Gaskin, F., Canton C. R. & Shelanski, M. L. (1974) *J. Mol. Biol. 89,* 737-758.
Herzog, W., & Weber, K. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 1860-1864.
Jacobs, M., & Kaplow, M. (1976) *Biochem. Biophys. Res. Commun. 68,* 127-135.
Jacobs, M., Smith, H., & Taylor, E. W. (1974) *J. Mol. Biol. 89,* 455.
Kobayashi, T. (1974) *J. Biochem. (Tokyo) 76,* 201-204.
Kobayashi, T., & Simizu, T. (1976) *J. Biochem. (Tokyo) 79,* 1357-1364.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14,* 5183-5187.
Lee, J. C. & Timasheff, S. N. (1977) *Biochemistry 16,* 1754-1764.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193,* 265-275.
Maccioni, R., & Seeds, N. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 462-466.
Olmsted, J. B., & Borisy, G. G. (1975) *Biochemistry 14,* 2996-3005.
Oosawa, F., & Asakura, S. (1975) in *Thermodynamics of the Polymerization of Protein,* Academic Press, New York, N.Y.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 765-768.
Tilney, L. G., Bryan, J., Bush, D. J., Fujiwara, K., Mooseker, M. S., Murphy, D. B., & Shyder, D. H. (1973) *Cell Biol. 59,* 267-275.
Weingarten, M. D., Lockwood, A. H., Hwo, S., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 1858-1862.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15,* 4248-4254.

The Blue Membrane: The 3-Dehydroretinal-Based Artificial Pigment of the Purple Membrane†

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ABSTRACT: A blue-colored artificial purple membrane pigment is formed from the recombination of retinal$_2$ with bleached apopigment. The dark- and light-adapted forms of this "blue membrane" have their λ_{max}'s at 593 and 603 nm, respectively, and their extinction coefficients relative to that of light-adapted purple membrane were 0.79 and 0.87, respectively. The circular dichroism spectrum of the blue membrane shows an exciton interaction between the chromophores, similar to that seen in the purple membrane. The ratios of maximum to minimum in the biphasic CD spectrum are larger than those of the purple membrane, the differences probably being due to a larger intrinsic CD band of the blue-membrane pigment. The rate of dark adaptation of blue membrane is faster than that of purple membrane, and the activation energy of dark adaptation of blue membrane is 19 kcal/mol, while that of purple membrane is 24 kcal/mol. The blue membrane's absorption spectrum is more readily affected by changes in pH than the purple membrane. In the purple membrane, irradiation leads to a ~410-nm intermediate designated M. An intermediate similar to this, M^B, is formed by irradiating the blue membrane at −60 °C in 1 M NaCl and at a slightly alkaline pH. The primary photoproduct was observed at liquid nitrogen temperatures, but the quantum efficiency seems to be temperature dependent. Many of the differences between the blue and the purple membrane are due to the differences in the length of π-electron conjugation of the two chromophores. However, other differences such as temperature-dependent photochemistry, different pH stability, and different rates of dark adaptation would suggest a different chromophore–protein relationship in the blue and purple membranes.

The purple membrane of *Halobacterium halobium* absorbs photons and converts the light energy to chemical free energy by the formation of a proton gradient across the membrane (Oesterhelt and Stoeckenius, 1973; Rosenfeld et al., 1977). The chromophore of purple membrane, like visual pigments such as rhodopsin and iodopsin, is retinal$_1$. Upon prolonged incubation in the dark, the purple membrane has its absorption λ_{max} at 558 nm (dark-adapted form). After illumination, the λ_{max} shifts to 568 nm; this species, the light-adapted form, can slowly revert back to the dark-adapted form (Oesterhelt and Stoeckenius, 1971). In the light-adapted form, the retinal$_1$ is in the all-trans conformation, while the dark-adapted form contains both the 13-cis and all-trans conformations (Oesterhelt et al., 1973; Pettei et al., 1977; Maeda et al., 1977). The individual purple-membrane protein molecules are arranged in a rigid, highly ordered, two-dimensional hexagonal lattice with groups of three molecules clustered about a symmetry axis (Henderson and Unwin, 1975). The chromophores of the pigment molecules interact with each other and show a typical biphasic CD spectrum due to exciton interaction (Heyn et al., 1975; Becher and Ebrey, 1976; Ebrey et al., 1977).

In nature, there is another type of retinal, retinal$_2$ (3-dehydroretinal), found in some visual pigments such as porphyropsins and cyanopsins (Wald, 1960); retinal$_2$ has one more double bond in the β-ionone ring than retinal$_1$. Because of this extra conjugation, retinal$_2$-based pigments have a number of altered spectroscopic properties, such as having their λ_{max} at

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longer wavelengths than the corresponding retinal$_1$-based pigments (Ebrey and Honig, 1977). In *Halobacterium,* retinal$_2$ has not yet been found as a naturally occurring chromophore. We have made a synthetic retinal$_2$-based pigment from the bleached purple membrane and free retinal$_2$. This pigment has a blue color, so we have called it the blue membrane. Here we report a number of spectroscopic and physical properties of the blue membrane and compare them to the corresponding ones of the purple membrane. While some of the differences seen can be attributed just to the extra conjugation of the second double bond on the ring, others must be due to more profound effects of the artificial chromophore on the pigment and its membrane.

Materials and Methods

Preparation of the Bleached Membrane. Purified purple membrane, prepared according to the method of Becher and Cassim (1975), was suspended in a 0.75 M hydroxylamine solution neutralized with NaOH and irradiated with intense yellow light until it had lost its color, due to the formation of retinal oxime. The bleached purple membrane was washed with distilled water four times and lyophilized, petroleum ether was added, and the sample was vigorously shaken, extracting most of the retinal oxime. The membrane was then pelleted, lyophilized, suspended in distilled water, and sonicated to reduce light scattering in the suspension.

Preparation of Regenerated Purple and Blue Membrane. all-trans-Retinal$_1$ was obtained from Sigma Chemical Co. and used without further purification; the major contaminant, 13-*cis*-retinal (less than 5%), combines with the apoprotein to yield the dark-adapted form of the retinal$_1$ pigment and was converted to the all-trans (light-adapted) form by irradiation in all our experiments. Retinal$_2$ was a gift from Hoffman-LaRoche and was purified for us by high-pressure liquid chromatography by Dr. Rosalie Crouch, Medical University of South Carolina.

