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BLOOD–BRAIN BARRIER: SELECTIVE CHANGES DURING MATURATION

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Abstract—The penetration of substances relative to freely-diffusible antipyrine was determined in regions of the maturing chick brain. Two amino acids were studied: tyrosine, which is essential to the brain and is a neurotransmitter precursor, and proline, a non-essential amino acid. During maturation the barrier toward proline developed to a much greater extent than that toward tyrosine. The penetrance of D-glucose, the main energy source for the brain, increased during maturation, while the penetrance of L-glucose, a metabolically inert stereoisomer of D-glucose, decreased.

The results suggest differing rates of development of the blood–brain barrier to various substances.

SUBSTANCES in the blood have to pass through capillary walls before entering the brain extravascular space. The process that selectively determines the degree of passage has been termed the blood–brain barrier. Penetration through this barrier appears to be related to the nature of the substances, but closely related molecular species penetrate to very different extents. Previous radioisotopic studies have shown that the barrier to most compounds is more developed in the mature animal. This is true for inulin (VERNADAKIS & WOODBURY, 1965; FERGUSON & WOODBURY, 1969), GABA (WOOD, 1970) and chloride LAJTHA, 1957; VERNADAKIS & WOODBURY, 1965). In adult brain non-essential amino acids are excluded to a greater extent than essential amino acids (OLDENDORF, 1971; YUDILEVICH, DEROSE & SEPULVEDA, 1972), and a non-metabolizable sugar (L-glucose) is excluded to a greater extent than a metabolizable sugar (D-glucose) (OLDENDORF, 1971). We have studied the development of these specificities in various regions of the chick brain.

The present study compares the penetration of tyrosine with proline, and D-glucose and L-glucose at different stages of development. Our results are presented as the degree of penetration relative to that of a freely-diffusible control substance.

MATERIALS AND METHODS

Materials

White Leghorn chicks ranging from the 11-day embryo to 14-day-old chicks were used. Isotopes were obtained from New England Nuclear Corp. (Boston, MA): (*N*-methyl-^{14}C) antipyrine 15–40 mCi/mmol and L-(1-^{14}C) glucose 0.05 mCi/mmol; and Amersham/Searle (Arlington Heights, IL): D-(1-^{3}H) glucose 5.5 Ci/mmol, L-(5-^{3}H) proline 33 Ci/mmol, L-(3,5-^{3}H) tyrosine 53 Ci/mmol and ^{3}H-inulin 875 mCi/mol.

Determination of penetration of substances into brain

A mixture was prepared containing approximately 17 μCi of a ^{3}H-labelled test substance and approximately 0.35 μCi of ^{14}C-labelled antipyrine in each 0.1 ml aliquot. Isotonic (0.14 M) NaCl was used as a diluent; no non-radioactive test substance was added.

A 0.1 ml intracardiac injection of the radioisotope mixture into 1- and 14-day-old chicks was completed within 2 s. The chicks were decapitated 15 s after injection. Care was taken with embryos so that blood loss due to hemorrhage was negligible. The shell was cracked, and the entire embryo with membranes intact was poured onto a dissecting tray. The embryo was removed from the sac by its feet and placed on a paper towel being careful not to rupture any major blood vessels and arranged so that the neck was straight and blood flow to the brain was not impaired. The chest cavity was opened; the heart exposed and 0.05 ml of the isotope mixture was injected. The needle remained in the heart to prevent excessive bleeding. Embryos were decapitated 15 s later. The brain was dissected out and rinsed with isotonic saline to remove any extraneous blood. In the case of 17-day-old embryos or older chicks, one optic lobe, one cerebral hemisphere and half a cerebellum were used from each animal. The whole brain of 11-day-old embryos was used.

