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Developmental Changes in Microheterogeneity of Foetal Plasma Glycoproteins of Mice

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Changes in microheterogeneity of foetal plasma glycoproteins during development of mouse embryos were investigated. Analysis of foetal plasma by polyacrylamide-gel electrophoresis indicated three major zones of proteins: (1) transferrins, (2) α-foetoproteins and (3) albumin. Three transferrins (Tr1, Tr2, Tr3) and five α-foetoproteins (Fp1, Fp2, Fp3, Fp4, Fp5) were resolved. Evidence for the presence of transferrins was the binding of ^{59}Fe to the three electrophoretic variants. By day 15.5 of gestation, there was a marked increase in the more-acidic components (Tr3, Fp4, Fp5) and a decrease in the less-acidic ones (Tr1, Tr2, Fp1, Fp2, Fp3). Treatment of foetal plasma with neuraminidase at this time of development converted the more acidic components into Tr1 and Tr2 and Fp1, Fp2 and Fp3. Furthermore, it was shown that early in development (day 12.5) only the less-acidic components of transferrin and α-foetoprotein were synthesized; at the later time in development (day 14.5) new synthesis of the acidic components of both groups occurred. That these more-acidic components of α-foetoprotein (Fp4, Fp5) were in fact electrophoretic variants of the less-acidic α-foetoproteins was shown by the immunoprecipitation of labelled Fp4 and Fp5 with anti-Fp1, anti-Fp2 and anti-Fp3. From these results it is postulated that the plasma glycoproteins that are synthesized later in development contain increased amounts of sialic acid and that the observed changes in microheterogeneity of these proteins represent regulation of glycoprotein biosynthesis at the level of carbohydrate attachment.

Very little is known about the regulation of glycoprotein synthesis. Many glycoproteins exhibit variations in the type and amount of carbohydrate attached to their polypeptide chain (microheterogeneity). Although synthesis of the polypeptide chain is under direct genetic control, the attachment of carbohydrate moieties probably is not (Spiro, 1969, 1970; Marshall & Neuberger, 1970). Rather, this process is one step removed: the glycosyltransferases are synthesized under direct genetic control and then their subsequent activities are somehow regulated (Spiro, 1969, 1970). Spiro (1969) has suggested that the various transferase activities form the basis for the microheterogeneity found in glycoproteins. Some examples of this microheterogeneity have been related to various amounts of sialic acid present in a glycoprotein: the electrophoretic forms of human prostatic acid phosphatase (Smith & Whitby, 1968) and organ-specific variations of human acid phosphatase (Beckman & Beckman, 1967) are thought to arise from a single protein by addition of increasing amounts of sialic acid. A similar relationship has also been suggested for two forms of *N*-acetyl-β-D-glucosaminidase from human spleen (Robinson & Stirling, 1968). Thus the regulation of sialic acid attachment may determine a range of proteins that differ in their net negative charge.

An understanding of the regulation of glycoprotein synthesis might be useful in explaining many other electrophoretic variants and genetic polymorphisms. As suggested by Beckman & Beckman (1967), enzyme (protein) variants could provide a useful marker system for the study of tissue differentiation. We have attempted to do this and report here changes in concentrations of foetal mouse plasma glycoproteins during embryonic development that are due to changes in sialic acid content.

Experimental

Materials

Breeding of mice. Inbred strain C3H/An mice (Cumberland View Farms, Clinton, Tenn., U.S.A.) were used in all experiments. Evidence of a vaginal plug the morning after mating of mice was used as the criterion for conception, and this time was considered to be day 0.5 of gestation.

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Preparation of foetal plasma. At the appropriate gestational age, one to three mice were killed by cervical dislocation; the embryos were removed *in utero* and placed in a Petri dish (room temperature). The uterine wall was carefully dissected away to expose the foetus, with the umbilical cord and placenta still intact. After removal of the membranes, amniotic fluid was collected, the embryos were blotted dry, the umbilical cord was severed and an incision was made in the thoracic region. Blood was collected in 1.2mm×75mm heparin-treated capillary tubes that were sealed and centrifuged at 1000*g* for 20min. The tubes were broken and the clear plasma was drained and pooled. Protein concentration was determined by the method of Lowry *et al.* (1951); bovine serum albumin was used as a standard.

Methods

Incubation of proteins with neuraminidase. Incubation mixtures, unless otherwise indicated, contained 400μg of plasma proteins, 40μg of neuraminidase (EC 3.2.1.18; *Clostridium perfringens*; 1.1 units/mg; Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 14μmol of sodium acetate (pH5.0) in a total volume of 0.4ml. The mixtures were incubated at 37°C for 30min. Controls were incubated without the enzyme.

Purification of mouse α-foetoprotein and calf fetuin. Mouse α-foetoprotein and calf fetuin were purified by the method of Spiro (1960) from 20ml of day 15.5 amniotic fluid and 10ml of foetal calf serum (Grand Island Biologicals, Grand Island, N.Y., U.S.A.) respectively. Gustine & Zimmerman (1972*a*) have established that the mouse α-foetoproteins present in amniotic fluid are identical with those present in the foetal plasma. Proteins were further purified by preparative disc-gel electrophoresis. Two gels each containing 2.4mg (0.2ml) of calf fetuin and 28 gels each containing 0.3mg of mouse α-foetoprotein (0.4ml) were subjected to electrophoresis. The absorbance of the gels was recorded by densitometry at 280nm with a Gilford model 2400 spectrophotometer, after which the portions of the gels corresponding to calf fetuin and mouse α-foetoprotein were cut. The appropriate gel slices were combined and homogenized in 0.05M-Tris–HCl (pH7.0) with a loose-fitting Dounce homogenizer, the gel particles were removed by centrifugation and the supernatant fraction was collected. The gel particles were washed four times with the same buffer and the washes were combined with the original supernatant. Calf fetuin and mouse α-foetoprotein were precipitated by addition of 4vol. of ethanol. After standing for 2h at −20°C the precipitated proteins were centrifuged and the protein pellets were dissolved in 0.05M-sodium acetate (pH5.0). Each preparation was >95% pure, as determined by polyacrylamide-gel electrophoresis of measured portions.

