of the species discussed in this report (*16*). Furthermore, if allele frequencies change as a function of geography (clinal variation), n_e will change accordingly. Evidence presently available indicates that PHI allele frequencies are uniform in *Mytilus edulis* from Virginia to Cape Cod. However, at the LAP locus, over this range there are significant departures from otherwise uniform allele frequencies, north of Cape Cod and within Long Island Sound (*14*).

Tables 1 and 2 show the values of these parameters for the species studied. In PHI, there is a steady diminution in both the absolute and effective number of alleles down the burrowing depth gradient discussed above. A high correlation exists between n_e and the number of alleles observed ($r_s = .96$, $N = 6$, $P < .05$). In LAP, this same phenomenon is observed for n_e and for A with a significant correlation between these two parameters ($r_s = .92$, $N = 5$, $P < .05$).

Despite the problems of the measures employed, it seems clear that species supposed to be experiencing more environmental variability are more polymorphic than those living in more constant environments. Two hypotheses, one involving selection for heterozygotes in a varying environment, the other involving diversifying selection in a heterogeneous environment with extensive gene flow, may explain the above data. Some data obtained by Koehn *et al.* (*17*) suggest that selection for heterozygotes in *Modiolus demissus* does occur in the high intertidal zone, relative to the low intertidal zone. However, environmental heterogeneity should not be dismissed as an important factor. *Mytilus edulis* occurs in a wide variety of substrata in Long Island Sound, including rock, gravel, sand, and wood pilings. With a planktonic larva, gene flow could extensively mix all of these populations. By contrast, *Nucula annulata* is collected in only subtidal muddy substrata (*18*).

It is significant that there is a correlation in n_e between the two different loci, over the species studied ($r_s = .95$, $N = 5$, $P < .05$). This suggests that epistatic interactions, environmental heterogeneity selecting for genetic diversity, or selection for heterozygosity is controlling genetic polymorphisms at these two loci. The latter two alternatives, taken singly or in combination, are consistent with the data presented above. Thus, environmental variability selects for increased A and n_e, independently at both loci. More work is needed to determine whether heterozygosity is selected in a fluctuating environment, thus increasing polymorphism, or whether diversifying selection increases polymorphism in a spatially heterogeneous habitat. Both processes are probably important in shallow-water marine bottoms. It is also of great importance to critically evaluate objective measures of environmental variability. It is here proposed that living depth within the sediment is an imperfect, but adequate, indicator.

Table 2. Genetic variation of bivalves at the LAP locus (most anodally migrating locus).

Species	A	n_e	N
1) *Mytilus edulis*	5	3.0	52
2) *Modiolus demissus*	4	2.5	66
3) *Mercenaria mercenaria*	4	2.6	60
4) *Macoma balthica*	4	2.2	50
5) *Mya arenaria*	3	1.1	64

JEFFREY LEVINTON
Department of Earth and Space Sciences, State University of New York, Stony Brook 11790

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Two Morphologically Distinct Blood-Brain Barriers Preventing Entry of Cytochrome c into Cerebrospinal Fluid

Abstract. *After intravenous injection, cytochrome c does not enter the cerebrospinal fluid. In most areas of the brain, the marker is prevented from leaving cerebral vessels by the capillary endothelium. In the choroid plexus, the marker passes freely out of capillaries into the extracellular space. However, it does not traverse tight junctions between epithelial cells and is rapidly incorporated into membrane-bound vesicles within the cell cytoplasm. Thereafter, cytochrome c is apparently removed by lysosomal degradation. These data suggest that there are at least two morphologically distinct blood-brain barriers to cytochrome c and that pinocytosis may be a mechanism for intracellular degradation rather than transcellular transport.*

Although it is firmly established that a major fraction of the cerebrospinal fluid (CSF) is formed constantly in the cerebral ventricles (*1*), the specific sites and mechanisms of CSF formation remain unclear. Among the many obstacles that confront investigators in this field is the lack of an electron-dense marker that can be injected into the blood and recovered in the CSF. With respect to the commonly employed intracerebral markers, ferritin and horseradish peroxidase, there is some question about tissue toxicity (*2*) and no proof that the markers enter the CSF.

