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Developing Blood Brain Barrier to Trypan Blue.* (23077)

FRED M. GRAZER AND CARMINE D. CLEMENTE
(Introduced by D. C. Pease)
Department of Anatomy, School of Medicine, University of California at Los Angeles and Veterans Administration Hospitals, Los Angeles and Sepulveda, Calif.

Ever since Ehrlich first observed that the central nervous system could not be stained by intravenous injections of acid aniline dyes (1), information dealing with the functional and structural embryonic development of the blood brain barrier has been of considerable importance to physiologists. Bakay(2) was able to show by use of radioactive P^{32} that embryonic rabbit brain was more permeable to this ion than adult brain, and that permeability to P^{32} is greatest in early intrauterine life but continues to decrease until about 7 weeks of postnatal development. Grontoft (3) perfused human and animal fetuses with trypan blue and found the brain normally impermeable to this vital dye. Ten-minute delivered human fetuses 5 to 30 cm long had already developed an impermeability to trypan blue. It appears, however, that no one has observed penetration of vital dyes into the embryonic nervous system *in vivo*, due to the permeability problem of placental barrier and the difficulty of injecting embryos directly, although Waddington and Carter(4) were able to induce abnormalities in mouse embryos by injection of trypan blue into pregnant females.

In this study, an attempt was made to show *in vivo* staining characteristics of the rat embryo with trypan blue, and special emphasis was placed on development of blood brain barrier with respect to developing CNS blood vessels. All observations are made *in vivo* with the exception of the youngest rat embryos (10½ days, about 3 mm), which were perfused with trypan blue in Ringer's Solution while the heart was still beating.

Methods. Four-month-old Wistar female rats were used, and exact gestation times were determined by vaginal smears. If insemination had occurred, the female was separated from the male and used for experimentation at the appropriate gestation time. An error of about 12 hours in embryo age is possible with this method. Rat embryos were injected intraperitoneally or via amniotic circulation with a saturated trypan blue solution. Gestation ages in litters studied ranged from 10½ days to birth. Embryos of 10½ and 11 days were perfused with dye after delivery of the fetuses into Ringer's Solution at 37°C since it was extremely difficult to make successful *in vivo* injections in embryos younger than 12 days. Following injection, these fetuses were placed immediately in 10% formalin. All *in vivo* injections were made through the wall of the uterus, which was rendered transparent with proper illumination. Intravascular injections of the amniotic circulation were suc-

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cessful in embryos of 13 days or older; but in 12-day-old fetuses, intraperitoneal injections of dye were administered. Mico-capillary pyrex pipettes were used for all injections. The pregnant females were prepared under ether anesthesia, and the uterine horns were exposed. Injections were controlled with oral pressure by means of a small rubber-blood pipete tube and were visualized under a dissecting microscope. This method was found most advantageous since it allowed greatest manipulation of the pregnant uterus. Six to 24 hours after *in vivo* injections, the embryos were delivered, and both injected and control embryos were fixed in 10% formalin. Only injected embryos that were delivered alive and living control embryos of the same litter were used for observation. Certain embryos were injected directly into the ventricular system of the developing brain to demonstrate the staining of the nervous system by dye not confined to blood vessels.

Results. Intrauterine injections of trypan blue were performed intraperitoneally or intravascularly in 100 rat embryos ranging from 10 days to birth. Usually 2 fetuses in a litter were injected. Out of the 100 embryos injected, 60 were successful; the remaining 40 died *in utero* and were not used in this study. In 20-day embryos injected on nineteenth day of gestation, there was no evidence of trypan blue in the central nervous system following a 24-hour dye equilibrium period, although all other organs showed intense staining with the vital dye. When trypan blue was injected into the ventricular system of developing brain, there was complete staining not only of the central nervous system but also of the rest of the embryo.

Trypan-blue injected rat embryos of 17, 15, and 12 days gave essentially the same results (Fig. 1). In each case, trypan blue had been administered intraperitoneally 6 hours prior to delivery of embryos. Although skin, viscera and cerebral vessels showed intense staining, there was no evidence of dye penetration within the central nervous system. In 12- and 10-day embryos the barrier phenomenon appears most distinctly in the developing spinal cord; however, it definitely existed in brain, even though in the earlier embryos there was still incomplete closure of the neural folds.

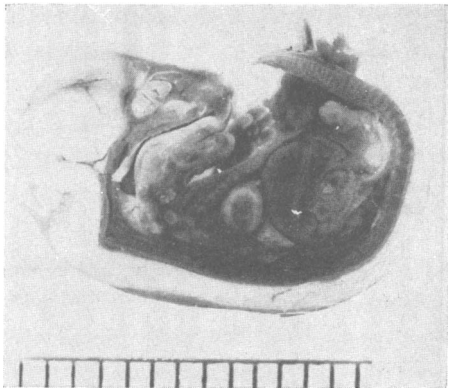

FIG. 1. Fifteen-day-old rat embryo injected with trypan blue via amniotic circulation and sacrificed 6 hr after injection. There is no evidence of dye in the central nervous system although dye penetrated the rest of the body. Scale in mm.

Discussion. The existence of a blood brain barrier phenomenon to trypan blue was demonstrated in this study in rat embryos ranging in age from the time blood vessels invade the brain until birth. Vascular invasion of the central nervous system occurs in the 3 to 4.5 mm rat embryo, which is approximately at 10 days of gestation(5). Our observations are in agreement with those of Grontoft(3), who perfused a great number of animal and human fetuses with trypan blue. When he perfused fetuses within 45 minutes after separation from the mother, the central nervous system remained impermeable to trypan blue. It was his belief that as long as the endothelium of CNS capillaries was intact, the central nervous system was impermeable to trypan blue.

Penfield(6) indicated that astrocytes make their appearance in the developing central nervous system at about the same time that blood vessels invade the brain. At birth, astrocytes are not well developed and have only a few fibrous processes, and it would appear that embryonic impermeability to trypan blue is unrelated to developing astrocytes but rather to vascular endothelium of the cerebral blood vessels. In fluorescent dye studies, the findings of Rodriguez(7) additionally support this hypothesis. It cannot be as-

sumed, however, that the mechanisms preventing all substances from entering the central nervous system are the same as those which retard protein-bound dyes.

Hess(8,9) believes that presence of ground substance in the central nervous system is necessary for the functional blood brain barrier. He observed that in neonatal mice and several other newborn animals the periodic acid Schiff reaction was negative, and he concluded that the absence of a chemical "ground substance" was causally related to the lack of blood brain barrier activity. We have observed that a blood brain barrier toward trypan blue definitely exists in neonatal rats. Other unpublished observations lead us to believe that this is also true in neonatal mice and cats. Behnsen(10) observed that areas which normally stain with trypan blue in the adult mouse were somewhat larger in the neonatal animal, but his findings have been misinterpreted by many investigators as pointing to an absence of blood brain barrier activity *in utero*.

As Bakay(2) points out, it seems that the blood brain barrier is not simply a screening agent but a complex mechanism which is functionally adapted to the needs of the central nervous system. The phenomenon toward trypan blue in the rat exists as soon as the vessels are laid down in the developing brain, but the same mechanism may not act for all substances. The selectivity for smaller molecular weight substances such as the electrolytes is decreased as the embryo grows older and might be correlated with neuroglial development.

Summary. Trypan blue injected into developing rat embryos (10 days to birth) fails to stain the central nervous system. This impermeability in the developing central nervous system exists at the time when blood vessels invade the brain. It cannot be assumed, however, that the mechanisms which prevent protein-bound dyes from penetrating the cerebral blood vessels are the same for all other substances.

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