Samples were dissolved in 2 ml NCS tissue solubilizer (Amersham/Searle) at 48°C. After cooling, 20 ml of toluene counting solution was added to each vial. The scintillation mixture consisted of 5 g PPO (2,5-diphenyloxazole) and 100 mg POPOP (1,4-bis-(5-phenyloxazolyl-2)-benzene) dissolved in 1000 ml toluene. Counting rates were determined in a Picker Liquimat scintillation counter using the external standard ratio method. Efficiency of counting was 6–9% for ^{3}H and 35–41% for ^{14}C. Corrections of ^{3}H-radioactivity for ^{14}C-crossover was always less than 2% of the ^{3}H-counts. The radioactivity due to (^{14}C)-antipyrine was between 700–2500 c.p.m./100 mg wet tissue.

An aliquot of the injected mixture was similarly counted. The ratio of ^{3}H to ^{14}C in the tissue was divided by the same ratio in the injected mixture, and the result was multiplied by 100 to give the penetration or uptake of the test substance as a percentage of the antipyrine penetration.

$$\% \text{ Penetration} = \frac{^{3}\text{H}/^{14}\text{C in tissue}}{^{3}\text{H}/^{14}\text{C in mixture}} \times 100.$$

Results and statistics were calculated on a Wang 462 programmable calculator. As (^{3}H)-L-glucose was not available, (^{14}C)-L-glucose penetration was indirectly determined by initial comparison to the (^{3}H)-proline penetration. The

Abbreviation: GABA, γ-aminobutyric acid.

TABLE 1. ENTRY OF INULIN INTO CHICK BRAIN REGIONS AT VARIOUS STAGES OF DEVELOPMENT

Age	% Apparent penetration Optic lobes	Cerebral hemispheres	Cerebellum
11-day embryo		Whole brain 47.34 ± 8.01	
17-day embryo	8.99 ± 1.03	9.65 ± 0.44	15.75 ± 5.14
1-day-old chick	12.97 ± 0.86	8.62 ± 0.41	12.76 ± 0.77
14-day-old chick	14.54 ± 1.06	9.59 ± 0.65	16·99 ± 2.03

Values shown are means ± S.E.M. Penetration was determined as described in the text.

apparent penetration of high molecular weight inulin was used as a control value. Each data point presented is a mean value derived from between 6 and 12 individual birds.

Hemoglobin determination

The optical density of a 500-fold dilution of blood in 0.1% ammonium hydroxide was determined at 417 mμ. After homogenizing each brain region in 10 vol of 0.1% ammonium hydroxide and centrifuging 9000 *g* for 10 min, the 417 mμ readings of the clear supernatants were compared to those of blood from animals of the same age.

RESULTS

Inulin

Inulin, a D-fructose polysaccharide, does not diffuse out of the mature vascular system to any significant extent when injected into the heart (OLDENDORF, 1971). This non-metabolizable carbohydrate can therefore be used as an indicator of background 'penetration', i.e. that due to radioactive mixture in the blood vessels of the brain. All other penetrations have had the corresponding inulin value (Table 1) subtracted from them.

There is a major decrease in the apparent penetration of inulin between 11 and 17 days of embryonic development from 47% to below 17%; this reduction does not seem to be correlated to the amount of blood in the brain (Table 2) which is relatively constant during development. If the circulation were sluggish or the heart stopped beating temporarily as happens frequently in the 11-day embryo, more of the inulin–antipyrine mixture would remain in the brain, thereby increasing the apparent penetration. Thus variations in the velocity of circulation of different animals could explain the high standard error seen at this age. Leakage of inulin from immature capillaries could also contribute to the high penetration.

Penetration of inulin into the cerebellum is consistently higher than that into either the optic lobe or cerebral hemisphere. This may be due in part to the slightly increased percentage of blood in the cerebellum, since even a small amount of blood would increase the apparent penetration significantly.

