by early or middle Eocene times and were approaching extinction. They can, therefore, be considered to be better representatives of archaic mammals at an early stage of evolution than the entire Eocene group. The dashed line in Fig. 1 is a least-squares fit to the "Eocene" points, 2, 3, 7, and 8, and the contemporary opossum, which was assumed to be representative of forms of that type. The equation of the dashed line is:

$$E = 0.026\,P^{0.657} \qquad (5)$$

It should be emphasized that the only restriction imposed by this analysis was that the lines in Fig. 1 be straight. They have slopes that are essentially 2/3 and are displaced as they are in the vertical dimension because these slopes and displacements fit the data best.

Despite the small number of points, the equations are relatively well determined. The exponents of Eqs. 3, 4, and 5, that is, the slopes of the lines in Fig. 1, stated with their standard errors, are: for the Recent sample, $\beta = 0.664 \pm 0.012$; for the Oligocene sample, $\beta = 0.655 \pm 0.020$; and for the archaic "Eocene" and the opossum, $\beta = 0.657 \pm 0.028$. The linear regression accounts for 97 percent of the variance of the Recent sample, 94 percent of the Oligocene, and 99 percent of the archaic "Eocene" and the opossum. We can conclude that for these specimens the likelihood of a true β much different from 2/3 is small, and that the linearity implied by assuming Eq. 1a is supported by the data.

In addition to confirming my speculations about the primitive relationship between brain and body weight in mammals (compare Eqs. 2 and 5), perhaps the most important aspect of the results is that they give simple, yet mathematically precise, statements about the evolution of the brain. The same general rule that describes brain-to-body relationships in contemporary mammals was found in the earlier evolutionary stages sampled here, and the parameter, k, differentiated those stages. This parameter is not as successful in differentiating subgroups of contemporary mammals (see *7*), but the present results support its consideration as an index of cephalization for the mammals as a class at different stages in evolution.

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5. Most of this research was performed under grant M-3145(a) from the National Institutes of Health and was supported in part by the U.S. Air Force under contract No. AF 49(638)-536 monitored by the Air Force Office of Scientific Research of the Air Research and Development Command. The results were included in a paper presented to the Psychonomic Society, 2 September 1960. Fossil specimens used in this work are from the departments of vertebrate paleontology at the American Museum of Natural History (AMNH), Harvard's Museum of Comparative Zoology (MCZ), the U.S. National Museum (USNM), and the Chicago Natural History Museum (CNHM). I wish to thank E. H. Colbert, G. G. Simpson, A. S. Romer, C. L. Gazin, and W. D. Turnbull of those museums for allowing me access to these materials. Special thanks are due Dr. Gazin for identifying *Subhyracodon* and *Hoplophoneus* at the U.S. National Museum on the basis of associated teeth, and for copies of endocranial casts of *Subhyracodon* and *Uintatherium*. My visit to Harvard's Museum of Comparative Zoology was made especially memorable by the gracious hospitality of Dr. Tilly Edinger. Her monograph *Evolution of the Horse Brain* [*Geol. Soc. Am., Mem. 25* (1948)] suggests important departures from the general evolutionary trend described here and includes an invaluable bibliography.
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Alterations in Sialic Acid Content of Human Transferrin

Abstract. Starch gel electrophoresis of human transferrin treated with neuraminidase revealed a pattern of five bands whose intensities varied with neuraminidase concentration. Sialic acid analysis after starch block electrophoresis suggested that the bands represented the stepwise removal of sialic acid from the transferrin molecule. Evidence was also obtained for the purification of a particular genetic transferrin variant.

Genetically determined polymorphism has been described in several proteins in man, and in certain instances the variation has been localized to a single aminoacid substitution (*1*). The experiments reported in the present study suggest that observed variations in electrophoretic mobility may also represent differences in carbohydrate content.

Poulik (*2*) has described alterations in the electrophoretic mobility of serum protein components after treatment with diphtheria toxin. Using the technique of immunoelectrophoresis, Schultze and Schwick (*3*) have shown a decrease in mobility of the "β_1-iron-combining globulin" upon incubation with neuraminidase, an enzyme which cleaves the glycosidic bond joining sialic acid to a protein molecule. After the introduction of starch gel electrophoresis by Smithies (*4*), several genetically controlled variations in human serum β-globulins were described and subsequently identified as transferrin, the iron-binding component of serum. Neuraminidase-treated transferrin has recently been reported to separate into two sub-units in the starch gel (*5*).

