THE ERYTHROCYTE MEMBRANE IN HUMAN MUSCULAR DYSTROPHY

PROEFSCHRIFT

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VOORWOORD

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ENZYME LIST

Name used in this thesis	Enzyme Commission number	Comments on specificity
Acetylcholinesterase	3.1.1.7	-
Adenylate cyclase	4.6.1.1	-
Aldolase	4.1.2.13	fructose-1,6-bisphosphate triosephosphate-lyase
Ca^{2+}-ATPase	3.6.1.3	Ca^{2+}-stimulated, Mg^{2+}-dependent adenosinetriphosphatase
$(Ca^{2+} + Na^{+}/K^{+})$-ATPase	3.6.1.3	Ca^{2+} plus Na^{+}/K^{+}-stimulated, Mg^{2+}-dependent adenosine-triphosphatase
Ca^{2+}-PNPPase	3.1.3.41	Ca^{2+}-stimulated *p*-nitrophenyl-phosphatase
Carnitine palmitoyltransferase	2.3.1.21	-
Creatine phosphokinase	2.7.3.2	-
K^{+}-PNPPase	3.1.3.41	K^{+}-stimulated *p*-nitrophenyl phosphatase
Long chain acyl-CoA synthetase	6.2.1.3	-
Mg^{2+}-ATPase	3.6.1.3	Mg^{2+}-stimulated adenosine-triphosphatase
$(Na^{+} + K^{+})$-ATPase	3.6.1.3	Na^{+} plus K^{+}-stimulated, Mg^{2+}-dependent adenosinetriphosphatase
Neuraminidase	3.2.1.18	-
Phosphoglycerate kinase	2.7.2.3	-
Phosphoglycerate mutase	5.4.2.1	monophosphoglycerate mutase
Phospholipase A_2	3.1.1.4	phosphatide 2-acyl-hydrolase
Phospholipase C	3.1.4.3	hydrolysing glycerol-phosphate bond of phospholipids
Phosphorylase	2.4.1.1	-
Pronase	3.4.24.4	contains trypsin
Protein kinase	2.7.1.37	-
Protein phosphatase	3.1.3.16	phosphoprotein phosphatase
Transglutaminase	6.3.2.-	cross-linking proteins by γ-glutamyl-ε-lysine bonds
Trypsin	3.4.21.4	-

CHAPTER I

INTRODUCTION

1.1. *Muscular dystrophies*

More than 250 different forms of human neuromuscular diseases are known. They differ in age of onset, severity of weakness, rate of progression, type of inheritance, groups of muscles affected, frequency of incidence. Sometimes the clinical symptoms are not restricted to nervous and/or muscular tissue. The *muscular dystrophies* form an extensive subdivision of the neuromuscular diseases. This group of disorders is inheritable and it is accompanied by progressive weakness and degeneration of skeletal muscle. The aetiology is not known. The most progressive type of the dystrophies is the Duchenne form, first described in detail by Duchenne de Boulogne in 1868 (83). Another representative, with several dissimilarities with the Duchenne type, is the myotonic dystrophy, also called dystrophia myotonica, myotonia atrophica or Steinert's disease. This disorder shows, apart from dystrophic characteristics, myotonic symptoms, i.e. there exists a delay in relaxation of the affected muscles after contraction. The myotonic and Duchenne types are the most frequently occurring forms of human muscular dystrophy. These types of muscular dystrophy have been the subject of this thesis, and details of the disorders will be given first.

1.2. *Clinical aspects of Duchenne muscular dystrophy* (18,131,224)

The mode of inheritance of the Duchenne disorder is X-chromosomal and recessive and isolated cases have been described (see also 249,280,389). Thus, boys carrying the gene are affected, and girls carrying the gene are

asymptomatic, but transmit the disease. Some cases of girls with the clinical symptoms of Duchenne dystrophy have been reported (79,116,146,240). Estimations of the rate of incidence vary between 2 and 4 cases per 10,000 male births (see 38,66,145,349).

The most clear sign in the first stage of the disease is the drastically increased activity of enzymes in the blood. Schapira and Schapira (299) first described a high aldolase activity and Ebashi et al. (85) a high creatine phosphokinase activity in Duchenne sera. These and other enzymes, originating from the cytosol of skeletal muscles, also have a high activity in serum (see 131).

At the end of the second year of life the muscles of the pelvic girdle and calves are weakened, resulting in difficulties in rising from the floor, climbing stairs, running and hopping. The patients frequently fall. They have a waddling gait and a pathological lordosis. Later, the proximal muscles of the arms become affected. Contractures and deformities of the extremities are often observed. The proximal skeletal muscles are affected in an earlier stage than the distal ones. The facial muscles remain intact during most stages except the final one of the disorder. Pseudohypertrophy of the calf muscles is seen in most patients, owing to abundant connective and adipose tissue in the wasting muscle. This can be clearly seen in microscopic preparations, in which also degenerating and regenerating fibres and macrophage infiltrations can be observed (37,205). The fibre size varies too much and central nuclei are present. Fibre type differentiation is not very pronounced, type I (oxidative) fibres may be predominant (80). Electron microscopic studies reveal many abnormalities of the intracellular organization. Different stages can be distinguished, also in a single biopsy (64). The Z-lines are often discontinuous, the volume of mitochondria is decreased, that of the cytoplasm increased. Later, the myofibrils are more disorganized and disappear

in some areas. Other areas contain overcontracted sarcomeres. The characteristic striations have disappeared or make uncommon curves. Undulation of the sarcolemma has been observed. An increase of glycogen granulae, lipid droplets and lysosomes takes place. Later stages show clumps of contracted material, which condense more and more. Fibres with structureless cytoplasm can be seen. The contractile apparatus is absent and mitochondria degenerate. The earliest detectable alteration is not well-defined. Mokri and Engel (221) reported focal lesions in Duchenne skeletal muscular plasma membranes in only mildly affected fibres, while Schotland *et al.* (312) reported less protein particles in these membranes. For detailed description of electron-microscopic alterations, see Stern *et al.* (338) and Markesbery (205). Most of the morphological alterations are not very specific for one neuromuscular disease.

Apart from the skeletal muscles, cardiac and smooth musculature may also be involved in Duchenne dystrophy (14,60,132,208,291). Mental retardation, already present in early stages of the disorders, has been described, but may be absent (102). The electromyographic pattern in Duchenne dystrophy is comparable with that in other myopathies. Involvement of the nervous system has been postulated by McComas and coworkers (200,326), but was rejected by others (15, see also 283).

The progression of the disease is rapid. The use of a wheelchair at about 10 years is often necessary. Later follows immobilisation in bed, and as no therapy exists for the muscular dystrophy hitherto (see 119) death usually occurs at the end of the second or beginning of the third decade. The cause of death is often pneumonia and/or heart failure.

As a rule the female carriers of the Duchenne gene do not show clinical symptoms. Abnormalities have been found in electromyographic, histological and electron-microscopic

studies. Furthermore in 60-80% of the carriers high levels of muscle enzyme activity are found in the blood, but they are much lower than those of their affected sons (see 372).

1.3. *Clinical aspects of myotonic muscular dystrophy* (224,390)

Myotonic dystrophy is inherited in an autosomal dominant trait. Several symptoms have been reported in this type of dystrophy. Muscles are affected in a characteristic pattern, being the facial, pharyngeal and neck muscles and the distal muscles of the extremities. Affected muscles become weak and atrophic, and also show myotonia. In addition to skeletal musculature, heart muscles and smooth muscles of the gastrointestinal tract may also be affected. In some cases the cardiac myofibres are degenerated; arrhythmia and electrocardiographic abnormalities are common (106). Involvement of eyes, scalp, bones, testes and brains is often observed. A low level of immunoglobulin G (269) and hyperinsulinism (see 225) have been reported (see 5.3.). Easily recognizable symptoms in this multi-systemic disease are facial paresis, open mouth, frontal baldness, cataracts, defective speech, difficult swallowing, infertility. As the expressivity of the mutant gene varies, the relative severity of these symptoms greatly varies from patient to patient and also between close relatives.

Characteristic in the electromyographic pattern for myotonia are delayed discharges. Microscopic studies of affected muscles reveal variation in fibre size, atrophy and predominance of type I (oxidative) fibres, rounded fibres, too many central nuclei, desorientation of the fibres relative to each other. The amount of connective tissue and re- and degenerating fibres is lower than in Duchenne muscular dystrophy.

Owing to the great variation in symptoms and age of onset, a reliable rate of incidence is not available.

Grimm (121) estimated it on 5.5 per 10,000 individuals. The progession of this disorder is much slower than in the Duchenne type, but varies greatly. Mean life span is shortened, due to heart failure and respiratory problems. The dystrophic process cannot be arrested, but myotonic symptoms can be relieved by drugs, which interact with membranes, for example diphenylhydantoin (120).

Since about 1965 several infants and children have been reported with symptoms of myotonic dystrophy from the day of birth. Characteristics of the *congenital variant of myotonic dystrophy* are: severe hypotonia, affected facial muscles, deformities of joints, mental retardation, speech retardation and difficult swallowing (19,124,125, 237,362). After some years the "classical" symptoms gradually appear. Strikingly, the congenital type is nearly always maternally inherited, possibly due to a special interaction between altered intra-uterine circumstances with the fetal mutant gene (126).

1.4. *The site of the lesion in muscular dystrophy*

For a long time the aetiology and pathogenesis of the human muscular dystrophies have been discussed. Three important theories have been postulated as to the site of the primary cause of the disorders: muscle, nerve and blood vessels. Most of the alterations in patients are found in the muscular tissue and that was the reason to believe that the primary cause was located in the muscle itself. But other workers ascribed the dystrophies to a primary defect in the nervous system. This concept was based upon some parameters of the electromyogram and upon the fact that the nerve fibres innervate the muscle fibres and determine the type of metabolism of them. The neural hypothesis is defended recently especially by McComas (90, 199-202,326). Other studies on the innervation of the muscle fibres in Duchenne patients do not support the

neural hypothesis (15,58,59). The vascular/biogenic amine theory was based upon the finding that a combination of inferior blood supply and serotonin induces in rat muscle a histological pattern, similar to that found in Duchenne patients (see 213). Also serotonin plus imipramine injection caused the lesions (215). Later Munsat *et al.* (226) reported that the changes were different from those in Duchenne dystrophy. It has now been established that the blood supply is normal in Duchenne patients (35,154, 177,178,235). The hypothesis is defended in spite of the normal blood supply, owing to alterations found in serotonin uptake by platelets from Duchenne patients (227) and to various influences of biogenic amines on cell membranes (91,92,233,234,365,366). None of the hypotheses can explain all aspects of the symptoms in muscular dystrophy. The various arguments pro and contra the concepts have been put forward repeatedly (9,34,73,99,153, 242,244,273,348,353,354,372). Recently, a combination of a neural and myogenic factor has been proposed for myotonic dystrophy (232).

Recently, a new hypothesis has gained ground. The human dystrophies should be caused by a *generalized membrane defect*, present not only in muscle cells but in other cell types, too. Thomson *et al.* (355) suggested already in 1960 that an increase in membrane permeability was the cause of the dystrophies. High serum activities were found, also in early stages of the Duchenne disease, of enzymes originating from muscle (see 1.2.). Whether the enzyme leakage can be explained by a simple hole-in-membrane-theory is doubtful as for other compounds, like amino acids, a normal serum value has been found (16). Kleine (173) found evidence for the release of enzymes from different tissues in Duchenne dystrophy. Further evidence came from abnormal features of erythrocyte membranes (10,39,136,186). Rowland (283) has summarized the arguments for the membrane concept in the Duchenne and

myotonic forms of muscular dystrophy, after Howland (136) and Attley (11) had pointed again at this theory. The reason why most symptoms (in Duchenne disease) are restricted to muscle tissue may be that skeletal muscle is very vulnerable towards a membrane defect by its long tubular form and its motion. The reason why not all muscles are affected to the same degree remains unexplained.

This thesis deals with studies in which the involvement of cell membranes in human muscular dystrophy has been the subject. In section 1.5., the alterations of the muscle membrane in human dystrophy are summarized. Chapters 2, 3 and 4 contain details of biochemical and biophysical properties in erythrocyte membranes of patients and controls. In section 5.3., some data for other cell types are summarized.

1.5. *Muscle membrane alterations in human dystrophy*

1.5.1. Duchenne dystrophy

Both biochemical and morphological changes have been described for muscle membranes in Duchenne dystrophy. Samaha and Gergely (290) reported a lowered capacity of sarcoplasmic reticular vesicles to accumulate Ca^{2+}-ions with a reduced speed of uptake and lowered ATPase activity. Takagi *et al.* (346) found less phosphatidylcholine in these vesicles, and proposed that the cause of this and of the low Ca^{2+}-uptake could be contamination by adipose and connective tissue. A normal rate of Ca^{2+}-uptake by the microsomal fraction from Duchenne patients was reported by Radu *et al.* (257). Peter and Worsfold (245) measured a lower Ca^{2+}-affinity and normal Ca^{2+}-accumulating capacity in less affected muscles. Indirect indication for an early manifesting impairment of sarcoplasmic reticulum came from measurements of tension generation (383).

Dhalla and coworkers (75) measured ATPase activities in Duchenne muscle sarcolemmal fractions. The Mg^{2+}-

stimulated and the Ca^{2+}-stimulated, Mg^{2+}-dependent components were increased, while the (Na^{+} + K^{+})-stimulated component was decreased. Three studies have been published about the membrane-bound adenylate cyclase. Mawatari *et al.* (209) found a normal, Canal *et al.* (52) a lower basal activity. Both studies, and that of Susheela *et al.* (341) show decreased activity after stimulation by epinephrine, isoproterenol and fluoride. Kar and Pearson (162) described a proteinase, which is likely to be membrane-bound and of increased activity in Duchenne muscle.

Further support for the membrane concept came from a study on glucose metabolism (88). Increased glucose-transport was suggested to explain the abnormalities. Mokri and Engel (221) reported morphologic lesions in the plasma membrane of non-necrotic fibres in Duchenne dystrophy, combined with hypercontraction of the adjacent myofibrils, as a proof of Ca^{2+}-influx. This lack of membrane barrier may form an early change of the Duchenne muscle cells. It may be that these holes cause the drastical Ca^{2+}-accumulation, described by this group (27). In a freeze fracture study Schotland *et al.* (312) detected less protein particles and a nonuniform distribution of them in plasma membrane. An irregular localization of binding sites for concanavalin A has recently been reported (30), while cluster formation of patient's myoblasts in culture may also point to muscle membrane impairment (353). The fact that in Duchenne carriers serum enzym activities are often elevated, while their muscles are not weak, may also point to an early impairment of the membrane in this disease.

An elevated sphingomyelin and lowered phosphatidyl-choline content in Duchenne muscle homogenates have been reported (143,185). Takagi *et al.* (344) found in phosphatidylcholine an increase of oleic acid at the cost of linoleic acid. Kunze and coworkers (187) reported a similar fatty acid alteration in phosphatidylcholine. They reported metabolic studies in order to investigate the

possibility that a defect in the phosphatidylcholine metabolism is the primary cause of Duchenne muscle dystrophy (for reviews, see 183,184).

1.5.2. Myotonic dystrophy

Strong evidence for a muscular site of the lesion in myotonic dystrophy is the fact that myotonia can be evoked in patients by percussion, i.e. without involvement of the nervous system (see 120). Based on the reduced Cl^- conductance in myotonic animals, the chloride hypothesis was applied to all forms of myotonic disorders and thus considered as muscle membrane disorders (see 17). However, normal Cl^- conductance was found in myotonic dystrophy (195). There exist better indications for a muscle membrane defect in this disorder. Hofmann and DeNardo (134) suggested an enhanced Na^+-permeability. In animals myotonia could be evoked by inhibition of the cholesterol synthesis (7). Samaha and Gergely (290) found a normal capacity for Ca^{2+}-accumulation and normal ATPase activity. Radu *et al.* (257), however, measured a decreased rate of Ca^{2+}-uptake. A normal affinity for Ca^{2+} of the microsomes has been reported (246). The unstimulated activity of adenylate cyclase seems normal, but the fluoride- and epinephrine-stimulated activity may be lowered (52,209). A lower degree of membrane protein phosphorylation by endogenous protein kinase under certain incubation conditions has been reported (274). In myotonic dystrophy hyperinsulinism has been established and it is likely that the muscles are less sensitive to this hormone (for a review, see 225). Whether the increased insulin secretion is the trigger for the insensitivity of the muscle receptor or the reverse, is not known (see also 350).

1.6. *Objective of this study*

The aim of the present study is to gather information

about the biochemical features of human muscular dystrophy. On the one hand, insight into the primary lesion of the disease could be obtained, possibly resulting in a therapeutic tool. On the other hand, the study may lead to a diagnostic tool, to be used to determine the carrier state incontrovertibly in the case of X-chromosome linked dystrophy. As it appears that membranes of various cell types are disordered in the muscular dystrophies (see 1.4.) we have used the general membrane defect hypothesis as starting-point. This theory predicts membrane lesions in, among others, erythrocytes, and some alterations were already described when the study was started. Analysing muscle membranes has many disadvantages above erythrocyte membranes:

a) Purification of muscle plasma membranes is rather difficult; contamination with reticular and tubular membranes easily occurs.
b) Muscle homogenates contain, in addition to muscle fibre components, adipose tissue, connective tissue and blood remnants; especially in severely affected muscles the contribution by connective and adipose tissue may be considerable.
c) In muscle disorders often one of the fibre types is predominantly affected; in addition, regenerating and degenerating fibres may be present.
d) The availability of muscle biopsy material is restricted.

These points make it difficult to interprete biochemical data from muscle membrane fractions. Furthermore, much basic knowledge about the red cell membrane is present. The cells are relatively easy to obtain, while very pure membranes can be isolated.

Therefore, we have used erythrocyte membranes from patient and control groups for this study in order to elucidate the cause of the red cell membrane abnormalities and after that extending the results to muscle. An interesting note may be that the first study on

erythrocytes in connection with dystrophy was already published in 1958 by Corsini and Cacciari (63).

We limited the experiments to two types of human muscular dystrophy. The Duchenne type is by far the most severe type. Myotonic dystrophy was chosen as the second object for analysis. The myotonias are considered as membrane diseases; myotonic dystrophy is the most frequent form of the myotonias. Restriction to the congenital type of myotonic dystrophy was made because its severity, to get a homogeneous group, and for age-matching reasons. Duchenne patients were aged from 3 till 18 years, the patients with congenital myotonic dystrophy between 6 and 14 years (all males). Controls were boys without any neuromuscular or hematological disorder, aged between 4 and 20 years. All blood donors, including controls, were fasted overnight before venapuncture, and did not use medicaments.

CHAPTER II

THE LIPID COMPOSITION OF THE ERYTHROCYTE MEMBRANE IN HUMAN MUSCULAR DYSTROPHY

2.1. *Lipid classes*

In 1973 Kunze and coworkers published a report about the amounts of the various lipid classes in erythrocytes from patients suffering from different kinds of muscular dystrophy (186). They found a slightly elevated level of sphingomyelin for Duchenne patients. The levels of cholesterol, phosphatidylcholine and phosphatidylethanolamine plus -serine were normal. In 2 myotonic dystrophic patients all levels were unchanged. Since the publication of this study, several other groups have analysed the lipid composition of erythrocytes from dystrophic humans. The results of the studies are summarized in Table 2-I. These values are comparable with control values, gathered by Nelson (228). Only Kalofoutis *et al.* (160) reported significant differences. They found 8% increased sphingomyelin, 7% decreased phosphatidylcholine, 51% increased lysophosphatidylcholine (only covering a very small part of total phospholipid content) and 104% increased cardiolipin (which has only rarely been detected in erythrocytes) in Duchenne membranes. The other studies suggest that the lipid composition of erythrocytes from patients with myotonic muscular dystrophy and patients and carriers of Duchenne dystrophy is normal. This does not necessarily implicate that the lipid microenvironment of any enzyme, which may be very important for the enzyme kinetics (166), is not altered (see Chapter 4). Such changes may be too small to detect in the bulk lipid fraction.

Special interest has to be paid to cholesterol and its precursors in myotonic dystrophy. It has been reported

TABLE 2-I LIPID COMPOSITION OF ERYTHROCYTES IN DUCHENNE AND MYOTONIC MUSCULAR DYSTROPHY.

Values are in μmoles/ml cells, the phospholipid ratio in % of total phospholipids. Between brackets: patient value as percentage of control value; n is the number of patients. For significancy, see text.

Lipid class	Duchenne muscular dystrophy					Myotonic muscular dystrophy	
	Patients				Carriers		
	Kunze et al. 1973 (n=14)	Kalofoutis et al. 1977 (n=11)	Koski et al. 1978 (n=5)	Kobayashi et al. 1978 (n=9)	Godin et al. 1978 (n=10)	Godin et al. 1978 (n=6)	Kunze et al. 1973 (n=2)	Thomas and Harper 1978 (n=5)
Cholesterol	3.75 (116%)	1.92[a] (88%)		1.3[d] (85%)	2.2[d] (112%)	2.4[d] (111%)	3.05 (94%)	3.02 (102%)
Triglycerides		0.27[a,b] (129%)						1.02 (109%)
Total phospholipids	4.00 (111%)	4.11[a,c] (93%)	3.41 (111%)	2.3[c,d] (89%)	2.7[d] (101%)	2.8[d] (88%)	3.77 (104%)	3.82 (99%)
Phospholipid ratio (in %):								
Phosphatidylcholine	36 (103%)	28 (93%)	30 (99%)	29 (97%)	28 (97%)	30 (114%)	32 (107%)	30 (100%)
" ethanolamine	27 (79%)	25 (95%)	28 (101%)	30 (95%)	40 (107%)	38 (86%)	35 (104%)	30 (102%)
" serine		12 (95%)	11 (115%)	15 (100%)	20 (99%)	19 (107%)		13 (100%)
" inositol		3.6 (116%)	1.5 (99%)					
Sphingomyelin	37 (119%)	19 (108%)	28 (95%)	26 (98%)	7.8 (55%)	8.5 (65%)	33 (90%)	28 (97%)
Lysophosphatidylcholine		2.3 (151%)	1.0 (159%)					
Cardiolipin		1.1 (204%)						

Some figures were obtained by converting the original data by using:

[a] Mean corpuscular volume = 94×10^{-15} l/cell

[b] Mean molecular weight of triglycerides = 350

[c] Mean molecular weight of phospholipids = 725

[d] Recovery of 4 mg membrane protein/ml cells

that 20,25-diazacholesterol, an inhibitor of cholesterol synthesis from 24-dehydrocholesterol, induces myotonia (7,248). In the presence of this drug, desmosterol (24-dehydrocholesterol) accumulates in plasma and red cell membranes, while plasma cholesterol decreases. This condition also leads to cataracts, another symptom often seen in myotonic dystrophy patients. It has been proposed to use the drug-induced disorder as model for human myotonic dystrophy (248). Analysis of plasma from myotonic dystrophy individuals indeed revealed a higher desmosterol level (368). But later in two studies unchanged levels in plasma and red blood cells were reported (8,352). Thus, at this moment no clear relationship between myotonic dystrophy and an impairment of the cholesterol metabolism has been established.

2.2. *Fatty acid composition*

Kunze *et al.* (186) determined the fatty acid composition of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine in a mixed group of patients with progressive muscular dystrophy (X-chromosomal and autosomal type). They reported increased stearic acid (from 13 to 27%) and decreased linoleic (from 13 to 5%) and nervonic acid (from 17 to 9%) in sphingomyelin. In the phosphatidylethanolamine (plus -serine) fraction the content of arachidonic acid was decreased (from 19 to 13%), while the dimethylacetals, originating from the plasmalogen form (1-alkenyl,2-acylphosphatidylethanolamine), were increased from 7 to 15%. However, direct measurement of the plasmalogen amount reveals no alteration. Incorporation experiments with labelled palmitic and linoleic acid did not result in an explanation for the abnormal fatty acid spectrum.

It seemed useful to check and extend the data of Kunze *et al.* (186), since they were not satisfactory in some

details (patient's heterogeneity, aberrant fatty acid composition of control sphingomyelin; compare 228). Therefore, we have set up a series of experiments in order to characterize the fatty acids in the various lipid fractions of erythrocytes from patients with either Duchenne or congenital myotonic muscular dystrophy. The length of the fatty acid chains and the amount of unsaturated carbon-carbon bonds are important for the fluidity of the lipid bilayer in the membrane. This parameter co-determines the mobility of the other membrane components like enzymes, ion-pumps, hormone receptors and structural proteins. Furthermore special phospholipid classes have their influence upon enzyme and transport systems (61,96). The results of our study can be found in appendix paper I (286). The main fatty acids in phosphatidylcholine, -ethanolamine, -serine, sphingomyelin, phosphatidic acid, lysophosphatidylcholine, glycosphingolipids and an unknown one have in both dystrophic groups normal levels. The major alteration in less occurring fatty acids was the decrease level of palmitoleic acid in diglycerides and triglycerides from 10% in controls to 2% in myotonic dystrophy. In Duchenne dystrophy the percentage of palmitoleic acid in diglycerides was reduced to 4%. The origin of these neutral lipid fractions may be not the erythrocyte membrane itself, but contaminating plasma (228). Reticulocytes form another source. They are able to synthesize neutral lipids *de novo* (192). Palmitoleic acid may come from dietary fats, especially animal fat (364) or as a product of palmitic acid desaturase (258). Children with essential fatty acid deficiency possess enhanced palmitoleic, oleic and eicosatrienoic acid levels in serum lipids, at the cost of linoleic and arachidonic acid (236). There are no indications for an abnormal diet in the case of patients, and it seems doubtful whether it could bring about such a major deviation in palmitoleic acid content (97). So, it is difficult to correlate the declined

palmitoleic acid concentration with any disturbed metabolic process.

Wakamatsu *et al.* (368) found decreased palmitoleic acid levels in various serum lipid fractions from patients with myotonic dystrophy or congenital myotonia. Howland and Iyer (137) reported a lowered content of palmitoleic acid in total lipid extracts from red blood cells of Duchenne patients and carriers. Yao *et al.* (387) detected abnormalities in the palmitoleic acid content in neutral lipids from serum of patients with various types of neuropathy. Apparently palmitoleic acid is involved in red cell metabolism of patients with neuromuscular disorders, but the mechanism is not known.

Recently, Kobayashi *et al.* (176) published normal fatty acid patterns in Duchenne erythrocytes for phosphatidylcholine, -ethanolamine, -serine plus -inositol and sphingomyelin.

From the normality of the fatty acid composition it cannot be concluded that the immature stages of the red blood cells also possess normal fatty acid levels. During the erythrocyte life span lipids are lost, are exchanged with blood lipids or are, after deacylation, reacylated (359). Phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, free fatty acids and cholesterol from the bloodstream are exchanged with their red cell membrane counterparts. Lysophosphatidylcholine and -ethanolamine are readily acylated to intact phospholipids. These processes are likely able to normalize the fatty acid pattern in erythroid cells. In order to elucidate a probable influence of blood phospholipids, the fatty acids in patient's serum phosphatidylcholine, -ethanolamine, sphingomyelin and lysophosphatidylcholine have been determined (appendix paper I; 286). Myristic acid levels of sphingomyelin and lysophosphatidylcholine were increased in plasma from myotonic dystrophy patients (from 3 to 5% and

from 4 to 9%, respectively). Stearic acid was diminished in sphingomyelin (from 19 to 13%) and eicosatrienoic acid in phosphatidylcholine was reduced (from 3 to 2%) in this group. In plasma phospholipids from Duchenne patients no alterations were found. It is clear that an abnormality of fatty acids in the early stages of the erythroid cells cannot be excluded definitely. It is noteworthy that reticulocyte counts in Duchenne blood have been reported to be normal (2,68). Reticulocytes have a fatty acid pattern different form mature red cells; especially the content of linoleic and arachidonic acid is dissimilar (360).

CHAPTER III

THE PHYSICO-CHEMICAL PROPERTIES OF THE ERYTHROCYTE MEMBRANE IN HUMAN MUSCULAR DYSTROPHY

An important stimulus to investigate physico-chemical characteristics of erythrocytes from dystrophic patients, was the altered morphology of Duchenne erythrocytes reported by Matheson and Howland (206). The following parameters will be discussed: morphology, electrophoretic mobility, microviscosity, viscoelasticity and osmotic stability.

3.1. *Morphology of erythrocytes*

In 1974 Matheson and Howland published a scanning electron microscopic study (206), in which they reported an increased percentage of echinocytes in the blood of Duchenne patients and carriers. (Echinocytes are red cells with many crenations at the surface, a sea-urchin appearance, which can be reversibly formed *in vitro* from normal erythrocytes (discocytes) (22,74,373).) Matheson and Howland ascribed the echinocyte formation to an abnormality of the membrane lipid, as fatty acids and lysophospholipids are able to induce this cell shape. The deviation was confirmed by Lumb and Emery (197[a]) in a light-microscopic study, although a smaller difference was found. Some papers followed, reporting no echinocytes in Duchenne blood (216,219). Matheson found, with other coworkers, that the way of preparation was important for the shape of the erythrocytes and could not reproduce the earlier finding (207). In contrast, Howells (135) reported significantly more echinocytes in blood samples from Duchenne patients, independent from the treatment. Korczyn *et al.* (181) reported abnormal morphology, but considered it as an

artifact that supported the membrane hypothesis (see 1.4.). In an extensive study Soltan (333) could not detect more echinocytes, while Grassi *et al.* (118) only found more abnormal erythrocytes in Duchenne patients when analysed immediately after blood withdrawal. The difference had disappeared after a storage period of 3 days. The amount of echinocytes in carrier blood was elevated in those cases, in which serum creatine phosphokinase level was also increased.

Morphology of red cells from patients with myotonic muscular dystrophy has been scarcely described. Miller and coworkers (220) counted more stomatocytes in patient's blood samples. (Stomatocytes are cup-shaped cells with the central biconcave region not circular, but elongated (22).) They found the same in Duchenne patients and carriers. Grassi *et al.* (118) reported an increased number of echinocytes in various types of muscular dystrophy, among others myotonic dystrophy. In neurogenic disorders no aberration was found.

There exist no indications that patient's red cells are odd-shaped *in vivo*; their life span is normal (2,68). A possible explanation for the described results may be that the erythrocytes in Duchenne and myotonic muscular dystrophy have an increased sensitivity to an unknown *in vitro* condition, leading to controversial results. Ca^{2+} may be considered to play this role. Weed and Chailley (373,374) reported that increasing Ca^{2+}-levels produce a higher oleate- or salicylate-induced echinocyte formation. Dise *et al.* (77) have demonstrated an enhanced sensitivity of Duchenne erythrocytes to intracellular Ca^{2+}. The crenations could have been formed after interaction of Ca^{2+} with an irregularly stimulated or localized contractile apparatus. High intracellular Ca^{2+} and/or low ATP concentrations may induce aggregate formation of dephosphospectrin molecules in the membrane. These aggregates may interact with actin, resulting in

"contraction" of the membrane and echinocyte transformation (190). However, hitherto there are too few indications in favour of this idea to exclude other mechanisms.

3.2. *Electrophoretic mobility of erythrocytes*

Bosmann and coworkers (33) measured the mobility of red cells, suspended in saline-sorbitol, under the influence of an electric field. The mean electrophoretic mobility of cells from myotonic and Duchenne dystrophy patients was elevated. However, 42% of the myotonic and even all the Duchenne values were within the normal range. The mobility is a measure for the average surface charge. In erythrocytes the negative charge is for a great part determined by the sialic acid moieties of glycoproteins and glycolipids (351). Neuraminidase treatment greatly reduces the electrophoretic mobility (375). Bosmann *et al.* (33) suggested that their results may be explained by an increase in negatively charged groups, e.g. sialic acid, in dystrophic erythrocyte samples. Godin *et al.* (112) demonstrated recently that the total sialic acid content of red cells from Duchenne patients was only lightly elevated (not significantly). The problem remains whether it can explain the small increase in electrophoretic mobility. An alternative explanation may be that the membrane charges are masked in a different way in patient and control red cells, for example by a more deep or superficial localization (see also 33,112).

Szibor *et al.* (343) measured just a lower mobility for Duchenne erythrocytes. Ouabain reduced the mobility of control cells, but enhanced it in 5 of 7 cell samples of Duchenne patients. This effect may be related to the (by some groups found) ouabain stimulation of $(Na^+ + K^+)$-ATPase of patient's membranes (see 4.2.).

3.3. *Fluidity or microviscosity of the erythrocyte membranes*

In addition to the investigations on the lipid and fatty acid composition of the red cell membrane in various types of muscle dystrophy (see Chapter 2), some studies have been published on the fluidity of the membrane in these diseases. The fluidity is important for cell metabolism, as it has its influence on the activity of membrane-bound enzymes and transport processes (61,166). The techniques used are: electron spin resonance (ESR) and fluorescence polarization.

3.3.1. Fluorescence polarization studies

This method is based on the determination of the degree of polarization of an organic fluorescent molecule, embedded in a membrane. The degree of polarization of the fluorophore (also designated as probe) is a measure for its rotational mobility, which is defined by the fluidity of its local environment, also called microviscosity (323; for a review, see 324). The hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) is frequently used to monitor the fluidity of lipids in natural and artificial membranes.

We have measured the degree of polarization of the probe DPH introduced into red cell membranes from patients suffering from either Duchenne or congenital myotonic muscular dystrophy, and from control boys. One portion of the membranes (about 0.2 mg protein), prepared according to the method described in appendix I (286), was washed with 5 mM EDTA, another portion with 5 mM $CaCl_2$, and both in 20 mM Tris-HCl, pH 7.4. The EDTA-membranes were incubated with 2×10^{-6} M DPH in 130 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.4. Ca^{2+}-membranes were incubated in the same medium while EDTA was replaced by 5 mM $CaCl_2$. The conditions for incubation and measurement have been published by Van Hoeven *et al.* (361). The results are shown in Table 3-I.

