STUDIES ON THE TRANSFERRINS OF ADULT SERUM, CORD SERUM, AND CEREBROSPINAL FLUID*

The Effect of Neuraminidase

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Recent population studies by starch gel electrophoresis have revealed an elaborate genetically determined polymorphism of human and primate transferrin, the iron-binding protein in serum. At the present time twelve different molecular species of human transferrin have been identified (1). Transferrins whose electrophoretic mobilities are faster than the common type C are labeled B, and slower moving variants are labeled D. With the exception of transferrin C, which is found in high frequency in all populations, particular variants are rare and appear to be restricted to particular populations. Thus transferrin B_{0-1} is found in Navajo Indians, B_2 in Caucasians, D_{Chi} in Chinese, and D_1 in Negroes. The gene frequency for each of these variants is approximately 0.05; the remaining seven variants have been reported only in isolated individuals.

The glycoprotein nature of transferrin has been investigated by Schultze *et al.* (2), who found a 5 per cent carbohydrate fraction composed of hexose, hexosamine, sialic acid, and fucose. Schultze and Schwick (3) had earlier described a reduction in the electrophoretic mobility of transferrin after incubation with neuraminidase, a bacterial and viral enzyme which cleaves the glycosidic bond joining sialic acid to a protein molecule. A similar effect had been observed by Perlmann, Tamm, and Horsfall (4) for urinary mucoprotein after incubation with influenza virus.

A brief report (5) previously described the stepwise action of neuraminidase in removing the four sialic acid residues from the transferrin molecule. The present study is concerned with an extension of the neuraminidase effect from transferrin C to certain genetic variants of human and primate transferrin. In addition, the transferrins of cord blood and cerebrospinal fluid are shown to be related by the neuraminidase effect to adult serum transferrin. Amino acid analyses of three human transferrins and a primate transferrin are also given.

Materials and Methods

Samples.—Sera of phenotypes B_0C, B_1B_1, B_1C, B_2C, and CD_3 were kindly provided by Dr. E. R. Giblett, King County Central Blood Bank, Seattle. Transferrin types $B_{0-1}C$,

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B_3C, CD_{Chi}, and D_1D_t were obtained through gene frequency studies in Navajo (6) and Japanese, Chinese, and Negro (1) populations. Rhesus and chimpanzee sera were kindly provided by Dr. M. Goodman and Dr. E. Poulik, Wayne State University, Detroit, and by Dr. A. J. Riopelle, Yerkes Laboratories of Primate Biology, Orange Park, Florida. Cynomolgus monkey and cattle sera were obtained from Cappel Laboratories, West Chester, Pennsylvania. Samples of cord blood were obtained from Dr. H. P. Goldberg, New York Hospital, and Dr. A. F. Guttmacher, Mount Sinai Hospital. Samples of cerebrospinal fluid were provided by Dr. B. Ray and Dr. F. S. Vogel, New York Hospital. The cerebrospinal fluid was concentrated 100- to 400-fold by vacuum dialysis in heavy membrane sacs (Membranfilter Gesellschaft, Göttingen).

Electrophoresis.—Starch gel electrophoresis was carried out in borate buffer according to the vertical system of Smithies (7). Prior to electrophoresis, radioactive iron as Fe^{59} citrate was added to each sample (8); electrophoresis was carried out at 5 v/cm for 16 hours, after which the gel was sliced into three layers. Transferrin was determined by autoradiography from the bottom slice, which was subsequently stained for ceruloplasmin (9). The middle slice was stained for haptoglobin by the benzidine reagent, and the top slice was stained for protein with amido black.

Immunoelectrophoresis was performed in agar gel by the method of Grabar and Williams (10) and in starch gel by the method of Poulik (11). Horse anti-human serum was obtained from the Pasteur Institute, and rabbit anti-human transferrin serum was obtained from the Behringwerke.

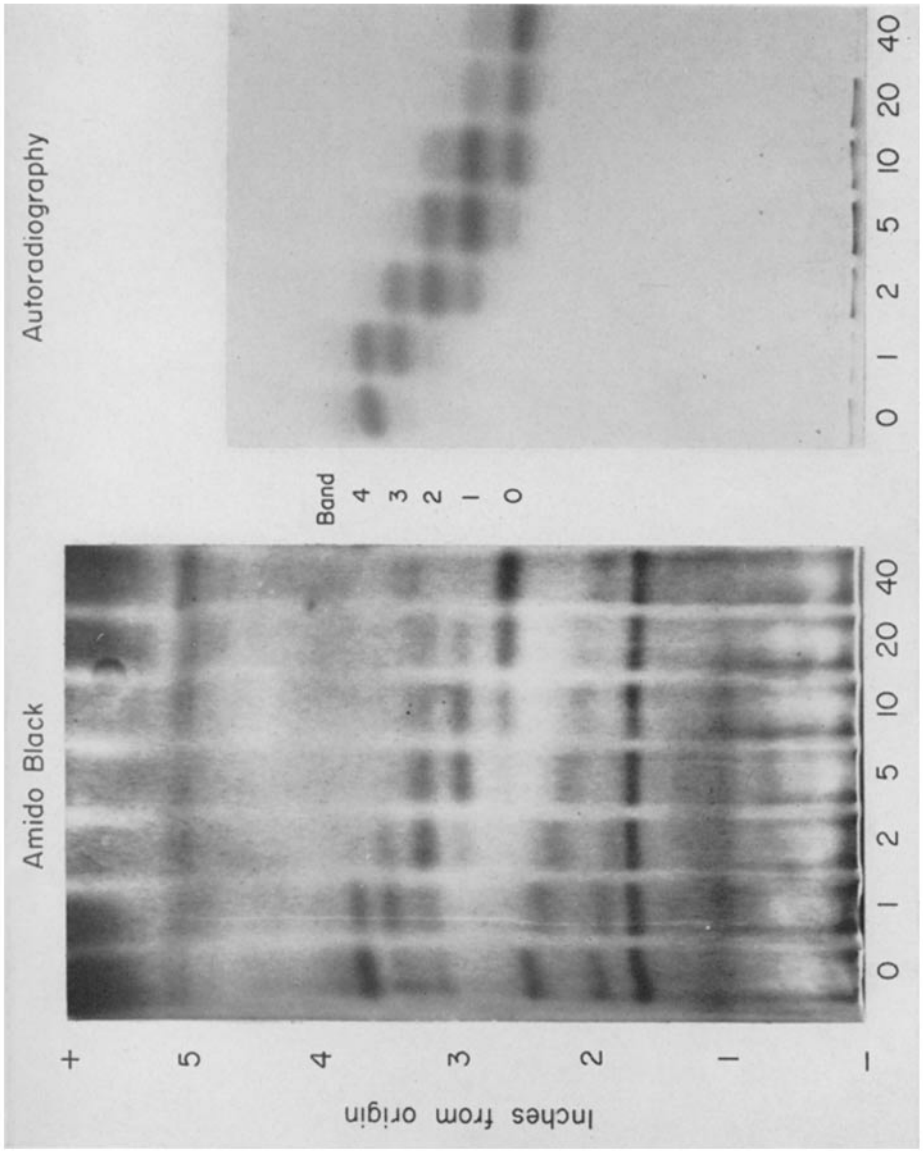
Treatment with Neuraminidase.—The neuraminidase used in these experiments was a purified preparation kindly supplied by Prof. H. E. Schultze, Behringwerke. The enzyme showed a titer of 1024 units when tested against the heme-agglutinating activity of the Lee strain of influenza B virus (12, 13). Serum and purified transferrin preparations were incubated at 37°C with neuraminidase at various concentrations and for various time intervals. No proteolytic activity could be detected in the enzyme apart from the neuraminidase activity.

Chemical Analyses.—Analyses for glucosamine and all amino acids except tryptophan were performed by ion exchange chromatography according to the method of Moore *et al.* (14) and Spackman *et al.* (15). Samples were hydrolyzed in 6 N HCl at 110°C in evacuated sealed tubes. Cysteic acid and methionine sulfone were determined by the performic acid oxidation procedure of Hirs (16), as modified by Moore (17). Tryptophan was determined by 16-hour alkaline hydrolysis in starch-barium hydroxide solution according to the method of Drèze (18), as modified by Moore (17); the hydrolysate was analyzed by chromatography on a 30 cm starch column in 0.1 N HCl by the method of Moore and Stein (19).