Incubation of whole embryos. Embryos at appropriate gestational ages were isolated with amniotic membranes and placenta intact as described above and placed in wells of sterile organ-culture dishes (Falcon Plastics, Oxnard, Calif., U.S.A.). Groups of three embryos were incubated in 1ml of triple Eagle's medium (Grand Island Biologicals) containing 10% foetal calf serum, 10% chick-embryo extract (Grand Island Biologicals), glutamine (5mM), penicillin (75 units/ml) and streptomycin (50μg/ml). Radioactive precursors were injected with a 30 gauge needle into the amniotic sac: 0.5μCi of L-[^{14}C]leucine (30mCi/mmol; New England Nuclear Corp., Boston, Mass., U.S.A.) and 1.1μCi of D-[^{3}H]glucosamine (1.3Ci/mmol; New England Nuclear Corp.) were injected in a total volume of 15μl (Figs. 4 and 5). In the experiment described in Fig. 6, 5μCi of L-[^{3}H]leucine (10μl, 54Ci/mmol, Schwarz Bioresearch, Orangeburg, N.Y., U.S.A.) was employed. Embryos were maintained at 37°C in $CO_2+N_2+O_2$ (1:7:12, by vol.).

Polyacrylamide-gel electrophoresis. Analytical separation of proteins was performed by polyacrylamide-gel electrophoresis as described by Ornstein (1964) and Davis (1964). Sample gels contained 50–150μg of total protein; proteins were separated in 6mm×90mm separating gels for about 2h at 3.5mA/gel unless otherwise indicated. Electrophoresis was performed in the cold room in an electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif., U.S.A.) until the tracking dye was about 1cm from the end of the gel, unless otherwise indicated. Extruded gels were stained in 1% Aniline Blue–Black in 7% acetic acid; unbound dye was removed by diffusion destaining overnight in 7% acetic acid. Absorbance patterns were then recorded at 600nm. To minimize differences in electrophoretic mobilities of transferrins and α-foetoproteins from different gels, replicate gels were usually run. Gels presented in the figures were of the same length, and albumin and tracking dyes had also moved to the same position.

In the ^{59}Fe-binding experiment (Fig. 3) electrophoresis was carried out for 3h, the unstained gels were scanned at 280nm to localize the presumed transferrin bands and the gels were sliced in a longitudinal gel-slicer (Canal Ind. Corp., Bethesda, Md., U.S.A.). One-half of the gel was stained and the other half was assayed for radioactivity as indicated below.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the radioactive immunoprecipitate (Fig. 6) was performed by the method of Weber & Osborn (1969) by using 10cm-length 5% polyacrylamide gels (final concn.) and one-half of the amount of cross-linker. Each appropriate precipitate was dissolved by incubation at 37°C for 2h in 50μl of 0.01M-

sodium phosphate (pH7.0), 1% sodium dodecyl sulphate, 1% β-mercaptoethanol and 8M-urea. To each sample was added 50μl of 0.01M-sodium phosphate (pH7.0), 0.1% sodium dodecyl sulphate and 0.1% β-mercaptoethanol, 5μl of Bromophenol Blue (0.04%) and 1 drop of glycerol. Gels were run for 4h at 8mA/gel at room temperature. The gels were stained with Coomassie Brilliant Blue, scanned at 600nm and the stained gel was analysed for radioactivity.

Glycoproteins were detected by their reaction with periodic acid–Schiff's reagent. The procedure used was a modification of the method of Kao *et al.* (1966). Gels were immersed in 7% acetic acid for 1h, in 1% periodic acid for 1h, and then excess of periodic acid was removed by four successive 15min washes in 7% acetic acid. The gels were then placed in capped tubes containing acid Schiff's reagent.

Radioactivity measurements. The unstained gel was sectioned into 1.5mm slices with a lateral gel-slicer (Canal Ind. Corp.); each slice was placed in a scintillation vial, 0.5ml of aq. NH_3 soln. (sp.gr. 0.880) was added, and the slices were incubated overnight at 37°C in the capped vials. Scintillation fluid (10ml) was added [4g of 2,5-diphenyloxazole and 0.15g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 600ml of toluene and 400ml of 2-methoxyethanol]. Radioactivity was measured in a scintillation spectrometer; dual-isotope-counting efficiencies were 24% for ^3H and 57% for ^{14}C, and background radioactivity of about 14 and 12c.p.m. respectively was subtracted. The counting efficiency of 49% was achieved for ^{59}Fe (high specific radioactivity; citrate form; International Chemical and Nuclear Corp., Waltham, Mass., U.S.A.).

The stained gel was also sectioned into 1.5mm slices. Each slice was placed in a counting vial and decolorized with 0.1ml of 30% H_2O_2 at 50°C for 16h. After cooling, 5ml of Aquasol (New England Nuclear Corp.) was added and radioactivity was determined. A counting efficiency of 25% was achieved for ^3H (Fig. 6).