TABLE 3-I. DEGREE OF POLARIZATION OF DPH IN ERYTHROCYTE MEMBRANES FROM PATIENTS WITH DUCHENNE AND CONGENITAL MYOTONIC MUSCULAR DYSTROPHY IN THE PRESENCE OR ABSENCE OF 5 mM Ca^{2+}, AT 25° AND 12°C (means ± SEM)

Preparation	25°C		12°C	
	EDTA	Ca^{2+}	EDTA	Ca^{2+}
Control	0.333±0.001 (n=3)	0.336±0.002 (n=3)	0.355±0.004 (n=2)	0.363±0.004 (n=2)
Duchenne dystrophy	0.333±0.001 (n=5)	0.334±0.002 (n=5)	0.354±0.001 (n=2)	0.358±0.002 (n=2)
Myotonic dystrophy	0.334±0.002 (n=4)	0.331±0.007 (n=4)	0.357±0.005 (n=2)	0.364±0.002 (n=2)

Control values are in agreement with those found by Van Hoeven *et al.* (361) for human red cell membranes. No differences have been found between patients and controls despite variation in Ca^{2+} and temperature. As we assumed Ca^{2+} playing a role in the alterations of the red cells of dystrophy patients, we measured the membrane fluidity in the absence and presence of Ca^{2+}. The same polarization values were obtained at both conditions at 25°C. Obviously, binding of Ca^{2+} to membrane components like phosphatidylserine and proteins has no effect on the fluidity of the hydrophobic core as measured by DPH polarization. These results are different from ESR-studies which showed that Ca^{2+} increased the fatty acid chain mobility in erythrocyte membranes (1). A Ca^{2+}-effect is also observed, when the ionophore A 23187 is applied as fluorophore in red cell membranes (376). A temperature decrease from 25° to 12°C gives rise to increase in polarization and, therefore, decrease in fluidity, with the same change in the three groups. From these results it may be concluded that the flow activation energy in patient's membranes is normal. Although not significantly, Ca^{2+} seems to have a little decreasing effect on the fluidity at 12°C.

The degree of polarization of the probe depends on several factors: the length of the fatty acid chain and number of unsaturated carbon-carbon bonds in it, the cholesterol concentration (or better the cholesterol/phospholipid ratio) and sphingomyelin content (62,222). Erythrocyte membranes and liposomes, prepared from the erythrocyte lipids, show about the same degree of fluorescence polarization or microviscosity, indicating a low to negligible effect of the proteins of the (mature) red cell membrane to the lipid fluidity (6,222,285). The lack of protein influence may be caused by:

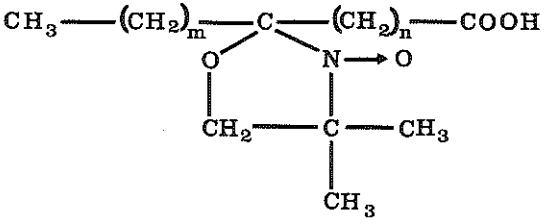
a) Proteins do not influence the structure of the lipid bilayer and have no effect on the microviscosity. This seems in contrast to studies applying other techniques for fluidity determination and is in disagreement with today's conceptions of membrane architecture (1,20,36,65,282,317,321,330,371,391).
b) The probe is embedded in the more apolar lipid regions of the membrane, where the lipid-protein interactions are not strong. The reason for the localization of the probe could be the lack of a polar group in the probe molecule.
c) The probe is localized in both apolar and polar regions of the membrane, while the contribution of the latter to the fluorescence polarization is negligible. This possibility seems not satisfactory, as more than half of the amount of phospholipids is in direct contact with proteins (65), containing for a great part polar moieties. Some contribution of DPH in the polar domains, and therefore influence of proteins, should be expected to be detectable.

The second possibility seems the most attractive one. The conclusion from the normal values for the degree of fluorescence polarization has to be that the average fluidity of the relative hydrophobic parts of the red cell membrane in Duchenne and myotonic dystrophy is unchanged. This points to

normal lipid, especially cholesterol, levels and fatty acid composition. The measurements described in Chapter 2 are thus supported by physical findings.

3.3.2. Electron spin resonance studies

In these studies probes are introduced into the membrane, from which the tumbling motion of the label spin reflects the motion of the probe. The probe motion is defined by the environment to which it is bound. Two important labels are: the nitroxide derivatives of stearic acid and the nitroxide derivative of N-ethylmaleimide. These probes, often applied in erythrocyte studies, give insight into the fluidity of the lipid and protein(-SH-moieties) of the membrane, respectively. The structure of the stearic derivative is given below. The m and n of the probe I(m,n) can be chosen, which makes it possible to vary the distance between the nitroxide spin and the carboxyl group, which is anchored in the polar layers of the membrane.



I(m,n)

By this method the various "depths" of the membrane can be monitored (21,44,138,164,313).

Butterfield *et al.* (46) concluded from ESR experiments, using I(12,3), that the fluidity of the lipids rather near the membrane surface was elevated in erythrocytes from patients with myotonic dystrophy. The lipid fluidity in these cells becomes more and more normal deeper in the

membrane, as tested with I(5,10) and I(1,14). The same group reported that phenytoin decreases the fluidity in myotonic cells to a normal value (277). This drug is applied for the stabilization of epileptic and myotonic syndromes (120). Tetany and other clinical symptoms of hypocalcemia are improved by phenytoin (298). It is bound to nervous tissue, probably to both proteins and lipids (115). It inhibits the Ca^{2+}-transport in brain (250) and synaptosomes (332) and has a stimulating effect on Ca^{2+}-binding to phospholipids (114). The membrane fluidity may be lowered by phenytoin in the same manner as cholesterol does (36,62), namely intercalating its apolar (phenyl) moieties between the fatty acid chains and the polar (hydantoin) group in the area of phospholipid bases. So, it may be that the packing of the myotonic erythrocyte membrane directly beneath the hydrophilic region is less condense. It is not clear whether the cholesterol-desmosterol hypothesis for myotonia (see 2.1.) may be applied here. It is obvious that desmosterol interacts in a similar way with the lipid bilayer as cholesterol does, but the interaction with the proteins may be different (43,70). The proteins in their turn may influence the fluidity of the lipid domains.

Another explanation for the higher fluidity may be a protein aberration. Butterfield and coworkers applied the nitroxide derivative of N-ethylmaleimide to erythrocytes from myotonic dystrophy patients (48). This probe reveals the presence of two types of SH-moieties, a strongly and a weakly bound group (329). The amount of strongly bound SH-groups is reduced in red cells in myotonic dystrophy, the amount of weakly bound groups is unaltered (48). The weakly bound SH-groups are located superficial, the strongly bound ones both superficial and deeper into the membrane layer. The exact reasoning for these changes is obscure.

ESR studies have also been performed on red cells from Duchenne patients. Spin labelling of the fatty acid chains with I(12,3) reveals no abnormality (47). The ratio of weakly bound to strongly bound SH-moieties is increased (45). Butterfield (44,45) concluded that dystrophy is accompanied with altered membrane protein features, and myotonia with abnormal lipid fluidity. Sato *et al.* (297) found with the I(12,3) probe also normal fluidity values for the lipids in Duchenne red cells. Using I(1,14), they indicated a decreased fluidity near the centre of the hydrophobic core. The fluidity of that region also possessed altered pH and temperature dependency. The authors suggested that the cause may be a more tight protein-lipid interaction in Duchenne cells. Wilkerson *et al.* (378) applied I(12,3) combined with the saturation transfer ESR technique, which detects slow motions of the label (107). They found abnormal intensities both after short and long labelling times. They concluded that those results were due to the spin exchange effect and concluded that in Duchenne erythrocytes the label was primarily bound to specific sites at a high concentration, followed by a delayed distribution to other sites. This idea may be in agreement with a stronger protein-lipid interaction, as suggested by Sato *et al.* (297). It is not excluded that the altered SH-groups (45) are involved in this interaction, too.

3.3.3. Conclusions from the fluidity measurements

From the foregoing studies it is clear that in some cases fluorescence polarization measurements give normal fluidity values, while with ESR studies abnormal values are found. Fluidity, measured by ESR, is influenced, as is the case with fluorescence polarization, by cholesterol, fatty acid chain length and -saturation degree and the nature of the polar headgroup of the phospholipids (98,

167,322). The reason for the discrepancies in the two techniques may be ascribed to the fact that the mobility of the ESR fatty acid probes is influenced by proteins (1,20, 282,317,321,371),while erythrocyte proteins do not affect the lipid fluidity as detected in fluorescence studies with DPH (see 3.3.1.). ESR offers the possibility to distinguish between fluidity of the lipid bilayer and the lipids, bound very tightly to proteins (5,23,157). The nitroxide label has a much more polar character than the acyl chains and may be more embedded in the polar lipid domains. Possibly the degree of DPH polarization reflects the fluidity of the bulk lipids, comparable with the chemically determined composition, and ESR probes predominantly monitor the fluidity of those lipids, which are in close contact with proteins. A second reason for the discrepancies between the two techniques may be a difference in sensitivity to certain membrane compounds.

Both types of microviscosity measurements make use of probes which may perturb the native state of the membrane to a certain extent. Some studies have revealed label-induced deviations, while other ones showed no changes (23,49,50,139,161; for a review, see 164). It is hardly possible to say to what extent the original fluidity has been influenced by the various probes.

With these notions in mind it can be concluded that:

a) there exist no changes in the composition of the bulk of the lipids in red blood cells of Duchenne and myotonic muscular dystrophy patients.
b) in both patient groups ESR studies have shown some alterations in lipid fluidity and protein architecture; the origin of the alterations may be a change in some protein property, although a primary lipid change cannot be excluded.
c) in Duchenne red cell membranes there exists a less uniform distribution of membrane proteins and lipids.

d) the nature of the membrane alterations is different in the two muscular dystrophies.

3.4. *Viscoelasticity of the erythrocyte membrane*

When an erythrocyte is forced to pass through a micropipette with an orifice diameter of 3 μm, it can do so only after deformation, as the erythrocyte diameter is about 8.3 μm (377). The underpressure, necessary to aspirate the red cell into the pipette is a measure for the deformability or viscoelasticity of the cell. The lower the elasticity the higher the aspirating force necessitated.

Percy and Miller (243) performed the described technique on red cells from Duchenne patients and carriers. They found for both groups that less cells could be aspirated by pressures under 100 mm Hg. The conclusion can be that these cells possess a larger volume, an altered geometry, an increased rigidity or a combination of these factors. In the case of patients, no indication for a larger volume exists (2). A (tendency to) geometric alterations has been discussed in section 3.1. The study of Kohn *et al.* (180) seems to support the possibility of an increased rigidity in Duchenne erythrocytes. They calculated a significantly increased elastic shear modulus from experiments, in which not the whole cell but only a part of it was sucked into a micropipette. The same deviation, although to a minor extent, was found in myotonic muscular dystrophy. Cells from patients suffering from neurological, non-dystrophic diseases, showed normal viscoelastic parameter values.

The elastic shear modulus of the cell, a measure for the extension of the cell membrane per pressure unit, is mainly defined by the membrane protein-protein interactions (94,130,375). Some of these interactions are, in their turn, influenced by lipids. Heusinkveld and coworkers (130) reported that crosslinking of the membrane proteins (by glutaraldehyde) drastically increased the elastic shear

modulus. Reduction of the spectrin content lowered the modulus value, while sulfhydryl reagents and glycophorin only had little influence. Spectrin, an extrinsic protein at the cytoplasmic side of the membrane, can form aggregates or polymers with other membrane proteins at increased Ca^{2+} concentrations (53,87,328). ATP is able to prevent the Ca^{2+}-induced aggregation (53,198). Low ATP and high Ca^{2+} concentrations are well-known circumstances related to reduced deformability (130,194,198,375). Thus, this type of spectrin-involved crosslinking may be responsible for the increased rigidity in Duchenne erythrocytes, and perhaps also for red cells in myotonic muscular dystrophy.

One may wonder whether the reduced deformability after ATP-depletion may be directly linked to the Ca^{2+}-induced aggregation. For, the former process is reversible (375), the latter being irreversible (53,328). It may be that the spectrin conformation is changed by a moderately increased Ca^{2+} and/or moderately decreased ATP concentration, leading to a rather small reduction of deformability, while more extreme circumstances induce (via spectrin) protein polymerization. The reversible condition may be compared with the discocyte-echinocyte transformation and the irreversible one to the glutaraldehyde-treated cells, with an almost normal and drastically increased elastic shear modulus, respectively (190). The deformability, however, of echinocytes is significantly decreased (194). Ca^{2+}-levels above 0.5 mM may stand for such extreme conditions. These concentrations induce the mentioned protein aggregation (53,168,328), while rigidity increased already at concentrations 10 times lower (375). Palek *et al.* (231) described two different crosslinking reactions: one being reversible and occurring at an intracellular Ca^{2+} concentration of 0.1 mM and the other being irreversible. This polymerization takes place at Ca^{2+} concentrations above 0.5 mM and may be catalyzed by a Ca^{2+}-stimulated, ATP-dependent transglutaminase (328). The influence of Ca^{2+}

upon membrane proteins is visible in the electrophoretic pattern (168). At 0.1 mM Ca^{2+} more hemoglobin is attached to the red cell membrane than at 10 mM or without Ca^{2+}. The hemoglobin binding may be concerned to the mildly increased rigidity of the membrane (375). So, an increased elastic shear modulus may be caused by an extrapolation of conditions, inducing reduced deformability.

The condition in Duchenne and myotonic dystrophy erythrocytes may be rather disordered, as revealed by the increased elastic shear modulus.

3.5. *Osmotic stability of erythrocytes*

The osmotic stability is the capacity of the cell to resist pressures, exercised by the increasing volume of the cell content. It is determined by several factors, including the viscoelasticity (190):

a) the surface/enclosed volume-ratio
b) the viscoelasticity (or rigidity) (see 3.4.)
c) the yield shear, the elastic limit of the membrane
d) the shear viscosity at irreversible (plastic) extension.

The osmotic stability of erythrocytes from Duchenne patients has been reported to be reduced in 5 studies (101, 112,196,289 (appendix II),334). Adornato *et al.* (2) reported normal stability. Godin *et al.* (112) and Lloyd and Nunn (196) found normal cell stability in Duchenne carriers. Patients with congenital myotonic muscular dystrophy have red cells which are less stable than control and Duchenne cells (289; appendix II). A serum factor, which eventually persists in causing fragility during hemolysis was excluded in our study by applying the NaCl-phosphate buffered solutions to washed cells instead of whole blood, which was taken in the other studies.

In order to obtain information about the cause of the fragility, we have incubated red cells in the presence of various phospholipases A. The phospholipase from bee venom

is able to hydrolyse erythrocyte phospholipids, while phospholipase from pancreas and Crotalus snake venom cannot (392). This difference may be due to the fact that the latter two enzymes are unable to phospholipid breakdown at surface pressures above 23 dynes/cm (71). Thus, the study may give insight into the surface architecture of patient's erythrocytes (289; appendix II). Only the bee venom phospholipase-treated cells of Duchennes, myotonics and controls were hemolysed to a greater degree when compared with untreated cells, after being brought into the same NaCl-phosphate solutions. The same order of stability was found in the three groups of cells, independently from the type of phospholipase A applied.

A surprising effect of the treatment of cells from Duchenne patients and controls was, that more cells lysed at isotonic NaCl-phosphate solutions than at about 0.50% ($^{w}/v$) solutions. This holds for all types of phospholipases tested. Erythrocytes from myotonic dystrophy patients hardly demonstrate this phenomenon. Omission of phospholipase from the pretreatment medium results in 10% lysis of erythrocytes from control men in isotonic medium. The addition of phospholipase causes 11, 36 and 78% extra lysis of these cells in isotonic medium, for pancreatic, snake venom and bee venom enzyme, respectively. When the Ca^{2+} concentration of the pretreatment medium was lowered from 14 to 6 mM, lysis was hardly detectable both with and without phospholipase-treatment. It turned out that omission of phosphate (present in the lysis medium, not during pretreatment) made lysis disappear, and that replacement of Na^{+} by other monovalent cations drastically reduced hemolysis. Furthermore, addition of 10 mM ribose, glucose or sucrose decreased the rate of lysis to less than half the amount, as did ouabain. These effects cannot simply be explained by an energy sparing effect, as sucrose is not metabolized by red cells (218). Moreover, we were unable to detect lowered amounts of ATP or

2,3-diphosphoglycerate (the most important high-energy compounds in erythrocytes) in the cells remaining after a high degree of lysis had taken place. The same lack of correlation was found between the Ca^{2+}content and rate of hemolysis. La^{3+}-ions and mersalyl, which suppress the Ca^{2+}-efflux and enhance passive Ca^{2+}-influx (308,342), greatly enhanced the lysis process. A high extracellular Ca^{2+} concentration (about 25 mM) and no phosphate after pre-treatment results in less lysis. Therefore, a high intra-cellular Ca^{2+}-level alone cannot explain the lytic process. The Na^{+}-transport inhibitor tetrodotoxin (222[a]), the serine protease inhibitor phenylmethylsulfonyl fluoride (168, this drug did not inhibit a postulated membrane-bound protease in red cells) and the cholinergic agent carbamyl choline (142) all reduce lysis by 60-70%.

It is clear, that the Ca^{2+}-, Na^{+}-, and phosphate-dependent process(es),causing the high degree of lysis in control red cells, and which is induced or stimulated by exogenous phospholipase, is less or not active in myotonic dystrophy cells. The results of this study can be compared with the findings of Huestis (142), who treated erythrocytes with liposomes and found an increase in Na^{+}-permeability and in lysis, which processes were diminished by tetrodotoxin. Treatment of the extracted cells with liposomes, which were loaded with proteins, previously extracted from the same cells, also reduces lysis and Na^{+}-permeability. The rate of hemolysis was reduced by sucrose. In another publication Huestis (141) reported that incubation of erythrocytes with carbamyl choline resulted in decreased lipid fluidity if external Ca^{2+} was present. The effect was absent when tetrodotoxin and Ca^{2+}-chelator were added. An acetylcholine receptor was isolated in this study. These studies suggest that, when the acetylcholine receptor is stimulated, a Na^{+}-specific ion channel is opened and hemolysis is induced. Ca^{2+} should be required for this process. Comparison of

these data with the phospholipase-treated cells in our study may suggest that the described liposome-treated cells have (partly) the same characteristics as the phospholipase-treated cells from controls. During pre-treatment one or more proteins, which are involved in the Na^+-channel (perhaps coupled to the acetylcholine receptor), may have been removed. The inhibition of the hemolysis by sucrose and EGTA and partly inhibition by tetrodotoxin are in agreement with this idea. Apart from Ca^{2+}- and Na^+-ions, phosphate plays also a role in the high degree of lysis in our experiments. This effect may be comparable with the somewhat enhanced phosphate uptake by the liposome-treated red cells (142). The abnormality of the red cells from patients with congenital myotonic muscular dystrophy may be caused by the unaccessibility of the Na^+-channel/acetylcholine receptor-complex to phospholipase attack. Whether this is due to an abnormal superficial structure, or to an alteration in the channel-receptor with its direct environment, is not to conclude. Obviously this concept cannot explain all results enumerated in appendix II. But it may reflect one of the processes that results in a high rate of lysis in control cells, or may be the process that triggers other pathways causing hemolysis.

3.6. *Discussion*

This chapter contains data obtained by techniques based on different principles. In spite of it, some aspects are repeatedly prominent. From measurements of the lipid fluidity (see 3.3.) and the viscoelasticity (see 3.4.) it is clear that the protein-protein or protein-lipid interactions may be altered in both myotonic and Duchenne dystrophy. The suggested tightened interactions (297) may be accompanied with a high rigidity of the membranes. Ca^{2+} and ATP play important roles in membrane-protein interaction. Both cytoplasmic and membrane-bound

proteins are involved in Ca^{2+}-dependent processes (see 3.4.). A role for Ca^{2+} has been proposed from the hemolysis (see 3.5.) and ESR data (see 3.3.2.), while the abnormal shape of erythrocytes, sometimes found (see 3.1.), also may point to a Ca^{2+}-involvement in red cells from dystrophic patients.

Thus, many of the deviations can be explained by an elevated Ca^{2+}-level or a diminished ATP content. These two conditions often coincide (130,357,375). The consequences are reduced deformability, high elastic shear modulus and aggregation of membrane proteins (see 3.4.). One can imagine that these conditions are linked with altered membrane sulfhydryl group properties, as demonstrated by ESR studies (see 3.3.2.). The osmotic stability of red cells also depends on the Ca^{2+} concentration, but not in a simple way. Lake *et al.* (191) described that intracellular Ca^{2+}-levels up to about 1 mM increased the stability, while extremely high Ca^{2+} concentrations lowered the stability. From these data it can also be seen that changes in osmotic stability are not negatively correlated with rigidity changes. For, the rigidity of red cells is increased at 0.05 mM and more Ca^{2+} (170), while the osmotic stability is optimal in the presence of 1 mM Ca^{2+} (191). ATP prevents excessive binding of Ca^{2+} to the membrane via complexation of Ca^{2+} or via hemoglobin-ATP interaction (55,271). Maybe ATP has a direct effect upon the membrane organization, which changes the phospholipid environment at the outside of the membrane (325). ATP may be required to maintain spectrin in its phosphorylated, monomeric form (24,169,188). The ratio of phosphorylated-dephosphorylated spectrin molecules is controlled by a protein kinase and protein phosphatase (24). Evidently, ATP has an important function as energy source for different anabolic pathways, like the renewal of membrane components.

There are no indications for a low ATP-level (32,67, 295) or a high Ca^{2+}-level (77) in Duchenne erythrocytes. It is conceivable that patient red cells are more sensitive

towards Ca^{2+} owing to, for example, an enhanced affinity of spectrin for Ca^{2+}. Dise *et al.* (77) reported that Duchenne erythrocytes, loaded with Ca^{2+} to the same level as control cells, reveal an enhanced response as to hemolysis and K^{+}-efflux.

Apart from ATP, 2,3-diphosphoglycerate can lower the free Ca^{2+}-levels. This compound can maintain a high degree of deformability of red cells (189). Bosia *et al.* (32) reported in red cells from Duchenne patients a normal level, but Sarpel *et al.* (295) found half the normal level of 2,3-diphosphoglycerate.

The physico-chemical properties of erythrocytes can also be changed by the binding of prostaglandins and catecholamines. Prostaglandin E_2 and epinephrine induce a lowering of lipid fluidity as measured by ESR (188). Cyclic AMP or cyclic GMP can induce this change in intact erythrocytes. The authors suggested that the fluidity change takes place via a phosphorylation-dephosphorylation mechanism of spectrin. Prostaglandin E_2 and epinephrine increased the viscoelasticity of the red cells (4). These hormones also cause a higher rate of hemolysis, an effect which can be induced by exogenous cyclic AMP in intact cells (260). The hormones, however, do not increase the (intra- or extracellular) cyclic AMP-levels. Prostaglandin E_2 diminishes the size of a rapidly exchangeable, oscillating calcium pool and reduces the amplitude of the concentration oscillations (260). The changes in the Ca^{2+}-pool may trigger the rigidity and osmotic stability of the cells. Ji and Nicholson (155) demonstrated a transmembrane effect by a lectin upon red cell protein crosslinking. One of these receptor-mediated mechanisms may be impaired in red cells from Duchenne (and perhaps myotonic) muscular dystrophy. In this context it is of interest that Wakayama *et al.* (370) recently published a freeze fracture study, showing a lower amount of intramembranous protein particles in both layers of the membranes. Furthermore, the particles were not

distributed uniformly, supporting the explanation of Wilkerson *et al.* (378) for their ESR data (see 3.3.2.). As the protein-lipid ratio (112,176,182) and the electrophoretic pattern (see 4.1.) are normal for Duchenne red cells, it can be concluded from these studies that protein aggregation has taken place. The pattern of protein distribution is comparable with the picture seen after a moderate degree of protein aggregation (stage 1, see 86).

It has to be stated that the above described suggestions especially refer to the Duchenne type of muscular dystrophy. For myotonic dystrophy much less data are available. For instance, the Ca^{2+}-level in erythrocytes in this disorder is unknown and the ESR and rigidity studies are more limited. Similarities with the Duchenne red cells are the normal lipid fluidity as measured by fluorescence polarization, the altered sulfhydryl group conformation, the increased elastic shear modulus and the reduced osmotic stability. Dissimilarities are the elevated lipid fluidity as measured by ESR and the behaviour of phospholipase-treated cells (see 3.5.). Furthermore, a higher ATP-level has been reported in erythrocytes in myotonic dystrophy (67), while no abnormality was found in freeze fracture pictures (369). Therefore, it may be that the trigger for the abnormalities in myotonic dystrophy cells has a nature different from that in Duchenne dystrophy, but that the consequences are partly similar.

Finally, based on the decreased osmotic stability and deformability, one should expect a decreased survival of the red cells in both types of dystrophy. However, Adornato *et al.* (2), taking into account reticulocyte counts (see also 68), hemoglobin measurements and decay of ^{51}Cr-labelled cells, concluded that Duchenne red cells have a normal time of survival. Additional support for this view can be found in the study of Campbell *et al.* (51).

CHAPTER IV

THE PROTEINS OF THE ERYTHROCYTE MEMBRANE IN HUMAN MUSCULAR DYSTROPHY

Activities of various membrane-bound enzymes have been determined in erythrocytes from patients suffering from muscular dystrophy. The determinations give insight into several aspects of cell metabolism. An altered enzyme property may be the clue to the primary cause of the disorders. It may point to an adaptation of the cell to changed conditions caused by a metabolic perturbation. It may be the consequence of an abnormal membrane structure. Especially the lipid environment of the enzymes is able to induce changes in enzyme properties. Some enzymes are surrounded by a lipid layer with a composition strongly different from the total membrane lipid composition. Destruction of that layer drastically affects the enzyme activity and other kinetic parameters. The effect of more moderate influences can be seen in diet and hormonal studies, which change the fluidity of the membrane lipids (for reviews, see 61,96,166).

This chapter deals with the red cell membrane protein composition and phosphorylation in the myopathies. Studies on activities and other kinetic parameters of membrane-bound enzymes in red cells will be summarized.

4.1. *Membrane proteins and their phosphorylation*

The protein pattern, as seen after SDS-polyacrylamide-gel electrophoresis, of membrane ghosts from Duchenne patients (278) and patients with myotonic muscular dystrophy (275) is quite normal. Godin *et al.* (112) reported normal values for the amount of sialic acid, and for superficial and total amino groups in Duchenne

erythrocytes. The same study showed that these properties were also normal in Duchenne carriers. Normal protein spectra and glycoprotein bands were reported by Kobayashi *et al.* (176).

Another way to study possible alterations in erythrocyte proteins is to separate them after phosphorylation with labelled ATP. The group of Roses and Appel has analysed the phosphorylation of the proteins of erythrocyte ghosts from dystrophic patients by endogenous, membrane-bound protein kinases. They measured a 50% decrease in phosphorylation of one component of band III in myotonia dystrophica (275; for enumeration of the electrophoretic pattern, see 336). They also reported enhanced phosphorylation of band II (spectrin) and band III in Duchenne erythrocyte membranes (276). The results of both studies can be explained by an altered substrate or ratio of protein kinase-protein phosphatase activities. Further purification of the band II reveals that some polypeptides in Duchenne membranes are phosphorylated to a higher degree, while other ones are not (279). Treatment of band II with trypsin yields fragments, of which the phosphorylation is abnormal in a pedigree-specific manner (281).

More detailed studies are required for determination of the exact alterations in the muscular disorders. Helpful may be the addition of stimulators (cAMP) or inhibitors (Ca^{2+} or Na^{+}) of the phosphorylation of band III in control membranes (95,123). Then band III kinase can be distinguished from the spectrin band II kinase, which is stimulated by Ca^{2+}, Mg^{2+} and monovalent cations and hardly stimulated by cyclic AMP (13,284). In this way it may be elucidated whether the altered phosphorylation is due to a structural change of the kinase or substrate, or to a reduced or enhanced phosphorylation degree of the substrate. That lack of details does not justify far-reaching conclusions from these experiments, is supported by Iyer *et al.* (150).

These investigators did not find increased and decreased protein phosphorylation in Duchenne and myotonic dystrophy, respectively. They just reported an enhanced band III phosphorylation in myotonic dystrophy and a normal phosphorylation pattern in Duchenne dystrophy.

4.2. *($Na^+ + K^+$)-ATPase and K^+-PNPPase; the ouabain effect*

The most widely studied enzyme in erythrocytes from Duchenne patients is the ($Na^+ + K^+$)-stimulated, Mg^{2+}-dependent ATPase. This activity, which correlates with the ATP-driven Na^+-extrusion and K^+-uptake of the cell, can be inhibited by the glycoside ouabain (see 31). Since Brown *et al.* in 1967 reported that ouabain stimulated ($Na^+ + K^+$)-ATPase of Duchenne erythrocytes (39), several studies have been published with controversial results. Table 4-I summarizes the data hitherto published. The results vary to a great extent, especially as to the ouabain effect. Several groups (including ours, see 287; appendix III) have assayed the enzyme in two different media, namely at optimal and sub-optimal cation concentrations, but the medium does not alter the ouabain effect. Of more importance could be the purification procedure of the membranes. Dystrophic serum has been reported to lower the ouabain inhibition of control membranes (247,327). In the study of Mawatari *et al.* (210) epinephrine normalized the ouabain sensitivity (cyclic AMP did not). The outside of the cells may be altered by a factor in the Duchenne plasma. The use of other ATPase or cation transport inhibitors does not result in a clear explanation of the underlying mechanism (238).

The differences of the ouabain effect either in control and patient membranes may be partly due to the orientation of the vesicles. Ouabain, which binds to the outside of the red cell membrane, is not able to reach its binding site in inside-out vesicles. Latency phenomena were not present in our study as a detergent was applied and a freeze-thawed

TABLE 4-I. ERYTHROCYTE ($Na^+ + K^+$)-ATPase ACTIVITY AND THE OUABAIN EFFECT UPON IT IN DUCHENNE PATIENTS AND CARRIERS, MYOTONIC DYSTROPHY PATIENTS, AND CONTROLS.

(Some data had to be estimated from the standard error of the mean.)

* Significantly different.

N.B. Ouabain inhibition by more than 100% means that the stimulation by Na^+ plus K^+ is lower than the ouabain inhibition.

References	Activity as % of control values	Number of dystrophic individuals in which ouabain: inhibits more than 10%	effect is less than 10%	stimulates more than 10%	Range of ouabain inhibition (in %)
	Duchenne patients				Controls
Brown et al., 1967	72	1	2	5	29 - 82
Klassen & Blostein, 1969	106	7	0	0	10 - 55
Peter et al., 1969	77*	0	1	12	0 - 50
Araki & Mawatari, 1971	?	0	1	5	4 - 27
Mawatari et al., 1976	82	0	10	0	4 - 26
Niebrój-Dobosz, 1976	38*	1	0	10	45 - 75
Hodson & Pleasure, 1977	115	11	0	0	44 - 225
Siddiqui & Pennington, 1977	102	7	6	0	16 - 49
Pearson, 1978	60*	0	0	7	18 - 40
Souweine et al., 1978	64*	10	3	0	1 - 37
Ruitenbeek, 1979; appendix III	108	4	0	0	45 - 170
	Duchenne carriers				Controls
Araki & Mawatari, 1971	?	2	0	0	4 - 27
Mawatari et al., 1976	95	3	1	0	4 - 26
Pearson, 1978	90	0	0	5	13 - 27
	Myotonia dystrophica patients				Controls
Araki & Mawatari, 1971	?	0	1	0	4 - 27
Roses & Appel, 1975	?	9	0	0	?
Niebrój-Dobosz, 1976	19*	0	4	0	55 - 77
Ruitenbeek, unpublished data	104; 229	2	0	0	19; 90

preparation was used (287; appendix III).

The influence of ouabain was also determined in other kinds of studies. Bosia *et al.* (32) incubated intact red cells in the presence of ouabain and glucose. In cells from Duchenne patients ouabain did not increase the levels of fructose-1,6-diphosphate and dihydroxyacetonphosphate as it did in control cells. Ouabain had also an abnormal effect upon the ATP level. At 5 mM K^+, ouabain reduced the ATP-level, while ouabain had no effect at 40 mM K^+. In control cells ATP was enhanced at both K^+-concentrations. These results point to an altered influence of ouabain upon the Na^+-K^+-pump of the membrane, resulting in changes in some of the glycolytic steps. The link between the pump and glycolysis is likely the phosphoglycerate kinase/ phosphoglycerate mutase-complex, which can bind to the membrane and thus provide the Na^+-K^+-pump with ATP (104, 105,318). The binding of this complex with the membrane can be suppressed by ouabain.

An altered ouabain effect should also be demonstrable in the K^+-stimulated *p*-nitrophenylphosphatase (K^+-PNPPase) activity. It correlates with the second, rate-limiting step of (Na^+ + K^+)-ATPase, at which the ATPase molecules are dephosphorylated (31,109). We have analysed the ouabain effect on K^+-PNPPase in erythrocyte membranes from patients with Duchenne and congenital myotonic dystrophy (287; appendix III). This study reveals normal ouabain sensitivity of K^+-PNPPase, as well as of (Na^+ + K^+)-ATPase in patients. In no case we found stimulation by ouabain. It would be of interest to determine the ouabain effect upon the easily measurable K^+-PNPPase in those preparations which show ouabain stimulation of the (Na^+ + K^+)-ATPase activity.

Ouabain influences the Na^+- and K^+-flux through the erythrocyte membrane. Probstfield *et al.* (254) showed normal uptake in Duchenne cells of a rubidium isotope, and thus of K^+-influx (31) in the presence and absence of ouabain either in washed cells and in cells plus plasma.

Lloyd *et al.* (197) reported for Duchennes a normal erythrocyte/plasma ratio for this isotope, indicating a normal K^+-permeability. Sha'afi *et al.* (319) found an unchanged ouabain-sensitive Na^+-efflux and a somewhat higher K^+-influx in red cells from Duchenne patients and carriers. A decreased Na^+-efflux was also reported (335). Hull and Roses (144) measured a lower ouabain-sensitive Na^+-efflux and normal K^+-influx in red cells from myotonic dystrophy patients. Thus, the results of the studies on ouabain influence on cation transport are as controversial as those found in (Na^+ + K^+)-ATPase studies. A clear explanation for the abnormal ouabain effect, found by some investigators, is not available.

4.3. *Ca^{2+}-ATPase and Ca^{2+}-PNPPase*

As has been discussed in section 3.6., Ca^{2+} may be involved in various abnormalities reported in erythrocytes of Duchenne and myotonic muscular dystrophy. An enhanced sensitivity of Duchenne red cells towards Ca^{2+} has been described (77). Therefore, we have determined the kinetic properties of two membrane-bound enzyme activities, which are stimulated by Ca^{2+}-ions, viz. Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase) and Ca^{2+}-stimulated *p*-nitrophenylphosphatase (Ca^{2+}-PNNPase). For a correct interpretation of the results, firstly a kinetic model of Ca^{2+}-ATPase will be presented.