Retinal$_1$ (2.5 mM) or retinal$_2$ (2.0 mM) in ethanol was added to an aqueous suspension of the bleached purple membrane at the molar ratio of 1:1 (retinal/apoprotein), and the mixture was incubated for at least 2 h at room temperature (see Results). To reduce light scattering, some samples were regenerated in 50% sucrose.

The samples for low-temperature spectrophotometry were prepared by adding glycerol to the regenerated samples at a final concentration of 75%.

Measurements of Absorption and CD Spectra. Absorption spectra were measured with a Cary 118, and CD spectra were measured with a JASCO J-40A. For measuring spectra at temperatures above 0 °C, a temperature-controlled cell with 5-mm light-path-length cell was used. For low-temperature spectra, a glass-windowed Dewar was used. The temperature was controlled by adding liquid nitrogen to ethanol.

For circular dichroism measurements, the same temperature-controlled cell as in the absorption measurements was used to maintain the samples at 10 °C to prevent dark adaptation (see below). Several precautions are necessary to obtain accurate CD spectra from these membrane suspensions. In order to reduce the scattering artifacts, the CD measurements were made with the samples in 50% sucrose using a 5-mm path-length cell placed close to the photomultiplier (see Ebrey et al., 1977).

Calculation of Activation Energies. The simplest possible model for the interrelationship between the dark- and light-adapted forms of the pigment which includes the thermal equilibrium between the different isomeric forms of the dark-adapted pigment is:

$$PM_c\ (13\text{-}cis\text{-retinal}) \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PM_t\ (all\text{-}trans\text{-retinal})$$

where PM_c are the purple membrane molecules which have 13-*cis*-retinal as the chromophore, and PM_t are the purple membrane proteins which have all-trans as the chromophore. We assume a similar equation can be written to represent dark-adaptation for the blue membrane. According to the reaction formula, the following equations can be written:

$$\frac{d[PM_t]}{dt} = k_1[PM_c] - k_{-1}[PM_t] \text{ and } [PM_c] = [PM]_0 - [PM_t]$$

where $[PM]_0$ is the total concentration of purple membrane. The solution is:

$$[PM_t] = [PM_t]^f - ([PM]_0 - [PM_t]^f)e^{-(k_1 + K_{-1})t}$$

where $[PM_t]^f$ is the final concentration of PM_t. The rate of dark adaptation is $K = k_1 + k_{-1}$. We can write:

$$K = k_1 + k_{-1} = A_1 e^{E_{-1}^{\ddagger}/RT} + A_{-1}e^{-E_{-1}^{\ddagger}/RT}$$

we now assume $E_1 = E_{-1} = E$; that is, we assume the activation energies of both directions, from all-trans to 13-cis and from 13-cis to all-trans, are equal. This has been shown to be the case for native purple membrane by Keen and Dencher (1976). Then:

$$\ln K = \ln (k_1 + k_{-1}) = \ln [A_1 e^{-E^{\ddagger}/RT} + A_{-1}e^{-E^{\ddagger}/RT}] = -\frac{2F^{\ddagger}}{RT} + \ln (A_1 + A_{-1})$$

Based on the above assumptions, the activation energies for the dark adaptation of regenerated purple and blue membrane can then be calculated from measurements of the rate constant K as a function of temperature.

Results

Regeneration. Retinal$_1$ and retinal$_2$ in ethanol were added to identical bleached purple membrane suspensions and the mixtures were shaken vigorously and incubated overnight at room temperature (~22 °C) (Figure 1). More than 80% of the final pigment concentration was regenerated within 15 min, but additional regeneration continued for several hours. The regeneration rate and final level in a buffered suspension (phosphate buffer 1, 10, or 100 mM) were almost the same as that in distilled water. The slow-regeneration phase was observed whether or not the retinal oxime was extracted from the bleached membrane.

When identical bleached membrane samples were regenerated with retinal$_1$ or retinal$_2$, the ratio of the regenerated dark-adapted purple membrane's final, maximum optical density to the final maximum optical density of the regenerated dark-adapted blue membrane at λ_{max} was 1.07.

Dark Adaptation. We noted that the rate of dark adaptation of blue membrane was faster than that of purple membrane. To study this phenomena, the rates of dark adaptation were measured at various temperatures (Figure 2). As the temperature was lowered, the rate of dark adaptation decreased for both the blue and the purple membrane samples. However, the rate at which they decreased was different; that is, the dark adaptation processes had different Q_{10}'s. The conversion rates just after turning off the irradiating light were always higher than the final rates. The latter part of the conversion curves were fitted to an exponential and, when extrapolated to zero time, accounts for ca. 85% of the change. We tentatively believe