Results

Changes in the concentrations of some mouse foetal plasma proteins with time of development are shown in Fig. 1. At day 14.5 there were three major groups of

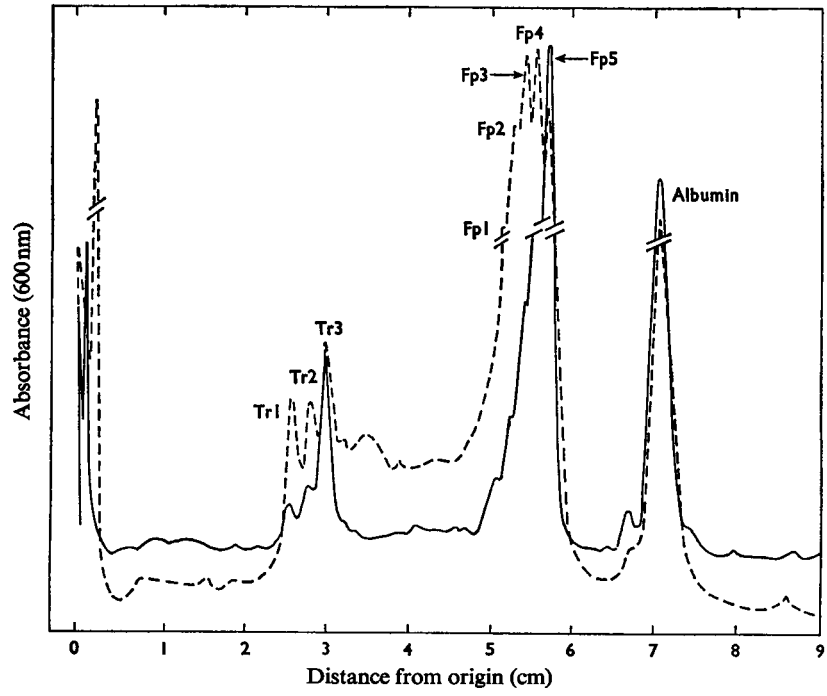

Fig. 1. *Polyacrylamide-gel electrophoresis of foetal plasma proteins at different times during development*

Preparation of foetal plasma and gel electrophoresis were performed as described in the Experimental section. The sample gels with foetal plasma for days 14.5 (---) and 15.5 (——) contained 96 and 102μg of protein respectively; full-scale absorbance was 2.0 and 3.0 respectively. Note that the baselines are different for each scan.

proteins: the transferrins (Tr1, Tr2, Tr3), the α-foetoproteins (Fp1, Fp2, Fp3, Fp4, Fp5) (Pantelouris & Arnason, 1967; Gustine & Zimmerman, 1972*a*) and albumin (Gustine & Zimmerman, 1972*b*). [In previous publications (Gustine & Zimmerman, 1972*a*,*b*) we have referred to the mouse α-foetoproteins as mouse β-proteins and numbered them β1, β2 etc. To conform with accepted practice in this paper

Table 1. *Changes in concentrations of foetal plasma proteins during development*

Total protein concentration in foetal plasma for each of the three periods of development was 14.5±1.7mg/ml (mean±S.D.). Volume of foetal plasma applied to each sample gel was 5μl. The numbers of determinations are shown in parentheses.

Protein peak	Foetal age (days) ...	% of total peak area 13.5 (2)	14.5 (2)	15.5 (5)
Tr1		6.2	4.6	2.7
Tr2		3.8	4.8	3.0
Tr3		6.0	8.3	6.7
Fp1		11.1	6.4	3.3
Fp2		13.8	12.2	4.9
Fp3		16.8	15.4	8.5
Fp4		13.0	16.4	16.0
Fp5		6.2	13.4	23.4
Tr1, Tr2, Tr3		16.0	17.7	12.4
Fp1, Fp2, Fp3, Fp4, Fp5		60.9	57.4	56.1
Albumin		18.0	18.4	23.9

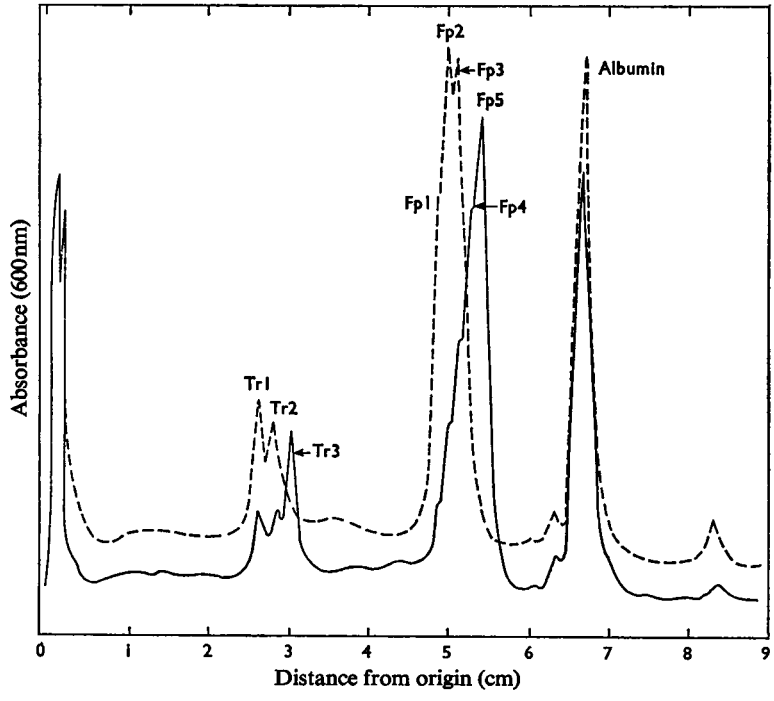

Fig. 2. *Effect of neuraminidase on the electrophoretic mobilities of foetal plasma proteins*

Plasma from foetuses of 15.5 days was treated with neuraminidase as described in the Experimental section. The samples (100μg of protein/gel) were subjected to gel electrophoresis and absorbance patterns were recorded. Full-scale absorbance for each scan was 3.0. ---, With neuraminidase; ——, without neuraminidase.

we refer to these foetal-specific proteins as α-foetoproteins and number them Fp1, Fp2 etc.] By day 15.5, Tr3 had increased in concentration relative to Tr1 and Tr2, and Fp4 and Fp5 had increased in concentration relative to Fp1, Fp2 and Fp3. Mouse α-foetoproteins are present in these high concentrations only in foetal tissues, and are not present in maternal tissues (Abelev *et al.*, 1963; Gustine & Zimmerman, 1972*a*).

