described for the CAM peptides (*10*), except that AE CN-3 and AE CN-4, unlike the CAM derivatives, were not resolved by gel filtration with Sephadex G-50, but were purified by ion-exchange chromatography with carboxymethyl-cellulose at 40°C. The purified fragments gave compositions in close agreement with those expected for their sequences. The amino terminal sequence of CN-4 was constructed from the sequences of overlapping peptides isolated from thermolysin and trypsin digests, and was confirmed by an automated sequencer analysis of CAM CN-4. The sequence from CN-1 is based largely on the structures of arginine cleavage fragments obtained by trypsin digestion of the citraconyl derivative of AE CN-1 and purified by ion-exchange chromatography and gel filtration. Peptides isolated from trypsin, chymotrypsin, or thermolysin digests of CAM CN-1 were also used in constructing the sequence. All peptides were sequenced by the combined dansyl chloride–Edman degradation procedure (*12*) or by the subtractive Edman degradation procedure. Amides were determined by amino acid analysis of Pronase aminopeptidase digests of appropriate peptides (*10*).

The peptides denoting the ordering of CN-7, CN-9, and CN-4 were isolated from a thermolysin digest of performic acid–oxidized transferrin by ion-exchange chromatography and gel filtration. Their sequences are shown in Fig. 1. Although only one residue of CN-4 is contained in this overlap (tyrosine), there is no ambiguity as CN-4 is unique in possessing amino terminal tyrosine. The two regions of sequence are shown in Fig. 2, having been aligned by the insertion of one gap of three residues in the CN-7,9,4 sequence and a gap of one residue in the CN-1 sequence. Of the 57 positions being compared, 23 residues in corresponding positions in the two sequences are identical. Many other amino acids in corresponding positions are chemically similar. The extent of homology based on identical residues (40 percent) is comparable to that seen in comparisons of pairs of proteins whose common ancestry is well established, for example, α-lactalbumin and lysozyme (*12*). As the two regions of sequence are well separated in the molecule, the region from CN-1 being at least 22 residues removed from the amino terminus of this fragment, this sequence alignment indicates the existence of internal homology in the polypeptide chain of transferrin, reflecting an ancestral gene doubling during the phylogenetic development of vertebrate transferrins.

We conclude from this study and the work of Williams (*9*) that transferrin is composed of two homologous regions, each having a single metal binding site and a degree of conformational independence, including an absence of disulfide cross-links between the domains (implicit in the limited proteolysis studies).

The necessity for conserving the metal binding sites and other functional areas in the two homologous regions of the molecule would be expected to restrict the sequence variation around such areas. These areas should be apparent in the internal homology as regions of strongly conserved sequence. The highly conserved region in the above sequence alignment (positions 31 to 44) is therefore a possible candidate for an area of functional significance. It is interesting that each metal binding site in transferrin contains two tyrosyl residues (*13*), which chelate with the metal, and a positively charged group, associated with anion binding. Such residues (two tyrosines and an arginine) are present in the conserved area in Fig. 2. When our current sequence studies of transferrin are completed, we hope to clarify the functional significance of this and other areas in the molecule by chemical modification.

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Estrogen Target Sites in the Brain of the Chick Embryo

Abstract. *Autoradiograms prepared from brains of chick embryos after injection of [^{3}H]estradiol demonstrate the existence of target cells for estrogen in the medial preoptic and ventral hypothalamic regions as early as day 10 of incubation. Target cells also appear in telencephalic, diencephalic, and mesencephalic locations during later stages of embryonic development. These hormone-concentrating cells probably are the anatomical substrate for the formative action of sex steroids during embryonic life on certain brain functions, such as control of sexual and aggressive behavior and gonadotropin secretion.*

The role of gonadal steroids in influencing the development of the brain and determining differences between males and females in behavior and hormonal regulation has been examined in numerous species, such as the rat, mouse, guinea pig, dog, rhesus monkey, chicken, and Japanese quail (*1–5*). It has been suggested that early gonadal secretions also affect the development of the brain in man (*6*). In some species, such as the rat, the critical period for sexual differentiation of the brain occurs perinatally or in the early postnatal period (*4*). In many species, the effect of gonadal steroids on brain development is initiated prenatally or restricted to a period during embryonic life, as is likely to be the case in primates (*1, 7*). Efforts to demonstrate steroid hormone "receptors" in the neonatal brain with biochemical techniques have been largely unsuccessful (*8*), and hitherto no report exists in the literature demonstrating steroid hormone receptors in embryonic nervous tissue. In contrast, the presence of target cells for estrogen and androgen in the brain of the 2-day-old neonatal rat has been demonstrated with autoradiography (*9*). In our study, we used autoradiography to investigate the ontogeny of estrogen target cells. The chick embryo was selected, since the presence of maternal and placental steroids would be expected to impair demonstration of binding sites in placental animals.