TABLE 2. PER CENT HEMOGLOBIN IN BRAIN REGIONS RELATIVE

Sample	Optical density at 417 mμ	% Hb
11-day embryo		
Blood	280	100.0
Whole brain	6.7	2.4
1-day-old chick		
Blood	490	100.0
Optic lobe	10.2	2.1
Cerebral hemisphere	10.5	2.1
Cerebellum	13.8	2.8
14-day-old chick		
Blood	542	100.0
Optic lobe	10.5	1.9
Cerebral hemisphere	10.2	1.9
Cerebellum	11.1	2.1

Values are the means of two determinations. Per cent hemoglobin was determined as described in the text.

Tyrosine and proline

In the 11-day embryo, tyrosine and proline penetrance do not differ significantly, both being in the range of 22–32% (Fig. 1a,b). By 17 days of embryonic development, a barrier to proline has started to develop; this being most marked in the optic lobes. In contrast, tyrosine penetration at this time has increased over the 11 day values. At 1 day after hatch,

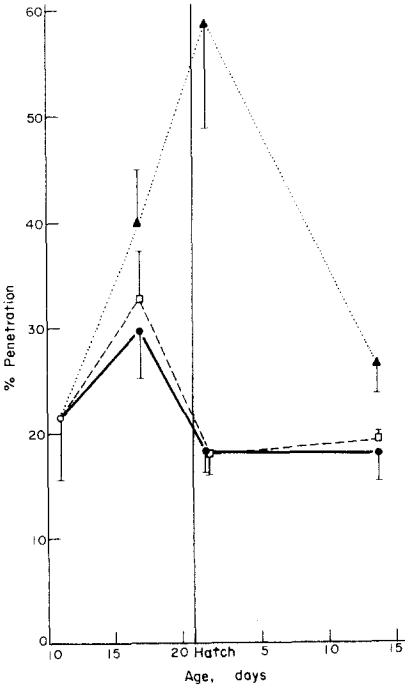

FIG. 1(a).

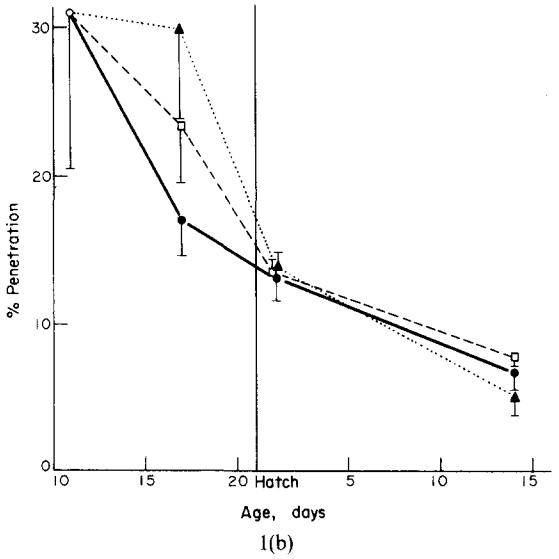

FIG. 1. Penetration of tyrosine (1a) and proline (1b) into the optic lobe (●——●), cerebral hemisphere (□-----□) and cerebellum (▲.....▲) of the developing chick brain. A mixture of ^{3}H-tyrosine or proline and ^{14}C antipyrine was injected into the heart 15 s before decapitation. The degree of penetration was determined by the procedure described in Methods. Values shown are means ± S.E.M.

there are regional differences in tyrosine penetrance, this being 58% for cerebellum and around 30% for the other regions. Since increasing the number of samples in the cerebellum group did not lower the standard error, the large variance could be due to rapid developmental changes that are occurring in individual birds at this time. Passage of proline in 1-day old birds is identical (13%) in all regions but lower than the passage of tyrosine into any region. In 14-day-old chicks, the penetration of proline has fallen to 10% in all brain regions while the penetration of tyrosine is between 18–27%.