To determine more precisely the extent of these changes, peak areas from gel-absorbance patterns of plasma from foetuses of 13.5, 14.5 and 15.5 days were calculated (Table 1). Since the protein concentration was constant in the plasma from the foetuses of 13.5, 14.5 and 15.5 days, and since the numbers presented in Table 1 are percentages of the total peak area, they therefore represent the protein concentrations that may be directly compared. During the 48h period Tr1 steadily decreased and Tr2 and Tr3 increased slightly at day 14.5 and returned to their original concentrations at day 15.5. On days 14.5 and 15.5, the concentration of Tr3 was about equal to that of Tr1 and Tr2 combined. Changes in the concentration of α-foetoproteins were complex: Fp1

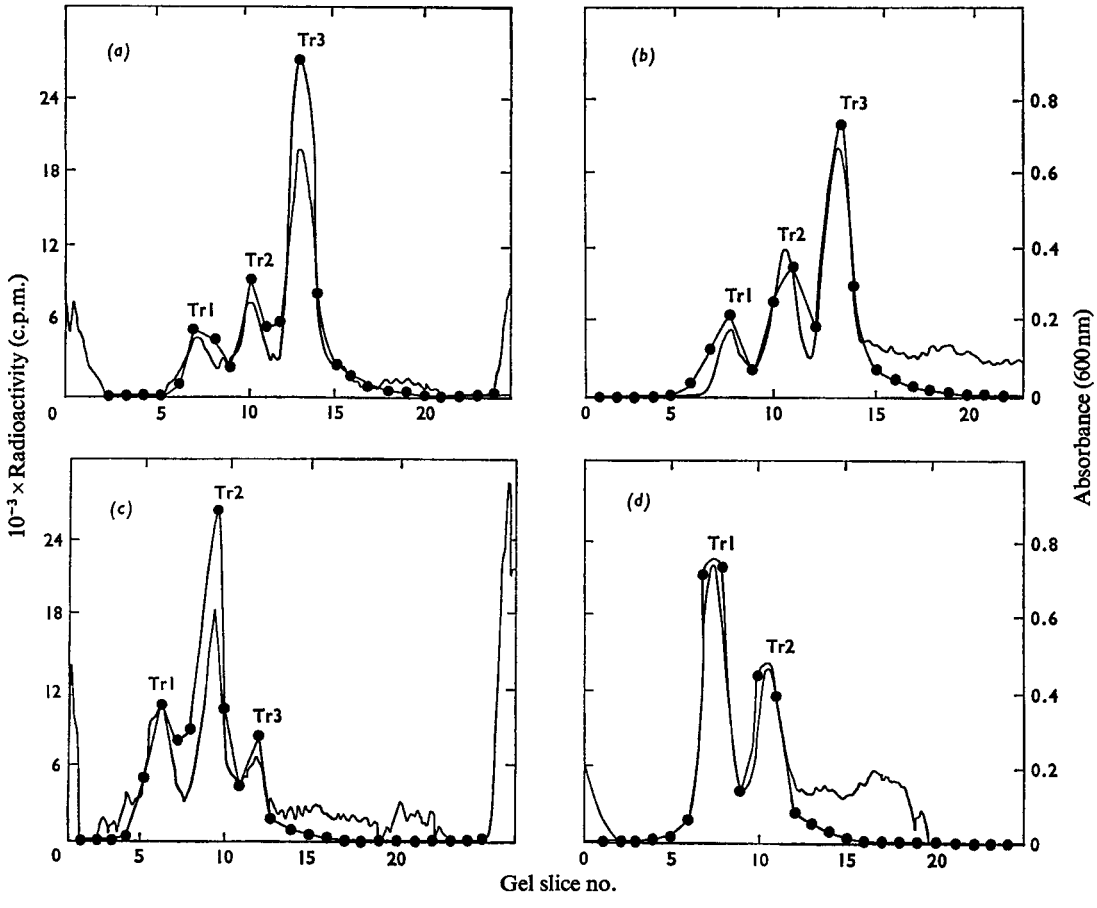

Fig. 3. *Binding of* ^{59}*Fe to day* 15.5 *foetal plasma proteins and effect of neuraminidase*

To each sample containing 180μg of plasma protein, 7μmol of sodium acetate (pH5.0) and 1μCi of ^{59}Fe (Giblett *et al.*, 1959) was added the indicated amount of neuraminidase. Each mixture (0.2ml final volume) was incubated as before, and the reaction was stopped by chilling at 5°C and adjusting the mixture to pH7.0–7.5 with 0.25M-Tris (un-neutralized). After electrophoresis, each gel was sliced longitudinally. The section of each half of the gel (3.0–5.5cm from the top) containing the presumed transferrin peaks was removed; one half was stained and scanned at 600nm (——) and the other half was assayed for radioactivity (●—●). (*a*) Control; (*b*) 0.18μg of neuraminidase; (*c*) 1.8μg of neuraminidase; (*d*) 18μg of neuraminidase.

concentration was almost halved each day, the concentrations of Fp2 and Fp3 were unchanged at day 14.5, but each was about halved by day 15.5; Fp4 was slightly increased at day 14.5 and remained constant at day 15.5, whereas Fp5 was almost doubled every 24h period. Albumin concentration was constant, except for a slight increase at day 15.5. The total concentrations of the transferrins and the α-foetoproteins were nearly constant during the 72h period. Thus Tr3 appeared to have increased at day 15.5 (Fig. 1) because Tr1 and Tr2 had decreased at that time. On the other hand, Fp5 appeared to increase because Fp1, Fp2 and Fp3 had decreased, in addition to an actual increase in Fp5. These results suggested that Fp1, Fp2 and Fp3 were converted into Fp5 and, to a lesser extent, into Fp4.