4.3.1. Kinetic model for Ca^{2+}-ATPase activity and Ca^{2+}-transport

The Ca^{2+}-ATPase activity is related to the pump, extruding Ca^{2+}-ions out of the red cells. This pump is capable to maintain the total calcium concentration at less than about 15 µmoles/liter cells and the free Ca^{2+}-level at less than 1 µM (304,367). The reaction mechanism

for Ca^{2+}-ATPase is less well established than for $(Na^+ + K^+)$-ATPase (331). However, recently many kinetic data about Ca^{2+}-ATPase have been published. The following scheme (Fig. 4-I) has been proposed to explain these data (see also 304,306). $R\text{-}C_1$ and $R\text{-}C_2$ are two different conformations of the enzyme, R being a regulatory subunit, C a catalytic subunit. The $R\text{-}C_1$ complex can be phosphorylated in the presence of Ca^{2+} (step II; 163,174,261,306). The complex may be

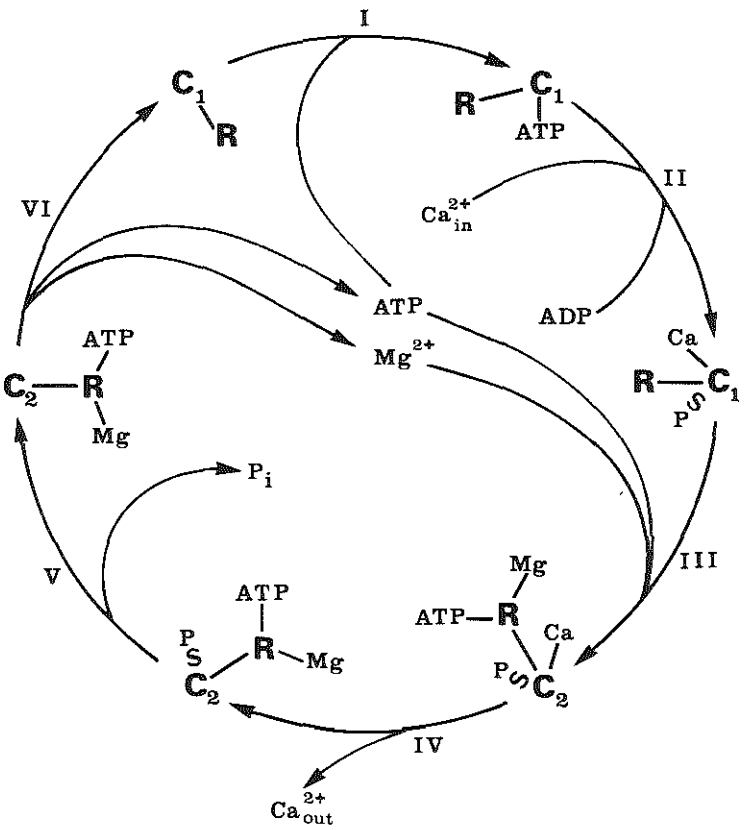


Fig. 4-I. Kinetic model for Ca^{2+}-ATPase and Ca^{2+}-transport in human erythrocytes.

dephosphorylated in the absence of Mg^{2+} to a very low degree, but Mg^{2+} greatly stimulates the Ca^{2+}-ATPase activity and the dephosphorylation of the enzyme (step V; 261,267,305). In the kinetic model Mg^{2+} is bound to R of the phosphorylated complex together with ATP (step III). This scheme thus accounts for 2 types of ATP binding sites (267). Binding of Mg^{2+} and ATP induces a conformational change from $R\text{-}C_1$ to $R\text{-}C_2$, resulting in a diminished affinity for Ca^{2+}. The complex looses Ca^{2+} at the outside of the cell (step IV) and is dephosphorylated (step V). Thereafter Mg^{2+} and ATP leave $R\text{-}C_2$, which is then converted into $R\text{-}C_1$ (step VI).

Originally, MgATP was supposed to be the substrate for Ca^{2+}-ATPase, but recently more data appear, in favour of free ATP (see 261,294,300,305,381). Richards *et al.* (267) suggested that R has a low affinity for ATP, which could only bind in the presence of Mg^{2+}. His study infers that binding of ATP to R is not strictly required, but that the ATPase activity is enhanced by ATP binding, as is the dephosphorylation (163).

It is likely that the scheme of Fig. 4-I demonstrates only a simplified model of the enzyme kinetics. Wolf *et al.* (382) reported in a purified Ca^{2+}-ATPase preparation three different subunits, one of which could be phosphorylated. Allosteric effects of Ca^{2+} and ATP may point to an enzyme complex with several catalytic and/or regulatory subunits. Furthermore, some reaction steps are reversible (306). One important question is the stoichiometry of the pump. Some authors have reported a Ca^{2+}-ATP ratio of 1:1 (193,303), while others found a 2:1 ratio (256,293). In spite of these restrictions, the scheme may be useful in understanding the following experiments.

4.3.2. Ca^{2+}-PNPPase

Ca^{2+}-stimulated hydrolysis of *p*-nitrophenylphosphate

(Ca^{2+}-PNPPase) has been described previously in erythrocyte membranes (110,253,262). Ca^{2+}-PNPPase needs Ca^{2+}, Mg^{2+} and ATP. The substrate *p*-nitrophenylphosphate inhibits the active Ca^{2+}-efflux (262). Analogous to K^{+}-PNPPase (see 4.2.), one could suppose that Ca^{2+}-PNPPase represents the dephosphorylation of Ca^{2+}-ATPase (step IV-V in Fig. 4-I; 266). But the dephosphorylation is not stimulated by Ca^{2+} (261), while Ca^{2+}-PNPPase is. The only Ca^{2+}-stimulated step in the scheme is step II. It is unlikely, that *p*-nitrophenyl-phosphate can replace ATP in forming the high energetic phosphorylated enzyme. Richard *et al.* (266) concluded from the results of phospholipase C-treated membranes that the R-C_1-ATP complex is required for Ca^{2+}-PNPPase activity. This complex, without forming the phospho-enzyme, could hydrolyse *p*-nitrophenylphosphate in the presence of Mg^{2+} under the influence of Ca^{2+}. The exact relationship between Ca^{2+}-stimulated ATPase and PNPPase has yet to be established. The PNPPase has the advantage of being easily determinable.

We have estimated the Ca^{2+}-PNPPase activity in erythrocyte membranes from patients with either the congenital myotonic type or Duchenne type of muscular dystrophy (287; appendix III). In Duchenne dystrophy the specific activity at 10 mM substrate was increased twofold, the maximal velocity being about 3 times higher than in controls. The pH optimum, concentration with half-maximal velocity of and the Hill-coefficient for *p*-nitrophenylphosphate, and the half-maximal activation by Ca^{2+} were unchanged. It seems that the enzyme molecules may have normal properties, but that the amount is increased in Duchenne membranes. The velocity of the enzyme in myotonic dystrophy was not significantly increased.

4.3.3. Ca^{2+}-ATPase

The increased Ca^{2+}-PNPPase activity in Duchenne dystrophy (see 4.3.2.) may be linked with the increased

activity of Ca^{2+}-ATPase, reported by Hodson and Pleasure (133). Therefore, we have also analysed the kinetic parameters of Ca^{2+}-ATPase (287; appendix III). The activities at 0.18 and 0.97 mM and at infinite MgATP concentration are about two times increased in both groups of patients, as compared with control erythrocytes. The apparent affinity of the enzyme for MgATP is normal in Duchenne preparations. The Duchenne enzyme reacts with Ca^{2+} and Ca^{2+}-dependent stimulator protein in a normal manner. This protein which is present in the soluble fraction of human erythrocytes (28,151) likely enhances the affinity of Ca^{2+}-ATPase for Ca^{2+} (117,302). Perhaps it binds to the catalytic subunit C (see Fig. 4-I), thereby regulating the Ca^{2+}-ATPase activity (302). It stimulates the Ca^{2+}-extrusion out of the red cell (203). It has been demonstrated to stimulate cyclic AMP phoshodiesterase and adenylate cyclase activities in other cell types (for a review, see 56;151). The stimulator interacts normally with Ca^{2+}-ATPase in Duchenne membranes. The ratio of protein stimulated to unstimulated activity is similar in Duchenne and control membranes (1.8 at 500 µM Ca^{2+} and 7.8 at 50 µM Ca^{2+}). The enhanced Ca^{2+}-ATPase activity in the Duchenne membranes can therefore not be ascribed to the presence of more stimulator protein. The cause of the high activity seems, as was concluded for Ca^{2+}-PNPPase, an increased amount of normal enzyme molecules. This is not in agreement with the finding of Hodson and Pleasure (133), who reported a decreased K_m of MgATP for the Duchenne enzyme. But for controls they reported a K_m which was more than 10 times higher than the literature (304) and our values.

The Ca^{2+}-ATPase activities have been determined in the presence of Na^+: 140 mM in the study of Hodson and Pleasure (133) and 70 mM in our study (287; appendix III). K^+ and Na^+ each stimulate an ATPase activity in the presence of Ca^{2+} (29,307). The authors of the latter study suggested that the stimulation by the monovalent cations was due to an

uncoupling of (Na^+ + K^+)-stimulated ATPase by Ca^{2+}. Recent studies, however, indicate that the (Ca^{2+} + Na^+/K^+)-stimulated ATPase shows parallelism with Ca^{2+}-ATPase after different treatments of red cell membranes (265,266). Na^+ and K^+ stimulate the Ca^{2+}-transport in membrane vesicles (294). These and other studies (301) suggest that Na^+ and K^+ can influence Ca^{2+}-ATPase *per se*. Na^+ and K^+ may stimulate both the phosphorylation and dephosphorylation of the enzyme molecule (263). Scharff (301) concluded from kinetic studies on stimulator associated and dissociated Ca^{2+}-ATPase, that monovalent cations interact with the Ca^{2+} binding sites.

In appendix III is reported that Ca^{2+}-ATPase in control membranes is activated by Na^+ according to the Michaelis-Menten kinetics with a half-maximal activating concentration of 26 mM, which is in agreement with the study of Scharff (301). The Duchenne enzyme shows a biphasic interaction with Na^+, resulting in half-maximal activating concentrations of 4 and 58 mM Na^+. The point of intersection in a reciprocal plot of the activity against the Na^+ concentration lies at 34 mM Na^+. Preliminary experiments demonstrate a comparable behaviour of Ca^{2+}-ATPase from membranes of Duchenne carriers (Scholte and Houwen, personal communication). Thus in the membranes of Duchenne patients and carriers an extra ATPase activity can be measured at low Na^+ concentrations. As the physiological Na^+ concentration is about 10 mM (241), this enzyme activity may have a role *in vivo*. At 1 mM MgATP and 0.5 mM Ca^{2+}, the Ca^{2+}-ATPase activity in controls is 2.2, and in Duchennes 4.2 nmoles/mg protein/min, both at 10 mM Na^+. This means that under these conditions the Ca^{2+}-ATPase activity in Duchenne membranes is elevated by 90%.

Table 4-II summarizes the results of the Na^+ stimulation of Ca^{2+}-ATPase in myotonic muscular dystrophy. The first two membrane preparations interact in a monophasic manner with Na^+, the other three in a biphasic manner. The first two

TABLE 4-II. Na^+-DEPENDENCY OF Ca^{2+}-ATPase IN ERYTHROCYTE MEMBRANES IN MYOTONIC MUSCULAR DYSTROPHY (CONGENITAL TYPE). K_a is the apparent concentration of Na^+, which stimulates half-maximal (for assay, see appendix III).

Patient	Point of intersection	K_a (low Na^+-affinity site)	K_a (high Na^+-affinity site)
I	absent	21.9 mM	absent
II	absent	12.6 mM	absent
III	55.5 mM	38.3 mM	1.6 mM
IV	40.0 mM	30.5 mM	2.0 mM
V	34.5 mM	45.7 mM	0 mM

membranes have a K_a within the control range. The second three ones have K_a-values which are dissimilar with that of Duchenne values. Especially the K_a's of the high Na^+ affinity site are lowered in these three myotonic patients.

Some experiments were performed in order to elucidate the origin of the abnormal influence of Na^+ upon Ca^{2+}-ATPase in Duchenne erythrocytes. Replacement of Na^+ by K^+ gives essentially similar results. This fact supports the idea that in our study we measure indeed the monovalent cation-stimulated Ca^{2+}-ATPase. Addition of 0.2 mM La^{3+}, an inhibitor of Ca^{2+}-ATPase (193,256,342), results in inhibition of the Duchenne enzyme, maintaining the biphasic behaviour to Na^+. The half-maximal activation constants for Na^+ remain unchanged, suggesting that the extra enzymatic activity in Duchenne membranes is related to the Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase. Thus, the involvement of Ca^{2+}-stimulated ATPase (not Mg^{2+}-dependent) present in spectrin preparations (171) in these studies can be excluded. Other arguments are the low activity of this ATPase under the conditions applied, and the fact that contractile proteins are largely removed at low ionic strength and in the presence of Ca^{2+}-chelators (12,171,272).

Table 4-III shows the effect of the stimulator protein, ethanol and epinephrine upon the Na^+-dependency of Ca^{2+}-

TABLE 4-III. INFLUENCE OF Na^+ UPON Ca^{2+}-ATPase ACTIVITY IN DUCHENNE AND CONTROL ERYTHROCYTE MEMBRANES IN THE PRESENCE OF SOME COMPOUNDS (for assay, see appendix III). K_a is the apparent concentration of Na^+ in mM, resulting in half-maximal stimulation (means ± SEM)

Type of membrane	Addition	Point of intersection ($[Na^+]$ in mM)	K_a for low Na^+-affinity site	K_a for high Na^+-affinity site
Duchenne (n=4)	-	34±2	58±9	4±1
Duchenne (n=2)	stimulator protein	28;35	32;66	3.6;6.7
Duchenne (n=3)	0.5 M ethanol	45±6	56±18	9±2
Control (n=4)	-	absent	26±9	absent
Control (n=3)	stimulator protein	absent	16±6	absent
Control (n=3)	0.5 M ethanol	38±3	42±8	5±2
Control (n=4)	10^{-6} M epinephrine	23±1	40±12	see text

The stimulator protein has no influence upon the activation coefficients in both types of membranes. In one experiment control membrane was assayed in the presence of phenytoin, as this drug stabilizes the membrane likely via a Ca^{2+}-effect (see 3.3.2.). A biphasic curve in a reciprocal plot of the activity against Na^+ concentration was the result, comparable with the curve of Duchenne membranes without phenytoin. The effect, however, was caused by the solvent of the drug: ethanol. As can be seen in Table 4-III, 0.5 M ethanol has hardly any effect upon the Duchenne ATPase, while the control enzyme is drastically influenced. We wondered whether the ethanol effect on control membranes was mediated by a receptor, as the effect was also found at 1 mM ethanol. As shown in Fig. 4-II, 1 µM epinephrine induces a biphasic behaviour of control Ca^{2+}-ATPase towards Na^+. The affinity at Na^+ concentrations above 23 mM is similar to that of the Duchenne enzyme without epinephrine, and also similar to that of the control enzyme, measured in the presence of 0.5 M ethanol (see Table 4-III). At lower Na^+ concentrations a stimulating effect of epinephrine was

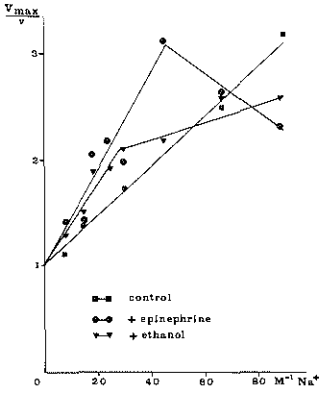


Fig. 4-II. Influence of epinephrine (10^{-6} M) and ethanol (0.5 M) on the Na^+-dependency of Ca^{2+}-ATPase in control red cell membranes.

found, which was increasing at decreasing Na^+ concentrations. Thus it seems that ethanol and epinephrine unmask a binding site for Na^+ at the Ca^{2+}-ATPase molecule, which cannot be occupied under standard assay conditions. This binding site is always accessable on the Duchenne enzyme. The difference between the curve for control ATPase plus epinephrine and the curve for Duchenne enzyme is not clear. It may be that the epinephrine effect is concentration-dependent.

It has to be mentioned that the erythrocyte membranes are washed several times. Therefore, it is likely that epinephrine or epinephrine-like substances have been removed from them. This suggests that the membrane properties are changed either during synthesis or later by an irreversible alteration in dystrophic blood.

4.4. *Adenylate cyclase*

Recently, a low activity of adenylate cyclase has been established in human red cell membranes (159,270). The activity is slightly stimulated by adrenergic agents

and prostaglandins, while F^--ions stimulate to a higher degree. Ca^{2+} at a concentration above 10 µM is inhibitory (260,270).

Mawatari and coworkers (210) reported that in membranes from Duchenne patients and carriers adenylate cyclase was somewhat more active than in control preparations. F^--ions stimulated the cyclase to control values. In the presence of epinephrine the activity in Duchennes was lower than in controls. Thus, the hormone sensitivity of the enzyme is diminished. The cause may be rather a membrane defect than a high *in vivo* stimulation by catecholamines. These compounds are excreted in the urine of patients to a normal level (214).

In myotonic dystrophy a normal basal activity and F^--stimulation was found, with a reduced epinephrine response (but higher than in Duchenne membranes) (210).

4.5. *Acetylcholinesterase*

Acetylcholinesterase activity in human erythrocytes is localized on the outer surface of the cell membrane (165). Its function is still obscure. It is likely that an acetylcholine receptor is also present in human erythrocytes (141) and it may be coupled to the Na^+ channel (142).

Das (69) reported that acetylcholinesterase in erythrocytes from Duchenne patients and carriers react abnormally to various inhibitors of the enzyme. He claimed that the inhibition rate of nialamide and eserine was a good tool for carrier detection. In another study a lowered activity was found in Duchenne dystrophy (113). However, in an extensive kinetic study, Scholte and Houwen (311) found a quite normal acetylcholinesterase activity and behaviour in erythrocyte membranes from Duchenne patients and carriers and from patients with congenital myotonic muscular dystrophy. The study included the interaction of various inhibitors with the enzyme, the

activation energy and heat stability measurements. Normal Hill-coefficients for F^- and imipramine inhibition likely point to an unchanged lipid fluidity in the vicinity of the enzyme (see 26,96).

4.6. *Phospholipase*

Iyer *et al.* (149) reported that in red cell membranes from Duchenne and myotonic muscular dystrophic patients the phospholipase A activity was increased by 60 and 240%, respectively. In spite of the low activity (3-9 nU/mg protein), the increment may point to an increased breakdown of the membrane. Phenytoin normalized the hydrolytic activity, probably via a Ca^{2+}-membrane interaction (see 3.3.2.).

4.7. *Long chain acyl-CoA synthetase and carnitine palmitoyltransferase*

Acyl-CoA synthetase produces acyl-CoA esters, the substrates for the incorporation of fatty acids into the membrane. This enzyme may determine the relative availability of the activated fatty acids for the renewal of the membrane. Furthermore, its activity may give insight into the (ab-)normality of the microenvironment of the enzyme.

We have tested the activation of palmitic, palmitoleic and linoleic acid in patient and control membranes (288; appendix IV). Red cell membranes from patients with congenital myotonic dystrophy reveal a twofold increase in acyl-CoA synthetase activity. Membranes of Duchenne patients were normally active. The activity was independent from the fatty acid in all groups of membranes. It is evident that these findings cannot explain the diminished palmitoleic acid content of the membrane neutral lipid fractions in Duchenne and myotonic dystrophy (286; appendix I). The increased fatty acid activation may, combined with the

enhanced phospholipase activity (see 4.6.) point to a more rapid turnover of the phospholipids in myotonic muscular dystrophy.

Leucocyte homogenates from patients with congenital myotonic dystrophy possess a normal acyl-CoA synthetase activity. This activity is higher with palmitate than with palmitoleate or linoleate, independently from the investigated group. The differences in properties of acyl-CoA synthetase in leucocyte homogenates and erythrocyte membrane may be due to a different subcellular localization, accompanied with a different microenvironment.

The presence of carnitine palmitoyltransferase in human erythrocyte membranes was first demonstrated by Wittels and Hochstein (380). The function of this enzyme is not known. It may be concerned in transport or storage of acylcarnitine (see also appendix IV).

We have tested the carnitine palmitoyltransferase I activity at 1.6 and 8.0 μM palmitoyl-CoA in patient's red cell membranes (288; appendix IV). No alterations were found, indicating a normal activity and normal affinity of the enzyme in erythrocytes in human muscular dystrophy.

4.8. *Discussion*

This chapter summarizes the features of the proteins of the red cell membranes in human muscular dystrophy. The enhanced phosphorylation rate of spectrin in Duchenne membranes (see 4.1.) may support the involvement of this structural protein as discussed in section 3.6. The abnormalities found for Ca^{2+}-ATPase, adenylate cyclase, phospholipase and acyl-CoA synthetase in one or both types of dystrophy can hardly be explained by a change in a single definite phospholipid, interfering with the micro-environments of the various enzymes. Neither can they easily be understood by an adaptation to one metabolic alteration. In section 4.3.3. the fact has been mentioned

that ethanol and epinephrine are able to uncover a high-affinity site for Na^+ in Ca^{2+}-ATPase of control membranes, which is comparable with Duchenne membranes. As pointed out in section 3.6., epinephrine exercises a radical influence upon red cell membranes. It seems reasonable to suppose that enzyme activities undergo changes under that condition. This may form the origin of the various deviations, described in this chapter. A possible role of epinephrine or related compounds and the consequences of it will be further discussed in chapter 5.

CHAPTER V

THE EPINEPHRINE-Ca^{2+} HYPOTHESIS; THE POSSIBLE INVOLVEMENT OF OTHER CELLS; FINAL REMARKS AND PERSPECTIVES

It may be clear from the foregoing chapters, that erythrocyte membranes from patients with myotonic and Duchenne muscular dystrophy are altered in several respects. Whether the alterations are caused by a change of one or more membrane characteristics is not clear, but it is likely that Ca^{2+} plays a role. The fact, that some alterations are different and other ones similar in both diseases may point to a different origin, causing several secondary, including common, effects. In the next two sections the possible origin of the modifications will be discussed separately for the two disorders.

5.1. *Erythrocytes in Duchenne muscular dystrophy; the epinephrine-Ca^{2+} hypothesis*

Several times an involvement of hormones (see sections 3.4., 3.6., 4.2., 4.3.3., 4.4. and 4.8.) or Ca^{2+} (see sections 3.1., 3.3.2., 3.4., 3.5., 3.6., 4.3.2., 4.3.3., 4.6. and 4.8.) is proposed to explain the abnormalities of Duchenne erythrocytes. Most of the observed changes can be explained by the following epinephrine-Ca^{2+} hypothesis.

1) In red cells of Duchenne patients the β-adrenergic receptor has a conformational state, which simulates a receptor, loaded with its effector epinephrine.
2) This state causes enhanced basal adenylate cyclase activity, reduced epinephrine stimulation, and normal F^--stimulation of adenylate cyclase. This situation has been described in Duchenne erythrocytes (see 4.4.).

3) The state is accompanied with increased phosphorylation of specific membrane components, e.g. of band III (see 4.1.), and with enhanced affinity of certain membrane moieties for Ca^{2+} (see 260). The change in the Ca^{2+}-membrane interaction may be responsible for the high sensitivity of Duchenne erythrocytes towards Ca^{2+} (77).
4) The β-adrenergic stimulated state leads to unmasking of a high affinity activating site for Na^{+} at Ca^{2+}-ATPase. The site is overt in Duchenne membranes (see 4.3.3.).
5) The epinephrine stimulated state explains the enhanced rigidity (4,188,260) and the reduced osmotic stability (260), which have been found in Duchenne cells (see 3.4. and 3.5.). These effects are the consequences of the intensified Ca^{2+}-membrane interaction (170,375). Another consequence of this interaction may be an augmented echinocyte formation (see 3.1.; 320,373).
6) The high Ca^{2+} affinity of certain membrane groups leads to increased Na^{+} levels and K^{+} permeability, effects described for control cells loaded with Ca^{2+} (84,170, 292). These changes in cation concentration and permeability have been observed in untreated Duchenne cells (78,136).
7) Probably the phosphorylation change causes the non-uniform distribution of proteins (370), the delayed energy transfer in saturating transfer ESR studies (378) and the alteration in protein SH-groups (45), reported in Duchenne membranes (see 3.3.2. and 3.3.3.).

Whether cyclic AMP plays a role in the phosphorylation and/or other changed properties of the Duchenne erythrocyte is uncertain. Although Rasmussen *et al.* (260) could not detect epinephrine-stimulated cyclic AMP synthesis, they were able to mimic the hormone effect with exogenous cyclic AMP. Maybe epinephrine alters the affinity of the nucleotide binding site of the membrane-bound, cyclic AMP-dependent protein kinase as they were detected by Fairbanks and Avruch (95) and Clari *et al.* (57). It is

hypothetical that cyclic AMP is involved in the rapid-exchangeable, oscillating Ca^{2+}-pool of red cells, as described by Rasmussen *et al.* (259,260).

It would be useful to check the hypothesis at various points. E.g. the normal fluidity observed by Butterfield *et al.* (47) does not support this hypothesis, since epinephrine induces an enhancement of the microviscosity (188). The pedigree-specific enhanced phosphorylation of some spectrin bands (281) is not explained by the concept. Measurements of the rapid-exchangeable Ca^{2+}-pool (260) in Duchenne erythrocytes may give useful information, as do studies on the affinity and capacity of membrane fractions to bind Ca^{2+} (see 103,223,296).

The primary cause of the hormone-stimulated state of the red cell in Duchenne dystrophy is not known. It is not likely, that it is caused by an increased level of catecholamines, as Mendell *et al.* (214) reported normal urine levels of these compounds and metabolic products in patients. Moreover, the hormone should be washed out from the cells during membrane isolation. This argument also holds for the various possible effectors which are present in the blood after muscle damage. The cause may rather be a true membrane defect. In this context it would be of interest to check the metabolism of phosphatidylinositol. This lipid plays a role in hormone-receptor interactions and ion transport (128,217). In these processes Ca^{2+} and cyclic AMP may also be involved. It is meaningful to mention here that erythrocyte membranes with a high content of polyphosphatidylinositide can bind more Ca^{2+} and reveal high Ca^{2+}-ATPase and normal $(Na^{+} + K^{+})$-ATPase activity (41). The phosphorylation of phosphatidylinositol, located at the inner side of the membrane (111), is drastically inhibited at elevated endogenous levels of Ca^{2+} (41). The breakdown of polyphosphatidylinositide may also be regulated by the intracellular Ca^{2+} level (3). There is no evidence suggesting that the reduced level of palmitoleic

acid in diglycerides of Duchenne red cells (286; appendix I) is involved in these metabolic pathways.

Further insight into the origin of the alterations may be obtained by treatment of the membranes with pronase and/or diethylether, which results in perturbation of well defined membrane portions (65). The treatment may elucidate whether and which type of lipids are important for the receptor conformation change. The importance of spectrin can be determined in cytochalasin-treated membranes; this drug interferes with the contractile apparatus (93,141).

Prostaglandin E_2 influences the rigidity, lysis, lipid fluidity and membrane-Ca^{2+} interaction of red cells in a similar manner as does epinephrine (188,260). But there also exist differences between effects of these hormones. The fact, that prostaglandin E_2 increases the 2,3-diphosphoglycerate level, while catecholamines do not (156) is in favour of a role for the epinephrine receptor.

5.2. *Erythrocytes in myotonic muscular dystrophy*

Research data about red cells in myotonic dystrophy are scarce. As pointed out already, some deviations are identical with those found in Duchenne dystrophy. However, the increased lipid fluidity (see 3.3.2.) and the aberrant behaviour after phospholipase-treatment (see 3.5.) cannot be explained by the hypothesis outlined above for Duchenne dystrophy. In section 3.5. the osmotic behaviour of the myotonic red cells has been suggested to be due to an acetylcholine receptor, which is masked or less active than in control cells. Little is known about the effect of cholinergic stimulation on erythrocyte metabolism. At the present, it cannot be concluded that the other abnormalities, like increased viscoelasticity and hemolysis (see 3.4. and 3.5.) can also be explained by a reduced influence of cholinergic compounds upon myotonic cells.

Indicative for an acetylcholine receptor impairment in

myotonia may be the finding that (drug-induced) myotonic rats show myotonic discharges which could be evoked by stimulators of the cholinergic receptor and silenced by tetrodotoxin, a Na^+-transport inhibitor (40). A coupling between the cholinergic receptor and Na^+-ion channel is widely spread in nature (140) and maybe also in erythrocytes (141).

Abnormal high fatty acid activation rates were found in this disorder (see 4.7.). A stimulation of fatty acid incorporation in normal erythrocytes by Ca^{2+} has been reported (76). This, combined with the elevated Ca^{2+}-ATPase activity (see 4.3.3.), the increased Ca^{2+} permeability (251), and the fact, that phenytoin normalizes the phospholipase activity and the microviscosity (as measured by ESR) (see 3.3.2. and 4.6.) point to an involvement of calcium in the red cell of myotonic muscular dystrophy patients. Whether acetylcholine receptor and calcium functional impairments are interrelated, and whether they are the real origin(s) of the alterations, remain to be established. Studies, in which various stimulatory and inhibitory effects upon normal and myotonic red cells are compared, may be useful in elucidating these problems.

5.3. *Non-erythroid cells in human muscular dystrophy*

After considering the red cells, the problem arises, whether the alterations are specific for this cell type. Apart from studies on muscular tissue only few data have been published about non-erythroid cells.

5.3.1. White blood cells, platelets, fibroblasts.

Pickard *et al.* (249) reported diminished cap formation in lymphocytes of Duchenne patients and of most carriers. An alteration of the membrane fluidity was suggested, probably combined with a defect of the contractile proteins or Ca^{2+}-metabolism. Normal cap

formation was observed in myotonic dystrophy. Abnormal chemotaxic activity and chemiluminescence have been described in polymorphonuclear leucocytes in myotonic dystrophy (316). A discrepancy exists about the number of insulin binding sites in monocytes in this disorder (100, 175). Normal fatty acid activation has been described in leucocytes from both Duchenne and congenital myotonic dystrophy patients (288; appendix IV). Normal ratio of active and inactive phosphorylase in leucocytes from Duchenne and myotonic patients has been found (310), suggesting normal cyclic AMP levels. The platelets seem also affected in muscular dystrophy. An increased aggregation of them by epinephrine, owing to abnormal α-receptors was found (54) in myotonic dystrophy. In Duchenne platelets a diminished serotonin uptake (227) and an augmented oxidation of epinephrine and dihydroxy-phenylalanin (72) was reported. The isoenzyme pattern of this oxidase is normal (230).

A few studies have been published, in which the characteristics of cultivated fibroblasts from dystrophic patients have been analysed. Kohlschütter *et al.* (179) found normal phospholipid and fatty acid composition. Pena *et al.* (239) found no abnormalities in the major protein fractions. However, Ionasescu *et al.* (147) reported differences in collagen and non-collagen protein synthesis. Characteristic inclusion bodies were described (386). Normal growth and acetate incorporation in various lipid classes were found in fibroblasts from patients with myotonic muscular dystrophy.

These results with white blood cells, platelets and fibroblasts do not clash with the suggested hypothesis proposed for patient's erythrocytes (see 5.1. and 5.2.), but on the other hand also do not support it.

5.3.2. Skeletal muscle cells

The epinephrine effect on skeletal muscle is rather complicated and fibre type dependent. The Ca^{2+}-pump of the sarcoplasmic reticulum may be enhanced by a cyclic AMP mediated stimulation of protein phosphorylation. Ca^{2+}-accumulation may be increased, and relaxation and tension parameters are changed (340). A higher Ca^{2+}-ATPase activity has indeed been found in Duchenne muscle (75).

In skeletal muscle of Duchenne and myotonic muscular dystrophy patients the stimulation of adenylate cyclase by epinephrine is reduced (see 1.5.1.), like in Duchenne red cells. However, in contrast to the red cell enzyme, the basal and fluoride-stimulated activities are normal and reduced, respectively. Thus, it is not allowed to conclude merely that in Duchenne muscle cells the epinephrine-like state is present (see 5.1.). But it is striking that a higher basal activity and a reduced stimulated activity of adenylate cyclase has been found in cultured myotubes from Duchenne tissue (211). Some evidence for a catecholamine involvement may be the enhanced fluorescence, possibly owing to catecholamine accumulation, which seems specific for Duchenne fibres (309,384). Takamori (347) reported a super-sensitivity of muscles from Duchenne carriers to epinephrine as to contraction-relaxation parameters. He considered this deviation to be a property of the dystrophic muscle.

Summarizing, we may conclude that the epinephrine-Ca^{2+} hypothesis may hold for muscle tissue in Duchenne patients, too, but the support for it is scarce. It looks reasonable to suspect an improvement by insulin, if the muscle cells of Duchenne patients are epinephrine-stimulated, as insulin often inhibits catecholamine-induced stimulations (264). In 1950, Mayerhofer already reported clinical improvement by insulin (212) but no other cases have since been reported. The fasting insulin level and secretion seems to be normal (129), while the glucose tolerance is normal

(345) or decreased (129). It may be that during glucose-insulin-infusion the leakage of muscle compounds into the bloodstream is diminished (25). After infusion serum enzyme activities return to the original levels (see also 268). Glycogen, the synthesis and breakdown of which is influenced by insulin and catecholamines, has a normal or somewhat lower concentration in skeletal muscle of Duchenne patients (131,337). Thus, these data also do not give a definite answer to the problem of the epinephrine receptor state in Duchenne muscular dystrophy.

Some aspects of red cells in myotonic dystrophy could be explained by an altered acetylcholine receptor structure or structural change in its environment (see 5.2.). Although the influence of cholinergic agents on skeletal muscle has been described (see 255), other available data for myotonic dystrophy do not allow to conclude whether the receptor plays a role of importance. It has to be established whether the hyperinsulinism, described in myotonic dystrophy (see 225), is important in the explanation of the pathogenesis of the disease. Its effect upon erythrocytes will be low, since glucose transport in red cells is insensitive to insulin (158).

Like in erythrocytes, Ca^{2+} may play a central role in Duchenne muscle tissue. The increased Ca^{2+}-ATPase and decreased capacity for accumulating Ca^{2+} are examples. The drastical accumulation of Ca^{2+} in Duchenne fibres (27) may lead to cell necrosis in later stages. The role of Ca^{2+} in fibre necrosis has been discussed in the literature (91,348, 385,388).

