types of myasthenia gravis such as the form restricted to the ocular muscles. The inhibitory globulin may also be sequestered and not be detectable in the circulation. Furthermore, the presence of inhibitory globulin may depend on secretion from the thymus.

Possibly the inhibitory globulins in our patients may represent only an epiphenomenon, similar to the antibodies against nuclei (*16*), muscle structural proteins (*3*), and other tissues in a proportion of patients with myasthenia gravis (*17*). The major immunological factor would then be related to delayed sensitivity directed against the neuromuscular junction and the acetylcholine receptor. Although we cannot exclude such a possibility, the demonstration of circulating inhibitory globulin certainly suggests the potential relevance of humoral factors to the pathogenesis. Furthermore, germinal center formation in the thymus suggests that an active synthesis of antibodies may occur in that organ (*18*).

Whether the observed changes in globulin represent primary or secondary factors in the pathogenesis of myasthenia gravis remains to be determined. However, these inhibitory globulins should be useful not only for understanding the disease process but also as a probe for investigating the acetylcholine receptor.

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12. The muscles of the lower extremity were removed and homogenized in four volumes of 0.05*M* tris • HCl, *p*H 7.4, 0.1*M* NaCl at 4°C with a Brinkmann Polytron homogenizer for 90 seconds at setting 9. After equilibration with room temperature, 10 percent Triton X-100 in 0.05*M* tris • HCl, *p*H 7.4, and 0.1*M* NaCl was added to a final volume of 1 percent Triton X-100 (by volume). The solutions were agitated at room temperature for 90 minutes, cooled to 4°C and centrifuged at 28,000*g* for 15 minutes. The supernatant was used as the source of acetylcholine receptor.
13. In the binding assay, 0.1 ml of the muscle fraction was added to 1 ml of serum and incubated for 30 minutes at 25°C. Thereafter the solution was divided into 0.5-ml portions and 1.0 ml of ^{125}I-labeled α-bungarotoxin (20 pmole) in 0.05*M* tris • HCl, *p*H 7.4, 0.1*M* NaCl, 1 percent Triton X-100 was added to each portion. After an incubation period of 16 hours at 4°C, the portions were applied to a Sephadex G-200 column (1.6 by 45 cm) and developed with 0.05*M* tris • HCl, *p*H 7.4, 0.1*M* NaCl, and 1 percent Triton X-100. The toxin bound to the receptor emerged at approximately one-third the column volume. Radioactivity was measured with a Packard model 500 C gamma spectrometer. Similar incubation conditions were used for globulins isolated from the patients' serums. The globulins were employed at concentrations indicated in the text. Under the same incubation conditions employed for serums and globulins, decamethonium, carbamyl choline, and *d*-tubocurarine were able to inhibit binding up to 90 percent.
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Urea Tolerance as a Molecular Adaptation of Elasmobranch Hemoglobins

Abstract. *Urea is maintained at moderately high concentrations in the blood and tissues of marine elasmobranchs. Functional properties of the hemoglobins from several elasmobranch species are unaffected by urea concentrations as high as 5 molar. This insensitivity to urea, which is not observed with human hemoglobin, is accompanied by an increased sensitivity to sodium chloride.*

High concentrations of urea are known to have a denaturing effect on many proteins (*1*). In the presence of urea, human hemoglobin binds oxygen with a much higher affinity (*2*). Since elasmobranchs maintain relatively high urea concentrations in their blood and tissues (*3*), the question arises whether elasmobranch proteins are particularly resistant to urea. To answer this we have studied the functional properties of several elasmobranch hemoglobins as a function of urea concentration. Our results indicate that the elasmobranch hemoglobins do show a molecular adaptation to the relatively high urea concentrations found in vivo. Indeed, their urea tolerance extends far beyond the physiological range of urea concentrations.

The specimens of clearnose skate *Raja eglanteria*, electric ray *Torpedo nobiliana*, and smooth dogfish *Mustelus canis* used in these studies were captured by trawl in the vicinity of Beaufort, North Carolina. Blood samples were obtained in heparinized saline from the caudal vein and washed three times with cold 3.5 percent NaCl. Packed red blood cells were lysed with cold, distilled water and centrifuged; the supernatant was dialyzed against 0.01*M* tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl), *p*H 8.0, to remove urea and salts. Hemoglobin solutions were stripped of organic and inorganic ions by treatment with a mixed-bed ion exchange resin (*4*). Protein concentrations were determined by using human hemoglobin extinction coefficients (*4*). Oxygen equilibriums were done spectrophotometrically (*5*); flash photolysis and rapid mixing experiments were done as previously described (*6*). Solutions of urea were deionized before they were used by treatment with a mixed-bed ion exchange resin.