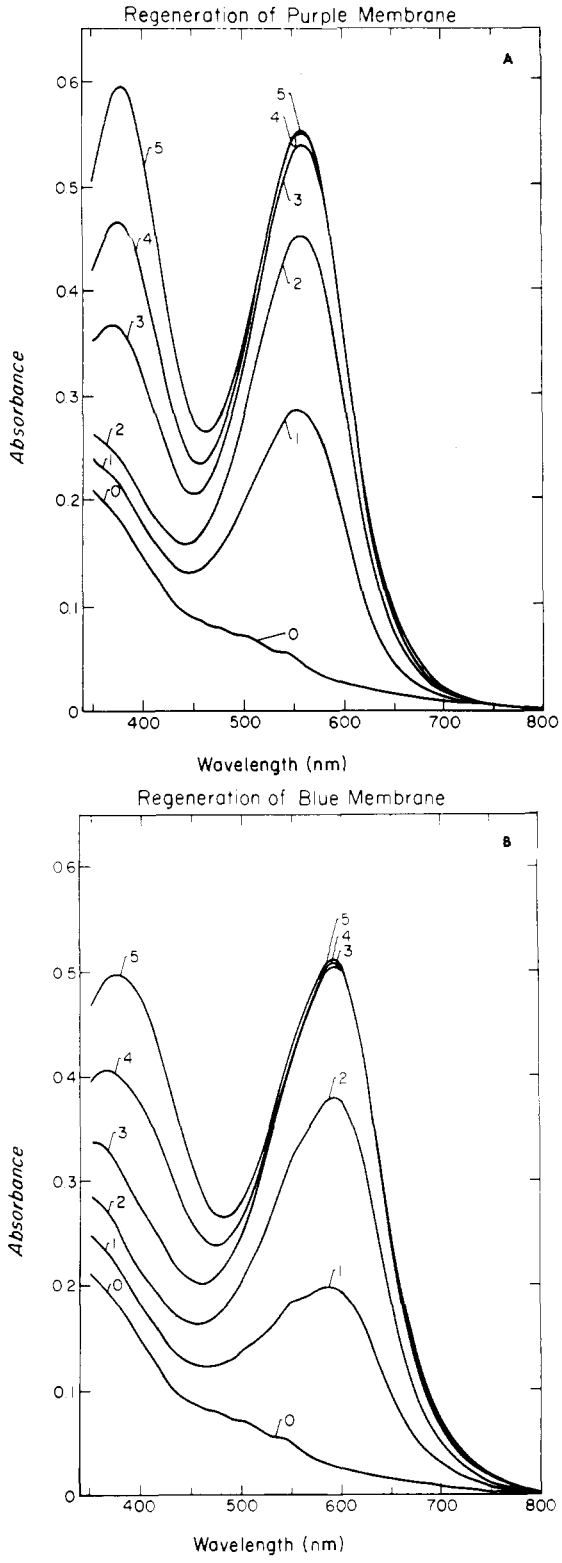


FIGURE 1: Regeneration of the purple and blue membrane. (A) Regeneration of purple membrane: curve 0, bleached purple membrane in water; curves 1 to ~5, the products incubated at room temperature overnight after the addition of 1.5, 4.6, 6, and 7.5 μL of retinal$_1$ in ethanol (2.0 mM) to 1 mL of the bleached purple membrane ($OD_{\lambda_{280}}$ 1.08). (B) Regeneration of blue membrane: curve 0, bleached purple membrane in water; curves 1 to ~5, the products were incubated at room temperature overnight after the addition of 2, 4, 6, 8, and 10 μL of retinal$_2$ in ethanol to 1 mL of the bleached purple membrane ($OD_{\lambda_{280}}$ 1.08).

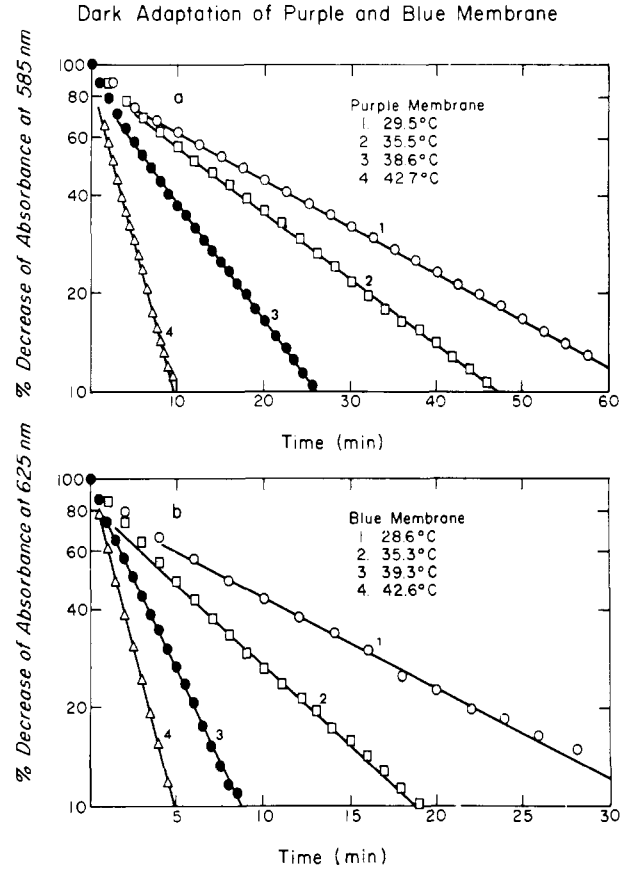


FIGURE 2: Dark adaptation of the purple and blue membrane. (a) Regenerated purple membrane in 50% sucrose-water. The sample was light adapted and then the absorbance at 585 nm was followed at the different temperatures (±0.3 °C): 1, at 29.5 °C; 2, at 35.5 °C; 3, at 38.6 °C; 4, at 42.7 °C. (b) Blue membrane in 50% sucrose-water. The sample was light adapted and then the absorbance at 625 nm was followed at the different temperatures: 1, at 28.6 °C; 2, at 35.3 °C; 3, at 39.3 °C; 4, at 42.6 °C.

the initial rapid conversion seen in both the purple- and blue-membrane samples is due to 10–15% of the pigment molecules being part of clusters of three apopigment molecules which do not totally regenerate. We have two reasons for this suspicion. First, on a similar semilog plot, the conversion curve of native purple membrane was a straight line while partially regenerated purple membrane (monomers and dimers) dark adapts faster than molecules in complete trimers (Ebrey et al., 1977). Moreover, we have found via an independent method utilizing the fluorescence from the pigments (Govindjee, Becher, and Ebrey, unpublished observations) that in many samples about 10–15% of the initial sites are not regenerated, leading to a few dimers. Nevertheless, none of the conclusions of this paper depend crucially on whether or not these 10–15% of the sites cannot be regenerated.

From the temperature dependence of the rate of dark adaptation, and making the assumptions outlined under Materials and Methods, we can construct an Arrhenius plot (Figure 3). The activation energy of dark adaptation of the regenerated purple membrane was 24 kcal/mol, which was very close to that reported for the native membrane (Keen and Dencher, 1976). The activation energy of dark adaptation of blue membrane was 19 kcal/mol.

Extinction Coefficients of Blue Membrane. In order to study the light-adapted blue membrane, the rate of dark adaptation was greatly slowed down by doing all experiments at 10 °C. At this temperature, the half-time of dark adaptation of blue membrane was about 50 min, while that of purple membrane was estimated to be about 5.5 h. To reduce scattering, all measurements were done in 50% sucrose. Figure 4 shows the absorption spectrum of the regenerated pigments.