Since many plasma proteins are known to be glycoproteins, we treated gels with plasma from foetuses of 14.5 and 15.5 days with periodic acid–Schiff's reagent (see the Experimental section) to stain the carbohydrate moieties. At each day all of the presumed transferrins and α-foetoproteins gave a positive reaction, indicating that they were glycoproteins; however, albumin, which contains little carbohydrate, gave a faint stain. Thus the observed changes in Tr1, Tr2 and Tr3 and Fp1, Fp2, Fp3, Fp4 and Fp5 during development could reflect changes in carbohydrate content.

Microheterogeneity of human transferrin was suggested by Parker & Bearn (1962) to be due to the presence of various amounts of sialic acid. The separation of the three transferrins as well as the five α-foetoproteins in our gels (Fig. 1) could be due to such a variation in sialic acid content. If this was the case, treatment with neuraminidase would remove negatively charged sialic acid residues and thus decrease the mobilities of some of these proteins in the gels. The results of incubation of plasma from foetuses of 15.5 days with the enzyme are presented in Fig. 2. The gel with plasma that was not exposed to neuraminidase had an absorbance pattern essentially identical with that with plasma from foetuses of 15.5 days shown in Fig. 1. In the neuraminidase-treated sample, Tr3, Fp4 and Fp5 were missing, with corresponding increases in Tr1, Tr2, Fp1, Fp2 and Fp3. We concluded that Tr3, Fp4 and Fp5 were probably converted into the slower-moving components by removal of sialic acid residues. In subsequent experiments, day 15.5 plasma was incubated with neuraminidase in enzyme–substrate ratios of 1:10, 1:100, 1:1000 and 1:10000. At the lower enzyme concentrations, proportionately greater amounts of the intermediate moving glycoproteins were observed, e.g. Tr 2 and Fp4 (results are not shown for α-foetoproteins; see Fig. 3 for transferrins). These results also support the notion that the faster-moving components are electrophoretic variants of the slower-moving proteins, and contain a greater amount of sialic acid.

Although it had been shown previously that the only plasma proteins that bound ^{59}Fe were in the Tr1–Tr3 region and were therefore transferrins, nevertheless resolution between the peaks was poor and only two peaks of radioactivity were observed (Gustine & Zimmerman, 1972*b*). Hence this experiment was repeated in the same way except that (1) the gels were run longer to increase separation of the presumed transferrins and (2) gels were cut longitudinally and one-half was stained for protein and the other half sectioned and analysed for radioactivity. Fig. 3(*a*) indicates that ^{59}Fe binds to the Tr1, Tr2 and Tr3 proteins in proportion to the concentration of protein in each peak. Furthermore, increasing the concentration of neuraminidase causes a proportional decrease in concentration of protein and ^{59}Fe binding, first in Tr3 and then in Tr2 (Figs. 3*b*, 3*c* and 3*d*). In this experiment, no Tr3, as measured by ^{59}Fe binding, remains after the highest concentration

Table 2. *Effect of neuraminidase on sialic acid bound to mouse α-foetoproteins and calf fetuin*

Mouse α-foetoproteins isolated from amniotic fluid from foetuses of 15.5 days contain an approximately equal concentration of the five α-foetoproteins (Gustine & Zimmerman, 1972*a*). The purified mouse and calf proteins were incubated in 0.5ml of 0.05M-sodium acetate (pH5.0) containing 0.2% Tween 40; the treated samples contained 100μg of neuraminidase. Sialic acid was measured as *N*-acetylneuraminic acid (Sigma Chemical Co., St. Louis, Mo., U.S.A.) bound to the respective protein; standards were determined under the same conditions by the method of Jourdian *et al.* (1971).

Sample	Amount (μg)	Bound sialic acid (nmol): Without neuraminidase	Bound sialic acid (nmol): With neuraminidase	Sialic acid released (%)
Mouse α-foetoproteins Fp1, Fp2, Fp3, Fp4, Fp5*	1250	21.7	8.8	60
Calf fetuin	103	35.6	2.0	94

* Both samples contained precipitates that were removed by centrifugation. Bound sialic acid was determined for the supernatant fraction.

of neuraminidase (Fig. 3*d*; enzyme–substrate ratio = 1:10). It is therefore concluded that Tr1, Tr2 and Tr3 are all electrophoretic variants of transferrin.

To establish that incubation with neuraminidase removed sialic acid, glycosidically bound sialic acid was determined for purified mouse α-foetoproteins (Fp1, Fp2, Fp3, Fp4 and Fp5 mixture) before and after incubation with neuraminidase (Table 2). Calf fetuin, which is known to contain sialic acid, was used as a positive control. The results showed that incubation with neuraminidase did remove sialic acid from mouse α-foetoprotein and calf fetuin. Further, the calculated number of bound sialic acid residues on calf fetuin that were released by neuraminidase was

