The difference in sorption and release of P between oxidized and reduced soils and sediments is very likely due to the difference in the capacity of oxidized and reduced forms of iron oxide to sorb and release orthophosphate-P. Ferric oxyhydroxide is apparently capable of binding orthophosphate ions more firmly than the ferrous form, but probably has less surface area exposed to the solution P than the gel-like hydrated ferrous oxide or ferrous hydroxide.

Other factors, such as *p*H and the concentrations of Ca^{2+} and Mg^{2+}, are known to influence P exchange in aerated soil systems. Their effect in anaerobic soils and sediments is not precisely known, but it is likely that they have much less effect than the amount and oxidation state of the iron compounds. Immobilization of P is described here in terms of sorption involving iron oxides instead of precipitation of ferrous phosphate compounds such as vivianite. For most soils and sediments containing appreciable amounts of poorly crystallized hydrated iron oxide, the P distribution between solid and solution is very likely largely dependent on the nature of the iron compounds. Changes in the hydrated iron oxides as a result of oxidation or reduction reactions can be expected to change the concentration of dissolved orthophosphate.

W. H. PATRICK, JR.
R. A. KHALID
Laboratory of Flooded Soils and Sediments, Agronomy Department, Louisiana State University, Baton Rouge 70803

References and Notes

1. C. H. Mortimer, *J. Ecol.* **29**, 280 (1940); *ibid.* **30**, 147 (1941).
2. M. Aoki, *J. Sci. Soil Anim. Fert. Japan* **15**, 182 (1941); R. E. Shapiro, *Soil Sci.* **85**, 190 (1958).
3. J. K. Syers, R. F. Harris, D. E. Armstrong, *J. Environ. Qual.* **2**, 1 (1973).
4. S. S. Shukla, J. K. Syers, J. D. H. Williams, D. E. Armstrong, R. F. Harris, *Soil Sci. Soc. Am. Proc.* **35**, 244 (1971).
5. J. D. H. Williams, J. K. Syers, S. S. Shulka, R. F. Harris, *Environ. Sci. Technol.* **5**, 1113 (1971).
6. D. Langmuir and D. O. Whittemore, *Adv. Chem. Ser. No. 106* (1971), p. 209.
7. S. Gotoh and W. H. Patrick, Jr., *Soil Sci. Soc. Am. Proc.* **38**, 66 (1974).
8. S. C. Chang and M. L. Jackson, *Soil Sci.* **84**, 133 (1957).
9. K. Kumada and T. Asami, *Soil Plant Food* **3**, 197 (1958).
10. V. Schwertmann, *Z. Pflanzenernaehr. Dueng. Bodenkd.* **105**, 194 (1964); J. A. McKeague and J. H. Day, *Can. J. Soil Sci.* **46**, 13 (1966).
11. Supported in part by Soils and Fertilizer Research Branch, Tennessee Valley Authority (contract TV-13627).

17 December 1973; revised 29 March 1974

Serum Globulin in Myasthenia Gravis: Inhibition of α-Bungarotoxin Binding to Acetylcholine Receptors

Abstract. *Serum factors that inhibit the binding of ^{125}I-labeled α-bungarotoxin to the acetylcholine receptor extracted in detergent from denervated rat muscle were detected by a sensitive assay. The serum of at least 5 and possibly 11 out of 15 patients with myasthenia gravis showed inhibitory activity that was localized to the globulin fraction. No controls showed inhibitory activity. The demonstration of inhibitory globulins may help explain the involvement of the immune system in the pathophysiology of the neuromuscular junction in patients with myasthenia gravis.*

Myasthenia gravis is a neuromuscular disease of man characterized by muscle weakness which increases with exertion and improves with rest (*1*). Although numerous electrophysiologic studies have demonstrated involvement of the neuromuscular junction, it is still not clearly resolved whether the primary site of pathology is the nerve terminal or the muscle end plate (*1*). Involvement of the immune system in myasthenia gravis has been suggested by the marked frequency of thymic hyperplasia (*2*), the presence of antibodies directed against muscle structural proteins (*3*), the increased frequency of lymphocytes toxic to muscle tissue (*4*), and the beneficial effects of repeated lymphocyte drainage (*5*). None of these immunologic studies adequately explain the physiologic alterations of the neuromuscular junction, and attempts to demonstrate the presence of antibodies directed against the neuromuscular junction have been negative (*6*).