TABLE I: Extinction Coefficients, Bandwidths, and Dipole Strengths for the Light- and Dark-Adapted Forms of Both the Purple and Blue Pigments.[a]

	λ_{max}	Max extinct. rel to light adapted purple membr.	Mol[b] extinct. coeff.	Dipole[c] strength (10^{-35})	Band-width (cm^{-1})
Light-adapted purple membrane	571	1.00	60 000	7.83	3300
Dark-adapted purple membrane	562	0.865	51 900	7.59	3700
Light-adapted blue membrane	603	0.870	52 200	7.58	3600
Dark-adapted blue membrane	593	0.792	47 500	7.56	3900

[a] All measurements were done at 10 °C. [b] The extinction coefficient of light-adapted purple membrane was assumed to be 60 000. [c] These values were calculated by the following formula: $D = (0.92 \times 10^{-38}/\lambda_{max})\sqrt{\pi}\epsilon_{max}\Delta$, where Δ is half-bandwidth at $0.368\epsilon_{max}$.

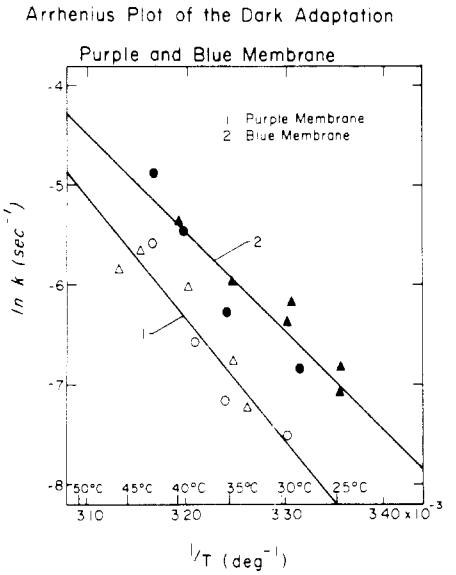


FIGURE 3: Arrhenius plot of the dark adaptation of the purple and blue membrane in a 50% sucrose-water solution. Circles were from Figure 2. Triangles were from another set of experiments. Open symbols show the purple membrane and closed symbols show the blue membrane.

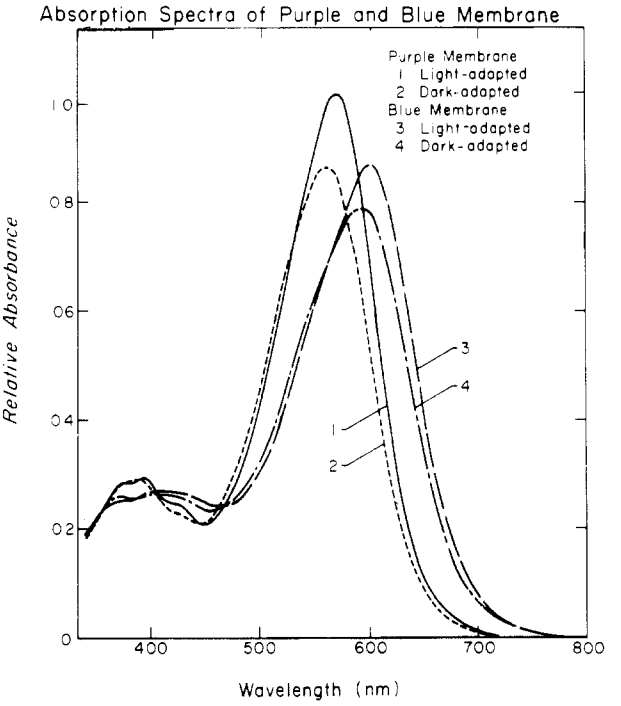


FIGURE 4: Absorption spectra of purple and blue membrane in 50% sucrose-water. All measurements were done at 10 ± 2 °C. In the reference cell was bleached membrane in 50% sucrose-water also at 10 °C: Curve 1, light-adapted purple membrane; curve 2, dark-adapted purple membrane; curve 3, light-adapted blue membrane [the sample was irradiated with orange light (Corning glass filter CS3-63) for 2 min, 50 nm was scanned and reirradiated, and another 50 nm was scanned (scanning speed 5 nm/s)]; curve 4, dark-adapted blue membrane.

The λ_{max}'s of dark- and light-adapted blue membrane were 593 and 603 nm, respectively, at 10 °C. The extinction coefficients, bandwidths, and dipole strengths for the light- and dark-adapted forms of both the purple and blue pigments are given in Table I. The bandwidths of both light-adapted pigments were narrower than those of dark-adapted forms, as would be expected if the dark-adapted species consisted of a mixture of two pigments while the light-adapted was a single pigment. As noted in the Introduction, this has been shown to be the case for the purple membrane and seems a reasonable hypothesis to extend to the blue membrane.

The spectrum of the blue membrane has a shoulder on the short-wavelength side. This shoulder was also clearly seen in the difference spectrum between the blue membrane and the M-type intermediate, which does not absorb past 500 nm (see below, Figure 7C) and thus is not due to a colored contaminant like the carotenoids. The shoulder becomes more prominent upon cooling to 77 K (Figure 9). A similar shoulder can also be seen in the retinal A_2 based visual pigments (Yoshizawa, 1972).

Circular Dichroism. In the purple membrane the chromophores are close to each other so that there is an exciton-type interaction between the transition dipoles, leading to a typical biphasic CD spectrum (Becher and Ebrey, 1976; Ebrey et al., 1977). The CD spectrum of the blue membrane also shows such an exciton interaction between the retinal$_2$ chromophores.

Figure 5 shows the CD spectra. Light adaptation of a dark-adapted sample shifted the CD spectra to longer wavelengths and increased the intensities. The ratios of the maximum to the minimum of the ellipticities of light- and dark-adapted blue membrane were 1.17 and 1.81, respectively, while those of purple membrane were 0.67 (dark adapted) and 0.87 (light adapted). A quantitative analysis of the CD spectrum of the blue membrane can be made after realizing that the CD spectrum is a mixture of two components—an "intrinsic" CD component, due to chromophore twisting, etc. (see Honig et al., 1973), and an exciton component, which must consist of two bands of exactly the same rotational strength but of opposite sign (see Ebrey et al., 1977). The larger positive CD portion of the blue membrane presumably is due to a much larger positive intrinsic CD band than that of the purple-membrane protein. The intrinsic CD of the purple membrane