In the continuing search for a suitable blood-CSF marker that can be used for examining mechanisms of CSF formation, we were recently led to a study of cytochrome c. This hemo-

chromogen, which is one of the cytochromes identified by Keilin (*3*) as essential to intracellular respiration, has the following theoretical advantages as an intravascular marker: (i) it is a naturally occurring protein, common to all aerobic organisms; (ii) it has specific photometric properties and can be quantitatively measured in body fluids; (iii) it can be visualized by electron microscopy after reaction with 3,3′-diaminobenzidine (*4*); and (iv) it is a considerably smaller protein (molecular weight 13,000, radius 15 Å) than ferritin (molecular weight 400,000, radius 50 Å) or horseradish peroxidase (molecular weight 40,000, radius 25 to 30 Å).

In the current study, cytochrome c (Calbiochem grade A) was administered intravenously to adult male Wistar rats and immature male pigs (7 to 8 weeks old) in doses ranging from 20 to 75 mg per 100 g of body weight. In pigs, lumbar or ventricular punctures, or both, were performed, and serial samples of CSF, urine, and blood were collected. The specimens were centrifuged, reacted with an excess of ascorbic acid, and viewed against blanks of deionized water in a Beckman DB spectrophotometer at 415 nm, one of the absorption maximums for cytochrome c (*3*). In control animals the absorbance for CSF was 0.040, the absorbance for urine was 0.260, and the absorbance for plasma was 0.215. In animals receiving exogenous cytochrome c, the CSF determinations were found to be invalid unless (i) the CSF tap had been atraumatic and (ii) the CSF samples remained completely free of blood during the interval of the study. Table 1 shows the findings in a technically successful experiment in which uncontaminated samples of CSF (total, 11 ml) were collected over a 9-hour interval. Note that whereas the absorbance for CSF never exceeded the control value of 0.040, the absorbance for plasma was 28 times the control plasma value at 6 hours.

A report of our technique for visualizing cytochrome c with the electron microscope is in preparation. The methods, with minor modifications, are those of Karnovsky and Rice (*4*). One shortcoming of this technique is the moderate degree of tissue swelling and lipid extraction that attends incubation at *p*H 3.9. In these experiments, all tissue was dehydrated in a standard ascending ethanol series terminating in *n*-butyl glycidyl ether with subsequent embedding in Epon-Araldite. Sections of silver-gold interference color were cut on an LKB Ultrotome III with diamond knives, mounted on bare 400-mesh copper grids, and visualized in a Forge Flo 4C electron microscope operating at 50 kv.

Figure 1, a and b, shows capillaries in the cerebral cortex (parietal lobe) of the rat after the intravascular injection of cytochrome c. Cytochrome activity is apparent as an electron-dense reaction product that fills the capillary lumina (Fig. 1a). Although occasional pinocytotic pits or vesicles or both are noted (Fig. 1b), no reaction product is present on the contraluminal side of the endothelium. Similar findings were encountered in other areas of the rat and pig brain, including the cerebral cortex and central white matter of the frontal, temporal, and occipital lobes, and the subependymal tissue surrounding the lateral ventricles.

Figure 1, c to g, shows the choroid plexus in the rat and pig at varying intervals after the intravascular injection of cytochrome c. Within 2 minutes, the reaction product is present in the perivascular space, the extracellular space between epithelial cells, and small intracytoplasmic membrane-bound vesicles adjacent to the lateral and basal plasmalemmas (Fig. 1c). No reaction product is noted beyond the tight junctions joining epithelial cells at their apical border (Fig. 1d). Between 1 and 9 hours, the reaction product is cleared from the extracellular space and is found in large and small vesicles ranging in size from 50 to 250 nm. In the rat, these vesicles are randomly distributed throughout the cell cytoplasm and in some instances resemble secondary lysosomes (Fig. 1e). In the pig, the intracytoplasmic distribution of the vesicles is less random. In particular, a predominant number are found in jux-

