ERYTHROCYTE MEMBRANE STUDIES IN HUMAN MUSCULAR DYSTROPHY

Susan J Lloyd

A thesis presented for the degree
of
Doctor of Philosophy
of the
University of Edinburgh
in the
Faculty of Medicine

March 1980

ABSTRACT

This study was designed to investigate some of the properties of the erythrocyte membrane in human muscular dystrophies.

Physico-chemical properties of the erythrocyte membrane were investigated. Echinocyte and stomatocyte formation did not reveal any major alterations in the deformability of the erythrocyte membrane in Duchenne muscular dystrophy. Osmotic fragility was increased in erythrocytes from patients with Duchenne muscular dystrophy. Results from carriers, however, were normal.

(Na^+, K^+) ATPase is one of the enzymes involved in the Na^+ and K^+ pump systems of the cell. The basal activity of the enzyme was studied, as well as the response to ouabain, a cardiac glycoside, in erythrocyte ghost membranes from healthy controls and patients with muscular dystrophies. (Na^+, K^+) ATPase activity was lower in patients with clinically confirmed Duchenne muscular dystrophy, and the response to ouabain was also altered. The results from carriers were less conclusive. However, this effect appeared to be specific to Duchenne muscular dystrophy. Control erythrocyte ghost membranes, subsequent to incubation in Duchenne plasma, also exhibited an altered response to ouabain, characteristic of Duchenne muscular dystrophy (Na^+, K^+) ATPase. This confirmed the existence of a possible 'circulating plasma factor' in Duchenne muscular dystrophy.

ACKNOWLEDGEMENTS

I am grateful to Professor A.E.H. Emery for his constant encouragement and supervision throughout the course of this project.

I should also like to thank all the staff of the Department of Human Genetics, and especially Dr. R. Skinner and Mrs M. Lewis who collected many samples on my behalf, and Dr. R. Anand for helpful discussions.

I am grateful to Mr. D. Henry, Haematologist at the Princess Margaret Orthopaedic Hospital for supplying control blood samples and Drs. K. Brown and C. Steer, of the Royal Hospital for Sick Children, for the referal of some patients. I should also like to thank Dr. D. Horn, of the Department of Clinical Chemistry, at the Western General Hospital, for the measurement of plasma levels of calcium, magnesium and total protein.

Finally, I should like to acknowledge financial support (Fullerton Research Scholarship) from the Faculty of Medicine Scholarship Fund of the University of Edinburgh, and the Muscular Dystrophy Group of Great Britain.

CONTENTS

CHAPTER 1

INTRODUCTION 1

Classification of the muscular dystrophies

Clinical description

Duchenne muscular dystrophy

Changes in muscle fibre structure detected
- by light microscopy
- by electron microscopy

Biochemical changes in dystrophic muscle
- Enzymic distribution and activity patterns
- Protein synthesis in dystrophic muscle
- Lipid characteristics
- Sarcoplasmic reticulum

Biochemical changes in body fluids
- Serum enzymes
- Other changes

Pathogenesis of muscular dystrophies

- Vascular theory
- Neurogenic theory
- Membrane theory

Carrier detection

Prenatal diagnosis

Introduction I

The muscular dystrophies are a group of genetically determined myopathies, characterised by progressive degeneration of voluntary muscle. The term 'myopathy' applies to any disorder with signs and symptoms attributable to a primary pathological, biochemical or electrical change in the muscle fibre or in the interstitial tissue of the voluntary musculature.

Extensive studies of the muscular dystrophies began in the mid 19th century, with the classical descriptions by Duchenne (1868), Leyden (1876), Hutchinson (1879), Erb (1884) and Landouzy and Dejerine (1884). These investigators relied on clinical features when diagnosing the specific types of myopathies, although familial tendencies were observed. The primary abnormalities which gave rise to the characteristic degeneration of muscle fibres remain unknown, and some form of classification is necessary for prognosis and genetic counselling. Both genetic and clinical criteria are used for the classification of the muscular dystrophies. Walton and Nattrass, in 1954, constructed a fairly comprehensive scheme of classification which, with some recent modifications, is still in use.

a) X-linked muscular dystrophy
   - Severe (Duchenne type)
   - Benign (Becker type)
     - (Emery - Dreifuss type)

b) Autosomal recessive muscular dystrophy
   - Limb-girdle type
   - Childhood muscular dystrophy (except Duchenne)

c) Facioscapulohumeral muscular dystrophy

d) Distal muscular dystrophy

e) Ocular muscular dystrophy

f) Oculopharyngeal muscular dystrophy

g) Congenital muscular dystrophies

Clinical description of the muscular dystrophies

a) Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is inherited as an X-linked recessive trait. The disorder affects young males and, in rare occasions, girls with Turner's syndrome and other X chromosome aberrations. The gene is transmitted by apparently healthy carriers. Clinical onset is normally between the ages of three and five, although occasionally as late as the seventh. There is symmetrical involvement of the muscles of the pelvic girdle, and later of the shoulder girdle. The earliest symptom is often clumsiness in walking with a tendency to fall, which is followed by difficulty in climbing stairs and rising from the floor. Pseudohypertrophy of the calves is usually present at this stage. These physical signs often seem to regress, but this is usually due to growth outstripping degeneration. However, the relentless progression of the disease leads to an inability to walk about 5 years after onset. When the patient becomes partially immobilised in a wheelchair, skeletal deformities and muscular contractures are inevitable. Death is usually in the late teens or early twenties, due to respiratory infections or cardiac failure.

Recent estimates of the incidence of DMD range from 180 to 330 per 1 000 000 live born males. Approximately one third of these cases will have a positive family history.

Becker muscular dystrophy

This is the more benign form of X-linked muscular dystrophy. Onset is usually between the ages of five and 25 years. There

is a gradual progressive weakness and wasting of the pelvic girdle muscles, with the later involvement of the pectoral muscles, and often calf pseudohypertrophy. Twenty five years or more after onset the patient becomes unable to walk. There is usually no cardiac involvement, and contractures and deformities associated with immobilisation are rare. This muscular dystrophy is often compatible with a normal life span (Emery and Skinner, 1976). The gene can be transmitted from affected males through their carrier daughters, to their grandsons.

The incidence of Becker muscular dystrophy is estimated to be 17.7 per 1 000 000 live born males.

Emery - Dreifuss muscular dystrophy

This is another benign form of X-linked muscular dystrophy. Onset is usually between the ages of four and five years, with weakness of the pelvic girdle musculature. The pectoral girdle musculature is involved at a later stage. The disease is very slowly progressive. Flexion contractures of the elbows often occur. The distal muscles are not affected, even late in the disease, and there is no pseudohypertrophy of the calf muscles. This muscular dystrophy is compatible with a normal life span (Emery and Dreifuss, 1966).

b) Limb-girdle muscular dystrophy

This type of muscular dystrophy is expressed in either sex, and transmission is usually as an autosomal recessive trait. Onset is commonly in the second or third decade, with the primary involvement of either the muscles of the shoulder or pelvic girdle.

Weakness usually spreads to the other girdle muscles after a period of time. There is variable severity and rate of progression, but severe disability is usually present 20 years after onset. Skeletal deformities and contractures are rare, but do occur in some cases late in the disease. Most patients become severely disabled in middle life, and normal life span is reduced. The limb-girdle type is one of the most variable categories of muscular dystrophies. Transmission in rare cases appears to be as an autosomal dominant trait. There is considerable variation in severity from case to case, which often results in the misclassification of other types of muscle disorders in this group.

The estimated incidence is 46.8 per 1 000 000 live births.

Childhood muscular dystrophy

In general, this type of muscular dystrophy is similar to DMD, but is more benign. Proof of autosomal recessive inheritance is difficult in most cases, except where consanguinity has occurred, but the occasional occurrence of muscular dystrophy in a girl suggests a distinct category for this type. Onset is usually between the ages of two and 14 years, with progressive muscle weakness leading to an inability to walk in the early twenties. Normal life span is reduced. The weakness is proximal and hypertrophy is frequent.

Incidence is estimated to be 29.6 per 1 000 000 live births.

c) Facioscapulohumeral muscular dystrophy

This type of muscular dystrophy is characterised by expression in either sex with transmission as an autosomal dominant trait.

Occasionally there is an apparent sex limitation in some families. Onset is at any age from childhood into adult life, but usually in the second decade, with the primary involvement of facial and shoulder girdle muscles, with weakness subsequently spreading to the pelvic girdle. Hypertrophy is rare, as are muscular contractures and skeletal deformaties. This type of muscular dystrophy is compatible with a normal life span, although in rare cases progression is unusually rapid.

The estimated incidence is 9.2 per 1 000 000 live births.

d) Distal muscular dystrophy

The muscles of the hands, feet and legs are involved in this benign disease, with weakness spreading proximally. The age of onset ranges from 20 to 77 years, and severe disability can occur 10 to 15 years after onset. Transmission appears to be as an autosomal dominant trait. The true status of the distal myopathies remains obscure.

e) Ocular muscular dystrophy

The involvement of the external ocular muscles in any of the other muscular dystrophies is extremely rare. However, ocular weakness has been described; the clinical symptoms begin with ptosis, and progress to complete bilateral ophthalmoplagia. The muscles of the face, neck, trunk and limbs, especially the arms, are often involved. The inheritance of this type is usually autosomal dominant and there is indecision as to whether this is a true muscular dystrophy.

f) Oculopharygeal muscular dystrophy

This type is probably a distinct entity from the ocular type, onset is in middle age with progressive dysphagia a prominent feature. Inheritance is believed to be as an autosomal dominant trait.

g) Congenital muscular dystrophy

Muscle hypotonia is present in these cases at birth. The severe muscle weakness can be rapidly progressive, leading to death after six months. There is little definite evidence of autosomal recessive inheritance, although sibs are often affected. The borderline remains obscure between cases of congenital and childhood dystrophy, and later in life these cases are often of the limb-girdle type with primary involvement of the pelvic girdle.

Duchenne muscular dystrophy

The Duchenne type is the most common and also the most severe of the muscular dystrophies. There is intellectual retardation in about one third of the patients (Emery et al., 1979). Karagan and Zellweger (1978) also reported verbal disability in patients with DMD, which they suggested was related to the physical influence of the disease rather than the psychosocial factors. There is usually cardiac involvement, which may not, however, be obvious in the early stages of the disease. Chronic cardiac failure is rare. The characteristic electrocardiogram (ECG) shows tall R waves in the right precordial leads, and deep Q waves in the limb leads and the left precordial leads. Emery (1972) considered that this ECG pattern may be of diagnostic value in distinguishing between various juvenile forms of neuromuscular disease. Perloff et al., (1967) reported pathological changes in the muscle of the left ventricular free wall. Sanyal et al., (1978) found ultrastructural alterations in the heart muscle of two patients with DMD, at autopsy. There was loss of thick and thin myofilaments and accumulation of mitochondria, as well as dilatation of the sarcoplasmic reticulum, and depletion of the glycogen particles.

There is no treatment known, at the present time, which has any definite effect on muscular dystrophy. Many drugs have been tried with little or no success. Of course, controlled double blind trials of drug administration are difficult, with many motivational factors involved. Among those prescribed and found to be unsuccessful have been vitamin E, corticosteroids, multiple amino acid and vitamin therapy, adenosine triphosphate, and anabolic steroids. Many of these have resulted in a reduction of SCK levels, but muscle power has not been increased. Recently,

Thomson and Smith (1978) reported that daily prescription of Allopurinol, which inhibits Xanthine oxidase activity, thereby preventing purine breakdown, resulted in improved muscle strength in 16 DMD patients. However, this was not confirmed by Mendell and Weichers (1979).

A great deal of interest has recently been shown in the properties of the antiproteinases, which inhibit cathepsin activities. McGowan et al., (1976) reported that the individual use of the antiproteinases pepstatin, leupeptin and antipain, delayed embryonic chick dystrophic muscle degeneration in culture.

Enomoto and Bradley (1977) investigated the effects of subcutaneous injections of antipain with pepstatin and leupeptin with pepstatin on murine muscular dystrophy. They reported little success, and suggested that the antiproteinases were unable to pass through the sarcolemma into the muscle fibres. The use of liposomes may be of value in further investigations.

Stracher et al., (1978), however, found that muscle degeneration of dystrophic chicks was inhibited by injections of leupeptin with pepstatin directly into the pectoralis major muscle. This positive effect was reflected in the ratio of muscle mass to total body weight, and by different pathohistological changes viewed under the light microscope, between treated and untreated dystrophic chicks.

However, results from studies on non human tissue must be viewed critically. There is no true animal model for DMD, either in the mode of inheritance or in the patterns of muscle degeneration and regeneration.

Duchenne muscular dystrophy is characterised by progressive degeneration of voluntary skeletal muscle. The alterations in muscle fibre structure have been investigated.

Changes in muscle fibre structure detected by light microscopy

There are a number of histopathological changes associated with DMD. Muscle biopsy findings vary with the stage of the disease at which the biopsy was taken, but a composite sequence of events can be constructed.

There is a marked diminution of total fibre complement, as well as pronounced variation in fibre size. The marked enlargement of muscle fibres is an early change. Degeneration of fibres, as well as necrosis and phagocytosis, are observed, with muscle fibre regeneration occurring early in the disease. A characteristic finding of DMD biopsy material taken at this early stage, is the grouped pattern of regenerating and degenerating muscle fibres. However, the process of fibre regeneration becomes less efficient as the disease progresses (Walton, 1973). Considerable emphasis is placed on the appearance of large, dark, rounded hyaline or opaque fibres. These have increased enzymic activity (Dubowitz, 1975). Sarcolemmal nuclei become enlarged, with central nucleation and subsequent formation of nuclear chains. Proliferation of perimysial and endomysial connective tissue are also associated with the histopathological changes in DMD (Engel, 1973). In the later stages of degeneration, necrosis becomes less active and hypertrophied fibres are common. During these later stages of muscle replacement by an abundance of fat and connective tissue, DMD is indistinguishable from other chronic myopathies. The almost total loss of muscle fibres presumably causes the progessive paralysis.

Changes in muscle fibre structure detected by electron microscopy

Milhorat et al., attempted, in 1966, to ascertain the primary structural lesions which initiated fibre breakdown in Duchenne

dystrophy, and to correlate these alterations with the changes in SCK activity. They divided the fibre degeneration into three sequential stages based on the condition of groups of fibres. Cullen and Fulthorpe (1975) suggested five stages of fibre breakdown. This approach is helpful in understanding the fine structural details involved in each stage, although the disease is a continuous process and does not occur in the series of discrete steps.

In the early stages of fibre degeneration in DMD, discontinuity of the Z line becomes apparent. The sarcoplasmic reticulum expands, and the myofibrils become disorganised. Consequently the intermyofibrillar space widens and there is an increase in intermyofibrillar glycogen. As the degeneration progresses, severe fragmentation of the myofibrils becomes obvious. Swollen and degenerated mitochondria are found between the myofibrils, as well as variable-sized vesicles. Some areas are completely devoid of contractile material. Connective tissue is found between the fibres. In the late stages of fibre degeneration, hyaline fibres are found. These are degenerated fibres containing dark, dense and homogenous contractile material. Eventually the fibre becomes a mass of structureless cytoplasm which is invaded by macrophages.

Although electron microscopists have been attempting to define the earliest detectable morphological change in dystrophic muscle, the majority have really only added fine details to the known pathology. Mokri and Engel (1975) described focal defects in the plasma membrane from patients with DMD. The basement membrane was always preserved, whilst the plasma membrane was disrupted or absent. Although these alterations could have been artefacts due to the method of sample preparation, the authors suggested the alterations in the plasma membrane were possibly an early and basic lesion.

Biochemical changes in dystrophic muscle

Cumings, in 1939, reported an altered K^+ content of muscle from one patient with 'pseudohypertrophic muscular dystrophy'. The K^+ concentration was markedly reduced when compared with controls. This finding was confirmed by Horvath and Proctor (1958), in patients with progressive muscular dystrophy. This was probably due to the degeneration of contractile fibres and infiltration of connective tissue and fat cells, which do not contain large quantities of K^+. This alteration in K^+ content, however, would be non specific to DMD, occurring in many other degenerative myopathies. The rate of K^+ exchange across the muscle membrane was measured by Blahd et al., in 1953 and 1954. In cases of childhood muscular dystrophy, the exchange rates of K^+ were very low, and there appeared to be a correlation with 24 hr urinary excretion of creatinine, and with the extent of physical disability. The authors suggested that as normal muscle function cannot be maintained with low K^+ exchange, this could be a cause of muscle degeneration. Howland, in 1974, reported raised K^+ conductance in muscle associated with genetic muscular dystrophy. The addition of a noncompetitive inhibitor of K^+ translocation resulted in a decrease in the level of SCK activity. These findings also suggested that muscle deterioration may be as a result of leaking K^+.

Enzymic distribution and activity patterns in dystrophic muscle

The majority of muscle enzymes can be expected to show altered levels of activity in dystrophic muscle. Many changes will be secondary to the progressive muscle degeneration. Others,

however, may represent aspects of a primary metabolic change.

Dreyfus et al., in 1956, published the results of an extensive study into the enzyme levels of dystrophic muscle. Specimens of transversus abdominis from muscular dystrophy patients were compared with the corresponding healthy muscle from controls. The results were expressed using the non collagen nitrogen as a reference base. The glycolytic enzymes phosphoglucomutase, aldolase and ∝ glucan phosphorylase had reduced levels of activity. These findings were confirmed by Kleine and Chlond in 1967. However, Dreyfus et al., (1956) considered these enzyme alterations to be a secondary defect, as they quoted similar changes in enzymic activity in experimental neurogenic atrophy. It could be assumed that decreased activities of enzymes in dystrophic muscle were due to slow leakage into the blood.

Dubowitz and Pearse (1961) reported no obvious differences between normal and dystrophic muscle enzyme levels measured histochemically. Ronzoni et al., (1958) also reported normal levels of lactate dehydrogenase (LDH) in dystrophic muscle, despite a marked increase in serum level. Altered electrophoretic patterns of LDH isozymes, however, have been reported. Wieme and Lauryssens (1962) found differences in the relative amounts of the isozymes between healthy and dystrophic muscle. The major component in human muscle, LDH5, which moves slowest on electrophoresis, was markedly reduced in dystrophic muscle. This has been confirmed by other investigators (Emery, 1964; Brody, 1964).

Pearson et al., (1965) confirmed a reduction of LDH5 in five patients with DMD and in one patient with Becker muscular

dystrophy, in the quadriceps muscle, but no reduction was reported in the gastrocnemius in the case of the Becker patient. Emery (1968) reported that LDH5 reduction was not specific to any particular type of muscular dystrophy. The deficiency was probably a nonspecific concomitant of pathological change in the muscular system. There appeared to be no effect of storage on LDH5, which suggested no specific destructive mechanism of the enzyme.

Pennington (1962) reported that adenosine 5'-phosphate deaminase had a lower activity in dystrophy compared with healthy muscle. The enzyme, which has been suggested to be involved in buffering lactate production, occurs in far greater concentration in skeletal muscle than any other tissues. Cathepsin, an intracellular proteolytic enzyme, activity was also increased in DMD. Pennington and Robinson (1968) were able to confirm the increased cathepsin activity. Increased activity of a Ca^{2+} activated neutral protease found in the muscle of five DMD patients, was reported by Kar and Pearson (1976). The authors suggested a specific role for this protease in muscle protein catabolism, but the role in normal muscle remains unknown. These authors confirmed the findings in 1978, and reported raised cathepsin activities as well, (Kar and Pearson, 1978).

Haymond et al., (1978) measured mild glucose intolerance in DMD patients. Plasma glutamine concentrations were not different from controls. There was a marked decrease in alanine turnover, which could not be wholly attributed to the reduction in muscle mass in DMD. The authors suggested this may be indicative of accelerated protein degradation in the muscle of patients with DMD. Ballard et al., (1979) reported that the

fractional rates of muscle degradation in DMD patients were two to three times higher than in controls. Protein synthesis was also increased in DMD.

Another hydrolytic enzyme which has been described as exhibiting higher activity in human dystrophic muscle is ribonuclease. Abdullah and Pennington (1968) reported an increased alkaline ribonuclease activity of muscle homogenates from DMD compared with controls. This increase may be due to the greater number of lysosomes in dystrophic muscle. The enzymic distribution in dystrophic muscle has been reviewed recently by Emery (1977).

Protein synthesis in dystrophic muscle

Monkton and Nihei, in 1969, reported that dystrophic muscle contained ribosomes with a far higher rate of activity than normal. This finding was confirmed by Ionasescu et al., who published evidence of increased protein synthesis in DMD, in 1971. Incorporation of amino acids was five times higher in DMD than in controls, yet the ribosomal content relative to noncollagen nitrogen was the same. It appeared that the heavy polyribosomes from dystrophic muscle were capable of producing four to five times more collagen than those from healthy muscle samples. The extent of the synthesis depended upon whether a complementary soluble enzyme fraction was present, from controls or DMD patients. This suggested that a substance, or substances, in the soluble fraction, which had the potential to arrest development of connective tissue, was absent in patients with DMD. The distribution pattern of polyribosomes on a sucrose gradient was a method of distinguishing between Becker and Duchenne muscular dystrophies (Ionasescu et al., 1973).

Distribution of ribosomes in DMD was abnormal compared with those from controls and Becker muscular dystrophy patients. The supplementation of dystrophic heavy polyribosomes with the soluble fraction from control biopsies reduced the level of collagen production to that of controls (Ionasescu, 1975).

Collagen production by polyribosomes from controls was enhanced by the addition of the enzyme fraction from DMD patients. The production of noncollagen proteins by DMD polyribosomes was increased with the use of diphenylhydantoin and orgotein in muscle cell cultures (Ionasescu et al., 1979).

Kar and Pearson, however, published their findings of glyoxalase I activity in 1975. The substrates of the enzyme were believed to inhibit cell growth with specific action on protein synthesis. The authors reported a decreased enzyme activity in DMD compared with controls. This appears to be in direct disagreement with the findings of Ionasescu.

Neerunjun et al., (1979) reported raised levels of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) activity in DMD muscle. This enzyme may be involved with enhanced protein synthesis by increasing intracellular levels of purine ribonucleotides. High levels of HGPRT have been associated with rapid cellular growth and differentiation.

Lipid characteristics of dystrophic muscle

The fatty acid composition of infiltrating fat in muscle from patients with progressive muscular dystrophies has been studied.

Pennington et al., (1966) reported no marked abnormality in the fatty acid composition of the triglycerides from three

different muscles at autopsy of a DMD patient. Susheela (1968) found that the types and concentrations of the fatty acids from four DMD patients surprisingly fell within normal limits. This suggested a rapid turnover rate of free fatty acids in the diseased muscle.

The fatty acid profiles of choline phosphoglycerides have also been investigated. The increased levels of oleic (18:1) acid and decreased levels of linoleic (18:2) acid reported by Takagi et al., (1968) were confirmed by Kunze et al., (1975). Takagi et al., (1968) also found increased levels of myristic (14:0), palmitic (16:0) and arachidonic (20:4) acids associated with choline phosphoglycerides. The exact role of the phospholipid fatty acids in maintaining the integrity of the membrane remains unclear, but both reports suggested that the alterations of fatty acid acylation of choline phosphoglycerides were of specific interest.

Kar and Pearson (1975a), measured an increased activity of muscle lipase, which rules out the possibility of fat build up in diseased muscle being due to a defect in that enzyme system. Increased levels of fat in the muscle are probably due to metabolic modifications. The swelling of the mitochondria and subsequent impairment of function could contribute to the accumulation of fat.

Sarcoplasmic reticulum

The early involvement of the sarcoplasmic reticulum in the degenerative muscle process was suggested by Peter and Worsfold in 1969. They reported reduced calcium uptake by sarcoplasmic vesicles in muscle samples from DMD patients. Samaha and Gergely (1969) confirmed these findings. Takagi et al., (1973) suggested

the reduced calcium uptake could be due to a membrane dysfunction, but they were unable to find any changes in the proteins and phospholipid patterns, apart from a decrease in choline phosphoglyceride content. However, major alterations in the sarcoplasmic reticulum protein profiles were reported by Samaha and Congedo (1977). They found a significant reduction in the quantity of 100 000 molecular weight protein in DMD samples compared with controls. This protein contains the ATPase enzyme systems and constituents of the calcium transport mechanism. Oberc and Engel (1977) and Bodensteiner and Engel (1978) reported increased abundance of calcium in degenerating muscle fibres in DMD. It was suggested in both reports that this calcium accumulation was probably due to abnormal sarcolemma membrane permeability. The influx of calcium would not be counteracted by increased sarcoplasmic reticulum accumulation and there would be precipitation of calcium within active organelles, which would result in impaired function.

Biochemical changes in body fluids

Serum enzymes

Dreyfus et al., (1958) reported raised levels of activities of several serum enzymes in patients with progressive muscular dystrophies when compared with controls. These included aldolase and pyruvic transaminase. However, Ebashi et al., showed in 1959 that creatine kinase was a much more sensitive indicator of muscle damage. Creatine kinase transfers a phosphate from creatine phosphate to ADP, forming creatine and ATP. Three soluble isozymes are found; type I (BB) which is found almost exclusively in the brain; type II (MB) mainly found in cardiac muscle but significant

activity has been reported in skeletal muscle; type III (MM) the predominant sources of which are skeletal and cardiac muscles. Van der Veen and Willebrands (1966) studied the isozyme pattern in serum from patients with DMD, and found large quantities of type III, and in some instances type II. This finding has been confirmed by other investigators (Goto and Katsuki, 1970; Cao et al., 1971; Goto, 1974; Takahashi et al., 1977; Tzvetanova, 1978; Yasmineh et al., 1978). Goto (1974) found no relationship between total SCK activity and the proportion of type II isozyme and suggested that this reflected type II was released following heart muscle damage. However, Takahasi et al., (1977), Tzvetanova (1978) and Yasmineh et al., (1978) did not confirm this and believed skeletal muscle was the predominant source of isozyme type II.

Values of activity several hundred times greater than normal are found in early cases of DMD, and the levels of SCK decline markedly as the disease progresses (Pearce et al., 1964).

Somer et al., (1973) published a comprehensive report of serum isozyme studies in 22 cases of DMD. SCK isozyme patterns were similar to those of Van der Veen and Willebrands (1966). Aspartate aminotransferase was increased, and glutamate dehydrogenase activity was also raised in later stages of the disease. This may be an indicator of liver damage, as raised levels of ornithine transcarbamylase were also reported in several cases. Lactate dehydrogenase isozyme patterns were also altered. The raised levels of activity in serum of patients with DMD were due almost entirely to increases in LDH 1 - 3 isozymes. A marked depression of LDH 5 was persistently noted. This finding was confirmed by Hooshmand in 1975.

Raised levels of pyruvic kinase in serum from patients with DMD have also been reported by several authors (Harano et al., 1973; Weinstock et al., 1977; Zatz et al., 1978). The majority of these enzymes are known to be in high concentrations in muscle, but it is difficult to explain raised serum levels of a number of enzymes on simple leakage (Pennington, 1974).

Kleine (1970) published a comprehensive study, reporting eleven enzymes which displayed raised levels in DMD sera samples. Not all these enzymes were specific to muscle, some were released from liver cells and erythrocytes. Kleine suggested there may be a generalised enzyme syndrome in DMD. Rosalki and Thomson (1971) reported a normal level of γ glutamyl transpeptidase in seven patients with DMD, which suggested no liver or pancreatic disorder.

Other changes in body fluids

Danowski et al., in 1956, published the results of a comprehensive investigation into levels of various solutes in the blood of patients with the childhood type of muscular dystrophy. No changes in total CO_2, Na^+, K^+, total protein, albumin and globulin were found. However, levels of Pi and Ca^{2+} were increased and Cl^- and cholesterol were reduced. There appeared to be no correlation between these alterations and the severity of the disease. Garst et al., (1977) confirmed the unchanged levels of Na^+ and K^+ in the blood of patients with DMD. Oppenheimer and Milhorat (1961) however, reported increased levels of α_2 globulin.

Askansas (1966) identified hemopexin in serum samples from 32 out of 41 DMD patients. Fibrinolytic activity of blood from DMD patients was also increased (Menon et al., 1971). This may

have been due to leakage from muscle cells, but the authors quoted increases in other diseases where muscle enzyme leakage was known not to occur. Myoglobinemia in children with progressive muscular dystrophy was reported by Ando et al., in 1978. The levels appeared to be correlated with the age of the patients and serum levels of CK.