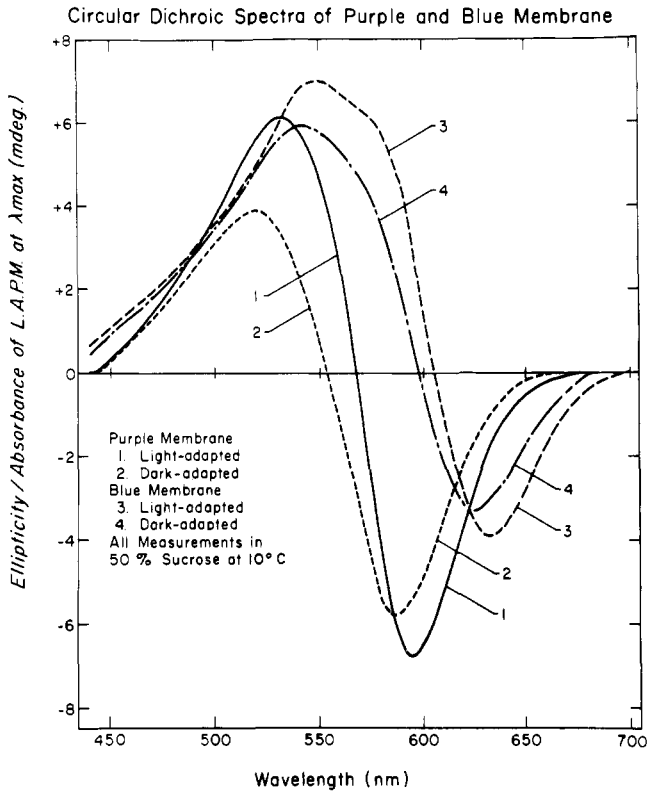


FIGURE 5: Circular dichroism spectra of purple and blue membrane. All measurements and irradiations were done in 50% sucrose at 10 °C. From each original spectrum was subtracted the spectrum of bleached purple membrane: Curve 1, light-adapted purple membrane (the sample was irradiated and then just 100 nm was scanned before reirradiation and scanning the next 100-nm segment); curve 2, dark-adapted purple membrane; curve 3, light-adapted blue membrane (the sample was irradiated and a 20-nm segment scanned; the sample was reirradiated, and the next 20-nm segment was scanned); curve 4, dark-adapted blue membrane.

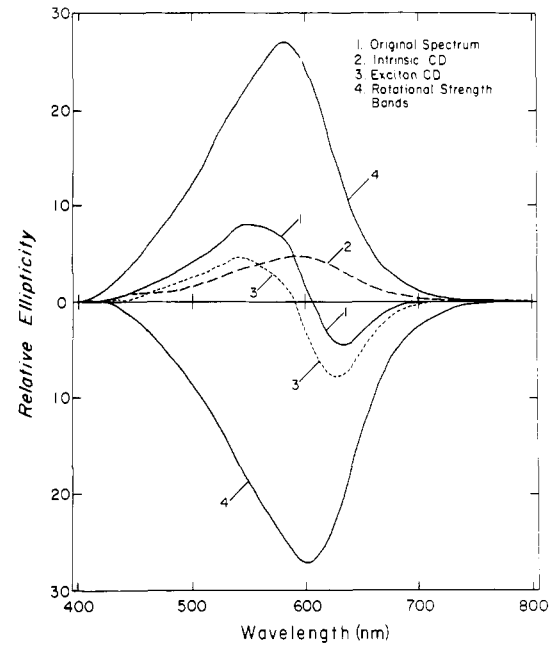


FIGURE 6: Curve fitting of CD spectrum of the light-adapted blue membrane. The original CD spectrum of 100% regenerated blue membrane (curve 1) was divided into two spectra; one was the intrinsic CD spectrum (curve 2), the shape and the maximum of which are the same as those of absorption spectrum of the light-adapted blue membrane, and another was the exciton CD spectrum (curve 3), which must have the same magnitude of rotational strength in positive and negative bands. The exciton CD spectrum was separated into a negative curve (curve 4) and a positive curve (curve 5), both of which have the same shape as that of the absorption spectrum of the light-adapted blue membrane and equal rotational strengths.

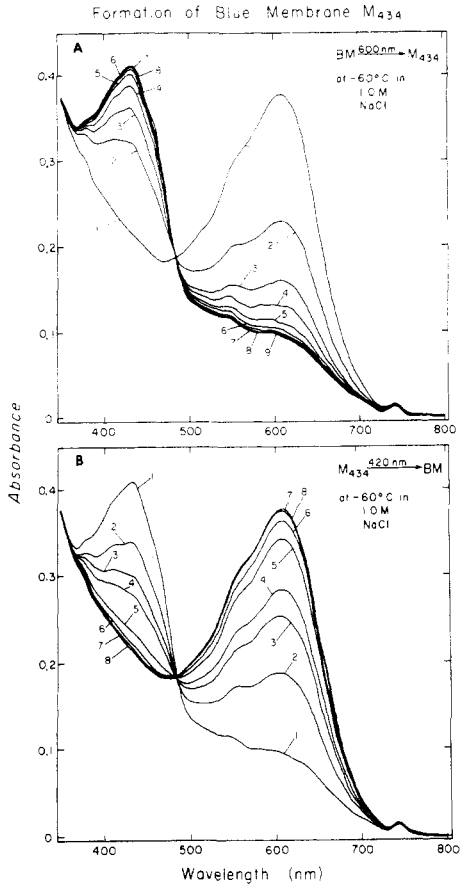
is very small (θ_{max}/OD $\simeq$ 0.02 mdeg) compared to its exciton CD (θ_{max}/OD $\simeq$ 6 mdeg) (Ebrey et al., 1977). A curve-fitting analysis of the exciton and intrinsic CD of the blue membrane is shown in Figure 6 and leads to a separation of the different CD components and a reliable estimate of the magnitude of the intrinsic band. The magnitude of the intrinsic CD band of the blue membrane was found to be 0.15 μ_D (debye magneton), three times larger than the purple membrane.

Having obtained the "pure" exciton CD spectrum, an estimate of the magnitude of the exciton splitting can be obtained from essentially the energy difference between the pure exciton CD crossover (λ^{CO} = 592 nm) and the absorption λ_{max} (603 nm). (For a detailed discussion of the curve fitting, see Ebrey et al., 1977.) The magnitude of the splitting of the two exciton bands giving rise to the CD is 600 cm^{-1}, similar to that observed in the purple membrane. This would place the blue-membrane exciton bands at 582 and 604 nm, with the longer wavelength band having about ten times greater dipole strength than the 582-nm band. In Figure 6, we extend the curve-fitting analysis to the exciton CD spectrum. By placing CD bands of opposite sign but equal magnitude and having the same shape as the absorption bands (curve 4) at the exciton CD λ_{max}'s, we can then vary their magnitude until their sum approximates the actual CD spectrum (curve 3). The magnitude of the rotational strength associated with each of the exciton CD bands is about 2 μ_D, which is close to that of the purple membrane, 2.3 μ_D (Ebrey et al., 1977).