RESULTS

Effect of Neuraminidase on Serum Proteins

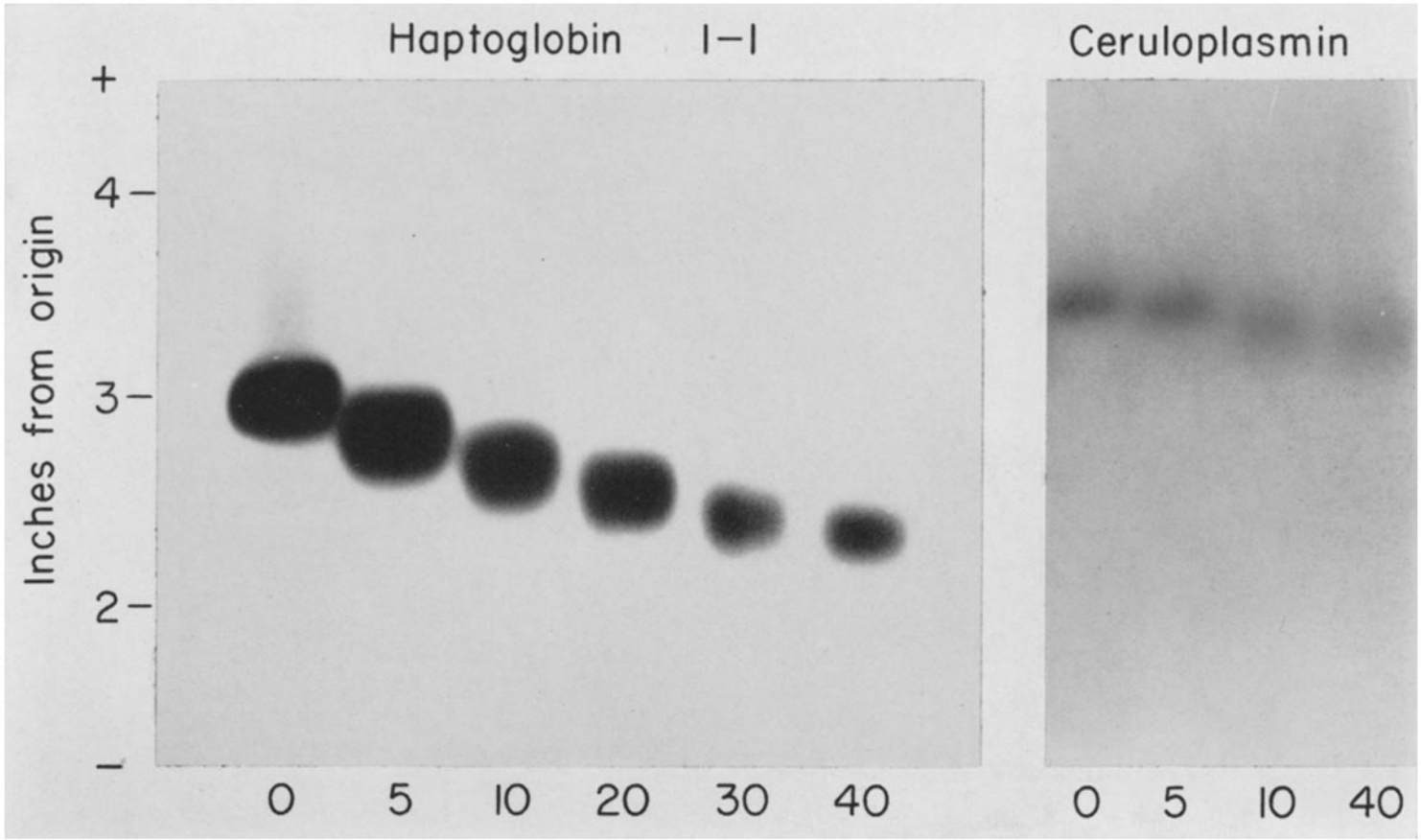
Treatment of normal human serum with neuraminidase for 16 hours at 37°C resolved the single transferrin C band into a stepwise pattern of four additional slower moving components (Fig. 1, Bands 4 to 0) whose relative intensities depended upon the concentration of neuraminidase. A similar pattern was obtained by varying the time of incubation. It was also found that the effect of neuraminidase could be increased if the serum was dialyzed during incubation with the enzyme. Large increases in neuraminidase concentration resulted in the appearance of no further bands, and virtually all of the transferrin appeared in the position of band 0. The serum in this experiment was of haptoglobin type



FIGS. 1*a* and 1*b*. Effect of neuraminidase on transferrin in serum. 0.1 ml serum (transferrin type CC, haptoglobin type 2-1) incubated 16 hours with enzyme. 0, 1, 2, 5, 10, 20, 40: relative concentrations of neuraminidase; 0.1 ml of the neuraminidase preparation is equivalent to a relative concentration of 20. Incubation mixtures adjusted to same final volume (0.3 ml) with isotonic saline.

FIG. 1*a*. Amido black stain after starch gel electrophoresis. The effect of neuraminidase on α_2-macroglobulin is not easily seen under the conditions of this experiment. The gradual reduction in mobility was most conveniently demonstrated when samples which had been incubated for longer periods of time with large and small concentrations of enzyme occupied adjacent slots in the gel.

FIG. 1*b*. Autoradiograph from gel of Fig. 1*a*. The amount of added Fe^{59} citrate was adjusted to give uniform exposures for each sample.



2 *a* 2 *b*

FIGS. 2*a* and 2*b*. Effect of neuraminidase on haptoglobin and ceruloplasmin in serum. 0.1 ml serum incubated 16 hours with enzyme. Neuraminidase concentration as in Fig. 1. Incubation mixtures not adjusted to same final volume.

FIG. 2*a*. Benzidine stain after starch gel electrophoresis of serum of haptoglobin type 1-1. The dilution of the serum at higher neuraminidase concentration obscures the increasingly more diffuse character of the haptoglobin bands.

FIG. 2*b*. Paraphenylene-diamine stain of ceruloplasmin after starch gel electrophoresis.

2-1; the effect of neuraminidase on the slower moving haptoglobins can be seen in the amido black stain of the gel (Fig. 1*a*). As opposed to the stepwise pattern obtained with transferrin, the electrophoretic mobility of the haptoglobins showed a gradual decrease with increasing neuraminidase concentration. A similar gradual decrease was observed for haptoglobin type 1-1 and ceruloplasmin (Fig. 2), as well as for α_2-macroglobulin, and it was found that the haptoglobin and ceruloplasmin bands became more diffuse with decreasing mobility.

