References and Notes

1. S. E. Glickman and B. B. Schiff, *Psychol. Rev.* **74**, 81 (1967); J. Olds and M. Olds, in *New Directions in Psychology, 2* (Holt, Rinehart & Winston, New York, 1965); E. S. Valenstein, in *Progress in Physiological Psychology*, E. Stellar and J. M. Sprague, Eds. (Academic Press, New York, 1966), vol. 1.
2. L. Stein, in *Proceedings of the 1968 American College of Neuropsychopharmacology*, D. H. Efron, Ed. (Government Printing Office, Washington, D.C., 1968).
3. R. M. Clavier and A. Routtenberg, *Brain Res.*, in press; G. Arbuthnott, K. Fuxe, U. Ungerstedt, *ibid.* **27**, 406 (1971); S. Ritter and L. Stein, *J. Comp. Physiol. Psychol.* **85**, 443 (1973); T. J. Crow and G. W. Arbuthnott, *Nat. New Biol.* **238**, 245 (1972).
4. H. C. Fibiger and A. G. Phillips, in *Catecholamines and Their Enzymes in the Neuropathology of Schizophrenia*, S. Matthysse and S. S. Kety, Eds. (Pergamon, New York, in press); J. M. Liebman and L. L. Butcher, *Naunyn-Schmiedebergs Arch. Pharmakol.* **227**, 305 (1973); C. D. Wise, B. D. Berger, L. Stein, *Biol. Psychiat.* **6**, 3 (1973); A. G. Phillips and H. C. Fibiger, *Science* **179**, 575 (1973); A. Routtenberg and C. Malsbury, *J. Comp. Physiol. Psychol.* **68**, 22 (1969); L. Hastings and R. M. Stutz, *Life Sci.* **13**, 1253 (1973).
5. A. S. Lippa, S. M. Antelman, A. E. Fisher, D. R. Canfield, *Pharmacol. Biochem. Behav.* **1**, 23 (1973).
6. L. Stein and C. D. Wise, *Science* **171**, 1032 (1971).
7. S. K. Roll, *ibid.* **168**, 1370 (1970).
8. R. Pickens and W. C. Harris, *Psychopharmacologia* **12**, 158 (1968).
9. J. R. McLean and M. McCartney, *Proc. Soc. Exp. Biol. Med.* **107**, 77 (1961); R. M. Ferris, F. L. M. Tang, R. A. Maxwell, *J. Pharmacol. Exp. Ther.* **181**, 407 (1972); L. A. Carr and K. E. Moore, *Biochem. Pharmacol.* **19**, 2361 (1970).
10. C. E. Moan and R. G. Heath, *J. Behav. Ther. Exp. Psychiatr.* **3**, 23 (1972).
11. G. Deneau, T. Yanagita, M. H. Seevers, *Psychopharmacologia* **16**, 30 (1969); M. C. Wilson, M. Hitomi, C. R. Schuster, *ibid.* **22**, 271 (1971); R. A. Yokel and R. Pickens, *ibid.* **34**, 255 (1974); *J. Pharmacol. Exp. Ther.* **187**, 27 (1973).
12. R. Pickens, R. A. Meish, J. A. Dougherty, *Psychol. Rep.* **23**, 1267 (1968); see also W. M. Davis and S. G. Smith, *J. Pharm. Pharmacol.* **25**, 174 (1973).
13. Some animals were tested with the more standard catheter exit from the skin of the back [R. Pickens and T. Thompson, *J. Pharmacol. Exp. Ther.* **161**, 122 (1968)].
14. J. Maj, H. Sowinska, Z. Kapturkiewicz, J. Sarnek, *J. Pharm. Pharmacol.* **24**, 412 (1972); P. A. J. Janssen, C. G. E. Niemegeers, K. H. L. Schellebens, A. Dresse, F. M. Lenaerts, A. Pinchard, W. K. A. Schaper, J. M. Van Nueten, F. J. Verbruggen, *Arzneim.-Forsch.* **18**, 261 (1968).
15. M. C. Wilson and C. R. Schuster, *Psychopharmacologia* **31**, 291 (1973).
16. ———, *ibid.* **26**, 115 (1972).
17. Pimozide does not simply increase the rate of amphetamine metabolism [W. Soudijn and I. Van Wijngaarden, *J. Pharm. Pharmacol.* **24**, 773 (1972)].
18. L. M. Gunne, E. Anggard, L. E. Jonsson, *Psychiatr. Neurol. Neurochir.* **75**, 225 (1972).

