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16. The absorption spectrums of whole isolated axolotl retinas were measured from 370 to 750 nm both before and at various times after a portion of the photopigment was bleached. These spectrums reflect mainly the absorption changes in the red rods because the red rod pigment predominates in the axolotl retina. The absorption maximums of axolotl red rod pigment and its photoproducts tend to be shifted to somewhat higher wavelengths than those of rhodopsin and its corresponding photoproducts, presumably because axolotl red rods contain a mixture of rhodopsin (based on vitamin A_1) and porphyropsin (based on vitamin A_2) (*15*). By obtaining difference spectrums at various times after the bleach, we were able to recognize the presence of at least two photoproducts, with peaks at 400 and 480 nm, respectively. The spectral positions and time courses of buildup and decay of these two absorption peaks were compared with the λ_{max}'s and kinetics of the more thoroughly studied rhodopsin photoproducts from frog and cattle. This comparison suggests that the peaks at 480 and 400 nm are due mainly to the presence of metarhodopsins III and II, respectively. Unfortunately, both metarhodopsin II and a later photoproduct—free retinal, which is subsequently reduced to vitamin A—absorb maximally near 400 nm. However, the following considerations make it unlikely that the reduction of free retinal to vitamin A makes a major contribution to the decay of the 400 nm peak during the first 25 minutes after the bleach. (i) One-third of the total decrease in absorbance at 400 nm occurs in the first 3 minutes after the bleach. Concurrently, absorbance at 480 nm increases, that is, metarhodopsin III accumulates, a result consistent with the interpretation that the decrease in absorbance observed at 400 nm during this time interval is associated with the decay of metarhodopsin II to metarhodopsin III. In any case, very little accumulation of the later photoproduct, free retinal, is expected during the first 3 minutes. (ii) Subsequently, metarhodopsin III decays to free retinal, which is in turn reduced to vitamin A. The concentration of free retinal at any instant, and thus its contribution to the absorbance at 400 nm, will depend on the relative kinetics of its production and removal. If reduction of free retinal is slow in comparison with its production, the kinetics describing decay in absorbance at 400 nm should gradually change with time as metarhodopsin II concentration decreases and free retinal becomes the dominant photoproduct in this region of the spectrum. However, in isolated axolotl retina at 24°C decay of the 400 nm peak can be described by a single first-order process (half-time of decay, 11.5 minutes) from 4 to 22 minutes after the bleach. Thus it appears that free retinal is removed as soon as it is formed during this period. (iii) Finally, our argument that the absorbance decrease at 400 nm corresponds mainly to metarhodopsin II decay is strengthened by the observation that the time course of absorbance change at 400 nm in axolotl closely parallels those absorbance changes identified as due mainly to decay of metarhodopsin II in frog by C. Gedney and S. Ostroy [*Biochim. Biophys. Acta* **256**, 577 (1972)] and by R. N. Frank [*Vision Res.* **9**, 1415 (1969)] and in rat by Frank and Dowling (*17*).
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18. Light stimuli at various intensities were presented in random order. Near threshold, stimuli were presented at 10-second intervals. After a high-intensity, saturating stimulus a prolonged hyperpolarization is observed, and presentation of a second stimulus before membrane repolarization results in a greatly diminished response. For that reason, the interval between consecutive stimuli was extended to about 50 seconds after high-intensity stimulation.
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22. Control experiments in dark-adapted retinas showed that when the membrane potential is stable, the sensitivity of the cell is also fairly stable (± 0.2 log units). Sudden or gradual changes in the cell's sensitivity were usually accompanied by corresponding erratic changes in the observed membrane potential. Therefore, if large, random fluctuations in the membrane potential occurred after a bleach, subsequent data were discarded.
23. We thank S. E. Ostroy for many helpful discussions and W. A. Cramer for the use of his Shimadzu spectrophotometer. Supported by PHS research grant EY 00033 and predoctoral fellowship GM 45860.