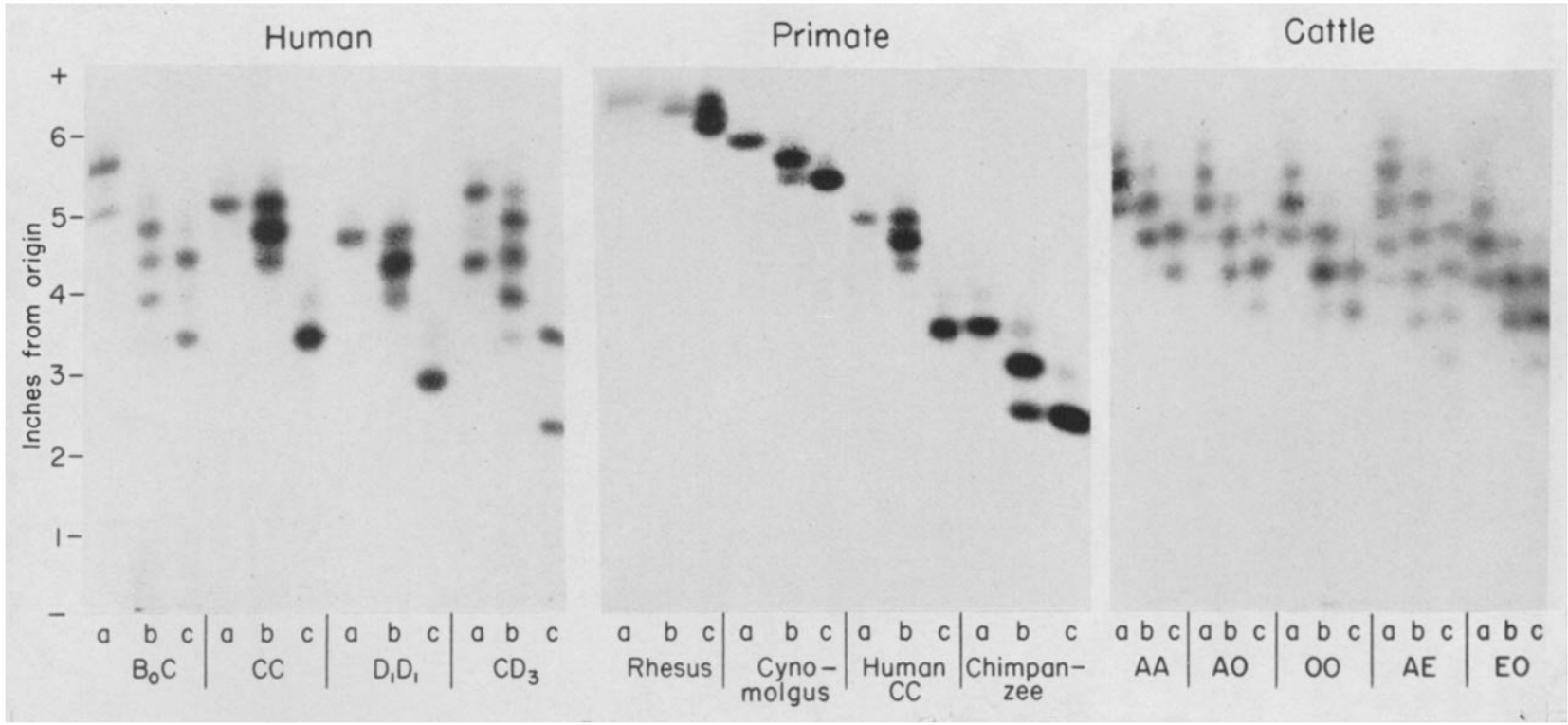
When available human transferrin variants were incubated with neuraminidase, a stepwise pattern was observed which was similar to that obtained for transferrin C. Fig. 3*a* illustrates the result obtained when sera of four different transferrin phenotypes were incubated with both a small concentration of neuraminidase and a concentration sufficiently large to reduce transferrin C to its slowest moving position, labeled band 0 in Fig. 1. In each case the characteristic stepwise pattern was obtained; each of the variants could be resolved into a pattern of four slower moving bands, and at the largest enzyme concentration only the slowest band was present in significant amount. In addition to the variants B_0, D_1, and D_3, similar experiments were carried out on transferrins B_{0-1}, B_1, B_2, B_3, and D_{Chi}, all of which revealed the characteristic pattern of four slower moving bands and all of which could be resolved into the slowest moving band at high enzyme concentration.

When sera from chimpanzees, rhesus and cynomolgus monkeys, and cattle were incubated with neuraminidase, a slightly different transferrin pattern was observed. The chimpanzee and monkey transferrins could be resolved into only two slower moving components (Fig. 3*b*), instead of the four additional components found in human transferrin. Similarly, neuraminidase treatment of sera from five of the six known cattle transferrin phenotypes appeared to yield a stepwise pattern of two slower moving components for each band in the untreated phenotype (Fig. 3*c*).

Purification and Analysis of Transferrins

Transferrin C was purified from human serum by starch block electrophoresis and cellulose chromatography. Twenty ml of serum from an individual homozygous for transferrin C was separated on a starch granule block according to the method of Kunkel (20). The beta fraction was isolated, concentrated to 2 ml, and chromatographed on a 2 × 14 cm TEAE cellulose column in 0.025 M Na_2HPO_4. Under these conditions transferrin C was obtained at the column volume.

Transferrins B_2 and D_1 were prepared by similar procedures. When the beta fraction from serum of transferrin type B_2C was chromatographed and eluted with 0.025 M phosphate, transferrin C appeared at the column volume, whereas the B_2 component was slightly retarded and formed a large proportion of the



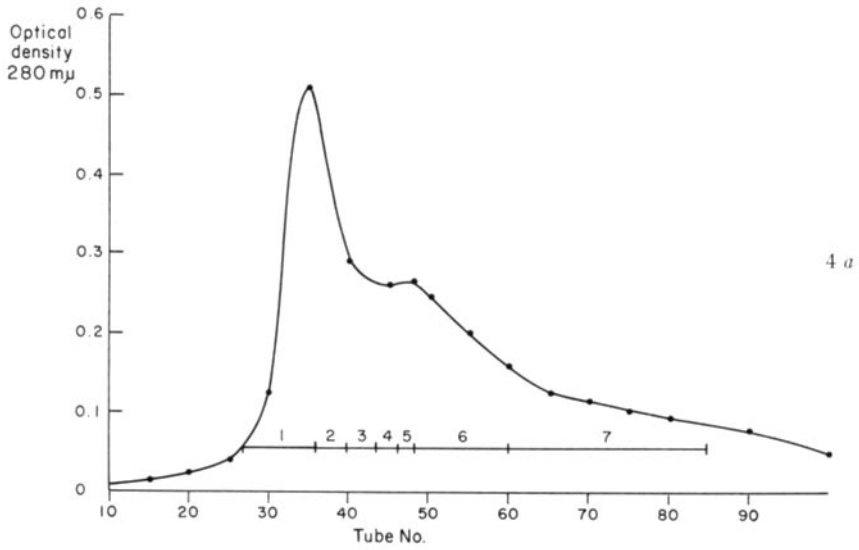
3 *a* 3 *b* 3 *c*

FIGS. 3*a*, 3*b*, and 3*c*. Effect of neuraminidase on human, primate, and cattle transferrins in serum. Autoradiography after starch gel electrophoresis. Incubation mixtures as in Fig. 1. *a*, *b*, *c*: relative enzyme concentrations of 0, 2, and 40, respectively.

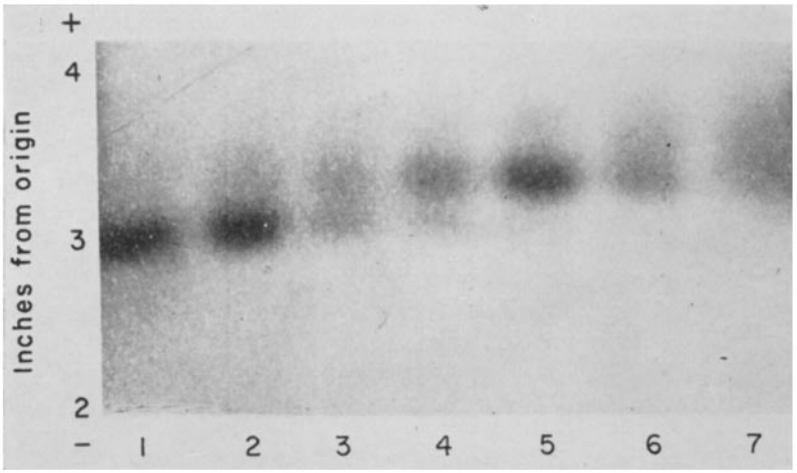
FIG. 3*a*. Comparison of four human transferrin phenotypes.

FIG. 3*b*. Comparison of rhesus, cynomolgus, human, and chimpanzee transferrins. Homozygous transferrin phenotypes were used.

FIG. 3*c*. Comparison of five cattle transferrin phenotypes. Phenotypes are labeled according to the system of Smithies and Hickman (*27*).



4 *a*



4 *b*

FIGS. 4*a* and 4*b*. Purification of transferrin B_2 from serum of phenotype B_2C.

FIG. 4*a*. Chromatography on TEAE cellulose of β-globulin fraction isolated by starch block electrophoresis. Elution in 0.025 M Na_2HPO_4. 2.5 ml was collected in each tube.