Milhorat (1953) reported increased creatine excretion in patients suffering from muscular dystrophies. Van Pilsum and Wolin (1958) confirmed these findings in patients with a variety of muscular disorders. Measurable creatine excretion in controls is rare, whereas creatinine can be excreted in large amounts (1 - 2 g per day). It is generally accepted that creatinuria is a non specific concomitant of muscle atrophy, which may be due to the muscle fibres' inability to retain creatine normally. Hydroxyproline excretion in urine of DMD patients was less than in controls (Kibrick et al., 1964). Blahd et al., (1955) measured increased levels of amino acid excretion in patients with muscular dystrophy. Normal sibs and female relatives of the patients also showed a high incidence of certain amino acids. However, Maskaleris et al., (1969) reported that several amino acid levels were decreased, but the number of the samples were too small for any significant conclusions to be made. Bank et al., (1971) measured an increased taurine excretion relative to levels of creatine, but no characteristic abnormality of amino acid patterns, either in urine or plasma, were apparent in 20 patients with DMD. However, Emery et al., (1979) reported increased urinary excretion of certain amino acids (ethanolamine, ornithine, leucine) in DMD patients with mental handicap compared with affected boys with normal intelligence.

Garst et al., (1977) were unable to detect any differences between DMD urinary excretion of Na^+ and K^+ and aldosterone, compared with controls. Di Mauro and Rowland (1976) reported normal levels of carnitine excretion in 21 patients with DMD. This was confirmed by Kobayashi et al., in 1979. Lundblad et al., (1979) have reported increased urine excretion of a glucose containing tetrasaccharide in patients with DMD. They speculated that glycogen was leaking out of the muscle cells, which was then degraded in the blood or urine to give this raised level of tetrasaccharide.

However, as these reports have suggested, not all muscle constituents have been found in raised amounts in the serum and urine of patients with DMD. This could be due to selective degradation, or the leakage being partially exclusive.

The pathogenesis of muscular dystrophy

By definition, DMD is an X-linked recessive disorder characterised by the progressive degeneration of voluntary muscle. The basic defect, however, remains unknown. There are three main theories which attempt to describe the pathogenesis of this disease.

The Vascular Theory

Démos published the results of his investigations into blood circulation in 1961. He reported a delayed arm to tongue circulation time in patients with DMD. The vascular theory was developed from this report, with additional evidence from Hathaway et al., (1970) and Mendell et al., (1971/2). Hathaway et al., (1970) injected spherical dextran particles and glass beads into experimental animals, which blocked the microcirculation. Muscle lesions which were characteristic of the histological pattern of DMD became evident. The authors accepted that although such similarities did not necessarily indicate a common pathogenic mechanism, an ischemic defect may be involved. Mendell et al., (1971/2) continued with this line of investigation.

However, more recent reports have suggested that there is no evidence of a structural or functional defect in the microcirculation of patients with DMD. Morphometric analysis of the microcirculation has failed to show any quantitative differences between DMD patients and controls (Jerusalem et al., 1974). Emery and Schelling (1965) reported that blood flow in patients was not decreased compared with controls. This finding was confirmed by Bradley et al., in 1975.

These results fail to support the theory of an ischemic defect in DMD.

The Neurogenic Theory

McComas et al., (1971) reported a reduction in the number of motor units per muscle cell, in extensor digitorum brevis muscles of patients with DMD. The motoneurones control excitation and the basic metabolic processes of the muscle fibre. Dysfunction of the motoneurone would result in an altered metabolism of the muscle fibre, characteristic of degeneration. McComas et al., (1971a) introduced the concept of 'sick motoneurones' which have difficulty in maintaining satisfactory synaptic connections with muscle fibres, and suggested that the underlying defect in DMD was neurogenic in origin. More extensive investigations into other muscles supported the theory that DMD was a neuropathy rather than a myopathy (McComas et al., 1974). The failure of neurones from dystrophic mice to support the functional regeneration of normal muscle in culture led Gallup and Dubowitz (1973) to agree with a neurogenic influence. Dystrophic muscles cultured with normal spinal cord resulted in good myotube formation. The authors suggested that there may be an aberration in the normal controlling influence by the nervous system on the muscle.

However, conflicting data have been published which does not support the theory of a basic neurogenic defect in DMD. Ballantyne and Hansen (1974) and Panayiotopoulis et al., (1974) reported finding normal numbers of motor units in DMD muscle. Nerve endings on dystrophic muscle fibres were also found to be ultrastructurally normal by Jerusalem et al., (1974a). The absence of a mosaic pattern in carriers of DMD also suggests the defect is not neurogenic (Thomson et al., 1974). Sica and McComas (1978) have published more evidence to support their neurogenic theory and reviewed the conflicting experimental data.

Membrane Theory

An impressive amount of evidence has emerged recently which indicates a primary membrane abnormality in DMD. This membrane theory, which was reviewed by Rowland in 1976 will be discussed, in detail, in Introduction II.

Carrier detection

Duchenne muscular dystrophy is inherited as an X-linked recessive trait, and is transmitted by apparently normal healthy female carriers. The genetic carriers are usually classified into three categories:-

1) Definite carriers are mothers of one or more affected sons, who have an affected brother or other male relative on the female side of the family.
2) Probable carriers are mothers of two or more affected sons with no other family history.
3) Possible carriers are mothers of one affected son where there is no other family history, or sisters of an affected boy.

It is not unreasonable to assume that the characteristic muscle patterns and raised protein levels measured in the affected males, should be detectable in the genetic carriers of the disease. Schapira et al., (1960) reported raised levels of SCK in carriers of DMD. Emery in 1963 studied the myopathic changes in two carriers of DMD, who exhibited raised levels of SCK. Muscle histology was abnormal, and consistent with the main changes observed in dystrophic patients. Similar observations in one carrier of DMD were also reported by Dubowitz (1963). Histopathological changes were found in muscle biopsies from seven out of nine carriers (Emery, 1965).

Pearce et al., (1964a) reported their findings of raised SCK levels in 74 female relatives of patients with DMD. 71% of the carriers defined as definite on the evidence of a family pedigree, had raised levels of SCK, compared with 62% of probable carriers and 50% of the possible carriers.

Walton and Pennington (1966) studied histopathological changes in muscle biopsies from carriers, as well as serum enzyme levels and electrophysiological responses. Raised SCK levels above the upper limit of normal were detected in 66% of the definite and probable carriers, whilst 50% of the possible carriers exhibited increased activity. A proportion of carriers showed myopathic changes assessed by electrophysiological methods, but this technique was considered of limited value. Histopathological changes were apparent in all 8 definite carriers studied, but alterations were also detected in some of the controls.

Emery (1965a) studied SCK levels, muscle LDH isozyme patterns and muscle histology in 25 definite and probable carriers of DMD. By combining the results of these three investigations, 70% of the carriers were detected. This final assessment could have been higher as not all the carriers had muscle biopsies.

Gardner-Medwin et al., published a comprehensive report in 1971 of the biochemical, histological and electromyographical (EMG) alterations in DMD carriers. Their results suggested that raised SCK levels were the most reliable indicator for carrier detection. Histological evidence of a myopathic pattern was often found in carriers, but alterations were uncommon in carriers where the SCK level was normal. More important, however, was the frequent absence of changes in carriers with raised SCK levels. EMG studies were as useful as the histopathological findings. 54% of the definite carriers, 64% of the probable and 20% of the possible carrier mothers exhibited raised SCK levels. The daughters of definite carriers were considered separately. 37% had raised SCK levels as did 14% of the sisters of isolated cases. Craig et al.,

(1978) confirmed that SCK levels were probably the best single index for carrier detection. However, they did suggest that a morphological examination would be useful as an additional discriminant if the SCK level was borderline.

Age related changes of creatine kinase levels in sera of carriers of DMD were considered by Moser and Vogt in 1974. The relationship of falling SCK levels with increasing age of the DMD patients was well known. Moser and Vogt (1974) suggested that SCK levels should be measured in possible carriers from early infancy. This suggestion was repeated by Gale and Murphy in 1978, who expressed concern at the variation in normal levels of SCK measured in different laboratories. However, they confirmed the need to study the effect of age on SCK levels, by repeatedly sampling carriers and controls over long periods of time. Nicholson et al., (1979) published their findings that the level of SCK did indeed drop with increasing age of the carrier, although individual variations in values were sometimes greater than the actual reduction in the level of activity. They suggested that the carriers should be tested as early as possible, as there may be a greater separation of SCK values between normal girls and carriers at a younger age. However, the distribution of SCK levels in carriers less than 20 years of age is unknown (Emery, 1979). Bundey et al., (1979) and Thomson et al., (1979) agreed in principle with this suggestion but not Carter (1979).

Attention has also been directed on other serum enzyme levels as a means of carrier detection. Pyruvate kinase has been studied by Alberts and Samaha (1974) who reported raised levels in 71% of the definite carriers, whilst SCK was raised in only 29%.

47% of possible carriers were detected by pyruvate kinase, whereas only 7% by SCK. The authors suggested that pyruvate kinase was a better, more sensitive test for detecting DMD carriers. This finding was not confirmed by Smith and Thomson (1977) nor by Hardy et al., (1977). Weinstock et al., (1977) also found that pyruvate kinase was not as sensitive as SCK, but more carriers were detected by combining the two enzymic activities rather than using only one determinant. Seay et al., (1978), however, found no benefit in combining the levels of SCK and pyruvate kinase for carrier detection.

Lactate dehydrogenase isozyme patterns have been studied in serum samples from carriers of DMD. Emery (1964) detected no differences in carriers compared with controls.

However, Roses et al., (1977) reported that the serum level of LDH5 was more sensitive than SCK as a test for carrier detection. Abnormal levels of LDH5 were detected in some of the mothers of isolated cases. These would usually have been considered to be possible new mutations. This finding confirmed a previous report (Roses et al., 1976) that the frequency of new mutations was lower in DMD than was previously thought. However, Davie and Emery (1978) failed to support this concept of a lower frequency of new mutations in DMD. Burt and Emery (1979) failed to confirm the suggestion that LDH5 was a better indicator of carrier status than SCK levels.

Askansas (1966a) reported finding increased hemopexin by an immunoelectrophoretic technique in 70% of the carriers studied. However, two of the definite carriers were not detected by this method. Danieli and Angelini (1976) confirmed raised levels of

hemopexin in carriers of DMD. Raised levels were detected in 33% of the possible carriers who had normal levels of SCK, and used concomitantly with SCK, 87% of carriers were detected.

Adornato et al., (1978) reported myoglobinaemia in nine out of 16 carriers of DMD, four of which had normal SCK levels. However, the authors detected only 78% of the DMD patients using this method.

Investigations into muscle metabolism have also been carried out. Ionasescu et al., (1971a) studied polyribosome synthesis of proteins in carriers of DMD, and reported that in eight out of 10, increased amino acid incorporation was evident. Only five had raised SCK levels. This preliminary finding was confirmed by Ionasescu et al., in 1973, in a follow up study of 63 female relatives of DMD patients. Ionasescu et al., (1975) suggested that muscle biopsy findings would be useful in complementing biochemical and genetic information in carrier detection.

Roses et al., (1976a) used endogenous phosphorylation of one of the major proteins of the erythrocyte membrane as a marker to identify carriers. Increased rates of erythrocyte peak 2 phosphorylation were demonstrated. The study included seven possible carriers whose serum enzyme levels were normal, but where raised peak 2 phosphorylation was measured.

Prenatal diagnosis

Toop and Emery, in 1974, suggested that abnormal muscle histology of foetuses at risk for DMD was apparent in utero. This was confirmed by Emery in 1977, who reported that DMD could be diagnosed in the 2nd trimester, using the parameters of muscle histology.

Mahoney et al., (1977) reported successful prenatal diagnosis of DMD using creatine kinase measurements of serum taken from the placental bed by aspiration. One foetus at risk for DMD had a low level of SCK measured in this way. The pregnancy, which went to term, resulted in the birth of an apparently normal, healthy boy. However, another at risk foetus had an abnormally high SCK level. At termination of the pregnancy, the foetus exhibited abnormal muscle histology characteristic of muscular dystrophy. Dubowitz et al., (1978) reported the birth of a normal healthy male to a carrier mother, after measuring the foetal SCK level using a foetoscope. However, Ionasescu et al., (1978) Emery et al., (1979) and Golbus et al., (1979) have indicated that foetal SCK levels are not a reliable test for prenatal diagnosis. Abnormal muscle histology in at risk foetuses was not always associated with raised SCK levels (Emery et al., 1979). Norregaard Hansen et al., (1978) suggested that a radioimmunoassay of myoglobin in amniotic fluid and serum may be a useful indicator of prenatal DMD. The protein has a lower molecular weight than creatine kinase, which may permit easier leakage.

Certainly a prenatal diagnostic test for DMD is of paramount importance. At the present time carriers receive genetic counselling with the option of selective termination of male pregnancies. Even

if the carrier status is definite there is still a 50% chance that the pregnancy would result in the birth of a normal healthy boy.

Prenatal diagnosis of an affected male foetus is therefore important, to prevent the termination of pregnancies which could have resulted in the birth of normal boys. However, as approximately two thirds of DMD cases have no positive family history, research into finding the basic treatment and thereby possibly a successful treatment for the patients is still necessary.

CHAPTER 2

MEMBRANE ABNORMALITIES IN DUCHENNE MUSCULAR DYSTROPHY

Pathogenesis of muscular dystrophy - Membrane theory

Erythrocyte membrane enzymes in muscular dystrophy

Lipid characteristics of erythrocyte membranes in muscular dystrophies

Erythrocyte membrane structure in muscular dystrophy

Physico-chemical properties of the erythrocyte membrane in muscular dystrophy

Membrane alterations in lymphocytes in muscular dystrophy

Muscle membrane alterations in muscular dystrophy

Erythrocyte membrane structure

Proteins of the erythrocyte membrane

Erythrocyte membrane enzymes
- Ca^{2+} ATPase
- (Na^{+}, K^{+}) ATPase
- Mg^{2+} ATPase
- Glyceraldehyde 3 phosphate dehydrogenase

Glycoproteins of the erythrocyte membrane

Lipid composition of the erythrocyte membrane

Membrane structure

Membrane abnormalities in Duchenne muscular dystrophy

A great deal of evidence has been published of abnormal membrane properties of erythrocytes, lymphocytes and muscle fibres, to suggest a generalised membrane defect in DMD.

Erythrocyte membrane enzymes in muscular dystrophy

Erythrocyte ghost membrane enzymes have been studied in detail. Brown et al., in 1967, reported an altered response of the Na^+ and K^+ dependent adenosine triphosphatase [(Na^+, K^+) ATPase] enzyme to the cardiac glycoside ouabain. Several groups of investigators have confirmed this finding, but not all. (This subject will be reviewed in Chapter 5).

Mawatari et al., (1976) as well as confirming the observations of an altered response of (Na^+, K^+) ATPase to ouabain, also reported abnormal adenyl cyclase activity in patients with DMD. Stimulation of the enzyme by epinephrine was not apparent in the Duchenne ghost membranes to the same extent as in controls, and the basal activity was higher. Das et al., (1976) observed alterations in erythrocyte acetylcholinesterase and other membrane proteins in DMD ghosts. Ruitenbeek (1979) although unable to confirm altered (Na^+, K^+) ATPase activity, reported that the Ca^{2+} stimulated Mg^{2+} dependent ATPase enzyme exhibited increased activity in DMD ghost membranes. Iyer et al., (1976) measured increased phospholipase A activity associated with DMD erythrocyte membranes. This elevation could be expected to lead to increased concentrations of lysophospholipids, which could contribute to membrane dysfunction. Roses et al., (1975) reported an alteration in erythrocyte ghost membrane protein kinase in DMD. There was increased phosphorylation of the membrane protein

spectrin. This was confirmed by Roses and Appel in 1976, Vickers, McComas et al., (1978), and Vickers, Rathbone et al., (1978) who also reported increased phosphorylation of other membrane proteins. However, Iyer et al., (1977), Fischer et al., (1978) and Falk et al., (1979) have been unable to confirm these findings. These differences could be partially explained by variations in the techniques employed to measure protein phosphorylation (Roses, 1979).

Lipid characteristics of the erythrocyte membrane in muscular dystrophy

The lipid constituents of the erythrocyte membrane have also been studied. Kunze et al., (1973) reported finding an increased cholesterol content as well as altered patterns of fatty acid incorporation in ethanolamine phosphoglyceride and sphingomyelin. In the former, the concentration of arachidonic acid (20:4) was diminished; in the latter, the concentrations of palmitic (16:0) and linoleic (18:2) acids were decreased whilst stearic acid (18:0) was increased. Kalofoutis et al., (1977) found that the amounts of lysophosphatidyl choline and sphingomyelin incorporated into the membrane were increased in DMD whereas choline phosphoglyceride was reduced. Howland and Iyer (1977) reported a diminished amount of palmitoleic acid (16:1) in the erythrocyte membranes of patients with DMD.

Ruitenbeek (1978) reported no major changes in phospholipid content, although a reduction in the level of palmitoleic acid (16:1) was observed. Kobayashi et al., (1978) and Koski et al., (1978), however, were unable to confirm any alterations in cholesterol and phospholipid content, nor any change in the pattern of fatty acid incorporation.

Erythrocyte membrane structure in muscular dystrophy

The structure of the membranes of erythrocytes from patients with DMD have been investigated using freeze fracture techniques (Wakayama et al., 1978/9). Alterations in the density and distribution pattern of intramembranous particles were found. In some cases, the cleaving behaviour of the extracellular face of the membrane bilayer resembled that of pure lipid. The authors suggested the observations may be due to altered properties of the intramembrane proteins, or changes in the nature of the lipid with which they interact.

Alterations in erythrocyte membrane structure have also been monitored by saturation transfer electron paramagnetic spectroscopy by Wilkerson et al., (1979). These results suggested changes in the protein conformation or organisation within the membrane.

Physico-chemical properties of the erythrocyte membrane in muscular dystrophy

Many physico-chemical properties of intact erythrocytes from patients with DMD have been investigated. Matheson and Howland (1974) reported finding increased numbers of deformed erythrocytes in samples from DMD patients viewed with a scanning electron microscope. However, not all investigators have been able to confirm this, and the subject will be reviewed in more detail in Chapter 3. Yet there is sufficient evidence in other reports of reduced deformability of the erythrocyte membrane to support the theory of a generalised membrane abnormality (Percy and Miller, 1975; Kohn et al., 1977; Brain et al., 1978).

Howland in 1974 reported that the rate of K^+ efflux from erythrocytes from DMD was five times higher than that from controls.

Sha'afi et al., (1975) confirmed the increased efflux in DMD erythrocytes.

Increased erythrocyte osmotic fragility has also been observed in muscular dystrophy (Fisher et al., 1976; Lloyd and Nunn, 1978; Ruitenbeek et al., 1979; Somer et al., 1979). This evidence will be reviewed in Chapter 4.

The technique of horizontal microelectrophoresis has proved useful in measuring the electrophoretic mobility of erythrocytes from patients with DMD (Bosmann et al., 1976). Szibor et al., in 1976 and Szibor et al., in 1979 confirmed altered erythrocytic mobilities in patients with DMD. They also measured different responses to ouabain, by incubation with the glycoside of erythrocytes from controls and patients prior to electrophoresis. In this way they confirmed the DMD erythrocytes' abnormal response to ouabain.

Although Solomons et al., (1977) reported an abnormal adenine metabolism of erythrocytes from patients with DMD, which could be expected to lead to a reduced viability, Adornato et al., (1977) found erythrocyte survival was normal.

These erythrocyte abnormalities are summarised in Table 1.

TABLE 1

ERYTHROCYTE ABNORMALITIES IN DUCHENNE MUSCULAR DYSTROPHY

Morphology

- Echinocyte formation
- Stomatocyte formation

} Light and electron microscopy ↑

Physico-chemical

- K^+ flux ↑
- Deformability ↓
- Osmotic fragility ↑
- Electrophoretic mobility ↑

Biochemical

- (Na^+, K^+) ATPase and ouabain inhibition ↓
- Adenyl cyclase ↓ and epinephrine stimulation ↓
- Phospholipids
- Protein kinase (2) ↑

Membrane alterations in lymphocytes in muscular dystrophy

Abnormal membrane properties of other tissues have also been reported. Verrill et al., (1977) found diminished cap formation in lymphocytes from patients with DMD. The capping process, studied with fluorescent antibodies, requires normal membrane fluidity. The authors suggested that the failure of cap formation reflected some basic defect in the cell membrane. Pickard et al., (1978) confirmed the earlier findings, and found that reduced cap formation was not specific to DMD. Hauser et al., (1979) were unable to detect any lymphocyte capping abnormality, and questioned the technique used by Pickard et al., (1978). However, in an extensive series of cases Horenstein and Emery (1979) have confirmed there is reduced lymphocyte capping in DMD but this seems to be related to the stage of the disease and may therefore be secondary to the disease process.

Muscle membrane alterations in muscular dystrophy

Dhalla et al., (1973) measured increased Ca^{2+} ATPase and Mg^{2+} ATPase activities and reduced (Na^{+}, K^{+}) ATPase activity of the muscle sarcolemma from patients with DMD. Mawatari et al., (1974) reported a normal level of adenyl cyclase activity, but an abnormal response to epinephrine and sodium fluoride. The enzyme was stimulated less, by the two compounds, compared to the stimulation in controls. Susheela et al., (1975) found that adenyl cyclase activity was much reduced in DMD muscle compared with controls. Samaha and Congedo (1977) investigated the proteins of the sarcoplasmic reticulum. They found, in DMD patients, a decrease in the 100 000 molecular weight protein, compared with controls. This reduction, which resulted in decreased activity of ATPase, was also divisible into two categories. One exhibited relatively high enzymic activity,

the other relatively low activity. The authors suggested on this evidence, that DMD could be subdivided into two biochemical types.

Mokri and Engel (1975) reported discontinuity in the plasma membrane of muscle fibres, which they suggested was an early or basic lesion.

Schotland et al., (1977) using the freeze fracture technique, found a non uniform distribution of particles in the muscle membrane, which they considered was an early sign of a membrane abnormality. Bonilla et al., (1978) studied, with an electron microscope, concanavalin A binding sites using a peroxidase labeling technique. Populations of muscle fibres were detected where the reaction at the cell surface was irregular and patchy. Concanavalin A binds specifically to ∝ D mannose, ∝ D glucose and ∝ D fructose residues on the surface of the membrane. Irregular distribution was obvious even when the plasma membrane was intact, which led the authors to suggest the abnormality may precede disruption of the plasma membrane of the muscle cell and formation of lesions in the muscle fibre.

Erythrocyte membrane structure

The erythrocyte membrane contains a diverse array of enzymes, transport systems, glycoproteins and proteins of unknown nature and activity, associated with a lipid bilayer. The interrelationships of these proteins and lipids are of importance for understanding the structure and function of the membrane system. There have been several extensive reviews on this subject, (Coleman, 1973; Juliano, 1973; Singer, 1974; Steck, 1974).

Erythrocyte ghosts have been utilised to study the properties of the membrane. Under certain conditions, the ghost closely mimic the permeability behaviour of intact erythrocytes. They can

be easily isolated, essentially free of contamination by other cells. The erythrocyte membranes are prepared by osmotic haemolysis in dilute alkaline buffers and repeated washings until the ghosts are pearly white. The proteins can be separated from the lipids by extraction with polar organic solvents. Approximately 52% of the membrane mass is protein, 40% is lipid and the remaining 8% is carbohydrate.

The proteins of the erythrocyte membrane

The polypeptides can be separated from the lipids, and from one another, by solubilisation of the membrane in sodium dodecyl sulphate,and the subsequent polyacrylamide gel electrophoresis. The proteins can be stained with Coomassie blue and densitometrically scanned. In this way the proportions can be derived for each major protein component.

Other components, such as the glycoproteins, are not readily detected with Coomassie blue, but some can be observed using the periodic acid-Schiff staining technique. The electrophoretic mobilities of these species are different from proteins of similar molecular weights, due to the glycosylated residues.

The major single protein of the erythrocyte membrane is spectrin. The two subunits of the protein, which are similar but not identical, have molecular weights of 240 000 and 220 000, and are bands 1 and 2 after a Coomassie blue stained polyacrylamide gel electrophoresis separation of membrane proteins. This protein has been extensively analysed since the report of its discovery by Marchesi and Steers in 1968. Spectrin dimers are believed to join end to end to form tetramers, which with other proteins form the skeletal core of the erythrocyte membrane (Lux, 1979).

The proteins of the membrane which exhibit enzymic activity, are of importance in maintaining the ionic content and integrity of the erythrocyte. The enzymes can be grouped into two categories based upon the relationship between membrane integrity and the expression of enzymic activity.

1. Primarily cytoplasmic enzymes, but which are also associated with the membrane, are released with haemoglobin. The activity is expressed in the supernatant after haemolysis, and there is no effect on the morphology of the ghosts. They dissociate free of any lipids, and are relatively soluble in neutral aqueous buffer. These criteria suggest these proteins are held to the membrane by weak electrostatic forces. The enzymes of the pentose phosphate pathway, glycolytic enzymes and adenylate kinase are examples.
2. The integral proteins require far more drastic treatment, such as detergents, to dissociate them from the membrane. Many of these enzymes lose their activities when outwith the membrane. It is not possible to remove them without disrupting the ghost morphology. The best example of this type is (Na^+, K^+) ATPase, although Ca^{2+} ATPase and acetylcholinesterase exhibit the same properties.

Erythrocyte membrane enzymes

Ca^{2+} Mg^{2+} ATPase

It has been demonstrated that the mechanical and permeability properties of the erythrocyte membrane depend upon the intracellular concentrations of Ca^{2+} and ATP. The metabolic depletion of ATP and the subsequent accumulation of Ca^{2+} converts the membrane from an

easily deformable state to a very rigid one. Low intracellular concentrations of Ca^{2+} are maintained by virtue of the erythrocyte's impermeability to the ion, as well as an active extrusion system. Jarrett and Penniston (1976) reported the partial purification of a protein activator, Calmodulin, of the Ca^{2+} ATPase enzyme. The authors suggested that the modulator protein was the intracellular Ca^{2+} receptor, which stimulated the Ca^{2+} ATPase. Gopinath and Vincenzi (1977) confirmed this report and suggested that Ca ions were necessary for the cytoplasmic activator to bind to the membrane. Cohen et al., (1978) reported that the modulator protein was very similar to a subunit of muscle phosphorylase kinase, as well as a subunit of Ca^{2+} dependent myosin light chain kinase of smooth muscle. The protein was also involved with activation of adenylate kinase, and cyclic nucleotide phosphodiesterases.

Roelofsen and Schatzmann (1977) studied the lipid requirement of the Ca^{2+} ATPase enzyme in the erythrocyte membrane. They concluded that only the glycerophospholipids in the membrane were involved in the maintenance of enzymic activity, and particularly the fraction located on the inner half of the membrane.

The enzyme requires both Ca^{2+} and Mg^{2+} for activation. The Ca^{2+} stimulated site of the enzyme must be located on the inner aspect of the erythrocyte membrane.

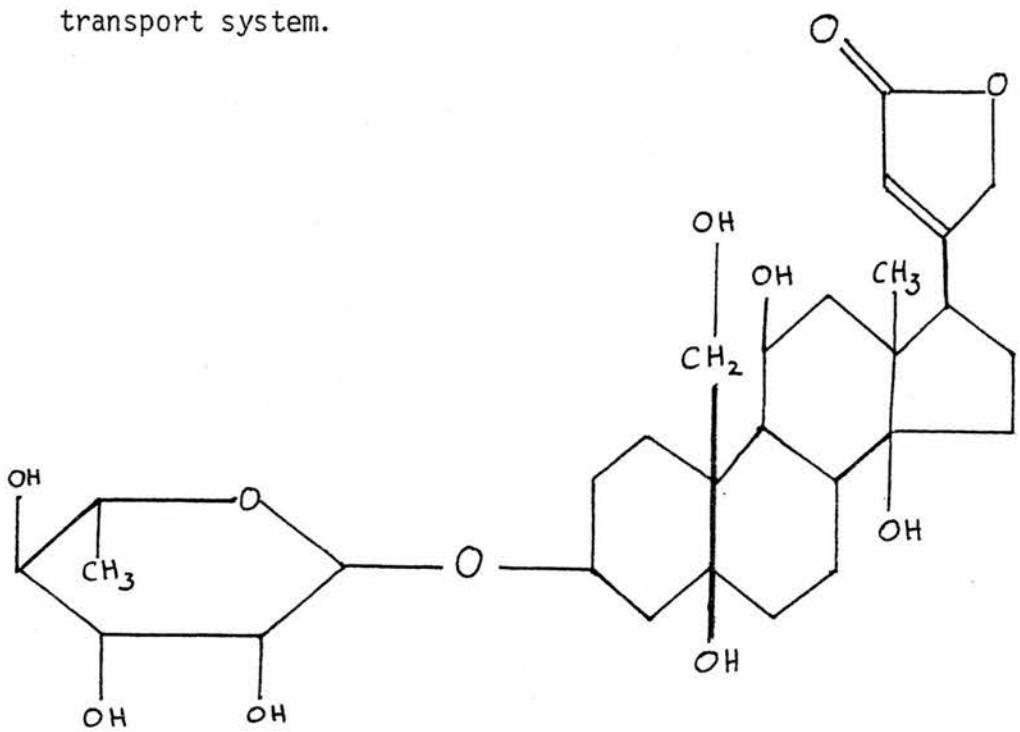
Na^+ and K^+ stimulated ATPase

The (Na^+, K^+) ATPase has been associated with the active alkali transport system (Dunham and Glynn, 1961). The purified enzyme has two types of polypeptide chains, the larger of which has a molecular weight of about 100 000. This protein carries the phosphate incorporated from ATP in the presence of Mg^{2+} and Na^+, and possesses

sulphydryl groups as well as the glycoside binding site. The small polypeptide has a molecular weight of about 55 000 and is a sialoglycoprotein. The exact role of this glycoprotein is unknown. Results from polyacrylamide gel electrophoresis suggest the enzyme is a complex of two large chains associated with one small chain (Jorgensen, 1974). The enzyme is believed to span the membrane of the cell, an idea made more appealing by the fact that almost half of the amino acids are hydrophobic in nature (Kyte, 1972).