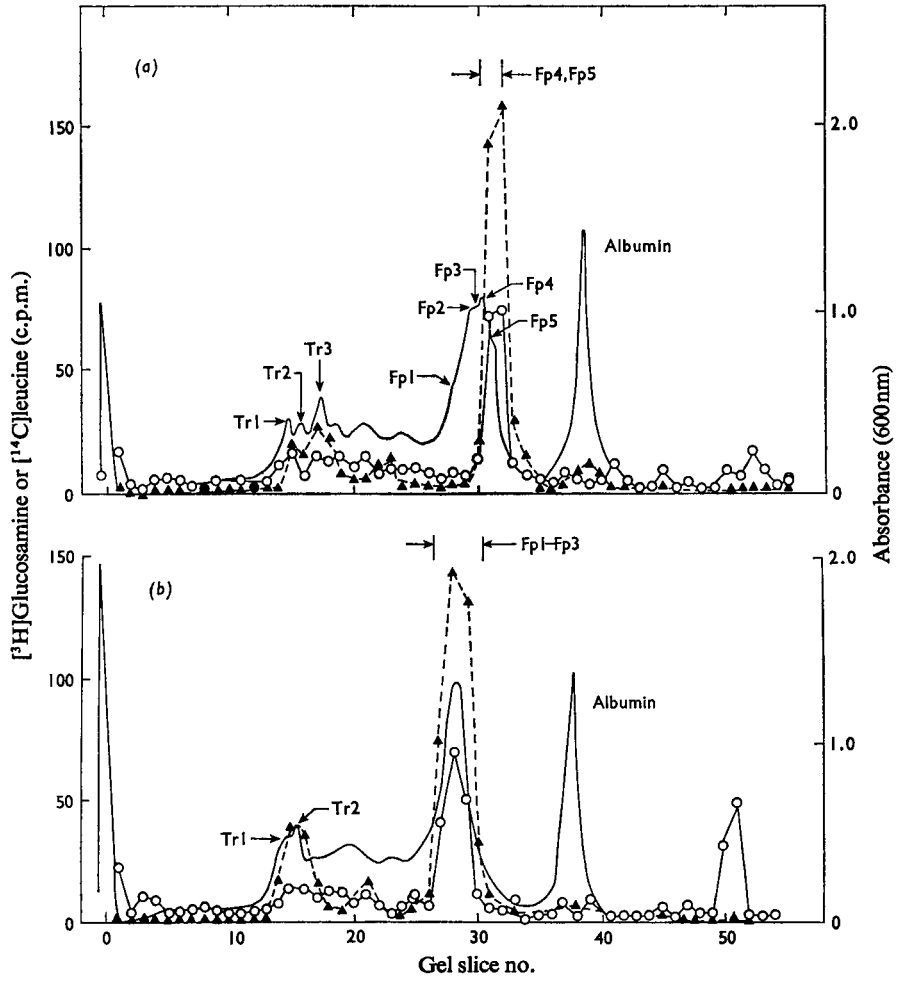

Fig. 4. *Electrophoretic separation of radioactive proteins synthesized by embryos of* 14.5 *days in vitro*

Embryos of 14.5 days (12 from two litters) were incubated in the presence of [^{14}C]leucine and [^{3}H]glucosamine for 24h (see the Experimental section). Amniotic fluid (including some foetal plasma) was collected, by breaking the amniotic membranes, and pooled. Proteins were precipitated by addition of ethanol to a final concentration of 80%, and centrifuged at 1000*g* for 10min. After three washes in aq. 80% ethanol, the precipitate was dissolved in 0.02M-Tris–HCl (pH7.6). This procedure removed more than 98% of unincorporated [^{14}C]leucine. About 50% of the protein was recovered. Portions were then incubated (*a*) without or (*b*) with neuraminidase as before. Duplicate gels of each were prepared; one was analysed for [^{3}H]glucosamine radioactivity (○) or [^{14}C]leucine radioactivity (▲) and the other was stained for protein and scanned at 600nm (——). Each sample gel contained 87μg of protein.

in close agreement with the number reported by Spiro (1960), establishing that the neuraminidase action was complete. In addition, longer incubations or higher concentrations of the enzyme in the experiment described in Fig. 2 did not result in further changes in the mobilities of Tr1, Tr2, Fp1, Fp2 and Fp3. These results prove that the decrease in electrophoretic mobilities of Tr3, Fp4 and Fp5 after neuraminidase action was due to the loss of at least some sialic acid (hence, negative charge).

Since the results in Fig. 1 suggested a specific developmental sequence in the synthesis of each of the transferrins and α-foetoproteins, we wanted to know which were synthesized early (day 12.5) in the developmental sequence and which were synthesized late (day 14.5). We have previously established that the transferrins and α-foetoproteins are synthesized in the foetus (Gustine & Zimmerman, 1972*a*) and that these synthesized proteins appear in the amniotic fluid. We therefore examined the synthesis

