2). The time dependent conversion of faster into slower migrating components of AFPa ( $R_{BPB}$ , 0.88  $\rightarrow$  0.83) and AFPb

( $R_{BFB}$ , 0.85  $\rightarrow$  0.76) was previously observed with neuraminidase upon incubation for varying periods of time, and no interconversion of these two components was confirmed (3).

Immunoelectrophoresis of liver AFP treated for 6 hr with neuraminidase revealed single precipitin lines with much slower mobilities, as obtained with purified serum AFP (Fig. 7). This is a further confirmation of the fact that purified AFP from both serum and liver similarly reacted with anti-AFP

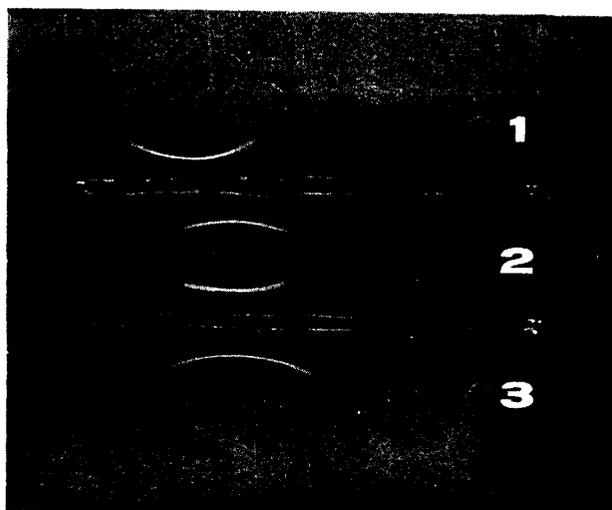


Fig. 7. Immunoelectrophoresis of neuraminidase-treated AFP purified from fetal serum and liver. Purified AFP was treated for 6 hr with neuraminidase. 1, untreated AFP purified from serum; 2, neuraminidase-treated AFP purified from liver; and 3, neuraminidase-treated AFP from serum. Electrophoresis was carried out at room temperature for 100 min on 1.5% agarose buffered at pH 8.6 with 12.5 mM barbital buffer. The electrophoresed plate received anti-AFP serum in the troughs.

serum even when AFP was treated with neuraminidase (see Fig. 5). A distinct immunoelectrophoretic separation of the two components in the purified serum AFP treated with neuraminidase for 12 hr was successful on the agarose plate (3).

Four bands of purified serum and liver AFP ( $R_{BFB}$ ; 0.85, 0.83, 0.78 and 0.76) obtained by treatment with neuraminidase for 6 hr upon electrophoresis were compared (Fig. 8). The relative amounts of protein in AFPa and AFPb were found to be 33% and 67%, respectively, for serum AFP and 36% and 64%, respectively, for liver AFP.

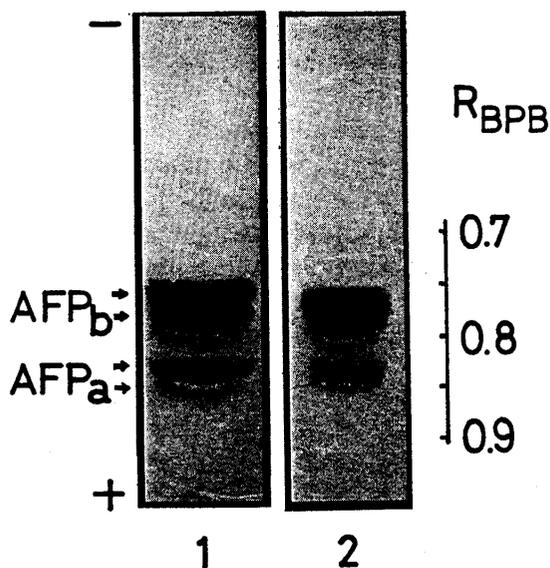


Fig. 8. Disc electrophoretic patterns of neuraminidase-treated AFP purified from fetal serum and liver. AFP was treated for 6 hr with neuraminidase. Five percent polyacrylamide gels were used for electrophoresis of treated AFP.

#### DISCUSSION

Carbohydrate analysis of rat AFP purified from fetal serum has revealed that AFP is a plasma glycoprotein (13). If attachment of carbohydrate moieties to AFP occurs by various glycosyltransferases after the release of newly synthesized AFP from the ribosome, the carbohydrate units of AFP present in liver may differ from those of circulating AFP in blood. This is an interesting problem to study since the different numbers of terminal sialic acid residues of AFP in liver and circulating AFP may give different half lives, functions and regulatory mechanisms in AFP synthesis. Thus, we have initiated a study on the microheterogeneity of AFP in liver, where it is mostly synthesized, in an attempt to determine whether there are differences between the liver and circulating plasma AFP. The results described above show that the fetal serum and liver AFP are immunochemically and electrophoretically indistinguishable from each other, even when the AFP was treated with neuraminidase for various periods of time.

Many glycoproteins exhibit microheterogeneity due to variations in the type and amount of carbohydrates attached to the polypeptide chains (14, 15). In some samples of glycoprotein microheterogeneity have been related to the variation in the amount of bound sialic acid (16, 17). Treatment of fetuin (17) and human AFP (16, 18) with neuraminidase reduced the number of the

electrophoretic bands, indicating that sialic acid is primarily responsible in these cases for the observed microheterogeneity. However, the complete removal of sialic acid from rat AFP revealed rather a more distinct separation of the two components of fetoprotein, suggesting that structural features other than those involving sialic residues must play an important role in determining these two components. The differences in the number or arrangement of amide groups, disulfide cross linkages or carbohydrate portions other than sialic acid have been suggested as being responsible for the observed microheterogeneity (3).

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