FIG. 4*b*. Analysis by starch gel electrophoresis of the seven fractions from the chromatogram in Fig. 4*a*. Fractions were concentrated by vacuum dialysis. Amido black stain.

drawn-out tail of the protein curve (Fig. 4*a*). Examination of successive pooled fractions in the starch gel (Fig. 4*b*) revealed that the major portion of the second peak was an essentially pure preparation of transferrin B_2. When the beta fraction from a CD_1 heterozygote was chromatographed in 0.020 M

Na_2HPO_4, transferrin D_1 appeared at the column volume, whereas under these conditions transferrin C was retarded and formed the tail of the curve. The isolation and purification of transferrin from a cynomolgus monkey of homozygous phenotype was accomplished by a similar procedure, except that the TEAE cellulose elution was performed in 0.035 M phosphate.

The purified transferrins C, B_2, and D_1 and the cynomolgus transferrin migrated as single components in starch gel electrophoresis. The slightly blurred gel fractions obtained from B_2 suggested a partial denaturation of this transferrin, which was the only one of the four transferrins retarded on the TEAE cellulose. When the purified transferrins were tested for purity by the Ouchterlony diffusion plate technique against anti-human serum, single precipitin lines were obtained. When transferrins B_2 and C were tested against anti-human serum by the double diffusion method of Preer (21), the pattern shown in Fig. 5 was obtained. The trace second component in the B_2 preparation may indicate a denatured B_2; it was estimated by dilution studies that this component could represent no more than 5 per cent of the total protein.

When transferrin C was compared in the ultracentrifuge with transferrin B_2 and the cynomolgus transferrin, each of the transferrins appeared monodisperse. No difference in sedimentation rate could be detected between C and B_2 and between C and the cynomolgus transferrin (Fig. 6, *a* and *b*). In addition, when purified transferrin C was incubated with neuraminidase at a concentration sufficient to produce bands in only the 1 and 0 positions, no difference in sedimentation rate was observed between the treated and untreated material (Fig. 6*c*).

Amino acid analysis (Table I) could detect no significant difference among the three human transferrins. However, the composition of the cynomolgus transferrin was approximately 20 per cent higher in serine and 50 per cent lower in glucosamine than transferrin C. In addition, the values for lysine, threonine, alanine, valine, and methionine were outside the range observed for the human transferrins.

Cord Blood and Cerebrospinal Fluid

Maternal and cord sera obtained at birth were examined from 18 mother-child pairs, and in each case the cord blood samples revealed a transferrin pattern consisting of a strong radioactive band in the position of transferrin C, accompanied by four faint slower moving bands of approximately equal intensity which coincided with the four transferrin bands produced by the action of neuraminidase on adult transferrin C. Fig. 7*a* illustrates the results obtained when three different specimens of cord blood were compared by starch gel electrophoresis with adult serum incubated with various amounts of neuraminidase. In no case was a similar pattern observed in the maternal serum.

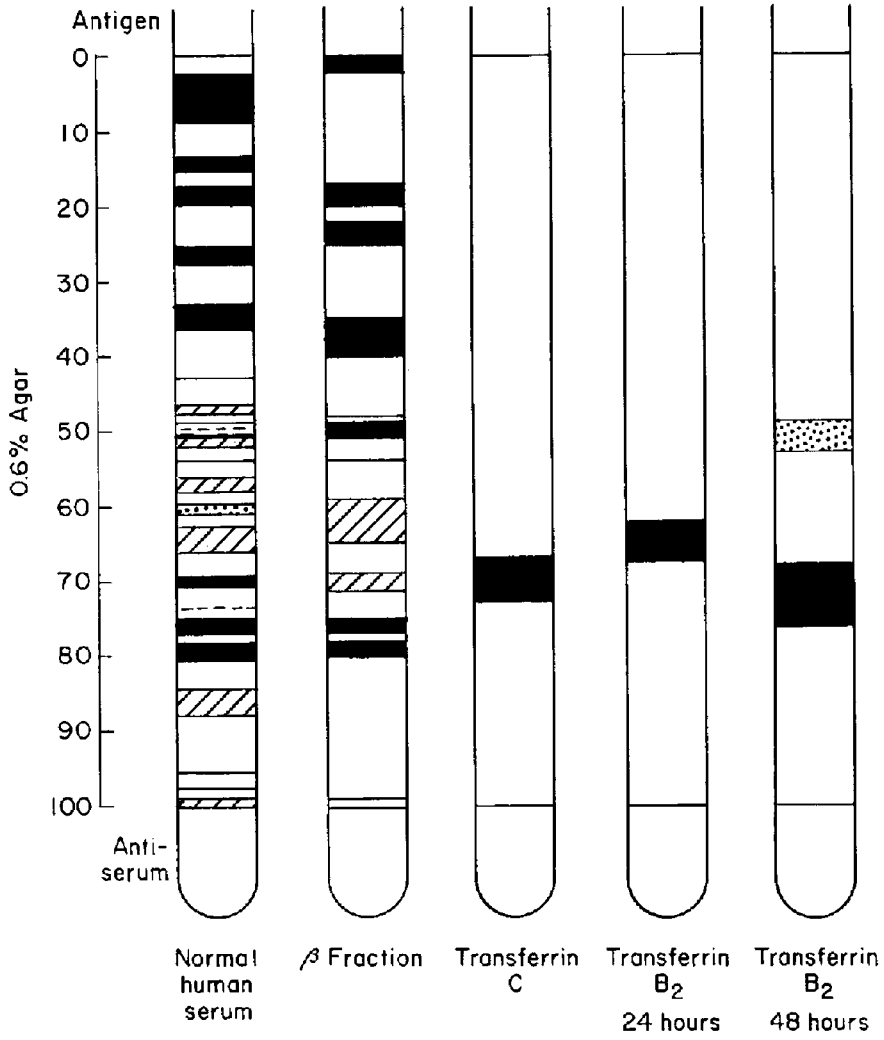
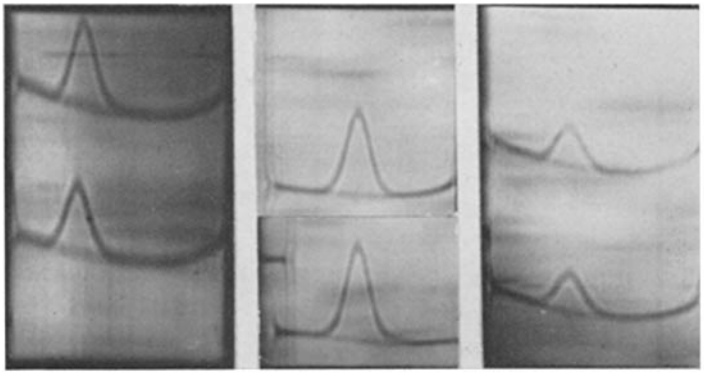


FIG. 5. Analysis of purified transferrins by the Preer double diffusion technique. Length of diffusion path normalized to 100 units agar. Antiserum: horse anti-human serum. The diagram represents observed bands, in order of decreasing intensity, as: solid band, hatched band, stippled band, solid line, dashed line.

In cerebrospinal fluid two principal transferrin bands of approximately equivalent intensity were observed by autoradiography (Fig. 7*b*); the faster moving of these bands corresponded to serum transferrin C and the slower moving band coincided with band 0 in the pattern obtained with neuraminidase-treated serum transferrin. The intensity of the bands in the autoradiograph was con-

siderably increased by incubation of the samples after the addition of the Fe^{59}. Faint bands in the 1 and 2 positions could also be detected in cerebrospinal fluid. The amido black stain of the gel (Fig. 8) indicated that the two principal CSF transferrins were not always of equal intensity.

Neuraminidase treatment of cerebrospinal fluid revealed the pattern shown in Fig. 7*b*. At large neuraminidase concentration all of the fast moving band was reduced to the 0 position and coincided with the slow band. No neuraminidase effect could be detected on the slow moving transferrin band. Although it



6*a* 6*b* 6*c*

FIGS. 6*a*, 6*b*, and 6*c*. Ultracentrifugal analysis of transferrins. Samples purified by starch block electrophoresis and TEAE cellulose chromatography. Solvent: 0.1 M barbital, pH 8.6. Speed: 52, 640 RPM. Time of photograph: 90 minutes. Phase plate angle: 45°.