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Chronic Effects of Osmotic Opening of the Blood-Brain Barrier in the Monkey

Abstract. *In the monkey, the blood-brain barrier and the blood-aqueous and blood-vitreous barriers of the eye can be opened by internal carotid perfusion of solutions of 2 molar urea in a way compatible with survival and, in some few cases, without detectable neurological deficits. Urea presumably acts by osmotically shrinking the endothelial cells of the cerebrovascular vessels and opening their tight junctions. The high incidence of brain necrosis with neurological sequelae after perfusion of urea by the present technique precludes the use of osmotic opening of the blood-brain barrier for pharmacotherapy at this time.*

The blood-brain barrier regulates brain homeostasis by controlling exchange of material between the intravascular fluid and the central nervous system (*1*). In short-term experiments on rabbits, this barrier can be opened reversibly for the passage of large molecules by exposure to concentrated solutions of electrolytes or relatively lipid-insoluble nonelectrolytes (*2*). The action of these substances on the barrier is consistent with the hypothesis that they osmotically shrink barrier cells (presumably the cells of the vascular endothelium) and open the tight junctions between them (*2*, *3*).

Since temporary barrier opening would be a useful tool to study barrier function and could have a possible clinical application for brain pharmacotherapy (*1*, *4*), we wanted to know the effect of osmotically induced barrier breakdown on long-term animal morbidity and mortality. Previous studies of barrier breakdown dealt with acute pathophysiology without being concerned with consequences on survival or neurological function (*1*, *5*). Perfusion of solutions of 2*M* (2 osmol) urea into the internal carotid artery of rabbits opens up the barrier reversibly to the Evans blue–albumin complex in short-term experiments (*2*). The monkey was chosen for long-term studies because a more meaningful neurological and behavioral examination is feasible. As in man and rabbit, the internal carotid arteries are distributed solely to the brain and do not anastomose with vessels from the external carotid system (*5*–*7*).

Twenty-eight monkeys (*Macaca mulatta*) weighing 3.5 to 5.5 kg were anesthetized with intravenous pentobarbital (20 to 30 mg per kilogram of body weight). The left external carotid, lingual, and thyroid arteries were ligated. The left common carotid was ligated caudally and catheterized for brain perfusion (*5*, *7*). To indicate barrier permeability, Evans blue dye (4 ml/kg; concentration being 2 g per 100 ml of saline) was injected intravenously. This quantity of dye is bound completely to blood albumin and does not normally cross the blood-brain barrier (*2*). In the first five monkeys, a 1-cm hole was trephined in the skull and the pia-arachnoid was exposed. The pial arterioles were observed under a dissecting microscope while a freshly prepared and filtered solution of 2*M* to 3*M* urea

Table 1. Effect of perfusion of urea and isotonic saline on blood-brain barrier.

Flow rate (ml/sec)	Solution	Staining			Normal neurologically	Monkeys (No.)
		None	Left eye only	Left eye + left hemisphere		
0.26	Urea	0	3	1	4	4
	0.9 percent NaCl	2	0	0	2	2
0.4–0.65	Urea	0	2	3*	3	5
	0.9 percent NaCl	3	0	1†	3	4
0.7–1.67	Urea	0	1	8*	2	9
	0.9 percent NaCl	2	0	0	2	2

* Only one in this group with normal neurological signs; the rest were abnormal. † Staining of brain only; abnormal neurological examination.

($pH \simeq 7.4$) was perfused manually for 30 seconds into the left internal carotid artery at a rate just necessary to replace the blood in the pial arterioles (*5*). This rate was between 0.7 and 1.67 ml/sec. Following this first series, 23 animals were studied without brain exposure. The left internal carotid was perfused for 30 seconds with a perfusion pump, at a constant rate between 0.26 and 1.05 ml/sec. In 9 of these 23 animals, carotid perfusion was performed 1 to 3 days after common carotid ligation, to rule out sequelae from ligation alone. After perfusion, the animal was permitted to recover from anesthesia and was observed for 3 to 14 days. It was killed under anesthesia by perfusing saline, followed by 10 percent formalin, through the heart. The brain was examined for extravasated dye, and frozen sections were stained for histological examination.

