best. However to offset this possible advantage, diazepam administration definitely acts to maintain the tendency to select alcohol. Such a result, if confirmed at the clinical level, has important implications for the pharmacological treatment of alcoholism. Diazepam is a drug which is very widely prescribed, often for alcoholism, which has assumed major proportions as a health hazard in this country. The use of diazepam to treat human alcoholism may be counterproductive.

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References and Notes

1. J. L. Mottin, *Q. J. Stud. Alcohol* **34**, 444 (1973).
2. K. S. Ditman, *Q. J. Stud. Alcohol Suppl.* **1**, 107 (1961); E. C. Hoff, *ibid.*, p. 138; B. Kissin and M. M. Gross, *Am. J. Psychiatry* **125**, 31 (1968); B. Kissin and A. Platz, *Public Health Serv. Publ. 1836* (1968), p. 835.
3. J. A. Deutsch and W. T. Hardy, *Behav. Biol.* **17**, 379 (1976).
4. J. A. Deutsch and N. Y. Walton, *ibid.* **19**, 349 (1977).
5. J. A. Deutsch and H. S. Koopmans, *Science* **179**, 1242 (1973).
6. The mean effective dose (ED_{50}) for diazepam in several behavioral measures is 5 mg/kg for the rat [L. O. Randall and B. Kappell, in *The Benzodiazepines*, S. Garattini and E. Mussini, Eds. (Raven, New York, 1973), pp. 27–51].
7. P. Soubrie, P. Simon, J. R. Boissier, Jr., *Experientia* **32**, 359 (1976).
8. Supported PHS grant AA000302 to J.A.D. while N.Y.W. was a trainee on NIAAA grant AA07129-01. We thank Dr. J. O. Miller for statistical advice and Hoffmann–La Roche, Inc., for the gift of diazepam (Valium).

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Erythrocyte Lipids in Heterozygous Carriers of Duchenne Muscular Dystrophy

Abstract. *Erythrocyte membranes from heterozygous carriers of Duchenne muscular dystrophy exhibit a diminished amount of palmitoleic acid when compared to membranes from normal subjects. A similar, but more variable, diminution is observed in the case of patients with this disorder. The change in fatty acid composition appears related to a low membrane triglyceride content and may provide both a possible technique for carrier detection and a clue regarding pathogenesis.*

Muscular dystrophy of the Duchenne type is transmitted in a recessive, sex-linked fashion, and the majority of affected children are borne by mothers who are heterozygous carriers. Rational genetic counseling requires reliable detection of such individuals, and the most commonly employed criterion, increased concentrations of creatine phosphokinase (E.C. 2.7.3.2) in the serum, fails to identify about 30 percent of the carriers examined (*1*). Therefore, alternative methods for detecting carriers are required. Moreover, the occurrence of a heterozygous dystrophic condition, in which the phenotype is generally functionally normal, allows the study of the pathogenesis of the disease under conditions that avoid a background of general deterioration of physical condition and abortive regeneration of muscle tissue. Here we report that the erythrocyte membrane lipids are altered in heterozygous individuals as well as in their hemizygous, affected offspring.

We have reported the occurrence of structural abnormalities in erythrocytes from mice and humans with various forms of muscular dystrophy (*2*). This alteration, an increased proportion of echinocytes, was also observed in several carriers of Duchenne dystrophy, suggesting that it might be used in the detection of carriers. Although the observations were confirmed in other laboratories (*3*), it became apparent that cell shape is too variable to permit reliable diagnosis or carrier detection (*4*).

On the basis of these and other abnormalities, we suggested that muscular dystrophies might be associated with a systemic membrane defect (*5*), and a similar suggestion was made by Appel and associates [see (*6*)], who reported that membrane protein kinase activity and membrane fluidity were altered in erythrocytes. A number of other abnormalities of the erythrocyte membrane have been observed in patients and carriers, and some of them have been suggested as having possible diagnostic utility. For instance, Percy and Miller (*7*) demonstrated decreased membrane deformability in cells obtained from patients and carriers of Duchenne muscular dystrophy. It appeared likely that such a varied collection of membrane alterations reflected some abnormality in membrane composition. If the abnormality were closely related to the primary genetic defect, then its identification would be of importance, both because it would provide information about the enzymic basis of the disease, and because it might be a more reliable measure of genotype than its later consequences, thus providing a carrier test of greater certainty.

