Isolated Retinas Synthesize Visual Pigments from Retinol Congeners Delivered by Liposomes

Abstract. *Isolated vertebrate retinas bathed in circulating Ringer solution cannot regenerate all of their bleached visual pigments. When dioleoyl-lecithin vesicles containing certain retinol congeners are added to the Ringer solution, such retinas begin to regenerate pigment immediately. The visual pigment of a bleached perfused retina can now be restored fully, making the isolated retina an independent unit for study. Liposomes can protect oxygen-sensitive, lipid-soluble substances and deliver them to living cells.*

A study of the metabolism of water-insoluble substances by isolated living tissues in an aqueous medium is hindered by the problem of delivering to the cells a sufficient amount of the substances without undue insult to either cells or substances. Retinol, a precursor to the prime component of visual pigments required by the retina in microgram amounts, is difficult to supply to a perfused isolated retina, because the solubility of retinol in water is less than 1 n*M* (*1*), and the retinol becomes extremely susceptible to oxidation when dispersed in water (*1, 2*).

Isolated retinas, if perfused by a Ringer solution containing glucose and oxygen, can continue to respond to light for many hours, but are unable to restore visual pigment after bleaching (*3*). The addition of retinol congeners to the circulating Ringer solution in order to effect pigment regeneration is futile for the reasons already mentioned.

However, as much as 57 percent of the visual pigment can be restored in an isolated and bleached skate retina kept moist in air when 0.11 ml of Ringer solution containing 20 percent ethanol and 15 m*M* 11-*cis* retinaldehyde is placed in drops on the rod surface. Similar addition of Ringer solution with less retinaldehyde and alcohol to partially bleached retinas increases their light sensitivity (*4*).

We report here that live, isolated retinas, sustained by perfusion with oxygenated Ringer solution, regenerate visual pigment from retinol or retinaldehyde supplied by liposomes.

A dark-adapted frog retina was mounted in a double window chamber (*3*) with an opal plate, perfused with circulating Ringer solution and placed in the sample beam of a Cary 17 spectrometer so that the light passed axially through the rods. Vesicles formed from L-α-dioleoyl-lecithin (DOL) and containing retinol congener were prepared by sonication in a manner modified from Weinstein *et al.* (*5*).

When DOL vesicles containing 9-*cis* retinaldehyde were added to the Ringer solution bathing a fully bleached live retina of the frog (*Rana pipiens*) (*6*), synthesis of pigment began immediately, with initially 1 percent of the original amount being formed per minute. The λ_{max} is shifted slightly toward lower wavelength, because the opal window is not a perfect scatterer (Fig. 1a). The λ_{max} value was therefore determined from the difference spectrum in Fig. 1b. The difference spectrum shows the new pigment to be the 9-*cis* retinaldehyde containing isorhodopsin (λ_{max} = 489.3 ± 1.6 nm) (*7*), thus indicating that it was created from the added retinaldehyde. Frog retinas treated in like manner with 11-*cis* retinaldehyde or retinol regenerated rhodopsin.

The vesicle-treated retina, even after 20 minutes of washing with Ringer solution, continued to show absorption attributable to retinol or retinaldehyde, in addition to some light scattering not observed in untreated retinas. This scattering was probably caused by vesicles adhering to the retina, since such vesicles are known to stick to cells (*8*), and we have observed that bleached retinas which have been briefly exposed to vesicles and then washed continue to regenerate pigment without further addition of vesicles.

These phospholipid vesicles can also deliver 11-*cis* retinaldehyde to mammalian (for example, rat) retinas which will then synthesize rhodopsin at 37°C (Fig. 1, c and d) (*9*).

On the basis of the amount of visual pigment regenerated by frog and rat retinas in this manner, we estimated that the quantity of retinol transferred into the rod cell by these vesicles was approximately 200 times greater than that of water-soluble, membrane-impermeable 6-carboxyfluorescein in similar vesicles (*10*), even though the amount of each compound per vesicle was approximately the same. This, and the difference in