High salt concentrations in the ocean create osmotic problems for most marine fish. Elasmobranchs have solved the problem of conserving water by maintaining their blood and tissues hyperosmotic to seawater. This adaptation to an existence in seawater is shared by all the cartilaginous fishes—the sharks, skates, rays, and chimeras. It is accomplished by retention of the nitrogenous end products, urea and trimethylamine oxide (*3*). Urea concentrations in elasmobranch blood and tissues range from 0.001*M* in freshwater species to about 0.45*M* in ocean-dwelling species (*3*). In clearnose skate red cells and serum, we determined the in vivo concentrations of urea to be 0.39 and 0.44*M*, respectively. The contrasting effects of urea on the oxygen-binding properties of clearnose skate hemoglo-

bin and human hemoglobin A (HbA) at these physiological concentrations can be seen in Fig. 1A, which also shows that the oxygen affinity of the skate hemoglobin is largely unaffected by urea at concentrations as high as 5*M*. High concentrations of urea affect neither the *p*H dependence of oxygen binding nor the degree of heme-heme interaction in this hemoglobin (*7*). In contrast to its insensitivity to urea, clearnose skate hemoglobin is markedly affected by NaCl. Figure 1B shows the effect of NaCl on the oxygen affinities of skate and human hemoglobins. In high concentrations of NaCl, the oxygen affinity of clearnose skate hemoglobin is increased but heme-heme interactions are not affected (*7*). Human hemoglobin shows no other effects than the well-known anion effect at low NaCl concentrations (*4*).

The results shown in Fig. 1, A and B, for hemoglobin from the clearnose skate are qualitatively the same as results we have obtained with hemoglobins from species representing two other major groups of elasmobranchs, the sharks and rays. Although we know of no data on amino acid sequences for elasmobranch hemoglobins, the species used in this study have tetrameric hemoglobins in vivo, as do most vertebrates (*8*). We find that oxygen-binding properties of hemoglobin from the electric ray are only slightly affected by urea. In buffer solution containing 0.05*M* tris and 0.1*M* NaCl, *p*H 7.5, the partial pressure of oxygen at which this hemoglobin is 50 percent saturated, P_{50}, is 14.4 mm-Hg and the Hill coefficient, n, is 1.45. In the same buffer, containing 5*M* urea, P_{50} is 9.6 mm-Hg and n is 1.35. Although the oxygen affinity is only slightly increased by 5*M* urea, the value of P_{50} at *p*H 7.5 drops to 2.9 mm-Hg in the presence of 4*M* NaCl. Similarly, hemoglobin from the smooth dogfish is only slightly affected by urea. In the buffer solution containing 0.05*M* tris and 0.1*M* NaCl, *p*H 7.5, P_{50} is 2.24 mm-Hg and n is 2.0. In the same buffer, containing 2.5*M* urea, P_{50} is 2.14 mm-Hg and n is unchanged.

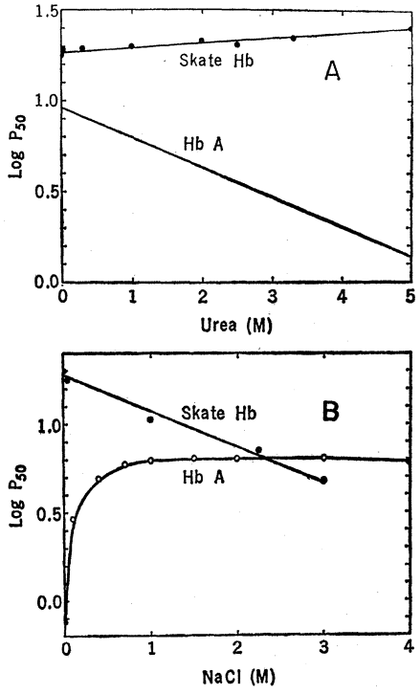

Fig. 1. Effects of (A) urea and (B) NaCl on the oxygen affinities of clearnose skate and human hemoglobins at 20°C. (A) The skate hemoglobin (●) concentration was 1.8 mg/ml in 0.05*M* tris, 0.1*M* NaCl at a nominal *p*H of 7.5. Human hemoglobin (HbA) data were taken from (*2*); the HbA concentration was 6 mg/ml in 0.3*M* phosphate, *p*H 7.1. (B) Skate and HbA concentrations in 0.05*M* tris, *p*H 7.5, were about 1.8 mg/ml.