The most promising strategy at this moment seems to elucidate first the primary cause of the red cell abnormalities. The same factor may be responsible in the diseased skeletal muscle. From the available data it is not possible to conclude that the aberrations are primarily caused by a membrane factor. The membrane is involved, but it cannot be excluded that a serum factor has induced the

membrane changes. The possible importance of a serum factor has been proposed by W.K. Engel and coworkers (see 1.4.), who obtained evidence predominantly from animal models. In few studies an influence of dystrophic serum has been reported. Sugita and Tyler (339) observed that serum from Duchenne patients induces leakage of enzymes from rat muscle *in vitro*. This finding has not been confirmed. Peter *et al.* (247) found an abnormal ouabain inhibition of $(Na^+ + K^+)$-ATPase in control red cells, incubated in Duchenne serum. Dubowitz *et al.* (81) observed abnormal growth of Duchenne muscle in culture only in the presence of dystrophic serum. An inhibitor of fibroblast outgrowth was found in urine of Duchenne patients (122). All these effects may be due to some compounds leaked from the affected muscle cells. Another explanation is, that it is the factor which attacks the various cell membranes, with the fatal consequence for the muscles.

5.4. *Treatment and prenatal diagnosis of patients; carrier detection*

Returning to the purpose of this study (see 1.6.) it is clear that with the present knowledge of the origin of Duchenne muscular dystrophy it is impossible to prevent the dystrophic process by some drug or other treatment. Further research with the catecholamine metabolism in the centre may be fruitful for basic knowledge and patient care. The same, yet gloomy, perspective has to be given for myotonic muscular dystrophy. A few starting-points for further research in this disorder have been mentioned (see 5.2.).

The only expedient of prenatal diagnosis in Duchenne dystrophy hitherto is the determination of the sex of the fetus. In cultivated fibroblasts our group is trying to find reliable alterations which may be used in cultivated

amniotic cells with a prospecting value for the condition of the fetus. Also determinations of the most promising features, found in our erythrocyte studies (see appendix III) will be performed in order to check their applicability in amniotic fluid cells and erythrocytes from fetal blood. Whether fetal blood also offers the possibility in distinguishing affected and healthy fetuses in the case of Duchenne dystrophy by determination of creatine phosphokinase activity is open to question (82,89,148,204). Muscle abnormalities in fetuses at risk for Duchenne dystrophy have been described (358,363,379).

Prenatal diagnosis in myotonic muscular dystrophy is feasible in special cases. The test is based on the determination of blood group substances in the amniotic fluid. These compounds are only found in the case of a secretor-positive fetus. The secretor locus is closely linked to the locus coding for myotonic dystrophy (314, 315).

The early detection of heterozygotes with the gene for myotonic dystrophy has been described (42,252). The possibilities of detecting Duchenne carriers have recently been reviewed (127). An extensive research publication has appeared (356). However, at this moment it is still hard to detect the carrier state with a security of more than about 75%. A screening program has been proposed for male children at the age of 18 months for detecting Duchenne dystrophy at an early time (108). We have now planned a study in which the applicability of the Ca^{2+}-ATPase deviations (see 4.3.3.) for Duchenne carriers will be checked.

REFERENCES

1. Adams, D., Markes, M.E., Leivo, W.J. and Carraway, K.L. (1976) Biochim. Biophys. Acta 426, 38.
2. Adornato, B.T., Corash, L. and Engel, W.K. (1977) Neurology 27, 1093.
3. Allan, D. and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277.
4. Allen, J.E. and Rasmussen, H. (1971) Science 174, 512.
5. Almeida, A.F. and Charnock, J.S. (1977) Biochim. Biophys. Acta 467, 19.
6. Aloni, B., Shinitzky, M. and Livne, A. (1974) Biochim. Biophys. Acta 348, 438.
7. Anderson, P.C. and Martt, J.M. (1965) Arch. Dermatol. 92, 181.
8. Andiman, R.M., Peter, J.B. and Dhopeshwarkar, G. (1974) Neurology 24, 352.
9. Appenzeller, O. and Ogin, G. (1975) Arch. Neurol. 32, 2.
10. Araki, S. and Mawatari, S. (1971) Arch. Neurol. 24, 187.
11. Attley, J. (1974) Lancet II, 42.
12. Avissar, N., De Vries, A., Ben-Shaul, Y. and Cohen, I. (1975) Biochim. Biophys. Acta 375, 35.
13. Avruch, J. and Fairbanks, G. (1974) Biochemistry 13, 5507.
14. Bajolet, A. (1975) Semaine Hop. 51, 370.
15. Ballantyne, J.P. and Hansen, S. (1974) J. Neurol. Neurosurg. Psychiat. 37, 1195.
16. Bank, W.J., Rowland, L.P. and Ipsen, J. (1971) Arch. Neurol. 24, 176.
17. Barchi, R.L. (1975) Arch. Neurol. 32, 175.
18. Beckmann, R. (1978) Klin. Pädiat. 190, 531.
19. Bell, D.B. and Smith, D.W. (1972) J. Pediat. 81, 83.
20. Berger, K.U., Barratt, M.D. and Kamat, V.B. (1970) Biochem. Biophys. Res. Commun. 40, 1273.
21. Berliner, L.J. (1976) Spin labeling; theory and applications; Academic Press, New York.
22. Bessis, M. (1972) Nouv. Rev. Franç. Hématol. 12, 721.
23. Bieri, V.G. and Wallach, D.F.H. (1976) Biochim. Biophys. Acta 443, 198.
24. Birchmeier, W. and Singer S.J. (1977) J. Cell Biol. 73, 647.
25. Blietz, R., Kruchten, J., Riedel, K. and Wagner, J. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 1609.
26. Bloj, B., Morero, R.D., Farías, R.N. and Trucco, R.E. (1973) Biochim. Biophys. Acta 311, 67.
27. Bodensteiner, J.B. and Engel, A.G. (1978) Neurology 28, 439.
28. Bond, G.H. and Clough, D.L. (1973) Biochim. Biophys. Acta 323, 592.
29. Bond, G.H. and Green, J.W. (1971) Biochim. Biophys. Acta 241, 393.
30. Bonilla, E., Schotland, D.L. and Wakayama, Y. (1978) Ann. Neurol. 4, 117.
31. Bonting, S.L. (1970) in: Membranes and Ion Transport (Bittar, E.E., ed.), vol. I, Wiley-Interscience, London, pp. 257.
32. Bosia, A., Pescarmona, G.P. and Arese, P. (1971) Eur. J. Clin. Invest. 1, 413.
33. Bosmann, H.B., Gersten, D.M., Criggs, R.C., Howland, J.L., Hudecki, M.S., Katyare, S. and McLaughlin, J. (1976) Arch. Neurol. 33, 135.

34. Bradley, W.G. (1974) Nature 250, 285.
35. Bradley, W.G., O'Brien, M.D., Walder, D.N., Murchison, D., Johnson, M. and Newell, D.J. (1975) Arch. Neurol. 32, 466.
36. Brockerhoff, H. (1974) Lipids 9, 645.
37. Brooke, M.H. and Kaiser, K.K. (1974) Ann. N.Y. Acad. Sci. 228, 121.
38. Brooks, A.P. and Emery, A.E.H. (1977) Clin. Genet. 11, 290.
39. Brown, H.D., Chattopadhyay, S.K. and Patel, A.B. (1967) Science 157, 1577.
40. Brumback, R.A., Bertorini, T.E., Engel, W.K., Trotter, J.L., Oliver, K.L. and Zirzow, G.C. (1978) Arch. Neurol. 35, 8.
41. Buckley, J.T. and Hawthorne, J.N. (1972) J. Biol. Chem. 247, 7218.
42. Bundey, S., Carter, C.O. and Soothill, J.F. (1970) J. Neurol. Neurosurg. Psychiat. 33, 279.
43. Butler, K.W., Smith, I.C.P. and Schneider, H. (1970) Biochim. Biophys. Acta 219, 514.
44. Butterfield, D.A. (1977) Accounts Chem. Res. 10, 111.
45. Butterfield, D.A. (1977) Biochim. Biophys. Acta 470, 1.
46. Butterfield, D.A., Roses, A.D., Cooper, M.L., Appel, S.H. and Chesnut, D.B. (1974) Biochemistry 13, 5078.
47. Butterfield, D.A., Chesnut, D.B., Appel, S.H. and Roses, A.D. (1976) Nature 263, 159.
48. Butterfield, D.A., Roses, A.D., Appel, S.H. and Chesnut, D.B. (1976) Arch. Biochem. Biophys. 117, 226.
49. Butterfield, D.A., Whisnant, C.C. and Chesnut, D.B. (1976) Biochim. Biophys. Acta 426, 697.
50. Cadenhead, D.A. and Müller-Landau, F. (1973) Biochim. Biophys. Acta 307, 279.
51. Campbell, J.C.W., Suelter, C.H. and Puite, R.H. (1977) Clin. Chim. Acta 79, 379.
52. Canal, N., Frattola, L. and Smirne, S. (1975) J. Neurol. 208, 259.
53. Carraway, K.L., Triplett, R.B. and Anderson, D.R. (1975) Biochim. Biophys. Acta 379, 571.
54. Castaigne, P., Bousser, M.G., Lecrubier, C., Conard, J. and Samama, M. (1977) Rev. Neurol. 133, 673.
55. Chau-Wong, M. and Seeman, P. (1971) Biochim. Biophys. Acta 241, 473.
56. Cheung, W.Y., Lynch, T.J. and Wallace, R.W. (1978) in: Advances in Cyclic Nucleotide Research (George, W.J. and Ignarro, L.J., eds.), vol. 9, Raven Press, New York, pp. 233.
57. Clari, G., Michielin, E. and Moret, V. (1978) Biochim. Biophys. Acta 539, 420.
58. Coërs, C. (1977) Arch. Neurol. 34, 258.
59. Coërs, C. and Telerman-Toppet, N. (1977) Arch. Neurol. 34, 396.
60. Cohen, J. (1977) in: Advances in Neurology (Griggs, R.C. and Moxley, R.T. III, eds.), vol. 17, Raven Press, New York, pp. 325.
61. Coleman, R. (1973) Biochim. Biophys. Acta 300, 1.
62. Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzky, M. and Shattil, S.J. (1978) Biochemistry 17, 327.
63. Corsini, F. and Cacciari, E. (1958) Clin. Pediatr. (Bologna) 40, 743.

64. Cullen, M.J. and Fulthorpe, J.J. (1975) J. Neurol. Sci. 24, 179.
65. Cullis, P.R. and Grathwohl, Ch. (1977) Biochim. Biophys. Acta 471, 213.
66. Danieli, G.A., Mostacciuolo, M.L., Bonfante, A. and Angelini, C. (1977) Hum. Genet. 35, 225.
67. Danon, M.J., Marshall, W.E., Sarpel, G. and Omachi, A. (1978) Arch. Neurol. 35, 592.
68. Danowski, T.S., Wirth, P.M., Leinberger, M.H., Randall, L.A. and Peters, J.H. (1956) Amer. J. Dis. Child. 91, 346.
69. Das, P.K. (1975) in: Abhandlungen der Akademie der Wissenschaften der DDR (Rapoport, S. and Jung, F., eds.), Akademie-Verlag, Berlin, pp. 643.
70. Demel, R.A. and De Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109.
71. Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97.
72. Démos, J. (1973) Clin. Genet. 4, 79.
73. Desmedt, J.E. and Borenstein, S. (1973) Nature 246, 500.
74. Deuticke, B. (1968) Biochim. Biophys. Acta 163, 494.
75. Dhalla, N.S., McNamara, D.B., Balasubramanian, V., Greenlaw, R. and Tucker, F.R. (1973) Res. Commun. Chem. Pathol. Pharmacol. 6, 643.
76. Dise, C.A., Lake, W.C., Goodman, D.B.P. and Rasmussen, H. (1976) J. Biol. Chem. 251, 4162.
77. Dise, C.A., Goodman, D.B.P., Lake, W.C., Hodson, A. and Rasmussen, H. (1977) Biochem. Biophys. Res. Commun. 79, 1286.
78. Dowben, R.M. and Holley, K.R. (1959) J. Lab. Clin. Med. 54, 867.
79. Dubowitz, V. (1960) Brain 83, 432.
80. Dubowitz, V. and Brooke, M.H. (1973) Muscle Biopsy: A Modern Approach, W.B. Saunders, London.
81. Dubowitz, V., Gallup, B. and Witkowski, J. (1973) J. Physiol. 231, 61P.
82. Dubowitz, V., Van Iddekinge, B., Rodeck, C.H., Campbell, S., Singer, J.D., Scheuerbrandt, G. and Moss, D.W. (1978) Lancet I, 90.
83. Duchenne de Boulogne, G. (1868) Archives générales de médicine 11, 5, 179, 305, 421, 552.
84. Dunn, M.J. (1974) Biochim. Biophys. Acta 352, 97.
85. Ebashi, S., Toyokura, Y., Momoi, H. and Sugita, H. (1959) J. Biochem. (Tokyo) 46, 103.
86. Elgsaeter, A. and Branton, D. (1974) J. Cell. Biol. 63, 1018.
87. Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101.
88. Ellis, D.A., Strickland, J.M. and Eccleston, J.F. (1973) Clin. Sci. 44, 321.
89. Emery, A.E.H. (1977) Nature 266, 472.
90. Emery. A.E.H. and Gosden, C. (1974) J. Med. Genet. 11, 76.
91. Engel, W.K. (1977) in: Advances in Neurology (Griggs, R.C. and Moxley, R.T. III, eds.), vol. 17, Raven Press, New York, pp. 197.
92. Engel, W.K. and Derrer, E.C. (1975) Nature 254, 151.
93. Estensen, R.D., Rosenberg, M. and Sheridan, J.D. (1971) Science 173, 356.
94. Evans, E.A. and Hochmuth, R.M. (1976) Biophys. J. 16, 1.
95. Fairbanks, G. and Avruch, J. (1974) Biochemistry 13, 5514.

96. Farías, R.N., Bloj, B., Morero, R.D., Sineriz, F. and Trucco, R.E. (1975) Biochim. Biophys. Acta 415, 231.
97. Farquhar, J.W. and Ahrens, E.H. Jr. (1963) J. Clin. Invest. 42, 675.
98. Feinstein, M.B., Fernandez, S.M., Sha'afi, R.I. (1975) Biochim. Biophys. Acta 413, 354.
99. Fenichel, G.M. (1975) Developm. Med. Child Neurol. 17, 527.
100. Festoff, B.W. and Moore, W. (1978) Ann. Neurol. 4, 164.
101. Fisher, E.R., Silvestri, E., Vester, J.W., Nolan, S., Ahmad, U. and Danowski, T.S. (1976) J. Amer. Med. Ass. 236, 955.
102. Florek, M. and Karolak, S. (1977) Eur. J. Pediatr. 126, 275.
103. Forstner, J. and Manery, J.F. (1971) Biochem. J. 124, 563.
104. Fossel, E.T. and Solomon, A.K. (1977) Biochim. Biophys. Acta 464, 82.
105. Fossel, E.T. and Solomon, A.K. (1978) Biochim. Biophys. Acta 510, 99.
106. Franks, A.J. (1978) J. Pathol. 125, 213.
107. Freed, J.H. (1976) in: Spin Labeling; Theory and Applications (Berliner, L.J., ed.), Academic Press, New York, pp. 53.
108. Gardner-Medwin, D., Bundey, S. and Green, S. (1978) Lancet I, 1102.
109. Garrahan, P.J., Pouchan, M.I. and Rega, A.F. (1969) J. Physiol. 202, 305.
110. Garrahan, P.J., Pouchan, M.I. and Rega, A.F. (1970) J. Membr. Biol. 3, 26.
111. Garrett, R.J.B. and Redman, C.M. (1975) Biochim. Biophys. Acta 382, 58.
112. Godin, D.V., Bridges, M.A. and McLeod, P.J.M. (1978) Res. Commun. Chem. Pathol. Pharmacol. 20, 331.
113. Goedde, H.W., Benkmann, H.G., Das, P.K. and Agarwal, D.P., Lang, H., Würzburg, U., Beckmann, R. (1977) Klin. Wochenschr. 55, 215.
114. Goldberg, M.A. (1977) Neurology 27, 827.
115. Goldberg, M.A. and Crandall, P.H. (1978) Neurology 28, 881.
116. Gomez, M.R., Engel, A.G., Dewald, G. and Peterson, H.A. (1977) Neurology 27, 537.
117. Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203.
118. Grassi, E., Lucci, B., Marchini, C., Ottonello, S., Parma, M., Reggiani, R., Rossi, G.L. and Tagliavini, J. (1978) Neurology 28, 842.
119. Griggs, R.C. (1977) in: Advances in Neurology (Griggs, R.C. and Moxley, R.T. III, eds.), vol. 17, Raven Press, New York, pp. 1.
120. Griggs, R.C. (1977) in: Advances in Neurology (Griggs, R.C. and Moxley, R.T. III, eds.), vol. 17, Raven Press, New York, pp. 143.
121. Grimm, T. (1975) J. Genet. Human 23, suppl. 172.
122. Gross, S. (1977) Clin. Chem. 23, 299.
123. Guthrow, C.E., Allen, J.E. and Rasmussen, H. (1972) J. Biol. Chem. 247 8145.
124. Harper, P.S. (1975) Arch. Dis. Child. 50, 505.
125. Harper, P.S. (1975) Arch. Dis. Child. 50, 514.
126. Harper, P.S. and Dyken, P.R. (1972) Lancet II, 53.
127. Hausmanowa-Petrusewicz, I., Niebrój-Dobosz, I., Borkowska, J., Lukasik, E. and Liszewska-Pfejfer, D. (1977) in: Pathogenesis of Human Muscular Dystrophies (Rowland, L.P., ed.), Exerpta Medica, Amsterdam pp. 32.

128. Hayashi, F., Sokabe, M., Takagi, M., Hayashi, K. and Kishimoto, U. (1978) Biochim. Biophys. Acta 510, 305.
129. Herschberg, A.D. and Coirault, R. (1965) in: Myopathieën (Beckmann, R., ed.), G. Thieme Verlag, Stuttgart, pp. 127.
130. Heusinkveld, R.S., Goldstein, D.A., Weed, R.I. and LaCelle, P.L. (1977) Blood Cells 3, 175.
131. Heyck, H. and Laudahn, G. (1969) Die Progessiv-dystrophischen Myopathien, Springer-Verlag, Berlin.
132. Heymsfield, S.B., McNish, T., Perkins, J.V. and Felner, J.M. (1978) Amer. Heart J. 95, 283.
133. Hodson, A. and Pleasure, D. (1977) J. Neurol. Sci. 32, 361.
134. Hofmann, W.W. and DeNardo, G.L. (1968) Amer. J. Physiol. 214, 330.
135. Howells, K.F. (1976) Res. Exp. Med. 168, 213.
136. Howland, J.L. (1974) Nature 251, 724.
137. Howland, J.L. and Iyer, S.L. (1977) Science 198, 309.
138. Hubbell, W.L. and McConnell, H.M. (1971) J. Amer. Chem. Soc. 93, 314.
139. Hubbell, W.L. Metcalfe, J.C., Metcalfe, S.M. and McConnell, H.M. (1970) Biochim. Biophys. Acta 219, 415.
140. Hucho, F. and Schiebler, W. (1977) Mol. Cell. Biochem. 18, 151.
141. Huestis, W.H. (1976) J. Supramol. Struct. 4, 355.
142. Huestis, W.H. (1977) J. Biol. Chem. 252, 6764.
143. Hughes, B.P. (1972) J. Neurol. Neurosurg. Psychiat. 35, 658.
144. Hull, K.L. Jr. and Roses, A.D. (1976) J. Physiol. 254, 169.
145. Hutton, E.M. and Thompson, M.W. (1976) Canad. Med. Ass. J. 115, 749.
146. Ionasescu, V. and Zellweger, H. (1974) Acta Neurol. Scand. 50, 619.
147. Ionasescu, V., Lara-Braud, C., Zellweger, H., Ionasescu, R. and Burmeister, L. (1977) Acta Neurol. Scand. 55, 407.
148. Ionasescu, V., Zellweger, H. and Cancilla, P. (1978) Lancet II, 1251.
149. Iyer, S.L., Katyare, S.S. and Howland, J.L. (1976) Neurosci. Lett. 2, 103.
150. Iyer, S.L., Hoenig, P.A., Sherblom, A.P. and Howland, J.L. (1977) Biochem. Med. 18, 384.
151. Jarrett, H.W. and Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210.
152. Jarrett, H.W. and Penniston, J.T. (1978) J. Biol. Chem. 253, 4676.
153. Jerusalem, F. (1976) J. Neurol. 213, 155.
154. Jerusalem, F., Engel, A.G. and Gomez, M.R. (1974) Brain 97, 115.
155. Ji, T.H. and Nicolson, G.L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2212.
156. Johnson, M. (1974) in: The Prostaglandins (Ramwell, P.W., ed.), vol. 2, Plenum Press, New York, pp. 75.
157. Jost, P.C., Griffith, O.H., Capaldi, R.A. and VanderKooi, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 480.
158. Jung, C.Y. (1974) in: The Red Blood Cell (Surgenor, D.MacN., ed.), vol. II, Academic Press, New York, pp. 705.
159. Kaiser, G., Quiring, K., Gauger, D., Palm, D., Becker, H. and Schoeppe, W. (1974) Blut 29, 115.
160. Kalofoutis, A., Jullien, G. and Spanos, V. (1977) Clin. Chim. Acta 74, 85.

161. Kaplan, J., Canonico, P.G. and Caspary, W.J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 66.
162. Kar, N.C. and Pearson, C.M. (1978) Clin. Chim. Acta 82, 185.
163. Katz, S. and Blostein, R. (1975) Biochim. Biophys. Acta 389, 314.
164. Keith, A.D., Sharnoff, M. and Cohn, G.E. (1973) Biochim. Biophys. Acta 300, 379.
165. Kiefer, H., Lindstrom, J., Lennox, E.S. and Singer, S.J. (1970) Proc. Nat. Acad. Sci. U.S.A. 67, 1688.
166. Kimelberg, H.K. (1977) in: Cell Surface Reviews (Poste, G. and Nicolson, G.L., eds.), vol. 3, North Holland Publ. Comp., Amsterdam, pp. 205.
167. King, M.E. and Spector, A.A. (1978) J. Biol. Chem. 253, 6493.
168. King, L.E. Jr. and Morrison, M. (1977) Biochim. Biophys. Acta 471, 162.
169. Kirkpatrick, F.H. (1976) Life Sciences 19, 1.
170. Kirkpatrick, F.H., Hillman, D.G. and LaCelle, P.L. (1975) Experientia 31, 653.
171. Kirkpatrick, F.H., Woods, G.M., LaCelle, P.L. and Weed, R.I. (1975) J. Supramol. Struct. 3, 415.
172. Klassen, G.A. and Blostein, R. (1969) Science 163, 492.
173. Kleine, T.O. (1970) Clin. Chim. Acta 29, 227.
174. Knauf, P.A., Proverbio, F. and Hoffman, J.F. (1974) J. Gen. Physiol. 63, 324.
175. Kobayashi, M., Meek, J.C. and Streib, E. (1977) J. Clin. Endocrinol. Metab. 45, 821.
176. Kobayashi, T., Mawatari, S. and Kuroiwa, Y. (1978) Clin. Chim. Acta 85, 259.
177. Koehler, J.P. (1974) Neurology 24, 354.
178. Koehler, J.P. (1977) Neurology 27, 861.
179. Kohlschütter, A., Wiesmann, U.N., Herschkowitz, N.N. and Ferber, E. (1976) Clin. Chim. Acta 70, 463.
180. Kohn, I., Missirlis, Y., McComas, A.J. and Brain, M.C. (1977) Clin. Res. 25, 708A.
181. Korczyn, A.D., Fishman, P., Djaldetti, M. and Berginer, V.M. (1977) Isr. J. Med. Sci. 13, 131.
182. Koski, C.L., Jungalwala, F.B. and Kolodny, E.H. (1978) Clin. Chim. Acta 85, 295.
183. Kunze, D. (1973) in: Basic Research in Myology (Kakulas, B.A., ed.), part 1, Exerpta Medica, Amsterdam, pp. 161.
184. Kunze, D. (1977) in: Pathogenesis of Human Muscular Dystrophies (Rowland, L.P., ed.) Exerpta Medica, Amsterdam, pp. 404.
185. Kunze, D., Olthoff, D. and Schellnack, K. (1967) Acta Biol. Med. Germ. 19, 1057.
186. Kunze, D., Reichmann, G., Egger, E., Leuschner, G. and Eckhardt, H. (1973) Clin. Chim. Acta 43, 333.
187. Kunze, D., Reichmann, G., Egger, E., Olthoff, D. and Döhler, K. (1975) Eur. J. Clin. Invest. 5, 471.
188. Kury, P.G. and McConnell, H.M. (1975) Biochemistry 14, 2798.
189. LaCelle, P.L., Kirkpatrick, F.H. and Udkow, M. (1973) in: Erythrocytes,

Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E., Wilmanns, W., eds.) Georg Thieme Publ., Stuttgart, pp. 49.
190. LaCelle, P.L., Evans, E.A. and Hochmuth, R.M. (1977) Blood Cells 3, 335.
191. Lake, W., Rasmussen, H. and Goodman, D.B.P. (1977) J. Membr. Biol. 32, 93.
192. Lane, T.A., Ballas, S.K. and Burka, E.R. (1976) Blood 47, 189.
193. Larsen, F.L., Hinds, T.R. and Vincenzi, F.F. (1978) J. Membr. Biol. 41, 361.
194. Leblond, P. (1972) Nouv. Rev. Franç. Hématol. 12, 815.
195. Lipicky, R.J. and Bryant, S.H. (1971) Trans. Amer. Neurol. Assoc. 96, 34.
196. Lloyd, S.J. and Nunn, M.G. (1978) Brit. Med. J. 2, 252.
197. Lloyd, R.D., Mays, C.W., McFarland, S.S., Zundel, W.S. and Tyler, F.H. (1973) Radiat. Res. 54, 463.
197a. Lumb, E.M. and Emery, A.E.H. (1975) Brit. Med. J. 3, 467.
198. Lux, S.E. and John, K.M. (1977) in: Progress in Clinical and Biological Research (Revel, J.P., Henning, U. and Fox, C.F., eds.), vol. 17, A.R. Liss, Inc., New York, pp. 481.
199. McComas, A.J. (1974) Nature 251, 569.
200. McComas, A.J., Sica, R.E.P. and Campbell, M.J. (1971) Lancet I, 321.
201. McComas, A.J., Sica, R.E.P. and Upton, A.M. (1974) Arch. Neurol. 30, 249.
202. McComas, A.J., Sica, R.E.P. and Brandstater, M.E. (1977) J. Neurol. Neurosurg. Psychiat. 40, 1147.
203. MacIntyre, J.D. and Green, J.W. (1978) Biochim. Biophys. Acta 510, 373.
204. Mahoney, M.J., Haseltine, F.P., Hobbins, J.C., Banker, B.Q., Caskey, C.T. and Golbus, M.S. (1977) N. Engl. J. Med. 297, 968.
205. Markesbery, W.R. (1977) in: Advances in Neurology (Griggs, R.C. and Moxley, R.T. III, eds.), vol. 17, Raven Press, New York, pp. 175.
206. Matheson, D.W. and Howland, J.L. (1974) Science 184, 165.
207. Matheson, D.W., Engel, W.K. and Derrer, E.C. (1976) Neurology 26, 1182.
208. Matsuda, M., Akatsuka, N., Yamaguchi, T., Saito, T., Takahashi, H., Tomomatsu, K., Tamura, T., Furukawa, T. and Marukami, T. (1977) Jap. Heart J. 18, 638.
209. Mawatari, S., Takagi, A. and Rowland, L.P. (1974) Arch. Neurol. 30, 96.
210. Mawatari, S., Schonberg, M. and Olarte, M. (1976) Arch. Neurol. 33, 489.
211. Mawatari, S., Miranda, A. and Rowland, L.P. (1976) Neurology 26, 1021.
212. Mayerhofer, E. (1950) Ann. Paediat. 174, 316.
213. Mendell, J.R., Engel, W.K. and Derrer, E.C. (1971) Science 172, 1143.
214. Mendell, J.R., Murphy, D.L., Engel, W.K., Chase, T.N. and Gordon, E. (1972) Arch. Neurol. 27, 518.
215. Mendell, J.R., Silverman, L.M., Verrill, H.L., Parker, J.M. and Olson, W.H. (1976) Neurology 26, 968.
216. Miale, T.D., Frias, J.L. and Lawson, D.L. (1975) Science 187, 453.
217. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81.
218. Miller, D.M. (1969) in: Red Cell Membrane; Structure and Function (Jamieson, G.A. and Greenwalt, T.J., eds.) J.P. Lippincott Comp., Philadelphia, pp. 240.
219. Miller, S.E., Roses, A.D. and Appel, S.H. (1975) Science 188, 1131.
220. Miller, S.E., Roses, A.D. and Appel, S.H. (1976) Arch. Neurol. 33, 172.
221. Mokri, B. and Engel, A.G. (1975) Neurology 25, 1111.
222. Montfoort, A. and Boere, W.A.M. (1979) submitted for publication.
222a. Moore, J.W. and Narahashi, T. (1967) Fed. Proc. 26, 1655.

223. Moore, R.B., Manery, J.F. and Dryden, E.E. (1978) in: Progress in Clinical and Biological Research (Kruckeberg, W.C., Eaton, J.W. and Brewer, G.J., eds.), vol. 20, A.R. Liss, Inc., New York, pp. 51.
224. Moosa, A. (1974) Devel. Med. Child Neurol. 16, 97.
225. Moxley, R.T. III (1977) in: Advances in Neurology (Griggs, R.C. and Moxley R.T. III, eds.), vol. 17, Raven Press, New York, pp. 161.
226. Munsat, T.L., Hudgson, P. and Johnson, M.A. (1977) Neurology 27, 772.
227. Murphy, D.L., Mendell, J.R. and Engel, W.K. (1973) Arch. Neurol. 28, 239.
228. Nelson, G.J. (1972) in: Blood Lipids and Lipoproteins; Quantitation, Composition, and Metabolism (Nelson, G.J., ed.), John Wiley, New York, pp. 317.
229. Niebrój-Dobosz, I. (1976) J. Neurol. 214, 61.
230. Pacold, I., Morgan, J. and Cohen, L. (1975) Clin. Genet. 7, 435.
231. Palek, J., Liu, S.C. and Liu, P.A. (1978) in: Progress in Clinical and Biological Research (Kruckeberg, W.C., Eaton, J.W. and Brewer, G.J., eds.), vol. 20, A.R. Liss. Inc., New York, pp. 75.
232. Panayiotopoulos, C.P. and Scarpalezos, S. (1977) J. Neurol. Sci. 31, 261.
233. Parker, J.M. and Mendell, J.R. (1974) Nature 247, 103.
234. Patten, B.M., Oliver, K.L. and Engel, W.K. (1974) Arch. Neurol. 31, 347.
235. Paulson, O.B., Engel, A.G. and Gomez, M.R. (1974) J. Neurol. Neurosurg. Psychiat. 37, 685.
236. Paulsrud, J.R., Pensler, L., Whitten, C.F., Stewart, S. and Holman, R.T. (1972) Amer. J. Clin. Nutr. 25, 897.
237. Pearse, R.G. and Höweler, C.J. (1979) Arch. Dis. Child., in press.
238. Pearson, T.W. (1978) Life Sci. 22, 127.
239. Pena, S.D.J., Vust, A., Tucker, D., Hamerton, J.L. and Wrogemann, K. (1978) Clin. Genet. 14, 50.
240. Penn, A.S., Lisak, R.P. and Rowland, L.P. (1970) Neurology 20, 147.
241. Pennell, R.B. (1974) in: The Red Blood Cell (Surgenor, D. MacN., ed.), vol. I, Academic Press, New York, pp. 93.
242. Pennington, R.J. (1971) in: Advances in Clinical Chemistry (Bodansky, O. and Latner, A.L., eds.), vol. 14, Academic Press, New York, pp. 409.
243. Percy, A.K. and Miller, M.E. (1975) Nature 258, 147.
244. Perry, S.V. (1971) J. Neurol. Sci. 12, 289.
245. Peter, J.B. and Worsfold, M. (1969) Biochem. Med. 2, 364.
246. Peter, J.B. and Worsfold, M. (1969) Biochem. Med. 2, 457.
247. Peter, J.B., Worsfold, M. and Pearson, C.M. (1969) J. Lab. Clin. Med. 74, 103.
248. Peter, J.B., Andiman, R.M., Bowman, R.L. and Nagatomo, T. (1973) Exp. Neurol. 41, 738.
249. Pickard, N.A., Gruemer, H.D., Verrill, H.L., Isaacs, E.R., Robinow, M., Nance, W.E., Myers, E.C. and Goldsmith, B. (1978) N. Engl. J. Med. 299, 841.
250. Pincus, J.H. and Lee, S.H. (1973) Arch. Neurol. 29, 239.
251. Plishker, G.A., Gitelman, H.J. and Appel, S.H. (1978) Science 200, 323.
252. Polgar, J.G., Bradley, W.G., Upton, A.R.M., Anderson, J., Howat, J.M.L., Petito, F., Roberts, D.F. and Scopa, J. (1972) Brain 95, 761.