Since a number of the membrane alterations noted above might reflect conditions in the lipid phase of membranes, we searched for changes in the lipid composition of erythrocyte membranes obtained from normal individuals, carriers, and patients. Kalofoutis *et al.* (*8*) reported that the concentrations of the major

Table 1. Total fatty acids of erythrocyte ghosts from patients with Duchenne muscular dystrophy, heterozygous carriers, and normal subjects. For this report, carriers are defined as mothers of a dystrophic son who have, in their family, in addition, at least one occurrence of dystrophy in their own or the preceding generation. The fatty acids listed are, with the exception of palmitoleic, those present in highest concentrations; 18 others were measured but they showed no significant differences between normal, carrier, and patient populations. The ghosts were prepared from washed erythrocytes by osmotic lysis in a medium containing 10 m*M* tris buffer, *p*H 7.4, and 1 m*M* $MgCl_2$. The cells were washed three times in isotonic saline; in other experiments it was evident that variations in the number of washes (from two to five) did not affect the results obtained. The ghosts were washed and suspended in distilled water at a concentration of about 4 mg of protein per milliliter and twice extracted with eight volumes of a mixture of chloroform and methanol (2 : 1). The chloroform phases were pooled and evaporated to dryness under nitrogen. The extract was incubated at 70°C for 30 minutes in 3 percent KOH in methanol; this was again extracted with petroleum ether, and the residue was acidified with HCl. The fatty acids were extracted with petroleum ether and their methyl esters prepared by incubation at 70°C for 30 minutes with borontrifluoride-methanol (Sigma). The esters were extracted with petroleum ether and evaporated to dryness under nitrogen. Methyl esters of fatty acids were estimated using a Perkin-Elmer model 3920 gas chromatograph equipped with 12 percent diethylene glycol succinate (DEGS) dual columns and a temperature program from 150° to 220°C at 2° per minute. Peaks were assigned through comparison with known standards and measured either manually (height and width) or with a CSI computing integrator. The number of carbon atoms relative to the number of double bonds is indicated beside each acid.

Fatty acid	Total fatty acids (%)		
	Normal (N = 16)	Carrier (N = 10)	Patient (N = 17)
Palmitic (16 : 0)	26.9 ± 1.6	26.0 ± 1.8	25.5 ± 1.2
Palmitoleic (16 : 1)	4.9 ± 1.1	1.4 ± 0.1*	1.2 ± 0.4*
Stearic (18 : 0)	13.9 ± 1.1	16.0 ± 2.3	17.9 ± 1.3
Oleic (18 : 1)	17.7 ± 0.7	15.7 ± 1.2	15.2 ± 0.8
Linoleic (18 : 2)	11.7 ± 1.0	12.5 ± 1.2	10.1 ± 0.7
Arachidonic (20 : 4)	10.0 ± 1.2	11.0 ± 0.8	10.6 ± 1.1

*Significant to $P < .005$ when compared with normal; carriers and patients did not differ significantly.

phospholipids and of cholesterol were within normal limits in cells from Duchenne patients. We carried out similar measurements and our results concurred with their findings, indicating that the major lipid classes in the erythrocyte membrane do not account for the membrane disorders described.

Therefore, we examined the total fatty acid composition of erythrocyte ghosts as a possible means of screening for an altered level of some other complex lipid. Our results (see Table 1) showed that, of the 24 fatty acids that we measured, only one was significantly altered ($P < .005$). This was palmitoleic acid, the 16-carbon acid with a single, Δ9-double bond. In no instance did we observe an overlap in the range of 16 carbon atoms and one double bond between the population of carriers (or patients) and normal subjects. It is noteworthy that two of our subjects had been identified as carriers by genetic criteria, although they exhibited serum creatine phosphokinase activities within the normal range. Thus, in this small sample of carriers, fatty acid composition appears more closely correlated with the carrier state than increased serum creatine phosphokinase activity. The diminution of palmitoleic acid was observed also in patients with the Becker form of the disease which is characterized by its late onset. It was not observed in muscle disorders of a purely neurogenic origin (for example, spinal muscular atrophies), while in three cases of limb girdle dystrophy, the palmitoleic acid content was increased about sixfold above the normal level. Thus, alteration in fatty acid composition appears specific for particular forms of muscular dystrophy and does not reflect generalized changes common to the entire class of these diseases.

It is possible to attribute the decline in palmitoleic acid to a decrease in concentration of some complex lipid particularly rich in it. This approach is simplified by the rarity of palmitoleic acid in most complex lipids, with most phospholipids, for instance, containing less than 1 percent of the fatty acid (*9*). Indeed, only such neutral lipids as intracellular cholesterol esters (*10*) and membrane-bound triglycerides contain a substantial amount of this compound (*9*). Since the cholesterol esters appear not to occur in erythrocyte ghosts (*11*), we directed our attention to acylglycerides and found a substantially decreased triglyceride content in membranes from Duchenne carrier red cells (Table 2). The decline in palmitoleic acid cannot be entirely the result of a lower triglyceride content because triglycerides constitute less than 1 percent of total membrane lipid, and contain no more than 10 percent of palmitoleic acid. Instead, we suggest that the fatty acid alteration reflects both a net loss of membrane triglyceride and a consequent inability of the triglyceride to serve as a conduit of fatty acids to other membrane lipids. It should be added that the triglyceride that remains in carrier membranes exhibits an altered fatty acid composition with, for example, less palmitoleic acid. Thus, it would appear to originate in part from a metabolic source different from that in normal membranes.