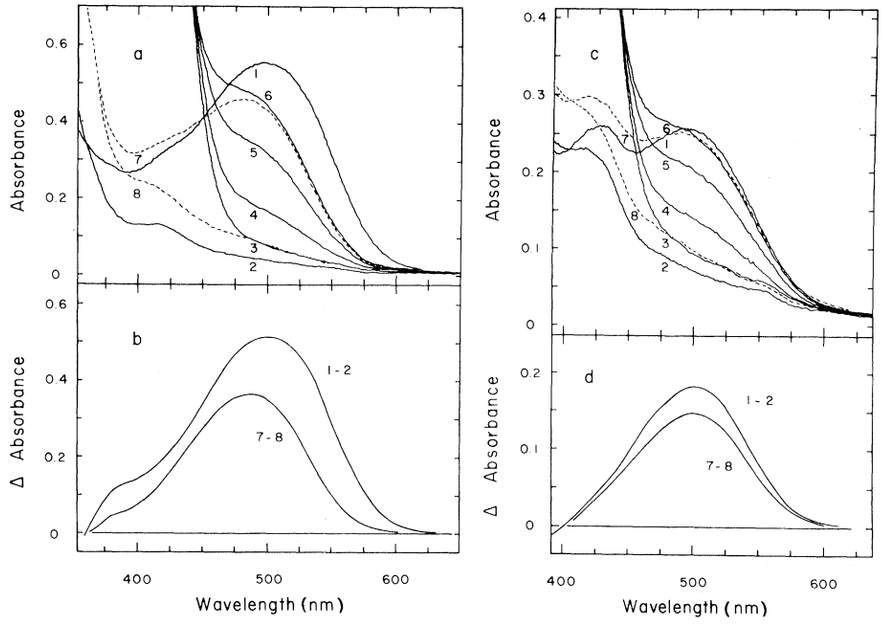

Fig. 1. (a) The rhodopsin, curve 1, in a dark-adapted frog retina at 25°C was bleached. Rhodopsin formed spontaneously up to 10 percent of the original amount and was removed with a second bleach, curve 2. Then, 6900 moles of 9-*cis* retinaldehyde in DOL vesicles per mole of opsin were added, curve 3, at time zero, and selected curves 4 through 6 show synthesis of isorhodopsin after 20, 120, and 300 minutes. The vesicles were removed, curve 7, and the retina was bleached, curve 8. (b) Difference spectra of the rhodopsin, curve 1 minus curve 2, with λ_{max} = 502.9 ± 3.5 nm, and the synthetic isorhodopsin, curve 7 minus curve 8, with λ_{max} = 489.3 ± 1.6 nm. (c) The rhodopsin in a rat retina at 37°C, curve 1, was bleached, curve 2, and reformed by the addition of 1200 moles of 11-*cis* retinaldehyde in DOL vesicles per mole of opsin, curve 7. Curves 4 through 6 were obtained after 13, 44, and 164 minutes, respectively, of exposure to the vesicles. (d) The difference spectra of the original difference spectra (curve 1 minus curve 2, λ_{max} = 501.5 ± 1.0 nm) and the new rhodopsin (difference spectra curve 7 minus curve 8, λ_{max} = 501.6 ± 1.0 nm).

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the partition of each compound within the vesicle (*5*), suggest that the way most of the fat-soluble material is transferred from unilamellar vesicles to cells may not necessarily involve vesicle fusion, unlike the situation with water-soluble substances. Lipid vesicles appear to transfer most of the lipid-soluble retinol to the cell by releasing it into the Ringer solution near the cell, which in turn acts as an intermediary source, because other sundry, nonfusing lipophilic particles such as multilamellar liposomes and Biobeads S-X12 can deliver large amounts of retinol congeners to the retina (*11*).

The shape of the regeneration curves from isolated retinas treated with vesicles resembles those in living animals (Fig. 2) (*12, 13*). Regeneration starts with a linear phase and is followed by a decrease in the rate of rhodopsin formation, with the rate approaching 100 percent as an asymptote as the process nears completion. Such kinetics could be described as a bimolecular reaction, where one reactant (opsin) is fixed in concentration and the other (11-*cis* retinaldehyde) is supplied at a constant rate by a zero order process such as diffusion from an inexhaustible source, or an enzyme working at saturation (*13*). The nonlinear rate at the start of pigment synthesis from 11-*cis* retinol in Fig. 2 may reflect enzymic oxidation of it to the aldehyde [see (*14*)].

The rates of regeneration from isolated retinas (see Fig. 2) are lower than those observed in the intact frog. They can be increased and full restoration of rhodopsin can be obtained with the addition of more reactant, but the maximum rate has not been determined (*15*). The rates in the isolated retinas deviate from linearity at a lower level of rhodopsin synthesis than in the live animal. This could be caused by vesicles adhering to the rod surface and serving as finite sources of retinol congener, while blocking access for fresh vesicles.