When the alkali cation pump is operating in its usual manner, three Na^+ and two K^+ are transported across the membrane for each ATP molecule hydrolysed. The overall (Na^+, K^+) ATPase reaction is believed to consist of three sequential steps. The first involves the transfer of a phosphate group from ATP to the protein, with the production of ADP. This reaction is dependent upon Mg^{2+} and Na^+, and is inhibited by ouabain. The second step is the translocation of the phosphate group to another site, this is inhibited by oligomycin. The final event is the dephosphorylation of the protein, dependent on Mg^{2+} and K^+ and is inhibited by ouabain.

Ouabain, a cardiac glycoside, is a highly specific inhibitor of the (Na^+, K^+) ATPase enzyme, and consequently of the alkali transport system.



Evidence of a tyrosyl residue at the active site of the (Na^+, K^+) ATPase enzyme has been published by Masiak and D'Angelo (1975). Acetylation of human erythrocytes by N-acetyl imidazole resulted in loss of enzymic activity. The effect of borate ions strongly suggests that an arginine residue is also involved (De Pont et al., 1977)

Freeze fracture studies of the membrane bound (Na^+, K^+) ATPase were reported by De Guchi et al., in 1977. Each surface particle consisted of one large catalytic polypeptide chain of molecular weight approximately 100 000. Two such surface particles were united to form the active unit of (Na^+, K^+) ATPase which bound one molecule of ATP and ouabain. The surface particles protruded from the surface membrane, which suggested the protein units spanned the membrane and were capable of lateral motion. The intracellular site bound the (Na^+, K^+) ATPase antibody (Jorgensen et al., 1973) and the outer aspect bound ouabain.

Mg^{2+} ATPase

This enzyme requires Mg^{2+}, and ATP. However, no specific physiological function has been attributed to Mg^{2+} ATPase.

Glyceraldehyde 3 phosphate dehydrogenase

The action of ouabain on the (Na^+, K^+) ATPase system causes a variation in the rate of alkali cation transport. This is reflected by variations in the rate of glycolysis of the erythrocyte. Glyceraldehyde 3 phosphate dehydrogenase, one of the most important enzymes in the glycolytic pathway, is located on the inner surface of the membrane, orientated towards the cytoplasm (Schrier, 1977).

Glycoproteins of the erythrocyte membrane

The major glycoprotein, which can be observed by polyacrylamide gel electrophoresis and periodic acid-Schiff staining, contains large quantities of sialic acid residues, which are the main contributors of surface charge of the erythrocyte. The main sialoglycoprotein contains 64% carbohydrate, which includes 28% N-acetylneuraminic acid. The carbohydrate side chains which stem from alkali labile linkages, are probably tetramers, which end in sialic acid. These glycoproteins are known to possess blood group antigens, and also lectin and influenza virus receptor sites. Freeze fracture studies have shown that the carbohydrate chains extend beyond the exterior face of the membrane, whilst the protein interacts with the other components in the interior of the membrane to form intramembranous particles (Marchesi et al., 1972).

Lipid composition of the erythrocyte membrane

The lipid, phospholipid and fatty acid composition of erythrocyte membranes have been investigated (Ways and Hanahan, 1964; Dodge and Phillips, 1967). The hydrophobic nature of the lipid bilayer has an effect on the activity of the intramembrane enzymes, as well as on passive permeability.

Membrane Structure

A great deal of information on membrane structure, particularly erythrocyte membrane structure, has been collected since 1935 when Davson and Danielli proposed their theory of the membrane being a continuous hydrocarbon phase contributed by the lipid components. The unit membrane hypothesis of Robertson, which extended that of

Davson and Danielli by including a mononuclear layer of proteins on either side of the lipid bilayer, no longer explains the structure of the membrane in such a way as to agree with recent experimental data. Singer and Nicholson put forward their theory of membrane structure in 1972. They suggested a fluid mosaic structure analogous to a two dimensional orientated solution of integral proteins in a viscous phospholipid bilayer solvent. This model agrees well with experimental data on the membrane (Figure 1).

In this model it is clear that the nature of the lipids can exert an effect on the microenvironment of the proteins. Spectrin tetramers lie attached to the inner surface of the membrane, providing the skeletal core. Any pertubation in proteins or lipids will effect the membrane as a whole (Figure 2).

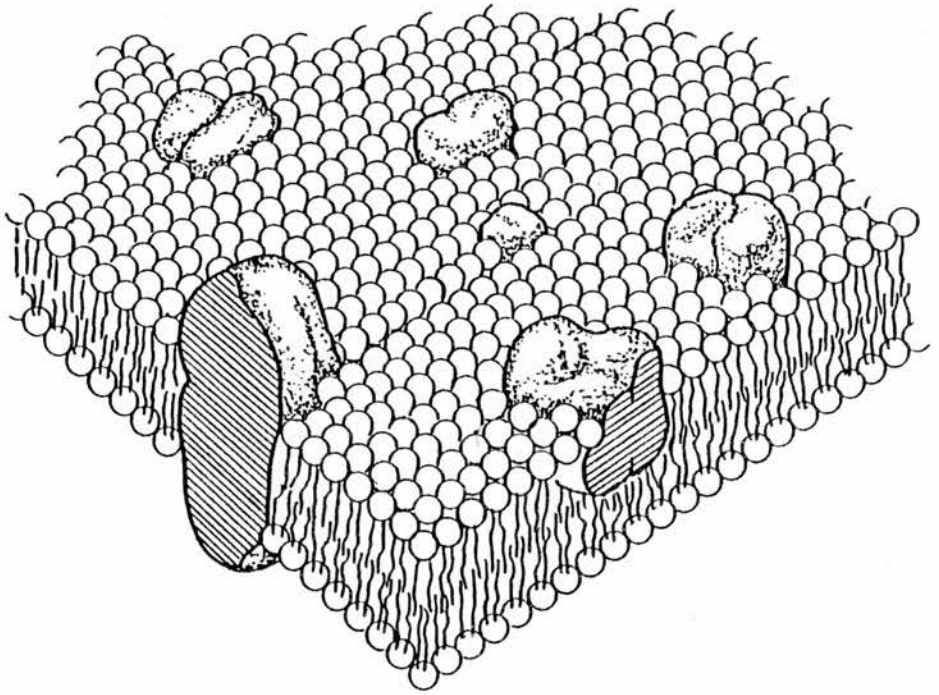
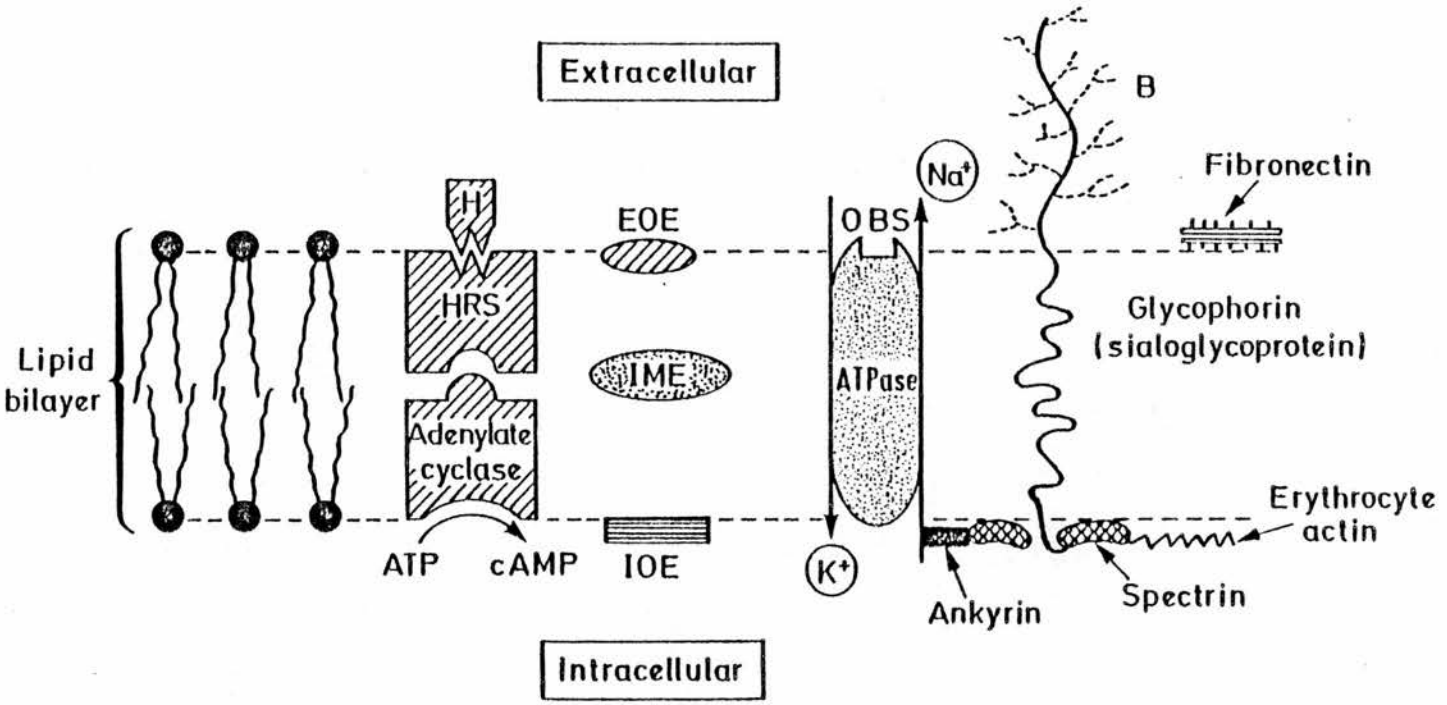


Figure 1 Diagrammatical representation of the fluid-mosaic model of membrane structure (from Singer and Nicholson, 1972). The solid bodies represent globular proteins within the lipid matrix.

Figure 2 Diagrammatical representation of the cross-section of the erythrocyte membrane (Emery, 1979, unpub)



HRS	Hormone receptor site
H	Hormone
EOE	Externally orientated enzyme
IME	Intra membrane enzyme
IOE	Internally orientated enzyme
OBS	Ouabain binding site
B	ABO blood group system

CHAPTER 3

ECHINOCYTE AND STOMATOCYTE FORMATION

Introduction

Materials and Methods

Results

Discussion

Introduction

The interrelationships between shape, structure and functional characteristics of normal and pathological erythrocytes have been under investigation in a variety of genetic disorders. With the advent of the scanning electron microscope three dimensional aspects of cell shape can be studied, with increased resolution and magnification.

The healthy mature erythrocyte, as viewed in the circulation, assumes a biconcave shape. This is the most appropriate for the erythrocyte's major function of transporting oxygen to the various tissues of the body, which requires optimum deformability and a smooth profile as well as exhibiting maximum surface area. In some diseases of the blood the functional characteristics of the erythrocytes are altered, with associated changes in shape, which can be detected with an electron microscope. In 1969, Bertles and Dobler utilised the electron microscope to distinguish between reversible and irreversible sickling of erythrocytes in sickle cell anaemia.

By 1972, there was some evidence to suggest altered membrane properties of erythrocytes in muscular dystrophy. Brown et al., (1967) and Araki and Mawatari (1971) reported an altered erythrocyte ghost membrane (Na^+, K^+) ATPase. These alterations in enzyme behaviour of the erythrocyte may affect cell shape. Morse and Howland (1973) studied erythrocyte shape in animal models of muscular dystrophy. The authors, using a scanning electron microscope, reported a high percentage (51.6%) of the cells from dystrophic mice were distorted when compared with cells from normal littermates (4.1%). These irregularities in shape were due to protrusions of

variable size from the cell surface. Similar irregularities in shape were found in patients suffering from congenital muscular dystrophies by Matheson and Howland (1974) who classified these erythrocytes as 'echinocytes' (Bessis, 1973). These echinocytes were also visible under the light microscope (Matheson and Howland, 1974; Lumb and Emery, 1975), and this suggests that they were not artefacts due to the methods of preparation and fixation. Increased proportions of echinocytes were also found in some carriers of the X-linked Duchenne dystrophy (Matheson and Howland, 1974; Lumb and Emery, 1975; Grassi et al., 1978). Lumb and Emery (1975) and Miale et al., (1975) however, doubt that this would be a more valuable test than SCK for carrier detection.

The preparation of the erythrocytes can induce echinocyte formation. Matheson and Howland (1974) described that prewashing the erythrocytes caused an increase in the number of echinocytes. This was confirmed by Howells in 1976, and could be due to the stripping of plasma proteins adhered to the membrane. Lumb and Emery (1975) reported that prewashing the erythrocytes had no such effect.

However, Matheson et al., (1976) and Soltan (1977) were unable to detect any significant abnormalities in the shape of the erythrocytes from patients with DMD. Matheson et al., (1976) measured the reproducibility of the procedure, not only between samples but environments as well. They reported great variations between repeated samples, and were unable to confirm the previous findings of Matheson and Howland (1974). They advised caution in the use of this procedure as a diagnostic test for carrier detection or clinical confirmation of DMD. The morphology of the cells was very sensitive to the various preparative treatments.

Another distinct population of erythrocytes was found by Miller et al., (1976) in myotonic and Duchenne muscular dystrophy. These erythrocytes were cup-shaped and classified as stomatocytes (Bessis, 1973). Although the percentage of stomatocytes in samples from dystrophic patients was very variable (20% - 80%) only 2% - 5% of the erythrocytes from controls were cup-shaped. In this study the scanning electron microscope rarely showed echinocytes.

More recently Tillman et al., (1979) confirmed increased echinocytes in DMD, measured by phase contrast microscopy.

In view of the discrepant findings of previous investigations, and because of the obvious relevance to the pathogenesis of DMD, the present study was undertaken to determine if significant morphological abnormalities of erythrocytes in patients with DMD and in carriers could be demonstrated.

Materials and Methods

Venous blood was collected (5ml - 10ml) from patients and their relatives when the families attended the Neuromuscular Clinic at the Western General Hospital in Edinburgh. Control blood samples were collected from healthy female hospital staff, with no family history of any neuromuscular disease. Blood was collected from young male controls prior to orthopaedic operations unrelated to any muscular disorder. The samples were studied within four hours of collection.

The majority of the patients suffered from Duchenne muscular dystrophy, diagnosed by clinical investigation, confirmed by abnormal muscle histology observed after biopsy. The female relatives of the patients were classified into five categories for these experimental investigations:-

1) Definite carriers - mothers of one or more affected males who had an affected brother or other male relative on the female side of the family.
2) Probable carriers - mothers of two or more affected sons, with no other family history.
3) Possible carriers I - mothers with one affected boy where there is no other family history.
4) Possible carriers II - sisters of affected boys.
5) Other females relative - aunts and grandmothers.

The patients and relatives are referred to, throughout the text, by the family number, followed by the generation number, and personal number within that generation.

The erythrocytes were prepared by a modification of the method of Matheson and Howland (1974).

20 µl of heparinised blood was diluted 10 fold with ice cold 0.9% saline, washed twice and centrifuged at 1 000 g for three minutes. 180 µl of cold glutaraldehyde fixative was added and the cells left at room temperature for one hour, then mixed and left for another hour, centrifuged and the supernatant removed. The cells were washed twice in buffered sucrose (10% cacodylate buffer). They were then dehydrated by suspending sequentially in 25%, 50%, 75%, 100% acetone. These samples were stored in 100 per cent acetone at 4°C overnight.

The volume of each sample was made up to 10 ml with 100% acetone, mixed and allowed to stand for five minutes. 2 ml of the cell suspension was filtered through a millipore membrane (5 µm). The cells adherent to the membranes were critical point dried, and coated with gold using the E5000 Diode Spluttering System. The samples were then stored in a desiccator prior to electron microscopy.

Five different areas of the membranes were scanned and photographed at a magnification of x3000. The cells exhibiting the characteristic shape changes associated with echinocyte and stomatocyte formation (Bessis, 1973) were counted and expressed as a percentage of the total cell count. All samples were studied 'blind' without knowledge as to the source of the material and the code was broken at the end of the study.

Results

Obvious shape changes were classified into three categories (Bessis, 1973):-

Echinocytes I

Echinocytes II and III

Stomatocytes

Duchenne patients

The results of erythrocyte deformation in samples from controls, patients with clinically confirmed DMD and their unaffected brothers are shown in Table 2 and individual values for patients are given in Table 3. There was no significant correlation between either age or SCK value with either percentage of echinocytes or percentage stomatocytes. There was also no significant difference in percentages of echinocytes and stomatocytes in affected boys and controls.

Two views of the erythrocytes after preparation as seen under the electron microscope are shown in Plate 1 depicting typical scans.

Carriers of DMD

The range and mean of echinocyte and stomatocyte formation in the controls and the carrier groups are expressed in Table 4. The individual results for each of the carrier groups are shown in Table 5. There was no correlation between SCK levels and percentage echinocytes or percentage stomatocytes in the mothers of the patients. There was no significant difference in the percentages of echinocytes or stomatocytes in the possible carriers I and controls. Only two of the values for echinocyte I formation were outside the control range, and none for echinocytes II and III. However, the

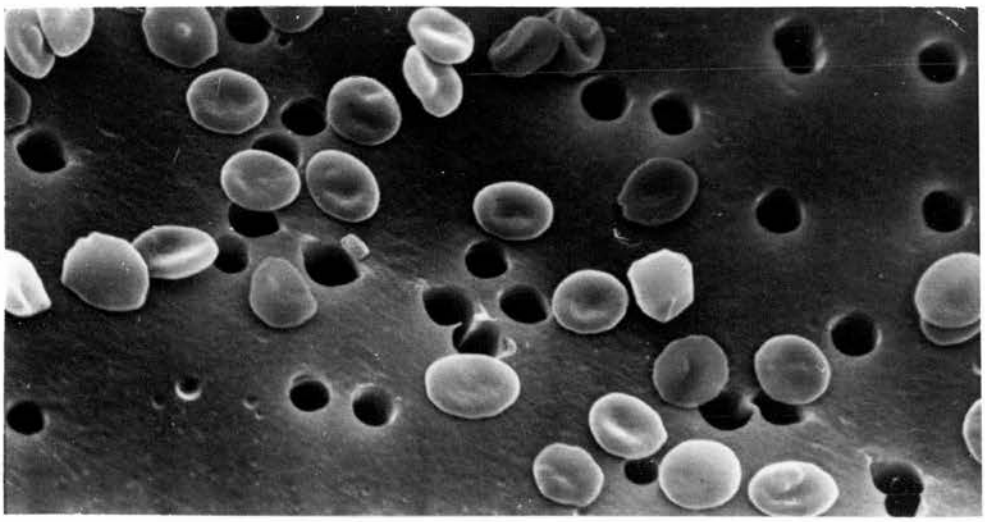
Table 2 Echinocyte and Stomatocyte formation in controls, patients with clinically confirmed DMD and their unaffected brothers. Actual range of values are given in brackets.

	Age years		SCK IU/l		Echinocytes (%) I		Echinocytes (%) II+III		Stomatocytes (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Controls (n = 8)	13.4 (11 - 15)	1.4	49.9 (32 - 69)	11.9	11.0 (5.2 - 14.2)	2.6	0.6 (0 - 2.3)	0.8	8.6 (4.2 - 16.9)	5.2
DMD (n = 8)	11.0 (6 - 18)	3.8	5901.0 (310 - 18,200)	6014.0	15.0 (9.3 - 27.3)	5.9	0.6 (0 - 3.7)	1.3	10.6 (2.8 - 17.5)	4.7
Brothers (n = 5)	10.8 (3 - 16)	4.9	61.8 (50 - 75)	11.2	13.2 (4.3 - 22.5)	6.6	3.3 (0 - 11.4)	4.6	11.0 (5.7 - 17.7)	4.5

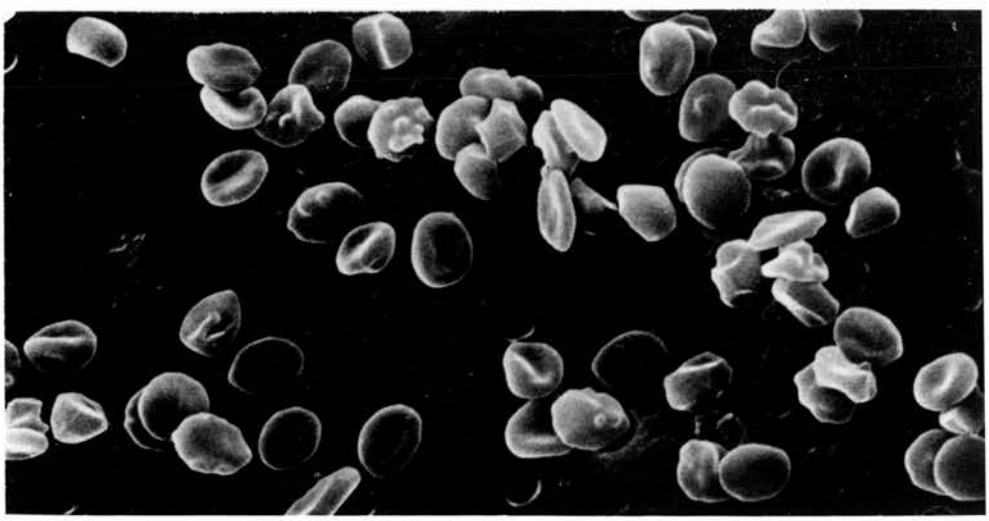
Table 3 Individual values of echinocyte and stomatocyte formation in patients with clinically confirmed DMD.

Patients	Age (yrs)	SCK IU/l	Echinocytes I	Echinocytes II+III	Stomatocytes
1. II_3	7	6 000	19.5	3.7	6.5
2. III_2	6	18 200	27.3	0.4	2.8
3. II_2	18	310	12.7	0.0	9.3
3. II_4	14	2 300	11.6	0.0	14.8
3. II_6	11	2 900	15.4	0.4	11.1
4. II_4	10	2 500	13.8	0.4	17.5
6. II_6	11	3 500	9.3	0.0	13.7
8. III_1	11	11 500	10.0	0.0	9.2

Plate 1 Control erythrocytes (a) and erythrocytes from a DMD patient (b) as seen under the scanning electron microscope.



(a)



(b)

Table 4 Echinocyte and Stomatocyte formation in controls, carriers of DMD and other female relatives. Actual range of values are given in brackets.

	Age years		SCK IU/l		Echinocytes (%) I		Echinocytes (%) II+III		Stomatocytes (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Controls (n = 28)	36.5 (17 - 62)	15.2	54.0 (30 - 88)	16.6	11.4 (6.6 - 19.3)	3.6	1.3 (0 - 5.5)	1.7	12.4 (4.4 - 19.4)	4.3
Definite/Probable Carriers (n = 1)	48		344		10.5		0		15.1	
Possible Carriers I (n = 6)	34.6 (29 - 41)	5.01	50.8 (36 - 82)	17.38	15.0 (6.7 - 24.5)	7.89	1.2 (0 - 3.3)	1.31	9.6 (5.4 - 18.3)	4.54
Possible Carriers II (n = 4)	15.3 (14 - 17)	1.3	71.8 (50 - 95.5)	23.6	14.8 (10.4 - 19.8)	4.5	0.3 (0 - 0.6)	0.3	6.0 (2.6 - 10.4)	3.3
Other Female relatives (n = 2)	77 24		148.5 642.0		14.6 8.3		0 0.4		14.6 10.2	

Table 5 Individual values of Echinocyte and Stomatocyte formation in carriers of DMD and other female relatives

	Age years	SCK IU/l	Echinocytes (%) I	Echinocytes (%) II+III	Stomatocytes (%)
Definite/Probable Carriers					
3. I 2	48	344.0	10.5	0	15.1
Possible Carriers I					
1. I 2	32	41.0	24.5	3.3	5.4
2. II 2	29	56.0	24.0	2.1	7.4
5. I 2	41	36.0	9.6	0	18.3
6. I 2	39	82.0	8.7	0	9.8
8. II 3	30	37.0	6.7	1.2	9.1
12. II 4	37	53.0	16.5	0.5	7.5
Possible Carriers II					
6. II 2	17	50.0	17.3	0.6	5.6
6. II 3	15	88.5	10.4	0	10.4
6. II 4	14	53.0	11.6	0	2.6
12. III 5	15	95.5	19.8	0.5	5.2
Other Female Relatives					
10. II 7	24	642.0	8.3	0.4	10.2
12. I 2	77	148.5	14.6	0	14.6

percentage of stomatocytes appeared to be decreased in the sisters of patients compared with controls but because of the small numbers no significance can be attached to this.

Discussion

The results of this study have failed to confirm some of the previous findings of an increased population of deformed erythrocytes by scanning electron microscopy in patients with DMD and in heterozygous carriers. There was no observable difference between echinocyte formation in pre- or un- washed erythrocytes. All these observations were made on coded samples to remove all chances of observer bias.

There are a number of factors involved in the maintenance of cell shape, some of which may be of importance when discussing the mechanism of echinocyte and stomatocyte formation. Nakao et al., (1960) measured the relationship between the levels of ATP and ADP, and erythrocyte shape. They reported that if the sum of the two concentrations of ATP and ADP were kept at a certain level, the erythrocyte assumed a biconcave shape. The erythrocyte became crenated at lower levels and completely spherical at very low levels of ATP. The authors, however, were not clear on the importance of absolute concentrations, or the level of turnover, of these adenine nucleotides. These results were confirmed by Weed et al., (1969) who studied the changes in concentration of ATP in the erythrocyte during incubation in serum. The level of ATP dropped with incubation, which paralleled a progressive rise in the calcium level of the erythrocyte and a change in the viscosity of the red cell suspensions. These changes were reversible by the addition of excessive ATP, even in the presence of ouabain. The authors suggested the major role of ATP in the maintenance of erythrocyte viability was related to the preservation of membrane deformability, and that calcium ions were also important. Any condition leading to a diminution of intracellular levels of ATP

was likely to result in leakage of enzymes and other proteins, as the ATP may exert a direct effect in maintaining the integrity of the cell membrane (Wilkinson and Robinson, 1974).

The protective role of ATP on membrane integrity and maintenance of cell shape could be mediated by the protein spectrin. This protein, which is situated on the intracellular side of the membrane, has ATPase activity (Kirkpatrick et al., 1975) and is capable of lateral movement. It has been described as erythrocyte myosin, and is postulated to be involved in the maintenance of cell shape and deformability. The removal of spectrin from erythrocyte ghosts causes them to lose their characteristic shape, and to become spherical and fragile (Elgsaeter et al., 1976). Sheetz and Singer reported their findings in 1977 on the relationship between ATP and the spectrin complex, with reference to erythrocyte shape changes. Intact erythrocytes and ghosts assumed a crenated shape when depleted of ATP. This change was reversible on incubation with ATP. Stomatocytes were formed on incubation of erythrocytes with excess ATP. Magnesium was required for the shape changes, which were completely inhibited in the presence of calcium. The addition of ouabain had no effect. The major enzyme involved was assumed to be the protein kinase of the erythrocyte membrane. The phosphorylation of the spectrin molecule was postulated to result in the polymerisation of the subunits on the inside of the membrane. The ratio of inner to outer surface area would then increase, with the subsequent formation of a cup-shaped erythrocyte. When the ratio decreases, with the absence of ATP for spectrin phosphorylation, the erythrocyte would assume the shape of a crenated sphere. The shape resulting from a balanced ratio would be that of a biconcave disc. The importance of this shape for the functional characteristics

of the erythrocytes rests on two points. Firstly, a biconcave disc exhibits a greater surface area than a sphere of the same volume; secondly, the biconcave disc is a less energy utilising shape than other non spherical forms.

From these results the authors suggested the ratio of surface areas is very important, and it is in this way that ATP exerts an effect on membrane integrity. Birchmeier and Singer (1977) hypothesised that the phosphorylation of component 2 of the spectrin molecule causes changes in shape, by realignment or polymerisation of the spectrin subunits. This would cause an increase in the inner surface area, thereby causing the formation of stomatocytes.