Although urea has a large effect on oxygen binding by human hemoglobin, urea concentrations below 4*M* probably have little effect on the tertiary structures of the α and β subunits (*1*, *9*). The optical rotatory dispersion curve and the intrinsic viscosity of human HbCO in 6*M* urea are essentially the same as for the native protein (*9*). At concentrations below 4*M* the dominant effect of urea on the liganded forms of human hemoglobin appears to be an increased dissociation into subunits (*1*, *9*).

In the absence of urea the relative proportion of dimers and tetramers in a solution of hemoglobin at neutral *p*H may be monitored by studying the kinetics of CO combination after flash photolysis (*10*). Dimers are characterized by a rapid combination (the so-called quickly reacting material) and tetramers by a slow combination with CO. As might be expected, addition of urea to human hemoglobin produces changes in the kinetics of CO binding after flash photolysis. Figure 2A shows the time course of CO combination after flash photolysis of the carbon monoxy forms of skate and human hemoglobin as a function of urea concentration. Even after several hours in 5.4*M* urea, skate hemoglobin shows only a slight increase in its rate of CO combination. Under similar conditions human hemoglobin recombines with CO at a greatly increased rate, giving a combination velocity constant of $6.7 \times 10^6 M^{-1}$ sec^{-1}. This rate is similar to that reported for the quickly reacting form of hemoglobin, $6.5 \times 10^6 M^{-1}$ sec^{-1} (*11*). Figure 2B shows a marked decrease in the percentage of slowly reacting material for human hemoglobin in urea and shows that the percentage of the slow phase remains high for skate hemoglobin even in 9*M* urea. Results qualitatively similar to those for skate hemoglobin were obtained in flash photolysis studies with hemoglobins from the electric ray and the smooth dogfish (*7*). Greater stability of the elasmobranch hemoglobin tetramers may be a partial explanation for the urea tolerance we observe in these hemoglobins. On the basis of sodium dodecyl sulfate (SDS) polyacrylamide gels of clearnose skate hemoglobin (*12*) and flash photolysis studies in the presence of urea and mercaptoethanol (see

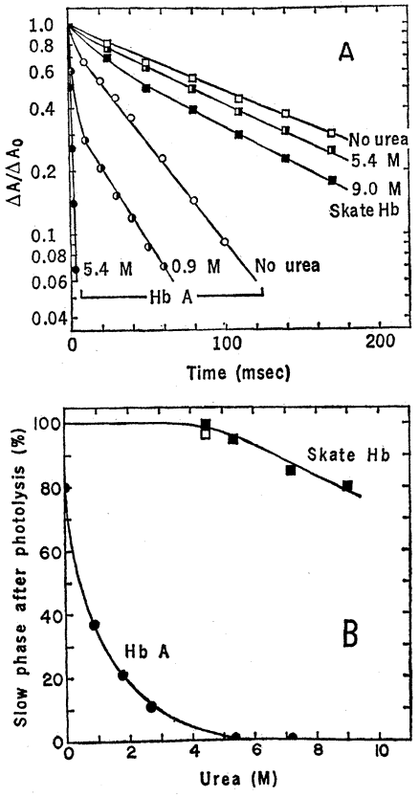

Fig. 2. (A) Effect of urea on the time course of CO recombination after flash photolysis for clearnose skate hemoglobin (squares) and human hemoglobin (circles) in 0.05*M* tris, *p*H 7.1, at 20°C. The recombination was followed by the change in absorbancy, *A*. Hemoglobins were $10^{-5}M$ (heme), CO was $10^{-4}M$, and the observation wavelength was 438 nm. (B) Percentage of slowly reacting material as a function of urea concentration. The open symbol corresponds to an experiment with a sample incubated for 4 hours in urea and 0.1*M* mercaptoethanol.

Fig 2B), we conclude that neither inter- nor intramolecular disulfides contribute to increased stability of elasmobranch tetramers.