253. Pouchan, M.I., Garrahan, P.J. and Rega, A.F. (1969) Biochim. Biophys. Acta 173, 151.
254. Probstfield, J.L., Wang, Y. and From, A.H.L. (1972) Proc. Soc. Exp. Biol. Med. 141, 479.
255. Quastel, D.M.J. and Hackett, J.T. (1973) in: The Structure and Function of Muscle (Bourne, G.H., ed.), vol. IV, Academic Press, New York, pp. 1.
256. Quist, E.E. and Roufogalis, B.D. (1975) FEBS Lett. 50, 135.
257. Radu, H., Gödri, I., Albu, E., Radu, A. and Robu, R. (1970) J. Neurol. Neurosurg. Psychiat. 33, 294.
258. Rao, G.A., Siler, K. and Larkin, E.C. (1978) Science 200, 1416.
259. Rasmussen, H., Goodman, D.B.P. and Tenenhouse, A. (1972) C.R.C. Crit. Rev. Biochem. 1, 95.
260. Rasmussen, H., Lake, W. and Allen, J.E. (1975) Biochim. Biophys. Acta 411, 63.
261. Rega, A.F. and Garrahan, P.J. (1975) J. Membr. Biol. 22, 313.
262. Rega, A.F., Richards, D.E. and Garrahan, P.J. (1973) Biochem. J. 136, 185.
263. Rega, A.F., Richards, D.E. and Garrahan, P.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 242, 317.
264. Renner, R., Kemmler, W. and Hepp, K.D. (1974) Eur. J. Biochem. 49, 129.
265. Richards, D.E., Rega, A.F. and Garrahan, P.J. (1977) J. Membr. Biol. 35, 113.
266. Richards, D.E., Vidal, J.C., Garrahan, P.J. and Rega, A.F. (1977) J. Membr. Biol. 35, 125.
267. Richards, D.E., Rega, A.F. and Garrahan, P.J. (1978) Biochim. Biophys. Acta 511, 194.
268. Richter, I., Pathenheimer, F. and Plath, Chr. (1972) Kinderärztliche Praxis 5, 216.
269. Roberts, D.F. and Bradley, W.G. (1977) J. Med. Genet. 14, 16.
270. Rodan, S.B., Rodan, G.A. and Sha'afi, R.I. (1976) Biochim. Biophys. Acta 428, 509.
271. Romero, P.J. (1976) J. Membr. Biol. 29, 329.
272. Rosenthal, A.S., Kregenow, F.M. and Moses, H.L. (1970) Biochim. Biophys. Acta 196, 254.
273. Roses, A.D. and Appel, S.H. (1974) Lancet II, 1400.
274. Roses, A.D. and Appel, S.H. (1974) Nature 250, 245.
275. Roses, A.D. and Appel, S.H. (1975) J. Membr. Biol. 20, 51.
276. Roses, A.D. and Appel, S.H. (1976) J. Neurol. Sci. 29, 185.
277. Roses, A.D., Butterfield, D.A., Appel, S.H. and Chesnut, D.B. (1975) Arch. Neurol. 32, 535.
278. Roses, A.D., Herbstreith, M.H. and Appel, S.H. (1975) Nature 254, 350.
279. Roses, A.D., Herbstreith, M.H., Metcalf, B.S. and Appel, S.H. (1976) J. Neurol. Sci. 30, 167.
280. Roses, A.D., Roses, M.J., Metcalf, B.S., Hull, K.L., Nicholson, G.A., Hartwig, G.B. and Roe, C.R. (1977) Ann. Neurol. 2, 271.
281. Roses, A.D., Herbstreith, M.H. and Hartwig, G.B. (1978) Neurology 28, 364.
282. Rottem, S. and Samuni, A. (1973) Biochim. Biophys. Acta 298, 32.
283. Rowland, L.P. (1976) Arch. Neurol. 33, 315.

284. Rubin, C.S. and Rosen, O.M. (1973) Biochem. Biophys. Res. Commun. 50, 421.
285. Rudy, B. and Gitler, C. (1972) Biochim. Biophys. Acta 288, 231.
286. Ruitenbeek, W. (1978) Clin. Chim. Acta 89, 99.
287. Ruitenbeek, W. (1979) J. Neurol. Sci., in press.
288. Ruitenbeek, W. and Scholte, H.R. (1979) J. Neurol. Sci., in press.
289. Ruitenbeek, W., Edixhoven, M.J. and Scholte, H.R. (1979) Clin. Chim. Acta, accepted for publication.
290. Samaha, F.J. and Gergely, J. (1969) N. Engl. J. Med. 280, 184.
291. Sanyal, S.K., Johnson, W.W., Thapar, M.K. and Pitner, S.E. (1978) Circulation 57, 1122.
292. Sarkadi, B., Szász, I. and Gárdos, G. (1976) J. Membr. Biol. 26, 357.
293. Sarkadi, B., Szász, I., Gerlóczy, A. and Gárdos, G. (1977) Biochim. Biophys. Acta 464, 93.
294. Sarkadi, B., MacIntyre, J.D. and Gárdos, G. (1978) FEBS Lett. 89, 78.
295. Sarpel, G., Danon, M.J. and Omachi, A. (1978) Neurology 28, 332.
296. Sato, T. and Fujii, T. (1974) Chem. Pharm. Bull. 22, 368.
297. Sato, B., Nishikida, K., Samuels, L.T. and Tyler, F.H. (1978) J. Clin. Invest. 61, 251.
298. Schaaf, M. and Payne, C.A. (1968) N. Engl. J. Med. 271, 1228.
299. Schapira, G. and Schapira, F. (1953) Sem. Hop. Paris 29, 1917.
300. Scharff, O. (1976) Biochim. Biophys. Acta 443, 206.
301. Scharff, O. (1978) Biochim. Biophys. Acta 512, 309.
302. Scharff, O. and Foder, B. (1978) Biochim. Biophys. Acta 509, 67.
303. Schatzmann, H.J. (1973) J. Physiol. 235, 551.
304. Schatzmann, H.J. (1975) in: Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), vol. 6, Academic Press, New York, pp. 125.
305. Schatzmann, H.J. (1977) J. Membr. Biol. 35, 149.
306. Schatzmann, H.J. and Bürgin, H. (1978) Ann. N.Y. Acad. Sci. 307, 125.
307. Schatzmann, H.J. and Rossi, G.L. (1971) Biochim. Biophys. Acta 241, 379.
308. Schatzmann, H.J. and Vincenzi, F.F. (1969) J. Physiol. 201, 369.
309. Schiffer, D., Fornatto, L., Giordana, M.T. and Peres, B. (1974) Acta Neurol. (Napoli) 29, 397.
310. Scholte, H.R. and Busch, H.F.M. (1979), in preparation.
311. Scholte, H.R. and Houwen, I.E.M. (1979), in preparation.
312. Schotland, D.L., Bonilla, E. and Van Meter, M. (1977) Science 196, 1005.
313. Schreier, S., Polnaszek, C.F. and Smith, I.C.P. (1978) Biochim. Biophys. Acta 515, 395.
314. Schrott, H.G. and Omenn, G.S. (1975) Neurology 25, 789.
315. Schrott, H.G., Karp, L. and Omenn, G.S. (1973) Clin. Genet. 4, 38.
316. Seay, A.R., Ziter, F.A. and Hill, H.R. (1978) J. Neurol. Sci. 35, 25.
317. Seelig, J. and Hasselbach, W. (1971) Eur. J. Biochem. 21, 17.
318. Segel, G.B., Feig, S.A., Glader, B.E., Müller, A., Dutcher, P. and Nathan, D.G. (1975) Blood 46, 271.
319. Sha'afi, R.I., Rodan, S.B., Hintz, R.L., Fernandez, S.M. and Rodan, G.A. (1975) Nature 254, 525.
320. Sheetz, M.P. (1977) in: Progress in Clinical and Biological Research

(Revel, J.P., Henning, U. and Fox, C.F., eds.), vol. 17, A.R. Liss, Inc., New York, pp. 559.
321. Shiga, T., Suda, T. and Maeda, N. (1974) Biochim. Biophys. Acta 466, 231.
322. Shimshick, E.J. and McConnell, H.M. (1973) Biochem. Biophys. Res. Commun. 53, 446.
323. Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652.
324. Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367.
325. Shukla, S.D., Billah, M.M., Coleman, R., Finean, J.B. and Michell, R.H. (1978) Biochim. Biophys. Acta 509, 48.
326. Sica, R.E.P. and McComas, A.J. (1978) J. Canad. Sci. Neurol. 5, 189.
327. Siddiqui, P.Q.R. and Pennington, R.J.T. (1977) J. Neurol. Sci. 34, 365.
328. Siefring, G.E. Jr., Apostol, A.B., Velasco, P.T. and Lorand, L. (1978) Biochemistry 17, 2598.
329. Simpkins, H., Tay, S. and Panko, E. (1971) Biochemistry 10, 3579.
330. Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720.
331. Skou, J.C. (1974) Quart. Rev. Biophys. 7, 401.
332. Sohn, R.S. and Ferrendelli, J.A. (1973) J. Pharmacol. Exp. Ther. 185, 272.
333. Soltan, H.C. (1977) J. Med. Genet. 14, 276.
334. Somer, H. and Olarte, M. (1977) Neurology 27, 361.
335. Souweine, G., Bernard, J.C., Lasne, Y. and Lachanat, J. (1978) J. Neurol. 217, 287.
336. Steck, T.L. (1974) J. Cell Biol. 62, 1.
337. Stengel-Rutkowski, L. and Barthelmai, W. (1973) Klin. Wochenschr. 51, 957.
338. Stern, L.Z., Payne, C.M., Gruener, R., Anderson, R.M. and Hannapel, L.K. (1975) J. Neurol. Neurosurg. Psychiat. 38, 900.
339. Sugita, H. and Tyler, F.H. (1963) Trans. Ass. Amer. Phys. 76, 231.
340. Sulakhe, P.V. and St. Louis, P.J. (1976) Gen. Pharmacol. 7, 313.
341. Susheela, A.K., Kaul, R.D., Sachdeva, K. and Singh, N. (1975) J. Neurol. Sci. 24, 361.
342. Szász, I., Sarkadi, B., Schubert, A. and Gárdos, G. (1978) Biochim. Biophys. Acta 512, 331.
343. Szibor, R., Redmann, K., Deike, H. and Müller, K. (1976) Helv. Paediat. Acta 31, 249.
344. Takagi, A., Muto, Y., Takahashi, Y. and Nakao, K. (1968) Clin. Chim. Acta 20, 41.
345. Takagi, A., Shimada, Y. and Mozai, T. (1970) Neurology 20, 904.
346. Takagi, A., Schotland, D.L. and Rowland, L.P. (1973) Arch. Neurol. 28, 380.
347. Takamori, M. (1975) J. Neurol. Neurosurg. Psychiat. 38, 483.
348. Takamori, M., Hazama, R. and Tsujihata, M. (1978) Neurology 28, 603.
349. Takeshita, K., Yoshino, K., Kitahara, T., Nakashima, T. and Kato, N. (1977) Jap. J. Human Genet. 22, 43.
350. Tell, G.P., Haour, F. and Saez, J.M. (1978) Metabolism 27, 1566.
351. Tenforde, T. (1970) in: Adv. Biol. Med. Phys. (Lawrence, J.H. and Gofman, J.W., eds.), vol. 13, Academic Press, New York, pp. 43.
352. Thomas, N.S.T. and Harper, P.S. (1978) Clin. Chim. Acta 83, 13.
353. Thompson, E.J., Yasin, R., Van Beers, G., Nurse, K. and Al-Ani, S. (1977) Nature 268, 241.

354. Thomson, W.H.S., Sweetin, J.C. and Elton, R.A. (1974) Nature 249, 151.
355. Thomson, W.H.S., Leyburn, P. and Walton, J.N. (1960) Brit. Med. J. 2, 1276.
356. Thomson, W.H.S., Sweetin, J.C. and Hilditch, T.E. (1975) Clin. Chim. Acta 63, 383.
357. Till, U., Petermann, H., Wenz, I. and Frunder, H. (1977) Acta Biol. Med. Germ. 36, 597.
358. Toop, J. and Emery, A.E.H. (1974) Clin. Genet. 5, 230.
359. Van Deenen, L.L.M. and De Gier, J. (1974) in: The Red Blood Cell (Surgenor, D. MacN., ed.), vol. I, Academic Press, New York, pp. 147.
360. Van Gastel, C., Van den Berg, D., De Gier, J. and Van Deenen, L.L.M. (1965) Brit. J. Haemat. 11, 193.
361. Van Hoeven, R.P., Van Blitterswijk, W.J. and Emmelot, P. (1979) Biochim. Biophys. Acta 551, 44.
362. Vanier, T.M. (1960) Brit. Med. J. 2, 1284.
363. Vassilopoulos, D. and Emery, A.E.H. (1977) J. Med. Genet. 14, 13.
364. Vergroesen, A.J. and Gottenbos, J.J. (1975) in: The Role of Fats in Human Nutrition (Vergroesen, A.J., ed.), Academic Press, London, pp. 1.
365. Verrill, H.L., Pickard, N.A. and Gruemer, H.D. (1977) Clin. Chem. 23, 2219.
366. Verrill, H.L., Gruemer, H.D. and Baba, N. (1977) Clin. Chem. 23, 2226.
367. Vincenzi, F.F. and Hinds, T.R. (1976) in: The Enzymes of Biological Membranes (Martonosi, A., ed.), vol. 3, John Wiley and Sons, London, pp. 261.
368. Wakamatsu, H., Nakamura, H., Ito, K., Anazawa, W., Okajima, S., Shigeno, K. and Goto, Y. (1972) Horm. Metab. Res. 4, 458.
369. Wakayama, Y., Hodson, A., Bonilla, E., Pleasure, D. and Schotland, D.L. (1978) Neurology 28, 363.
370. Wakayama, Y., Hodson, A., Pleasure, D., Bonilla, E. and Schotland, D.L. (1978) Ann. Neurol. 4, 253.
371. Wallach, D.F.H., Verma, S.P., Weidekamm, E. and Bieri, V. (1974) Biochim. Biophys. Acta 356, 68.
372. Walton, J.N. (1977) Isr. J. Med. Sci. 13, 152.
373. Weed, R.I. and Chailley, B. (1972) Nouv. Rev. Franç. Hématol. 12, 775.
374. Weed, R.I. and Chailley, B. (1973) in: Erythrocytes, Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds.) Georg Thieme Publ., Stuttgart, pp. 45.
375. Weed, R.I., LaCelle, P.L. and Merrill, E.W. (1969) J. Clin. Invest. 48, 795.
376. Weidekamm, E., Schudt, C. and Brdiczka, D. (1976) Biochim. Biophys. Acta 443, 169.
377. Weinstein, R.S. (1974) in: The Red Blood Cell (Surgenor, D. MacN., ed.), vol. I, Academic Press, New York, pp. 213.
378. Wilkerson, L.S., Perkins, R.C. Jr., Roelofs, R., Swift, L., Dalton, L.R. and Harting Park, J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 838.
379. Winn, K.J. and Heller, R.H. (1978) Clin. Genet. 13, 335.
380. Wittels, B. and Hochstein, P. (1967) J. Biol. Chem. 242, 126.
381. Wolf, H.U. (1972) Biochim. Biophys. Acta 266, 361.
382. Wolf, H.U., Dieckvoss, G. and Lichtner, R. (1977) Acta Biol. Med. Germ. 36, 847.

383. Wood, D.S., Sorenson, M.M., Eastwood, A.B., Charash, W.E. and Reuben, J.P. (1978) Neurology 28, 447.
384. Wright, T.L., O'Neill, J.A. and Olson, W.H. (1973) Neurology 23, 510.
385. Wrogemann, K. and Pena, S.D.J. (1976) Lancet I, 672.
386. Wyatt, P.R. and Cox, D.M. (1977) Lancet I, 172.
387. Yao, J.K., Ellefson, R.D. and Dyck, P.J. (1976) J. Neurol. Sci. 29, 161.
388. Yarom, R. (1976) Lancet I, 1242.
389. Zatz, M., Lange, K. and Spence, M.A. (1977) Lancet I, 759.
390. Zellweger, H. and Ionasescu, V. (1973) Acta Neurol. Scand. 49, suppl. 55, 1.
391. Zwaal, R.F.A. and Van Deenen, L.L.M. (1970) Chem. Phys. Lipids 4, 311.
392. Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83.

SUMMARY

The subject of this study is to characteristic biochemical changes in persons carrying the gene of Duchenne muscular dystrophy or myotonic dystrophy. Probably the findings ultimately will contribute to understanding the primary cause of human muscular dystrophy.

Recently, data became available, supporting the hypothesis that muscular dystrophy is caused by a generalized membrane defect. Thus, not only muscular membranes are impaired, but also the membranes of other cell types. We performed research on the erythrocyte membrane of patients suffering from Duchenne dystrophy or congenital myotonic muscular dystrophy.

Lipids (Chapter II)

The major fatty acids have normal levels in each of eight phospholipid classes in erythrocyte membranes from dystrophy patients. Other investigators found unchanged concentrations of the different phospholipids in isolated erythrocyte membranes of patients. From these facts we conclude that there are no alterations in the major lipids of red cells from patients with muscular dystrophy of both types. The normal lipid fluidity, as measured by fluorescence polarization (see 3.3.1.) is in agreement with this conclusion.

We did find alterations in minor lipid components of the isolated membranes. Palmitoleic acid in neutral lipid fractions of patients is decreased. In Duchenne patients the level in diglycerides is reduced by 66% and in myotonic patients by 86%. In the latter group of patients triglyceride palmitoleic acid is reduced by 75%. This does not necessarily points to an abnormality of the membrane.

The fatty acid composition of phospholipids in blood plasma is normal in muscular dystrophy.

Osmotic stability (Chapter III)

Washed red cells of dystrophic patients have a diminished osmotic stability. An abnormal feature of red cells in myotonic dystrophic patients was observed after phospholipase treatment. These cells, like untreated ones, are more stable in isotonic than in hypotonic solutions. On the contrary, phospholipase-treated red cells from controls and Duchenne patients lyse to a greater extent in isotonic than in 0.5% phosphate-buffered NaCl solutions. This phospholipase-induced process is stimualted by Ca^{2+}, Na^{+} and phosphate. The nature of the normal osmotic behaviour of myotonic cells may be, that the acetylcholine receptor and/or the Na^{+}-ion channel in these cells is not destructed by phospholipase, in contrast to control cells.

Membrane-bound enzymes (Chapter IV)

Other indications for a membrane defect may be changes in kinetic properties of membrane-bound enzymes.

Long chain fatty acid activation in red cell membranes is increased in congenital myotonic dystrophy only. The activation in leucocytes is not changed. Erythrocyte fatty acid activation has the same activity with palmitate, palmitoleate and linoleate. Leucocytes activate palmitate with a higher velocity than the other two fatty acids.

Carnitine palmitoyltransferase in myotonic red cell membranes is not much increased. Its affinity for palmitoyl-CoA is the same in patients and controls.

The activities of $(Na^{+} + K^{+})$-ATPase and K^{+}-stimulated *p*-nitrophenylphosphatase, both related to the Na^{+}-K^{+}-pump, are not changed in dystrophy. Also the ouabain sensitivity is unaltered.

The activities of Ca^{2+}-ATPase and Ca^{2+}-stimulated *p*-nitrophenylphosphatase are twofold increased in both types of human muscular dystrophy. The interaction of Ca^{2+}-ATPase from Duchenne membranes with MgATP and Ca^{2+} is the

same as in controls, but the Ca^{2+}-ATPase activation by Na^{+} is different. Ca^{2+}-ATPase from Duchenne red cells has two activating sites for Na^{+}, while control cells have only one, low affinity, site. The high affinity site is responsible for an extra enzyme activity below 34 mM Na^{+} in Duchenne red cells. At a physiological intracellular Na^{+}-concentration of 10 mM, the activity of Ca^{2+}-ATPase is nearly doubled. In control membranes, a second Na^{+}-activation site is uncovered by ethanol or epinephrine. The Na^{+}-activation of control membranes in the presence of ethanol is the same as in Duchenne membranes without ethanol. Epinephrine stimulates control activity even more at decreasing Na^{+}-concentrations than ethanol.

Hypothesis

Other investigators reported an increased basal activity of adenylate cyclase in Duchenne red blood cells, and a reduced epinephrine stimulation of the enzyme. These and our findings on Ca^{2+}-ATPase suggest that the alterations in the membrane of Duchenne erythrocytes are caused by an epinephrine receptor, the conformation of which is identical to a normal receptor loaded with its effector. This stimulated β-adrenergic state can explain the diminished osmotic stability of Duchenne erythrocytes, and also the increased rigidity reported in the literature. This conformational change presumably induces augmented affinity of certain parts of the membrane for Ca^{2+}. This may explain the increased sensitivity of Duchenne red cells towards Ca^{2+}, and the impaired membrane protein phosphorylation reported by others.

The hypothesis of a conformational change of the β-adrenergic receptor explains many erythrocyte membrane alterations in Duchenne muscular dystrophy. This change may play a role in the pathogenesis of the disease.

SAMENVATTING

Het onderwerp van deze studie is het vinden van karakteristieke biochemische veranderingen bij personen, die het gen van Duchenne dystrofie of myotonische dystrofie dragen. Hopenlijk zullen deze onderzoekingen uiteindelijk een bijdrage leveren aan het begrijpen van de primaire oorzaak van spierdystrofie bij de mens.

Kortgeleden kwamen gegevens beschikbaar, die de hypothese ondersteunen, dat spierdystrofie veroorzaakt wordt door een algemeen voorkomend membraandefekt. Niet alleen de spiermembranen van de spiercellen zijn dus aangetast, maar ook die van andere soorten cellen. Wij hebben erytrocietmembranen onderzocht van patiënten, die lijden aan Duchenne spierdystrofie of congenitale myotonische spierdystrofie.

Lipiden (Hoofdstuk II)

Ieder van de acht fosfolipideklassen in erytrocietmembranen van dystrofische patiënten hebben normale concentraties van de belangrijkste vetzuren. Andere onderzoekers vonden onveranderde concentraties van de verschillende fosfolipiden in geïsoleerde erytrocietmembranen van patiënten. Uit deze feiten concluderen we, dat er geen afwijkingen bestaan in de belangrijkste lipiden van de erytrocieten van patiënten met een van de twee vormen van spierdystrofie. De onveranderde vloeibaarheid van de lipiden, gemeten met behulp van fluorescentie polarizatie (zie 3.2.1.), stemt met deze conclusie overeen.

We vonden wel verschillen in beperkt voorkomende lipidecomponenten van geïsoleerde membranen. Palmitoliezuur in neutrale lipidefrakties van de patiënten is verminderd. Bij Duchenne-patiënten is het gehalte in de diglyceriden afgenomen met 66% en bij myotonische patiënten met 86%. In deze laatste patiëntengroep is palmitoliezuur in triglyceriden verminderd met 75%. Dit hoeft niet per se op een afwijking in het membraan te duiden.

De vetzuursamenstelling van fosfolipiden in bloedplasma is normaal bij spierdystrofie.

Osmotische stabiliteit (Hoofdstuk III)

Gewassen erytrocieten van dystrofische patiënten hebben een verminderde osmotische stabiliteit. Een abnormale eigenschap van rode bloedcellen van patiënten met myotonische dystrofie werd waargenomen na behandeling met fosfolipase. Deze cellen blijken, evenals onbehandelde cellen, stabieler te zijn in isotone dan in hypotone oplossingen. Dit in tegenstelling tot de met fosfolipase behandelde erytrocieten van controles en Duchenne-patiënten. Deze hemolyseren meer in isotone oplossingen dan in 0,5% met fosfaat gebufferde NaCl-oplossingen. Het door fosfolipase veroorzaakte proces wordt door Ca^{2+}, Na^{+} en fosfaat gestimuleerd. De aard van het afwijkende osmotische gedrag van de myotonische erytrocieten zou kunnen zijn, dat de acetylcholine receptor en/of het Na^{+}-ion kanaal in deze cellen niet door fosfolipase wordt vernietigd en bij controles wel.

Membraan-gebonden enzymen (Hoofdstuk IV)

Andere aanwijzingen voor een membraandefekt kunnen veranderingen zijn in de kinetische eigenschappen van membraangebonden enzymen.

Aktivering van lang-keten vetzuren in de erytrocietmembranen is alleen bij congenitale myotonische dystrofie verhoogd. De aktivering in leukocieten is onveranderd. De aktivering van palmitaat, palmitoleaat en linoleaat in erytrocieten is even snel. Leukocieten aktiveren palmitaat sneller dan de beide andere vetzuren.

Carnitine palmitoyltransferase is in myotonische erytrocieten niet veel verhoogd. De affiniteit voor palmitoyl-CoA is bij patiënten en controles gelijk.

De aktiviteiten van $(Na^{+} + K^{+})$-ATPase en K^{+}-gestimuleerde *p*-nitrofenylfosfatase, beide betrokken bij de

Na^+-K^+-pomp, zijn niet verschillend bij dystrofie. Ook gevoeligheid voor ouabaine is ongewijzigd.

De aktiviteiten van Ca^{2+}-ATPase en Ca^{2+}-gestimuleerde *p*-nitrofenylfosfatase zijn verdubbeld voor beide vormen van spierdystrofie. De interaktie van Ca^{2+}-ATPase van Duchenne membranen met MgATP en Ca^{2+} is identiek aan die bij controles, maar de aktivering van de Ca^{2+}-ATPase door Na^+ verschilt. Ca^{2+}-ATPase in Duchenne erytrocieten heeft twee aktiveringsplaatsen voor Na^+, terwijl controle cellen slechts één plaats bezitten met lage affiniteit. De plaats met hoge affiniteit leidt tot extra enzymaktiviteit in Duchenne erytrocieten bij Na^+-concentraties lager dan 34 mM. Bij de fysiologische intracellulaire Na^+-concentratie van 10 mM is de aktiviteit van Ca^{2+}-ATPase bijna verdubbeld ten opzichte van controles. Bij controle membranen wordt een tweede plaats voor Na^+-aktivering vrijgemaakt door ethanol of epinefrine. De Na^+-aktivering in controle membranen in aanwezigheid van ethanol is dezelfde als in Duchenne membranen zonder ethanol. Epinefrine stimuleert de aktiviteit bij afnemende Na^+-concentraties zelfs nog meer dan ethanol.

Hypothese

Andere onderzoekers hebben een verhoogde basale aktiviteit beschreven van adenylcyclase in Duchenne erytrocieten, en een verminderde epinefrine stimulatie van het enzym. Deze en onze resultaten met Ca^{2+}-ATPase doen veronderstellen, dat de veranderingen in de membranen van Duchenne erytrocieten veroorzaakt worden door een epinefrine receptor, waarvan de struktuur identiek is aan een normale receptor opgeladen met zijn effektor. Deze gestimuleerde β-adrenergische staat kan de verminderde osmotische stabiliteit van de Duchenne erytrocieten verklaren, evenals de toegenomen starheid, die de literatuur vermeldt. De struktuurverandering veroorzaakt waarschijnlijk verhoogde affiniteit van bepaalde membraangroepen voor Ca^{2+}. Dit verklaart mogelijk de verhoogde ge-

voeligheid van Duchenne erytrocieten voor Ca^{2+} en de door anderen beschreven veranderde fosforylering van membraaneiwitten.

De hypothese van een verandering in struktuur van de β-adrenergische receptor verklaart veel afwijkingen in membraaneigenschappen van erytrocieten bij Duchenne spierdystrofie, die een rol zouden kunnen spelen bij het ontstaan van de ziekte.

CURRICULUM VITAE

W. Ruitenbeek werd geboren 28 oktober 1948 te Nijkerk. Na aldaar de lagere school te hebben gevolgd, werd van 1961 tot 1966 het Corderius Lyceum te Amersfoort bezocht, wat leidde tot het behalen van het diploma H.B.S.-B. In 1966 begon de studie scheikunde aan de Rijksuniversiteit te Utrecht. Na het kandidaatsexamen werd biochemie als hoofdrichting gekozen. Het hoofdvak metabolisme van fosfolipiden werd bestudeerd onder leiding van dr. H. van den Bosch aan het laboratorium Biochemie (hoofd prof. L.L.M. van Deenen). In 1972 werd zowel het doctoraaldiploma behaald, als de wapenrok aangetrokken. Van september 1974 tot november 1978 werd het onderhavige proefschrift bewerkt aan de afdeling Biochemie I van de Erasmus Universiteit te Rotterdam (hoofd prof. W.C. Hülsmann). Een subsidie van het Prinses Beatrix Fonds maakt het de auteur mogelijk het onderzoek tijdelijk voort te zetten.

APPENDIX PAPERS

PAPER I

THE FATTY ACID COMPOSITION OF VARIOUS LIPID FRACTIONS ISOLATED FROM ERYTHROCYTES AND BLOOD PLASMA OF PATIENTS WITH DUCHENNE AND CONGENITAL MYOTONIC MUSCULAR DYSTROPHY

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Summary

Ten lipid fractions, both neutral and phospholipids, were isolated from erythrocytes of patients with Duchenne and congenital myotonic dystrophy. These fractions were: phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin, lysophosphatidylcholine, phosphatidic acid, glycosphingolipids, triglycerides, diglycerides and one unknown. The fatty acid compositions were compared with control values. The major deviation was a decreased level of palmitoleic acid (16 : 1ω7) in three neutral lipid fractions. It was lowered from 9.1 to 1.3% in diglycerides and from 10.6 to 2.6% in triglycerides in myotonic dystrophy. In Duchenne muscular dystrophy this percentage was decreased from 9.1 to 4.0 in diglycerides.

From blood plasma were isolated phosphatidylcholine, lysophosphatidylcholine, sphingomyelin and phosphatidylethanolamine. The myristic acid (14 : 0) content was raised in lysophosphatidylcholine (from 4.4 to 8.6%) and in sphingomyelin (from 3.3 to 5.0%) in myotonic dystrophy. In the latter phospholipid the stearic acid level was decreased from 18.6 to 12.8%. In phosphatidylcholine from plasma in myotonic dystrophy the level of eicosatrienoic acid (20 : 3ω6) was lowered from 3.1 to 2.0%. Plasma fractions from Duchenne dystrophy showed no deviation.

The results do not constitute an explanation for the various biochemical abnormalities found in dystrophic erythrocytes. Neither do they point to a defect in a major pathway in lipid metabolism. However, an aberration in the de novo synthesis of lipids in immature red blood cells cannot be excluded.

Introduction

One of the most clear phenomena found in Duchenne muscular dystrophy is the drastically elevated activity of creatine phosphokinase in serum. It seems

likely that a muscle membrane defect leads to leakage of compounds originally localized in the cytosol of the fibers. This hypothesis has been confirmed by abnormal characteristics of erythrocyte membranes. Erythrocytes from Duchenne patients have a lower mechanical resistance [1,2], an altered membrane conformation [3], a higher potassium influx [4] and show a changed electrophoretic mobility [5]. Furthermore, many membrane bound enzymes possess kinetic properties different from control values; among them acetylcholinesterase, adenylate cyclase, ($Na^+ + K^+$)ATPase, protein kinase (for review, see Rowland [6]). The fatty acids of the membrane lipids have been analysed; the relative content of palmitoleic acid (16 : 1ω7) was decreased in Duchenne patients and carriers [7]. In another study [8] this fatty acid level was found normal in the three major phospholipid fractions, but other acids had abnormal levels in sphingomyelin and phosphatidylethanolamine. Among them stearic (18 : 0), linoleic (18 : 2ω6), arachidonic (20 : 4ω6) and nervonic (24 : 1ω9) acid.

The fatty acid composition of membrane lipids plays an important role in the physical state of the membrane, which can influence metabolic processes. The minor phospholipids such as phosphatidylserine and phosphatidylinositol might be of great importance for special enzymes and transport processes [9]. We have studied the fatty acid pattern in six different phospholipid and four neutral lipid fractions from erythrocyte membranes in two types of muscle dystrophy, namely Duchenne dystrophy and myotonic dystrophy. These forms are genetically different. The first is X-chromosomal, recessive, the latter autosomal dominant.

The fatty acids of four phospholipids isolated from plasma have also been determined. Some of these compounds are exchangeable with erythrocyte lipids. Therefore, they may have influenced the composition of them. Moreover, serum lipid abnormalities are indicative of the involvement of other organs.

Abnormal fatty acid composition can either be used as a diagnostic tool or may be a clue to the metabolic origin of muscle dystrophy.

Materials and methods

1. Preparation of erythrocyte membranes

Peripheral blood was taken from overnight fasted patients suffering from Duchenne dystrophy or the congenital form of myotonic dystrophy, or male controls not suffering from a neuromuscular or hematological disorder. The age of all donors varied between 4 and 18 years (mean 9 years). 10—25 ml blood was collected in a siliconized glass cylinder with 1 ml 67 mM EDTA (Na salt) in 0.8% NaCl, pH 7.4, per 25 ml blood. Red and white blood cells were separated according to the method of Wyss et al. [10] by addition of 50 ml 3% Dextran mol. wt. approx. 250 000) in isotonic NaCl per 25 ml blood. The erythrocytes were sedimented after standing 45 min at room temperature. The pellet was washed two times in 1 volume 172 mM Tris-HCl, pH 7.6, at 0—4°C. The cells were lysed with 6 volumes 11 mM Tris-HCl, pH 7.6, for 10 min in an ice-bath. The suspension was centrifuged for 35 min at 37 000 $\times g_{max}$ at 0—4°C. The pellet was washed until the pink colour disappeared (2—4 times) [11]. The

membranes were stored at −70°C in the hypotonic Tris-HCl buffer at a protein concentration of about 2 mg per ml.

2. Preparation of the plasma

The upper phase of the dextran-blood mix (see above) was centrifuged for 10 min at 600 × g_{max} and after that for 10 min at 10 000 × g_{max} at 0—4°C in order to remove leukocytes and thrombocytes, respectively. The remaining supernatant, which contains the serum lipoproteins, was stored at −20°C.

3. Extraction, purification and analysis of the lipids

About 2 ml of the membranes or plasma were treated with half that volume of dry *n*-butanol at room temperature during 15 min. After centrifugation (10 min, 9000 × g_{max}) the organic phase was pipetted into a tube. The water phase and protein layer were extracted another 3 times with 1 ml of butanol, saturated with water. The combined organic phases were washed 3 times with the same volume of water, saturated with butanol [12]. The butanol phase was dried with a stream of nitrogen at 45°C, the residue taken up in chloroform/methanol (1 : 1, v/v) and stored at −20°C under nitrogen atmosphere.

For the separation of the different phospholipid classes the system of Siakotos and Rouser [13] was applied: Silicagel 60 plates (Merck, G.F.R.), 20 × 20 cm, thickness 0.25 mm with the developing system for the first dimension chloroform/methanol/aqueous ammonia (280 g/l) (13 : 7 : 1, v/v) and for the second dimension chloroform/acetone/methanol/acetic acid/water (10 : 4 : 2 : 2 : 1, v/v). Between the two runs the plates were dried in a vacuum desiccator for 50 min.

The neutral lipid classes were separated on the same type of plate with the developing system toluene/ethyl acetate/diethyl ether/acetic acid (80 : 10 : 10 : 0.2, v/v).

Lipid spots became visible under ultraviolet light after spraying with concentrated ammonia and 2.7-dichlorofluorescein (20 mg per 100 ml ethanol) [14]. The spots were scraped off and the lipids eluted by chloroform/methanol (1 : 2, v/v). The solution was dried under nitrogen at 45°C and hydrolysed and methylated with boron trifluoride (14% in methanol; Sigma) at 100°C, in accordance with Morrison and Smith [15], as was the extraction procedure for the methyl esters with pentane. The methyl esters of the fatty acids were analysed with a gas chromatograph (type F & M 402, Hewlett-Packard), equipped with an ECNSS-S column (10% on Gaschrom P, 100—120 mesh, Applied Science; length 6 ft, inner diameter 3 mm) and a flame ionization detector. Nitrogen was used as carrier gas (flow rate 20 ml/min); temperature was 155°C. Area of the peaks was measured by an electronic integrator (Hewlett-Packard 3370 B). Retention times were compared with standard esters (Supelco) and mixtures of known composition (kindly supplied by Dr. U.M.T. Houtsmuller, Unilever Research, Vlaardingen, The Netherlands).