19. Supported by the Canadian Non-Medical Use of Drugs Directorate. We thank Jane Stewart, Peter Milner, and Nancy Taylor for helpful comments and criticism. Pimozide was donated by Jannsen Pharmaceuticals, Beerse, Belgium; phentolamine, by Ciba Pharmaceuticals, Montreal; and *l*-propranolol, by Ayerst Laboratories, Montreal.

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Myogenic Defect in Acetylcholinesterase Regulation in Muscular Dystrophy of the Chicken

Abstract. *To determine whether inherited muscular dystrophy of the chicken is neurogenic or myogenic in origin, limb buds from homozygous normal and dystrophic chick embryos were exchanged prior to muscle differentiation and innervation. Biceps muscles of hatched chicks, in which muscle of the donor was innervated by nerves of the host, were analyzed for embryonic properties of muscle acetylcholinesterase and for fiber diameter, two distinctive markers for expression of the dystrophic gene. The results indicate that muscular dystrophy of the chicken is caused by an initial biochemical lesion in the limb and its muscle rather than in its innervating nerve.*

Recently there has been much interest in whether inherited muscle abnormalities, particularly the muscular dystrophies, are myogenic or neurogenic in origin (*1*). Inherited muscular dystrophy of the chicken is one of the models that has been studied to determine the nature of the primary biochemical defect and its cellular site of expression. This disorder is a progressive abnormality involving a single codominant gene, and affecting mainly fast twitch, glycolytic muscle fibers (*2, 3*). Many of the properties that are altered in dystrophic chick muscle are known to be regulated by neural activity. A good example is the enzyme acetylcholinesterase (AChE); embryonic properties of muscle AChE that disappear after hatching in normal muscle are maintained in dystrophic chick muscles and return with denervation but not tenotomy of normal chick muscles (*3*). This report presents evidence that nerves of genetically dystrophic chickens are capable of regulating muscle AChE and that dystrophic muscle cells lack the ability to respond normally to their nerves (*4*).

The experimental approach used was limb bud transplantation in which primordial limb regions were exchanged at an early embryonic age between genetically different embryos, producing muscles of one genotype innervated by nerves of another genotype (*5*). In the experiments reported here, right wing limb buds were removed from stage-19 to stage-20 embryos (3½ days of incubation) and replaced by limb buds of the same or different genotype. Normal limb buds were grafted onto normal hosts and dystrophic hosts; dystrophic limb buds were grafted onto normal hosts. Only birds with morphologically normal, healthy wings were used for analysis. Transplants were done before the motor nerve axons had reached the primordial limb tissue (*6*) so that muscles of the transplanted wings became innervated by neurons of the host and subject to the host's systemic regulation. Chicks were killed 5 to 14 weeks after hatching, and biceps muscles of the donor and host limbs were examined for AChE activity and muscle fiber diameter. In the 15 birds analyzed, AChE-positive motor end plates and spindle fibers were seen in all transplant muscles, no fiber degeneration was observed, and chicks could voluntarily contract muscles in their transplanted wings.

The strain of dystrophic chickens used in this study exhibits pronounced muscle fiber hypertrophy in afflicted muscles, making this parameter a useful marker for expression of the dystrophic gene (*7*). The AChE properties studied differ greatly between normal and dystrophic chick muscle. Adult dystrophic muscle maintains high levels, extrajunctional localization, and small molecular weight isozymes of AChE characteristic of embryonic muscles; normal muscle has low levels, no extrajunctional localization, and only a single high molecular weight isozyme of AChE (*2*).