The stability and the increased urea tolerance may be due to minor structural differences since studies with abnormal human hemoglobins have shown that single changes in primary sequence at key positions can greatly alter both the oxygen affinity and the equilibrium between dimers and tetramers (*13*). The sensitivity of elasmobranch hemoglobins to NaCl strengthens the possibility that increased electrostatic interactions between their subunits may provide the structural integrity to withstand the denaturing effect of urea. Experiments with human hemoglobin show this to be a real possibility. In its unliganded form, human hemoglobin possesses additional salt bridges between its subunits (*13*). These electrostatic interactions hold the unliganded hemoglobin in a conformation which is much more resistant to dilution-induced dissociation into subunits (*10, 13*). Our kinetic experiments show that the unliganded form is also more resistant to urea-induced dissociation. When the deoxy form of human hemoglobin is rapidly mixed with a solution containing CO, the time course of the reaction shows no evidence of quickly reacting material, even in the presence of 4*M* urea (*14*).

Urea has recently been proposed as a therapeutic agent for the treatment of sickle cell disease. Suggested dosages yield blood urea concentrations of 0.1*M* (*15*). As can be seen from Fig. 1, this concentration of urea has little direct effect on the oxygen affinity of HbA. Aqueous urea preparations, however, are known to form appreciable amounts of cyanate spontaneously. It has been proposed that the "urea effect" comes about by slow carbamylation of the $-NH_2$ termini of the chains by the cyanate (*16*). The ubiquitous presence of urea in blood and tissues of elasmobranchs suggests that their proteins might be highly carbamylated. If this is not the case, elasmobranchs may have evolved biochemical systems for the decarbamylation of amino groups or the removal of cyanate as it is formed.

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14. When the same 4*M* urea solutions used in rapid mixing experiments are manually mixed in a 1 to 1 ratio and then subjected to flash photolysis, the recombination with CO is almost all fast. This is in marked contrast with the slower rate of CO combination observed in rapid mixing experiments with previously unliganded hemoglobin.
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Methionine Adenosyltransferase Deficiency: New Enzymatic Defect Associated with Hypermethioninemia

Abstract. *A specific deficiency of methionine adenosyltransferase has been demonstrated in the liver of an infant with hypermethioninemia. Since the enzymatic activity was below that in fetal liver and the metabolic abnormality has persisted (the infant now being 1 year of age), there is probably a genetic mutation. Mass screening for hypermethioninemia may uncover more such cases.*

Several enzymatic deficiencies on the pathway of metabolism of methionine (Fig. 1) have been described in humans; clinical and biochemical aspects of these deficiencies have been reviewed (*1*). The most well-studied and the most common is cystathionine

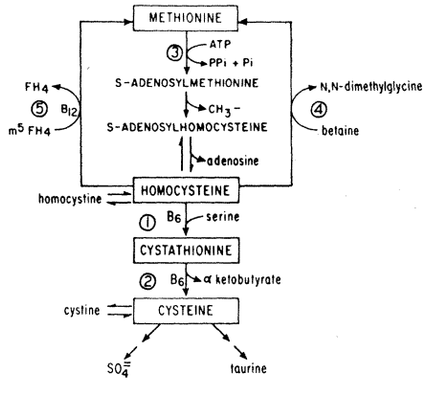

Fig. 1. Transsulfuration and remethylation pathways. (1) Cystathionine β-synthase; (2) cystathionase; (3) methionine adenosyltransferase; (4) betaine-homocysteine methyltransferase; and (5) N^5-methyltetrahydrofolate-homocysteine methyltransferase; FH_4, tetrahydrofolate; m^5FH_4, N^5-methyltetrahydrofolate. [Reproduced with permission of *Pediatric Research*]

β-synthase deficiency, which is associated with hypermethioninemia, homocystinemia, and hypocystinemia. So-called hereditary tyrosinemia and a variety of other diseases accompanied by cirrhoses of the liver have been associated secondarily with nonspecific deficiencies of both methionine adenosyltransferase and cystathionine β-synthase (*2*). Hypermethioninemia occurs in infants, especially in prematurely born infants and in infants fed formulas containing more than 5 percent protein, a concentration well above the 1 percent found in human milk (*3*). In these infants hypermethioninemia usually is accompanied by cystathioninuria (*4*) and is thought to result from a delay in the maturation of cystathionase. Cystathionase normally is absent from human fetal liver and reaches full activity some time after birth (*5*).

In a mass screening survey, a newly born infant with hypermethioninemia was identified. Methionine in the plasma was 128 μmole/100 ml ($>$ 30 times the concentration in normal plasma) and was unaccompanied by homocystinemia, hypocystinemia, or cystathioninuria. Hyperthioninemia was still