Two recent studies suggest physiological and immunological involvement of the muscle acetylcholine receptor in myasthenia gravis. Lindstrom and Patrick demonstrated that injection of purified torpedo acetylcholine receptor into rabbits results in a flaccid paralysis with electromyographic and pharmacologic similarity to myasthenia gravis (*7*). Fambrough, Drachman, and Satyamurti demonstrated that myasthenic muscle biopsies had only 11 to 30 percent the binding capacity of normal muscle for α-bungarotoxin, a specific inhibitor of the acetylcholine receptor (*8*). However, as pointed out by the authors, the reduction in the α-bungarotoxin binding and presumably in the number of available acetylcholine receptors may reflect a secondary effect of drugs or of the disease process.

A reasonable approach to both immunologic alterations and neuromuscular pathophysiology would be provided by the demonstration of circulating globulins that interact with the muscle acetylcholine receptor. We have devised a sensitive assay and have used it to demonstrate the presence in myasthenia gravis of circulating globulins with affinity for the muscle acetylcholine receptor. The basis of our assay is the interference of serum factors with the binding of ^{125}I-labeled α-bungarotoxin to the acetylcholine receptor (*9*).

Lyophilized crude venom of *Bungarus multicinctus* (Miami Serpentarium Laboratories) was fractionated by ion-exchange chromatography on carboxymethyl-Sephadex G-25 (*10*). The isolated α-bungarotoxin was desalted on Sephadex G-50, labeled to high specific activity (10^3 c/mole) with ^{125}I (*11*), and separated from free ^{125}I by chromatography on Dowex 1 and Sephadex G-50. The resultant ^{125}I-labeled α-bungarotoxin was homogeneous by both gel permeation chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. When the labeled α-bungarotoxin (100 n*M*) was applied to a frog sartorius neuromuscular preparation, we noted a rapid and effective elimination of miniature end plate potentials. After both nerve stimulation and the iontophoretic application of acetylcholine, end plate potentials were reduced. The muscle fiber was still excitable by direct stimulation.

The muscle preparation was derived from adult female rats (150 to 200 g) that were unilaterally denervated by removal of a segment of the sciatic nerve 10 days before the animals were killed (*12*). The preparation contained approximately 0.6 to 1.0 pmole of α-bungarotoxin binding units in each 0.1 ml. Serum was fractionated into various globulin subfractions by sodium sulfate precipitation (*11*).

The acetylcholine receptor extracted by Triton X-100 from denervated

skeletal muscle was able to bind approximately 100 pmole of ^{125}I-labeled α-bungarotoxin per gram of muscle (wet weight). The high affinity of the α-bungarotoxin for the receptor complex ($K_A = 1 \times 10^9$ liter/mole), the greater than tenfold increase in binding with denervated as compared to innervated preparations, and the almost complete inhibition with decamethonium, carbamyl choline, and *d*-tubocurarine, all suggest that the binding assay is monitoring specific interactions with the acetylcholine receptor (*9*). When normal serums were introduced into the binding assay, no alteration either in the number of binding sites (N) or in the affinity of binding (K_A) could be demonstrated (*13*) (Table 1). When serum from patients with myasthenia gravis were introduced into the assay, the binding of ^{125}I-labeled α-bungarotoxin was reduced up to 48 percent. Fifteen patients with myasthenia gravis were investigated for the presence of serum factors that inhibited α-bungarotoxin binding. Five of these patients demonstrated the presence of factors that interfered with the α-bungarotoxin acetylcholine receptor interaction (patients 1, 2, 4, 7, and 9). Out of the remaining ten patients, the data on six additional patients suggest the presence of the factor (patients 8, 11, 12, 13, 14, and 15). However, the level of inhibition with these serums was sufficiently small that we preferred to designate them as negative.

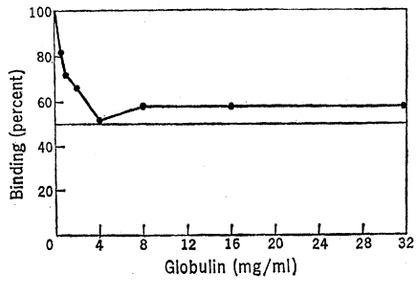

Fig. 1. Globulin inhibition of ^{125}I-labeled α-bungarotoxin binding to extracted acetylcholine receptor. The globulin from myasthenic patient 2 was prepared as described, and the binding assay was performed at a toxin concentration of 20 nM. The data represent results of an experiment performed in duplicate. Other experiments gave identical results.