In the present experiments transferrin was detected by its characteristic position in vertical starch gel electrophoresis in borate buffer, and its iron-binding property was confirmed by autoradiography (*6*). Transferrin C was prepared from the beta fraction of 20 ml of normal serum isolated by starch block electrophoresis in *p*H 8.6 barbital buffer (*7*). The beta fraction was chromatographed on TEAE cellulose and eluted with 0.025*M* Na_2HPO_4. The purified transferrin migrated as a single component in starch gel electrophoresis. Transferrin concentration was determined by the Folin-Ciocalteau procedure, and a standard curve was calculated from a transferrin sample dried to constant weight. Neuraminidase experiments on normal human serum and on the purified transferrin preparations were carried out by incubating samples at 37°C at various enzyme concentrations for 12 hours. The neuraminidase preparation was a ten times concentrated filtrate of *Vibrio cholerae*. The unconcentrated filtrate inhibited the agglutination of chicken erythrocytes at a 1:128 dilution when titrated against the Lee strain of influenza B virus (*8*). Sialic acid was determined by a modification of Bial's orcinol reaction (*9*), and optical density was measured in a Beckman Model DU spectrophotometer at 570 and 670 mμ, as suggested by Svennerholm (*10*). A standard curve was calculated from sialic acid purified from human ovarian cyst fluid.

Treatment of normal transferrin with neuraminidase split the single transferrin C (Fig. 1, band 4) into four additional slower-moving, iron-binding components (bands 3 to 0) whose relative intensities depended upon concentration of neuraminidase. A similar pattern was obtained by varying incubation time. No further bands appeared with an additional tenfold increase in neuraminidase concentration.

Starch block electrophoresis of purified transferrin treated with neuraminidase (Fig. 2) showed that the protein peak of the treated material migrated slower than an untreated control. Ultracentrifugal analysis could detect no difference in *S*-rate between the treated and untreated material. Sialic acid analysis revealed that the treated material contained approximately one to two sialic acid residues per molecule of

transferrin, with the fewest residues located in the slowest-moving portion of the peak, whereas the sialic acid content of the untreated control remained constant at approximately four residues per molecule throughout the peak, in agreement with previously reported values (*11*).

A comparison of the results obtained by starch gel and starch block electrophoresis suggested that the stepwise changes in mobility (Fig. 1) reflected variations in the number of sialic acid residues. Each of the four sialic acid residues of the transferrin molecule was readily accessible to the enzyme, and each residue contributed a definite increment to the electrophoretic mobility of the intact protein. As the glycosidic bonds were hydrolysed by the enzyme, an increasing but random removal of sialic acid took place until the four residues had been removed, after which there was no further change in mobility. Band 4 represented the untreated transferrin and band 0 appeared to represent the complete removal of sialic acid from the molecule, while bands 1 to 4 contained one to four sialic acid residues, respectively. With increased amounts of enzyme, most of the transferrin appeared in band 0, with only a small quantity in band 1.

In certain sera examined by autoradiography, normal transferrin C was accompanied by a faint slower-moving component in the position of band 3. This previously reported "shadow" band (*12*) may represent either a naturally occurring heterogeneity in transferrin C or the loss of a sialic acid residue from transferrin as a result of aging or contamination. A splitting of transferrin similar to the stepwise pattern obtained with neuraminidase has been observed in the starch gel upon treatment of transferrin with diphtheria toxin (*13*).

Neuraminidase treatment of haptoglobin and ceruloplasmin in serum resulted in a gradual decrease in mobility instead of the stepwise pattern obtained for transferrin. Since the ceruloplasmin molecule contains 10 sialic acid residues and the haptoglobin molecule at least 15 (*14*), it was unlikely that small differences in sialic acid content would yield a stepwise pattern in the gel. However, the decrease in mobility was accompanied by a spreading of the protein band which suggested that the leading portion of the band contained two or three more sialic acid residues per molecule than the trailing portion.

Because of the somewhat similar electrophoretic patterns obtained for abnormal transferrin variants and neuraminidase-treated normal transferrin (Fig. 1), it was of interest to determine the sialic acid content of an available