D- *and* L-*glucose*

In the 11-day-old embryo there is no significant difference in the penetration of D- and L-glucose (Fig. 2a,b). At this age a barrier to glucose probably does not exist, and the 17–18% penetration may be due to simple diffusion. The penetration of D-glucose into all three regions does not change significantly between 11 and 17 days. After 17 days of incubation, the penetrance of D-glucose begins to increase. The greatest uptake of D-glucose (around 30%) is found in the cerebral hemispheres and cerebella of 14-day-old birds. Penetration into optic lobes reaches a plateau of around 20% at one day post hatch. By 17 days of development there are differences in the ability of various brain regions to exclude L-glucose with optic lobes excluding more than the other regions. The barrier to L-glucose is complete by 1 day after hatch, penetration being at background level.

DISCUSSION

Many substances readily migrate into the brain of the early embryo. This process does not seem to be very specific and may be largely due to diffusion. During development, a series of sophisticated uptake and exclusion mechanisms appear. Thus the mature brain has a varied response toward substances carried in the vascular system. In addition to allowing the passive inward diffusion of chemicals, it is also capable of either excluding or actively taking up substances from the blood stream. These latter two systems appear at around the same developmental stage.

In the 11-day-old embryo, all substances seem to penetrate into the brain to the same extent. As tight junctions become numerous in the choroidal epithe-

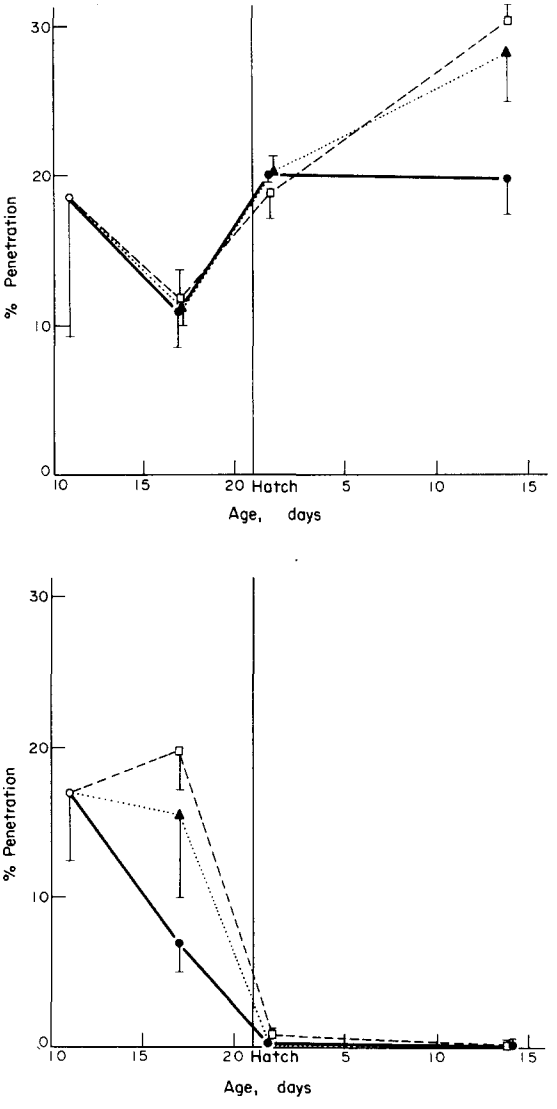

FIG. 2. Penetration of D-glucose (2a) and L-glucose (2b) into the optic lobe (●——●), cerebral hemisphere (□-----□) and cerebellum (▲.....▲) of the developing chick brain. Penetration was determined as described in Methods. Values shown are means ± S.E.M.

lium at 15–18 days of incubation (DOOLIN & BIRGE, 1969), the capacity to selectively exclude substances also appears. Inulin has reached its definitive penetration by 17 days of development; protein by 20 days (BIRGE, ROSE, HAYWOOD & DOOLIN, 1974). However, when tight junctions in the monkey were osmotically opened, protein was again able to extravasate into the brain (RAPOPORT, HORI & KLATOZO, 1971; RAPOPORT, 1974). Tight junctions are, therefore, requisite for a barrier, but the selectivity that evolves after their formation implies more than merely mechanical exclusion.