Results

Fig. 1 shows the separation of phospholipids from erythrocyte membranes. The pattern agrees with that of Siakotos and Rouser [13] and Marggraf [16].

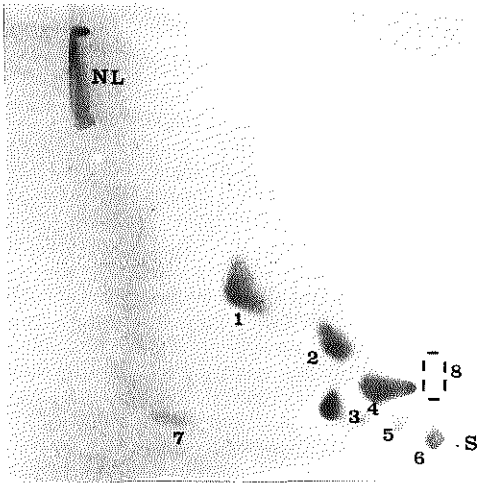

Fig. 1. Separation on TLC-plates of erythrocyte phospholipids, using the 2-dimensional system of Siakotos and Rouser [13]. 1, phosphatidylethanolamine (PE); 2, phosphatidylcholine (PC); 3, phosphatidylserine (PS); 4, sphingomyelin (SPH); 5, lysophosphatidylcholine (LPC); 6, glycosphingolipids; 7, phosphatidic acid (PA); 8, unknown. NL, neutral lipids; S, start.

In contrast to these workers, we found the lysophosphatidylcholine spot just beneath the sphingomyelin spot, and not to the right of it. The same pattern was obtained with plasma. Spot 6 may contain two types of glycospingolipid, because sometimes there were visible two partly separated spots. These spots were resorcinol-positive [14], pointing to gangliosides. Spot 8 was not detectable every time. It probably does not contain phosphorus, but may contain traces of complex sugar-lipids.

One of the lipids present in low concentrations in erythrocytes is phosphatidylinositol. We were not able to detect this phospholipid, but it is plausible that its location is in the environment of phosphatidylserine or sphingomyelin [15]. A few times we have scraped off these spots and, after elution, developed them two dimensionally on thin-layer chromatographic plates according to Peter and Wolf [17]. Nevertheless, phosphatidylinositol was never detected by the periodic acid-Schiff method [14]. The reason for this may be the low concentration of phosphatidylinositol or an incomplete extraction of the cell membranes. However, the extraction used has been proven to be useful for different kinds of phospholipid [12].

The separation pattern of plasma phospholipids was totally comparable with Fig. 1. Four lipids were always detectable, namely phosphatidylethanolamine, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine.

The di- and triglyceride fractions from erythrocytes were not visible on the plates when developed as described under Materials and methods. The amount

of neutral lipids, except cholesterol, is very low in these cells [18]. We have scraped off areas with R_f-values corresponding to standard di- and triglycerides, which may have been contaminated with some free fatty acids and cholesterol esters.

The fatty acid profiles of the various lipids are given in Tables I—VII. The fatty acids are coded as follows: (number of carbon atoms) : (number of double C-C bonds) ω (number of carbon atoms until the first double bond, starting from the methyl end).

Table I shows the fatty acids in the negatively charged phosphatidylethanolamine and -serine from erythrocytes. Both lipids are partly present in the plasmalogen form, in which one ester bond is replaced by a vinyl ether bond [14]. During methylation dimethylacetals (DMA) are formed instead of methyl esters of fatty acids. The quantities of the dimethylacetals are included in the figures of 16 : 0 and 18 : 0. There was no difference between the 3 groups with respect to these compounds. In phosphatidylethanolamine the mean for 16 : 0 DMA was 3.2%, for 18 : 0 DMA 7.1%. In phosphatidylserine only a few tenths of a percent were present as acetals. Kunze et al. [8] did not analyse the serine compound, but found a two-fold increase in dimethylacetals (16 : 0 DMA + 18 : 0 DMA?) in phosphatidylethanolamine in Duchenne patients. However, they found a normal content of plasmalogens in this fraction. We did not find

TABLE I

FATTY ACID COMPOSITION OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE FROM ERYTHROCYTE MEMBRANES

All data expressed as percentage (mean ± S.E.M.). Numbers in parentheses: number of samples analyzed. n.d., not detectable.

Acid	Phosphatidylethanolamine			Phosphatidylserine		
	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (7)	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (6)
14 : 0	0.6 ± 0.1	0.9 ± 0.4	1.0 ± 0.2	1.0 ± 0.2	1.5 ± 0.4	1.3 ± 0.2
15 : 0	0.3 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
16 : 0	17.9 ± 0.8 §	18.4 ± 2.0 §	18.2 ± 0.5 §	4.0 ± 0.2 §-***	5.1 ± 1.1 §	6.7 ± 0.6 §
16 : 1ω7	1.2 ± 0.3	2.0 ± 0.5	1.6 ± 0.4	1.0 ± 0.1	2.0 ± 0.6	1.9 ± 0.5
18 : 0	18.8 ± 1.3 §	18.5 ± 1.4 §	18.0 ± 1.0 §	42.4 ± 0.6 §	40.2 ± 2.3 §	39.4 ± 1.3 §
18 : 1ω9	18.3 ± 1.8	18.7 ± 1.0	18.3 ± 0.3	8.4 ± 0.4 *	9.1 ± 1.1	10.3 ± 0.6
18 : 2ω6	6.6 ± 0.6	6.8 ± 0.9	6.3 ± 0.3	2.3 ± 1.2	2.7 ± 1.3	2.1 ± 0.2
20 : 2ω9	0.4 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
20 : 3ω6	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.4 ± 0.2	1.6 ± 0.4	3.0 ± 1.2
20 : 4ω6	17.6 ± 1.4	18.2 ± 2.0	19.5 ± 0.6	21.4 ± 0.3 *	20.4 ± 1.1	18.4 ± 1.0
22 : 0	trace	trace	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
22 : 4ω6	6.7 ± 0.8	5.6 ± 1.1	5.8 ± 0.6	3.4 ± 0.5	3.2 ± 0.2	2.7 ± 0.2
22 : 5ω3	3.4 ± 0.4	2.7 ± 0.7	2.9 ± 0.2	3.0 ± 0.3	2.2 ± 0.3	2.3 ± 0.3
22 : 6ω3	4.2 ± 0.7	2.8 ± 0.7	3.9 ± 0.4	6.6 ± 1.0	5.0 ± 0.8	5.7 ± 0.7
Others	3.1	1.5	2.7	3.8	6.0	5.1

* Difference significant in t-test, $P < 0.05$.
** Difference significant in t-test, $P < 0.02$.
*** Difference significant in t-test, $P < 0.01$.
**** Difference significant in t-test, $P < 0.002$.
§ Including the dimethylacetals (DMA).

a lower arachidonic acid (20 : 4ω6) level. In phosphatidylserine from myotonic patients we found a decrease in palmitic acid (16 : 0).

Table II gives the results for the major choline-containing phospholipids, namely phosphatidylcholine and sphingomyelin. No important abnormality was found, in contrast to the findings of Kunze [8] who reported sphingomyelin from dystrophic patients as having too much stearic acid (18 : 0) and too little linoleic (18 : 2ω6), nervonic (24 : 1ω9) and palmitic (16 : 0) acid.

Table III contains the fatty acid pattern of lysophosphatidylcholine and phosphatidic acid, and Table IV the composition of the glycosphingolipid fraction and of spot 8, an unidentified lipid. The fatty acid profiles of the last four compounds have never been reported in muscle dystrophies. The only difference from normal was a lower content of 22 : 0 in lysophosphatidylcholine from Duchenne erythrocytes. Control values for these lipids are scarce in literature, although higher levels of palmitic acid (16 : 0) have been reported in lysophosphatidylcholine than we found [18]. Suitable values for the other three lipids are not available.

The most astonishing deviations were found in the di- and triglycerides (Table V). The palmitoleic acid (16 : 1ω7) content was drastically lowered, both in Duchenne and myotonic dystrophy. This supports to some extent the findings of Howland and Iyer [7], who reported less palmitoleic acid in total lipid extract.

The results for the blood plasma phospholipids are given in Tables VI and VII. Duchenne phospholipids do not show any aberration. In myotonic dystrophy the content of myristic acid (14 : 0) is raised in sphingomyelin and lysophosphatidylcholine. Very significant is the decrease in eicosatrienoic acid

TABLE II

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN FROM ERYTHROCYTE MEMBRANES

Legends: see Table I.

Acid	Phosphatidylcholine			Sphingomyelin		
	Myotonic dystrophy (4)	Duchenne dystrophy (6)	Control (6)	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (5)
14 : 0	1.1 ± 0.4	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.3	1.1 ± 0.4	1.4 ± 0.3
15 : 0	0.5 ± 0.1	0.5 ± 0.1	1.1 ± 0.5	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
16 : 0	33.3 ± 0.8	31.2 ± 0.7	32.6 ± 0.4	25.7 ± 0.9	24.0 ± 1.2	25.9 ± 0.9
16 : 1ω7	1.3 ± 0.7	1.9 ± 0.4	1.5 ± 0.4	1.3 ± 0.7	1.5 ± 0.4	1.9 ± 0.4
18 : 0	12.8 ± 0.6	15.5 ± 1.0	14.0 ± 0.7	9.8 ± 1.0	16.3 ± 3.1	10.1 ± 1.0
18 : 1ω9	18.2 ± 0.6	19.3 ± 0.6	19.9 ± 0.3	4.2 ± 0.6	7.1 ± 1.4	6.1 ± 0.6
18 : 2ω6	22.6 ± 0.5 *	19.1 ± 1.3	20.3 ± 0.4	0.8 ± 0.1	2.3 ± 0.7	1.0 ± 0.2
20 : 2ω9	0.8 ± 0.3	0.6 ± 0.2	0.3 ± 0.2	0.7 ± 0.3 **	0.4 ± 0.1	0.2 ± 0.1
20 : 3ω6	1.3 ± 0.3	1.5 ± 0.2	1.5 ± 0.1	n.d.	n.d.	n.d.
20 : 4ω6	4.0 ± 0.4	4.6 ± 0.7	4.0 ± 0.2	1.5 ± 0.5	2.0 ± 0.5	1.2 ± 0.3
22 : 0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	8.7 ± 0.6	7.7 ± 0.6	8.3 ± 0.8
22 : 4ω6	0.4 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	n.d.	n.d.	n.d.
24 : 0	trace	trace	trace	15.2 ± 0.5	11.8 ± 1.7	13.5 ± 1.2
24 : 1ω9	n.d.	n.d.	n.d.	21.6 ± 1.2	17.2 ± 2.4	19.5 ± 1.1
22 : 6ω3	0.7 ± 0.2	0.7 ± 0.2	0.9 ± 0.2	1.1 ± 0.2	0.9 ± 0.3	1.5 ± 0.5
Others	2.6	3.1	2.1	7.8	7.2	8.9

TABLE III

FATTY ACID COMPOSITION OF LYSOPHOSPHATIDYLCHOLINE AND PHOSPHATIDIC ACID FROM ERYTHROCYTE MEMBRANES

Legends: see Table I.

Acid	Lysophosphatidylcholine			Phosphatidic acid		
	Myotonic dystrophy (4)	Duchenne dystrophy (6)	Control (6)	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (4)
14 : 0	4.6 ± 0.4	5.3 ± 0.6	5.7 ± 0.6	3.2 ± 0.4	4.0 ± 0.7	3.6 ± 0.8
15 : 0	2.0 ± 0.2	1.6 ± 0.1	1.8 ± 0.2	3.9 ± 2.1	1.4 ± 0.2	0.8 ± 0.4
16 : 0	24.2 ± 3.7	21.1 ± 1.0	23.7 ± 2.6	21.0 ± 1.3	21.8 ± 1.5	23.0 ± 2.3
16 : 1ω7	5.0 ± 0.9	3.6 ± 0.8	3.7 ± 1.2	4.3 ± 1.4	4.3 ± 0.7	4.4 ± 0.7
18 : 0	32.4 ± 2.6	36.0 ± 3.4 *	25.1 ± 7.5	24.8 ± 1.9	26.3 ± 2.3	21.9 ± 4.4
18 : 1ω9	12.2 ± 1.0	13.8 ± 1.4	16.0 ± 2.1	18.1 ± 1.7	18.8 ± 2.5	21.9 ± 3.7
18 : 2ω6	1.8 ± 0.2	2.8 ± 0.7	2.1 ± 0.2	7.1 ± 1.1	6.2 ± 1.3	6.2 ± 1.2
20 : 2ω9	2.6 ± 1.1	2.2 ± 0.4	2.3 ± 0.4	3.1 ± 0.8	3.3 ± 1.5	1.7 ± 0.5
20 : 4ω6	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	2.1 ± 0.9	1.7 ± 0.7	2.5 ± 0.4
22 : 0	3.9 ± 0.4	2.0 ± 0.4 **	5.0 ± 1.0	2.5 ± 0.7	1.8 ± 0.8	1.5 ± 0.5
22 : 4ω6	1.6 ± 0.3 *	0.1 ± 0.1	0.3 ± 0.2	3.7 ± 1.8	1.0 ± 0.4	0.4 ± 0.3
24 : 0	2.1 ± 0.7	1.0 ± 0.7	2.0 ± 1.1	n.d.	n.d.	n.d.
24 : 1ω9	0.5 ± 0.3	0.6 ± 0.5	1.6 ± 1.4	n.d.	n.d.	n.d.
Others	6.7	9.5	10.4	6.2	9.4	12.1

(20 : 3ω6) in phosphatidylcholine. The dimethylacetal contents included in the figures of 16 : 0 and 18 : 0 of phosphatidylethanolamine were not significantly different between the 3 groups. The values were (on an average) 1.9% for 16 : 0 DMA and 3.9% for 18 : 0 DMA. Wakamatsu et al. [19] measured a decreased level of palmitoleic acid and more stearic acid in the total phospholipid fraction of myotonic plasma. Phillips and Dodge [20] reported fatty acid

TABLE IV

FATTY ACID COMPOSITION OF SPOT 8 (SEE FIG. 1 AND TEXT) AND GLYCOSPHINGOLIPIDS FROM ERYTHROCYTE MEMBRANES

Legends: see Table I.

Acid	Spot 8			Glycosphingolipids		
	Myotonic dystrophy (2)	Duchenne dystrophy (4)	Control (3)	Myotonic dystrophy (4)	Duchenne dystrophy (4)	Control (5)
14 : 0	8.6 ± 2.3 *	5.1 ± 0.4	4.9 ± 0.1	4.8 ± 2.4	3.6 ± 0.5	3.9 ± 0.2
15 : 0	1.3 ± 0.5	1.9 ± 0.3	2.1 ± 0.4	1.8 ± 0.2	1.4 ± 0.3	1.5 ± 0.3
16 : 0	16.3 ± 1.0	21.9 ± 3.0	19.9 ± 1.3	16.3 ± 1.6	15.2 ± 1.9	17.7 ± 0.4
16 : 1ω7	3.2 ± 1.0	7.4 ± 1.0	6.7 ± 1.5	4.9 ± 0.7	3.6 ± 0.7	6.1 ± 1.1
18 : 0	20.9 ± 5.2	28.5 ± 3.0	35.0 ± 2.6	18.4 ± 2.3	22.9 ± 2.7	20.4 ± 4.5
18 : 1ω9	11.4 ± 0.2	15.8 ± 1.1	16.8 ± 1.9	12.6 ± 2.7	11.2 ± 0.7	15.5 ± 2.4
18 : 2ω6	1.3 ± 0.4	3.1 ± 1.0	3.4 ± 0.6	1.6 ± 0.4	2.6 ± 0.4	2.4 ± 0.7
20 : 2ω9	2.3 ± 1.1	1.9 ± 0.6	1.0 ± 0.2	1.6 ± 0.7	2.2 ± 0.7	1.4 ± 0.2
20 : 4ω6	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	n.d.	trace	n.d.
22 : 0	1.2 ± 0.7	1.7 ± 0.2	1.7 ± 0.5	7.9 ± 0.5	7.4 ± 1.4	8.3 ± 2.1
22 : 4ω6	0.8 ± 0.4	0.8 ± 0.2	1.1 ± 0.5	n.d.	n.d.	n.d.
24 : 0	n.d.	n.d.	n.d.	10.4 ± 1.1	9.1 ± 2.5	6.7 ± 1.5
24 : 1ω9	n.d.	n.d.	n.d.	8.6 ± 1.1	7.8 ± 2.6	5.2 ± 1.8
Others	32.6	11.7	7.3	11.1	12.9	10.9

TABLE V

FATTY ACID COMPOSITION OF TRIGLYCERIDES AND DIGLYCERIDES FROM ERYTHROCYTE MEMBRANES

Legends: see Table I.

Acid	Triglycerides			Diglycerides		
	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (5)	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (4)
14 : 0	6.2 ± 1.5	5.6 ± 1.0	3.7 ± 1.6	5.0 ± 1.6	4.4 ± 1.4	5.3 ± 0.9
15 : 0	1.7 ± 0.2	2.5 ± 0.2	1.5 ± 0.3	5.7 ± 3.8	1.8 ± 0.3	3.0 ± 0.6
16 : 0	17.0 ± 1.2	20.4 ± 2.9	20.0 ± 1.6	16.9 ± 2.1	15.8 ± 0.8	20.3 ± 2.7
16 : 1ω7	2.6 ± 0.3 **	8.2 ± 1.3	10.6 ± 2.3	1.3 ± 0.5 ***	4.0 ± 0.7 *	9.1 ± 2.5
18 : 0	11.4 ± 1.6	9.0 ± 1.0	9.7 ± 0.8	12.8 ± 1.8	10.3 ± 0.9	9.2 ± 0.9
18 : 1ω9	9.0 ± 3.6	10.5 ± 0.7	12.7 ± 0.9	9.2 ± 4.4	6.5 ± 1.4	10.3 ± 2.4
18 : 2ω6	2.3 ± 0.2	1.4 ± 0.2	1.7 ± 0.4	1.5 ± 0.5	1.2 ± 0.2	1.0 ± 0.2
19 : 0	6.0 ± 2.2 *	2.1 ± 1.3	1.2 ± 0.6	4.7 ± 3.7	3.0 ± 1.1	3.8 ± 2.7
18 : 3ω6	2.3 ± 0.5	2.2 ± 0.7	3.0 ± 1.5	1.2 ± 0.5	2.8 ± 1.2	2.8 ± 1.0
20 : 2ω9	7.6 ± 1.6	12.2 ± 2.4	8.8 ± 2.0	7.9 ± 1.9	14.0 ± 2.5	9.9 ± 2.2
20 : 3ω6	7.9 ± 1.5	3.6 ± 1.5	5.5 ± 2.0	10.4 ± 2.4	5.4 ± 1.7	3.6 ± 1.3
22 : 0	10.2 ± 1.6	10.1 ± 3.5	9.6 ± 1.3	15.6 ± 2.2	13.9 ± 2.5	9.1 ± 1.2
Others	15.8	12.2	21.6	7.8	16.9	12.6

values for phospholipid fractions in controls. Our phosphatidylcholine profile agrees well with their values, the other three profiles less well for some fatty acids.

It may be observed that the standard deviations in myotonic material are rather small, those in Duchenne dystrophy are higher than in controls. The significance is unclear, but it may point to a difference in homogeneity between the groups.

TABLE VI

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE AND LYSOPHOSPHATIDYLCHOLINE FROM BLOOD PLASMA

Legends: see Table I.

Acid	Phosphatidylcholine			Lysophosphatidylcholine		
	Myotonic dystrophy (4)	Duchenne dystrophy (4)	Control (6)	Myotonic dystrophy (3)	Duchenne dystrophy (5)	Control (6)
14 : 0	2.1 ± 0.7	1.9 ± 0.6	1.0 ± 0.2	8.6 ± 1.5 ***	5.8 ± 1.5	4.4 ± 0.4
15 : 0	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	1.5 ± 0.7	2.2 ± 0.5	1.9 ± 0.5
16 : 0	28.0 ± 0.8	28.3 ± 0.9	26.9 ± 0.4	30.8 ± 0.5	28.0 ± 1.9	27.6 ± 2.4
16 : 1ω7	2.2 ± 0.7	2.4 ± 0.8	1.6 ± 0.7	5.7 ± 1.9	4.8 ± 1.0	6.1 ± 1.5
18 : 0	13.3 ± 0.3	13.4 ± 0.5	15.3 ± 0.5	13.5 ± 0.9	16.9 ± 2.9	23.8 ± 3.7
18 : 1ω9	13.9 ± 0.3	14.8 ± 0.1	14.1 ± 0.5	14.6 ± 1.4	17.6 ± 4.6	17.8 ± 0.9
18 : 2ω6	23.2 ± 1.7	20.5 ± 1.3	23.7 ± 0.8	6.0 ± 1.4	6.4 ± 0.4	7.0 ± 0.8
20 : 2ω9	0.7 ± 0.1	0.7 ± 0.4	0.5 ± 0.2	1.4 ± 0.5	2.2 ± 0.6	1.5 ± 0.4
20 : 3ω6	2.0 ± 0.2 ****	3.0 ± 0.6	3.1 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20 : 4ω6	6.6 ± 0.4	8.0 ± 0.6	7.6 ± 0.5	0.5 ± 0.2	0.5 ± 0.2	0.7 ± 0.5
22 : 0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.9 ± 0.8	1.7 ± 0.2	1.5 ± 0.6
22 : 6ω3	2.0 ± 0.6	2.1 ± 0.2	2.2 ± 0.3	n.d.	n.d.	n.d.
Others	5.2	4.2	3.4	15.1	13.5	7.3

TABLE VII

FATTY ACID COMPOSITION OF SPHINGOMYELIN AND PHOSPHATIDYLETHANOLAMINE FROM BLOOD PLASMA

Legends: see Table I.

Acid	Sphingomyelin			Phosphatidylethanolamine		
	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (6)	Myotonic dystrophy (4)	Duchenne dystrophy (5)	Control (4)
14 : 0	5.0 ± 0.4 **	3.5 ± 0.5	3.3 ± 0.4	6.7 ± 1.5	7.1 ± 1.6	4.6 ± 1.0
15 : 0	2.7 ± 0.9	1.5 ± 0.4	1.1 ± 0.2	1.6 ± 0.5	1.7 ± 0.5	1.8 ± 0.3
16 : 0	26.9 ± 1.3	24.8 ± 2.8	24.4 ± 0.6	22.7 ± 2.2 §	24.3 ± 3.1 §	17.2 ± 2.6 §
16 : 1ω7	3.3 ± 2.0	4.6 ± 1.0	3.5 ± 0.5	7.2 ± 0.9	4.2 ± 1.3	4.4 ± 2.0
18 : 0	12.8 ± 1.3 *	15.6 ± 2.1	18.6 ± 2.3	17.9 ± 2.5 §	18.8 ± 4.2 §	25.9 ± 3.5 §
18 : 1ω9	7.1 ± 2.4	12.3 ± 2.3	9.9 ± 0.8	14.0 ± 1.4	14.1 ± 4.7	15.5 ± 1.0
18 : 2ω6	2.8 ± 0.8	2.6 ± 0.3	3.5 ± 0.4	6.1 ± 0.7	4.6 ± 0.7	8.1 ± 1.8
20 : 2ω9	0.3 ± 0.1	0.8 ± 0.2	0.9 ± 0.4	2.5 ± 0.8	1.5 ± 0.2	2.2 ± 0.5
20 : 4ω6	1.6 ± 0.6	2.4 ± 0.6	2.8 ± 0.4	6.5 ± 0.7	2.4 ± 1.0	5.6 ± 1.0
22 : 0	10.2 ± 2.5	8.0 ± 0.6	7.8 ± 0.8	1.1 ± 0.2	2.5 ± 1.0	1.6 ± 0.7
24 : 0	2.4 ± 0.8	3.3 ± 0.5	5.0 ± 0.9	n.d.	n.d.	n.d.
24 : 1ω9	5.3 ± 1.5	6.1 ± 0.9	6.5 ± 2.0	n.d.	n.d.	n.d.
22 : 6ω3	1.2 ± 0.7	0.3 ± 0.1	0.2 ± 0.1	2.3 ± 0.2	1.9 ± 0.9	1.6 ± 0.5
Others	18.4	14.2	12.5	11.4	16.9	11.5

Discussion

Although the clinical syndromes in Duchenne dystrophy are very different from those in myotonic dystrophy, in both diseases the plasma membrane may be impaired. Rowland [6] has summarized many indications for this hypothesis, coming from both muscle and erythrocyte membrane data. Recently, a few other reports have been published, which point to the possibility of a general membrane defect. Mokri et al. [21] demonstrated lesions in muscle plasma membranes in an early stage of the Duchenne disease. Furthermore, the membrane of Duchenne erythrocytes responds abnormally to high cellular calcium concentrations [22]. Interestingly in this context, Roses et al. [23] found that phenytoin normalizes the increased membrane fluidity in myotonic erythrocytes. Phenytoin stimulates calcium binding to phospholipids [24]. This drug also lowers the increased phospholipase A activity in both myotonic and Duchenne red blood cells [25]. Whether this effect is due to increased calcium affinity of the membrane lipids, is not clear; phospholipase A is stimulated by calcium [26].

It seems likely to ascribe many of the aberrations to a defect in the architecture of the cell membrane of various tissues, among them the red blood cells. Structural proteins or lipids, or both, may be affected in a different manner in the various types of muscular dystrophy. Apart from the abnormal phosphorylation of membrane proteins, there has existed hitherto no clear indication of impairment of erythrocyte structural proteins (see also Roses [27]).

With respect to erythrocyte lipids in muscle disease some papers have been published. Andiman et al. [28] and Roses and coworkers [29] found the same phospholipid and ganglioside content for myotonic and control cells without

giving details. Thomas and Harper [30] found no deviation in neutral and phospholipid classes in dystrophia myotonica. Kunze et al. [8], too, reported no change in this disease but found that sphingomyelin content in Duchenne erythrocytes was increased. This was also found by Kalofoutis [31]. The latter study shows lowered lecithin and raised lysolecithin and cardiolipin levels. The fatty acid pattern of the total lipid extract from Duchenne erythrocytes has been analysed by Howland and Iyer [7]. They found four times less palmitoleic acid (16 : 1), ascribing this partly to a lower triglyceride content. Kunze and coworkers [8] have reported the fatty acid composition of the three phospholipids, with the highest concentration in red blood cells. They have detected changes in phosphatidylethanolamine and sphingomyelin. However, their fatty acid pattern of control sphingomyelin differed greatly from literature values (for example [18]). Furthermore, they do not distinguish between autosomal and X-recessive progressive muscle dystrophy and found no palmitoleic acid in any group.

Therefore we studied the fatty acid composition of isolated neutral and phospholipids in erythrocytes from both Duchenne patients and another well-defined group, namely patients suffering from the congenital form of myotonic dystrophy.

In Duchenne erythrocytes was found only a raised content of 18 : 0 and less 22 : 0 in lysophosphatidylcholine and less 16 : 1 in diglycerides. In myotonic cells various significant differences exist, the most striking ones being the five-fold increased level of 22 : 4ω6 in lysophosphatidylcholine and the 19 : 0 level in triglycerides, less 16 : 0 in phosphatidylserine and the drastically lowered content in di- and triglycerides of palmitoleic acid (16 : 1).

Therefore it is clear that we were not able to reproduce the findings of Kunze et al. [8]. Especially, their fatty acid pattern of sphingomyelin both in controls and in dystrophic patients is quite different from our results. The control values we found agree well with those in the literature [18].

The decreased level of palmitoleic acid in two neutral lipid fractions from both groups of patients is of interest. This fatty acid was mentioned by Howland and Iyer [7] to be decreased to a great extent in total lipid extract from Duchenne patients and carriers. In some other types of neuromuscular disorder increased or normal levels were measured. The decreased level we found in diglycerides cannot explain their findings, because this fraction covers only traces in erythrocytes [18]. Combination of decreased di- and triglyceride contents with lower palmitoleic acid concentrations in both fractions could account for their results. However, they did not report a lowered diglyceride content. When a calculation is based on the lipid levels in control cells [18] and our fatty acid compositions, we find for palmitoleic acid roughly only 2.5% in total lipids, which is in good agreement with other figures [18], but two times lower than those of Howland and Iyer. There is hardly any knowledge about the metabolism of palmitoleic acid so that further conclusions had to be omitted.

To obtain insight into possible involvement of other tissues in lipid metabolism and to analyse its consequence for the erythrocyte fatty acid pattern, we have studied the fatty acid composition of four phospholipids present in the lipoproteins of plasma. The only abnormalities found were an increased level of

14 : 0, both in sphingomyelin and lysosphosphatidylcholine, less 18 : 0 in sphingomyelin and less 20 : 3ω6 in phosphatidylcholine, all in myotonic plasma. The origin of these aberrations is unclear, with no indication of a special metabolic pathway or organ which could be involved. In Duchenne plasma fractions no deviation was found. To our knowledge only one report has been published which shows fatty acid composition of dystrophic serum [19]. More stearic and less palmitoleic acid in total phospholipids were found. A lower percentage of palmitoleic acid in all lipid fractions, measured by these authors, was observed.

From these studies we cannot exclude a membrane lipid deviation present only in the early stages of the dystrophic red blood cell lifespan. From diet, incorporation and cell-plasma exchange studies [32] we have to conclude that deviations due to de novo lipid synthesis in bone marrow or circulating reticulocytes may have been normalized in mature blood cells. The extent to which fatty acid patterns originally present and possibly abnormal, have disappeared by these mechanisms cannot be established. Anyhow, we have not found any phospholipid type possessing more deviations than others.

Di- and triglycerides can be synthesized de novo by reticulocytes [33], although most of these fractions found in erythrocytes may have been derived from contamination with serum lipoproteins [18]. Therefore abnormalities in these lipids can hardly be interpeted. Recently, attention has been paid to diglycerides, which may be involved in hormone and calcium action [34]. Together with the described effects of phenytoin, an important metabolic starting-point for research in the field of muscle dystrophy may exist here.

We have to conclude that the various deviations found in both dystrophic groups cannot simply be explained by influences of the fatty acids in erythrocyte lipids. It may be suggested that it is not only the bulk of the phospholipids which regulates cell metabolism. The compounds constituting the microenvironment of enzymes or ion channels are of great importance [35,17]. The influence of fatty acids on this has scarcely been reported [9], but Bloj and coworkers have clearly demonstrated a correlation between the double bond index of membrane lipids and changes in allosteric transitions of $(Na^+ + K^+)$-ATPase and acetylcholinesterase in rat erythrocytes [36]. Nevertheless, it is difficult to connect the many metabolic differences with a deviation in one micro-environment.

Obviously, if there exists a lipid abnormality in muscle dystrophies, it is not manifested in the phospholipid fatty acid patterns of erythrocytes, but aberrations in lipid metabolism cannot be excluded from this study.

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After the acceptance of this paper two studies have been published in which are reported the lipid content and fatty acid composition of some phospholipids in erythrocytes from Duchenne patients [37,38]. Both papers give further evidence in support of our conclusion that the phospholipids in Duchenne muscular dystrophy are unaltered. The levels of other membrane components of red blood cells from these patients were also recently found by Godin et al. [39] to be normal.

References

1 Percy, A.K. and Miller, M.E. (1975) Nature 258, 147—148
2 Fisher, E.R., Silvestri, E., Vester, J.W., Nolan, S., Ahmad, U. and Danowski, T.S. (1976) J. Am. Med. Assoc. 236, 955
3 Butterfield, D.A. (1977) Biochim. Biophys. Acta 470, 1—7
4 Sha'afi, R.I., Rodan, S.B., Hintz, R.L., Fernandez, S.M. and Rodan, G.A. (1975) Nature 254, 525—526
5 Szibor, R., Redmann, K., Deike, H. and Müller, K. (1976) Helv. Paediatr. Acta 31, 249—256
6 Rowland, L.P. (1976) Arch. Neurol. 33, 315—321
7 Howland, J.L. and Iyer, S.L. (1977) Science 198, 309—310
8 Kunze, D., Reichmann, G., Egger, E., Leuschner, G. and Eckhardt, H. (1973) Clin. Chim. Acta 43, 333—341
9 Coleman, R. (1973) Biochim. Biophys. Acta 300, 1—30
10 Wyss, S.R., Koster, J.F. and Hülsmann, W.C. (1971) Clin. Chim. Acta 35, 277—280
11 Hanahan, D.J. and Ekholm, J.E. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. 31, pp. 168—172, Academic Press, New York
12 Bjerve, K.S., Daae, L.N.W. and Bremer, J. (1974) Anal. Biochem. 58, 238—245
13 Siakotos, A.N. and Rouser, G. (1965) J. Amer. Oil Chem. Soc. 42, 913—919
14 Kates, M. (1972) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. and Work, E., eds.), North-Holland Publishing Company, Amsterdam
15 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600—608
16 Marggraf, W.D. (1976) J. Lipid Res. 17, 622—628
17 Peter, H.W. and Wolf, H.U. (1973) J. Chromatogr. 82, 15—30
18 Nelson, G.J. (1972) in Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism (Nelson, G.J., ed.), pp. 317—386, John Wiley, New York
19 Wakamatsu, H., Nakamura, H., Ito, K., Anazawa, W., Okajima, S., Shigeno, K. and Goto, Y. (1972) Horm. Metab. Res. 4, 458—462
20 Phillips, G.B. and Dodge, J.T. (1967) J. Lipid Res. 8, 676—681
21 Mokri, B. and Engel, A.G. (1975) Neurology 25, 1111—1120
22 Dise, C.A., Goodman, D.B.P., Lake, W.C., Hodson, A. and Rasmussen, H. (1977) Biochem. Biophys. Res. Commun. 79, 1286—1292
23 Roses, A.D., Butterfield, D.A., Appel, S.H. and Chestnut, D.B. (1975) Arch. Neurol. 32, 535—538
24 Goldberg, M.A. (1977) Neurology 27, 827—833
25 Iyer, S.L., Katyare, S.S. and Howland, J.L. (1976) Neurosci. Lett. 2, 103—106
26 Brockerhoff, H. and Jensen, R.G. (1974) Lipolytic Enzymes, pp. 229 and 250, Academic Press, New York
27 Roses, A.D. (1977) in Pathogenesis of Human Muscular Dystrophies (Rowland, L.P., ed.), pp. 648—655, Excerpta Medica, Amsterdam
28 Andiman, R.M., Peter, J.B. and Dhopesh-Warkar, G. (1974) Neurology 24, 352—354
29 Roses, A.D. and Appel, S.H. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1855—1859
30 Thomas, N.S.T. and Harper, P.S. (1978) Clin. Chim. Acta 83, 13—23
31 Kalofoutis, A., Jullien, G. and Spanos, V. (1977) Clin. Chim. Acta 74, 85—87
32 Van Deenen, L.L.M. and De Gier, J. (1974) in The Red Blood Cell (Surgenor, D.McN., ed.), Vol. 1, pp. 147—211, Academic Press, New York
33 Lane, T.A., Ballas, S.K. and Burka, E.R. (1976) Blood 47, 189—195
34 Allen, D. and Michell, R.H. (1977) Biochem. Soc. Trans. 5, 55—59
35 Roelofsen, B. and Van Deenen, L.L.M. (1973) Eur. J. Biochem. 40, 245—257
36 Bloj, B., Morero, R.D., Farías, R.N. and Trucco, R.E. (1973) Biochim. Biophys. Acta 311, 67—79
37 Kobayashi, T., Mawatari, S. and Kuroiwa, Y. (1978) Clin. Chim. Acta 85, 259—266
38 Koski, C.L., Jungalwala, F.B. and Kolodny, E.H. (1978) Clin. Chim. Acta 85, 295—298
39 Godin, D.V., Bridges, M.A. and MacLeod, P.J.M. (1978) Res. Comm. Chem. Pathol. Pharmacol. 20, 331—349

PAPER II

OSMOTIC STABILITY OF ERYTHROCYTES IN HUMAN MUSCULAR DYSTROPHY BEFORE AND AFTER PHOSPHOLIPASE TREATMENT

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OSMOTIC STABILITY OF ERYTHROCYTES IN HUMAN MUSCULAR DYSTROPHY BEFORE AND AFTER PHOSPHOLIPASE TREATMENT

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SUMMARY

The stability of washed erythrocytes from patients with muscular dystrophy was determined in hypotonic phosphate buffered sodium chloride. Control cells were more stable than cells from Duchenne and myotonic patients.