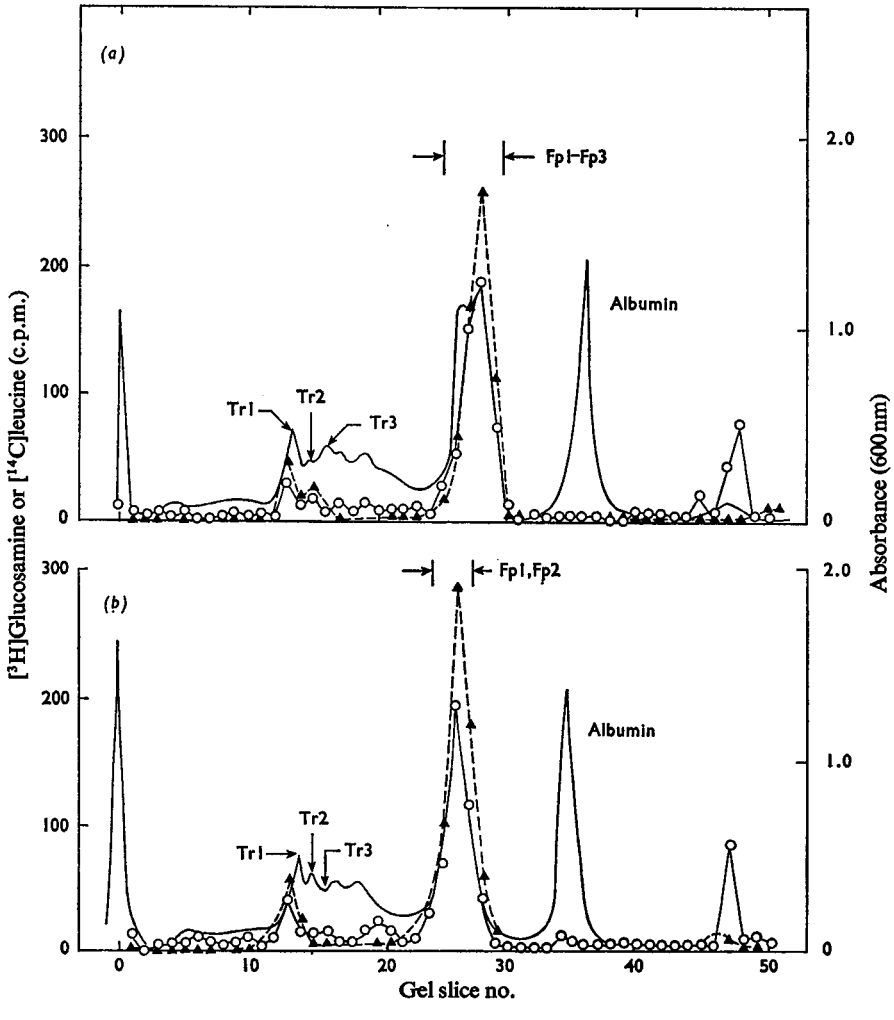

Fig. 5. *Electrophoretic separation of radioactive proteins synthesized by embryos of* 12.5 *days in vitro*

Embryos of 12.5 days (18 from three litters) were incubated with [^{14}C]leucine and [^{3}H]glucosamine for 8h. Amniotic fluid proteins were analysed as described in Fig. 3 and portions were incubated (*a*) without neuraminidase or (*b*) with neuraminidase. Each sample gel contained 82μg of protein. ——, Absorbance at 600nm; o, [^{3}H]glucosamine; ▲, [^{14}C]leucine.

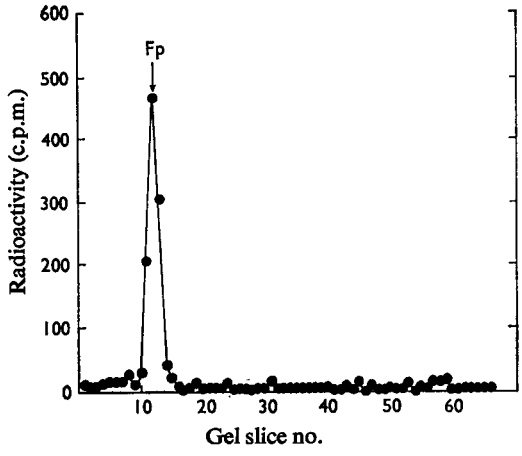

Fig. 6. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the immunoprecipitate of radioactive Fp4 and Fp5 and anti-(Fp1–Fp3)*

Embryos of 14.5 days (three) were incubated in the presence of [^{3}H]leucine for 20h after preincubation for 1h. Embryos in their amniotic sacs were washed with cold 0.15M-NaCl and amniotic fluid was collected; additional fluid was recovered by washing embryos and broken membranes with solution containing 0.02M-Tris (pH7.6), 0.01M-NaCl, 0.001M-$MgCl_2$, 0.25M-sucrose and 0.01M-leucine (2 drops/embryo). Erythrocytes were removed from the pooled sample by centrifugation at 600*g* for 10min (final volume was 0.25ml). A portion (5μl; 36μg of protein) was mixed with 0.4ml of 0.15M-NaCl and 0.1ml of antiserum prepared against Fp1–Fp3 proteins (day 12.5 amniotic fluid) (Gustine & Zimmerman, 1972*b*). After 2h at 25°C, incubation was continued for 40h at 5°C. The immunoprecipitate was centrifuged at 1000*g* for 15min and washed twice with 0.4ml of 0.15M-NaCl. The pellet was dissolved and the proteins were separated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis as described in the Experimental section. In addition, the following controls were carried out to localize the position of α-foetoprotein: purified Fp1–Fp5 mixture (20μg) (day 15.5 amniotic fluid) (E. F. Zimmerman, M. Madappally, J. R. Wilson & D. Bowen, unpublished work), Fp1–Fp5 immunoprecipitated with anti-(Fp1–Fp3) (200μl) and Fp1–Fp5 mixed with non-specific goat IgG. The location of Fp is indicated by the arrow.

of transferrins and α-foetoproteins in the foetus by incorporation of [^{14}C]leucine and [^{3}H]glucosamine into embryos in culture, followed by gel electrophoresis of amniotic fluid. The results for embryos of 14.5 days are shown in Fig. 4. These embryos synthesized mostly Fp4 and Fp5 (Fig. 4*a*); incorporation of both radioactive precursors indicated that protein assembly, including carbohydrate attachment, was complete *in vitro*. There was also some incorporation of [^{14}C]leucine into Tr2, Tr3 and albumin. Incubation of a portion of the amniotic fluid with neuraminidase converted radioactive Tr3 into Tr1 and Tr2, and radioactive Fp4 and Fp5 into Fp1, Fp2 and Fp3, thus establishing that Tr1 and Fp1, Fp2 and Fp3 were not synthesized (Fig. 4*b*). It should be noted also that the absorbance peaks for transferrin and α-foetoprotein were decreased in width by neuraminidase treatment. In addition, electrophoretic analysis of both foetal plasma and amniotic fluid from foetuses of 18.5 days indicated that the only α-foetoprotein remaining was Fp5, which is consistent with the synthesis of Fp4 and Fp5, and then only Fp5 at the later times of development. In Fig. 5(*a*) the results from a labelling experiment with embryos of 12.5 days indicated that proteins Fp1, Fp2 and Fp3 were synthesized; again, both labelled precursors were incorporated. The absorbance pattern also revealed the presence of Fp1, Fp2 and Fp3 and absence of Fp4 and Fp5. There seemed to be little synthesis of albumin, although at this early time Tr1 and Tr2 were synthesized. Incubation with neuraminidase (Fig. 5*b*) confirmed that Fp4 and Fp5 were not synthesized, as only a slight shift in mobility was seen (to Fp1, Fp2). However, radioactive Tr2 was decreased and Tr1 was increased in peak area. These experiments confirmed that the foetal transferrins and the α-foetoproteins are synthesized at specific times during development: that is, at day 12.5 Tr1, Tr2, Fp1, Fp2 and Fp3 were synthesized; at day 14.5 Tr2, Tr3, Fp4 and Fp5 were synthesized.