Lux et al., (1978) found that spectrin extraction from ATP depleted cells was more difficult. They suggested this may have been due to an increased binding between spectrin subunits, or between spectrin and integral membrane proteins or lipids. Such complexes could have resulted from intermolecular disulphide couplings (Palek et al., 1978).

Antibodies to the spectrin protein have been very useful in investigating these relationships further. Ferritin conjugated antibodies have been used to study the distribution of spectrin along the membranes of normal and echinocytic erythrocytes (Ziparo et al., 1978). With frozen sections of non haemolysed erythrocytes, the label of the spectrin antibodies was seen to follow the contours of the membrane surface, although the distance from the edge of the membrane varied, probably due to different planes. It is not known whether the spectrin is bound to lipid directly, or at some distance from the inner half of the layer. The pattern for echinocytic cells was identical. No antibodies were found at the bases of the echinocytic spines. The spiny processes did not

appear to have been formed by contraction of the spectrin meshwork, which would have resulted in extrusion of cytoplasm. This finding is not in agreement with the theory that the ratio of surface areas is of primary importance (Sheetz and Singer, 1977), as this would suggest that no spectrin meshwork should be found in the echinocytic protrusions.

Lin and Macey (1978) suggest that high intracellular levels of divalent metal ions result in linking of the acidic lipid head groups, causing echinocyte formation.

The involvement of spectrin phosphorylation and calcium ion concentrations in hereditary spherocytosis and sickle cell anaemia have been investigated. Rubin and Rosen (1975) and Matsumoto et al., (1977) have reported a decrease in phosphorylation of membrane proteins in spherocytosis. Lux et al., (1976) reported a defect in the 'spectrin-actin lattice', which may be the primary abnormality of the irreversibly sickled cell. Steinberg et al., (1978) suggest that rises in calcium concentrations in the erythrocyte associated with potassium efflux (Gardos effect) may be related to the sickling process.

The observations on spectrin phosphorylation and metabolism of erythrocytes in the congenital muscular dystrophies are contradictory.

A reduction of intracellular ATP and ADP levels and a rise in the concentration of AMP were measured by Solomons et al., (1977) in cases of DMD and myotonic dystrophy. However, Danon et al., (1978) reported that intracellular ATP has a higher concentration in erythrocytes from patients with DMD, and an increased production of lactate.

The first report of an altered rate of phosphorylation of spectrin in DMD was published by Roses et al., (1975). Both

phosphorylation of band 2 and 3 was significantly raised ($p < 0.01$) when compared with controls. This finding was not confirmed by Iyer et al., (1977) who studied protein kinase activity in three patients with DMD. These authors also found differences in enzyme behaviour upon freezing and storage, than those published by Roses et al., (1975). This was due to an altered method of membrane preparations and assay technique (Vickers, Rathbone et al., 1978). Vickers, McComas et al., (1978) confirmed the earlier findings of Roses et al., (1975) of an increased phosphorylation of band 3 protein and spectrin, in patients with DMD and heterozygous carriers. Vickers, McComas et al., (1978) reported increased rates of phosphorylation, but the mean increases were not different. The results of Falk et al., (1979) were similar in that peak 2 phosphorylation was slightly increased, but with the wide range of values from male and female controls (which were not considered separately), such differences were not so clear cut.

The theories which attempt to explain the echinocyte and stomatocyte formation of normal erythrocytes are interdependent upon one another. However, it is generally accepted that these deformed cells do not exist in vivo. The methods of preparation and fixation of the erythrocytes for study with light and electron microscopes are capable of inducing the changes which are seen. Therefore, it is more plausible that erythrocytes from patients with DMD are more likely to deform under the conditions of preparation, than are controls. If this can be accepted, the observed discrepancies can be explained by the different conditions of the preparative techniques.

The mechanism by which the erythrocyte shape does alter could be due to a generalised membrane defect which becomes apparent under certain conditions; age or metabolic deprivation may make the

erythrocytes more susceptible to deformation under the specific method of preparation for microscopic investigation.

CHAPTER 4

OSMOTIC FRAGILITY

Introduction

Materials and Methods

Results

Discussion

Introduction

The erythrocyte membrane acts as a barrier between the cytoplasm and extracellular fluid. There is passive transport of water, and active transport of other molecules and ions across this membrane in an attempt to maintain an equilibrium of thermodynamic energy. This phenomenon of osmosis is necessary for the cell to maintain its integrity whilst the composition of the extracellular fluid changes. This occurs continuously in the blood plasma, with the erythrocyte compensating by intracellular volume changes. There is an upper limit to which the membrane is able to expand. This occurs when the extracellular fluid has a much lower molecular concentration than the cytoplasm. There is excessive transport of water into the erythrocyte which results in cell lysis.

The erythrocyte can regulate the intracellular conditions not only by enzymic control of active transport but also by expansion and contraction of the membrane. The extensibility of the membrane is related to its fluidity and deformability.

Percy and Miller (1975) reported reduced deformability of the erythrocyte membrane from patients with DMD. They found that a greater negative pressure was required to aspirate erythrocytes from DMD patients into a micropipette, than those from controls. These findings were confirmed by Kohn et al., (1977) who measured the visco-elastic resistance to deformation. Brain et al., (1978) studied the sheer modulus of the erythrocyte membrane, which was higher in DMD patients than controls. The erythrocytes were subjected to a negative pressure which caused budding of the membrane rather than total aspiration. This was a more direct method of assessing membrane deformability, rather than involving the cytoplasm as well.

Sheer modulus is inversely related to deformability. Butterfield et al., (1976) however, came to the conclusion that DMD erythrocytes have normal membrane fluidity, as measured by electron spin labelling.

Fisher et al., (1976) measured the osmotic fragility of erythrocytes from patients with 'pseudohypertrophic muscular dystrophy'. They found that erythrocytes from patients with muscular dystrophy were more fragile than those from controls. This was confirmed with cases of clinically confirmed DMD by Lloyd and Nunn (1978). Ruitenbeek et al., (1979) studied the effects of phospholipase treatment on control, DMD and myotonic erythrocytes. They confirmed the earlier reports of increased osmotic fragility in DMD and myotonic dystrophy, and also found that lysis of control erythrocytes with pancreatic phospholipase could be partially inhibited. The addition of K and Li ions, or ouabain, or glucose, or lowering Ca^{2+} concentration, partially prevented lysis. Somer et al., (1979) reported increased osmotic fragility in 15 cases of DMD, but found no significant difference in membrane deformability between controls and DMD erythrocytes. They also found that the erythrocyte volume was lower in DMD than controls. Adornato et al., (1977) designed a study to investigate erythrocyte turnover in the blood. They found that the erythrocyte lifespan was normal, measured by labelling the cells with ^{51}Cr. The osmotic fragility was also found to be normal. Their results failed to support the theory of a significant erythrocyte membrane defect in DMD.

A larger study was designed to investigate osmotic fragility of erythrocytes from DMD patients and carriers, after a pilot study (Lloyd and Nunn, 1978) indicated some differences.

Materials and Methods

The osmotic fragility of erythrocytes was determined by the method of Dacie and Lewis (1975).

A stock solution of buffered sodium chloride was made up, osmotically equivalent to 100 g/l, pH 7.4.

Serial dilutions were prepared:-
1.0, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 9.0 g/l.

50 µl of heparinised whole venous blood was added to 5ml of these saline dilutions and immediately mixed by inversion. The solutions were left to stand at room temperature for 30 minutes, then reinverted and centrifuged at 1 000 g for five minutes.

The optical density of the supernatants were determined spectrophotometrically at 540 nm.

There was no erythrocyte lysis in 9.0 g/l saline, this was used as the blank for the spectrophotometer. Complete lysis was measured in 1.0 g/l saline.

The lysis at each dilution was expressed as a percentage of the optical density of the 1.0 g/l solution (complete lysis).

Results

Duchenne Patients

The results of studies into the osmotic fragility of erythrocytes from clinically confirmed DMD patients, their unaffected brothers, and age matched controls are shown in Table 6. At various concentrations of buffered saline (from 4.2 g/l to 5.0 g/l) there was a significant difference ($p < 0.01$) between the percentage lysis in DMD erythrocytes compared with controls.

However, there was an overlap of the individual values between the DMD samples and controls (Figure 3). The threshold of lysis was the most sensitive indicator of measurable differences between erythrocytes from DMD patients, and normal healthy boys. None of the controls, or unaffected brothers showed any signs of lysis at 5.0 g/l, whereas at this concentration fragility was detected in 15 out of the 18 DMD samples studied.

There was no correlation between the amount of lysis in DMD samples (at 4.2 g/l and 4.4 g/l) and age or SCK level. This suggests there was no direct relationship between the severity and the duration of the disease, and the osmotic behaviour of the erythrocytes.

At low concentrations of buffered saline (3.8 g/l and 4.0 g/l) the differences between unaffected brothers and controls just reached a level of statistical significance ($p < 0.05$) but this was probably due to the wide variation in measured values for the brothers and the small number involved in the study.

Carriers of DMD

The results of investigations into the osmotic fragility of erythrocytes from carriers and other female relatives are shown

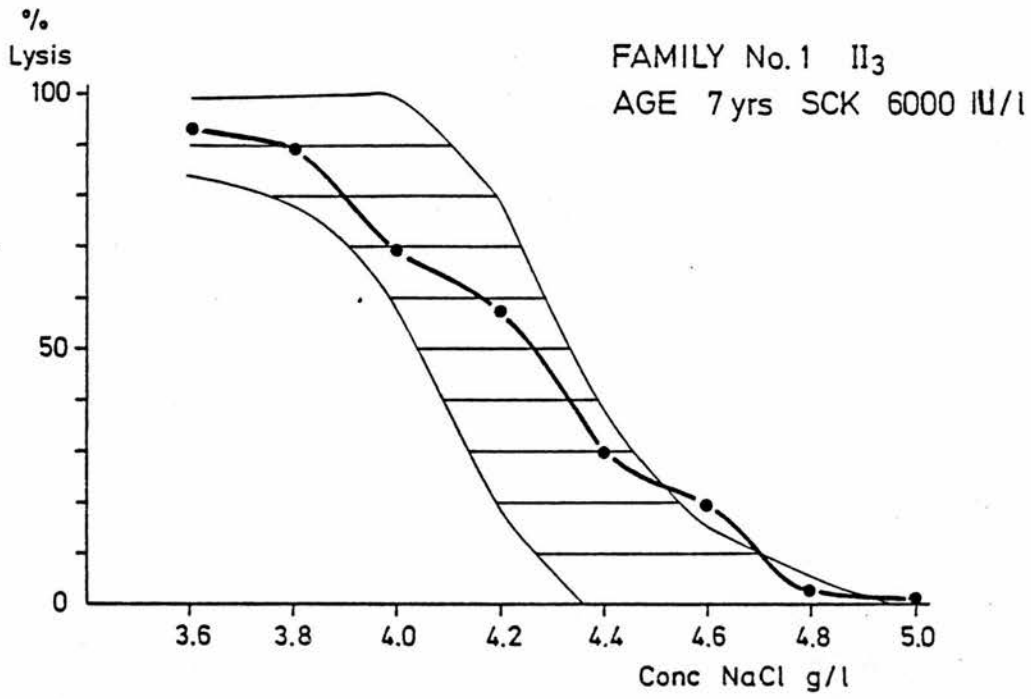
Table 6 Percentage lysis of controls, patients with clinically confirmed DMD and their unaffected brothers.

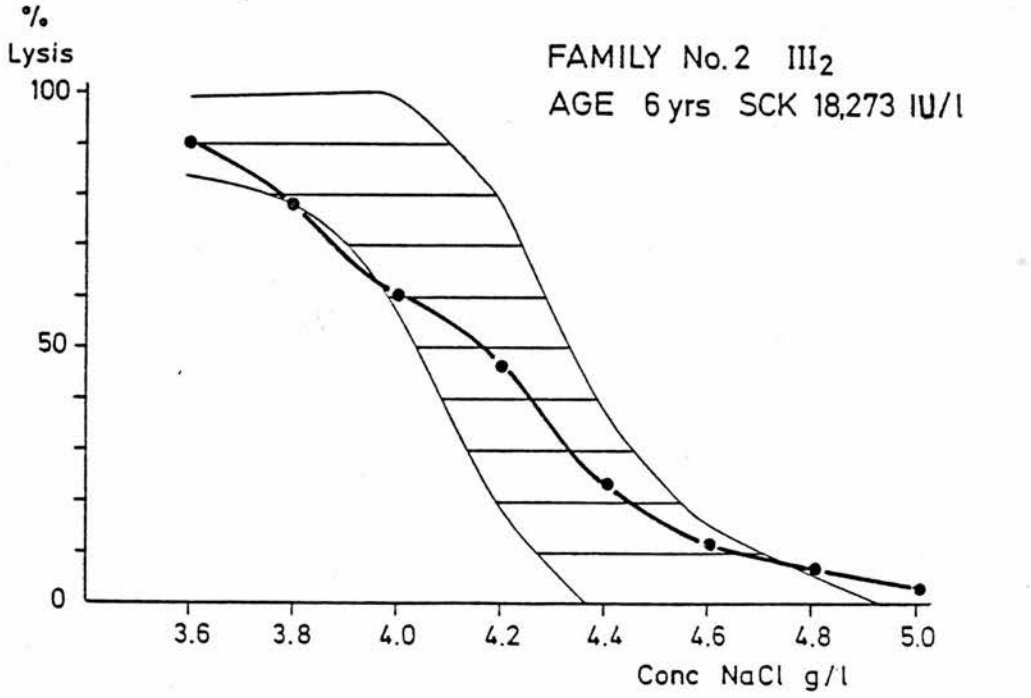
NaCl conc. g/l	Controls (n = 15) Mean	SD	DMD (n = 18) Mean	SD	Brothers (n = 5) Mean	SD
1.0	100.0		100.0		100.0	
3.6	95.5	2.45	94.1	5.03	93.4	6.01
3.8	91.2	2.92	91.1	5.86	83.2*	13.16
4.0	80.1	10.32	84.7	10.38	63.5*	18.40
4.2	50.3	15.09	69.9**	14.55	38.5	11.87
4.4	16.8	11.25	39.3**	18.43	9.6	2.11
4.6	6.3	5.37	18.7**	10.55	4.6	2.07
4.8	1.7	1.76	7.3**	5.22	1.1	0.86
5.0	0		1.8**	1.73	0	

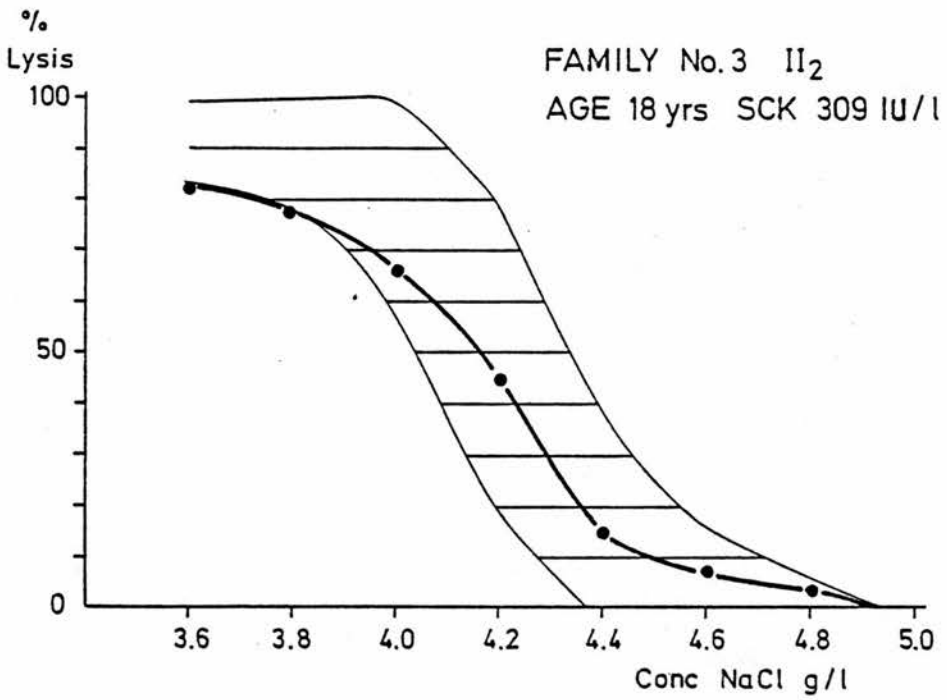
NB * $p < 0.05$ compared with controls

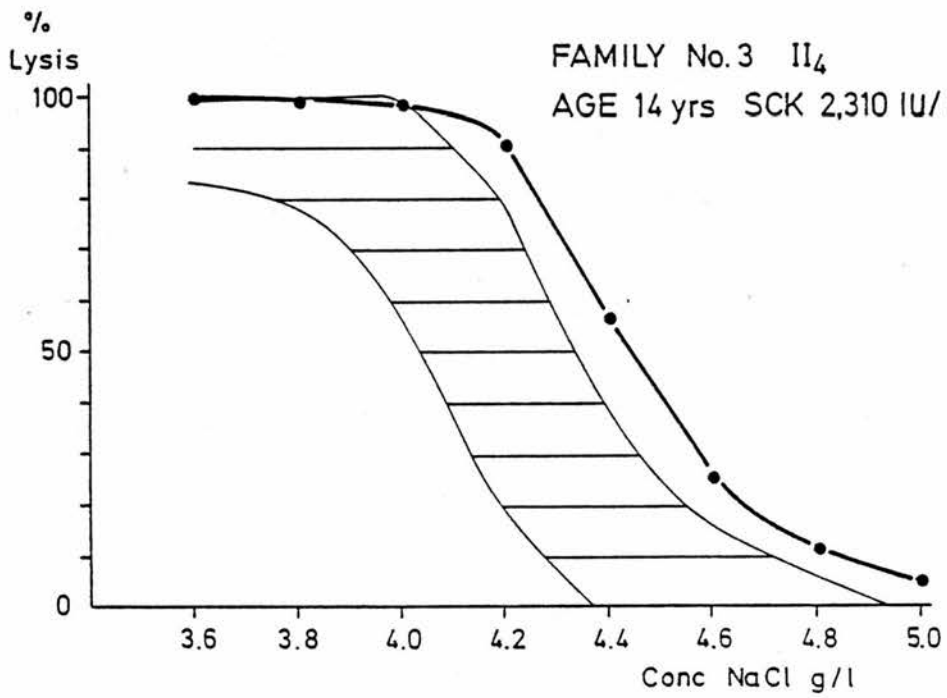
** $p < 0.01$

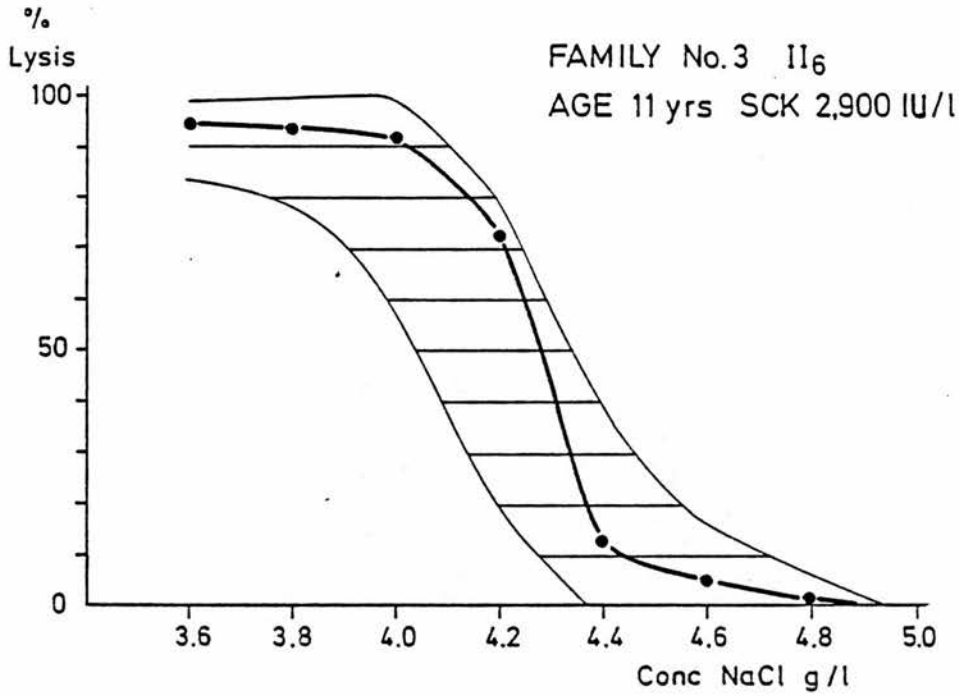
Figure 3a Individual graphs of osmotic fragility of erythrocytes from patients with DMD. Hatched area represents mean ± 2SD for controls.

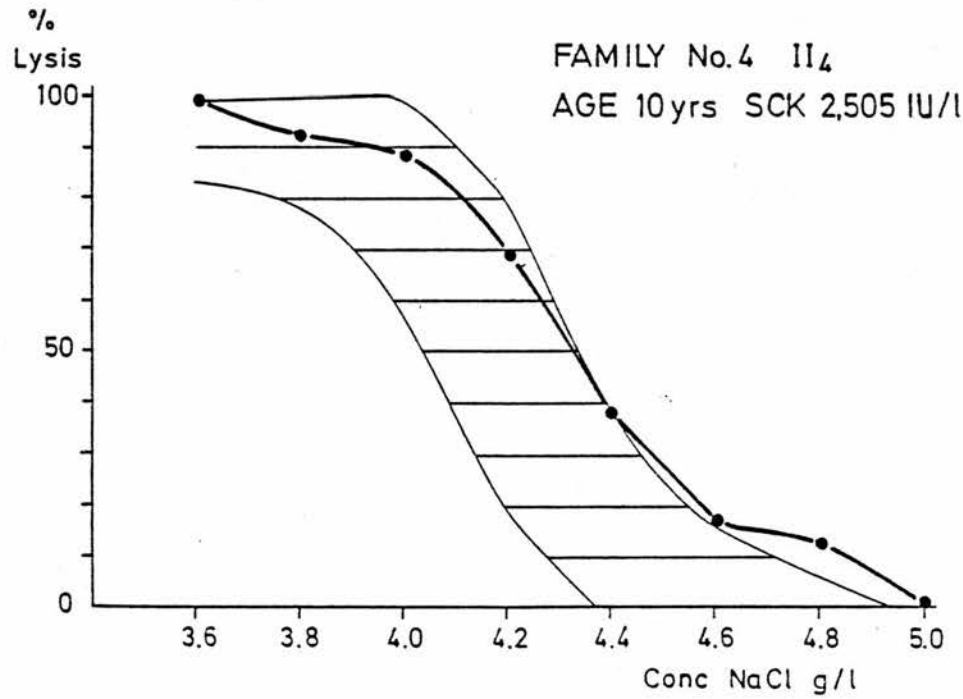

%
Lysis
100
50
0
FAMILY No. 1 II_3
AGE 7 yrs SCK 6000 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

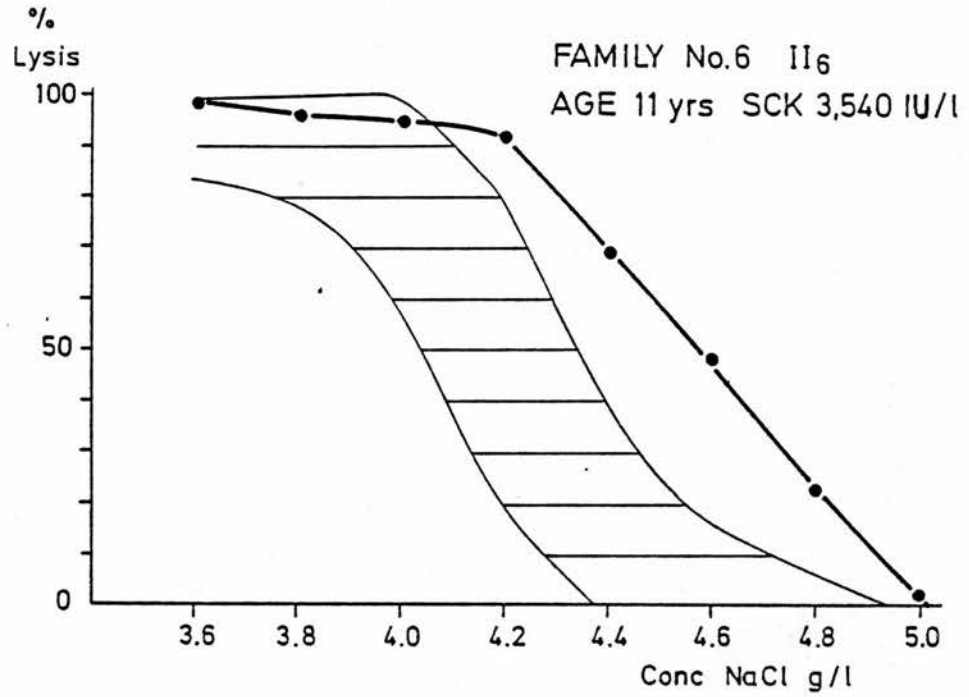

%
Lysis
100
50
0
FAMILY No. 2 III_2
AGE 6 yrs SCK 18,273 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

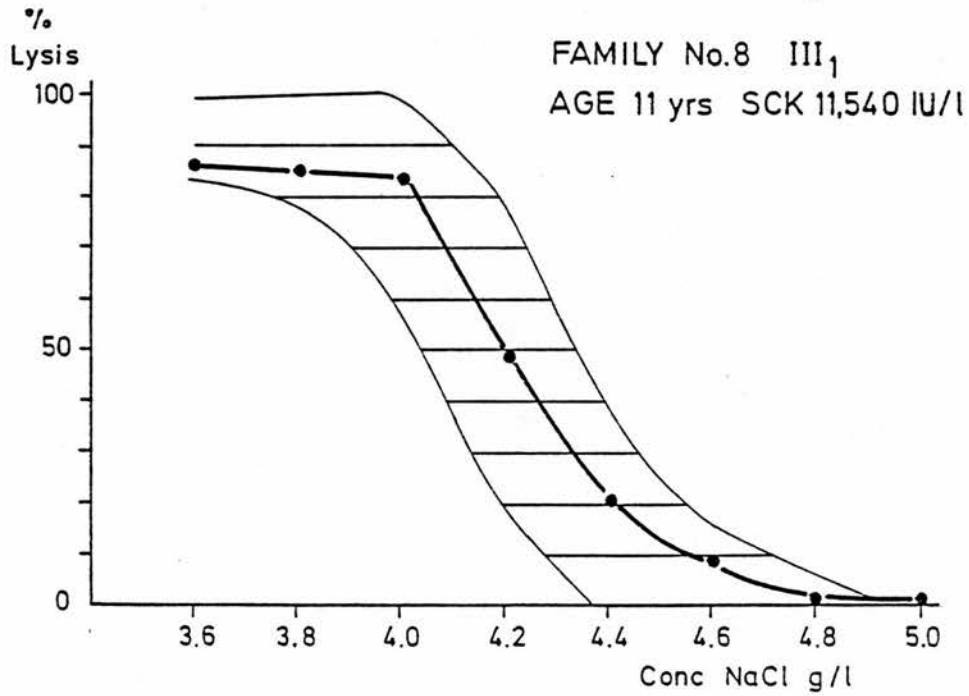

%
Lysis
100
50
0
FAMILY No. 3 II2
AGE 18 yrs SCK 309 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

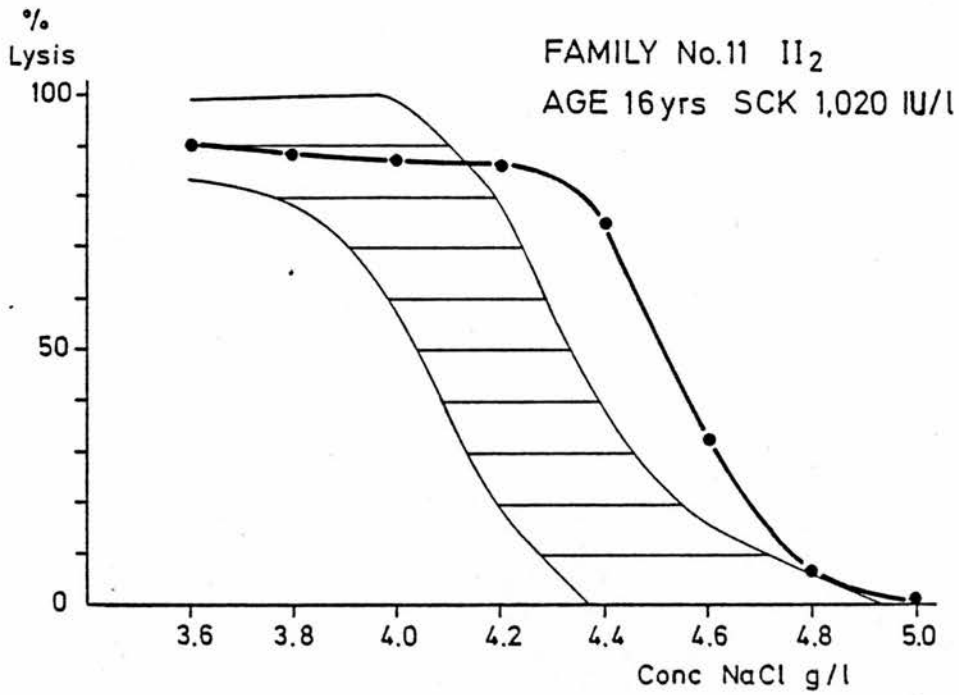

%
Lysis
100
50
0
FAMILY No. 3 II4
AGE 14 yrs SCK 2,310 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