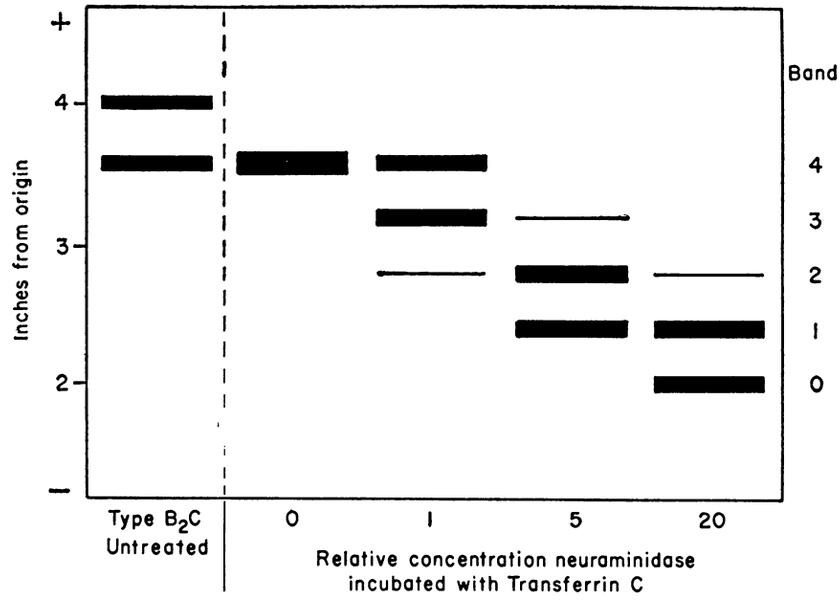

Fig. 1. Diagram of autoradiograph after starch gel electrophoresis of serum transferrin type C incubated with various concentrations of neuraminidase and of untreated type B_2C.

variant. Since individuals homozygous for an abnormal transferrin are extremely rare, a procedure was developed for the separation of an abnormal type from the serum of a heterozygote. The genetic transferrin variant B_2 was isolated by chromatography of the beta fraction from serum of transferrin type B_2C. Transferrin C appeared at the column volume in 0.025*M* Na_2HPO_4 whereas the B_2 component was slightly retarded. The purified B_2 and C components migrated as single bands in starch gel electrophoresis and as single peaks in the ultracentrifuge with no detectable difference in *S*-rate.

A single precipitin arc was obtained with each component by immunoelectrophoresis against antihuman serum although the precipitin line was displaced toward the anode for type B_2. Immunological studies in Ouchterlony plates confirmed that both transferrin B_2 and neuraminidase-treated type C were antigenically indistinguishable from normal transferrin C. When tested for purity against antihuman serum by the double diffusion method of Preer (*15*), transferrin C showed a single band, whereas 15 bands were observed with the original beta fraction. The B_2 preparation, however, revealed a trace

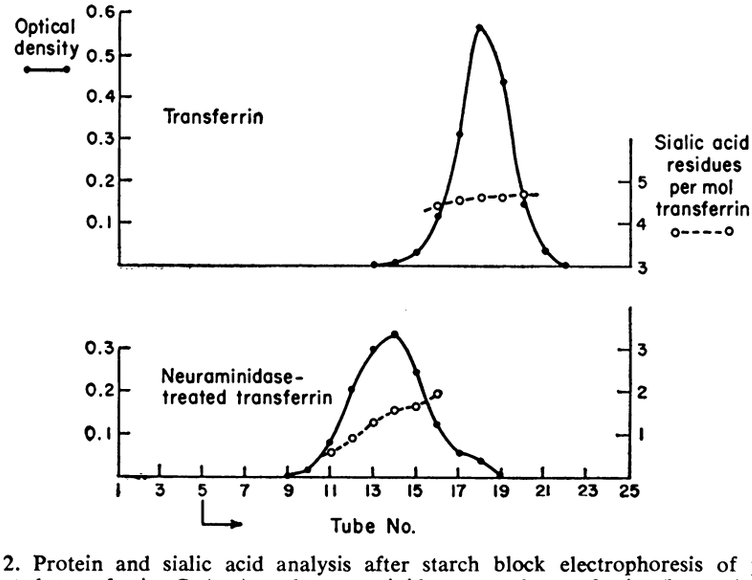

Fig. 2. Protein and sialic acid analysis after starch block electrophoresis of purified untreated transferrin C (top) and neuraminidase-treated transferrin (bottom). Starch gel electrophoresis of the untreated transferrin showed a single band in the 4 position. The treated material showed bands in the 2, 1, and 0 positions.

component which appeared only after 48 hours. It was estimated by dilution studies that this component could represent no more than a maximum of 5 percent of the total protein. Sialic acid analyses on transferrins B_2 and C could detect no significant difference. Further experiments are being conducted to determine the nature of the structural difference between these two components. Although differences in sialic acid content would be sufficient to account for observed variations in electrophoretic mobility of certain transferrins, the variation would be expected to extend to a specific genetically determined amino acid difference in the protein backbone. Although no definite selective advantage is known which could maintain the transferrin polymorphism (*16*), the genetic variation may influence resistance to infectious disease (*17*). The variation in charged groups may also affect the dissociation of the iron-transferrin complex according to the equilibrium postulated by Laurell (*18*) and alter the transfer of iron to sites of storage or synthesis (*19*).