During maturation the penetrations of proline and tyrosine gradually diverge, that of tyrosine increasing initially then decreasing, and that of proline consistently decreasing. The penetration of this amino acid through the blood–brain barrier may be related to the need for tyrosine, an essential amino acid in the brain. There is a significant elevation in tyrosine penetration into all regions in the 17 day embryo; the penetration into the cerebellum continuing to rise through 1 day post hatch. Between 15–20 days of incubation, rapid developmental changes are occurring in the cerebellum as indicated by the marked rise in the number of parallel fibers (MUGNAINI, 1969). These changes may create a physiological need for tyrosing either as an amino acid or as a neurotransmitter precursor. Extracellular levels of tyrosine have been shown to influence the rate of catecholamine synthesis in a variety of systems (LEVITT, SPECTOR, SJOERDSMA & UDENFRIEND, 1965; MAINS & PATTERSON, 1973; RICHELSON, 1974; LLOYD & BREAKEFIELD, 1974; WURTMAN, LARIN, MOSTAFAPOUR & FERNSTROM, 1974), and perhaps the increased tyrosine penetration may be correlated with the induction of catecholamine synthesizing enzymes at this time. The rate at which amino acids are transported into the brain may also be a determining factor. A more rapid tyrosine transport system may account for part of the increased tyrosine penetration. In the 14-day-old chick, the penetration of tyrosine is not significantly different in various regions. In contrast to tyrosine, the penetration of proline, an amino acid that can be made in the brain, steadily decreases. Any physiological need for this amino acid can probably be satisfied by cerebral synthesis.

A stereospecific barrier as well as a stereospecific transport system for glucose develops between 17 days of incubation and 1 day post hatch. The D-form of glucose is the main energy source for the brain; the L-form cannot be metabolized by vertebrates. In the 11-day embryo, the penetrations of both forms of glucose are similar. By one day post-hatch, D-glucose penetration is increased while L-glucose penetration is at background level. The complete exclusion of L-glucose can be explained by the development of the blood-brain barrier; the concomitant increase in D-glucose penetration implies the maturation of a transport system. Mechanisms for glucose transport have been postulated (CRONE, 1965; CUTLER & SIPE, 1971; LEFEVRE & PETERS, 1966) and its kinetics studied (BETZ, GILBOE, YUDILEVICH & DREWES, 1973; PARDRIDGE & OLDENDORF, 1975). As the brain matures and as complex functional activity begins after hatch, an increase in energy supply may be needed. This may be reflected by an increase in D-glucose uptake and also by an increased velocity of cerebral blood flow. Blood flow has been shown to be related to functional activity (BONDY, LEHMAN & PURDY, 1974; KENNEDY, DES ROSIERS, JEHLE, REIVICH, SHARPE & SOKOLOFF, 1975) and to be proportional to cerebral glucose consumption (KENNEDY, DES ROSIERS, PATLAK, PETTIGREW, REIVICH & SOKOLOFF, 1974). At one day after hatch, the exclusion of L-glucose is complete; therefore all structures necessary for a barrier must exist at this time. Any further modifications in the barrier are probably in response to the physiological needs of the brain (BONDY & PURDY, 1974).

In the optic lobes, the barriers toward L-glucose and proline are pronounced as early as 17 days of incubation, preceding the appearance of similar mechanisms in the cerebellum and cerebral hemisphere. Since optic lobes mature earlier than either of the other regions, this suggests a relation between regional maturity and barrier development.

The development of selective exclusion mechanisms in the chick brain is well established at the time of onset of complex cerebral function. This is coincident with the appearance of systems enabling the uptake of certain chemicals to be specifically enhanced. These two processes may be interrelated and subject to modulation dictated by physiological needs.

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