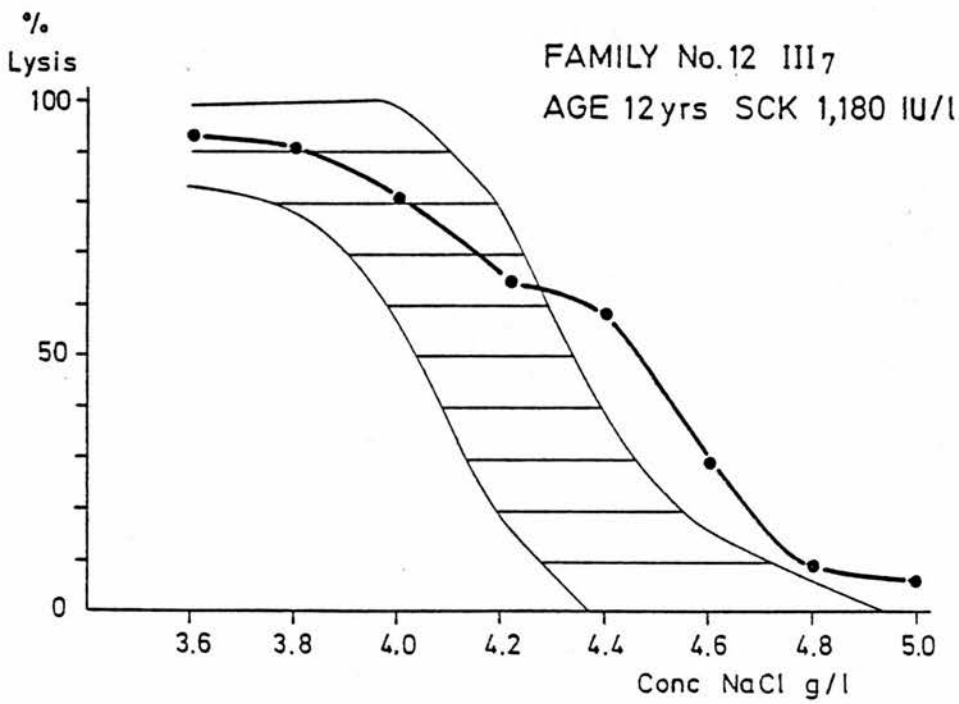

%
Lysis
100
50
0
FAMILY No. 3 II6
AGE 11 yrs SCK 2,900 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

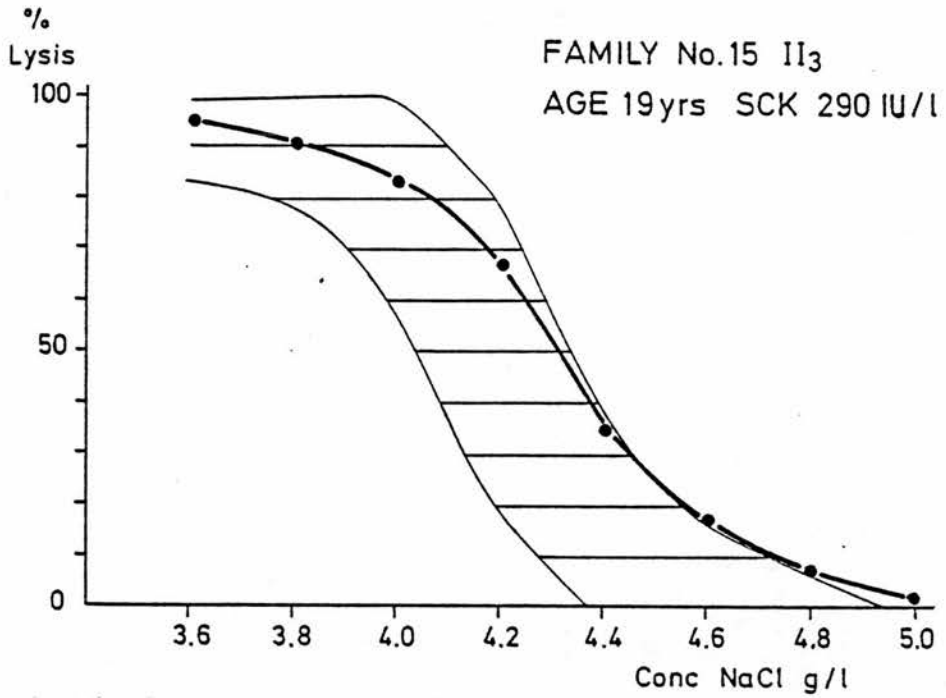

%
Lysis
100
50
0
FAMILY No. 4 II4
AGE 10 yrs SCK 2,505 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

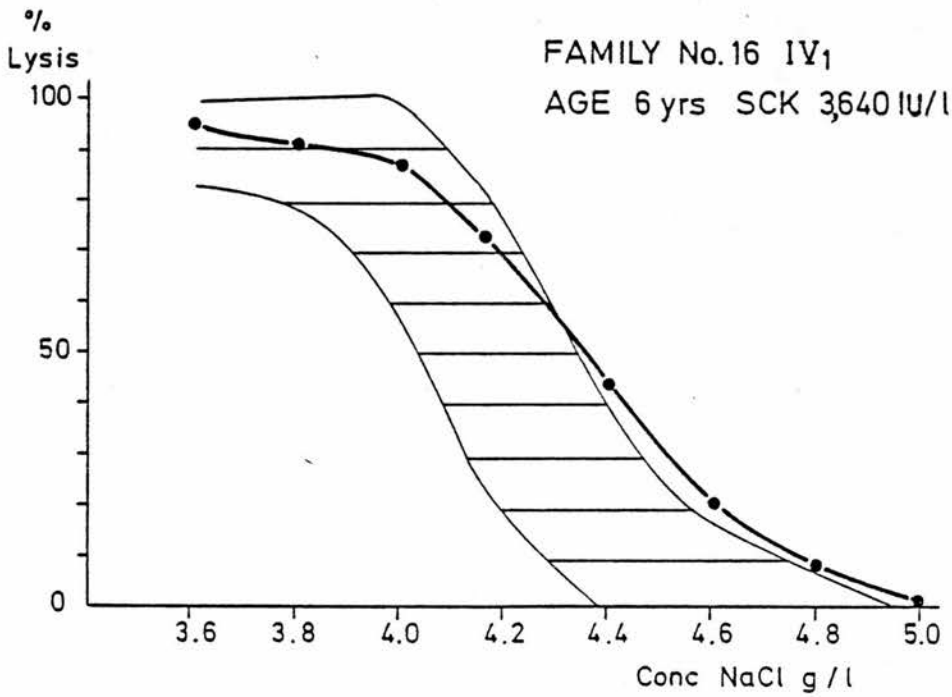

%
Lysis
100
50
0
FAMILY No.6 II6
AGE 11 yrs SCK 3,540 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

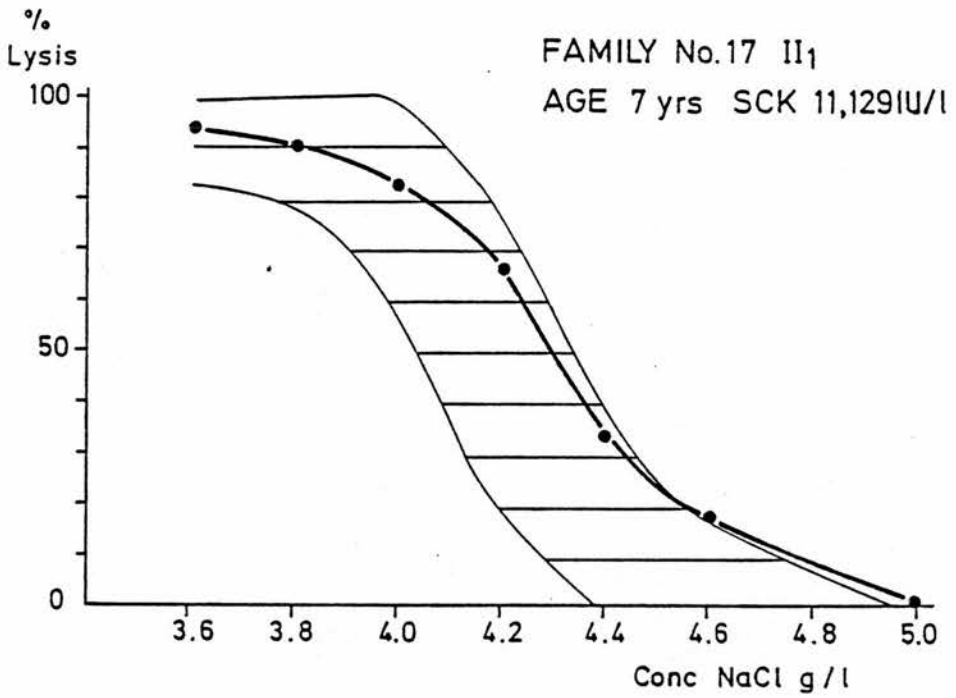

%
Lysis
100
50
0
FAMILY No.8 III1
AGE 11 yrs SCK 11,540 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

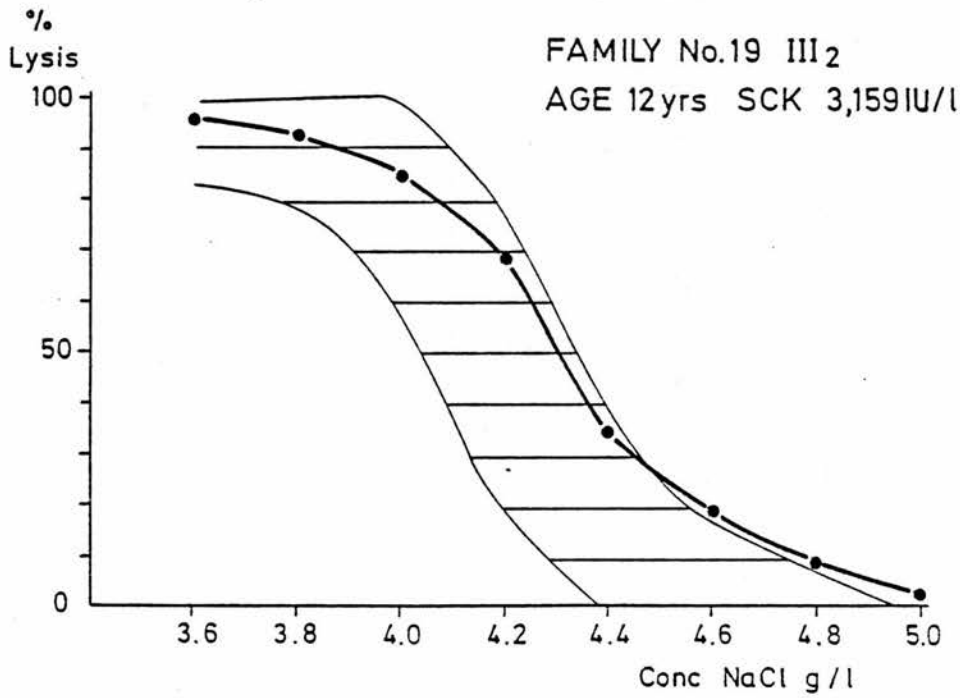

%
Lysis
100
50
0
FAMILY No.11 II2
AGE 16yrs SCK 1,020 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

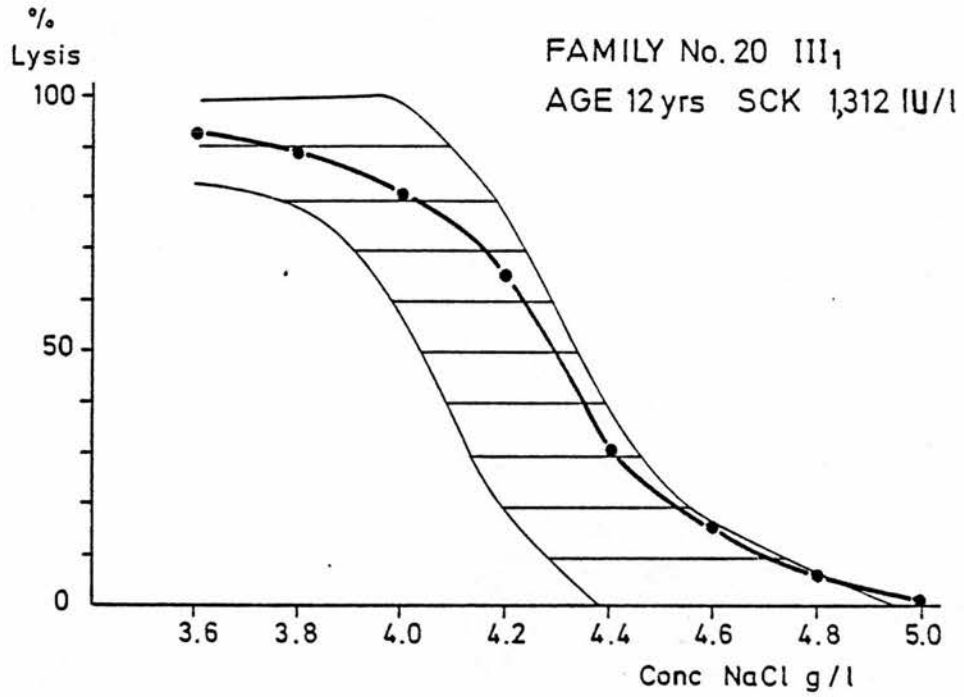

%
Lysis
100
50
0
FAMILY No.12 III7
AGE 12yrs SCK 1,180 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

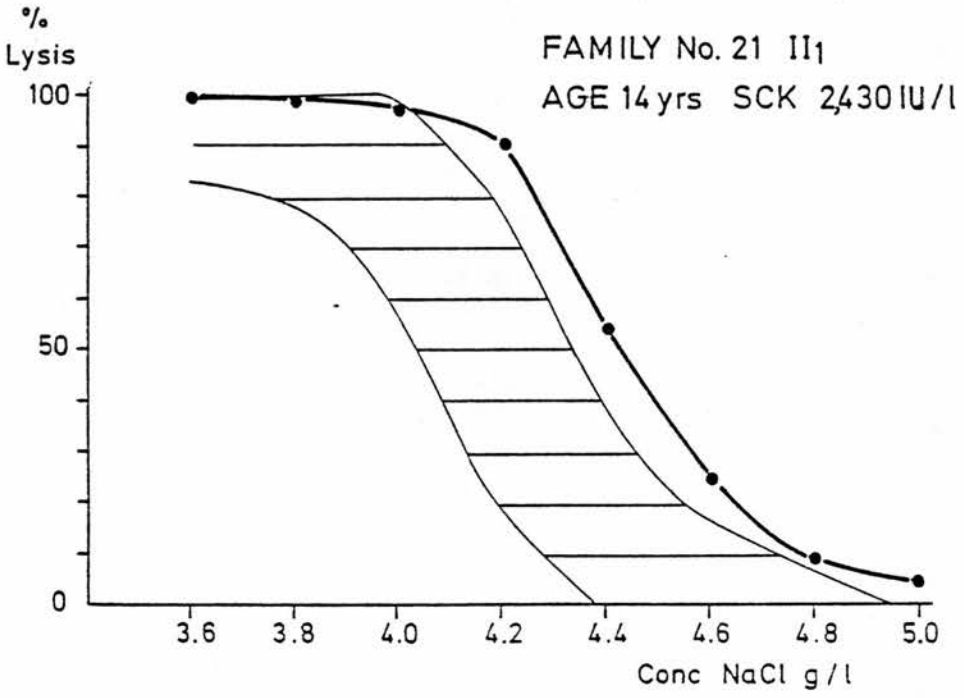

%
Lysis
100
50
0
FAMILY No. 15 II3
AGE 19 yrs SCK 290 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

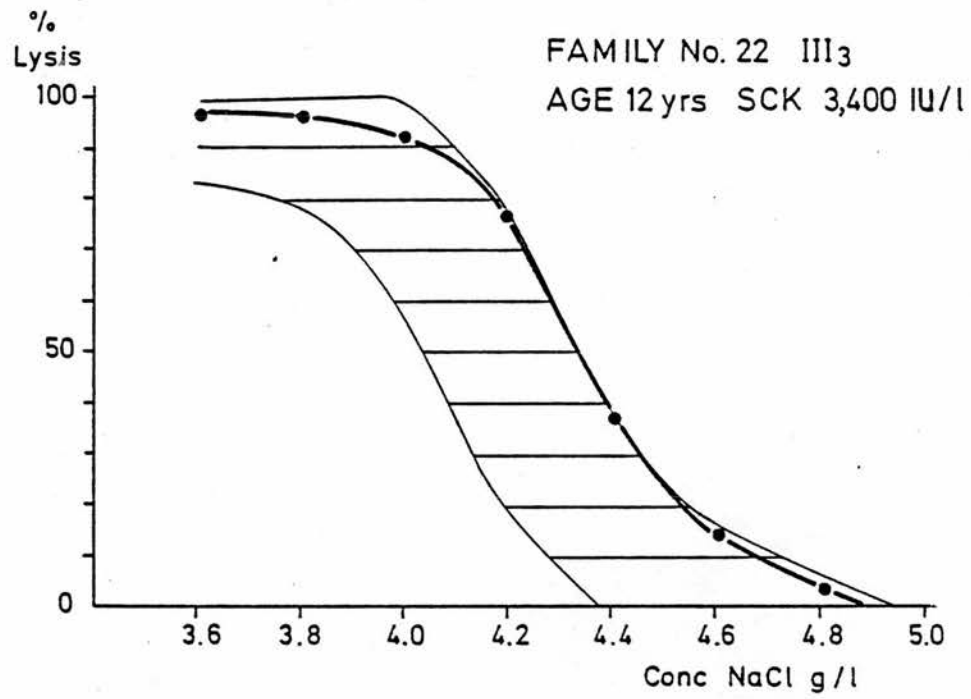

%
Lysis
100
50
0
FAMILY No. 16 IV1
AGE 6 yrs SCK 3,640 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l


%
Lysis
100
50
0
FAMILY No.17 II1
AGE 7 yrs SCK 11,129IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l


%
Lysis
100
50
0
FAMILY No.19 III2
AGE 12yrs SCK 3,159IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l


%
Lysis
100
50
0
FAMILY No. 20 III1
AGE 12 yrs SCK 1,312 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l


%
Lysis
100
50
0
FAMILY No. 21 II1
AGE 14 yrs SCK 2,430 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l


%
Lysis
100
50
0
FAMILY No. 22 III3
AGE 12 yrs SCK 3,400 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

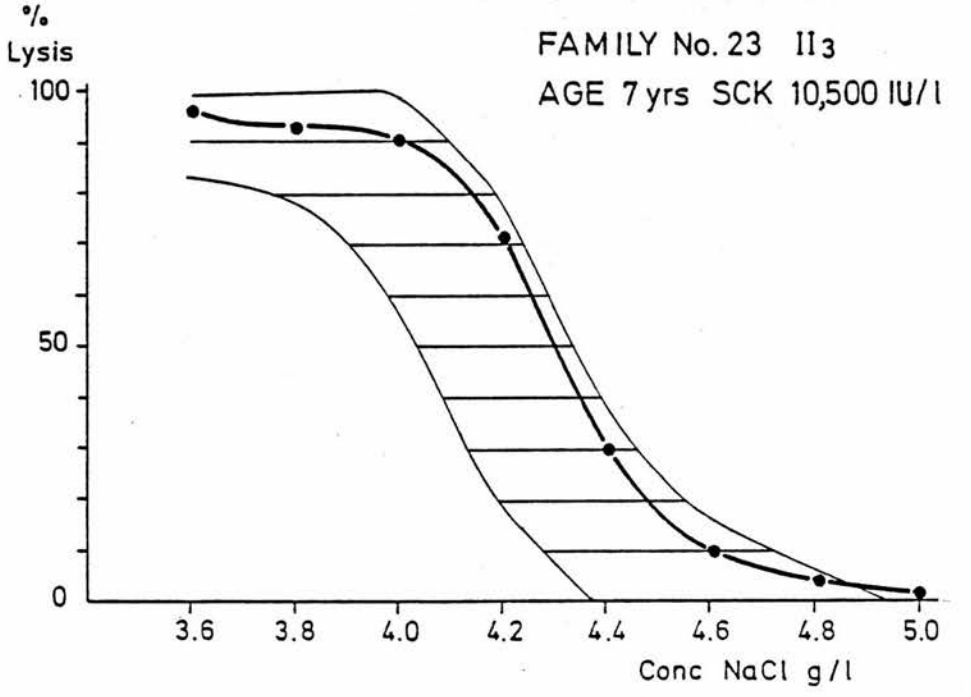
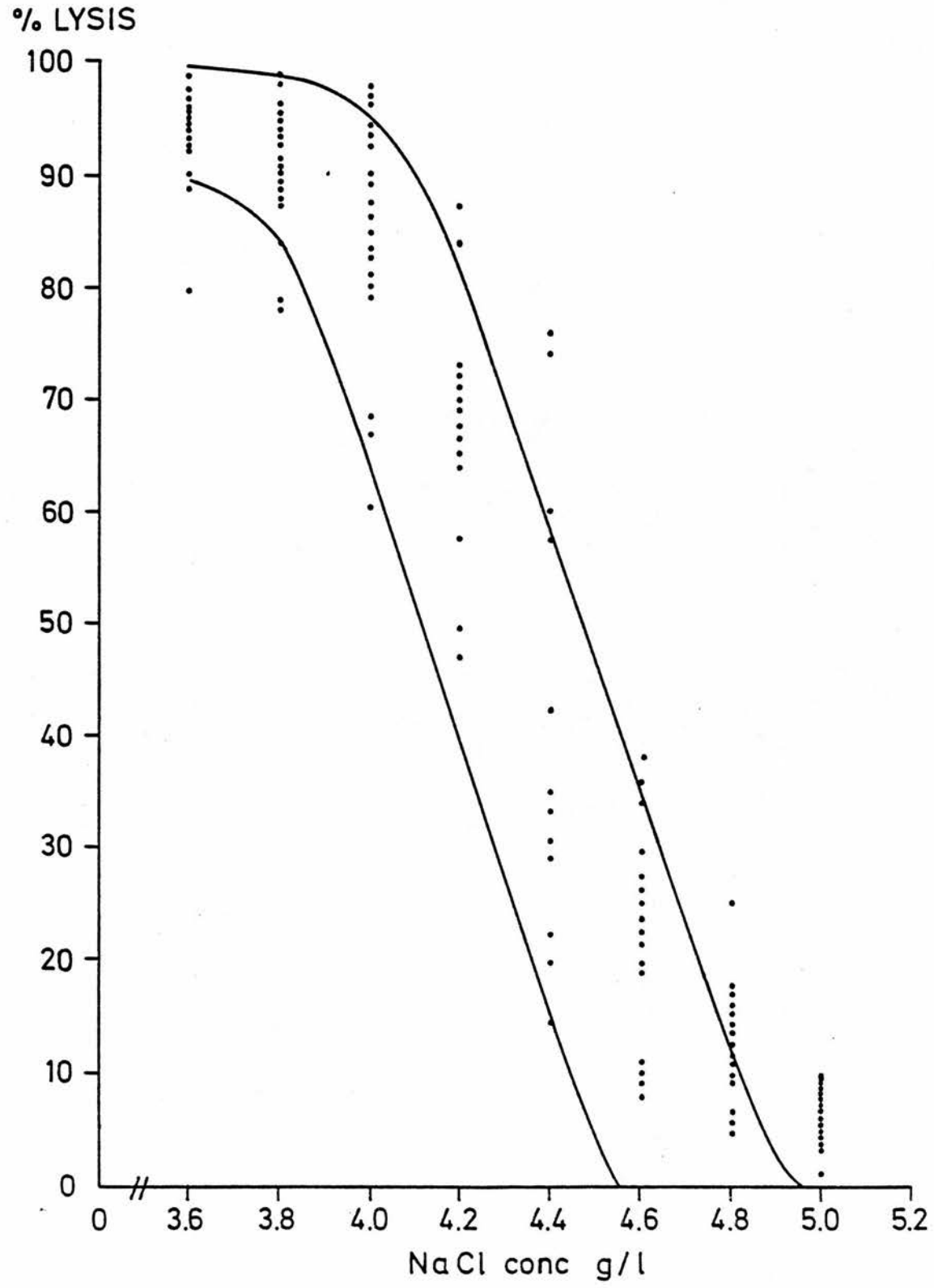

%
Lysis
100
50
0
FAMILY No. 23 II3
AGE 7 yrs SCK 10,500 IU/l
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Conc NaCl g/l

Figure 3b. Graph of osmotic fragility of all DMD patients compared with controls (mean $\pm$ 2SD).


% LYSIS
100
90
80
70
60
50
40
30
20
10
0
0
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
NaCl conc g/l

in Table 7. There were no significant differences between controls and any of the groups of female relatives.

In contrast to the young male controls, 58% of female controls exhibited lysis at 5.0 g/l buffered saline. In female controls there was no correlation between age and lysis.

Table 7 Percentage lysis of controls, and carriers of DMD

Conc. NaCl g/l	Controls (n = 36) Mean	SD	Definite/ Probable Carriers (n = 2)	Possible Carriers I (n = 11) Mean	SD	Possible Carriers II (n = 8) Mean	SD
1.0	100.0		100.0 100.0	100.0		100.0	
3.6	95.4	3.52	94.0 97.9	94.1	4.27	93.2	7.41
3.8	89.8	8.40	89.0 92.5	89.4	3.56	86.8	11.11
4.0	78.6	14.37	61.8 90.4	81.8	10.06	78.1	21.26
4.2	64.4	17.89	35.4 74.7	65.2	13.75	63.6	24.75
4.4	40.4	17.39	7.7 50.0	30.1	17.41	29.1	17.21
4.6	18.6	11.09	1.1 14.0	14.1	11.81	13.7	8.44
4.8	6.8	6.59	0 6.0	4.2	3.20	5.6	3.98
5.0	1.1	1.98	0 0.7	1.0	1.32	0.6	1.02

Discussion

The results of the present study have shown that there was a marked increase in the osmotic fragility of erythrocytes from patients with clinically confirmed DMD when compared with controls. There was no such difference between female controls and heterozygous carriers. There was a sex effect between the amount of lysis in the threshold region (5.0 g/l) exhibited by male and female controls.

The fluidity and deformability of the erythrocyte membrane is determined by many variables. The nature of the lipids is very important, not only the functional head groups but also the types of fatty acids associated with them. The strength of lipid and protein interaction is one of the main determinants of membrane integrity, thereby controlling the cytoplasmic volume.

The composition and fluidity of the membrane lipids is of critical importance in the normal functioning and growth of the cell. If the deformability of the membrane is reduced, the erythrocytes are unable to undergo the necessary shape changes whilst they circulate in vivo. The contours of the erythrocyte become folded, there will be a reduced ability to flow and a progressive loss in surface area. These erythrocytes will eventually be destroyed.

The unsaturated nature of the lipids, as well as cholesterol content are important determinants of membrane fluidity. Cooper (1977) suggested that cholesterol binding imposes degrees of immobility on the outer membrane, and thereby increases the freedom of motion deeper within the membrane core. Blau and Bittman (1978) concluded from their studies that cholesterol was distributed symmetrically between the inner and outer halves of the lipid bilayer. Up to

half of the total cholesterol has been removed fairly easily from the erythrocyte membrane by incubation in plasma (Gottlieb, 1976; Lange and D'Alessandro, 1977). Any increase in cholesterol concentration, which would effect crosslinking between lipids, would change the permeability characteristics of the membrane. There would be a decrease in mediated transport and facilitated diffusion of substances across the membrane (Cooper, 1977).

Any major alterations in the unsaturated nature of the lipids would also result in permeability changes of the membrane. The first report of an altered lipid composition of the erythrocyte membrane in progressive muscular dystrophy was published by Kunze et al., in 1973. They found that the sphingomyelin content was increased, and the pattern of fatty acid incorporation of phosphatidylethanolamine was altered. The unsaturated acids were decreased with a concomitant increase in the stearic acid (unsaturated) level. The lipid composition of muscle biopsies were also reported as being altered. The authors related these changes to the primary defect being the specific incorporation of linoleic acid (18:2) into phosphatidyl choline during the de novo synthesis of muscle.