The act of transplantation did not affect AChE activity or fiber diameters of either normal or dystrophic muscles. Both parameters were unchanged when normal muscles were transplanted to normal hosts (Table 1). When transplants were made between genetically different embryos, the transplanted muscles retained the properties of their origins and did not take on the characteristics of their hosts. Dystrophic muscle in a normal host had high levels of AChE activity and large muscle fiber diameters, and normal muscle in a dystrophic host had low AChE and normal muscle fiber diameters.

The cytochemical distributions of AChE and the number of AChE isozymes in transplanted muscles confirmed that normal and dystrophic muscle transplants retained the AChE properties characteristic of their genotypes (Fig. 1). In the birds studied, dystrophic muscle, whether of host or transplant origin, always had high extrajunctional AChE activity, and em-

Table 1. Acetylcholinesterase activity and fiber diameter of donor and host biceps muscles. Specific AChE activities in homogenates of biceps muscles from donor (right) and host (left) wings were determined as described (*2*), with the use of acetylthiocholine substrate and iso-OMPA (tetraisopropyl-pyrophosphoramide, Sigma, St. Louis, Missouri), an inhibitor of nonspecific cholinesterase. Values are averages, ± standard deviations. Numbers of birds and their ages are given in parentheses. Fiber diameter values, ± standard deviations, are averages from two birds; minimum diameters of 100 fibers in each biceps were measured in cryostat cross sections and combined for statistical analysis.

Combination	Limb	AChE activity*	Fiber diameter (μm)
Normal limb, normal host (4; 6 to 10 weeks)	Donor	1.50 ± 0.96	33.3 ± 9.3
	Host	1.03 ± 0.33	33.0 ± 7.3
Dystrophic limb, normal host (6; 6 to 14 weeks)	Donor	16.6 ± 13.1†	56.6 ± 15.2†
	Host	1.16 ± 0.29	27.0 ± 5.5
Normal limb, dystrophic host (5; 5 to 6 weeks)	Donor	2.06 ± 0.70†	25.6 ± 3.7†
	Host	13.1 ± 4.7	45.7 ± 11.1

* Acetylcholinesterase activity is the change in absorbancy at 412 mμ per minute per gram wet weight. † Significant differences ($P < .001$) between donor and host muscles, determined by Student's t distribution.

bryonic isozyme forms. Normal muscle, whether of host or transplant origin, had high AChE activity only at motor end plates, and a single isozyme form.

The results demonstrate that the defect in AChE regulation and the muscle fiber hypertrophy of dystrophic chick muscles are properties of the limb tissues, not of the nerves or systemic functions of the dystrophic animals. Most likely the lesion is in the muscle fibers themselves (although theoretically the experiments do not exclude expression of the gene in other limb tissues). Analysis of host and donor muscles for other properties which are altered in dystrophic chick muscle leads to the same conclusion (*8*).

Cosmos and Butler transplanted muscle minces between normal and dystrophic chicks after hatching (*9*). Although dystrophic muscle minces failed to regenerate well in normal hosts, normal muscle minces regenerated in dystrophic hosts and retained normal lipid distribution and succinic dehydrogenase activity. Because dystrophic muscles did not regenerate well, the results supported but did not prove that muscular dystrophy of the chicken was myogenic in origin. Cell culture studies have not revealed any clear-cut biochemical differences between normal and dystrophic chick embryo cells (*10*).

The myogenic and/or neurogenic origins of muscular dystrophies of mammals are unclear. Studies involving the degree and nature of innervation and transplants of muscles or muscle fragments between animals have had difficulty distinguishing between clear-cut expressions of the dystrophic genotypes and other effects such as those due to degeneration, regeneration, and incomplete or abnormal development of the muscles (*1*, *11*).

To date, the evidence indicates that dystrophy of the chicken is due to a defect in neurally mediated muscle maturation (*2*, *3*, *12*). The results of this study show that dystrophic muscle is unable to respond to neural influences that regulate the maturation of the muscle. It is still possible that nerves of the dystrophic chicken may become abnormal at a later time owing either directly to expression of the dystrophic gene or secondarily to action of the dystrophic muscle. The mechanisms of nerve-muscle interaction disrupted by the dystrophic gene remain to be determined. Perhaps they involve an inability of the muscle to respond to acetylcholine, or to contractile activity, or to other unknown "neurotrophic factors" proposed to be involved in neural regulation of muscle (*13*).

