tions are not precise enough to ascertain that there is a day-night rhythm for this catecholamine.

A 24-hour rhythm in norepinephrine turnover in nerves innervating the pineal gland probably reflects diurnal variations in the release of the neurotransmitter. The daily rhythm in stimulation by norepinephrine of β-adrenergic receptors on pineal cells appears to be responsible for the circadian cycle in pineal indoleamine metabolism. That the rhythmic changes in pineal indoleamines persist in blinded rats but can be abolished by interrupting nerve impulses from the brain to the superior cervical ganglia suggests the presence of a "clock" in the central nervous system of the rat. Recent work (*21*) suggests that this clock resides in the suprachiasmatic nucleus of the hypothalamus.

MICHAEL BROWNSTEIN
JULIUS AXELROD
Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014

References and Notes

1. W. B. Quay, *Gen. Comp. Endocrinol.* **3**, 473 (1963).
2. D. C. Klein and I. L. Weller, *Exerpta Med. Int. Congr. Ser. No. 256* (1972), p. 52.
3. H. J. Lynch, *Life Sci.* **10**, 791 (1971).
4. D. C. Klein and J. L. Weller, *Science* **169**, 1093 (1970).
5. M. J. Brownstein, R. Holz, J. Axelrod, *J. Pharmacol. Exp. Ther.* **186**, 109 (1973).
6. M. J. Brownstein, J. M. Saavedra, J. Axelrod, *Mol. Pharmacol.* **9**, 605 (1973).
7. S. H. Snyder, M. Zweig, J. Axelrod, J. E. Fischer, *Proc. Natl. Acad. Sci. U.S.A.* **53**, 301 (1965).
8. D. C. Klein, R. J. Reiter, J. L. Weller, *Endocrinology* **89**, 1020 (1971).
9. J. A. Kappers, *Z. Zellforsch. Mikrosk. Anat.* **52**, 163 (1961).
10. S. H. Snyder, M. Zweig, J. Axelrod, *Life Sci.* **3**, 1175 (1964).
11. D. C. Klein, J. L. Weller, R. Y. Moore, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3107 (1971).
12. T. Deguchi and J. Axelrod, *ibid.* **69**, 2208 (1972).
13. ———, *ibid.*, p. 2547.
14. P. H. Volkman and A. Heller, *Science* **173**, 839 (1971).
15. R. J. Wurtman and J. Axelrod, *Life Sci.* **5**, 665 (1966).
16. ———, G. Sedvall, R. Y. Moore, *J. Pharmacol. Exp. Ther.* **157**, 487 (1967).
17. One week after bilateral superior cervical ganglionectomies were performed, rats were injected with [^{3}H]norepinephrine and killed 5 minutes later. Sham-operated rats were treated in an identical manner.
18. L. G. Whitby, J. Axelrod, H. Weil-Malherbe, *J. Pharmacol. Exp. Ther.* **132**, 193 (1961).
19. D. C. Klein and J. L. Weller, *Science* **177**, 532 (1972).
20. J. T. Coyle and D. Henry, *J. Neurochem.* **21**, 61 (1973).
21. R. Y. Moore and D. Klein, unpublished observations; M. Brownstein and J. Axelrod, unpublished observations.
22. M. J. Brownstein, thesis, University of Chicago (1971).
23. We thank H. Hunt for excellent technical assistance and T. Deguchi for sharing with us his unpublished observations and useful criticism. M.B. is a research associate in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences.

22 June 1973; revised 3 December 1973

Erythrocyte Deformation in Human Muscular Dystrophy

Abstract. *Erythrocytes from patients with congenital muscular dystrophy exhibit dramatic surface deformation when observed with a scanning electron microscope. A similar alteration, but one affecting a smaller proportion of cells, occurs in the case of female carriers of the sex-linked Duchenne dystrophic condition. These observed changes in the erythrocyte surface may reflect a systemic defect in membrane properties.*

The fundamental lesion underlying congenital muscular dystrophy has been variously suggested to reflect a purely myopathic (*1*), neuronal (*2*), vascular (*3*), or autoimmune (*4*) mechanism. Investigations of the congenital disease in the laboratory mouse have provided evidence that effects of the lesion are systemic and are, moreover, discernible in altered membrane properties, including permeability to cations by liver mitochondria (*5*) and structural irregularities in the surface of erythrocytes (*6*). It is possible to question the comparability of the mouse disease, which is autosomal, to human Duchenne muscular dystrophy, which is sex-linked (*7*); it is therefore desirable to examine the human disease, as well, for signs of possible membrane alteration. We report here the results of a scanning electron microscopic examination of erythrocytes from patients suffering from several categories of human muscular dystrophy as well as erythrocytes obtained from carriers of the sex-linked (Duchenne) form.