Photocycle Intermediates of the Blue Membrane. The first photoproduct which was observed after light absorption in the purple membrane has been designated the bathoproductor the "K" intermediate because it is shifted to the red end of the spectrum (Lozier et al., 1975). This photoproduct then reverts to the original light-adapted purple membrane through a set of intermediates called L, M, N, and O (Lozier et al., 1975). While the pigment is a protonated Schiff base, M is an unprotonated Schiff base (Lewis et al., 1974; Aton et al., 1977).

A blue-membrane intermediate spectrally analogous to the M intermediate of the purple membrane (M^B) was found. This short-wavelength-absorbing intermediate was formed and was stable at −60 °C in 1 M NaCl and slightly alkaline pH, ~8.0. After adjusting the pH but before cooling, the blue membrane was irradiated by yellow light for a few minutes until light adapted. After waiting 15 s from the end of the irradiation to allow the pigment cycling to be completed, the sample was cooled to −60 °C. The sample was then irradiated with 420-nm light to form a photo-steady-state containing M^B and BM (Figure 7a, curve 1). When the sample was irradiated with 600-nm light, the absorption near 600 nm decreased and the absorption near 430 nm increased with an isosbestic point at 485 nm (Figure 7A). When the irradiated sample was reirradiated by 420-nm light, the absorption near 600 nm increased and that near 430 nm decreased (Figure 7B) with the isosbestic point also at 485 nm. Thus, the blue membrane and its M intermediate were interconvertible by light. When the blue membrane was irradiated at 640, 680, or 700 nm, the spectral change showed the same isosbestic point (485 nm). These results strongly suggest that only one photointermediate, M^B, is observed under these conditions.

The difference spectrum between blue membrane and M^B has its λ_{max} at 608 nm, λ_{min} at 434 nm, and shoulders near 500



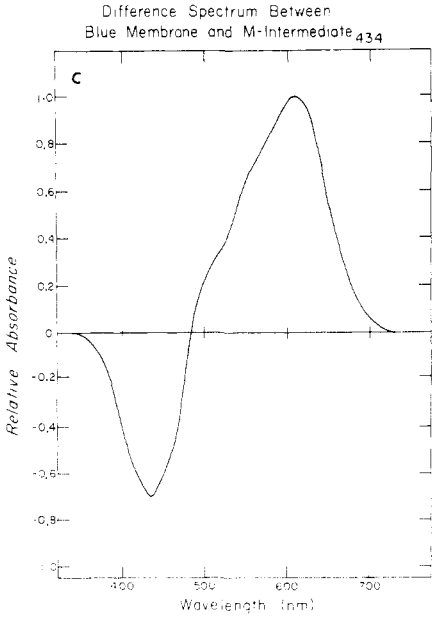


FIGURE 7: Formation of the M^B intermediate of the blue membrane. The blue membrane in 1.0 M NaCl and 75% glycerol was cooled to −60 °C after it had been light adapted. The sample was irradiated at 420 nm for 2 min to form a photo-steady-state containing mostly BM with some M^B. (A) Formation of M^B. The sample was irradiated at 600 nm: curve 1, the preirradiated blue membrane sample; curves 2 to ~9, the products formed by successively irradiating for 0.5, 0.5, 1, 2, 4, 8, 16, and 32 min. (B) Photoconversion of the M^B intermediate to the blue membrane: curve 1, the photo-steady-state produced by irradiation at 600 nm (same as curve 9 in a); curves 2 to ~7; the products formed by successively irradiating for 0.5, 0.5, 1, 2, 4, 8, and 16 min. (C) Difference spectrum between the blue membrane and its M-type intermediate, M^B, calculated from curves 1 and 6 in Figure 8.

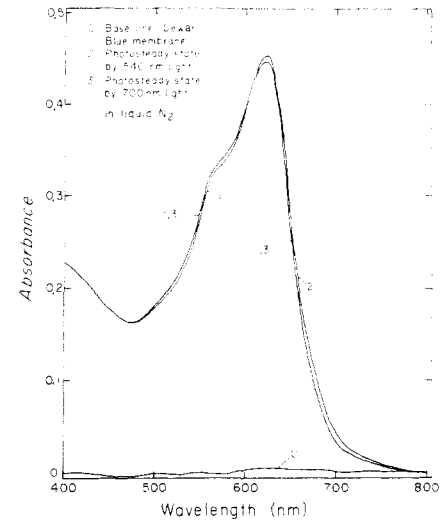


FIGURE 8: Formation of a bathoproduct of the blue membrane. The blue membrane in 1.0 M NaCl and 75% glycerol was cooled in liquid nitrogen after it had been light adapted: curve 1, the blue membrane; curve 2, the photo-steady-state mixture produced by irradiating with 540 nm light for 8 min; curve 3, the product by irradiating the photo-steady-state mixture (curve 2) with 700-nm light.

and 550 nm (Figure 7C). The spectrum of the blue membrane appears to be almost flat near 434 nm, so the true λ_{max} of the M intermediate is very close to that seen in the difference spectrum, 434 nm.

The formation of the bathoproduct from the blue membrane was also studied. Blue membrane in 0.1 M NaCl, pH ~7, containing 75% glycerol was cooled to 77 K (Figure 8, curve 1). Upon cooling, the shoulder on the short-wavelength side of the blue-membrane spectra became more prominent and the absorption maximum shifted to 606 nm.

When the blue membrane was irradiated with 540-nm light for 4 min, the absorption spectrum was shifted slightly to the red (Figure 8, curve 2). Further irradiation did not cause any further spectral shift. The spectra of the photo-steady-states, produced by irradiating the blue membrane with 500-, 560-, 600-, 640-, and 700-nm light and light of wavelengths longer than 670 nm (CS2-60 filter), were located between the spectra of the original blue membrane and the photo-steady-state produced by 540-nm light. So it may be concluded that no photoproduct other than the bathoproduct was produced in liquid nitrogen. From the spectral changes in Figure 8, compared to those seen in purple membrane at liquid nitrogen temperature (Tokunaga et al., 1976), the amount of bathoproduct in the photo-steady-state mixture was estimated to be very small. In order to evaluate the actual amount of the blue-membrane bathoproduct formed, the photo-steady-state mixture was warmed to −60 °C, a temperature at which M^B is stable. Assuming all of the bathoproduct converts into the M^B intermediate, the amount of pigment converted is estimated to be 4%.