Since circulating polypeptides have been implicated in the pathogenesis of myasthenia gravis (*14*), isolation of the factor responsible for the inhibition of the α-bungarotoxin acetylcholine receptor was undertaken. After dialysis through Diaflo XM 50 membranes (Amicon), no inhibitory activity passed through the filter even after concentration. Since all inhibitory activity was retained above the filter, possibly the pertinent factor was associated with globular components with a molecular weight greater than 50,000 or with nonglobular proteins. After salt fractionation and dialysis, the inhibitory factor was associated with the material which precipitated at a concentration of 13 percent sodium sulfate. After precipitation and dialysis of the remaining fractions no inhibitory activity could be detected. Globulins from three myasthenia gravis patients with positive serums inhibited the binding of α-bungarotoxin to the acetylcholine receptor, while globulin fractions from normal individuals including a patient with polymyositis had no inhibitory activity.

To assess the extent of inhibitory activity, the effect of increasing concentrations of globulin from myasthenia gravis patients on the toxin binding was investigated [at saturation levels of toxin (20 nM)] (Fig. 1). The maximum inhibition observed was 40 to 50 percent. Although the titers of the antibody differed in three globulins tested the maximum inhibition did not exceed 48 percent. The optimum concentration of globulin was independent of the α-bungarotoxin concentration. At no time could excesses of α-bungarotoxin overcome the globulin inhibition. Such data suggest that globulin is interacting in a noncompetitive fashion with the α-bungarotoxin acetylcholine receptor binding.

It is not clear exactly what role inhibitory globulins play in the pathogenesis of myasthenia gravis. The presence of a circulating inhibitor has been implicated as the best explanation for several clinical phenomena, especially the myasthenia gravis noted in infants born of myasthenic mothers (*15*). Other facets of the disease process can be similarly explained by circulating globulins that interact with the acetylcholine receptor. The failure of previous studies to demonstrate such circulating globulins with affinity for the neuromuscular junction is probably related to the lack of sensitivity of the particular assays employed.

Several factors may explain the absence of circulating inhibitory globulins in all patients with myasthenia gravis. The actual amount of inhibitory globulins may be below the sensitivity of our binding assay in many limited

Table 1. Inhibitory activity of serum or globulin from patients with myasthenia gravis or other disease on the binding of ^{125}I-labeled bungarotoxin to extracted acetylcholine receptor. The data represent assays of ^{125}I-labeled α-bungarotoxin binding to detergent-extracted acetylcholine receptor in the presence of 1 ml of control or myasthenic serums or 5 mg of control or myasthenic globulin; N refers to the number of assays, each performed at least in duplicate. The data have been normalized because of the variation in acetylcholine receptor units extracted from 50 muscle preparations [128 ± 32 pmole of toxin binding per gram (wet weight) of muscle, mean ± S.D.], and the variation in receptor units in the same preparation from week to week (10 percent decrease in bungarotoxin binding activity per week). All test serums and globulins were evaluated in pairs, one from a myasthenia gravis patient and one from a control, and were compared to a buffer control without serum or globulin. The data are presented as means ± S.D. Control patients consisted of four normal individuals, two patients with polymyositis, one with carcinomatous myopathy, and ten with brain tumor, cerebrovascular disease, meningitis, or dementia of unknown etiology. Patients 1, 2, 4, 7, and 9 were considered to show definite inhibition. Patients 8 and 11 to 15 were considered to have possible inhibition.