Table 1 summarizes the data on perfusion with urea and with isotonic saline solutions; it excludes two animals that did not recover after urea perfusion. Urea concentrations were 2*M* for 15 animals, 2.5*M* for 2 animals, and 3*M* for 1 animal. Opening of the blood-brain barrier by urea produced diffuse blue staining of the left hemisphere and of the aqueous and vitreous humors of the left eye (*8*) and occurred usually at perfusion rates above 0.7 ml/sec. At lower rates, the eye was stained often without brain involvement, probably because the ophthalmic artery is perfused from the internal carotid before urea can be mixed with blood at the circle of Willis (*7*). Eye staining represents breakdown of the blood-aqueous and blood-vitreous barriers, which are located at the tight junctions between endothelial cells of the retinal vessels and between cells elsewhere in the eye (*1*, *9*).

Of the 12 animals treated with urea and in which staining of both eye and brain occurred, 9 had detectable neurological defects. These ranged from slight right-sided forelimb weakness and flaccidity to profound right-sided paresis of the extremities. Some monkeys were less active and appeared withdrawn, when compared to the active, aggressive normal animals or saline-perfused controls. Most deficits distinctly improved with time. [A left-sided Horner's syndrome in some animals, produced by surgical damage to the cervical sympathetic trunk (*10*), was not considered a sequel of perfusion.] Three of 12 animals with left hemispheric and eye staining had no discernible motor or behavioral impairment, and appeared entirely normal. One of the eight saline controls had staining of the left hemisphere but not of the left eye, and very minimal weakness of the right arm. The rest were neurologically normal and did not have brain staining.

On pathological examination we found evidence of neuronal damage in monkeys with severe motor sequelae. The brains did not show midline displacement and were symmetrical. In the three animals without motor sequelae but with diffuse and even staining with Evans blue of the left hemisphere, cresyl violet preparations of the brain were histologically normal, except for a rare microembolus. Therefore, barrier opening in itself does not necessarily produce neuronal damage. This agrees with the demonstration that the barrier in the rabbit can be opened reversibly with perfusion of urea into the internal carotid artery without necessarily damaging vascular or brain cells (*2*).

As perfusion flow rate increased, the percentage of monkeys with breakdown of the blood-brain barrier increased (Table 1), but the percentage of animals with neurological sequelae also rose. Parameters other than flow must be important, since three monkeys with diffuse staining of the left hemisphere had no discernible neurological deficit. The biological variability in collateral circulation through the circle of Willis, the amount of arterial spasm or dilatation, and the volume of runoff into the ophthalmic artery probably affect the flow and the concentration of urea that actually reaches the left cerebral vessels. Those animals with severe brain necrosis and neurological deficit often had a period of bradycardia for more than 30 seconds following urea perfusion. Although ligating the left common carotid artery did not in itself, or with saline perfusion, produce necrosis, ligation may have predisposed the brain to anoxia or to toxic damage by urea.

It may be that a better combination of perfusion pressure, rate, and time, combined with only temporary occlusion of the common carotid artery, will decrease the incidence of sequelae. Furthermore, reversibly acting electrolytes, such as sodium chloride, and nonelectrolytes such as lactamide, may prove to be less toxic than urea (*2*). Indicators smaller than the Evans blue–albumin complex, which has a radius of about 38 Å (*11*), might show the barrier to be opened at the lower flow rates, at which Evans blue did not stain the left hemisphere and at which neurological sequelae were infrequent.

Contrast media used in cerebral angiography break down the blood-brain barrier in rabbits and produce neurological sequelae in man (*12*). Their osmolality, 1.5 to 2 osmol, is close to that of the urea solutions used in this experiment, which suggests that they may osmotically open the barrier.

We have demonstrated that, under certain experimental conditions, the blood-brain, blood-aqueous, and blood-vitreous barriers can be opened unilaterally by hypertonic urea solution without producing long-term neurological damage. The dye-albumin complex remains in the brain parenchyma and eye for at least 2 weeks. However, the high incidence of brain necrosis with neurological sequelae, following perfusion of urea with the present technique, precludes the use of osmotic opening for brain or eye pharmacotherapy (*4*) at this time.