Iyer et al., (1976) studied the activity of erythrocyte phospholipase A. The membranes from DMD patients exhibited higher levels of activity than those from controls. This high activity could be expected to lead to high concentrations of lysophospholipids in the membrane, which could contribute to membrane dysfunction. Diphenylhydantoin reduced the level of enzymic activity to normal. Further abnormalities in lipid composition of the erythrocyte membrane in DMD were reported by Kalofoutis et al., (1977) who also found increased sphingomyelin as well as lysophosphatidyl

choline. Howland and Iyer (1977) reported an altered fatty acid composition, which was obvious in the erythrocyte membranes from heterozygous carriers of DMD, but more variable in cases of patients. They also found low membrane triglyceride content, and suggested that these lipid abnormalities may give some clues to the primary defect of the disease.

Kobayishi et al., (1978) were unable to confirm these alterations. When comparing erythrocyte membrane lipids from DMD patients with age and sex matched controls they reported no differences in total cholesterol and phospholipid content, or the fatty acid components of the phospholipids. Koski et al., (1978) also found the DMD phospholipids normal. They suggested that the discrepancies between controls and patients that were previously measured by Kunze et al., (1973) and Kalofoutis et al., (1977) were perhaps due to lack of precautions against auto oxidation. This could have resulted in the observed increases in sphingomyelin content.

However, Di Stefano and Bosmann (1977) have studied the erythrocytes of mice with genetic muscular dystrophy, using the technique of aqueous polymer partioning. They found measurable differences between dystrophic mice and their normal littermates. This technique relies on alterations in the configuration and/or conformation of primarily lipids, and also proteins, as well as net organisation changes deep within the membranes, as a means to distinguish between different membrane systems. Wakayama et al., (1978) found an uneven distribution of particles on the protoplasmic and extracellular faces of the erythrocyte membranes from DMD patients, measured by freeze fracture. In some instances, the cleaving behaviour of the extracellular face resembled that of pure lipid.

The authors suggested this may be due to the proteins which constitute band 3 on SDS gel electrophoresis, having altered properties. Hidalgo et al., (1978) investigated the effect of the lipid environment on protein motion and enzymic activity of the sarcoplasmic reticulum Ca^{2+} ATPase. Any decrease in lipid fluidity, attained either by lipid replacement or temperature changes, resulted in inhibition of the ATPase and reduction in protein rotational mobility. These results were consistent with the proposal that both protein rotational mobility and lipid fluidity are essential for enzymic activity. A decrease in elasticity and deformability resulting in increased osmotic fragility was measured by Williamson et al., (1975) in erythrocytes at high temperatures.

The integral membrane proteins are also involved in regulation of cell shape and membrane deformability. Smith and LaCelle (1979) reported that the pH induced reduction of membrane deformability and elasticity was paralleled by a pronounced change in the solubility of the membrane protein spectrin.

Quantitative observations of altered phospholipid content of erythrocyte membranes are contradictory, but the ratio of lipid to protein may be a more valuable comparison. Although actual levels of phospholipids, cholesterol and sphingomyelin may be normal, the protein levels may be altered. This would lead to differences in specific lipid-protein interactions which would be detectable by freeze fracture or electron spin resonance (Wilkerson et al., 1979). Thus, the findings in the present study of increased osmotic fragility in DMD could be accounted for by the reported reduced deformability of erythrocytes; in this disorder probably secondary to protein-lipid alterations in the membrane. However, osmotic

fragility of the erythrocytes is not governed solely by the deformability of the membrane. The surface area to volume ratio is also of importance. Somer et al., (1979) found that erythrocyte volume was lower in DMD than controls. They also reported no differences in deformability measured by microsieving, when taking the reduced volume into account.

CHAPTER 5

ERTHROCYTE MEMBRANE (Na^+, K^+) ATPase AND THE EFFECT OF PLASMA INCUBATION ON THE ENZYME.

Introduction

Materials and Methods

Results

Discussion

Introduction

Relatively high concentrations of internal K^+ are required for a variety of cellular processes such as glycolysis and protein synthesis. If the high internal concentration of K^+ were not compensated by loss of some other cation, there would be a net increase in the internal osmotic pressure causing the cell to swell and burst. The active transport of Na^+ out of the cell by means of the Na^+ pump leaves the intracellular contents low in this cation and the extracellular concentration high. This inward gradient is believed to be the driving force of the active transport of K^+, glucose and amino acids into the cell. The enzyme responsible for the coupled transport of Na^+ and K^+ across the erythrocyte membrane, (Na^+, K^+) ATPase, is directional in its action.

The action of the cardiac glycoside ouabain supports the concept of Na^+ extrusion driving K^+, glucose and amino acid uptake. This compound specifically inhibits, in normal cells, Na^+ transport out of the cell, which thereby affects the transport of K^+, glucose and amino acids across the membrane.

However, Brown et al., in 1967 reported an unusual response to ouabain of (Na^+, K^+) ATPase in erythrocyte ghosts from patients with myopathies. Their investigations showed that (Na^+, K^+) ATPase of erythrocyte ghost membranes prepared from seven out of eight clinically confirmed cases of DMD had abnormal responses to ouabain. Consistent stimulation of activity by the cardiac glycoside was also found in patients described as having 'spontaneous muscular dystrophy' and myotonia. These results were not confirmed by Klassen and Blostein (1969) who found no enhancement of activity by ouabain in seven cases of DMD. Peter et al., in 1969, supported

the earlier findings of Brown et al., (1967) in reporting stimulation of erythrocyte ghost (Na^+, K^+) ATPase activity by ouabain in 12 out of 13 cases of clinically confirmed DMD. An altered response of (Na^+, K^+) ATPase to ouabain has been confirmed by other investigators (Araki and Mawatari, 1971; Niebroj-Dobosz, 1976; Mawatari et al., 1976; Siddiqui and Pennington, 1977; Pearson, 1978) but not by all (Hodson and Pleasure, 1977; Souweine et al., 1978; Wacholtz et al., 1979; Ruitenbeek, 1979).

The present investigation was undertaken in an effort to determine if the reported effects of ouabain could be confirmed and if so, whether or not this was specific for DMD; or occurred in other neuromuscular disorders (such as myotonia, facioscapulohumeral muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, peroneal muscular atrophy and periodic paralysis). Experiments were also designed to determine what effect dystrophic plasma may have on normal erythrocytes regarding (Na^+, K^+) ATPase activity and the effect of ouabain.

Materials and Methods

Preparation of ghost membranes

Erythrocyte ghost membranes were prepared by a modification of the method of Mawatari et al., (1976).

The packed red cells (10 ml) were haemolysed for 20 minutes at $4^{o}C$ in 10 mM Tris buffer (pH 7.4 containing 1 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol). The lysate was centrifuged at 10 000 g for 30 minutes, and the separated erythrocyte ghosts were washed in the same buffer three times. After each centrifugation, the lysozyme button, which sedimented below the ghost membranes was removed. All operations were carried out at $2^{o}C$ to $4^{o}C$.

The erythrocyte ghost membranes were resuspended in 0.5 ml - 1.0 ml of the same buffer and stored overnight at $-23^{o}C$.

Protein estimation

The protein concentration of the ghost membrane suspension was measured by the method of Lowry et al., (1951).

Reagent A (see Appendix II)	2% sodium carbonate and 0.5% copper sulphate and 1% sodium tartrate in 0.1M sodium hydroxide.
Reagent B (see Appendix II)	Folin - Ciocalteu Reagent
Bovine serum albumin stock (see Appendix II)	

The ghost membrane samples were thawed, gently homogenised in a hand-held homogeniser, and then 0.1 ml was dissolved in 0.9 ml of solubilising fluid.

Bovine serum albumin stock was dissolved in the solubilising fluid for calibration.

Each sample was measured in duplicate.

To 1 ml of protein solution was added 5 ml of reagent A, mixed by inversion and allowed to stand at room temperature for 10 minutes. 0.5 ml of reagent B was then added, immediately mixed by inversion and the extinction coefficient read at 700 nm after 30 minutes at room temperature. Bovine serum albumin was used for the standard calibration curve.

Inorganic phosphate estimation

The inorganic phosphate released by the (Na^+, K^+) ATPase was measured by the method of Fiske et al., (1925). Details of the reagents and proportion are given in Appendix II.

After the addition of 50% trichloroacetic acid (TCA), the reaction mixture was centrifuged at 1 500 g for 15 minutes. 0.5 ml of the supernatant was pipetted into a 1 ml graduated stoppered cylinder. 0.1 ml of molybdate 2 was added and mixed. Then 0.04 ml of aminonaphtholsulphonic acid was added and the solution made up to 1 ml.

A standard was made up using dilutions of a standard phosphate stock. 0.1 ml of molybdate 1 was added to 0.5 ml of standard phosphate and mixed. 0.04 ml of aminonaphtholsulphonic acid was added, and made up to 1 ml with water. After five minutes the extinction coefficient was measured at 680 nm. Standard phosphate dilutions were used for the calibration curve.

Assay procedure for (Na^+, K^+) ATPase enzyme

The activity of the (Na^+, K^+) ATPase enzyme was measured by a modification of the method of Peter et al., (1969).

All incubations were carried out at 37°C. The incubation mixture consisted of 0.1 ml of ghost membrane suspension and 1 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ in 0.1 M Tris (pH 7.4) in a final volume of 1 ml.

The incubation time and the concentrations of Na_2 ATP and ghost membrane protein were varied to optimise conditions for (Na^+, K^+) ATPase activity. Control ghost membranes were used throughout the following procedures.

(Na^+, K^+) ATPase activity vs. Na_2 ATP concentration

The activity of the (Na^+, K^+) ATPase was measured at various concentrations of Na_2 ATP:-

1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6 mM

After 10 minutes the reaction was stopped by the addition of 0.2 ml of 50% TCA. The inorganic phosphate released was measured by the methods of Fiske et al., (1925). The results are expressed graphically in Figure 4.

(Na^+, K^+) ATPase activity vs. ghost membrane protein concentration

Various protein concentrations were prepared by dilution of ghost membrane stock and assayed with 4 mM Na_2 ATP in the incubation mixture. After 10 minutes the reaction was stopped with TCA and the inorganic phosphate measured. The results are expressed graphically in Figure 5.

(Na^+, K^+) ATPase activity vs. time of incubation

5 ml of incubation mixture was taken containing 4 mM Na_2 ATP and including a suspension of ghost membranes equivalent to 300 $\mu g\ ml^{-1}$. The reaction mixture was incubated at 37°C. At time

Figure 4 Effect of increasing Na_2 ATP concentration on the activity of (Na^+, K^+) ATPase

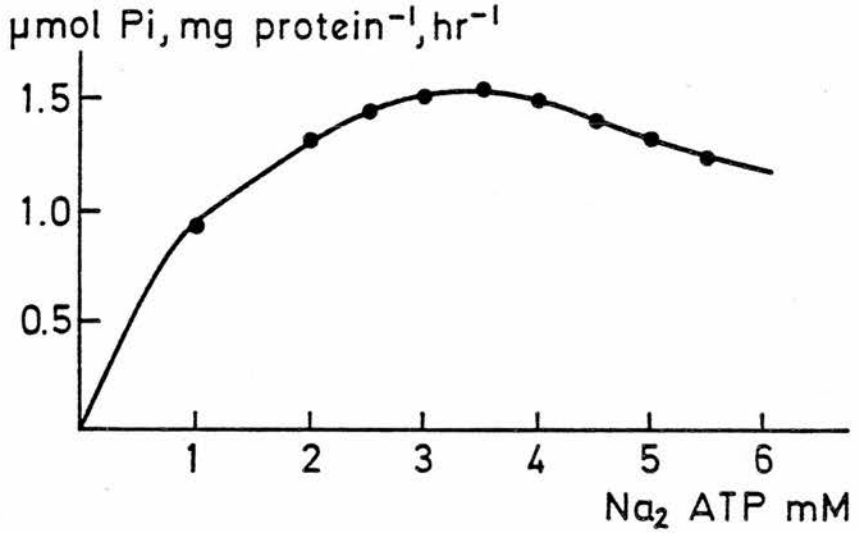
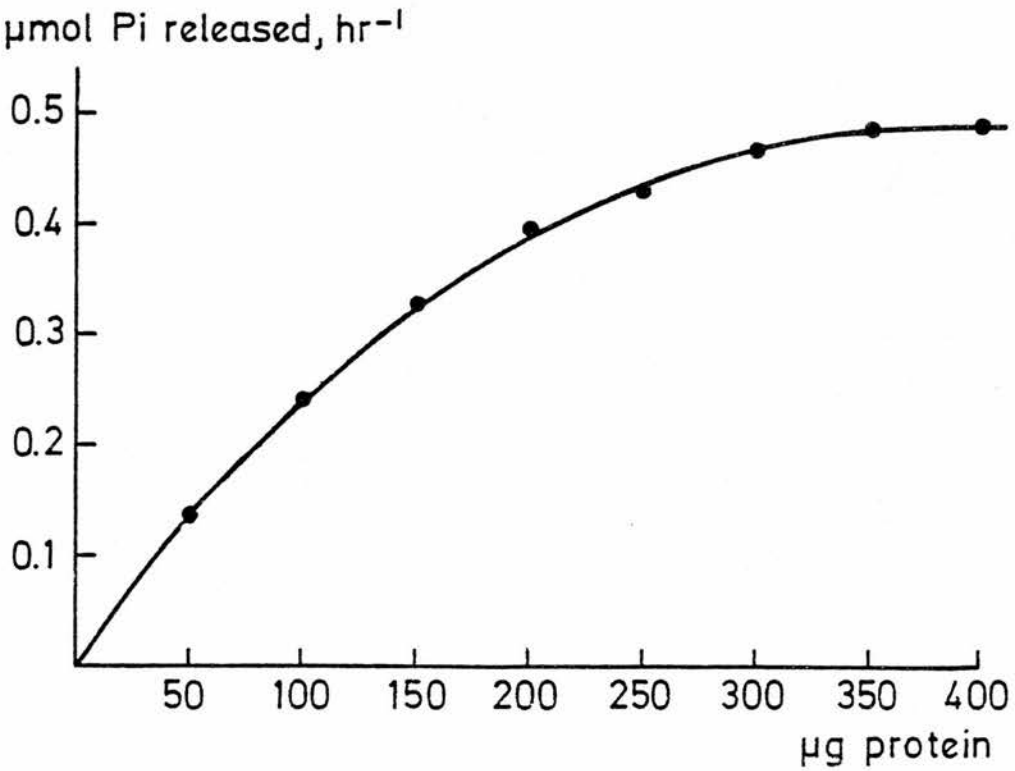


Figure 5 Effect of increasing ghost membrane protein concentration on the activity of (Na^+, K^+) ATPase



intervals 0.5 ml aliquots were withdrawn, to which 0.2 ml of 50% TCA was added. The inorganic phosphate released was assayed. The effects are shown graphically in Figure 6.

(Na^+, K^+) ATPase activity vs. time of storage

Ghost membranes were prepared and divided into 1 ml aliquots, all except one were frozen and stored at -23°C. The enzymic activity of the unfrozen sample was measured, and the other samples assayed at 24 hour intervals. The ghost membranes were thawed and kept on ice for 30 minutes prior to assay. The enzymic activity was reduced (7%) after storage for 24 hours. The drop in activity after storage is shown in Figure 7, expressed as the percentage loss compared with the 24 hour frozen sample.

(Na^+, K^+) ATPase activity vs. ouabain concentration

The effect of ouabain, at various concentrations, on the enzyme was studied:-

10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} 10^{-2} M.

The results are shown in Figure 8.

Measurement of the activity of (Na^+, K^+) ATPase and effect of ouabain

The erythrocyte membranes were stored, frozen at -23°C, for a maximum of seven days.

The ghost membranes were thawed at 10°C and then stored on ice for 30 minutes prior to assay. From the results of the above experiments, assays were carried out under what were considered optimal conditions:

The incubation mixture consisted of 1 mM NaCl, 1 mM $MgCl_2$,

Figure 6 · Effect of increasing incubation time on the activity of (Na^+, K^+) ATPase

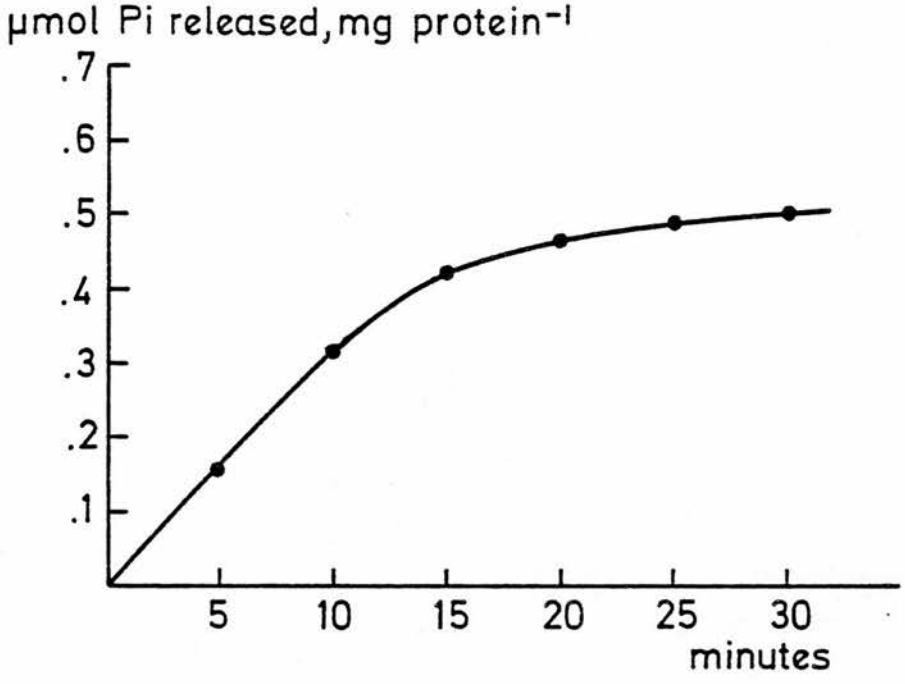


Figure 7 Effect of freezing and storage on the activity of (Na^+, K^+) ATPase

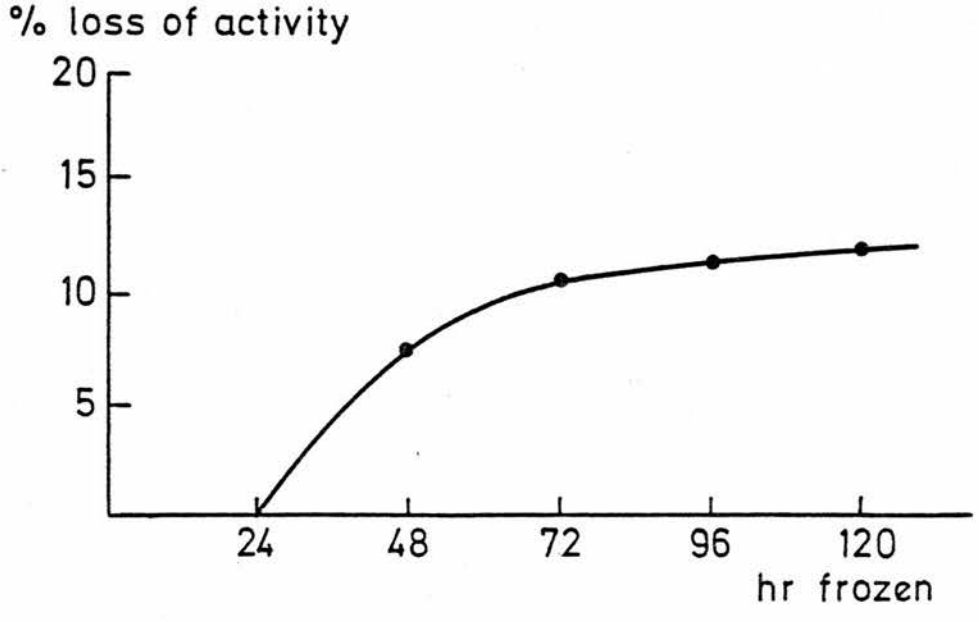
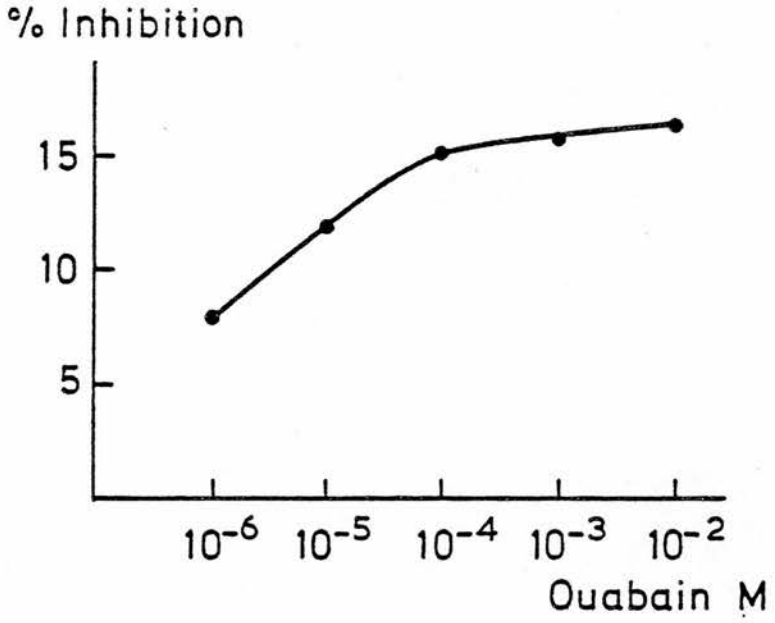


Figure 8 Effect of increasing concentration of ouabain on the activity of (Na^+, K^+) ATPase



2 mM KCl, 4 mM Na_2 ATP in 0.1 M Tris (pH 7.4) and a suspension of erythrocyte ghost membrane equivalent to 250 to 350 µg protein, in a final volume of 1 ml. After incubation at $37^{o}C$ for 15 minutes, protein was precipitated by adding 0.2 ml 50% TCA. The inorganic phosphate released was measured by the method of Fiske et al., (1925) as outlined earlier.

The enzyme activity determinations were measured in duplicate, and in two series, one with freshly prepared ouabain added to a final concentration of 10^{-4} M, the other without.

An enzyme blank was assayed with each sample, with 50% TCA added prior to the incubation.

The enzymic activity was expressed in µmoles inorganic phosphate released per mg ghost membrane protein per hour (µmoles Pi, mg protein^{-1}, hr^{-1}).

The effect of ouabain (10^{-4} M) on the enzyme was expressed as a percentage of the difference between the activity without and with the addition of the cardiac glycoside:-

$$\% \text{ effect} = \frac{(\text{activity - ouabain}) - (\text{activity + ouabain})}{(\text{activity - ouabain})} \times 100\%$$

Coefficient of Variation

100 ml of venous blood was donated by one control on a particular day, collected into 10 separate heparinised containers, and the ghost membranes prepared from each of the packed cell volumes as described previously. The activity of the (Na^+, K^+) ATPase enzyme, and the effect of ouabain was determined, and the 10 separate results compared. The coefficient of variation (SD/mean x 100) was 3.6%. The coefficient of variation of samples obtained on different days from the same individual was 4.8%.

Results

DMD patients

Individual (Na^+, K^+) ATPase activities with SCK levels and ages, are shown in Table 8 for controls and Table 9 for patients. There was no correlation between the level of (Na^+, K^+) ATPase activity and age, SCK or percent effect of ouabain, nor between the percent effect of ouabain and age or SCK level.

The basal activities are shown in the form of a histogram in Figure 9. The distribution was normal.

The basal activity of (Na^+, K^+) ATPase from 20 clinically confirmed cases of DMD was significantly lower ($p < 0.01$) than that from 13 age and sex matched controls (Table 10). Without exception, the addition of ouabain resulted in stimulation of enzymic activity in the samples from DMD patients, and inhibition in the controls.

Female relatives compared with controls

The female relatives were divided into 4 groups as described previously:-

1) Definite and probable carriers
2) Possible carriers I
3) Possible carriers II
4) Other female relatives

The individual basal activities of the ghost membrane (Na^+, K^+) ATPase are expressed in Table 11 for female controls and Table 12 for carriers as well as the response of the enzyme to ouabain. The distribution of enzymic activities are shown in Figure 10. Ouabain uniformally inhibited the (Na^+, K^+) ATPase in controls. In carriers

Table 8 Individual values of (Na^+, K^+) ATPase activity (μmoles Pi, mg protein^{-1}, hr^{-1}), and response to ouabain in age and sex matched controls.

Age years	SCK IU/l	ATPase -ouabain	ATPase +ouabain	% effect of ouabain
7	98	1.30	1.20	- 7.7
11	49	1.13	1.04	- 8.0
12	48	1.28	1.20	- 6.3
13	51	1.52	1.29	-15.1
13	32	1.66	1.50	- 9.6
14	58	1.57	1.21	-22.9
14	36	1.06	0.98	- 7.5
15	56	1.58	1.37	-13.3
15	69	1.86	1.75	- 5.9
18	50	1.40	1.32	- 5.7
18	64	1.30	1.19	- 8.5
19	66	1.35	1.22	- 9.6
20	60	1.50	1.20	-20.0

Table 9 Individual values of (Na^+, K^+) ATPase activity (μmoles Pi, mg protein^{-1}, hr^{-1}), and response to ouabain in patients with confirmed DMD.