Lower frame: human transferrin C.

Upper frame:

FIG. 6*a*. Human transferrin B_1.

FIG. 6*b*. Cynomolgus monkey transferrin.

FIG. 6*c*. Human transferrin C incubated with neuraminidase to produce bands in the 0 and 1 positions (*cf.* Fig. 1). Neuraminidase removed by starch block electrophoresis.

was difficult to determine the effect of neuraminidase on the four faint bands of cord blood, almost all of the transferrin was reduced to band 0 at large enzyme concentration and no slower moving bands were observed.

In addition to transferrin in CSF, it was possible to identify haptoglobin, ceruloplasmin, and α_2-macroglobulin in the starch gel pattern, as well as certain other proteins also found in serum (Fig. 8). CSF from each individual revealed the presence of a ceruloplasmin band in the position of normal serum ceruloplasmin. Two of three individuals examined showed a haptoglobin pattern of the 2-2 type found in normal human serum; the mobilities of the haptoglobin bands coincided with those found in the serum of the subject. In each of the CSF samples examined, a considerably increased protein concentration was observed in the region corresponding to the tryptophan-rich pre-albumin$_1$ of

TABLE I

Amino Acid Composition of Transferrin Variants

Amino acid	Residues*	Micromoles amino acid‡			
		C	B_2	D_1	Cynomolgus
Lysine	60	1.10	1.08	1.07	0.984
Histidine	19	0.353	0.350	0.341	0.351
Ammonia	62	1.14	1.15	1.18	1.17
Arginine	28	0.508	0.490	0.469	0.469
Cysteic acid	—	0.732	—	—	—
Aspartic acid	82	1.50	1.50	1.49	1.52
Methionine sulfone	—	0.180	—	—	—
Threonine	32	0.553	0.562	0.563	0.593
Serine	42	0.715	0.726	0.734	0.888
Glutamic acid	62	1.13	1.15	1.10	1.19
Proline	36	0.655	0.662	0.623	0.658
Glycine	51	0.932	0.967	0.931	0.896
Alanine	59	1.08	1.08	1.08	1.19
Half-cystine	40	0.608	0.628	0.577	0.590
Valine	47	0.758	0.771	0.793	0.860
Methionine	10	0.166	0.165	0.168	0.189
Isoleucine	15	0.270	0.254	0.259	0.263
Leucine	60	1.10	1.10	1.10	1.10
Tyrosine	26	0.477	0.479	0.491	0.496
Phenylalanine	28	0.522	0.526	0.519	0.549
Tryptophan§	8	0.143	0.124	0.130	0.125
Glucosamine	9	0.095	0.097	0.110	0.059

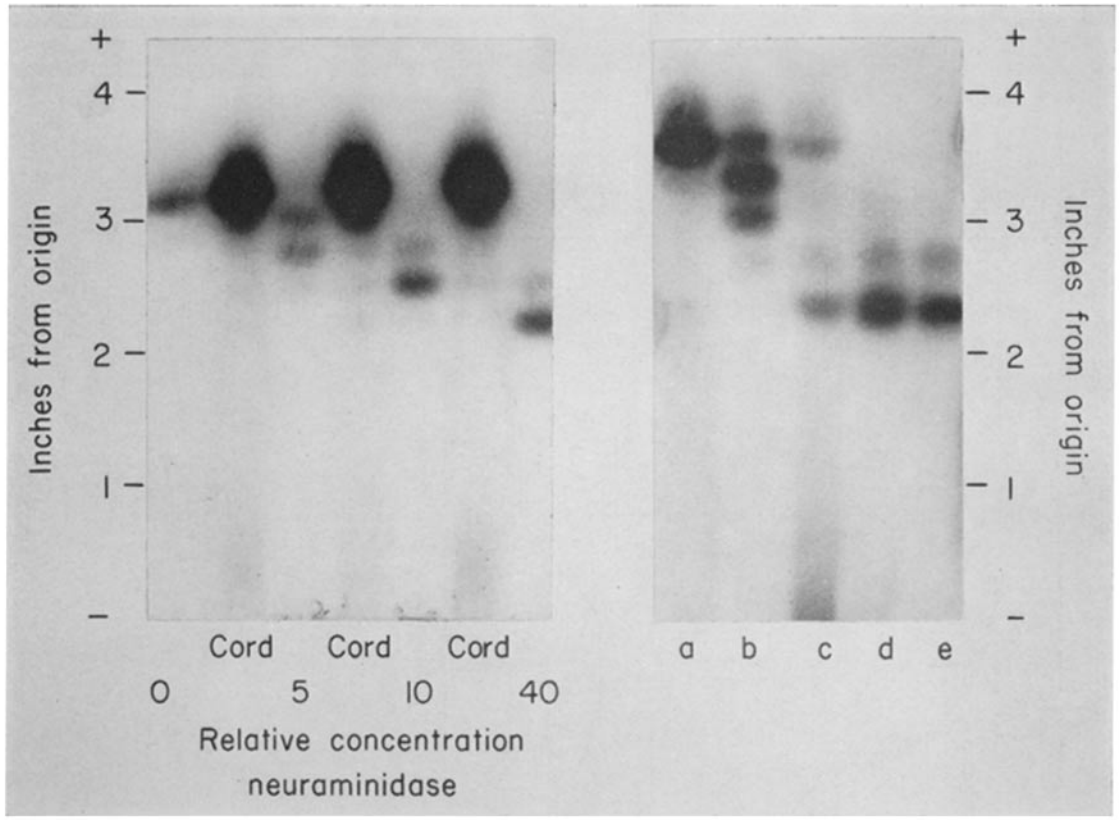
* Residues calculated for transferrin C molecule of molecular weight 90,000. Residues half-cystine and methionine determined as cysteic acid and methionine sulfone. Residues threonine, serine, and glucosamine determined for six hydrolysis times from 12 hours to 190 hours and extrapolated to zero time. Residues valine calculated from maximum observed value for these hydrolysis times.

‡ Observed micromoles for 1.596 mg transferrin C, corrected for ash (0.4 per cent) and moisture. Micromoles for B_2, D_1, and cynomolgus transferrins adjusted to leucine = 1.10. Cysteic acid and methionine sulfone determined after performic acid oxidation of transferrin C and adjusted to leucine = 1.10. 22 hour acid hydrolysis.

§ Tryptophan determined by alkaline hydrolysis.

normal serum. Pre-albumin$_2$, a thyroxine-binding globulin, was also present and appeared to have a proportion similar to that of pre-albumin$_2$ in serum. The gamma globulin fraction of cerebrospinal fluid could not be distinguished from that of serum.

In fetal serum a faint ceruloplasmin band was observed in a sample obtained at 17 weeks of development, and more prominent bands were observed in samples obtained at 24, 28, and 33 weeks of development, as well as in cord



7 a 7 b

FIGS. 7*a* and 7*b*. Analysis of transferrins in cord blood and cerebrospinal fluid. Autoradiography after starch gel electrophoresis.

FIG. 7*a*. Three different samples of normal cord blood compared to adult serum incubated with various concentrations of neuraminidase. Incubation mixtures as in Fig. 1. The amount of radioactive iron added to the samples of adult serum was adjusted to avoid intense exposure of the film.

FIG. 7*b*. Effect of neuraminidase on transferrin in cerebrospinal fluid:

(*a*) Human serum, transferrin phenotype CC.

(*b*) Human serum incubated with neuraminidase. Relative enzyme concentration = 2.

(*c*) Cerebrospinal fluid.

(*d*) Cerebrospinal fluid incubated with neuraminidase. Relative enzyme concentration = 40.

(*e*) Human serum incubated with neuraminidase. Relative enzyme concentration = 40.