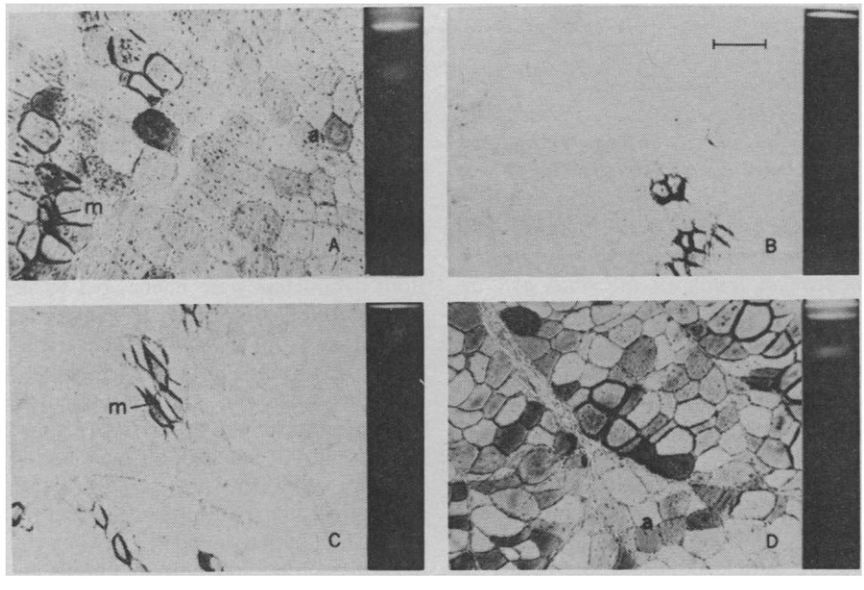

Fig. 1. Acetylcholinesterase localization and isozyme pattern of donor and host biceps muscles. Acetylcholinesterase activity appears as dark staining in cross section of muscle; as opaque, white bands in polyacrylamide gels. (A) Dystrophic limb transplanted onto a normal host, shows extrajunctional AChE localization (*a*) in many fibers and high activity at motor end plates (*m*). Three AChE isozyme forms are present. (B) Normal unoperated host limb has AChE activity only at motor end plates, and only one isozyme. (C) Normal limb transplanted onto a dystrophic host resembles the normal host limb. (D) Dystrophic unoperated host limb shows extrajunctional activity and three isozymes. Biceps muscles of normal limbs transplanted onto normal hosts (not shown) had AChE localization and isozyme patterns indistinguishable from those of normal host biceps. Acetylthiocholine substrate and iso-OMPA were used to demonstrate AChE activity, as described (*2*). Acetylcholinesterase isozymes were electrophoretically separated on 10 percent polyacrylamide gels, *p*H 8.5. Bar represents 100 μm. Electrophoretic migration is toward the cathode, bottom.

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References and Notes

1. L. P. Rowland, *Ann. N.Y. Acad. Sci.* **228**, 244 (1974); A. J. McComas, R. E. P. Sica, A. R. M. Upton, F. Petito, *ibid.*, p. 261; A. C. Peterson, *Nature (Lond.)* **248**, 561 (1974).
2. See B. W. Wilson, S. G. Linkhart, P. S. Nieberg, *J. Exp. Zool.* **186**, 187 (1973).
3. B. W. Wilson, M. A. Kaplan, W. C. Merhoff, S. S. Mori, *ibid.* **174**, 39 (1970).
4. Preliminary results have been presented at the 3rd International Conference on Isozymes (Yale University, April 1974) and at the 4th Annual Meeting of the Society for Neuroscience (St. Louis, Missouri, October 1974).
5. Eggs incubated at 37.5°C for 3½ days were laid on their sides and cooled to room temperature, and 1.5 ml of albumen was re-

moved. Through a 1-cm² window, right wing limb buds were removed and replaced by right wing limb buds from donor embryos; normal orientation was maintained. Cutting was done with fine glass needles. Grafted limbs were held in place by fine glass tacks and allowed to heal 2 to 3 hours under a warming lamp. Tacks were removed; 2.5 μg of amphotericin B and 50 units each of penicillin and streptomycin (Gibco, Grand Island, N.Y.) in Tyrode's solution were added per egg; the eggs were sealed with Parafilm and incubated on their sides to hatching. Approximately 10 percent of the operated embryos hatched. In the embryos which did not hatch the morphology of the transplanted limbs was normal.