Although isolated retinas were given what appeared to be a great excess of one reactant if referred to a homogeneous solution (6900 moles of retinol congener per mole of opsin), the mole ratio in the outer segment may have been much less than unity since the retinol congener was added in the phospholipid vesicle which was excluded from the outer segment by the plasma membrane. The supply of retinol congener from vesicles would be continuous, but at a rate limited by diffusion through membrane and water.

When nearly equal amounts of 11-*cis* retinol and retinaldehyde were given to

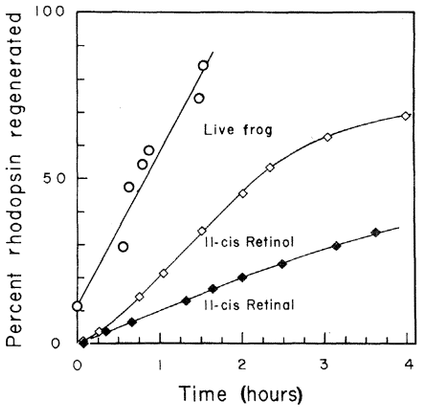

Fig. 2. Light-adapted frogs were dark adapted at time zero. The retinas were assayed for rhodopsin by absorption spectroscopy on Triton X-100 extractions made at the times indicated (○). Isolated and bleached frog retinas were exposed to DOL vesicles containing either 1160 moles of 11-*cis* retinol (◇) or 1260 moles of 11-*cis* retinaldehyde (*Retinal*) (◆) per mole of opsin at time zero, and the change in absorbance at 500 nm was used as the index of rhodopsin synthesis.

isolated retinas, retinol caused more rhodopsin to be regenerated at a higher rate (the time for 50 percent, $t_{1/2}$, of the rhodopsin to be regenerated was 130 minutes) than retinaldehyde ($t_{1/2}$ was 365 minutes) (Fig. 2) (*14*). Although 11-*cis* retinol and retinaldehyde were added in similar amounts at the beginning of the experiment, their concentrations began to differ with time; the retinaldehyde concentration in the vesicles decreased by 30 percent in 2 hours while that of retinol remained constant. The difference in their rates of regenerating rhodopsin is not due solely to the disappearance of retinaldehyde, since in experiments in which we used one-third less 11-*cis* retinol, the retina produced rhodopsin with a $t_{1/2}$ of 180 minutes, which was still more than twice as fast as the $t_{1/2}$ with retinaldehyde at the higher average concentration.

When 11-*cis* retinaldehyde is added to isolated retinas, its effective concentration near the opsin is lower than that of its precursor, retinol, added under the same conditions. There are more diversionary reactive sites for retinaldehyde, such as amino groups other than opsin's chromophoric site in the outer segment, than for retinol, which suggests that 11-*cis* retinol must be oxidized very near the opsin. Conversely, the all-*trans* retinaldehyde, a product of rhodopsin bleaching, must also be reduced there.

Oxygen-sensitive, water-insoluble retinol congeners can thus be effectively delivered to perfused isolated retinas, making it possible to follow their metabolism in visual pigment regeneration and photoexcitation (*1*). Phospholipid vesicles should be useful to deliver oxygen-sensitive lipid-soluble materials to other cells.