Family No.	Age years	SCK IU/l	ATPase -ouabain	ATPase +ouabain	% effect of ouabain
1. II 3	7	6 000	1.17	1.35	+15.4
2. III 2	6	18 273	1.06	1.18	+11.3
3. II 2	18	309	1.00	1.14	+14.0
3. II 4	14	2 310	1.01	1.08	+ 6.9
3. II 6	11	2 900	1.07	1.18	+10.3
4. II 4	10	2 505	0.70	0.82	+17.1
6. II 6	11	3 540	0.68	0.73	+ 7.4
8. III 1	11	11 540	1.33	1.58	+18.8
11. II 2	16	1 020	1.47	1.69	+15.0
12. III 7	12	1 180	1.10	1.23	+11.8
15. II 3	19	290	1.04	1.25	+20.2
16. IV 1	6	3 640	0.87	0.91	+ 4.6
17. II 1	7	11 129	1.33	1.39	+ 4.5
18. III 4	12	1 320	1.18	1.46	+23.7
19. III 2	12	3 159	0.86	1.01	+17.4
20. III 1	12	1 312	0.88	1.01	+14.8
21. II 1	14	2 430	0.89	1.01	+13.5
22. III 3	12	3 400	0.95	1.02	+ 7.4
24. II 2	11	1 180	1.03	1.16	+12.6
25. II 1	17	580	0.94	1.02	+ 8.5

Figure 9a Histogram of (Na^+, K^+) ATPase activity in DMD patients

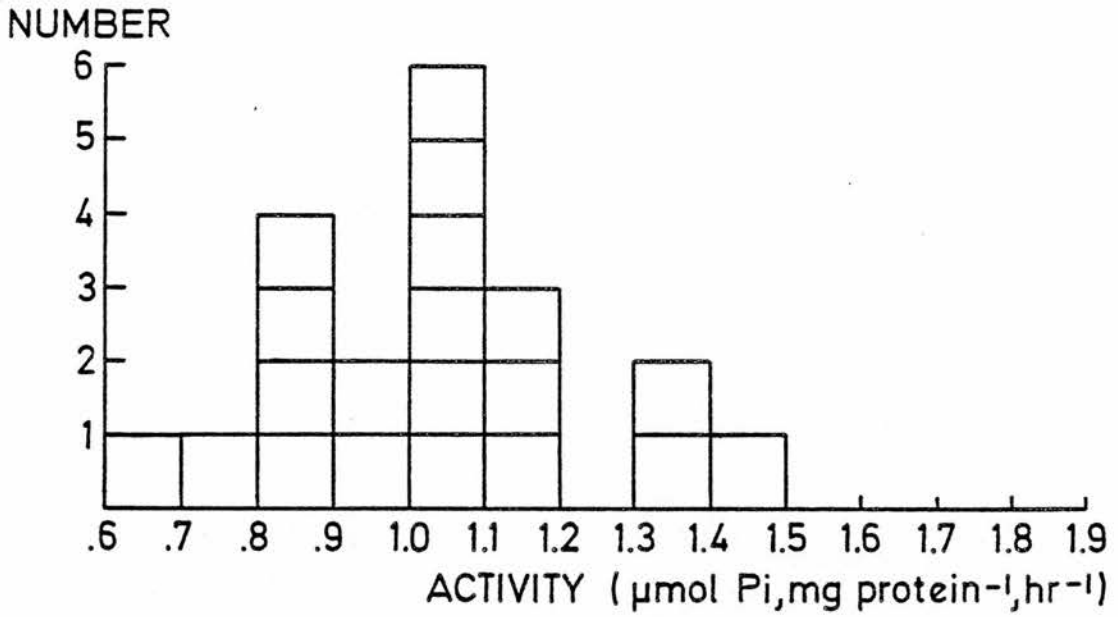


Figure 9b Histogram of (Na^+, K^+) ATPase activity in male controls

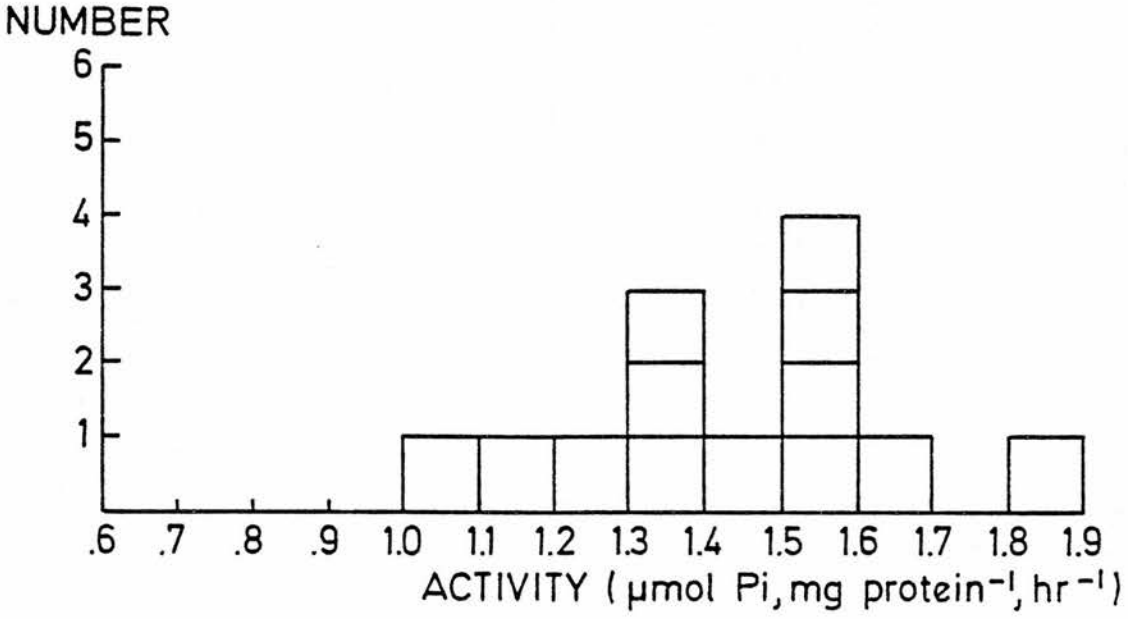


Table 10 (Na^+, K^+) ATPase activity (μmoles Pi mg protein^{-1}, hr^{-1}) and response to ouabain of erythrocyte ghost membranes from patients with DMD, compared with age and sex matched controls

	Age		ATPase -ouabain		ATPase + ouabain		% Effect	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DMD (n = 20)	11.9	3.73	1.03	0.201	1.16	0.243	+12.8	5.25
Controls (n = 13)	14.5	3.60	1.42	0.220	1.27	0.196	-10.8	5.50
Significance of difference			$p < 0.001$		N.S.		$p < 0.001$	

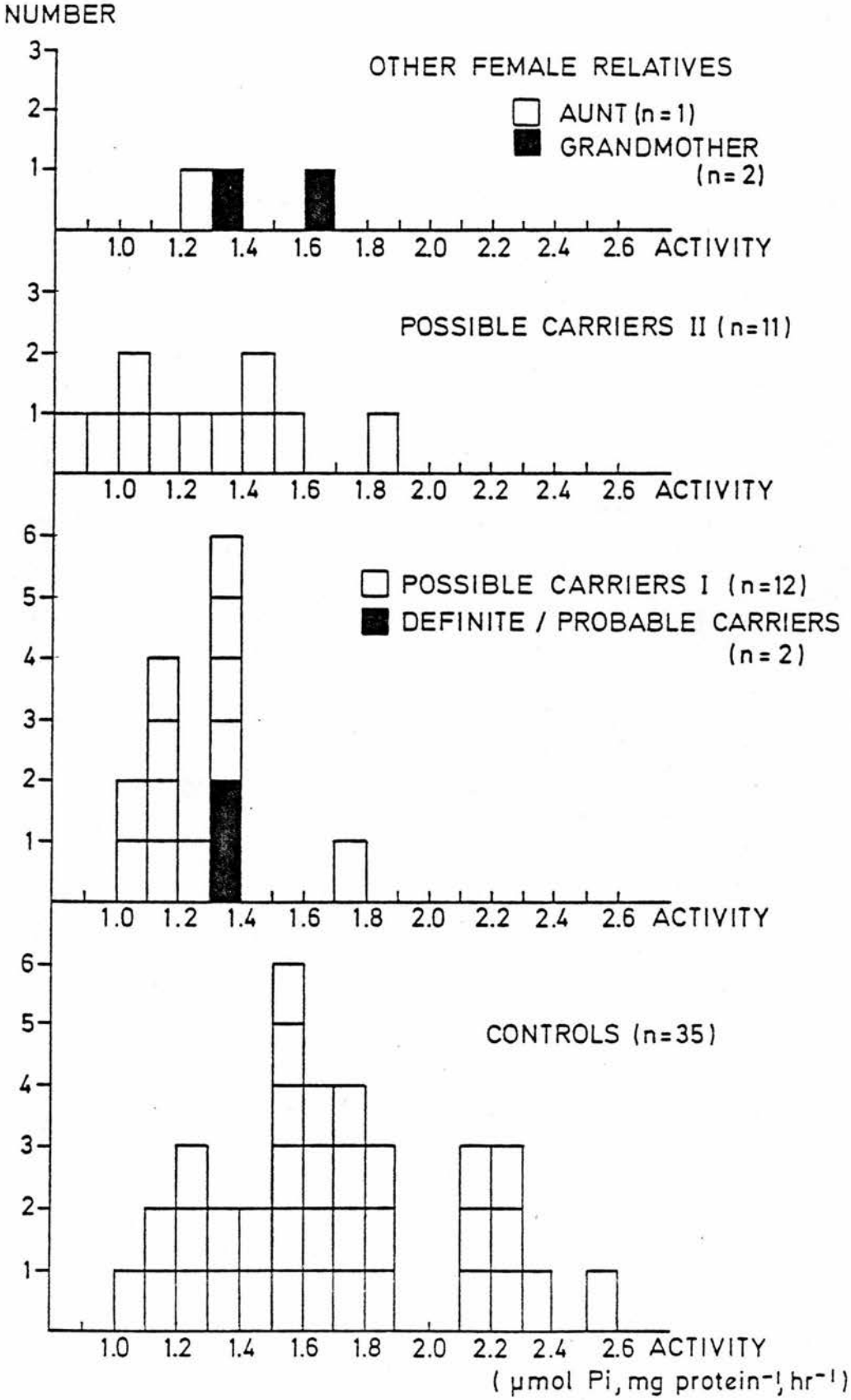
Table 11 Individual values of (Na^+, K^+) ATPase activity (μmoles Pi, mg protein^{-1}, hr^{-1}) and response to ouabain, in female controls.

Age years	SCK IU/l	ATPase -ouabain	ATPase +ouabain	% effect of ouabain
23	34	1.18	1.07	- 9.3
18	45	1.56	1.38	-11.5
17	47	2.26	2.11	- 6.6
27	79	2.55	2.19	-14.1
23	52	2.28	2.02	-11.4
17	35	2.29	1.99	-13.1
62	39	1.88	1.79	- 4.8
44	65	1.63	1.36	-16.6
22	55	1.57	1.43	- 8.9
20	39	1.51	1.32	-12.6
38	34	2.15	2.02	- 6.1
26	47	1.40	1.14	-18.6
49	64	2.10	1.86	-11.4
33	53	1.68	1.27	-24.4
46	52	1.89	1.44	-23.8
35	55	1.08	0.76	-29.6
54	45	1.37	1.19	-13.1
49	53	1.60	1.33	-16.9
23	42	1.39	1.15	-17.3
45	30	1.56	1.30	-16.7
45	52	1.77	1.58	-10.7
44	39	1.21	0.86	-28.9
44	39	1.76	1.19	-32.4
55	78	1.85	1.53	-17.3
57	60	1.26	1.11	-11.9
28	57	2.31	1.79	-22.5
19	50	1.74	1.51	-13.2
51	31	1.65	1.46	-11.5
21	72	1.47	1.28	-12.9
27	46	1.58	1.38	-12.7
25	40	1.28	1.01	-21.1
22	88	1.51	1.36	- 9.9
53	86	1.74	1.62	- 6.9
31	48	1.13	1.01	-10.6
17	75	2.15	1.85	-14.0

Table 12 Individual values of (Na^+, K^+) ATPase activity (μmoles Pi, mg protein^{-1} hr^{-1}) and response to ouabain in carriers of DMD and other female relatives

	Age years	SCK IU/l	ATPase -ouabain	ATPase +ouabain	% Effect of ouabain
Controls (range)	17 - 62	30 - 88	1.08 - 2.55	0.76 - 2.19	-4.8 to -32.4
Definite/Probable Carriers					
3. I_2	48	344.0	1.38	1.36	- 1.4
15. I_2	48	272.0	1.36	1.23	- 9.6
Possible Carriers I					
1. I_2	32	41.0	1.14	0.89	-21.9
2. II_2	29	56.0	1.17	0.93	-20.5
5. I_2	41	36.0	1.29	1.19	- 7.8
6. I_2	39	82.0	1.09	1.00	- 8.3
8. II_3	30	37.0	1.11	0.90	-18.9
11. I_2	47	38.0	1.37	1.37	0
12. II_4	37	53.0	1.78	1.70	- 4.5
14. I_2	50	60.0	1.33	1.32	- 0.8
16. III_1	31	90.0	1.35	1.41	+ 4.4
17. I_2	31	59.0	1.08	1.05	- 2.8
18. II_2	51	192.0	1.34	1.25	- 6.7
23. I_2	42	47.0	1.17	1.07	- 8.5
Possible Carriers II					
6. II_2	17	50.0	1.03	0.98	- 4.9
6. II_3	15	88.5	0.89	1.02	+14.6
6. II_4	14	53.0	0.99	1.01	+ 2.0
7. II_2	24	642.0	1.01	0.92	- 8.9
9. II_7	26	46.0	1.43	1.57	+ 9.8
12. III_5	16	95.5	1.23	1.60	+30.1
13. II_1	18	840.0	1.83	2.07	+13.1
14. II_2	20	20.0	1.46	1.32	- 9.6
15. II_1	23	355.0	1.57	1.73	+10.2
17. II_3	3	58.0	1.16	1.13	- 2.6
18. III_2	24	34.0	1.35	1.26	- 6.7
Other Female Relatives					
10. II_7	30	39.5	1.29	1.24	- 3.9
16. II_2	67	368.0	1.37	1.43	+ 4.4
12. I_2	77	148.5	1.68	1.46	-13.1

Figure 10 Histogram of (Na^+, K^+) ATPase activity in carriers of DMD and female controls



sisters and other female relatives however, the responses were less consistent. Some preparations were inhibited as normal, whilst others were inhibited less and stimulation occurred in several cases (Table 12).

There was no correlation between (Na^+, K^+) ATPase activity and SCK, age or % effect of ouabain, nor was the effect of ouabain correlated with SCK or age. Of the two definite carriers, one had an abnormal response to ouabain below the normal range, the other was normal. Five out of the 12 possible carriers had abnormal responses to ouabain, either less inhibition or stimulation. Seven out of the 11 sisters exhibited abnormal responses as did two of the other female relatives.

However, there was no obvious relationship between (Na^+, K^+) ATPase activity and response to ouabain with respect to carrier status.

Other Muscle Diseases

The (Na^+, K^+) ATPase activity of erythrocyte ghost membranes from patients with a variety of other muscle diseases was consistently inhibited by ouabain. The individual values are shown in Table 14.

Table 13 (Na^+, K^+) ATPase activity (umoles Pi, mg $protein^{-1}$, hr^{-1}) and the response to ouabain in age and sex matched controls, in carriers of DMD and other female relatives.

	AGE years		SCK IU/l		ATPase -ouabain		ATPase +ouabain		% Effect of ouabain	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Controls (n = 35)	34.6	13.93	52.2	15.52	1.70	0.378	1.45	0.363	-15.0	6.73
Definite/Probable Carriers (n = 2)	48.0		344.0		1.38		1.36		- 1.4	
	48.0		272.0		1.36		1.23		- 9.6	
Possible Carriers I (n = 12)	38.3	7.97	65.9	43.25	1.27 ***	0.195	1.17 *	0.247	- 8.0	8.42
Possible Carriers II (n = 11)	17.5 ***	6.77	207.5 ***	282.78	1.27 ***	0.290	1.33	0.371	+ 4.3 ***	12.41
Other Female Relatives (n = 3)	67.0	368.0			1.37		1.43		+ 4.4	
	77.0	148.5			1.68		1.46		-13.1	
	30.0	39.5			1.29		1.24		- 3.9	

NB * Significant at $p < 0.05$ when compared with controls
** $p < 0.01$
*** $p < 0.001$

Table 14 Individual values of (Na^+, K^+) ATPase activity (μmoles Pi, mg protein^{-1}, hr^{-1}) and response to ouabain of erythrocyte ghost membranes from patients suffering from a variety of muscle diseases other than DMD.

Male	Age years	SCK IU/l	ATPase -ouabain	ATPase +ouabain	% Effect of ouabain
Myotonic dystrophy	45	55	1.54	1.40	- 9.1
Periodic paralysis	18	198	0.97	0.78	-19.6
Facioscapulohumeral muscular dystrophy	26	218	1.09	1.01	- 7.3
Becker muscular dystrophy	33	533	1.01	0.88	-12.9
Limb-girdle muscular dystrophy	38	1 260	1.43	1.34	-20.9
Peroneal muscular atrophy	26	510	1.30	1.12	-13.8
Controls range (n = 4)	21 - 40	43 - 75	1.50 - 2.12	1.45 - 1.92	-6.3 to -15.1
Female					
Myotonic dystrophy	27	97.5	0.90	0.83	- 7.8
Facioscapulohumeral muscular dystrophy	42		1.46	1.32	- 9.6
Controls (n = 35) mean SD	34.6 13.93	52.2 15.52	1.70 0.378	1.45 0.363	-15.0 6.73

Incubation of plasma and ghost membranes

These experiments were designed to investigate the effects of plasma incubation on ghost membranes (Na^+, K^+) ATPase determined by the effect of ouabain on the enzyme.

Materials and Methods

1 ml of ghost membrane was incubated with 1 ml of plasma for 2 hours at 37°C. 40 ml of lysis buffer was added and the suspension centrifuged at 10 000 g for 30 minutes. The sedimented ghost membranes were resuspended in 1 ml of lysis buffer and stored at -23°C prior to ATPase assay.

Both stored and unstored plasma was incubated with stored and unstored ghost membranes (-70°C and -23°C respectively), and there was no detectable effect of storage. All samples were stored for a maximum of seven days prior to examination, except those plasma samples used as controls for the deproteination and dialysis experiments. These samples were fresh (within 2 hours of collection) and stored at 4°C whilst the other samples were prepared.

Dialysis of plasma samples

Fresh plasma was used (within 2 hours of collection) in this preparation. 1 ml of plasma was dialysed against 5 litres of distilled deionised water at 4°C overnight. Then the dialysis bag containing the sample was totally submerged in 20% polyetheleneglycol at 4°C, thereby reducing the volume of the sample. After 2 hours when the volume was slightly less than 1 ml the sample was removed, made up to 1 ml with deionised distilled water, and incubated with ghost membranes as previously described.

Deproteination of plasma samples

Fresh plasma was used (within 2 hours of collection) in this procedure. 3 ml of plasma was deproteinated in such a way that the final filtrate contained proteins of molecular weight $< 10\ 000$. This was achieved with two stages of ultrafiltration.

Firstly, proteins of m. wt. $> 25\ 000$ were removed by centrifugation at 1 500 g through an ultrafiltration cone filter, at $4^{o}C$. The second filtration was through a millipore cellulose membrane, pore size 0.005 μm, at $4^{o}C$ under 7 bar pressure with gaseous nitrogen.

The final filtrate was incubated with ghost membranes as previously described.

Effects of plasma incubation on the response of ghost membrane (Na^{+}, K^{+}) ATPase to ouabain

Ghost membranes were prepared from one healthy donor, and incubated with plasma samples from other controls and DMD patients. The effect of ouabain on the (Na^{+}, K^{+}) enzyme was then measured. These results are shown in Table 15. There was consistent ouabain inhibition of the (Na^{+}, K^{+}) ATPase enzyme in ghosts that had been incubated in control plasma (-7.4% $\pm$ 3.49) whereas enzyme stimulation was apparent in the membranes incubated in DMD plasma (+8.0% $\pm$ 2.99).

The abnormal response of DMD (Na^{+}, K^{+}) ATPase to ouabain was partially inhibited by incubation of DMD ghost membranes in control plasma (Table 16). The mean stimulation prior to incubation (+13.5 $\pm$ 2.98) had been reduced (+1.8 $\pm$ 1.77).

There was no obvious effect of DMD plasma incubation on other DMD ghost membranes (Table 17).

Table 15 Effect of ouabain on control ghost membrane (Na^+, K^+) ATPase after incubation with plasma from DMD patients and other controls.

% effect after incubation in DMD plasma (n = 7)	% effect after incubation in control plasma (n = 11)
+ 7.1	- 8.7
+ 5.8	- 9.0
+ 6.3	- 3.8
+ 4.8	- 6.5
+ 9.4	- 3.8
+ 8.8	- 3.7
+13.7	- 9.9
	-15.5
	- 8.3
	- 5.3
	- 6.8
MEAN SD	MEAN SD
+8.0 2.99	-7.4 3.49

Table 16 Percent effect of ouabain (10^{-4}M) on DMD ghost membrane (Na^+, K^+) ATPase (n = 8) after incubation in control plasma (n = 1).

+0.2	
+0.5	
+0.0	
+0.9	
+4.8	
+3.1	
+3.5	
+1.7	
MEAN	SD
+1.8	1.77

Table 17 Percent effect of ouabain (10^{-4}M) on DMD ghost membrane (Na^+, K^+) ATPase (n = 7) after incubation with DMD plasma samples (n = 7).

+ 5.3	
+ 2.9	
+ 3.3	
+11.5	
+13.8	
+11.6	
+ 6.7	
MEAN	SD
+7.9	4.39

The effects of dialysis and deproteination of the DMD plasma samples prior to incubation with the donor ghost membranes are shown in Table 18. These results indicate a partial loss of effect.

Ca^{2+}, Mg^{2+}, and total protein concentrations of plasma

The concentrations of Ca^{2+} and Mg^{2+} ions and total protein were measured in patients with DMD and controls. These results are expressed in Table 19. The individual values are shown in Table 20, from which it will be seen that there was no significant difference in the amounts of any of these between controls and patients.

Table 18 Effects of ouabain (10^{-4}M) on (Na^+, K^+) ATPase activity in ghost membranes from a control incubated with plasma samples from patients with DMD (n = 8), and after dialysis (n = 8) and deproteination (n = 3) of DMD plasma. Results expressed as percent effect on activity of exposure to ouabain.

DMD plasma		Dialysed DMD plasma		Deproteinated DMD plasma	
+18.6		- 7.0			
+ 7.0		-11.9			
+ 9.3		- 8.8			
+ 4.0		- 4.7			
+ 8.1		- 8.2			
+ 8.2		-16.1		- 6.5	
+11.3		-11.7		- 7.8	
+ 6.6		-13.5		-11.5	
MEAN	SD	MEAN	SD	MEAN	SD
+9.1	4.37	-10.2	3.73	-8.6	2.59

Table 19 Ca^{2+}, Mg^{2+} and total protein levels in plasma from DMD patients and controls.

	Ca mmoles/l		Mg mmoles/l		Protein g/l	
	Mean	SD	Mean	SD	Mean	SD
DMD	2.40	0.848	0.87	0.301	77.2	16.18
	(n = 13)		(n = 13)		(n = 10)	
Controls	2.38	0.487	0.84	0.128	73.3	12.81
	(n = 11)		(n = 11)		(n = 11)	

Table 20 Individual values of Ca^{2+}, Mg^{2+} and total protein concentrations in plasma samples from DMD patients.

DMD patient	Ca (mmol/l) (n = 13)	Mg (mmol/l) (n = 13)	Protein (g/l) (n = 10)
1. II_3	2.60	0.91	77.0
2. III_2	2.91	1.03	87.0
4. II_4	2.28	0.86	75.0
11. II_2	2.40	0.80	75.0
12. III_7	2.41	0.86	-
16. IV_1	2.47	0.84	-
17. II_1	1.86	0.68	70.0
18. III_4	2.51	0.93	76.0
19. III_2	2.28	0.88	75.0
21. II_1	2.28	0.85	-
22. III_3	2.48	0.95	85.0
24. II_2	2.34	0.81	74.0
25. II_1	2.42	0.88	78.0
	MEAN SD	MEAN SD	MEAN SD
	2.40 0.848	0.87 0.301	77.2 16.18
Control Range (n = 11)	2.18 — 2.69	0.77 — 0.89	68.0 — 79.0

SDS - polyacrylamide gel electrophoresis

The effects of plasma incubation on the ghost membrane proteins were studied using the technique of gel electrophoresis.

The method of gel preparation and run are outlined in Appendix II (5% acrylamide with 0.2% SDS). The gels were photographed with a Pentax Spotmatic camera, fitted with a 55 mm, f1.8 lens. The gels were underlit, the aperture was f11, exposure time 1.0 second, using Kodalith orthofilm type III, and an orange filter.

The protein profiles are shown in Plate 2. There were no detectable differences, as investigated by a densitometric scan, between the protein profiles of the DMD ghost membranes compared with those of the control. Plasma incubation resulted in a marked alteration in these protein profiles. However, there were no detectable differences between the patterns which could be related to the source of the ghost membranes or plasma samples.

Plate 2 SDS-PAGE protein profiles of ghost membranes from DMD patient (A), control (B), DMD ghosts incubated in DMD plasma (C), DMD ghosts incubated in control plasma (D), control ghosts incubated in DMD plasma (E) and control ghosts incubated in control plasma (F).

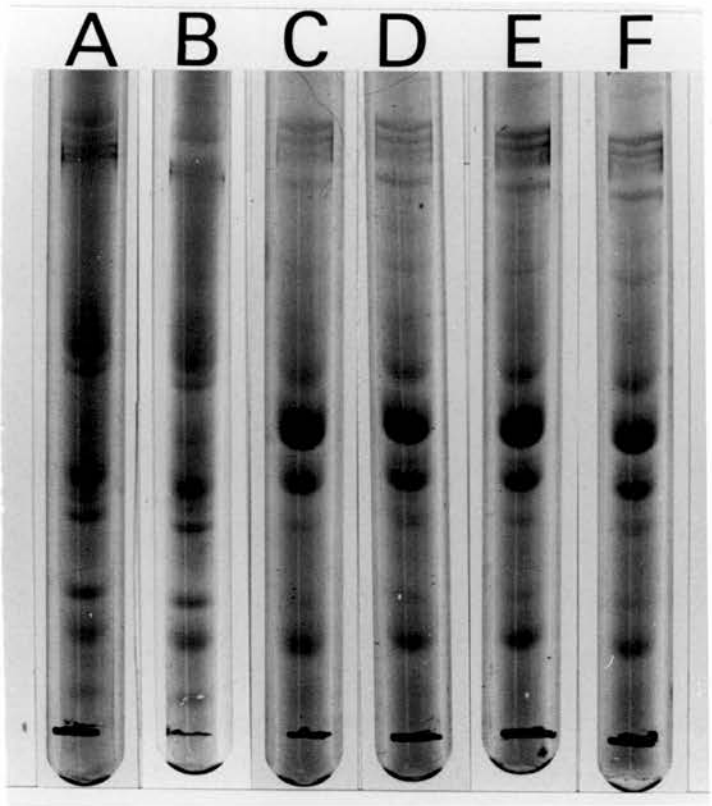


Table 21 Published values of (Na^+, K^+) ATPase activity, (expressed as mean, SD μmoles Pi, mg protein^{-1}, hr^{-1}), in controls and DMD patients, and % effect of ouabain in DMD.

Authors	Control Mean	Control SD	DMD Mean	DMD SD	% Effect of Ouabain on DMD Mean	% Effect of Ouabain on DMD SD
Brown et al., (1967) 'low salt'	25.1 (n = 7)	14.50	18.2 (n = 8)	6.99	+ 21.3	48.10
Peter et al., (1969) 'low salt'	0.85 (n = 6)	0.367	0.37 (n = 9)	0.162	+ 12.0	5.22
Klassen & Blostein (1969) 'high salt'	1.52 (n = 8)	0.936	1.70 (n = 7)	0.804	- 33.6	12.84
Araki & Mawatari (1971)					+ 18.5 (n = 6)	
Niebroj-Dobosz (1976) 'low salt'	1.41 (n = 10)	0.455	0.86 (n = 10)	0.668	+200.2	130.0
Mawatari et al., (1976) 'low salt'	2.45 (n = 12)	0.727	1.83 (n = 10)	0.759	- 6.4	3.7
Siddiqui & Pennington (1977) 'low salt'	0.91 (n = 10)	-	1.00 (n = 10)	-	+ 10.1	8.2
Hodson & Pleasure (1978) 'low salt'	0.20 (n = 11)	0.099	0.23 (n = 11)	0.099	-100.0	-
Pearson (1978) 'high salt'	15.0 (n = 7)	6.35	9.0 (n = 7)	6.35	+ 26.5	13.23
Wacholtz et al., (1979) 'low salt'	0.25 (n = 12)	0.014	0.23 (n = 12)	0.034	- 55.0	-
Ruitenbeek (1979)	0.20 (n = 5)	0.07	0.21 (n = 5)	0.04	- 63.0	22.0
Present study	1.42 (n = 13)	0.220	1.03 (n = 20)	0.201	+ 12.8	5.25

Klassen and Blostein (1969) and Pearson (1978) used a high salt medium, with at least 50 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, in 50 mM Tris HCl. Several authors (Niebroj-Dobosz, 1976; Mawatari et al., 1976; Siddiqui and Pennington, 1977; Wacholtz et al., 1979) have measured the (Na^+, K^+) ATPase activity under both salt conditions. Niebroj-Dobosz (1976) and Siddiqui and Pennington (1977) found a significantly higher ($p < 0.01$) activity under the 'high salt' conditions. Mawatari et al., (1976) reported a lower activity ($p < 0.01$) in the 'high salt' medium, and Wacholtz et al., (1979) found no difference.

Under the assay conditions employed the activity of the ouabain sensitive (Na^+, K^+) ATPase was not the only enzyme measured, Mg^{2+} ATPase activity would also be registered. By omitting K ions from the incubation media, the activity due to Mg^{2+} ATPase alone can be assayed. By subtracting the activities with and without K^+, the activity due to (Na^+, K^+) ATPase can be calculated. Hodson and Pleasure (1978) and Pearson (1979) used this method to measure the (Na^+, K^+) ATPase activity and its response to ouabain. Their published results differ, in basal activity of control samples, 75 fold.

Some investigators included sucrose (0.25 M) in the incubation mixture, which is believed to contain trace amounts of calcium. In this case, the activity of the measured enzyme should rise, as the Ca^{2+} ATPase will be activated, but there should be no effect on the response to ouabain. There is no obvious correlation between sucrose addition and greater enzymic activity. Incubation times also vary, from 10 minutes to 60 minutes, during which time the kinetics of the enzyme will change. However, the differences in sucrose and metal ion concentrations on the incubation media,

and assay time, are not sufficient to explain the wide variation in published values of activity of the (Na^+, K^+) ATPase enzyme.

The variability is more probably due to the different methods of ghost membrane preparation. Hanahan et al., (1973) and Hanahan and Ekholm (1978) have investigated (Na^+, K^+) ATPase and Ca^{2+} ATPase activities of ghost membranes prepared by a variety of techniques. Their results have illustrated the importance of different lytic conditions on ghost membrane preparations for the exposure and recovery of ATPase activity. The majority of investigators who reported an abnormal response to ouabain, prepared the ghost membranes by lysis in Tris buffer, concentrations ranging from 1 mM to 10 mM, pH 7.4, usually with the addition of EDTA. Subsequent washings were in the same buffer with NaCl added. The basal activities measured under this basic method of preparation, however, vary from 0.85 to 25.1 μmoles Pi, mg protein^{-1}, hr^{-1}.