Patients	Sex	Age	Time since onset	Tensilon test*	Serum		Immunoglobulin G	
					N	Mean ± S.D.	N	Mean ± S.D.
1. T.T.	M	65	3 months	+	3	0.79 ± 0.017		
2. J.F.	M	32	8 years	+	4	0.56 ± 0.06	7	0.56 ± 0.06
3. C.S.	M	40	7 months	+	2	0.97 ± 0.006	3	1.01 ± 0.06
4. D.S.	M	34	1 year	+	2	0.57 ± 0.06	3	0.58 ± 0.03
5. B.J.	M	32	8 years	+	2	1.03 ± 0.01	2	1.02 ± 0.11
6. T.S.	M	68	15 years	+	2	1.05 ± 0.001		
7. L.V.	F	54	1 year	+			2	0.76 ± 0.02
8. J.P.	M	75	2 years	+	2	0.91 ± 0.007		
9. R.Y.	F	74	4 months	−	3	0.75 ± 0.04	1	0.88
10. E.V.	F	62	14 years	+	2	0.97 ± 0.006		
11. L.B.	M	36	4 years	−	2	0.89 ± 0.02		
12. A.L.	F	19	9 years	−	2	0.90 ± 0.005		
13. J.S.	M	53	10 years	−	2	0.87 ± 0.006		
14. A.D.	F	31	12 years	−	2	0.90 ± 0.001		
15. E.R.	F	38	3 years	−	2	0.88 ± 0.003		
Controls	M/F	24–66			24	1.00 ± 0.027	26	1.00 ± 0.03

* The plus sign indicates the transient disappearance of muscle weakness upon injection of Tensilon.

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.

RICHARD R. ALMON
CLIFFORD G. ANDREW
STANLEY H. APPEL

Division of Neurology,
Duke University Medical Center,
Durham, North Carolina 27710

References and Notes

1. W. Fields, Ed., *Myasthenia Gravis* (Annals of the New York Academy of Sciences, New York, 1971), vol. 183, pp. 1–386.
2. B. Castleman, *Ann. N.Y. Acad. Sci.* **135**, 496 (1966).
3. A. J. Strauss, *Adv. Intern. Med.* **14**, 241 (1968); ——— and P. G. Kemp, Jr., *J. Immunol.* **99**, 945 (1967).
4. R. M. Armstrong, R. M. Nowak, R. E. Falk, *Neurology* **23**, 1078 (1973); L. I. Alpert, A. Rule, M. Nario, E. Kott, P. Kornfield, K. E. Osserman, *Am. J. Clin. Pathol.* **58**, 647 (1972); E. F. Field, D. Bates, D. A. Shaw, S. G. Griffin, B. K. Shenton, K. Smith, *Lancet* **1973-II**, 675 (1973); E. Kott, G. Genkins, A. H. Rule, *Neurology* **23**, 374 (1973).
5. K. Bergstrom, C. Franksson, G. Matell, G. Von Reis, *Eur. Neurol.* **9**, 157 (1973); S. C. Tindall, B. H. Peters, J. R. Caverley, H. Sarles, J. C. Fish, *Arch. Neurol.* **29**, 202 (1973).
6. D. E. McFarlin, W. K. Engel, A. J. Strauss, *Ann. N.Y. Acad. Sci.* **135**, 656 (1966).
7. J. Patrick and J. Lindstrom, *Science* **180**, 871 (1973).
8. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *ibid.* **182**, 294 (1973).
9. C. G. Andrew, R. Almon, S. H. Appel, *J. Biol. Chem.*, in press.
10. D. Mebs, K. Narita, S. Iwanaga, Y. Samejima, C. Y. Lee, *Biochem. Biophys. Res. Commun.* **44**, 711 (1971).
11. D. D. Mickey, P. N. McMillan, S. H. Appel, E. D. Day, *J. Immunol.* **107**, 1599 (1971).
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.
14. G. Goldstein and W. W. Hofmann, *Clin. Exp. Immunol.* **4**, 181 (1969); G. Goldstein and A. Manganero, *Ann. N.Y. Acad. Sci.* **183**, 230 (1971).
15. T. Namba, S. B. Brown, D. Grob, *Pediatrics* **45**, 488 (1970).
16. J. N. Whitaker and W. K. Engel, *Neurology* **24**, 61 (1974).
17. R. G. White and A. H. E. Marshall, *Lancet* **1962-II**, 120 (1962).
18. J. D. Sherman, M. M. Adner, W. Dameshek, *Ann. N.Y. Acad. Sci.* **124**, 105 (1965).
19. Supported by grants NS-07872 and GM-01238 from the National Institutes of Health and by grant 558-D-5 from the Multiple Sclerosis Society. We thank W. Clingenpeel for expert technical assistance.

8 March 1974; revised 9 July 1974

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-