6. G. Filogamo and G. Gabella, *Arch. Biol.* **78**, 9 (1967); L. Roncali, *Monit. Zool. Ital.* **4**, 81 (1970).
7. S. L. McMurty, L. M. Julian, V. S. Asmundson, *Arch. Pathol.* **94**, 217 (1972). Homozygous dystrophic embryos were from line 413, derived recently from line 304, maintained by the Department of Avian Sciences, University of California, Davis. Normal embryos were from a commercial White Leghorn flock, Donsing Hatcheries, Rio Linda, California.
8. T. A. Linkhart, G. W. Yee, B. W. Wilson, P. S. Nieberg, in preparation.
9. E. Cosmos and J. Butler, in *Research in Muscle Development and the Muscle Spindle*, B. Q. Banker, R. J. Przybylski, J. P. Van der Meulen, M. Victor, Eds. (Excerpta Medica, Amsterdam, 1972), p. 149.
10. V. Askanas, S. A. Shafiq, A. T. Milhorat, *Arch. Neurol.* **24**, 259 (1971); J. H. Peacock and P. G. Nelson, *J. Neurol. Neurosurg. Psychiatry* **36**, 389 (1973).
11. J. B. Harris and M. W. Marshall, *Exp. Neurol.* **41**, 331 (1973); W. B. Douglas and E. Cosmos, in *Exploratory Concepts in Muscle* (11), A. T. Milhorat, Ed. (Proceedings of the International Conference, Carefree, Arizona, 15 to 19 October 1973, Excerpta Medica, Amsterdam, in press).
12. C. R. Ashmore and L. Doerr, *Exp. Neurol.* **30**, 431 (1971); E. X. Albuquerque and J. E. Warnick, *Science* **172**, 1260 (1971); J. Jedrzejczyk, J. Wieckowski, T. Rymaszewska, E. A. Barnard, *ibid.* **180**, 406 (1973).
13. S. A. Cohen and G. D. Fischbach, *Science* **181**, 76 (1973); D. B. Drachman, *Ann. N.Y. Acad. Sci.* **228**, 160 (1974); E. X. Albuquerque, J. E. Warnick, F. M. Sansone, R. Onur, *ibid.*, p. 224.
14. Supported in part by PHS grant NS 01957 and by a grant from the Muscular Dystrophy Associations of America, Inc. T.A.L. is an NSF predoctoral trainee in physiology. We thank Dr. U. K. Abbott for her help and encouragement in development of the transplantation technique, Ms. P. S. Nieberg and Mr. F. Lantz for technical assistance, and Mr. J. Schenkel for his initial work in the study.

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Nitrogen Fixation in Marine Shipworms

Abstract. *Nitrogen fixation is associated with four shipworm species. A bacterium capable of fixing nitrogen under anaerobic conditions and of liquefying cellulose in culture has been isolated from the gut of one species. High fixation rates (up to 1.5 micrograms of nitrogen per milligram dry weight per hour), which resulted in a doubling of cellular nitrogen in as little as 1.4 days, was associated with* Teredora malleolus *from the Sargasso Sea. Three species from coastal waters were assayed, and of these juveniles showed the highest fixation rates. Nitrogen fixation activity appeared to be inversely related to the ability of shipworms to obtain combined-nitrogen compounds in their diet. It could be a significant source of nitrogen for shipworms and perhaps other oceanic organisms that ingest terrestrial plant material.*

Wood is notoriously low in combined-N compounds, and this presents a nutritional problem to any organism feeding on it. Woody plant tissue contains only 0.03 to 0.10 percent N, and the C : N ratio of most wood species is about 300 to 500 (*1*). In contrast, the C : N ratio of phytoplankton, the major food of herbivores in the sea, is about 5 to 10 (*2*).