Samples of blood were donated at the muscular dystrophy clinic at the Milton S. Hershey Medical Center by patients, their normal siblings and parents, and laboratory personnel. Blood was obtained by a finger stab and drawn into a heparinized capillary tube. A 10-μl portion was diluted tenfold in cold 0.9 percent NaCl and centrifuged at 900*g* for 3 minutes. Sedimented cells were resuspended in 100 μl of the same medium and centrifuged again. The sedimented cells were suspended in 3 percent glutaraldehyde containing 10 m*M* sodium cacodylate buffer (*p*H 7.4) and incubated for 2 hours at 22°C. Cells were then centrifuged, washed in 0.9 percent NaCl, and dehydrated in 70 percent ethanol and, after 5 minutes, 95 percent ethanol. Cells were finally diluted with 95 percent ethanol, spread

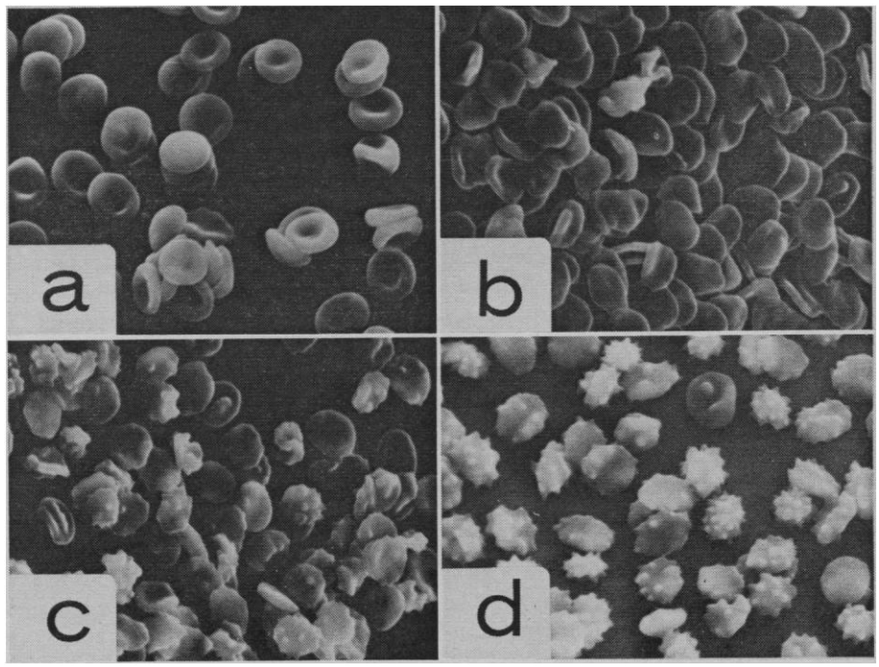

Fig. 1. Scanning electron micrographs of erythrocytes from normal subjects, patients with Duchenne muscular dystrophy, and probable carriers of Duchenne muscular dystrophy (×1500). Preparation of cells is described in the text. (a) Cells from normal subject; no saline wash. (b) Cells from normal subject; twice washed. (c) Cells from probable Duchenne carrier; twice washed. (d) Cells from Duchenne patient; twice washed.

on glass microscope slides, and dried in air. (In other samples, critical-point drying was employed, and this had no effect on the observations.) The slides were cut into 1-cm squares and glued to aluminum stubs, shadowed to a depth of 200 Å with gold-palladium (60 percent–40 percent), and examined in an AMR-900 scanning electron microscope at 21 kv and 30° tilt. Three areas on each stub were selected at random and photographed at a magnification of about 600, and cell counts were made directly from such photographs. In some instances, the two initial washings with NaCl were omitted (Fig. 1a) and the cells were immediately fixed in glutaraldehyde, dehydrated, and coated as described above.

Figure 1 illustrates the extensive modification seen in erythrocytes from the circulating blood of Duchenne dystrophic patients and carriers. Figure 1a shows the biconcave character of normal erythrocytes which have not been subjected to a saline wash. After two such washes, cells from normal individuals become somewhat flattened but still maintain their disklike shape (Fig. 1b). In contrast, a high percentage of cells from individuals with Duchenne muscular dystrophy are drastically deformed with many surface projections. This "echinocyte" configuration (*8*) is also observed with unwashed cells but is more pronounced in cells that have been subjected to the stress of saline washing (Fig. 1d). In such washed cell samples from dystrophic patients, the number of distorted cells approaches 100 percent, while samples from normal individuals contain no more than about 7 percent.

Table 1 is a summary of the results of scanning electron microscopic examination of blood from normal individuals, probable carriers, and patients exhibiting several clinical categories of dystrophy. In all instances, clinical dystrophy is associated with an elevated proportion of distorted cells. Since the distortion is visible under a light microscope when unfixed cells are diluted with saline solution and examined at a magnification of 450, alterations in the surface do not appear to reflect damage from fixation or coating. The higher resolution obtainable with scanning electron optics permits detection of intermediate degrees of distortion and thus a more accurate measure of the proportion of altered cells.