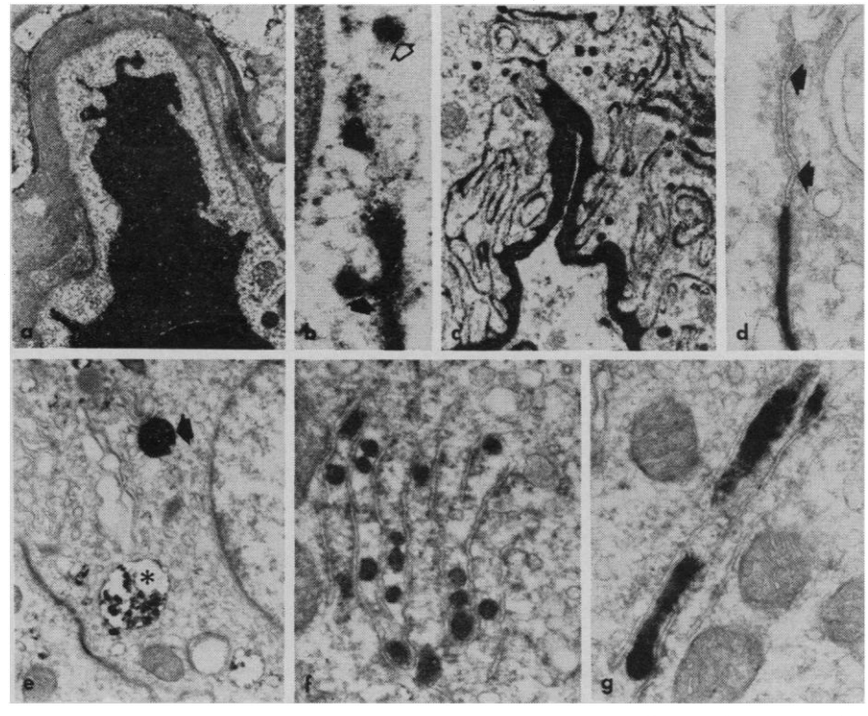

Fig. 1. Intravascularly injected cytochrome c in cerebral capillary of rat; reaction product is confined to the lumen of the capillary (× 7,200). (b) Cytochrome c in pinocytotic pit (black arrow) formed by the luminal plasmalemma of rat cerebral endothelium; upper (white) arrow points to probable pinocytotic vesicle containing reaction product (× 36,900). (c) Reaction product in perivascular and extracellular spaces of rat choroid plexus 2 minutes after intravascular injection of cytochrome c (× 10,900). (d) Intercellular space of rat choroid plexus 2 minutes after intravascular injection of cytochrome c; reaction product does not pass beyond tight junctions joining epithelial cells at apical surface (× 21,800). (e) Epithelial cell of rat choroid plexus 1 hour after intravascular injection of cytochrome c; note reaction product in vesicles resembling primary (arrow) and secondary (asterisk) lysosomes (× 13,000). (f) Epithelial cell of pig choroid plexus 1 hour after intravascular injection of cytochrome c; note cluster of vesicles containing reaction product in juxtaposition to apical concentration of endoplasmic reticulum (× 11,600). (g) Epithelial cell of pig choroid plexus 9 hours after intravascular injection of cytochrome c; note reaction product in elongated forms representing apparent fusion of individual vesicles (× 25,300).

taposition to the apically concentrated rough endoplasmic reticulum (Fig. 1f). Thereafter, the reaction product is eventually confined to large elongated vesicles (Fig. 1g). In this study, there was no evidence that cytochrome c is emptied into the cerebral ventricles by the fusion of vesicles with the apical plasmalemma.

Taken together, the foregoing data indicate that circulating cytochrome c does not enter the CSF up to 9 hours after intravenous injection. Thus, the original objective of this study, to find a suitable blood-CSF marker for examining the mechanisms of CSF formation, was not met. Since most of the commonly used intravascular markers are considerably larger than cytochrome c (for example, ferritin, horseradish peroxidase, glycogen, dextran), it is inferred that these molecules are similarly excluded from the CSF. In the future, the search for an electron-dense marker capable of leaving the blood and entering the CSF should probably be focused on molecules smaller than cytochrome c or on those with special lipid-solubility properties.