To be certain that Fp4 and Fp5 proteins synthesized later in development are related to the slower-moving components of Fp1, Fp2 and Fp3 synthesized earlier, the following experiment was performed. Embryos of 14.5 days were cultured *in vitro* in the presence of [^{3}H]leucine to label Fp4 and Fp5 in the amniotic fluid. Anti-(Fp1–Fp3) antibody, prepared against purified Fp1, Fp2 and Fp3 (day 12.5 amniotic fluid) in a goat, was shown to be monospecific against mouse α-foetoprotein (Gustine & Zimmerman, 1972*a*,*b*; E. F. Zimmerman, M. Madappally, J. R. Wilson & D. Bowen, unpublished work). When anti-(Fp1–Fp3) was incubated with the radioactive amniotic fluid and the immunoprecipitate was subjected to sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Fig. 6), only one radioactive peak was found in the gel, and that peak had the same mobility as mouse α-foetoprotein. Thus, since anti-(Fp1–Fp3) formed an immunoprecipitate with ^{3}H-labelled Fp4 and Fp5 synthesized later in development, Fp4 and Fp5 are antigenically similar to Fp1, Fp2 and Fp3 synthesized early in development.

Discussion

Results reported by other investigators suggest that glycoprotein biosynthesis is a two-step process: (1) synthesis of a polypeptide chain and (2) addition of carbohydrate units to the protein, possibly beginning just before its release from the ribosome. Evidence has been presented that the peptide portion of thyroglobulin is synthesized before and independently of carbohydrate attachment (Spiro & Spiro, 1966); additional evidence for this conclusion has been presented for other glycoproteins (Molnar *et al.*, 1965; Sinohara & Sky-Peck, 1965; Lawford & Schachter, 1966). Little is known about the site of attachment of carbohydrate units to the polypeptide, but it is thought to occur in the endoplasmic reticulum in a stepwise manner as the protein moves along the channels of the rough to the smooth endoplasmic reticulum. Some of the glycosyltransferases studied (Bosmann & Eylar, 1968; Spiro & Spiro, 1968; Hagopian & Eylar, 1969) are present in the membranes of the endoplasmic reticulum (Eylar & Cook, 1965; Sarcione & Carmody, 1966; Wagner & Cynkin, 1969). Sialic acid appears to be added at the terminal positions of carbohydrate units when the incomplete glycoprotein enters the region of the Golgi apparatus (Wagner and Cynkin, 1971).

Very little information is available on the regulation of glycoprotein synthesis. Two obvious sites of regulation are at the level of polypeptide synthesis and at the stage of carbohydrate attachment. Our results suggest that the changes in microheterogeneity of transferrin and α-foetoprotein were due to regulation at the stage of sialic acid attachment. On the other hand, even if there is a difference in the sialic acid content of the members of each group, there may also be differences in the more internally located sugars to which the sialic acid is attached. Differences in these sugars would not be apparent upon electrophoresis, as they contribute no charges to the molecule. In either case, these changes during development observed by us and by Pantelouris & Hale (1962) probably represent control with respect to attachment of carbohydrate. The elucidation of mechanisms for such a control system will require much further research; however, some possibilities can be suggested: (1) the control of the synthesis and degradation of glycosyltransferases (translational control), (2) control of transferase activities (post-translational control) by means of hormone interactions or mediation of substrate concentrations by feed-back control of sugar nucleotide biosynthesis (Kornfeld *et al.*, 1964), and (3) changes in the physical location of the transferases within microsomal membranes.

The mechanism by which sialic acid attachment is initiated in foetal mice could include any of the above possibilities. Since neuraminidase treatment of day 15.5 foetal plasma converted Tr3 into Tr1 and Tr2 and Fp4 and Fp5 into Fp1, Fp2 and Fp3 (Fig. 2), then Tr1, Tr2, Fp1, Fp2 and Fp3 must have contained less sialic acid. Our experiments clearly showed that at day 12.5 Tr1, Tr2, Fp1, Fp2 and Fp3 were synthesized (Fig. 5), but at day 14.5 only Tr3, Fp4 and Fp5 were synthesized (Fig. 4). It seems possible that, at the early time, transferrin and α-foetoprotein were synthesized to completion, except for the addition of the full complement of sialic acid and possibly other sugars. By day 15.5 the remaining sialic acids and other possible sugars were attached; thus the plasma glycoproteins were synthesized to completion. This would seem likely since our results show that α-foetoprotein synthesized early is antigenically similar to α-foetoprotein synthesized later (Fig. 6), but the early proteins contain less sialic acid than the later proteins (Figs. 4 and 5). Our experiments do not differentiate between possible mechanisms for sialic acid transfer to Tr1, Tr2, Fp1, Fp2 and Fp3, but they do establish an explanation for changes in microheterogeneity of proteins during foetal development, which is in agreement with the current concepts for glycoprotein synthesis.

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