The small amount of the blue membrane converted to the bathoproduct may be due to a small ratio of the quantum yield of the blue membrane to bathoproduct to that of its back reaction. At room temperature, a picosecond laser flash sets up a photo-steady-state which contains at least 25% bathoproduct at room temperature (K. Smith, K. Kaufmann, F. Tokunaga, and T. Ebrey, in preparation). This is similar to what is found in the purple membrane (Hurley et al., 1977). The discrepancy between the amount of bathoproduct found in a photo-steady-state at room and at liquid nitrogen temperatures strongly suggests that the quantum yield of the photoreactions

of the blue membrane depends on temperature. This resembles the behavior of the artificial visual pigment isorhodopsin and is in contrast to the purple membrane where the photochemistry is independent of temperature down to −196 °C (Hurley et al., 1977).

Spectral Changes Caused by pH Changes. The absorption spectrum of purple-membrane fragments is unchanged when the pH is varied from pH 3.5 to 10. For comparison, the pH stability of blue-membrane fragments was determined; as the pH decreased from pH 5.8, the spectrum shifted to longer wavelengths with an isosbestic point at 613 nm (Figure 9A). The spectral shift stopped between pH 3.2 and 2.8. The pK of this reaction was estimated to be ca. 4.0. When NaOH was added successively to the sample at pH 3.2, this reaction was reversible. When the pH was decreased below 2.8, the spectrum shifted to the blue, but the isosbestic point of this reaction was not the same as that of pH change from pH 6 to 2.8.

On the other hand, when a small amount of NaOH was added successively to the blue-membrane solution at pH 8.3, the spectrum shifted to the blue with an isosbestic point at 534 nm (Figure 9B). This spectral change was almost complete at pH 10.5. The pK of this reaction was ca. 9.7. When a small amount of HCl was added stepwise to the blue-membrane solution at pH 11.3, the spectrum reverted to the original one with the same isosbestic point. So this reaction was also reversible. When the pH was increased to more than pH 11.3, the spectrum shifted to shorter wavelengths, and the absorbance near 380 nm increased. This may be due to formation of deprotonated Schiff base.

In summary, the spectrum of the blue membrane is stable over a much narrower range of pHs than that of the purple membrane.

Discussion

The properties of the retinal$_2$-based synthetic purple-membrane pigment (blue membrane) that we have studied here and elsewhere (Tokunaga et al., 1977) can be tentatively divided into two categories. The first category concerns those properties which seem to be due primarily to the longer length of π-electron conjugation of the retinal$_2$ chromophore. These include the longer λ_{max} of the retinal$_2$ pigments' absorption band and exciton circular dichroism bands and the broader bandwidths. Very similar phenomena are seen in visual pigments in going from A_1 to A_2 chromophores and detailed explanations have been provided (Greenberg et al., 1975; Honig et al., 1976; Ebrey and Honig, 1976). Other differences between the blue and the purple membrane which probably are related to just the conjugation properties of the different chromophores are the lower extinction coefficients and the larger intrinsic CD of the blue membrane. Both of these differences are also seen in the retinal$_1$ vs. retinal$_2$-based visual pigments (Brown et al., 1963; Parkes et al., 1976), but the exact origin of these properties of the pigments is obscure (Bridges, 1967; Honig et al., 1973).

In contrast to these differences between the retinal$_1$- and retinal$_2$-based membranes, there were several properties which were rather unexpectedly different. These are: (1) the rate of dark adaptation of the blue membrane (perhaps this is related to the different Q_{10}'s for the dark-adaptation process); (2) the temperature dependence of the quantum efficiency for the photochemistry

$$\text{BM603} \xrightarrow{h\nu} K^{B} \text{ and/or } K^{B} \rightarrow \text{BM603}$$

which is not seen in the purple membrane (Hurley et al., 1977); and (3) the much greater sensitivity of the blue membrane pH

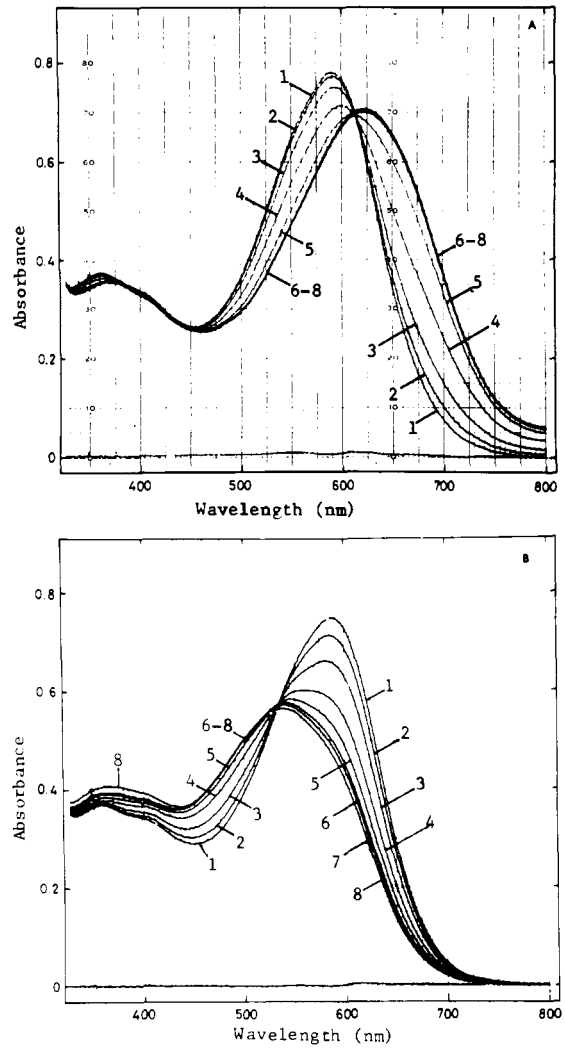


FIGURE 9: Spectral changes of the blue membrane caused by pH changes. The blue membrane was suspended in 1.5 mL of distilled water. Less than 10 μL of 0.01, 0.1, or 1.0 N HCl or NaOH was added in the dark, and the absorption spectra of the suspension were measured after the samples remained in the dark for at least 1 h. (A) Spectral change by the addition of HCl. Curves 1 to ~8 were at pH 5.8, 5.1, 4.6, 4.1, 3.7, 3.2, 3.0, and 2.8, respectively. (B) Spectral change by addition of NaOH. Curves 1 to ~8 were at pH 8.3, 8.8, 9.4, 9.9, 10.3, 10.6, 10.9, and 11.3, respectively.