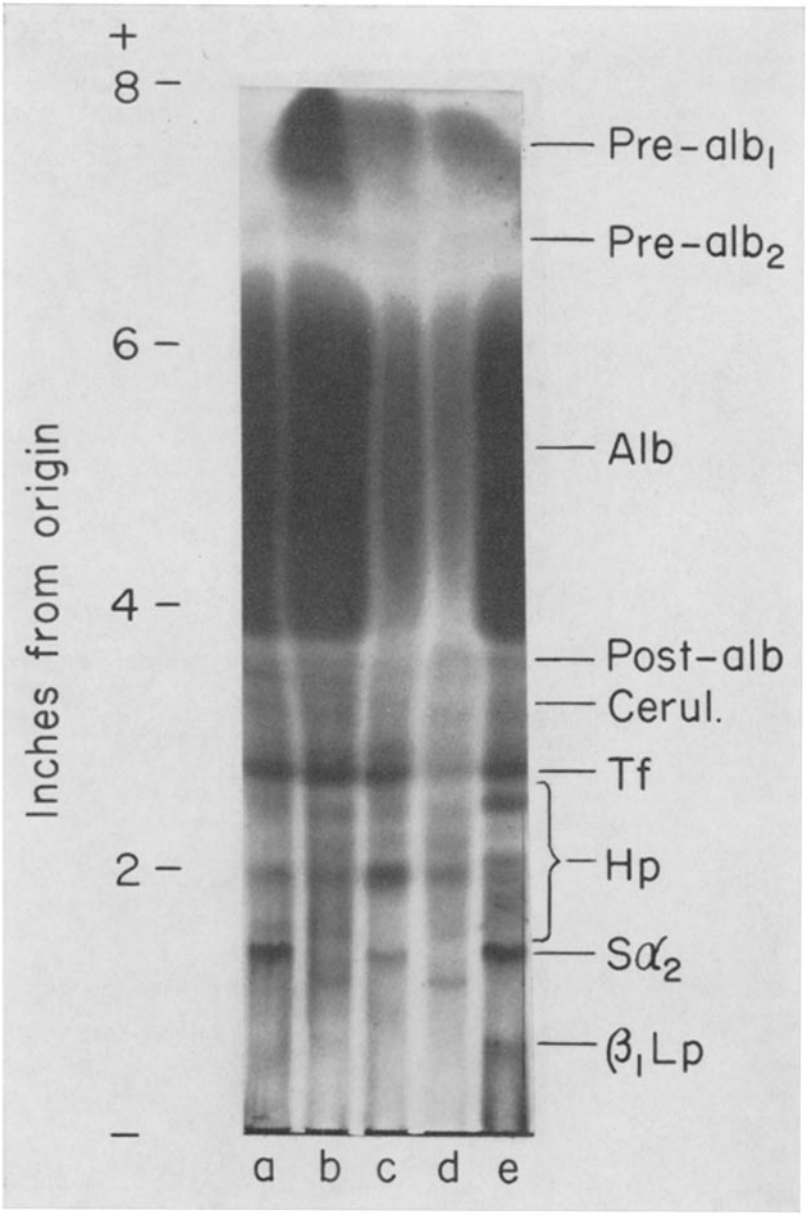
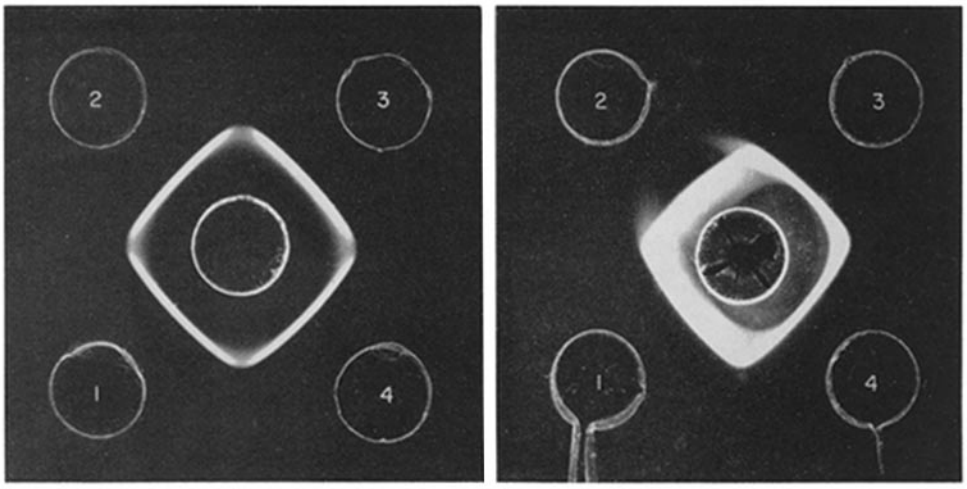


FIG. 8. Starch gel electrophoresis of cerebrospinal fluid. (*a*) human serum, transferrin phenotype CC; (*b*), (*c*), (*d*) cerebrospinal fluid from three different individuals; (*e*) human serum, transferrin phenotype CD_1. Tf represents transferrin C and $S\alpha_2$ represents α_2-macroglobulin (slow α_2). Amido black stain.

blood taken at birth. A clear haptoglobin 2-1 pattern was present in the 28 week sample; the maternal serum was of haptoglobin type 1-1. Haptoglobins could not be detected in the 17, 24, and 33 week samples or in the majority of the cord blood samples obtained at birth. The tranferrin patterns of the 17, 24, and 28 week samples revealed bands only in the position of transferrin C; the 33 week sample contained in addition the four faint transferrin bands observed in the cord blood samples obtained at full term.



9*a* 9*b*

FIGS. 9*a* and 9*b*. Immunological analysis of purified transferrin variants by Ouchterlony diffusion plate technique. Center wells: anti-human transferrin serum.

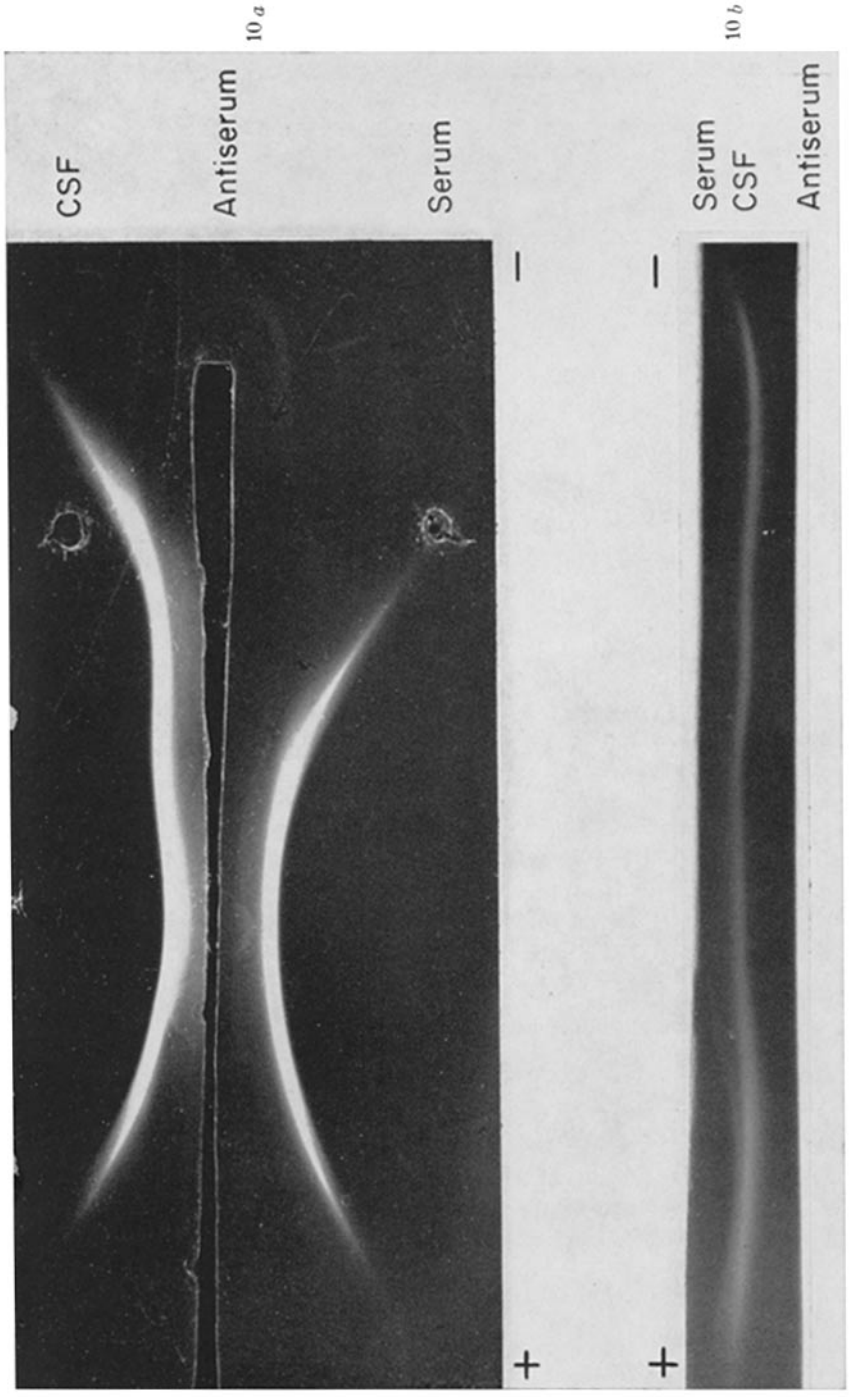
FIG. 9*a*. 1, transferrin C incubated with neuraminidase to produce bands in the 0 and 1 positions. 2, transferrin C. 3, transferrin B_2. 4, transferrin C.