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5. J. N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal, W. A. Hagins, *ibid.* **195**, 489 (1977). Synthetic L-α-dioleoyl-lecithin (25 mg; Applied Sciences) was mixed with 0.25 to 1.2 mg of retinol or retinaldehyde in 1 ml of ethanol containing 150 μg of DL-α-tocopherol (Sigma) and freeze-dried. Vesicles were diluted with oxygen-saturated Ringer solution at 25°C to make 6 ml of incubating solution which was circulated over both sides of the retina at 1.2 ml per minute. Ringer compositions are from W. A. Hagins and S. Yoshikami [*Ann. N.Y. Acad. Sci.* **264**, 314 (1975)], except that Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as the buffer.
6. Retinas were bleached twice for 20 minutes with a 550- to 700-nm band of light and given a 1-hour dark interval between the bleaches before the liposomes were added. The plasma membrane of the rod cells remained intact even after 10 hours of treatment with vesicles containing retinol, as determined by the fluorescent dye uptake test [S. Yoshikami, W. E. Robinson, W. A. Hagins, *Science* **185**, 1176 (1974)], and by electron microscopy. The dark current and photocurrent of a retina is not impaired by vesicle treatment (*10*), in fact we find the retinal light sensitivity can be restored repeatedly in a bleached rat retina with vesicles containing 11-*cis* retinaldehyde.
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9. Isolated rat retinas (Sprague-Dawley), unlike those of the frog, cannot oxidize and use added 11-*cis* retinol for pigment synthesis (*1*).
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11. Multilamellar DOL liposomes were made by vortexing the lipid retinaldehyde mixture in Ringer solution, and selected with a 0.2-μm pore diameter filter (Nuclepore). These vesicles, with 100 μg of 11-*cis* retinaldehyde, were added to Ringer solution lacking glucose; 10 m*M* NaCN and 5 m*M* 2-deoxy-D-glucose, which do not affect regeneration with 11-*cis* retinaldehyde, were added to prevent possible endocytosis of liposomes. In 1 hour, one-fourth of the original amount of rhodopsin was formed. Seventy-five milligrams of Biobeads S-X12 (Bio-Rad), 37 to 74 μm in diameter, containing 2 mg of 9-*cis* retinaldehyde and 150 μg of DL-α-tocopherol were placed upon a 10-μm thick, 0.2-μm pore diameter Nuclepore filter covering the rods of a bleached frog retina, and were kept in a darkened, moist chamber. The high concentration of retinaldehyde close to the outer segments, with the less than 0.5 μl of Ringer solution calculated to be between them, caused the synthesis of isorhodopsin in amounts up to two-thirds of the original amount of rhodopsin in 10 hours.
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15. When 11-*cis* retinaldehyde in DOL was added in

a ratio of 6900 moles per mole of opsin to a bleached frog retina at 27°C it caused full rhodopsin regeneration in 8 hours with a $t_{1/2}$ of 95 minutes. Rat rod outer segments have 3.5 times the surface to volume ratio of frog. Assuming a temperature coefficient (Q_{10}) of 2 for pigment regeneration [see Baumann (*14*)], one would expect rhodopsin regeneration to be seven times faster in rat than in frog. We found the rat retina at 37°C regenerated its rhodopsin completely in 1 hour (with a $t_{1/2}$ of 14 minutes) from equivalent amounts of reactants.

16. We thank W. A. Hagins for discussions and suggestions, W. E. Scott of Hoffmann–La Roche and W. Sperling of Kernforschungsanlage-Jülich for gifts of 11-*cis* retinaldehyde. G.N.N. thanks the Deutsche Forschungsgemeinschaft for travel support.

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Blood-Brain Neutral Amino Acid Transport Activity Is Increased After Portacaval Anastomosis

Abstract. *In rats after portacaval anastomosis (an animal model of chronic liver disease), transport of tryptophan and other members of the large neutral amino acid group from blood to brain was markedly enhanced. Increased transport activity was apparently restricted to the neutral amino acid transport system, since brain uptake of glucose, inulin, and tyramine was unaffected while blood-brain arginine transport was significantly reduced. These results strikingly confirm the hypothesis that carrier-mediated blood-brain transport is the limiting factor determining the availability of the neutral amino acids to the brain. The encephalopathy associated with cirrhosis may be the result of abnormal neurotransmitter metabolism and neurotransmission secondary to increased neutral amino acid transport activity and an increased brain content of members of the neutral amino acid group.*

Cirrhosis of the liver, usually related to alcohol abuse, is the sixth ranking cause of death in the United States. Chronic liver disease is frequently accompanied by symptoms of neurological impairment termed hepatic encephalopathy. The cause of the symptoms, which may progress to hepatic coma and death, is unknown. Encephalopathic episodes are associated with such diverse factors as protein ingestion, overdiuresis, or sepsis-induced catabolic states (*1*). In the cerebrospinal fluid of patients with cirrhosis, tryptophan (TRY) concentrations are two to three times normal and the serotonin metabolite 5-hydroxyindoleacetic acid is also elevated (*2*), suggesting that serotonin may play a role in hepatic encephalopathy. Fischer and Baldessarini (*1*) suggested that the other aromatic neutral amino acids, phenylalanine and tyrosine, may also be involved since their decarboxylated metabolites, phenethylamine and tyramine, can affect catecholamine metabolism. In a model of cirrhosis, the rat with a surgically constructed portacaval anastomosis (PCA), brain TRY and serotonin are markedly elevated, although total plasma TRY is unchanged (*3*). Since plasma leucine, isoleucine, and valine are frequently decreased in chronic liver disease, it has been suggested that blood-brain TRY transport is facilitated by decreased concentrations of the branched-chain neutral amino acid (NAA) competitors (*3, 4*). However, no evidence has yet been presented that brain TRY content may be influenced by modification of the blood-brain NAA transport system itself or that such alterations may play a role in any disease process.