After pretreatment of the cells with phospholipase from pancreas, snake venom or bee venom in the presence of 14 mM Ca^{2+}, the order of osmotic stability in the 3 groups was not changed. In isotonic phosphate buffered NaCl, however, the erythrocytes of the myotonic patients were much more stable than the cells of the Duchenne and the control group.

The lytic process was further studied in control cells with pancreatic phospholipase. 21±3 (SEM) % of the cells were lysed. This process was (partly) prevented by omitting the phospholipase, by replacement of Na^+ by K^+ or Li^+, by lowering the Ca^{2+}-concentration, by omitting phosphate, by ouabain, by glucose, by sucrose and by tetrodotoxin, a Na^+-transport inhibitor. Blocking of the Ca^{2+}-transport by La^{3+} or mersalyl greatly stimulated the lytic process.

INTRODUCTION

Since Matheson and Howland [1] reported an enhanced percentage of echinocytes in blood of Duchenne patients and carriers, several studies

have been published about morphology of patient erythrocytes. Although the results were often controversial [2 and its references], it seems legitimate to conclude from these studies that erythrocytes of Duchenne patients have an increased chance to be transformed *in vitro* to abnormally shaped cells [3,4], which perhaps also holds for cells of myotonic muscular dystrophic patients [5]. However, the survival of dystrophic red blood cells is normal [6]. The same study reports a normal resistance of Duchenne erythrocytes to hypotonic solutions, in contrast to three other publications, reporting a decreased osmotic stability [7,8,9].

We were interested in the osmotic stability because it may offer a chance to check the hypothesis, which postulates a general membrane defect in human muscular dystrophy [10]. Interactions between membrane proteins and phospholipids can be modified by treatment of cells by phospholipases. These enzymes might affect patient erythrocytes different from control cells, if the mozaic of membrane components would be altered. It is likely that this then might be reflected by a change in osmotic resistance.

In the present study we have measured the osmotic stability of erythrocytes, both before and after treatment by various phospholipases, of patients suffering from either congenital myotonic muscular dystrophy or Duchenne muscular dystrophy. The phospholipases used differ in activity when the compression state of the membrane lipid layer is different [11]. Therefore the study of the effects of various phospholipases on different erythrocyte sources may reveal possible variations in interaction between membrane components of red blood cells from patients.

MATERIALS AND METHODS

Blood samples were taken by venapuncture from boys, aged between 3 and 19 years after an overnight fast period. Patients suffered from myotonic muscular dystrophy (congenital form) or from Duchenne muscular dystrophy; controls did not suffer from any neuromuscular or hematological disease. Erythrocytes were purified as described before [12]. The washed cells were suspended in 172 mM Tris-HCl, pH 7.6, to yield a

hematocrit of about 50%. Part of the suspension was diluted with 0.90% (^{W}/v) NaCl, 10 mM phosphate (Na-salt), pH 7.3, to a hematocrit of 4.5%. When pretreatment with added phospholipase was carried out, the dilution medium contained only 0.90% (^{W}/v) NaCl.

The *pretreatment* step of erythrocytes by various phospholipases (EC 3.1.1.4) was carried out as follows [13]: to 1 ml medium, containing 0.90% (^{W}/v) NaCl, 25 mM $CaCl_2$ and 0.6 mM $MgCl_2$, either 8 µg phospholipase A (Crotalus terr.terr.; Boehringer), 0.3 mg phospholipase A (bee venom; Calbiochem) or 0.7 mg phospholipase (porcine pancreas; Boehringer) was added. 50 µl phospholipase mixture was then added to 50 µl erythrocyte suspension and incubated for 45 min at 37°C under shaking. Thereafter, 1 ml of one of the solutions, used in the lysis step, was added. Hemolysis was determined as described below.

The *lysis* step was performed according to Beutler [14]. To 50 µl of the untreated erythrocyte suspension or to 100 µl of the phospholipase treated erythrocyte suspension, 1 ml of isotonic or hypotonic phosphate buffered saline was added. After 30 min at 37°C hemolysis was measured at 540 nm in the supernatant. 100% hemolysis is defined as the extinction of the cells incubated with water, the blank contains isotonic saline.

ATP was measured with the luciferin-luciferase assay [15]. 2,3-Diphosphoglycerate was measured with the method of Krimsky [16].

RESULTS

The osmotic stability of erythrocytes from Duchenne and congenital myotonic muscular dystophy patients in hypotonic solutions is shown in Fig. 1. Both patient groups are more fragile than controls. This can be expressed by the NaCl concentration at which half the amount of the erythrocytes is lysed. The most accurate way to calculate this is to transform the sigmoid curves to straight logistic curves [17], from which the half maximal lytic concentration can be calculated. This yields for controls 0.341±0.006% (^{W}/v) NaCl, for Duchennes 0.372±0.008 and for myotonics 0.392±0.007 (mean ± SEM). The value for Duchenne dystrophy patients is significantly different from

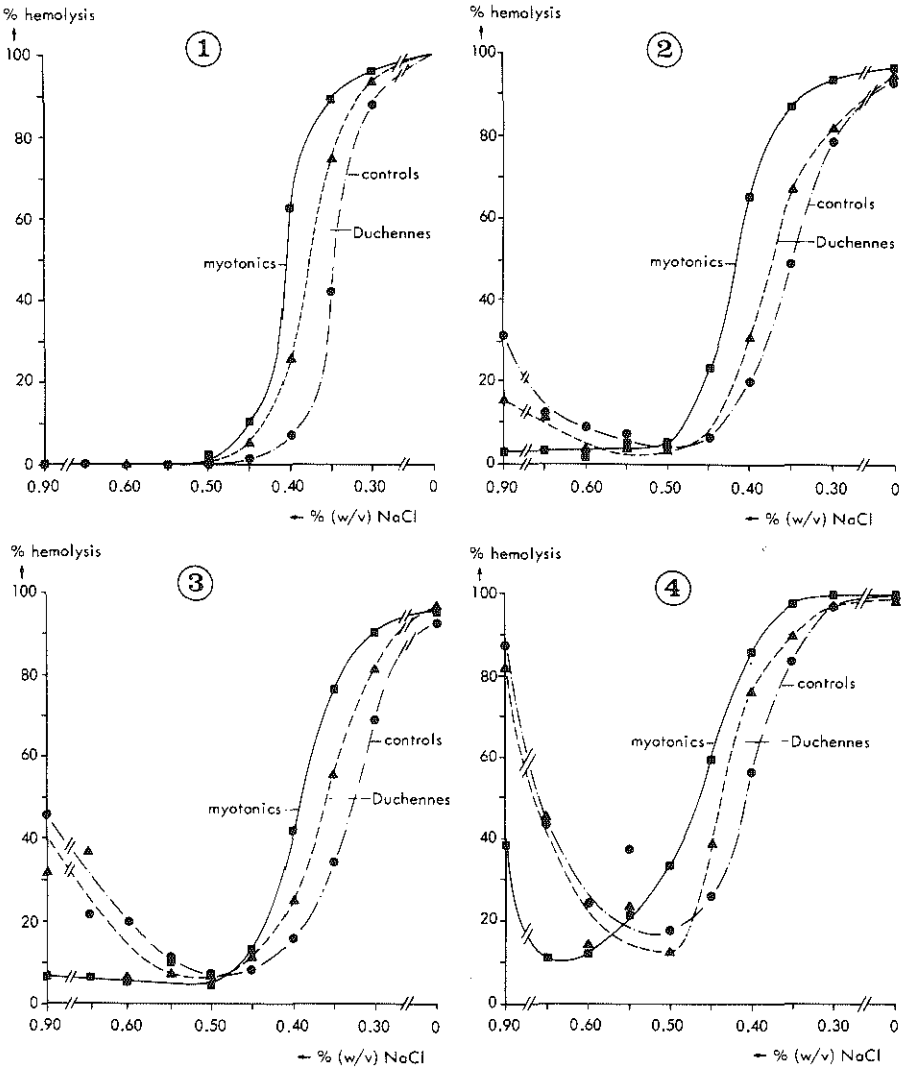

Fig. 1. Lysis of erythrocytes from patients with myotonic dystrophy (congenital form) and with Duchenne muscular dystrophy, and from controls.

Fig. 2. Lysis of erythrocytes, treated with pancreatic phospholipase, from patients with myotonic muscular dystrophy (congenital form) and with Duchenne muscular dystrophy, and from controls.

Fig. 3. Lysis of erythrocytes, treated with snake venom phospholipase (Crotalus terr.terr.), from patients with myotonic muscular dystrophy (congenital form) and with Duchenne muscular dystrophy, and from controls.

Fig. 4. Lysis of erythrocytes, treated with bee venom phospholipase, from patients with myotonic muscular dystrophy (congenital form) and with Duchenne muscular dystrophy, and from controls.

that of controls with $p<0.02$ (Student's t-test), the value for myotonia with $p<0.001$. The difference between Duchenne and myotonic values is not significant ($p>0.1$).

Hemolysis patterns of phospholipase treated erythrocytes are shown in Figs. 2, 3 and 4 for pancreatic phospholipase, snake venom phospholipase and bee venom phospholipase, respectively. The S-shaped curves of untreated cells have been turned into biphasic curves after attack by any of the phospholipases used. At tonicities of 0.45% saline or less the curves resemble that of untreated cells. Red blood cells of myotonic patients are more fragile at 0.35 and 0.40% saline than control cells. Statistical significance followed from the Wilcoxon's signed rank test [see 18]. Transformation of the biphasic curves to logistic curves does not result in straight lines. Although the average hemolysis at 0.35 and 0.40% NaCl is higher for Duchenne than for control cells, the differences of the individual points are not significant according to the Wilcoxon test. We can say that all three groups of cells have become more fragile by about the same degree after phospholipase treatment. This is the case for all the phospholipases tested.

It was surprising to find that treated cells of Duchenne patients and controls lyse to a greater extent in isotonic than in 0.5-0.6% NaCl, while cells from patients with myotonic muscular dystrophy do not (see Figs. 2, 3 and 4). We have tried to identify the cause of this difference by determining a number of factors which may be responsible for the high hemolysis of treated cells from controls. As the difference seems rather independent of the type of phospholipase used, pancreatic phospholipase has been used through the following experiments (Table I). Erythrocytes treated with this enzyme in the standard manner (see Materials and Methods) are lysed to 21±3%. This percentage has been taken as 100% reference to express the influence of the various incubation conditions upon hemolysis. From Table I it can be seen that omission of the enzyme in the pretreatment step results in half the amount of lysis detected in the presence of the lipase. Furthermore, lowering of the Ca^{2+}-concentration from 14 to 6 mM results in almost complete suppression of the hemolysis, while replacement of Na^{+} by

TABLE I

THE INFLUENCE OF VARIOUS CONDITIONS ON THE RATE OF HEMOLYSIS OF ERYTHROCYTES FROM CONTROLS, PRETREATED WITH PANCREAS PHOSPHOLIPASE.

Hemolysis in the changed medium is expressed as percentage (mean ± SEM) of the hemolysis after standard pretreatment with pancreas phospholipase in the same series of experiments in order to determine the relative influences. Hemolysis under standard conditions is 21±3%.

(P) : alteration in the pretreatment step (see Materials and Methods); in the lysis step the compound concentration is 11-fold lower.

(L) : alteration in the lysis step (absent during pretreatment step).

(P+L): compound concentration equal in pretreatment and lysis step.

n : number of experiments.

Change in incubation conditions	Relative hemolysis		
standard method	100	%	(n=18)
no pancreas phospholipase (P)	52±7	%	(n=13)
17.0 instead of 14 mM Ca^{2+} (P)	119	%	
8.5 " " " " " (P)	32	%	
6.0 " " " " " (P)	1	%	
10.0 " " " " " , no pancreas phospholipase (P)	16	%	
8.5 " " " " " , " " " " (P)	1	%	
+ 5 mM EGTA (L)	9±3	%	(n=6)
+ 10 mM EGTA (L)	0;1	%	
+ 0.1 mM $LaCl_3$ (P+L)	330±70	%	(n=3)
+ 50 µM mersalyl (P)	266;422	%	
choline chloride instead of 0.90% NaCl (L)	4±3	%	(n=3)
KCl " " " " (L)	8±3	%	(n=9)
LiCl " " " " (L)	7±1	%	(n=4)
Tris-HCl " " " " (L)	6;17	%	
+ 25 nM tetrodotoxin (P+L)	34±8	%	(n=6)
3.7 instead of 5.5 mM phosphate (L)	49	%	
1.8 " " " " " (L)	10	%	
0 " " " " " (L)	2	%	
Tris-HCl " " " " " (L)	4	%	
pretreatment medium instead of NaCl-phosphate (L)	5±4	%	(n=5)
+ 1 µM carbamyl choline (P+L)	38±23	%	(n=3)
+ 20 mM phenylmethylsulfonyl fluoride (P)	31±22	%	(n=3)
+ 10 mM glucose (P)	47±14	%	(n=8)
+ 10 mM ribose (P)	22±8	%	(n=3)
+ 10 mM sucrose (P)	6;28	%	
+ 1 mM ouabain (P)	49±15	%	(n=9)

other monovalent cations like K^+ and Li^+ results in less than 15% of the original lysis. Omission of phosphate results in hardly detectable hemolysis. La^{3+} and mersalyl, both inhibitors of the Ca^{2+}-efflux, stimulate hemolysis. Tetrodotoxin, which inhibits Na^+-uptake, and the protease inhibitor phenylmethylsulfonyl fluoride suppress lysis, as does carbamyl choline, a cholinergic agent. The sugars glucose, ribose and sucrose depress the rate of lysis to 47%, 22% and 17%, respectively.

DISCUSSION

Increased [7-9,19] and unchanged [6] fragility of Duchenne erythrocytes in the presence of plasma has been reported. Our results with washed red cells suggest that the erythrocyte itself had a lower resistance to osmotic shock, both in Duchenne and congenital myotonic dystrophy.

The aim of the phospholipase treatment was to obtain information about the phospholipid architecture of the membrane. Pure bee venom phospholipase causes breakdown of 20% of phospholipids of intact erythrocytes [13]. Purified pancreatic and snake venom (Crotalus) phospholipase do not hydrolyse these phospholipids, due to the inability of these enzymes to hydrolyse phospholipids at surface pressures above 23 dynes/cm [11]. From Figs. 1-4 it is clear that bee venom phospholipase induces the highest extent of lysis. The order of stability between patient and control groups remains the same at 0-0.5% NaCl, and is not dependent on the kind of phospholipase used. We conclude that the phospholipid packing in patient red cells is not much altered.

At 0.90 and 0.65% NaCl, pretreated cells from myotonic patients are significantly more stable than cells from controls and Duchenne patients. This lability of control erythrocytes was further investigated (Table I). Incubation in pretreatment medium without lipases results in 11% lysis, being 52% of the value found under standard conditions in the presence of pancreatic phospholipase. Addition of EGTA, or lowering of Ca^{2+} from 14 to 6 mM in the presence (or absence) of phospholipase strongly reduces the rate of lysis. A role for Ca^{2+} in the promotion of lysis is further supported by the lysis stimulating effect of La^{3+} and mersalyl, being inhibitors of Ca^{2+}-extrusion, and

stimulators of the passive Ca^{2+}-uptake [see 20,21]. Hemolysis is higher when La^{3+} is present during the pretreatment and the lysis step, compared with its presence during one of these steps. These findings suggest the importance of extracellular Ca^{2+}. Experiments with $^{45}CaCl_2$ do not reveal a correlation between extent of lysis and $^{45}Ca^{2+}$-level in the non-lysed cells. Cellular radioactivity disappears by washing with EGTA.

Also Na^+ promotes lysis. When it is replaced by monovalent cations with a smaller or larger radius, lysis decreases to less than 15%. Tetrodotoxin, a selective suppressor of the Na^+-uptake [22], reduces lysis to 34%.

Decrease of phosphate below the standard level of 5.5 mM results in decreased lysis. No lysis occurs in the pretreatment medium (containing no phosphate).

Which process is responsable for the Ca^{2+}-, Na^+- and phosphate-dependent lysis? It has been reported that Ca^{2+} and Na^+ are involved in the response of red cells to cholinergic agents [23]. In our study carbamyl choline reduced hemolysis. The rate of lysis is optimal between pH 7.6 and 7.8 (with or without pancreas lipase). Maybe an enzymatic process stimulated by Ca^{2+}, Na^+ and phosphate is involved. A serine protease inhibitor, which does not inhibit Ca^{2+}-stimulated erythrocyte protease [24], inhibits lysis in our experiments.

Energy depletion of red cells, causing high Ca^{2+}-permeability [25], has to be considered as probable cause of hemolysis. However, both metabolizable and non-metabolizable sugars (sucrose) strongly reduce lysis. Ouabain has the same effect. Probably this is not due to inhibition of the Na^+-K^+-pump, since this pump cannot function at exogenous K^+-level of 0.5 mM [26], which was measured in the incubation medium by flame photometry. Moreover, we were not able to detect a correlation between levels of ATP or 2,3-diphosphoglycerate in the remaining non-lysed cells and the degree of lysis.

In conclusion we can say that a combination of Ca^{2+}, Na^+ and phosphate causes lysis of phospholipase treated erythrocytes from controls and Duchenne patients under isotonic conditions. This process, likely not due to lack of energy, is much less active in red cells from patients with congenital myotonic dystrophy.

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REFERENCES

1. Matheson, D.W. and Howland, J.L. (1974) Science 184, 165
2. Matheson, D.W., Engel, W.K. and Derrer, E.C. (1976) Neurology 26, 1182
3. Grassi, E., Lucci, B., Marchini, C., Ottonello, S., Parma, M., Reggiani, R., Rossi, G.L. and Tagliavini, J. (1978) Neurology 28, 842
4. Korczyn, A.D., Fishman, P., Djaldetti, M. and Berginer, V.M. (1977) Isr. J. Med. Sci. 13, 131
5. Miller, S.E., Roses, A.D. and Appel, S.H. (1976) Arch. Neurol. 33, 172
6. Adornato, B.T., Corash, L. and Engel, W.K. (1977) Neurology 27, 1093
7. Fisher, E.R., Silvestri, E., Vester, J.W., Nolan, S., Ahmad, U. and Danowski, T.S. (1976) J. Amer. Med. Ass. 236, 955
8. Somer, H. and Olarte, M. (1977) Neurology 27, 361
9. Lloyd, S.J. and Nunn, M.C. (1978) Brit. Med. J. 2, 252
10. Rowland, L.P. (1976) Arch. Neurol. 33, 315
11. Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97
12. Ruitenbeek, W. (1978) Clin. Chim. Acta 89, 99
13. Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83
14. Beutler, E., (1972) In: Hematology (Williams, W.J., Beutler, E., Erslev, A.J. and Rundles, R.W., eds.), McGraw-Hill Book Comp., New York, p. 1375
15. Kimmich, G.A., Randles, J. and Brand, J.S. (1975) Anal. Biochem. 69, 187
16. Krimsky, I.(1970) in: Methoden der Enzymatischen Analyse (Bergmeyer, H.U., ed.), vol. II, Verlag Chemie, Weinheim, p. 1397
17. Bliss, C.I. (1970) Statistics in Biology, McGraw-Hill Book Comp., New York, p. 167
18. Forrester, J.C. and Ury, H.K. (1969) The Lancet i, 239
19. Godin, D.V., Bridges, M.A. and MacLeod, P.J.M. (1978) Res. Commun. Chem. Pathol. Pharmacol. 20, 331
20. Schatzmann, H.J. and Vincenzi, F.F. (1969) J. Physiol. 201, 369
21. Schatzmann, H.J. and Bürgin, H. (1978) Ann. N.Y. Acad. Sci. 307, 125
22. Moore, J.W. and Narahashi, T. (1967) Fed. Proc. 26, 1655
23. Huestis, W.H. (1976) J. Supramolec. Struct. 4, 355
24. King, L.E. Jr. and Morrison, M. (1977) Biochim. Biophys. Acta 471, 162
25. Romero, P.J. and Whittam, R. (1971) J. Physiol. 214, 481
26. Hoffman, J.F. (1966) Amer. J. Med. 41, 666

PAPER III

MEMBRANE-BOUND ENZYMES OF ERYTHROCYTES IN HUMAN MUSCULAR DYSTROPHY; $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase, K^+- and Ca^{2+}-p-Nitrophenylphosphatase

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SUMMARY

Four enzyme activities were studied in erythrocyte membranes from patients with Duchenne and congenital myotonic muscular dystrophy. $(Na^+ + K^+)$-stimulated, Mg^{2+}-dependent adenosinetriphosphatase, measured in two different media, showed normal activity and ouabain inhibition, as did K^+-stimulated *p*-nitrophenylphosphatase. The specific activity of Ca^{2+}-stimulated *p*-nitrophenylphosphatase was twice normal in Duchenne membranes. Ca^{2+}-stimulated, Mg^{2+}-dependent adenosinetriphosphatase was augmented in membranes from both Duchenne and congenital myotonic muscular dystrophic patients. The cause of the increased activities may be the necessity for compensating an alteration in the calcium metabolism in the dystrophic erythrocytes.

Several kinetic parameters of the two Ca^{2+}-stimulated enzyme activities were studied in Duchenne and control membranes. Most were not changed, with the exception of the Na^+-stimulation of Ca^{2+}-ATPase. In Duchenne membranes two affinity sites were present with half maximal activating concentrations of 58 ± 4 and 4 ± 1 mM Na^+. In control membranes only one affinity site was found with $K_a = 26 \pm 9$ mM Na^+.

INTRODUCTION

There is increasing evidence that a general membrane defect is the primary cause of human muscular dystrophy (Rowland 1976). Not only

the membranes of muscle, but also those of other tissues may have altered properties. In the last decade, many data have been published on erythrocyte membranes. Much attention has been paid to the membrane-bound ($Na^+ + K^+$)-stimulated, Mg^{2+}-dependent adenosinetriphosphatase (($Na^+ + K^+$)-ATPase; EC 3.6.1.3) and the effect of ouabain on it. In Duchenne erythrocytes, ouabain has been reported to stimulate the ATPase activity (Brown et al. 1967; Peter et al. 1969; Araki and Mawatari 1971; Niebrőj-Dobosz 1976; Pearson 1978). Others reported ouabain inhibition, although to a slight degree (Mawatari et al. 1976; Siddiqui and Pennington 1977), while normal inhibition has also been described (Klassen and Blostein 1969; Hodson and Pleasure 1977; Souweine et al. 1978). Instead of determining the influence of ouabain on the rate of ATP hydrolysis, the measurement of its effect on the hydrolysis of *p*-nitrophenylphosphate may be studied. The K^+-stimulated component of this hydrolytic activity (K^+-PNPPase), which is sensitive to ouabain, probably reflects the dephosphorylation step of ($Na^+ + K^+$)-ATPase (Bonting 1970).

Ca^{2+}-stimulated, Mg^{2+}-dependent adenosinetriphosphatase (Ca^{2+}-ATPase; EC 3.6.1.3) is the enzymatic activity corresponding to the active Ca^{2+}-extrusion from the (red blood) cell (Schatzmann 1975). One group has reported an increased activity of this enzyme in Duchenne erythrocytes (Hodson and Pleasure 1977). An enzyme with some characteristics identical to those of Ca^{2+}-ATPase is the Ca^{2+}-stimulated *p*-nitrophenylphosphatase (Ca^{2+}-PNPPase). It is also membrane-bound and it has been suggested (Rega et al. 1973) that both enzyme activities are catalyzed by the same molecular system.

In this study the K^+-stimulated and the Ca^{2+}-stimulated PNPPase activities have been measured in erythrocytes of patients suffering from congenital myotonic and Duchenne muscular dystrophy. These enzymes are compared with ($Na^+ + K^+$)-ATPase and Ca^{2+}-ATPase, respectively. Deviations from control values may indicate changes in membrane architecture and may reflect differences in capacity to pump Na^+, K^+ and Ca^{2+}-ions.

MATERIALS AND METHODS

Blood donors were Duchenne patients and male myotonic muscular

dystrophy patients (congenitally manifest type). Controls were boys without neuromuscular or hematological disease. Their age varied between 2.5 and 19.5 years. All children were fasted overnight. Erythrocyte membranes were isolated immediately after venapuncture according to the procedure described previously (Ruitenbeek 1978). Membranes were stored at $-70^{o}C$ and assayed from 1 day to 1½ years later (the length of the storage period had no detectable influence on the properties of the enzymes studied). Protein was determined according to Lowry et al. (1951).

K^+-PNPPase activity was determined in a medium (modification of procedure of Garrahan et al. 1969 and of Lamers et al. 1978) containing 100 mM Tris-HCl, pH 7.5 ($37^{o}C$), 0.25 mM ATP, 1 mM disodium ethylenediamine tetraacetic acid (EDTA), 40 mM KCl, 5 mM $MgCl_2$, 4.5 mM NaCl and 10 mM *p*-nitrophenylphosphate (Tris-salt). The blanks contained Tris-HCl instead of KCl. If present, the ouabain concentration was 0.1 mM. Incubation was carried out at $37^{o}C$ during 50 min in a volume of 1.1 ml with 0.1-0.4 mg protein. Reactions were stopped with 0.1 ml 40% ($^{w}/v$) trichloroacetic acid (TCA). The *p*-nitrophenol formed was measured at 412 nm ($E = 13.1\ mM^{-1}cm^{-1}$) after neutralization of the supernatant to pH 8.5.

($Na^+ + K^+$)-ATPase was assayed in two different media. The first one (minor modification of Mawatari et al. 1976) with *optimal Na^+ and K^+* contains: 20 mM Tris-HCl, pH 7.4 ($37^{o}C$), 3 mM γ-[^{32}P]-ATP (labeled ATP: Amersham, England; 70 mCi/mol; Tris-salt prepared from the disodium salt (Boehringer, G.F.R.) according to Garrahan et al. 1969), 13 mM KCl, 4 mM $MgCl_2$, 80 mM NaCl. From the blanks Na^+ and K^+ were omitted. Ouabain (Merck, G.F.R.) was tested at 0.1 mM. Membranes (about 0.1 mg protein per test) were pretreated with 0.04% ($^{w}/v$) deoxycholate during 10 min at $21^{o}C$ (this sometimes increased the activity). After incubation at $37^{o}C$ (30 min) 20 µl 20% ($^{w}/v$) TCA was added to the incubation mix (200 µl), followed by 20 µl ammonium molybdate (30% ($^{w}/v$) in 5 M H_2SO_4), 10 µl 5 mM K_2HPO_4 and 1.2 ml isobutanol. After rapid mixing and centrifugation the radioactivity of 1 ml of the organic phase was counted. The ($Na^+ + K^+$)-ATPase activity was calculated as the difference in the amount of [^{32}P]-phosphate liberated in the presence and absence of Na^+ and K^+.

The second medium contained *low Na^+ and K^+* as described by Brown et al. (1967; see also Niebrőj-Dobosz 1976 and Mawatari et al. 1976). The procedure followed was similar as described for the optimal Na^+ and K^+ medium.

Ca^{2+}-PNPPase was measured in the following mixture (see Rega et al. 1973): 40 mM Tris-HCl, pH 7.8 (37°C), 1 mM Na_2-ATP (Boehringer, G.F.R.), 0.3 mM $CaCl_2$, 100 mM KCl, 5 mM $MgCl_2$, 10 mM Na-*p*-nitrophenylphosphate, about 0.2 mg protein, which was washed 1 time with 0.6 mM neutralized EDTA before use, to remove traces of Ca^{2+}. In the blanks $CaCl_2$ was replaced by 0.5 mM Na_2-ethyleneglycolbis (beta-aminoethylether)-N,N'-tetraacetic acid (EGTA). The reaction (50 min at 37°C) was stopped by adding 0.1 ml 40% TCA to the incubation medium of 1 ml. The amount of *p*-nitrophenol was measured as described under K^+-PNPPase.

Ca^{2+}-ATPase activity was determined in a medium (see Wolf 1972) with 40 mM Tris-maleic acid, pH 7.2 (37°C), 1.32 mM γ-[^{32}P]-ATP (labeled ATP: Amersham, England; 0.5 Ci/mol; Tris-salt prepared from the disodium salt (Boehringer, G.F.R.; Garrahan et al. 1969), 1.68 mM $CaCl_2$, 1 mM EDTA, 2.03 mM $MgCl_2$, 70 mM NaCl, 0.5 mM ouabain (Merck, G.F.R.). Under these conditions MgATP, free Ca^{2+}, and free Mg^{2+}-concentrations were calculated (Wolf 1973; Sillén and Martell 1964) to be 0.97, 0.60 and 1.00 mM, respectively. In some experiments 0.26 mM ATP and 1.23 mM $MgCl_2$ was used, yielding 0.18 mM MgATP. Membranes were pretreated as for Ca^{2+}-PNPPase. In an incubation volume of 0.2 ml about 0.1 mg protein was used. Blanks did not contain $CaCl_2$. Incubations (20 min at 37°C) were stopped and analysed as described for (Na^+ + K^+)-ATPase.

RESULTS

Table 1 shows the K^+-PNPPase and (Na^+ + K^+)-ATPase activities of the 3 groups of erythrocyte membranes. Both enzymes had normal activities in the two dystrophic groups. Furthermore the influence of ouabain was also normal. Both enzymes were inhibited for 63-100%. In no individual case stimulation by ouabain was found. The K^+-PNPPase activity (y in nmoles PNP formed/min) seems to be positively correlated with the age (x in years; $y = 0.0867\ x + 0.184$ with $r = 0.94$) of

the control children. This correlation was hardly visible in the Duchenne membranes nor for $(Na^+ + K^+)$-ATPase in control membranes. One would expect to find age-dependency for the ATPase, because K^+-PNPPase reflects the dephoshorylating, rate-limiting step of $(Na^+ + K^+)$-ATPase (Bonting 1970). The mean activity of K^+-PNPPase of the two dystrophic groups was not significantly different from control activity with or without regard to the effect of age.

TABLE 1

ACTIVITIES OF K^+-PNPPase AND $(Na^+ + K^+)$-ATPase AND THE OUABAIN INFLUENCE IN ERYTHROCYTE MEMBRANES OF DUCHENNE, MYOTONIC MUSCULAR DYSTROPHY AND CONTROL BOYS

Activities in nmoles/min/mg protein are expressed as mean ± SEM; the ouabain influence as percentage of the activity in the absence of ouabain.

Enzyme	Duchennes		Myotonics		Controls	
	activity	ouabain inhibition	activity	ouabain inhibition	activity	ouabain inhibition
K^+-PNPPase	1.15±0.14 (n=6)	87± 3%	0.91±0.13 (n=3)	95±3%	1.03±0.17 (n=7)	86± 7%
$(Na^+ + K^+)$-ATPase,						
in low $Na^+ + K^+$	3.5 ±0.7 (n=4)	63±22%	n.d.	n.d.	3.4 ±1.1 (n=5)	74±12%
in high $Na^+ + K^+$	4.6 ±0.5 (n=4)	74± 5%	n.d.	n.d.	4.1 ±0.7 (n=5)	100±31%

n.d. = not determined.

In Table 2 some properties of Ca^{2+}-PNPPase are summarized. Duchenne membranes possessed a higher specific activity at 10 mM and at infinite PNPP concentration than control membranes. Although the average activity of myotonic membranes was increased, the difference was not significant. Apart from the activity, other kinetic parameters of Ca^{2+}-PNPPase of Duchenne cells had the same values as control cells. The affinity for *p*-nitrophenylphosphate was similar, while this compound exerted an identically positive allosteric effect on the erythrocyte membrane enzyme from both groups (see also Fig. 1), resulting in a Hill coefficient of + 1.8. The sensitivity to Ca^{2+}-ions and the optimal pH were also the same.

TABLE 2

PROPERTIES OF Ca^{2+}-PNPPase OF ERYTHROCYTE MEMBRANES FROM DUCHENNE AND CONGENITAL MYOTONIC MUSCULAR DYSTROPHIC PATIENTS

Activities (mean ± SEM) are expressed in nmoles/min/mg protein. $K_{0.5a}$ represents the concentration of Ca^{2+} resulting in half-maximal stimulation by Ca^{2+}.

	Duchennes	Myotonics	Controls
Activity at 10 mM PNPP	4.4±0.6 (n=9)[a]	3.6±0.6 (n=4)	2.1±0.5 (n=10)
V_{max}	9.2;9.5		2.8;3.8
$K_{0.5}$ for PNPP (mM)	6.2;7.5		5.7;6.9
Hill coefficient for PNPP	+1.7;+1.9		+1.5;+1.7
$K_{0.5a}$ for Ca^{2+} (μM)	12		10
pH-optimum	7.8		7.8

[a]Significantly different from control value: $P<0.01$.