Reliable identification of carriers of the Duchenne form of dystrophy has proved difficult. Thus, it is of interest that deformed erythrocytes are also observed in the blood of probable carriers, being present in a proportion generally intermediate between that of dystrophic patients and that of normal individuals (see Table 1 and Fig. 1c). Mothers of dystrophic offspring who are likely carriers are identified on the basis of a history of the disease in the family and elevated serum concentrations of such enzymes as creatine phosphokinase (CPK). One mother of a dystrophic child (not included in Table 1) was found to have normal erythrocytes; she had neither a familial history of the disease nor elevated CPK activity. The occurrence of dystrophy in her child is thus probably the result of mutation, such events giving rise to about one-third of Duchenne cases.

Table 1. Erythrocyte distortion in several categories of muscular dystrophy. Cells were prepared and counted as described in the text. Only subjects who could be unambiguously placed in the several categories were included here.

Age of donor	Sex	Cells	
		Counted (No.)	Distorted (%)
	Normal donors		
43	Male	640	3.4
45	Male	773	3.3
36	Male	605	7.4
32	Female	544	4.0
20	Female	752	3.2
	Dystrophic (Duchenne)		
12	Male	686	65.4
15	Male	191	98.4
14	Male	590	40.6
1	Male	431	20.6
	Dystrophic (limb-girdle)		
54	Female	708	17.3
51	Female	314	26.7
	Dystrophic (facioscapulohumeral)		
34	Male	581	17.5
12	Male	356	36.5
47	Female	838	27.3
	Carriers (Duchenne)		
32	Female	686	35.1
37	Female	901	39.9
32	Female	600	34.0

Thus, it appears that the erythrocyte surface is significantly altered in cases of human muscular dystrophy, an observation that both strengthens the analogy between the human and murine dystrophic condition and supports the view that the diseases are associated with systemic changes in membranes. The observed red cell alterations are similar to those associated with a number of clinical conditions, including severe liver disease, splenectomy, uremia, abnormal hemoglobin (beta thalassemia), and congenital deficiency of serum beta lipoprotein (*8*). In no instance is there evidence of a correlation between the presence of any of these conditions and congenital muscular dystrophy (*9*).

The morphological changes described here are similar to those observed in vitro when normal cells are incubated with fatty acids or lysophosphatides (*8*). Red cell membranes from patients with muscular dystrophy have been shown to differ from normal ones with respect to fatty acid and phospholipid composition (*10*), with, for example, an increase in sphingomyelin. Thus, it is likely that the observed morphological changes reflect alterations in the lipid component of the membrane.

Finally, since treatment of normal erythrocytes with calcium at a high *p*H leads to similar transformed surface morphology (*11*), and since we have already observed altered cation movement in liver mitochondria from dystrophic mice (*5*), we are attracted to the possibility that changes in membrane lipid produce an abnormal pattern of cation distribution which, in turn, is responsible for the dramatic membrane distortion described here.

DALE W. MATHESON*
Department of Anatomy, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey 17033

JOHN L. HOWLAND
Committee on Biochemistry, Bowdoin College, Brunswick, Maine 04011

References and Notes

1. J. N. Walton,, *Br. Med. J.* **1**, 1271 (1964); W. West and E. D. Murphy, *Anat. Rev.* **137**, 279 (1960).
2. B. Gallup and V. Dubowitz, *Nature (Lond.)* **243**, 287 (1973).
3. P. W. Hathaway, W. K. Engel, H. Zellweger, *Arch. Neurol.* **22**, 365 (1970).
4. G. Jasmin and F. Bokdawala, *Rev. Can. Biol.* **29**, 197 (1970).
5. J. L. Howland and M. D. Challberg, *Biochem. Biophys. Res. Commun.* **50**, 574 (1973).
6. P. F. Morse and J. L. Howland, *Nature (Lond.)* **245**, 156 (1973).
7. A. M. Michelson, E. S. Russell, P. J. Harman, *Proc. Natl. Acad. Sci. U.S.A.* **41**, 1079 (1955).
8. G. Brecher, J. E. Haley, R. O. Wallerstein, in *Red Cell Shape*, M. Bessis, R. I. Weed, P. F. Leblond, Eds. (Springer-Verlag, New York, 1973), p. 31.
9. K. L. Zierler, in *Biochemical Disorders in Human Diseases*, R. H. S. Thompson and I. D. P. Wootton, Eds. (Academic Press, New York, ed. 3, 1970), p. 489.
10. D. Kunze, G. Reichmann, E. Egger, G. Leuschner, H. Eckhardt, *Clin. Chim. Acta* **43**, 333 (1973).
11. R. I. Weed and B. Chailley, in *Red Cell Shape*, M. Bessis, R. I. Weed, P. F. Leblond, Eds. (Springer-Verlag, New York, 1973), p. 55.
12. Supported by NIH grant GM 17955 and a grant from the Muscular Dystrophy Associations of America to J.L.H., who is also recipient of PHS research career development award 5 K04 GM 11854. We thank those who donated blood for their cooperation, D. C. Berlin for assistance in obtaining blood samples, and V. Mumaw for assistance in operating the scanning microscope. Correspondence should be addressed to J.L.H.
* Present address: Litton Bionetics, Kensington, Maryland 20795.

19 November 1973; revised 8 January 1974