In the areas of the brain examined in this study, two morphologically distinct barriers preventing the CSF entry of cytochrome c were encountered. The first, and most widely dispersed, was the capillary endothelium of the cerebral vessels. This limiting surface, which has been recently identified by Reese and Karnovsky (*5*) as the probable site of the blood-brain barrier to proteins, has the following characteristics, according to these authors: (i) the presence of circumferential tight junctions between endothelial cells that prohibit the intercellular movement of horseradish peroxidase, and (ii) a limited number of plasmalemmal pits or vesicles or both that have been linked to transcellular protein transport in skeletal and cardiac endothelium. In view of the current findings, it is likely that circulating cytochrome c is prevented from entering most areas of the brain (and consequently the CSF) by the same endothelial barrier system described by Reese and Karnovsky (*5*), and subsequently others (*6–8*).

Since the original experiments of Ehrlich (*9*) and Goldmann (*10*), it has been known that, although basic dyes injected into the blood are largely excluded from the brain, certain areas, including the choroid plexus, basal hypothalamus, and area postrema, will be stained. It has been subsequently demonstrated that these dyes are bound to plasma proteins (*11*), so that the movement of the markers reflects the movement of dye-protein complexes. In more recent studies, intravenously injected horseradish peroxidase has been found to move across the endothelium of capillaries in the choroid plexus, median eminence, and area postrema with relative ease (*7, 12*). However, as noted by Brightman *et al.* (*13*), each of these areas of "functional leakage" is covered by a relatively impervious ependymal lining. This epithelium, unlike ependyma found elsewhere, possesses tight junctions between adjoining cells that are capable of halting the extracellular movement of peroxidase.

Table 1. Spectrophotometric analysis of lumbar CSF, urine, and blood in pig following intravascular injection of cytochrome c (25 mg per 100 g of body weight).

Specimens	Absorbance at 415 nm
Control CSF	0.040
Control urine	.260
Control plasma	.215
CSF, 15 minutes	0.020
CSF, 30 minutes	.030
CSF, 60 minutes	.020
CSF, 90 minutes	.020
CSF, 120 minutes	.018
CSF, 150 minutes	.020
CSF, 3 hours	.035
CSF, 6 hours	.020
CSF, 9 hours	.020
Urine, 150 minutes	6.60
Urine, 6 hours	6.64
Plasma, 6 hours	6.00

In the current study, the movement of circulating cytochrome c across the choroidal capillaries was found to be much more rapid but similar in distribution to the movement of circulating horseradish peroxidase (*7, 8, 12*). However, since we have shown that cytochrome c does not enter the CSF, we cannot agree with the speculation that larger proteins such as peroxidase may enter the CSF in small amounts by vesicular transport across the choroidal epithelium (*8*) or by diffusion through the perivascular basement membrane and stromal space to a point just beyond the root of the choroid plexus where the choroidal epithelium joins the ventricular ependyma (*14*). On the contrary, one may infer that proteins larger than cytochrome c are prevented from entering the CSF across the choroid plexus and other areas of "functional leakage" by an epithelial barrier that is just as selectively restrictive as the endothelial barrier of the cerebral capillaries. It is likely that one function of this dual barrier system is to maintain the CSF as an ultrafiltrate of blood. The means by which small but detectable amounts of labeled albumin and globulin enter the CSF after intravascular injection (*15*) remains unclear.

Finally, it is appropriate to question the ultimate fate of exogenous proteins entering the brain in areas of "functional leakage." As noted earlier for the choroid plexus, the extracellular migration of cytochrome c is abruptly halted by tight junctions between epithelial cells. Simultaneously, the marker is incorporated into membrane-bound vesicles having many of the features of primary and secondary lysosomes. In view of the fact that cytochrome c does not enter the CSF even in trace amounts, it is possible that pinocytosis represents an initial step in the degradation of certain proteins within the brain.

THOMAS H. MILHORAT
DONALD A. DAVIS
BOLIVAR J. LLOYD, JR.
Department of Neurosurgery, Children's Hospital, National Medical Center, Washington, D.C. 20009, and *Department of Neurosurgery, George Washington University School of Medicine, Washington, D.C. 20037*

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