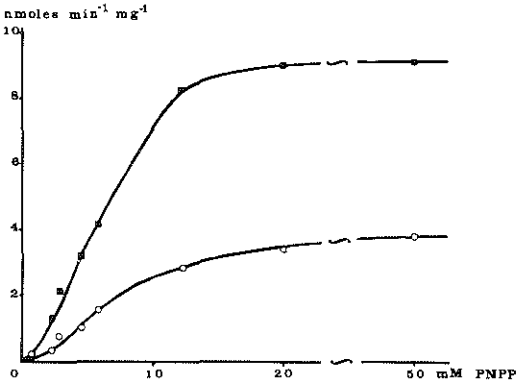

Fig. 1. Activity of Ca^{2+}-PNPPase as function of the concentration of *p*-nitrophenylphosphate in erythrocyte membranes from a control (O-O) and a Duchenne patient (■-■). From this curve and an identical one, the K_m values and Hill coefficients were calculated (see Table 2).

Table 3 contains kinetic characteristics of Ca^{2+}-ATPase of dystrophic and control membranes. Its velocity has been determined at 0.18 and 0.97 mM MgATP. At both substrate concentrations, the membrane preparations of Duchenne and myotonic dystrophic patients were more active

TABLE 3

PROPERTIES OF Ca^{2+}-ATPase IN ERYTHROCYTE MEMBRANES FROM PATIENTS WITH DUCHENNE AND CONGENITAL MYOTONIC MUSCULAR DYSTROPHY

Activities (mean ± SEM) are expressed in nmoles/min/mg protein. See text for explanation of the data. K_a is the concentration of activator at which half-maximal stimulation is reached.

	Duchennes	Myotonics	Controls
Activity at 0.18 mM MgATP	4.0 ±0.5 (n=11)[a]	4.9±0.6 (n=6)[c]	2.3 ±0.6 (n=8)
Activity at 0.97 mM MgATP	5.6 ±0.6 (n=11)[b]	5.9±0.7 (n=6)[a]	2.9 ±0.6 (n=8)
V_{max}	6.1	6.2	3.1
K_m for MgATP (mM)	0.10	0.05	0.06
K_a for Ca^{2+} (mM)	0.11±0.01 (n=4)		0.08±0.03 (n=4)
Hill coefficient for Ca^{2+}	+0.8 ±0.3 (n=4)		+0.8 ±0.2 (n=4)
Hill coefficient for stimulator protein	+0.8		+0.8
K_a for Na^+ (low affinity site) (mM)	58 ±9 (n=4)		26 ±9 (n=4)
K_a for Na^+ (high affinity site) (mM)	4 ±1 (n=4)		absent

[a]Significantly different from control value: P<0.02.

[b]Significantly different from control value: P<0.01.

[c]Significantly different from control value: P<0.002.

in hydrolysing MgATP than control membranes. Calculation of the V_{max}, using the average activities at the two mentioned concentrations, revealed values of 6.1 and 6.2 nmoles phosphate/min/mg protein for Duchenne and myotonic membranes, respectively, which is twofold higher than the control value of 3.1. The same calculation gives K_m-values of 0.10 and 0.05 mM for membranes of Duchenne and myotonic dystrophy patients, respectively, and a control value of 0.06 mM MgATP. These calculations are only allowed when the Lineweaver-Burk plots are straight, which was so in the 3 groups. The activation by Ca^{2+} of the enzyme from Duchenne patients was found to be the same as for control enzyme which results in a normal value for the activation constant $K_{0.5a}$ and for the Hill coefficient: 0.11 mM and 0.8, respectively. Application of a soluble protein, which stimulates the Ca^{2+}-ATPase activity (Gopinath and Vincenzi 1977; isolated from bovine brain according to Cheung 1971), resulted in the same Hill coefficient

of both types of enzymes for this protein. The values, measured at 1 mM MgATP, were + 0.8 both at 50 and 500 μM Ca^{2+}. At fixed Ca^{2+}-concentration the protein stimulates Ca^{2+}-ATPase from Duchenne and control membranes by the same factor, namely 7.8-fold at 50 μM Ca^{2+}, and 1.8-fold at 500 μM Ca^{2+}. The ratio in specific activity between patient and control membranes was therefore not influenced by the protein. In the presence of saturating amounts of stimulator protein the same velocity was obtained at 50 and 500 μM Ca^{2+} in each preparation. The blank values were not increased by the protein, which points to the absence of calcium in the membrane preparations.

Apart from the increased activity, only one other kinetic parameter of the Ca^{2+}-ATPase of Duchenne red blood cells was found to be abnormal. It is the Na^{+}-dependency, as shown in Fig. 2. Control enzyme gives a straight line in a Lineweaver-Burk plot, while Duchenne enzyme shows a biphasic curve, the point of intersecting being 34±2 mM Na^{+}. The K_a for Na^{+}-ions of the control enzyme is 26±9 mM, that for Duchenne membranes 58±9 and 4±1 mM, above and under 34 mM Na^{+}, respectively.

DISCUSSION

Many reports have been published on (Na^{+} + K^{+})-ATPase in erythrocyte membranes from Duchenne patients. The results are highly variable with regard to ouabain influence on enzyme activity, some workers finding remarkable stimulation by this glycoside (Brown et al. 1967; Peter et al. 1969; Araki and Mawatari 1971; Niebrój-Dobosz 1976; Pearson 1978). The present study describes the ouabain influence on K^{+}-PNPPase activity, both in Duchenne and myotonic muscular dystrophy. Ouabain inhibits the phosphatase activity in all individual cases, as is the case for the (Na^{+} + K^{+})-ATPase activity measured under different assay conditions. It seems likely that the isolation procedure for erythrocyte membranes is more important for the ouabain influence than the assay medium (see also Niebrój-Dobosz 1976); for example, serum contamination may play a role. In this study ouabain had the same effect on K^{+}-PNPPase as on (Na^{+} + K^{+})-ATPase in membranes of both groups of dystrophy, so it would be interesting to determine its effect on K^{+}-PNPPase in those cases in which ouabain stimulates (Na^{+} + K^{+})-ATPase.

erythrocyte cytoplasm, because addition of the protein stimulated both control and Duchenne ATPase by the same factor.

A second kinetic parameter of the Ca^{2+}-ATPase from Duchenne cell membranes appears to be altered. The enzyme has a two-phase dependency on Na^{+}-ions (see Fig. 2). It is probable that in these dystrophic cells an extra enzymatic activity is present at Na^{+}-concentrations below 34 mM. This activity may have a physiological significance as the sodium concentration in erythrocytes is about 9 mM (Natelson and Natelson 1978). Preliminary experiments suggest the presence of the Na^{+}-effect also in Duchenne carriers, an interesting point for further research.

One study has been published on Ca^{2+}-ATPase in Duchenne muscle dystrophy. Hodson and Pleasure (1977) reported a higher activity as was found in this study and an increased affinity for MgATP, which disagrees with the results in Table 3. Their K_m value for controls of 0.83 mM, however, was very high in comparison with other reports (Schatzmann 1975) of values of 0.04-0.05 mM. The K_m values of Table 3 are of the same order of magnitude as reported data.

The high activity of Ca^{2+}-ATPase may form a mechanism, necessary to compensate for changes in calcium metabolism in dystrophic red blood cells.Plishker et al. (1978) reported normal calcium levels in erythrocytes of patients suffering from (non-congenital) myotonic muscular dystrophy, which were able to extrude Ca^{2+} at a higher rate. The study of Dise et al. (1977) suggests a normal level also in Duchenne erythrocytes, but demonstrated that a high Ca^{2+}-level had a greater influence on stability and K^{+}-permeability of Duchenne erythrocytes than of control cells. An increased Ca^{2+}-stimulated K^{+}-efflux was described previously (Appel and Roses 1976) for Duchenne cells. All these effects may point to an abnormality of the erythrocyte metabolism with Ca^{2+} playing a causative or intermediary role. If the same defect is present in dystrophic muscle, its function may be impaired to a great extent, in view of the key role of calcium in muscle contraction, relaxation and metabolism. In this context it is of interest that Bodensteiner and Engel (1978) reported calcium accumulation in Duchenne muscle fibres.

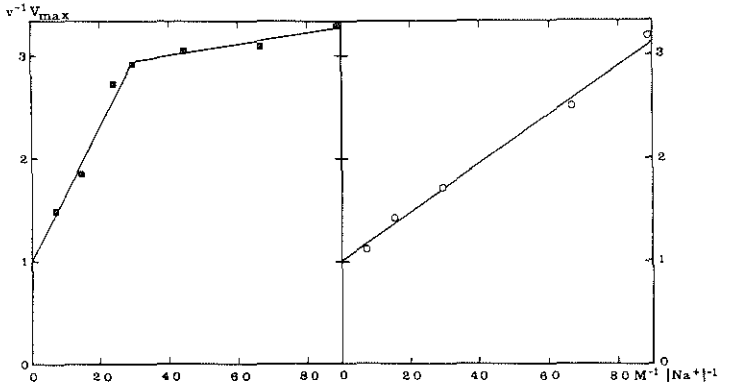

Fig. 2. Reciprocal plot of the Ca^{2+}-ATPase activity against Na^+-concentration. At lower Na^+-concentrations NaCl was replaced by choline chloride. Erythrocyte membranes from Duchenne patients (■-■) and from control (O-O). Curves are the mean of 4 control and 4 Duchenne membrane activities. The relationship is shown between the calculated velocity at infinite Na^+, V_{max}, divided by the measured velocity, against the reciprocal Na^+-concentration in M^{-1}.

Another membrane-bound enzyme activity, of which determination in human muscular dystrophic erythrocytes has not been reported, is Ca^{2+}-PNPPase. Table 2 shows that it is twofold increased in Duchenne dystrophy, while other kinetic parameters are unchanged. These results are comparable with the data of Ca^{2+}-ATPase measurements at 70 mM Na^+ (see Table 3), although this enzyme also has a significantly augmented activity in myotonic dystrophy. The kinetic characteristics of Ca^{2+}-ATPase in red blood cell membranes from Duchenne patients have been further analysed in order to find differences which may be useful for diagnosis of the carrier status in this disease. Enzyme from patients demonstrates normal interaction with MgATP and Ca^{2+}-ions. The K_a-values for Ca^{2+} of 80 and 110 µM are higher than reported for the K_m (see Schatzmann 1975), but in agreement with studies in which the ATPase activity was measured at 80 mM Na^+ (Schatzmann and Rossi 1971). The interaction between the enzyme and a calcium-dependent regulator protein which stimulates Ca^{2+}-ATPase, probably by enhancing the affinity of the enzyme to bind Ca^{2+} (Gopinath and Vincenzi 1977) was also normal for patient's membranes. This protein has been found to stimulate the transport of Ca^{2+}-ions from erythrocytes (MacIntyre and Green 1978). It seems unlikely that the cause of the higher activity could be contamination of the membranes with stimulator protein from

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REFERENCES

Appel, S.H. and A.D. Roses (1976) Membrane biochemical studies in myotonic muscular dystrophy. In: L. Bolis, J.F. Hoffman and A. Leaf (Eds.), *Membranes and Disease*, York, NY, pp. 183-195.

Araki, S. and S. Mawatari (1971) Ouabain and erythrocyte-ghost adenosine triphosphatase - Effects in human muscular dystrophies, *Arch. Neurol. (Chic.)*, 24: 187-190.

Bodensteiner, J.B. and A.G. Engel (1978) Intracellular calcium accumulation in Duchenne dystrophy and other myopathies - A study of 567,000 muscle fibers in 114 biopsies, *Neurology (Minneap.)*, 28: 439-446.

Bonting, S.L. (1970) Sodium-potassium activated adenosinetriphosphatase and cation transport. In: E.E. Bittar (Ed.), *Membranes and Ion Transport, Vol. 1*, Wiley-Interscience, London, pp. 257-392.

Brown, H.D., S.K. Chattopadhyay and A.B. Patel (1967) Erythrocyte abnormality in human myopathy, *Science*, 157: 1577-1578.

Cheung, W.Y. (1971) Cyclic 3',5'-nucleotide phosphodiesterase - Evidence for and properties of a protein activator, *J. biol. Chem.*, 246: 2859-2869.

Dise, C.A., D.B.P. Goodman, W.C. Lake, A. Hodson and H. Rasmussen (1977) Enhanced sensitivity to calcium in Duchenne muscular dystrophy, *Biochem. Biophys. Res. Commun.*, 79: 1286-1292.

Garrahan, P.J., M.I. Pouchan and A.F. Rega (1969) Potassium activated phosphatase from human red blood cells - The mechanism of potassium activation, *J. Physiol. (Lond.)*, 202: 305-327.

Gopinath, R.M. and F.F. Vincenzi (1977) Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+}-Mg^{2+})ATPase, *Biochem. Biophys. Res. Commun.*, 77: 1203-1209.

Hodson, A. and D. Pleasure (1977) Erythrocyte cation-activated adenosine triphosphatases in Duchenne muscular dystrophy, *J. neurol. Sci.*, 32: 361-369.

Klassen, G.A. and R. Blostein (1969) Adenosine triphosphatase and myopathy, *Science*, 163: 492-493

Lamers, J.M.J., J.T. Stinis, W.J. Kort and W.C. Hülsmann (1978) Biochemical studies on the sarcolemmal function in the hypertrophied rabbit heart, *J. mol. cell. Cardiol.*, 10: 235-248.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent, *J. biol. Chem.*, 193: 265-275.

MacIntyre, J.D. and J.W. Green (1978) Stimulation of calcium transport in inside-out vesicles of human erythrocyte membranes by a soluble cytoplasmic activator, *Biochim. biophys. Acta*, 510: 373-377.

Mawatari, S., M. Schonberg and M. Olarte (1976) Biochemical abnormalities of erythrocyte membranes in Duchenne dystrophy - Adenosinetriphosphatase and adenylcyclase, *Arch. Neurol. (Chic.)*, 33: 489-493.

Natelson, S. and E.A. Natelson (1978) The erythrocyte - Chemical composition and metabolism. In: *Principles of Applied Clinical Chemistry, Vol. 2*, Plenum Press, New York, NY, p. 27.

Niebrój-Dobosz, I. (1976) Erythrocyte ghosts ($Na^+ + K^+$)ATPase activity in Duchenne's dystrophy and myotonia, *J. Neurol.*, 214: 61-69.

Pearson, T.W. (1978) ($Na^+ + K^+$)ATPase of Duchenne muscular dystrophy erythrocyte ghosts, *Life Sci.*, 22: 127-132.

Peter, J.B., M. Worsfold and C.M. Pearson (1969) Erythrocyte ghost adenosine triphosphatase (ATPase) in Duchenne dystrophy, *J. Lab. clin. Med.*, 74: 103-108.

Plishker, G.A., H.J. Gitelman and S.H. Appel (1978) Myotonic muscular dystrophy: altered calcium transport in erythrocytes, *Science*, 200: 323-325.

Rega, A.F., D.E. Richards and P.J. Garrahan (1973) Calcium ion-dependent *p*-nitrophenyl phosphate phosphatase activity and calcium ion-dependent adenosine triphosphatase activity from human erythrocyte membranes, *Biochem. J.*, 136: 185-194.

Rowland, L.P. (1976) Pathogenesis of muscular dystrophies, *Arch. Neurol. (Chic.)*, 33: 315-321.

Ruitenbeek, W. (1978) The fatty acid composition of various lipid fractions isolated from erythrocytes and blood plasma of patients with Duchenne and congenital myotonic muscular dystrophy, *Clin. chim. Acta*, 89: 99-110.

Schatzmann, H.J. (1975) Active calcium transport and Ca^{2+}-activated ATPase in human red cells. In: F. Bronner and A. Kleinzeller (Eds.), *Current Topics in Membranes and Transport, Vol. 6*, Academic Press, New York, NY, pp. 125-168.

Schatzmann, H.J. and G.L. Rossi (1971) ($Ca^{2+} + Mg^{2+}$)-activated membrane ATPases in human red cells and their possible relations to cation transport, *Biochim. biophys. Acta*, 241: 379-392.

Siddiqui, P.Q.R. and R.J.T. Pennington (1977) Effect of ouabain upon erythrocyte membrane adenosine triphosphatase in Duchenne muscular dystrophy, *J. neurol. Sci.*, 34: 365-372.

Sillén, L.G. and A.E. Martell (1964) *Stability Constants of Metalion Complexes*, The Chemical Society, London, pp. 634-641 and 650-652.

Souweine, G., J.C. Bernard, Y. Lasne and J. Lachanat (1978) The sodium pump of erythrocytes from patients with Duchenne muscular dystrophy - Effect of ouabain on the active sodium flux and on (Na^+,K^+)ATPase, *J. Neurol.*, 217: 287-294.

Wolf, H.U. (1972) Studies on a Ca^{2+}-dependent ATPase of human erythrocyte membranes - Effects of Ca^{2+} and H^+, *Biochim. biophys. Acta*, 266: 361-375.

Wolf, H.U. (1973) Divalent metal ion buffers with low pH-sensitivity, *Experientia (Basel)*, 29: 241-249.

PAPER IV

FATTY ACID ACTIVATION AND TRANSFER IN BLOOD CELLS OF PATIENTS WITH MUSCULAR DYSTROPHY

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FATTY ACID ACTIVATION AND TRANSFER IN BLOOD CELLS OF PATIENTS WITH MUSCULAR DYSTROPHY

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SUMMARY

Two membrane-bound enzymes, concerned in repair of erythrocyte membranes, have been investigated in patients with muscular dystrophy. The activation of long-chain fatty acids is normal in erythrocytes from Duchenne patients, but increases twofold in cells from myotonic dystrophy patients (congenital form). This alteration is not present in leucocytes.

In all leucocytes tested palmitate was the preferred substrate while palmitoleate and linoleate were activated at a lower rate. In the erythrocytes the 3 fatty acids were activated at the same rate.

Carnitine palmitoyltransferase was not significantly altered in erythrocytes of both groups of patients.

INTRODUCTION

Erythrocyte membranes can easily be purified. These preparations are useful for measurements of general membrane properties, and have been used as a model in attempts to determine the primary biochemical defect in human muscular dystrophy. Both in the Duchenne and myotonic forms of muscular dystrophy indications of abnormalities in cell membranes have been found (Rowland 1976). In addition to muscle membranes (Mokri and Engel 1975; Rowland 1976; Schotland et al. 1977), erythrocyte membranes also possess altered morphological (Matheson and

Howland 1974), physical (Percy and Miller 1975; Bosmann et al. 1976; Butterfield 1977) and biochemical (Appel and Roses 1976; Iyer et al. 1976; Dise et al. 1977; Plishker et al. 1978) characteristics.

In this study we have tested long-chain acyl-CoA synthetase and carnitine palmitoyltransferase. These enzymes are not only bound to membrane, but also play a role in the synthesis of membrane lipids. A change in these enzymes could cause the lowered content of palmitoleic acid in erythrocyte membrane lipids from Duchenne patients and carriers found by Howland and Iyer (1977). We have found the same reduction in red cell di- and triglycerides from dystrophic patients (Ruitenbeek 1978). Abnormal membrane architecture may be reflected in altered enzyme properties.

MATERIALS AND METHODS

Patients were suffering from either Duchenne dystrophy or the congenital form of myotonic muscular dystrophy. Controls were boys without any neuromuscular or hematological disorder. All individuals were aged between 2 and 19 years and did not use any medication. Peripheral blood was taken after overnight fast. The isolation procedure of the erythrocyte membranes has been described before (Ruitenbeek 1978).

Leucocytes were isolated from the upper phase of the dextran-blood mixture according to Wyss et al. (1971). For lysis of the contaminating erythrocytes 50 mM NaCl was used instead of NaF. The cells, isolated at $0^{\circ}C$, were homogenized with a Potter-Elvehjem homogenizer in 0.25 M sucrose, 1 mM EDTA, 10 mM tricine-KOH (pH 7.4). Preparations were stored at $-70^{\circ}C$.

Acyl-CoA synthetase (EC 6.2.1.3) was measured according to Farstad et al. (1967) as modified by Van Tol and Hülsmann (1969) at $37^{\circ}C$ during 30 min. The reaction volume was 0.25 ml, and contained 0.1-0.6 mg protein.

Carnitine palmitoyltransferase (EC 2.3.1.21) was determined by method II of Van Tol and Hülsmann (1969) with modifications resulting in the following final concentrations: 2 mM ATP, 1 mM L-[^{3}H]carnitine (0.2 Ci/mole), 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM KCN, 2.5 mM

$MgCl_2$, 0.25 mM palmitate, 36 µM bovine serum albumin, 1.6 or 8.0 µM palmitoyl-CoA, 5 mM phosphoenolpyruvate, 80 mM Tris-HCl (pH 7.4), 0.4 U adenylate kinase (EC 2.7.4.3) and 0.4 U pyruvate kinase (EC 2.7.1.40).

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Pennington as described by Meijer et al. (1977).

Protein was determined according to Lowry et al. (1951).

RESULTS

The rate of activation of 0.5 mM palmitate, linoleate or palmitoleate in the presence of 70 µM bovine serum albumin by erythrocyte membranes is given in Table 1. The membranes of patients with congenital myotonic muscular dystrophy show a twofold increase in the rate of activation of the 3 fatty acids tested, while membranes of Duchenne patients do not. The range in myotonic cells is 0.42-0.73 nmoles acyl-CoA formed/mg protein/min, that in control cells 0.09-0.51. In all the membranes tested, the rate of activation is the same for the 3 fatty acids tested.

TABLE 1

ACYL-CoA SYNTHETASE ACTIVITIES OF ERYTHROCYTE MEMBRANES

The activities are given in nmoles acyl-CoA formed/min/mg protein ± the standard error of the mean. The significance of the difference is given between patients and controls (Student's t-test).

	Fatty acid		
	Palmitate	Palmitoleate	Linoleate
Controls	0.26±0.04 (n=9)	0.25±0.05 (n=6)	0.28±0.04 (n=9)
Duchenne dystrophy	0.25±0.04 (n=10; N.S.)	0.27±0.06 (n=6; N.S.)	0.29±0.04 (n=10; N.S.)
Myotonic dystrophy	0.55±0.05 (n=5; P<0.001)	0.56±0.05 (n=5; P<0.005)	0.55±0.04 (n=5; P<0.002)

N.S. = not significant.

TABLE 2

ACYL-CoA SYNTHETASE ACTIVITIES IN LEUCOCYTE HOMOGENATES

Activities are in nmoles acyl-CoA formed/min/mg protein ± the standard error of the mean. The significance of the difference is given between the rate of palmitoleate or linoleate activation and that of palmitate. There was no significant difference between the rates with palmitoleate and linoleate.

	Fatty acid		
	Palmitate	Palmitoleate	Linoleate
Controls (n=4)	1.04±0.11	0.66±0.05	0.55±0.04
		(P<0.02)	(P<0.01)
Duchenne dystrophy (n=4)	1.02±0.08	0.64±0.06	0.58±0.04
		(P<0.01)	(P<0.005)
Myotonic dystrophy (n=4)	1.12±0.16	0.79±0.10	0.67±0.07
		(P<0.2)	(P<0.05)

TABLE 3

CARNITINE PALMITOYLTRANSFERASE ACTIVITIES IN ERYTHROCYTE MEMBRANES

Activities are given in nmoles palmitoylcarnitine formed/min/mg protein ± the standard error of the mean. Between Duchenne and myotonic patients there was a significant difference with P<0.01 at 8 μM palmitoyl-CoA and P<0.05 at 1.6 μM palmitoyl-CoA.

	Palmitoyl-CoA (μM)	
	1.6	8.0
Controls (n=10)	0.064±0.008	0.13±0.02
Duchenne dystrophy (n=9)	0.054±0.005	0.11±0.01
Myotonic dystrophy (n=6)	0.083±0.015	0.17±0.04

Table 2 shows the activity of acyl-CoA synthetase in homogenates of leucocytes. There is no difference between fatty acid activation in cells from patients and controls. The pattern of activation of the 3 fatty acids was different from that of erythrocytes. The 3 groups of preparations show a significantly greater velocity in palmitate activation than in palmitoleate and linoleate activation, the latter being 50-70% of the activity with palmitate of 1.0±0.1 nmoles acyl-CoA formed/mg protein/min.

Table 3 shows the carnitine palmitoyltransferase activity in erythrocyte membranes of patients and controls at 1.6 and 8.0 μM palmitoyl-CoA. Respectively 0.064±0.008 and 0.13±0.02 nmoles palmitoylcarnitine were formed/mg protein/min in controls. At both substrate concentrations no abnormality could be detected, either in membranes from Duchenne or in those of congenital myotonic muscular dystrophic patients. But when the patient groups are compared the enzyme is significantly more active in myotonic than in Duchenne patients.

DISCUSSION

In order to maintain the stability of the cell, renewal of its membranes, and thus phospholipid synthesis, is necessary. Mature erythrocytes are unable to synthesize phospholipids *de novo*, but they can acylate lysophospholipids (Van Deenen and De Gier 1974). The substrate for this acylation is long chain acyl-CoA, synthesized by acyl-CoA synthetase. Oliveira and Vaughan (1964) reported the presence of the synthetase in erythrocytes by demonstrating incorporation of fatty acids into phospholipids. We have measured the activity of the enzyme in erythrocyte membranes of patients suffering from two types of muscular dystrophy. In congenital myotonic muscular dystrophy the fatty acid activating capacity is increased, independently of the fatty acid. This may point to the necessity of more intensive renewal of the membranes in this disease. Kunze et al. (1973) have measured the incorporation of palmitate and linoleate into various lipid fractions in intact erythrocytes from Duchenne and control subjects. They found a somewhat higher level of incorporation of the fatty acids in dystrophic erythrocytes. In both groups they measured less linoleate than palmitate incorporation, in contrast to other studies (Oliveira and Vaughan 1964; Waku and Lands 1968), reporting that linoleate was more rapidly incorporated in intact erythrocytes and isolated membranes from controls than palmitate.

We did not find a slower rate of palmitoleate activation compared with palmitate and linoleate activation. This implies that the low content of palmitoleic acid in red blood cell lipids from Duchenne and myotonic muscular dystrophic patients (Howland and Iyer 1977; Ruitenbeek 1978) is not due to relative impairment of the activation of this fatty acid.

The fatty acid activation pattern in homogenates of leucocytes is different from that in erythrocytes. In the congenital form of myotonic muscular dystrophy the activation in leucocytes is not augmented as is the case in erythrocytes (Table 2). The velocity with palmitoleate and linoleate is 50-70% of that with palmitate. This distinction may be explained by the different intracellular localization of acyl-CoA synthetase in erythrocytes and leucocytes. In the latter cells the enzyme is mainly localized in the mitochondria (Scholte 1974). The activity observed in erythrocyte membranes (Table 1) cannot be attributed to contamination with mitochondria, since succinate dehydrogenase (EC 1.3.99.1) was hardly detectable. So, it is likely that the synthetase activity in erythrocytes is localized in the plasma membranes. This membrane may have undergone a fusion at the end of the reticulocyte stage with mitochondrial and/or reticular membrane components. Acyl-CoA synthetase is confined to mitochondria and endoplasmic reticulum in the investigated tissues (Groot et al. 1976). A difference in intracellular localization may imply a different microenvironment for the acyl-CoA synthetase, affecting acyl-specificity.

Carnitine palmitoyltransferase activity in erythrocyte membranes was found by Wittels and Hochstein (1967). They assumed it to be involved in the transport of activated fatty acids, as it is in mitochondria (Bremer 1962; Fritz and Yue 1963). In this context it is of interest to know that acylation of lysophospholipids largely takes place at the inner side of the erythrocyte membrane (Renooij et al. 1974). Recently, Scholte et al. (1979) found that erythrocyte membranes, in contrast to mitochondria, only contain carnitine palmitoyltransferase I. This enzyme also catalyzes the reverse reaction, e.g. the formation of acyl-CoA from acylcarnitine, and may also be involved in acyl-delivery for erythrocyte membrane repair.

The transferase, like acyl-CoA synthetase, is membrane-bound, and could have a different activity if membrane architecture is changed in disease. It is likely to have its origin in mitochondria from reticulocytes, of which membranes may have undergone fusion with the plasma membrane during maturation. We found that carnitine palmitoyltransferase does not have a significantly higher activity in patients with myotonic dystrophy, in contrast to acyl-CoA synthetase. The enzyme was

tested at 1.6 and 8 μM palmitoyl-CoA. Calculation of Michaelis constant yields 2.5-2.8 μM palmitoyl-CoA for the 3 groups, which suggests an unaltered affinity for palmitoyl-CoA, which is of the same order of magnitude as in adult human erythrocytes and other cells (Scholte et al. 1979).

The increased acyl-CoA synthetase activity in the mature erythrocyte membrane in congenital myotonic muscular dystrophy may reflect a need for intensified membrane production or repair at an unknown stage of erythroid development. The anomaly is absent in Duchenne dystrophy. Carnitine palmitoyltransferase activity is normal in erythrocytes in both groups of muscular dystrophy.

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REFERENCES

Appel, S.H. and A.D. Roses (1976) Membrane biochemical studies in myotonic muscular dystrophy. In: L. Bolis, J.F. Hoffman and A. Leaf (Eds.), *Membranes and Disease*, Raven Press, New York, NY, pp. 183-195.

Bosmann, H.B., D.M. Gersten, R.C. Griggs, J.L. Howland, M.S. Hudecki, S. Katyare and J. McLaughlin (1976) Erythrocyte surface membrane alterations - Findings in human and animal muscular dystrophies, *Arch. Neurol. (Chic.)*, 33: 135-138.

Bremer, J. (1962) Carnitine in intermediary metabolism - The metabolism of fatty acid esters of carnitine by mitochondria, *J. biol. Chem.*, 237: 3628-3632.

Butterfield, D.A. (1977) Electron spin resonance studies of erythrocyte membranes in muscular dystrophy, *Accounts Chem. Res.*, 10: 111-116.

Dise, C.A., D.B.P. Goodman, W.C. Lake, A. Hodson and H. Rasmussen (1977) Enhanced sensitivity to calcium in Duchenne muscular dystrophy, *Biochem. biophys. Res. Commun.*, 79: 1286-1292.

Farstad, M., J. Bremer and K. Norum (1967) Long-chain acyl-CoA synthetase in rat liver - A new assay procedure for the enzyme, and studies on its intracellular localization, *Biochim. biophys. Acta*, 132: 492-502.

Fritz, I.B. and K.T.N. Yue (1963) Long chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine, *J. Lipid Res.*, 4: 279-288.

Groot, P.H.E., H.R. Scholte and W.C. Hülsmann (1976) Fatty acid activation - Specificity, localization, and function. In: R. Paoletti and D.Kritchevsky (Eds.), *Advances in Lipid Research, Vol. 14*, Academic Press, New York, NY, pp. 75-126.

Howland, J.L. and S.L. Iyer (1977) Erythrocyte lipids in heterozygous carriers of Duchenne muscular dystrophy, *Science*, 198: 309-310.

Iyer, S.L., S.S. Katyare and J.L. Howland (1976) Elevated erythrocyte phospholipase A associated with Duchenne and myotonic muscular dystrophy, *Neurosci. Letters*, 2: 103-106.

Kunze, D., G. Reichmann, E. Egger, G. Leuschner and H. Eckhardt (1973) Erythrozytenlipide bei progressiver Muskeldystrophie, *Clin. chim. Acta*, 43: 333-341.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent, *J. biol. Chem.*, 193: 265-275.

Matheson, D.W. and J.L. Howland (1974) Erythrocyte deformation in human muscular dystrophy, *Science*, 184: 165-166.

Meijer, A.E.F.H., D. Benson and H.R. Scholte (1977) The influence of freeze-drying of tissue specimens on enzyme activity, *Histochemistry*, 51: 297-303.

Mokri, B. and A.G. Engel (1975) Duchenne dystrophy - Electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber, *Neurology, (Minneap.)*, 25: 1111-1120.

Oliveira, M.M. and M. Vaughan (1964) Incorporation of fatty acids into phospholipids of erythrocyte membranes, *J. Lipid Res.*, 5: 156-162.

Percy, A.K. and M.E. Miller (1975) Reduced deformability of erythrocyte membranes from patients with Duchenne muscular dystrophy, *Nature (Lond.)*, 258: 147-148.

Plishker, G.A., H.J. Gitelman and S.H. Appel (1978) Myotonic muscular dystrophy - Altered calcium transport in erythrocytes, *Science*, 200: 323-325.

Renooij, W., L.M.G. Van Golde, R.F.A. Zwaal, B. Roelofsen and L.L.M. Van Deenen (1974) Preferential incorporation of fatty acids at the inside of human erythrocyte membranes, *Biochim. biophys. Acta*, 363: 287-292.

Rowland, L.P. (1976) Pathogenesis of muscular dystrophies, *Arch. Neurol. (Chic.)*, 33: 315-321.

Ruitenbeek, W. (1978) The fatty acid composition of various lipid fractions isolated from erythrocytes and blood plasma of patients with Duchenne and congenital myotonic muscular dystrophy, *Clin. chim. Acta*, 89: 99-110.

Scholte, H.R. (1974) *Biochemie van Spierdystrofie*, Erasmus University Press, Rotterdam, p. 14.

Scholte, H.R., F.G.I. Jennekens and J.J.B.J. Bouvy (1979) Carnitine palmitoyltransferase II deficiency with normal carnitine palmitoyltransferase I in skeletal muscle and leucocytes, *J. neurol. Sci.*, 40: 39-51.

Schotland, D.L., E. Bonilla and M. Van Meter (1977) Duchenne dystrophy - Alteration in muscle plasma membrane structure, *Science*, 196: 1005-1007.

Van Deenen, L.L.M. and J. De Gier (1974) Lipids of the red cell membrane. In: D. MacN. Surgenor (Ed.), *The Red Blood Cell, Vol. 1*, Academic Press, New York, NY, pp. 147-211.

Van Tol, A. and W.C. Hülsmann (1969) The localization of palmitoyl-CoA:carnitine palmitoyltransferase in rat liver, *Biochim. biophys. Acta*, 189: 342-353.

Waku, K. and W.E.M. Lands (1968) Control of lecithin biosynthesis in erythrocyte membranes, *J. Lipid Res.*, 9: 12-18.

Wittels, B. and P. Hochstein (1967) The identification of carnitine palmityltransferase in erythrocyte membranes, *J. biol. Chem.*, 242: 126-130.

Wyss, S.R., J.F. Koster and W.C. Hülsmann (1971) Choice of leucocyte preparation in the diagnosis of glycogen storage disease type II (Pompe's disease), *Clin. chim. Acta*, 35: 277-280.

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