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Critical Period in the Social Development of Dogs

Abstract. Litters of puppies were isolated, with the bitch, in fenced acre fields from 2 to 14 weeks of age. They were removed indoors at different ages, played with for a week, and returned to the field. The pups manifested an increasing tendency to withdraw from human beings after 5 weeks of age and unless socialization occurred before 14 weeks of age, withdrawal reactions from humans became so intense that normal relationships could not thereafter be established.

The term *imprinting* is generally defined as a capacity of some species of birds to develop a permanent attachment to any species, including man, made available to it during a critical period in its early development. This period of primary socialization usually ends with a mounting tendency to flee from strange species (*1*). The present experiment demonstrates a similar phenomenon in a mammalian species.

This study derived from the observation that purebred cocker spaniels exhibited intense flight responses to humans after they had been raised in an acre field with a minimum of human contact prior to 14 weeks of age. Our unsuccessful attempts to tame or socialize them led us to examine the age when human contact would most effectively reduce the withdrawal response at 14 weeks of age.

Five litters of cocker spaniels ($N = 18$) and three litters of beagles ($N = 16$) were raised in acre fields bounded by an 8-foot high wooden fence. The mother and her litter were raised alone in the pen and received food and water through drops in the fence. Pups from each litter were taken from the field for a week of socialization at 2 weeks of age ($N = 6$), 3 weeks, of age ($N = 6$), 5 weeks of age ($N = 7$), 7 weeks of age ($N = 7$), and 9 weeks of age ($N = 3$), and then returned to the field. During this week indoors, pups were played with, tested, and cared for throughout three daily half-hour periods (*2*). Controls remained in the field ($N = 5$) until the entire litter was taken indoors for final testing at 14 weeks of age.

At the start of the week of socialization pups removed from the field at 5 weeks of age scored significantly higher on a test of attraction to a handler [Handling Test (*3*)] than those removed at 2, 3, or 9 weeks of age ($p = 0.01$ to 0.05; t, tests). The low scores of 2- and 3-week-olds were due simply to their physical and motor immaturity, while 9-week-olds exhibited low scores because they had a marked tendency to avoid the handler. By the end of the week of socialization, however, all save the still immature 2-week-olds were equally attracted to the handler.

The progressive development of avoidance responses was evident in daily, 10-minute tests of the amount of time a puppy spent in physical contact with a passive, reclining human. Two-week-olds, again, were too immature to do much but sleep, eat, or crawl about randomly; 3-week-olds were immediately attracted to the experimenter and spent most of the 10-minute period pawing, mouthing, and biting him and his garments; 5-week-olds exhibited wariness at first, but they became comparable to 3-week-olds before the end of the first play period; 7-week-old pups, however, were frightened and wary of contacting the experimenter over the first *two* days of socialization, while 9-week-old pups exhibited these reactions over the first three days. No p values are given since this test was administered only to the last three litters; however, notes taken on all animals reveal no exceptions to this pattern of progressive avoidance, and it was persistently observed in the other situations of the socialization period.

After removal from the field at 14 weeks of age a series of tests was administered over a 2-week period. The handling test was administered to all pups at the start and at the end of this period of testing, a period involving daily contact with humans. At 14 weeks the pups socialized in their second week, and the controls scored significantly lower in "attraction to the handler" than did the fifth, seventh, and ninth week groups ($p \leqq 0.05$, t tests). By the 16th week, however, only the control group remained significantly low, and it scored lower than all other groups ($p \leqq 0.02$, t tests). These results are illustrated in Fig. 1.

Since the control animals appeared as timid on the final day of testing as

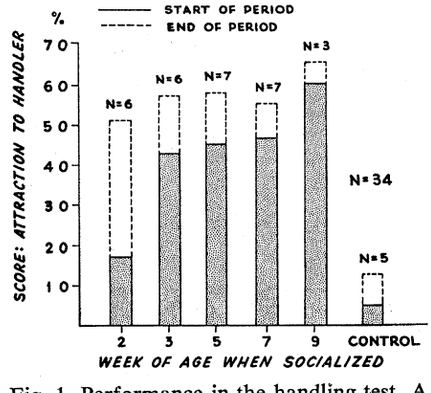

Fig. 1. Performance in the handling test. A measure of attraction to the handler, comparing performance at the start and at the end of the final period of testing (14 and 16 weeks of age).