changes. Since there is no increase in the number of atoms in going from a retinal$_1$ to the retinal$_2$ chromophore, no obvious steric effects of the chromophore itself are responsible for the changes. Rather, it seems clear that a very subtle change in the chromophore, the addition of one additional double bond to the β-ionone ring, *has significantly altered the relationship between the chromophore and the protein.* This could happen either by the retinal$_2$ chromophore assuming a different conformation than the retinal$_1$ chromophore so that sterically it fits differently into an unchanged apoprotein binding site or by the retinal$_2$ chromophore changing the conformation of the binding site. Either of these alternatives is quite interesting.

Finally, it is important that, despite these differences in chromophore–protein interactions, at room temperature the photochemistry of the blue membrane appears to be very similar to that of the purple membrane in that light absorption leads to a deprotonated Schiff-base ("M") intermediate being formed and that protons can be pumped across a vesicle membrane containing the blue-membrane pigment (Tokunaga et al., 1977). Moreover, the similarity in the exciton CD spectra of the purple-membrane pigments suggests that the pigments

have a similar packing arrangement with respect to each other in their membranes.

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References

Aton, B., Doukas, A. G., Callender, R. H., Becher, B., and Ebrey, T. G. (1977), Biochemistry 16, 2995.
Becher, B., and Cassim, J. Y. (1977), *Prep. Biochem. 5,* 161.
Becher, B., and Ebrey, T. G. (1977), *Biophys. J. 17,* 185.
Bridges, C. D. B. (1967), *Vis. Res. 7,* 349.
Brown, P., Gibbons, I. R., and Wald, G. (1963), *J. Cell. Biol. 19,* 79.
Ebrey, T. G., Becher, B., Mao, B., Kilbride, P., and Honig, B. (1977), *J. Mol. Biol. 112,* 377.
Ebrey, T. G., and Honig, B. (1977), *Vis. Res. 17,* 147.
Greenburg, A. D., Honig, B., and Ebrey, T. G. (1975), *Nature (London) 257,* 823.
Henderson, R., and Unwin, P. N. T. (1975), *Nature (London) 257,* 28.
Heyn, M., Bauer, P., and Dencher, N. (1975), *Biochem. Biophys. Res. Commun. 67,* 897.
Honig, B., Kahn, P., and Ebrey, T. G. (1973), *Biochemistry 12,* 1637.
Honig, B., Greenburg, A. D., Dinur, U., and Ebrey, T. G. (1976), *Biochemistry 15,* 4593.
Hurley J., Ebrey, T., Honig, B., and Ottolenghi, M. (1977) *Nature (London) 270,* 540.
Keen, M. E., and Dencher, N. A. (1976), EMBO Workshop on Transduction (Jülich), p 64.
Lewis, A., Spoonhower, J., Bogomolni, R., Lozier, R. H., and Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4462.
Lozier, R. H., Bogomolni, R. A., and Stoeckenius, W. (1975), *Biophys. J. 15,* 955.
Maeda, A., Iwasa, T., and Yoshizawa, T. (1977), *J. Biochem. (Tokyo), 82,* 1599.
Oesterhelt, D., Meentzen, M., and Schuhmann, L. (1973), *Eur. J. Biochem. 40,* 453.
Oesterhelt, D., and Stoeckenius, W. (1971), *Nature (London), New Biol. 233,* 149.
Oesterhelt, D., and Stoeckenius, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 2853.
Parkes, J. H., Rockey, J. H., and Liebman, P. A. (1976) *Biochim. Biophys. Acta 428,* 1.
Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, R., and Stoeckenius, W. (1977), *Biochemistry 16,* 1955.
Rosenfeld, T., Honig, B., Ottolenghi, M., Hurley, J., and Ebrey, T. G. (1977), *Pure Appl. Chem. 49,* 341.
Tokunaga, F., Iwasa, T., and Yoshizawa, T. (1976), *FEBS Lett. 72,* 33.
Tokunaga, F., Govindjee, R., and Ebrey, T. G. (1977), *Biophys. J. 19,* 191.
Wald, G. (1960), *Comp. Biochem. 1,* 311.
Yoshizawa, T. (1971), *Handb. Sens. Physiol. 7(1),* 146–179.

Ferric Enterobactin Transport System in *Escherichia coli* K-12. Extraction, Assay, and Specificity of the Outer Membrane Receptor†

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ABSTRACT: An outer membrane preparation from cells of *Escherichia coli* K-12 grown in low iron medium was found to retain ferric enterobactin binding activity following solubilization in a Tris-HCl, Na_2EDTA buffer containing Triton X-100. Activity was measured by means of a DEAE-cellulose column which separated free and receptor bound ferric enterobactin. The binding activity was greatly reduced in preparations obtained from cells grown in iron rich media or from cells of a colicin B resistant mutant grown in either high or low iron media. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis enabled correlation of this lack of activity to a single band missing in the outer membrane profile of the colicin B mutant. Evidence was obtained for in vitro competition between ferric enterobactin and colicin B for the extracted receptor. The binding specificity of the extracted receptor was examined by competition between ferric enterobactin and several iron chelates including a carbocyclic analogue of enterobactin, *cis*-1,5,9-tris(2,3-dihydroxybenzamido)cyclododecane. The ferric form of the latter compound supported growth of siderophore auxotrophs, apparently without hydrolysis to dihydroxybenzoic acid and resynthesis into enterobactin. These data may require revision of the accepted mechanism of enterobactin mediated iron utilization.

Several active transport systems have been identified in *Escherichia coli* which require an outer membrane component to bind the specific substrate, generally a hydrophilic molecule of molecular weight exceeding 600. Each such binding protein thus far identified also acts as a receptor site for bacteriophage and/or colicins. Those systems known are for vitamin B-12 (Bradbeer et al., 1976), maltose (Hazelbauer, 1975), ferrichrome (Wayne & Neilands, 1975), nucleosides (Hantke, 1976), and ferric enterobactin (Wayne & Neilands, 1976). Studies of these receptors relate to mechanisms of phage in-

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