FIG. 9*b*. 1, transferrin C. 2, Cynomolgus monkey transferrin. 3, transferrin C. 4, Transferrin D_1.

Immunological Relations of Transferrins

When transferrins C, B_2, and D_1, and neuraminidase-treated transferrin were examined in Ouchterlony plates, a reaction of identity was obtained between each of the human transferrins (Fig. 9). A purified preparation of transferrin B_{0-1} and serum of transferrin phenotype B_1B_1 also showed reactions of identity with purified transferrin C. However, a reaction of only partial identity was obtained between human transferrin C and the cynomolgus transferrin (Fig. 9*b*); the pattern indicated that the human transferrin possessed more antigenic determinants than the primate transferrin.

Agar gel immunoelectrophoresis of cerebrospinal fluid revealed the characteristic double arc shown in Fig. 10*a*. Starch gel immunoelectrophoresis also showed a continuous double arc (Fig. 10*b*) and the peaks of the two arcs co-



FIGS. 10*a* and 10*b*. Immunoelectrophoresis of serum and cerebrospinal fluid, developed with anti-human transferrin serum.

FIG. 10*a*. Precipitin pattern after agar gel electrophoresis.

FIG. 10*b*. Precipitin pattern after starch gel electrophoresis of serum and cerebrospinal fluid in adjacent slots of the gel; the precipitin arcs from each migration path appear superimposed on the same plane, but are actually separated in the third dimension of the photograph. In addition to the two principal transferrin arcs in cerebrospinal fluid, which correspond to bands 4 and 0 in the starch gel pattern, a faint precipitin arc corresponding to band 1 is also present (*cf.* Fig. 7-*b*).

incided with the two intense transferrin bands observed in the autoradiograph and in the protein stain. Starch gel immunoelectrophoresis of cord blood revealed in some cases a faint extension of the precipitin arc toward the cathode, which appeared to coincide with the faint bands observed by autoradiography.

DISCUSSION

Serum Transferrins

The characteristic five-band pattern obtained by the action of neuraminidase appears to represent the stepwise removal of sialic acid from the transferrin molecule. A previous report (5) has shown that the decrease in mobility after neuraminidase treatment is accompanied by a corresponding reduction in the sialic acid content of transferrin. Since ultracentrifugal analysis could detect no difference in sedimentation rate between treated and untreated transferrin, it is unlikely that sizeable units were split from the molecule; the loss of four sialic acid residues, with an accompanying reduction of 1200 units in an overall molecular weight of 90,000, would not have been detectable in the ultracentrifuge.

Thus far we have examined the effect of neuraminidase on nine of the twelve known polymorphic forms of human transferrin. Each variant showed a stepwise pattern similar to that observed for transferrin C; each variant could be resolved into four slower moving bands, and at increased enzyme concentration each could be resolved almost completely into the slowest moving component. It therefore appears that variations in sialic acid content are not involved in the genetic variation of human transferrin.

The starch gel experiments indicated that almost all of the sialic acid could be removed from human transferrin. At enzyme concentrations above which no further effect of neuraminidase could be observed, it was estimated by autoradiography (Fig. 1) that 90 per cent of the transferrin was present in band 0, with the remainder in band 1. This pattern suggested that all of the sialic acid had been removed from 90 per cent of the transferrin molecules, and that three out of four sialic acid residues had been removed from the remaining 10 per cent of the molecules. At this enzyme concentration, therefore, 97.5 per cent or virtually all of the sialic acid had been removed; equilibrium considerations could account for the residual percentage.

A splitting of transferrin after treatment with diphtheria toxin has been observed by Poulik (11). Using various concentrations of toxin, the same author has recently reported (22) a transferrin pattern similar to that obtained with neuraminidase, although even at large toxin concentrations two principal transferrin bands remained present. The author favored an interpretation of the toxin effect in terms of the presence of neuraminidase activity in the toxin preparation. In contrast to the present results, however, Warren and Blumberg (23) have reported that transferrin is split by neuraminidase into two bands of equal intensity. These authors used a single enzyme concentration, and it is

probable that their bands correspond to bands 0 and 1 of the stepwise pattern; such a result could be obtained by removal of approximately 88 per cent of the sialic acid from transferrin. The estimates by Blumberg and Warren of the amount of sialic acid removed vary from 78 to 107 per cent, and they consider the removal essentially complete.

The polymorphism of transferrin observed in rhesus monkeys (24, 1), chimpanzees (25), and cynomolgus monkeys (26) indicates that the inheritance of primate transferrin is similar to that of human transferrin. The genetics of transferrin in cattle appears to be different from that in humans, since cattle homozygous for a particular allele have been shown to possess more than one transferrin (27, 28). The untreated cattle phenotypes were suggestive of the stepwise patterns obtained with neuraminidase-treated human transferrin. However, it has not yet been established whether the multiple bands of cattle transferrin within a single phenotype represent a spectrum of molecules with slight variations or whether the slower moving bands represent associations of a basic unit analogous to the human haptoglobin series (29). The stepwise pattern of three bands obtained after neuraminidase treatment of the rhesus, cynomolgus, and chimpanzee sera suggested that the primate transferrins contained only two sialic acid residues accessible to the enzyme. The electrophoretic mobilities of the five cattle phenotypes were also reduced by incubation with neuraminidase (Fig. 3*c*). It was difficult to assess the effect of the enzyme on the intermediate bands in the untreated phenotypes; however, the fastest and slowest moving bands in each phenotype appeared to contain two sialic acid residues accessible to neuraminidase, and the relative proportions of the transferrin bands within each phenotype remained approximately constant during treatment with the enzyme.

A possible explanation of the differing patterns obtained after neuraminidase treatment of transferrin as compared to ceruloplasmin, haptoglobin, and α_2-macroglobulin is the low sialic acid content of transferrin relative to these other serum glycoproteins; ceruloplasmin, haptoglobin-1, and α_2-macroglobulin contain respectively 10, 15, and 50 residues of sialic acid per molecule (30). It is unlikely that the stepwise removal of sialic acid from a molecule such as haptoglobin would provide a sufficient decrease in mobility for the treated protein to appear in the form of separate components which differed in sialic acid content. However, the slower and more diffuse haptoglobin and ceruloplasmin bands obtained after incubation with neuraminidase suggested that these bands represented a group of distinct components differing slightly from each other in sialic acid content. Thus the leading portion of the neuraminidase-treated haptoglobin band may contain two or three more sialic acid residues per molecule than the trailing portion.