It has been proposed that carrier-mediated transport of the NAA's across the blood-brain barrier may be the limiting factor in determining their availability for brain cell metabolism (*5*). The introduction of a single-injection, tissue sampling technique for studying blood-brain transport has permitted the tabulation of estimates of the maximum initial velocity, V_{max}, and the Michaelis constant, K_m, for transport of individual amino acids in rats (*6*). We have now used this technique in combination with amino acid analysis to investigate the blood-brain transport of various substances including TRY in rats after a PCA is formed. We find that the activity of blood-brain NAA transport is selectively increased after the PCA operation and that increased TRY transport activity is well correlated with increased brain TRY.

Portal blood flow was surgically diverted into the systemic circulation of female Sprague-Dawley rats (300 to 350 g) by creating a PCA, as previously described (*7*). Rats with a PCA and controls of the same age and weight were fed Purina rat chow and water ad libitum until they were killed by decapitation. The blood and brain concentrations of the large NAA's excluding TRY were determined by using a Beckman 121-MB amino acid analyzer in pentobarbital-anesthetized rats 14 days after PCA and in unoperated controls (Table 1). After PCA, plasma valine, isoleucine, and leucine decreased to about 80 percent of control values. Plasma methionine was not significantly elevated, while tyrosine, phenylalanine, and histidine all increased significantly. Plasma tyrosine, which showed the greatest increase after PCA, was about twice the control value. In contrast, brain valine, isoleucine, and

Table 1. Effect of portacaval anastomosis on plasma and brain neutral amino acids. Groups of 8 control rats and 11 rats with a PCA were anesthetized with pentobarbital and 10 minutes later were decapitated. Plasma and brain NAA's were determined by ion-exchange chromatography, using a Beckman 121-MB amino acid analyzer. Data are presented as mean ± standard error (S.E.) and were analyzed by Student's *t*-test. The last column shows the concentration of each amino acid in the PCA rats as a percentage of that in the control rats.

Amino acid	Concentration		Percentage of control
	Control rats	PCA rats	
	Plasma (nmole/ml)		
Valine	242 ± 21	187 ± 11	77.3*
Methionine	60 ± 4	70 ± 4	116.7
Isoleucine	99 ± 7	77 ± 6	77.8*
Leucine	180 ± 17	144 ± 11	80.0
Tyrosine	61 ± 8	120 ± 8	196.7†
Phenylalanine	69 ± 4	119 ± 8	172.5†
Histidine	67 ± 5	107 ± 5	159.7†
	Brain (nmole/g)		
Valine	95 ± 6	92 ± 7	96.8
Methionine	51 ± 5	84 ± 3	164.7†
Isoleucine	39 ± 1	37 ± 2	94.9
Leucine	79 ± 3	91 ± 5	115.2
Tyrosine	53 ± 5	185 ± 8	349.1†
Phenylalanine	47 ± 2	151 ± 8	321.3†
Histidine	58 ± 3	166 ± 6	286.2†

*$P < .05$ compared to control values. †$P < .001$ compared to control values.

Table 2. Effect of portacaval anastomosis on rat brain/plasma concentration ratios of the large neutral amino acids. Data are presented as the mean ± S.E. for the brain/plasma ratio (nanomoles per gram divided by nanomoles per milliliter) of each amino acid in the groups of 8 control rats and 11 rats with a PCA. Statistical significance was determined by Student's *t*-test.

Amino acid	Brain/plasma ratio	
	Control rats	PCA rats
Valine	0.42 ± 0.04	0.51 ± 0.04
Methionine	0.88 ± 0.12	1.28 ± 0.08*
Isoleucine	0.41 ± 0.03	0.49 ± 0.02*
Leucine	0.46 ± 0.03	0.65 ± 0.04†
Tyrosine	0.91 ± 0.06	1.55 ± 0.09†
Phenylalanine	0.70 ± 0.05	1.30 ± 0.08†
Histidine	0.89 ± 0.06	1.58 ± 0.09†

*$P < .05$ compared to control values. †$P < .01$ compared to control values.

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