Table 2. Membrane triglycerides of erythrocyte ghosts. Preparation of ghosts and initial lipid extraction was carried out as described in Table 1. Neutral lipids were separated by thin-layer chromatography on activated silica gel G with a solvent containing benzene, ethyl acetate, diethyl ether, and acetic acid (80 : 10 : 10 : 0.2 by volume). The triglyceride region was extracted twice with chloroform and evaporated to dryness under nitrogen. Triglycerides were estimated by gas chromatography on columns packed with Chromosorb W, with temperatures programmed to increase from 230° to 300°C at 4° per minute. Tripalmitin was used as standard for both thin-layer and gas chromatography.

Subject	Membrane triglycerides (mole/mg of protein)	
	Mean	Range
Normal ($N = 7$)	1.54 ± 0.4	1.23 to 2.13
Carrier ($N = 6$)	0.40 ± 0.2*	0.15 to 0.90
Patient ($N = 6$)	1.00 ± 0.5	0.05 to 2.94

*Significant to $P < .05$.

The data in Table 2 emphasize the utility of studies of heterozygous carriers. In the case of Duchenne patients, the mean triglyceride content was intermediate between those of carriers and normal subjects, with a range extending from well below carrier values to some high level in the normal range. Similarly, Table 1 shows that the palmitoleic acid content does not differ substantially between carriers and patients and that, with the patients, there is a considerably elevated standard error. In the cases of both triglyceride and fatty acid content, it is likely that the increased variability in membrane preparations from patients, as well as the failure of the values to conform to conventional gene-dosage predictions, reflects variability in the degree of such secondary features of the disease as fat cell invasion of muscle tissue and consequent alteration in systemic lipid metabolism. Such problems are not encountered in most heterozygous carriers.

In view of the specific alterations in lipid composition reported here, we suggest that membrane triglyceride metabolism is defective in Duchenne muscular dystrophy and that the several membrane abnormalities observed in erythrocytes from carriers and patients (*2, 3, 8*) may reflect that defect. We cannot say with any certainty that the defect in triglyceride metabolism is the primary enzymic lesion in this disease, but the following considerations suggest that such might indeed be the case. (i) The defect, as revealed by fatty acid composition, is specific for the Duchenne sort of dystrophy and quite distinct fatty acid profiles are observed with membranes from subjects with other categories of dystrophy or myopathy. (ii) The lipid changes are observed in heterozygous carriers, where secondary effects of muscle degeneration, fat cell invasion, and generalized physical wasting are minimal. (iii) While these diseases produce systemic effects, their primary influence is on muscle, and any hypothesis concerning pathogenesis should have the capacity to explain this tissue selectivity. It is relevant, therefore, that the sarcolemma is distinctive in its high content of membrane triglycerides, which account for over 15 percent of all lipids (*12*), compared with less than 1 percent for many other cell types. Thus, a presumed defect in membrane triglyceride metabolism would necessarily produce more grave results in muscle than in cells where such compounds are present at much lower concentrations.

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References and Notes

1. J. N. Walton and D. Garner-Medwin, in *Disorders of Voluntary Muscle*, J. N. Walton, Ed. (Churchill Livingstone, London, 1974), p. 573.
2. P. F. Morse and J. L. Howland, *Nature (London)* **245**, 156 (1973); D. W. Matheson and J. L. Howland, *Science* **184**, 165 (1974).
3. E. J. Lumb and A. E. H. Emery, *Br. Med. J.* **1975**, 467 (August 1975); K. F. Howells, *Res. Exp. Med.* **168**, 213 (1976).
4. T. D. Miale, J. L. Frias, D. L. Lawson, *Science* **187**, 453 (1975); D. W. Matheson, W. K. Engle, E. C. Derrer, *Neurology* **26**, 1182 (1976).
5. J. L. Howland, *Nature (London)* **251**, 724 (1974).
6. A. D. Roses and S. H. Appel, *ibid.* **250**, 245 (1974).
7. A. K. Percy and M. E. Miller, *ibid.* **258**, 147 (1975).
8. A. Kalofoutis, G. Jullien, V. Spanos, *Clin. Chim. Acta* **74**, 85 (1977).
9. R. P. van Hoeven *et al.*, *Biochim. Biophys. Acta* **380**, 1 (1975).
10. M. S. Brown and J. L. Goldstein, *Science* **191**, 150 (1976).
11. G. L. Nelson, in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*, G. J. Nelson, Ed. (Wiley-Interscience, New York, 1972), p. 323.
12. W. Fiehn and J. B. Peter, *J. Biol. Chem.* **246**, 5617 (1971).
13. We thank A. Sherblom and D. McAlister for advice on the manuscript and B. Hess and M. Macul for skillful technical assistance. The investigation was supported by the Muscular Dystrophy Association of America and NIH grant NS/GM 11815.

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