Chick embryos of the White Rock (Arbor Acre) strain were incubated at 38°C in 60 to 90 percent relative humidity in a still air incubator. Prior to incubation the eggs had been stored at 18°C. Nine chick embryos at different stages of development and two newly hatched chicks received 0.2 μg of [2,4,6,7-^{3}H]17β-estradiol (specific activity, 105 mc/mmole) in a mixture of 10 percent ethanol and saline. One additional

chick was injected with 0.4 μg of [^{3}H]estradiol. Embryos were killed on day 6 (N = 2), day 10 (N = 6), or day 17 (N = 4) of incubation. In order to test for specificity of the radioactive labeling, a group of four embryos—two on day 10 and two on day 17 of incubation—and two newly hatched chicks were injected with 2 μg of unlabeled estradiol 10 minutes before administration of 0.2 μg of [^{3}H]estradiol. The injections were administered via the chorio allantois in 6- and 10-day embryos and intramuscularly in 17-day embryos and newly hatched chicks. When the animals were killed (1 or 2 hours after administration of [^{3}H]estradiol) all embryos were staged according to the Hamburger and Hamilton staging series (*10*). The brains were removed, mounted on tissue holders, frozen in liquid propane, and processed for thaw-mount autoradiography (*11*). The autoradiograms were exposed for 4 to 12 months.

Nuclear concentration of radioactivity was detected in brain cells as early as day 10 of incubation (Fig. 1a), but was not found in embryos examined at day 6, although radioactivity was present throughout the brain at this stage. Our 10-day embryos ranged from stage 33 to stage 35 and included both males and females (*12*). The areas of distribution of labeled cells appeared identical at these stages and in embryos of both sexes. Possible quantitative differences cannot be excluded at this time, however. Prior injection of a dose of unlabeled estradiol ten times higher than that of [^{3}H]estradiol prevented nuclear concentration of radioactivity in 10-day and older embryos (Fig. 1, b and d); this indicates that cells which concentrate radioactivity are characterized by a concentrating mechanism of limited capacity, such as is known to be present in cells carrying estradiol receptors. Estrogens are secreted in female chick embryos (*13*). It appears that endogenous estrogens do not saturate the full complement of receptors available in the brain of 10-day and older embryos.

In the 10-day embryo, nuclear concentration of radioactivity was seen in cells of the medial preoptic nucleus, in the lateral portion of the nucleus hypothalamicus posterior medialis, and in the ventrolateral portion of the nucleus inferior (*14*). In the medial preoptic nucleus, labeling was most intense in the dorsal portion and extended into the bed nucleus of the stria terminalis.

On day 17 of incubation (stages 43 and 44) nuclear concentration of radioactivity was present in the nucleus preopticus medialis (Fig. 1c), the bed nucleus of the stria terminalis, the lateral septum, the tuberculum olfactorium, the lateral portion of the nucleus hypothalamicus posterior medialis, the ventrolateral portion of the nucleus

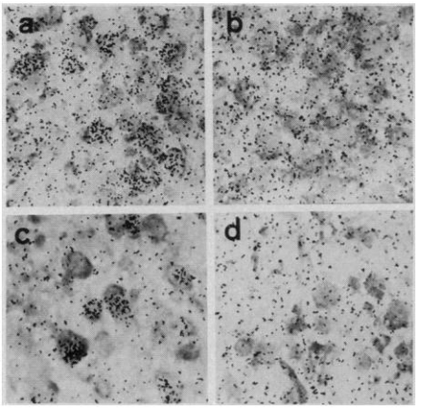

Fig. 1. Autoradiograms (prepared 1 hour after injection of [^{3}H]estradiol) showing nuclear concentration of radioactivity in cells of the nucleus preopticus medialis in 10-day embryo (a) and 17-day embryo (c). In the 10-day embryo, cells are more densely arranged and less intensely labeled than in the 17-day embryo. Prior injection of a dose of unlabeled estradiol ten times larger than that of [^{3}H]estradiol prevents nuclear uptake of radioactivity in the 10-day embryo (b) and 17-day embryo (d). Exposure times: 156 days (a and c) and 129 days (b and d). Sections 4 μm thick were stained with methyl green pyronine (× 700).

inferior, the area surrounding the nucleus mesencephalicus lateralis, pars dorsalis, and in small neurons interspersed between the large neurons of the nucleus of the mesencephalic tract of the fifth nerve. In the newly hatched chick, labeled cells were detected, in addition, in the infundibular nucleus and in the taenial nucleus.

The demonstration of estrogen target cells in specific regions of the brain of the 10-day embryo correlates well with the finding that estrogen administered during early incubation in birds depresses later performance of male copulatory behavior. Japanese quail males injected with estradiol benzoate or testosterone propionate on day 10 of incubation fail to display male copulatory behavior when mature (*5*). In the male chicken, mating behavior is suppressed if estradiol benzoate or testosterone propionate are administered before day 13 of incubation (*3*). Experimental evidence suggests that the medial preoptic region is involved in controlling male copulatory behavior in the chicken. In young and mature gonadectomized chickens, implantation of testosterone propionate in the medial preoptic area promotes male copulatory behavior (*15*), and lesions cause impairment (*16*). The ventral hypothalamus may also be involved in the control of male copulatory performance (*17*); additional evidence indicates that this area may play a role in the control of gonadotropin secretion (*18*). It is therefore likely that the presence of estrogen target cells during embryonic development in the preoptic region and ventral hypothalamus is related to an effect of early gonadal secretions on both imprinting of sex behavioral characteristics and the neural control of pituitary gonadal functions.

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