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Hybridization of Burkitt Lymphoblastoid Cells

Abstract. *Burkitt lymphoblastoid cell lines have been fused to mouse and human cell lines with the use of inactivated Sendai virus. The heterokaryons have developed into somatic cell hybrids of both parental cell types. Chromosome analyses confirm that cells now growing in selective medium are hybrids. Initial observations of preparations of the hybrid cells reveal that 5'-iododeoxyuridine can induce continued synthesis of Epstein-Barr virus antigens by these hybrid cells.*

Heterokaryons of several cell types have been produced by using inactivated Sendai virus to initiate cell fusion (*1*). When cells transformed by certain oncogenic viruses, such as simian papovavirus 40 (SV40), were fused to potentially susceptible cells, virus synthesis was initiated, and infectious SV40 was recovered from the cells (*2*).

A herpesvirus, the Epstein-Barr virus (EBV), has been associated with a number of lymphoblastoid cell lines derived from patients with Burkitt's lymphoma (BL) (*3*). A small percentage of the cell population of several of these lines contain antigens that can be detected with serums from BL patients by the indirect immunofluorescent test (*4*). In addition, there is evidence from cloning and nucleic acid hybridization experiments that the EBV genome is at least partially integrated in the host cell genome (*5*, *6*).

The fusion of Burkitt lymphoblastoid cells has previously been found to be difficult in this laboratory and others (*7*). We have attempted to produce heterokaryons of Burkitt lymphoblastoid cells that grow only as suspension cultures in vitro, with cells capable of growing on glass to determine if somatic cell hybrids can be developed. We have also investigated the possibility of recovering the EBV from the heterokaryons of human-human and human-mouse cells. It would be useful to obtain infectious virus in order to study the relation between EBV and Burkitt's lymphoma.

The Burkitt lymphoblastoid cell line studied was the EB_3 line containing EBV antigens in 1 to 4 percent of the cells. The other parental cell line was a mouse L cell line, LM(TK$^-$)CL1D (CL1D) (*8*), which is unable to grow in selective medium [Eagle's or F-12 medium supplemented with 10 percent fetal calf serum, $1 \times 10^{-4}M$ hypoxanthine, $4 \times 10^{-7}M$ aminopterine, and $1.6 \times 10^{-5}M$ thymidine (HAT)].

The fusion procedure was as follows: 2×10^6 CL1D cells were seeded into tissue culture plates, and were grown for 24 hours at 37°C. The EB_3 cells (1×10^7 cells in 0.2 ml of F-12 medium containing no serum) were washed with saline buffered with phosphate. Sendai virus (400 hemagglutinating units in 0.2 ml), inactivated with β-propiolactone, was added to the center of the cell monolayer and to the EB_3 cell suspension. Both the CL1D and EB_3 cells were cooled on ice for 5 minutes. The cell suspension was then placed on the monolayer cultures, and cooled for 5 minutes on ice; the cultures were then placed in an atmosphere of 5 percent CO_2 for 3 to 4 hours at 37°C. Normal F-12 medium supplemented with 10 percent fetal calf serum was added, and was replaced 3 days later by HAT medium. The cultures were maintained in selective HAT medium which was renewed every 3 days.

Chromosome spreads were prepared by a modification (*9*) of the method of Moorhead *et al.* (*10*). Sixty coded metaphases were analyzed for each cell line. The chromosomes of the parental human cell line EB_3 were diploid, consistent with previous reports (*11*).

There were wide variations in the total chromosome number and in the number of biarmed chromosomes, especially in the CL1D line (Table 1). The CL1D cells had chromosome numbers of 48 to 52, with a modal number of 51; the number of biarmed chromosomes generally had a range of 10 to 13, although a few cells contained 14 or 15. The modal number of biarmed chromosomes was 11 to 12. The chromosome number of the EB_3/CL1D hybrid, passage 15 (10 months after fu-

Table 1. Chromosome analysis of parental mouse (CL1D) and hybrid mouse-human (EB_3/CL1D) cell lines. Chromosome counts in italics were not considered to be significant.

	Number of cells with chromosome numbers of											Number of cells with biarmed chromosome numbers of							
	44	46	47	48	49	50	51	52	53	54	55	10	11	12	13	14	15	16	17
LM(TK$^-$) CL1D		*1*	*2*	3	*5*	5	7	4		*2*		8	21	16	9	*2*	*2*		
EB_3/CL1D						*1*	2	8	13	4	2		*3*	14	13	9	11	7	*3*