No significant difference could be detected by amino acid analysis of the human transferrins B_2, C, and D_1. Analysis of D_1 from a D_1D_1 homozygote and of two preparations of transferrin purified by the Cohn fractionation pro-

cedure[1] also gave results which could not be distinguished from the present values. However, the present analyses showed considerable differences from the analysis of Cohn-fractionated human transferrin performed by Hannig and reported by Schultze in 1957 (31). Hannig gave no value for methionine; values for lysine, serine, glycine, and alanine differed by 10 per cent from the present results, values for threonine, half-cystine, isoleucine, and leucine differed by 20 per cent, values for proline and tyrosine differed by 30 per cent, and the value for histidine differed by 40 per cent.

The general similarity in amino acid composition of the human and cynomolgus transferrins is noteworthy. The large differences in serine and glucosamine content appear significant. The differences in lysine, threonine, alanine, valine, and methionine cannot be regarded as of definite significance because of difficulties in the interpretation of small differences involving these amino acids after acid hydrolysis. The antigenic difference between the human and cynomolgus transferrins is also of interest. A similar reaction of partial identity has been observed by Beckman *et al.* (31 *a*) after immunoelectrophoresis of mixed human and cynomolgus sera.

Transferrin in Cerebrospinal Fluid and Cord Blood

Accurate determination of the protein components in normal cerebrospinal fluid has been difficult because of the low CSF protein concentration. Paper electrophoretic studies by Wallenius (32) suggested that serum and CSF proteins were generally similar. Subsequent electrophoretic studies revealed the presence of a CSF component, labeled the phi (33) or tau (34) fraction, which migrated between the β- and γ-regions and which was not detectable in serum. The presence in normal CSF of proteins not found in serum has also been suggested by Lowenthal *et al.* (35) who observed several gamma globulin components, and by Clausen (36), who reported specific beta and gamma globulins by immunoelectrophoresis with anti-CSF serum. In addition, Pert and Kutt (37) have observed unusual starch gel patterns in the gamma globulin and prealbumin regions of pathological CSF samples. The tau fraction was clarified by the immunoelectrophoretic studies of Gavrilesco *et al.* (38), who showed a characteristic β_2-globulin in CSF whose precipitin line appeared as a double arc extending from the β- to the γ-region. Burtin (39) identified the CSF double bow as a protein with the antigenic properties of serum transferrin, and Clausen and Munkner (40) found that both arcs of the double bow bound radioactive iron. Pette and Stupp (41) showed that, after neuraminidase treatment of CSF, the faster migrating part of the double bow disappeared and the transferrin arc of treated CSF coincided with the arc of treated serum.

[1] These two samples were kindly supplied by Dr. J. L. Oncley, Harvard University Medical School, and Dr. B. E. Sanders, Merck Institute for Therapeutic Research, West Point, Pennsylvania.

In agreement with the conclusions reached by Pette and Stupp (41) on the basis of immunoelectrophoresis, the starch gel patterns obtained in the present study suggest that the transferrin of CSF exists in two principal components, one of which contains the full serum complement of four sialic acid residues per molecule, and the other of which contains no sialic acid accessible to neuraminidase. The antigenic identity of bands 4 and 0 in the CSF as shown by starch gel immunoelectrophoresis suggested that the two CSF transferrins were very similar. Ouchterlony analysis showed that removal of sialic acid from transferrin in serum did not affect its antigenic properties, and autoradiography showed that the slower moving bands of neuraminidase-treated serum transferrin retained their iron binding capacity.

Although there is no clear explanation for the occurrence of the two principal transferrins in CSF, it is possible that band 4 enters the CSF from the plasma and that band 0 represents a protein synthesized within the central nervous system. Studies on sialic acid–containing units in glycoproteins (42, 43), and the recent isolation of a UDP-galactose-glucosamine–sialic acid sugar nucleotide (44) suggest that the biosynthesis of sialic acid prosthetic groups may proceed by the unit addition of prefabricated multi-membered chains. A step involving enzymatic addition of a sialic acid–containing unit may be lacking in the synthesis of transferrin band 0 in the CSF. It is of interest that in the present study the cynomolgus transferrin contained half the human complement of sialic acid accessible to neuraminidase and half the amount of glucosamine. Although the faint bands in the 1 and 2 positions in normal CSF clearly suggest neuraminidase activity, the presence of neuraminidase in CSF cannot easily explain the pattern of CSF transferrin, since a stepwise effect has been uniformly observed for neuraminidase-treated serum transferrin. In no case have bands 4 and 0 been observed simultaneously after neuraminidase treatment of serum. In addition, incubation of the concentrated CSF for 12 hours gave no indication of neuraminidase activity. The removal of sialic acid from transferrin by contact with enzymes of the central nervous system and the subsequent return of the stripped transferrin to the CSF is unlikely, since both haptoglobin and ceruloplasmin in CSF failed to show the reduction in mobility characteristic of their serum counterparts after neuraminidase treatment.

The observed CSF transferrin pattern in individuals of serum phenotype CC suggested that the CSF of individuals heterozygous for transferrin variants should contain four principal iron-binding components. Examination of CSF from an individual of serum phenotype CD_1 revealed the four expected transferrins; the two faster moving components coincided in mobility with the serum transferrins C and D_1, and the two slower moving components corresponded to transferrins C and D_1 after the complete removal of sialic acid by neuraminidase.

The transferrin patterns in untreated cord blood suggest that the faint bands

in the 3, 2, 1, and 0 positions differ in sialic acid content. As with cerebrospinal fluid, the observed pattern was not characteristic of the action of neuraminidase. Although the development of serum proteins in the human embryo is obscure, it has recently been shown that both haptoglobin (45, 46) and transferrin (47) can be synthesized by the fetus at least by the time of birth, and the present study has demonstrated haptoglobin synthesis as early as 28 weeks in development. Since the four faint bands of fetal serum were not detectable in the adult, it is possible that at a particular stage of embryonic development an enzyme mechanism begins to function in the liver which is capable of adding sialic acid to transferrin. Since a small proportion of the transferrin in fetal serum does not contain the full adult complement of sialic acid, it appears that the enzyme mechanism has not begun to function completely at the time of birth. The absence of the four faint bands in fetal samples obtained at periods of development earlier than 33 weeks raised the possibility that the transferrin C bands in these early samples may have derived from the maternal circulation. Further studies are in progress to clarify these results.

SUMMARY

Nine of the twelve known variants of human transferrin have been resolved by the action of neuraminidase into stepwise patterns of four additional slower moving components whose relative intensities depended upon the concentration of enzyme. These components appeared to represent the stepwise removal of the four sialic acid residues from the transferrin molecule, and at large enzyme concentrations, almost all of the transferrin was reduced to the position of the slowest moving component. In contrast, the electrophoretic mobilities of haptoglobin, ceruloplasmin, and α_2-macroglobulin showed a gradual decrease with increasing neuraminidase concentration.

The transferrins of chimpanzees, rhesus and cynomolgus monkeys, and cattle were resolved by neuraminidase into two slower moving components. These experiments suggested that the primate and cattle transferrins contained only two sialic acid residues accessible to the enzyme.

Transferrins C, B_2, and D_1 and a cynomolgus monkey transferrin were purified from serum by starch block electrophoresis and cellulose chromatography. Ultracentrifugal analysis could detect no difference in sedimentation rate between transferrin C, the primate transferrin, and neuraminidase-treated transferrin C. The human transferrins showed no variation in amino acid composition, but the cynomolgus transferrin was approximately 20 per cent higher in serine content and 50 per cent lower in glucosamine than human transferrin C. Reactions of antigenic identity were obtained among five human transferrin variants but a reaction of only partial identity was obtained between transferrin C and the cynomolgus transferrin.

The transferrin pattern of cord blood showed a prominent band in the posi-

tion of transferrin C, accompanied by four faint slower moving bands which coincided with the four transferrin components produced by the action of neuraminidase on transferrin C.

The transferrin pattern of cerebrospinal fluid in individuals homozygous for serum transferrin C showed two principal components, one of which appeared to contain no sialic acid. Haptoglobin, ceruloplasmin, and α_2-macroglobulin were also present in cerebrospinal fluid.

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