Table 1. Nitrogen fixation rates, dry weights, and lengths of nine individuals of *Teredora malleolus* collected from a 30-m-long pine log in the Sargasso Sea, September 1973. Three of the shipworms assayed (17, 18, and 38 mm long) had no measurable N_2 fixation. On later inspection it was noted that these worms had been damaged on extraction from the log.

Dry weight (mg)	Length (mm)	N_2 fixation (μg mg^{-1} hour^{-1})
11.2	12	1.56
11.4	9	0.57
21.7	15	1.51
58.9	23	0.21
151.3	29	0.0003

Shipworms are bivalve mollusks (family Teredinidae), the adults of which are obligatory wood borers and cellulose metabolizers (*3*). They have a functional gill (ctenidium) capable of filtering plankton for food (*4*); however, shipworms have been raised to sexual maturity on wood in filtered seawater with no plankton available for feeding (*5*). Since cellulose constitutes a major portion of the shipworm diet, and juvenile shipworms can grow extremely rapidly [from 0.2 mm to 6 cm in length in 1 month (*6*)], how they obtain combined-N compounds sufficient for protein metabolism is puzzling. We present data showing that N_2 fixation is associated with shipworms and suggest that this process may contribute to the nitrogen metabolism of these organisms.

Nitrogen fixation was demonstrated in juveniles and adults of four shipworm species (Fig. 1, Table 1). The acetylene reduction technique (*7*) was used to assay for N_2 fixation (*8*). Three species, *Psiloteredo megotara, Lyrodus pedicellatus*, and *Teredo navalis*, were obtained from wood held in a running seawater system in Woods Hole, Massachusetts. The fourth species, *Teredora malleolus*, typically found inhabiting floating wood in offshore waters, was obtained from a pine log found floating at 32°18′N, 60°21′W in the Sargasso Sea in September 1973.

For the three coastal species, N_2 fixation was inversely related to shipworm dry weight (Fig. 1). All individuals weighing less than 2.0 mg and 65 percent of those weighing more than 10 mg had measurable N_2 fixation rates. The relation between shipworm body weight and rate of N_2 fixation fits an inverse hyperbolic curve. On a logarithmic plot (Fig. 1) the data exhibit a statistically significant linear relation ($P < .05$) with a correlation coefficient of .76. Juvenile shipworms had the highest N_2 fixation rates: one *L. pedicellatus* juvenile fixed up to 81 ng of N_2 per milligram dry weight per hour; and three *L. pedicellatus* and one *T. navalis* juveniles, each less than 600 μg (dry weight), averaged 31 ng mg^{-1} hour^{-1}. The only larvae tested, those of *L. pedicellatus*, had no associated N_2 fixation.

Adult *Teredora malleolus* from the Sargasso Sea had N_2 fixation rates of up to 1.5 μg mg^{-1} hour^{-1}, exceeding the fixation rates of coastal shipworms by a factor of almost 20 (Table 1). These data suggest an N_2 fixing system which assumes greater importance when there is a dietary deficiency of combined N, for example, in areas of low phytoplankton density like the Sargasso Sea (*9*), or when the animals

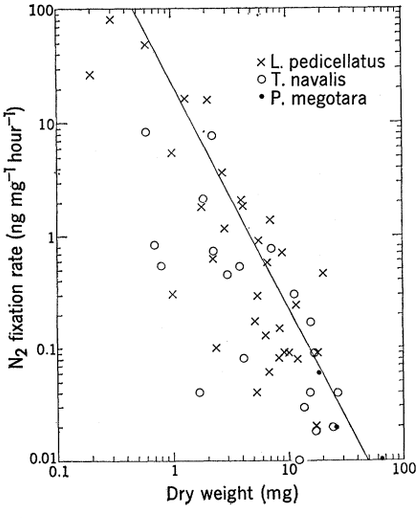

Fig. 1. Rate of nitrogen fixation associated with three coastal shipworm species.