Klassen and Blostein (1969) lysed the erythrocytes in distilled water, and washed the ghosts in Tris HCl, pH 7.4, but without the addition of NaCl. Despite the use of the same preparative technique, the basal activity of the enzyme from control ghosts reported by Klassen and Blostein in 1969 was three times greater than that published by Blostein in 1968.

Ruitenbeek (1979) used 172 mM Tris, pH 7.6, and also excluded NaCl from the buffer for subsequent washings of the ghost membranes. Hodson and Pleasure (1978) haemolysed the erythrocytes in NaCl solutions of varying isotonic strengths in Tris maleate buffer, pH 7.6.

Wacholtz et al., (1979) reported preparing ghost membranes by the different methods of Brown et al., (1967) and Blostein (1968). The enzymic activity of each preparation was measured by both assay techniques. In all cases the response to ouabain was normal.

However, the Blostein method resulted in a normal ghost membrane (Na^+, K^+) ATPase activity of 0.464 μmoles Pi, mg protein^{-1}, hr^{-1}, whereas Klassen and Blostein (1969) reported activity of 1.52 μmoles Pi, mg protein^{-1}, hr^{-1}. The activity of the Brown procedure was 0.253 μmoles Pi, mg protein^{-1}, hr^{-1}, whilst Brown et al., (1967) published their basal activity as 25.1 μmoles Pi, mg protein^{-1}, hr^{-1}.

The mechanism by which ouabain inhibits (Na^+, K^+) ATPase is unknown. Albers et al., (1968) suggested a model for (Na^+, K^+) ATPase action and postulated that ouabain acted at the dephosphorylation stage of the K^+ bound enzyme. The cardiac glycoside was believed to lower the free energy difference between the phosphorylated and dephosphorylated forms of the enzyme, or induce a change in the conformational potential of the system. Siegel et al., (1969) postulated that the enzyme - ouabain complex had a higher stability than the enzyme alone in either of its phosphorylated or dephosphorylated forms. By binding with the (Na^+, K^+) ATPase and reducing the energy difference, ouabain would inhibit the transport of Na^+ and K^+ across the membrane.

The 'local' concentrations of Na^+ and K^+ have an effect on ouabain binding. Increases in Na^+ concentration directly resulted in an increased rate of ouabain binding, whilst K ions were clearly antagonistic to the formation of the cardiac enzyme complex, (Gardner and Conlon, 1972). These authors suggested that there were a finite number of receptors in the cell surface, each composed of a glycoside binding site and a cation binding site. Na^+ and K^+ competed for the cation binding site, thereby affecting the ouabain affinity. Lindemayer and Schwartz (1973) also considered that Na and K ions competed for one site. However, Hobbs and Dunham (1978) concluded that there were two sites at which the alkali metal ions

could exert effects on ouabain binding. External Na ions facilitated ouabain binding, whatever the concentration of K^+, but the kinetics were different from inhibition of the K^+ pump by Na ions alone. K, Li and Cs ions, in the presence of Na^+, decreased ouabain binding. However, in the total absence of Na^+, only K^+ inhibited ouabain complexing with the enzyme, both Li^+ and Cs^+ promoted glycoside binding. The authors suggested one ouabain binding site was the loading area from which K ions were translocated inward, and the other site was that from which Na ions were discharged after outward translocation.

Lishko et al., (1972) reported that Pi increased the rate of ouabain binding in the presence of Mg^{2+} and that this effect was antagonised in the presence of K ions. Van Winkle et al., (1972) suggested that this ouabain - enzyme complex formed in the presence of Pi and Mg^{2+} may represent a different conformation than that formed in the presence of ATP, Na^+ and Mg^{2+}. This may have been due to different levels of enzyme phosphorylation, or enzyme conformation prior to binding.

Taniguchi and Iida, in 1972, reported that there were two related sites for the binding of ouabain, one with a higher affinity for the glycoside than the other. This was ascertained by varying the concentrations of ouabain and studying the effects on the (Na^+, K^+) ATPase enzyme. Further evidence was published by the same investigators in 1973, when they found that phospholipase A differentially affected the high affinity site, where the ouabain-enzyme complex was induced by the presence of Mg^{2+} and Pi. Heller and Beck (1978) confirmed these findings, and described two ouabain binding sites. Type I was detected at very low concentrations of ouabain (10^{-9} M), was not easily saturated (concentrations greater

than 10^{-3} M necessary) and was suggested to be the K^+ insensitive site. Type II required the inclusion of Mg^{2+} and Pi to form complexes with ouabain, and was saturated at 10^{-7} M ouabain, and was reported to be the K^+ sensitive site of the intact cell.

This recent evidence suggests that there are two sites at which ouabain complexes with the (Na^+, K^+) ATPase enzyme. At one of the sites, ouabain binding is facilitated by Na^+, and requires ATP and Mg^{2+}, whilst at the other site Mg^{2+} and Pi are prerequisites. Binding at this second site is antagonised by K ions.

Albers et al., (1968) reported a very slow rate of ouabain combination with (Na^+, K^+) ATPase. This binding is controlled not only by the conformation of the enzyme but also the balance of Pi to ATP, Na to K ions, and the concentrations of Mg^{2+} and Ca^{2+} present. The action of these three effectors could be explained in model terms, by loosening or tightening some form of steric barrier which would alter the rate of ouabain binding. However, once formed, the ouabain enzyme complex is very stable. This binding induces a conformational change that the uncomplexed enzyme never exhibits. Farley (1979) investigated the action of chymotrypsin and trypsin on (Na^+, K^+) ATPase before and after ouabain binding. No cleavage products were found before or after incubation with ouabain with trypsin. However, chymotrypsin treatment, subsequent to ouabain binding, resulted in the cleavage of the glycoprotein associated with the (Na^+, K^+) ATPase enzyme. One of the catalytic subunits was also altered in the presence of ouabain. The chymotrypsin treatment resulted in the further cleavage of one of the fragments which was produced in the absence of the glycoside. It can be concluded that ouabain binding resulted in a cleavage site previously hidden, becoming accessible to protease action.

Zachowski et al., (1977) reported that a protein or proteins (m. wt. 30 000) on the inside of the plasma membrane may exert some effect on the accessibility of the (Na^+, K^+) ATPase to ouabain action. Ouabain does not usually have access to the inner surface of the membrane in vivo. However, with the technique of resealing ghost membranes, it is possible to prepare 'inside out' vesicles, where the previously inaccessible inside membrane becomes the outer face of a resealed vesicle. EDTA treatment, which elutes 35% of the total membrane proteins, resulted in the 'inside out' vesicles becoming 350 times more sensitive to the action of ouabain. There was no change in the 'right side out' vesicles. Addition of the eluted proteins to the stripped 'inside out' vesicles resulted in restoration of the original resistance to ouabain. The proteins which were detached from the inside of the membrane were probably distinct from the (Na^+, K^+) ATPase but may still have exerted some effect on the enzyme, perhaps in the form of a steric barrier.

Erdmann and Hasse in 1975, studied the quantitative aspects of ouabain binding to human erythrocyte membranes. They reported the binding was a time and temperature dependant process, and speculated that there were in the region of 200 - 256 receptor sites per erythrocyte. If there was equal distribution, this would mean each site occupied less than 1 μm^2 of surface membrane area. However, Joiner and Lauf (1978) used whole erythrocytes and calculated between 450 and 500 sites per cell. This discrepancy may be due to the use of whole erythrocytes in one case and ghost membranes in the other. Preparation of the ghost membranes could have caused the inactivation of several receptor sites, but this is unlikely to account for the complete difference.

The proteins of the erythrocyte membrane can be separated, under certain conditions of gel electrophoresis, by molecular size and charge. (Na^+, K^+) ATPase migrates with band 3 proteins, as do the other enzyme systems associated with transmembrane ion transport. SDS gel electrophoresis has failed to show any major differences in the profiles of ghost membranes prepared from DMD patients compared with controls (Roses et al., 1975). This suggests that there have been no significant alterations in the structure of the membrane proteins in DMD. However, Das et al., (1976) have observed different protein profiles in triton treated ghosts from patients with DMD on slab gel electrofocusing.

The activity of the (Na^+, K^+) ATPase enzyme is dependent upon the environment in the membrane. The hydrophobic nature of the surrounding lipids, as well as the balance between the fluid and crystalline states, are of importance. The fluidity of the membrane may be an important determinant of lipid-protein interactions and may also influence protein-protein interactions, thereby affecting the function of the intermembrane transport systems. Alterations in cholesterol content and fatty acid composition of the phospholipids have been reported (Kunze et al., 1973; Howland and Iyer, 1977). However, the evidence of changes in the lipid composition of the erythrocyte membrane in DMD is contradictory (Introduction II). Although such alterations could explain the changes in basal activity, it is more difficult to interpret the abnormal response to ouabain.

A small change of amino acid sequence could result in an alteration of enzymic activity without affecting the physical characteristics of the protein. A substitution along the amino acid backbone of the protein could alter the configuration of the enzyme,

and indirectly affect the receptor and active sites. However, the substitution could occur at the receptor or active site, directly altering the ability of the enzyme to recognise and successfully bind its substrates, cofactors and inhibitors. In this way, the basal activity of (Na^+, K^+) ATPase could be altered, and the abnormal response of the enzyme to one of its inhibitors, ouabain, explained. The different configurations that the enzyme displays during its catalytic cycle may be unable to complex with ouabain, although this would fail to explain a stimulation of activity. However, ouabain may be irreversibly bound to a receptor site, inducing a local conformational change in the vicinity of the active site, and thereby make the active site more recognisable or accessible to the substrate or cofactors. The directional nature of the (Na^+, K^+) ATPase would almost certainly require movement of the enzyme within the membrane. The binding of ouabain, with a possible subsequent local configurational change, may remove some form of steric barrier to this enzymic movement.

However, the finding that incubation of normal ghost membranes in DMD plasma elicits an abnormal response of ghost (Na^+, K^+) ATPase to ouabain suggests that some fraction in DMD plasma may be responsible (Peter et al., 1969; Siddiqui and Pennington, 1977). After incubation in plasma, the profiles of the ghost membrane proteins showed an obvious shift in band 3. This was apparently the same whatever the source of ghost membranes or plasma. The partial reversal of ouabain effect on DMD ghosts by incubation in control plasma was unusual. This suggested that the membrane was capable of reverting back to a 'normal' condition once the 'factor' in the plasma was removed.

This so called 'circulating plasma factor' was rendered inactive by dialysis and deproteination of the plasma. This suggests that the 'factor' is a protein which may require ions for activation. However, there was no significant difference in the calcium or magnesium concentrations or level of total proteins in the DMD plasma.

The effect of the 'circulating plasma factor' however, does tend to rule out the possibility of an amino acid substitution in the enzyme, unless the action of the 'factor' on the membrane elicits a similar response by a different mechanism. The evidence does, on the other hand, suggest that there may be some form of steric barrier affecting normal functioning of (Na^+, K^+) ATPase of ghost membranes in DMD patients. This barrier inhibits normal activity of the enzyme in DMD under certain conditions. The binding of ouabain alters the barrier in such a way as to promote enzymic activity. The incubation in control plasma removes the barrier, but may do so by affecting the membrane as a whole, as shown by SDS gel electrophoresis. The 'circulating plasma factor' in DMD may be a protein which binds to the control membrane and thereby induces a conformational change near the enzyme. This would, in turn, elicit the abnormal response, as with the DMD ghost membranes.

The abnormal ouabain responses of control ghost membrane (Na^+, K^+) ATPase elicited by incubation in DMD plasma does not support the theory that the pathogenesis of DMD is a primary generalised membrane abnormality. Also, the partial reversal of the abnormal ouabain effect in DMD ghost membranes after incubation in control plasma suggests the membrane alteration in DMD may be secondary.

CHAPTER 6

SUMMARY

Summary

This study was designed to investigate certain properties of the erythrocyte membrane in human muscular dystrophies, particularly Duchenne muscular dystrophy, the most severe and common form.

Echinocyte and stomatocyte formation in patients with DMD was not significantly different from in controls. This suggests that if there were any alteration in the deformability of the erythrocyte membrane, the conditions of preparation in this study were not such as to induce the abnormality. However, erythrocytes from patients with DMD were found to be osmotically more fragile than those from controls. This was particularly obvious in the threshold of lysis region.

Investigations into the activity and properties of the ghost membrane (Na^+, K^+) ATPase enzyme were of most interest. In DMD patients, basal activity was lower than in controls, and the cardiac glycoside ouabain had a stimulatory effect on the enzyme, whereas in controls (Na^+, K^+) ATPase was inhibited by ouabain. However, this abnormal response to the cardiac glycoside was induced in control ghost membrane preparations by prior incubation in Duchenne plasma. This suggests that there is a possible 'circulating plasma factor' effect in DMD. Dialysis and deproteination of the Duchenne plasma resulted in partial loss of effect. After incubation of DMD ghost membranes in control plasma (Na^+, K^+) ATPase stimulation by ouabain was reduced. This suggests that if the abnormal response obtained in DMD erythrocytes is due to a plasma factor then the effect is at least partially reversible.

SDS - PAGE failed to reveal any significant differences between the protein profiles of DMD and control ghost membranes.

Incubation in control and Duchenne plasma resulted in major changes of these protein profiles, but these alterations were not significantly different between the DMD and control ghost membranes.

The results of the present investigation add evidence to the theory of a generalised membrane defect in DMD. However, the fact that there may be a 'circulatory plasma factor' in patients which can induce comparable changes in normal cell membranes, raises the possibility that some of the observed membrane changes in DMD may, in fact, be secondary to the disease process and not necessarily a reflection of a primary defect.

BIBLIOGRAPHY

Abdullah, F., and Pennington, R.J.T. (1968). Ribonuclease Activity in Normal and Dystrophic Human Muscle. Clinica Chimica Acta, 20, 365 - 371

Adornato, B.T., Corash, L., and King, W.K. (1977). Erythrocyte Survival in Duchenne Muscular Dystrophy. Neurology 27, 1093 - 1094

Adornato, B.T., Kagen, L.J., and Engel, W.K. (1978). Myoglobinaemia in Duchenne Muscular Dystrophy Patients and Carriers: A New Adjunct to Carrier Detection. Nature ii, 499 - 501.

Albers, R.W., Koval G.J., and Siegel, G.J., (1968). Studies on the Interaction of Ouabain and Other Cardioactive Steroids with Sodium - Potassium-activated adenine triphosphatase. Molecular Pharmacology, 4, 324 - 336

Alberts, M.C., and Samaha, F.J. (1974). Serum Pyruvate Kinase in Muscle Disease and Carrier States. Neurology, 24, 462 - 464

Ando, T., Shimizu, T., Kato, T., Onsawa, M., Fukuyama, Y., (1978). Myoglobinaemia in Children with Progressive Muscular Dystrophy. Clinica Chimica Acta, 85, 17 - 22

Araki, S., and Mawatari, S., (1971). Ouabain and Erythrocyte Ghost Adenosine Triphosphatase. Archives of Neurology, 24, 187 - 190

Askansas, W. (1966). Identification of the Agent Responsible for the Abnormal Immunoelectrophoretic Pattern of Serum in Duchenne's Progressive Muscular Dystrophy. Life Sciences, 5, 1767 - 1773

Askansas, W. (1966a). Immunoelectrophoretic Investigations of the Serum of Carriers of Duchenne's Progressive Muscular Dystrophy. Life Sciences, 5, 1775 - 1780

Ballantyne, J.P. and Hansen, S. (1974). New Method for the Estimation of the Number of Motor Units in a human Muscle. 2. Duchenne, Limb-girdle, Facioscapulohumeral and Myotonic Muscular Dystrophies. Journal of Neurology, Neurosurgery and Pyschiatry, 37, 1195 - 1201

Ballard, F.J., Tomas, F.M., and Stern, L.M. (1979). Increased Turnover of Muscle Contractile Proteins in Duchenne Muscular Dystrophy as assessed by 3-methylhistidine and Creatinine Excretion. Clinical Science, 56, 347 - 352

Bank, W.J., Rowland, L.P. and Ipsen, J. (1971). Amino Acids of Plasma and Urine in Diseases of Muscle. Archives of Neurology, 24, 176 - 186

Bertles, J.F. and Dobler, J. (1969). Reversible and Irreversible Sickling: A distinction by electron microscopy. Blood, 33, 884 - 898

Bessis, M. (1973). Red Cell Shape. Ed. Bessis, M. Weed, R.I., Leblond, P.F. Pub. Spinger-Verlag pl - 24

Birchmeier, W. and Singer, S.J. (1977). On the Mechanism of ATP Induced Shape Changes in Human Erythrocyte Membranes II. The Role of ATP. Journal of Cell Biology, 73, 647 - 659

Blahd, W.H., Bauer, F.K., Libby, R.L. and Rose, A.S. (1953). Studies in Neuromuscular Diseases with Radioactive Potassium. Neurology, 3, 604 - 608

Blahd, W.H., Bauer, F.K., Libby, R.L. and Rose, A.S. (1954). Radioisotope Studies in Neuromuscular Disease. Neurology, 5, 201 - 207

Blahd, W.H., Bloom, A. and Drell, W. (1955). Quantitative Study of Aminoaciduria in Muscular Dystrophy and Myotonia Dystrophica. Proceedings of the Society of Experimental Biology and Medicine, 90, 704 - 706

Blau, L. and Bittman R. (1978). Cholesterol Distribution between the Two Halves of the Lipid Bilayer of Human Erythrocyte Ghost Membranes. Journal of Biological Chemistry, 253, 8366 - 8368

Blostein, R. (1968). Relationships between Erythrocyte Membrane Phosphorylation and Adenosine Triphosphate Hydrolysis. Journal of Biological Chemistry, 243, 1957 - 1965

Bodensteiner, J.B. and Engel, A.G. (1978). Intracellular Calcium Accumulation in Duchenne Dystrophy and Other Myopathies: A Study of 567,000 Muscle Fibres in 114 Biopsies. Neurology, 28, 439 - 446

Bonilla, E., Schotland, D.L., and Wakayama, Y. (1978). Duchenne Dystrophy: Focal Alterations in the Distribution of Concanavalin A Binding Sites at the Muscle Cell Surface. Annals of Neurology, 4, 117 - 123

Bosmann, H.B., Gersten, D.M., Griggs, R.C., Howland, J.L., Hudecki, M.S. Katyare S. and McLanghlin, J. (1976). Erythrocyte Surface Membrane Alterations. Findings in Human and Animal Muscular Dystrophies. Archives of Neurology, 33, 135 - 138

Bradley, W.G., O'Brien, M.D., Walder, D.N., Murchison, D., Johnson, M and Newell, D.J. (1975). Failure to Confirm a Vascular Cause of Muscular Dystrophy. Archives of Neurology, 32, 466 - 473

Brain, M.C., Kohn, I., McComas, A.J., Missirlis, Y.F., Rathbone, M.P., and Vickers, J. (1978). Red Cell Stability in Duchenne's Syndrome. New England Journal of Medicine, 298, 403

Brody, I.A. (1964). The Significance of Lactate Dehydrogenase Isozymes in Abnormal Human Skeletal Muscle. Neurology, 14, 1091 - 1100

Brown, H.D., Chattopadhyay, S.K. and Patel, A.B. (1967). Erythrocyte Abnormality in Human Myopathy. Science, 157, 1577 - 1578

Bundey, S., Edwards, J.H. and Insley, J. (1979). Carrier Detection in Duchenne Muscular Dystrophy. Lancet i, 881

Burt, D. and Emery, A.E.H. (1979). Serum LDH5 in Carriers of Duchenne Muscular Dystrophy. Neurology, 29, 239 - 241

Butterfield, D.A., Chesnut, D.B., Appel, S.H. and Roses, A.D. (1976). Spin Label Study of Erythrocyte Membrane Fluidity in Myotonic and Duchenne Muscular Dystrophy and Congenital Myotonia. Nature, 263, 159 - 161

Cao, A., De Virgilis, S., Lippi, C. and Coppa, G. (1971). Serum and Muscle Creatine Kinase Isoenzymes and Serum Aspartate Aminotransferase Isoenzymes in Progressive Muscular Dystrophy. Enzyme, 12, 49 - 62

Carter, C.O. (1979). Carrier Detection in Duchenne Muscular Dystrophy. Lancet, i, 979

Cohen, P., Burchell, A., Foulkes, J.G., Cohen, P.T.W., Vanaman, T.C., and Nairn, A.C. (1978). Identification of the Ca^{2+} Dependent Modulator Protein as the Fourth Subunit of Rabbit Skeletal Muscle Phosphorylase Kinase. FEBS letters, 92, 287 - 293

Coleman, R. (1973). Membrane Bound Enzymes and Membrane Ultrastructure. Biochimica Biophysica Acta, 300, 1 - 30

Cooper, R.A. (1977). Abnormalities of Cell Membrane Fluidity in the Pathogenesis of Disease. New England Journal of Medicine, 297, 371 - 377

Craig, I.D., Allen, I.V. and McCormick, D. (1978). A Biochemical, Histological and Electromicroscopical Study of Possible Carriers in Duchenne Muscular Dystrophy. Irish Journal of Medical Science, 147, 125 - 133

Cullen, M.J., and Fulthorpe, J.J. (1975). Stages in Fibre Breakdown in Duchenne Muscular Dystrophy. An Electron-Microscopic Study. Journal of the Neurological Sciences, 24, 179 - 200

Cumings, J.N. (1939). The Potassium Content of Muscle in Disease. Brain, 62, 153 - 156

Dacie, J.V. and Lewis, S.M. (1975). Practical Haematology. 5th Edition. London, Churchill Livingstone

Danieli, G.A. and Angelini, C. (1976). Duchenne Carrier Detection. Lancet, ii, 90

Danon, M.J., Marshall, W.E., Sarpel, G. and Omachi, A. (1978). Erythrocyte Metabolism in Muscular Dystrophy. Archives of Neurology, 35, 592 - 595

Danowski, T.S., Wirth, P.M., Leinberger, M.H., Randall, L.A. and Peters, J.H. (1956). Muscular Dystrophy: III Serum and Blood Solutes and Other Laboratory Indices. American Journal of Diseases of Children, 91, 346 - 355

Das, P.K., Graesslin, D. and Goedde, H.W. (1976). Apparrent Alterations of Erythrocyte Acetylcholinesterase and Other Membrane Proteins in Duchenne Muscular Dystrophy: A Further example of a Generalised Membrane Defect Associated with Hereditary Muscular Dystrophy. Biochemical Society Transaction's, 4, 723

Davie, A.M. and Emery, A.E.H. (1978). Estimation of Proportion of New Mutants among Cases of Duchenne Muscular Dystrophy. Journal of Medical Genetics, 15, 339 - 345

Deguchi, N. Jørgensen, P.L. and Maunsbach, A.B. (1977). Ultrastructure of the Sodium Pump. Comparison of Thin Sectioning, Negative Staining and Freeze Fracture of Purified Membrane Band (Na^{2+}+ K^{+})ATPase. Journal of Cell Biology, 75, 619 - 634

Démos, J. (1961). Mésure des Temps de Circulation chez 79 Myopathies. Revue Francaise d'Études, Cliniques et Biologiques, 6, 876 - 887

De Pont, J.J.H.H.H., Schoot, B.M., Van Prooijen - Vaneeden, A. and Bonting, S.L. (1977). An Essential Arginine Residue in the ATP Binding Centre of (Na^{2+}+ K^{+}) ATPase. Biochimica Biophysica Acta, 482, 213 - 227

Dhalla, N.S., McNamara, D.B., Balasubramanian, V., Greenlaw, R. and Tucker, F.R. (1973). Alterations of Adenosine Triphosphatase Activities in Dystrophic Muscle Sarcolemma. Research Communications in Chemical Pathology and Pharmacology, 6, 643 - 650

Di Mauro, S. and Rowland, L.P. (1976). Urinary Excretion of Carnitine in Duchenne Muscular Dystrophy. Archives of Neurology, 33, 204 - 205

Distefano, P. and Bosmann, H.B. (1977). Erythrocyte Membrane Abnormality in Muscular Dystrophy. Cell Biology, 1, 375 - 378

Dodge, J.T. and Phillips, G.B. (1967). Composition of Phospholipids and of Phospholipid Fatty Acids and Aldehydes in Human Red Cells. Journal of Lipid Research, 8, 667 - 675

Dreyfus, J.C., Schapira, G. and Schapira, F. (1958). Serum Enzymes in the Physiopathology of Muscle Annals of the New York Academy of Sciences, 75, 235 - 249

Dreyfus, J.C., Schapira, G., Schapira, F. and Démos, J. (1956). Activitis enzymatiques du Muscle Humain. Clinica Chimica Acta, 1, 434 - 449

Dubowitz, V. (1963). Myopathic Changes in a Muscular Dystrophy Carrier. Journal of Neurology, Neurosurgery and Psychiatry, 26, 322 - 325

Dubowitz, V. (1975). Neuromuscular Disorders in Childhood. Archives of Diseases of Childhood, 50, 335 - 346

Dubowitz, V. and Pearse, A.G.E. (1961). Enzymic Activity of Normal and Dystrophic Human Muscle: A Histochemical Study. Journal of Pathology, 81, 365 - 378

Dubowitz, V., Van Iddekinge, B., Rodeck, C.H., Campbell, S., Singer, J.D., Scheurbrandt, G. and Moss, D.W. (1978). Prenatal Diagnosis in Duchenne Muscular Dystrophy: Salvage of Normal Male Foetus. Lancet, i, 90

Duchenne, G.B. (1868). Récherches sue la Paralysie Musculaire Pseudo-Hypertrophique ou Paralysie Myo-Sclérosique. Archives générales de Médicine, 11, 5, 179, 305, 421, 552

Dunham, E.T. and Glynn, I.M. (1961). Adenosine Triphosphatase Activity and the Active Movements of Alkali Metal Ions. Journal of Physiology, 156, 274 - 293

Ebashi, S., Toyokura, Y., Momoi, H. and Sugita, H. (1959). High Creatine Phosphokinase Activity of Sera of Progressive Muscular Dystrophy. Journal of Biochemistry, 46, 103 - 104

Elgsaeter, A., Shotton, D.M., and Bronton, D. (1976). Intramembrane Particle Aggregation in Erythrocyte Ghosts, II The Influence of Spectrin Aggregation. Biochimica Biophysica Acta, 426, 101 - 122

Emery, A.E.H. (1963). Clinical Manifestations in Two Carriers of Duchenne Muscular Dystrophy. Lancet, i, 1126 - 1128

Emery, A.E.H. (1964). Electrophoretic Pattern of Lactic Dehydrogenase in Carriers and Patients with Duchenne Muscular Dystrophy. Nature, 201, 1044 - 1045

Emery, A.E.H. (1965). Muscle Histology in Carriers of Duchenne Muscular Dystrophy. Journal of Medical Genetics, 2, 1 - 7

Emery, A.E.H. (1965a). Carrier Detection in Sex Linked Muscular Dystrophy. Journal de Génétique Humain, 14, 318 - 329

Emery, A.E.H. (1968). Muscle Lactate Dehydrogenase Isoenzyme in Hereditary Myopathies. Journal of Neurological Sciences, 7, 137 - 148

Emery, A.E.H. (1972). Abnormalities of the Electrocardiogram in Hereditary Myopathies. Journal of Medical Genetics, 9, 8 - 12

Emery, A.E.H. (1977). Biochimica Delle Miopatie Dell 'uomo'. Clinica e Laboratorio, 1, 106 - 113

Emery, A.E.H. (1977a). Muscle Histology and Creatine Kinase Levels in the Foetus in Duchenne Muscular Dystrophy. Nature, 266, 472 - 473

Emery, A.E.H. (1979). In Press

Emery, A.E.H., Burt, D., Dubowitz, V., Rocker, I., Donnai, D., Harris, R., and Donnai, P. (1979). Antenatal Diagnosis of Duchenne Muscular Dystrophy. Lancet, i, 847 - 849

Emery, A.E.H. and Dreifuss, F.E. (1966). Unusual Type of Benign X-linked Muscular Dystrophy. Journal of Neurology, Neurosurgery and Psychiatry, 29, 338 - 342

Emery, A.E.H. and Schelling, J.L. (1965). Limb Blood Flow in Patients and Carriers of Duchenne Muscular Dystrophy. Acta Genetica (Basel) 15, 337 - 344

Emery, A.E.H. and Skinner, R. (1976). Clinical Studies in Benign (Becker Type) X-linked Muscular Dystrophy. Clinical Genetics, 10, 189 - 201

Engel, W.K. (1973). Duchenne Muscular Dystrophy: A Histologically Based Ischemic Hypothesis and Comparison with Experimental Ischemic Myopathy. The Striated Muscle. Ed. Pearson, C.M. and Mostofi, F.K. Pub. Williams and Wilkins. Baltimore

Enomoto, A. and Bradley, W.G. (1977). Therapeutic Trials in Muscular Dystrophy. Archives of Neurology, 34, 771 - 773

Erb, W. (1884). Uber die 'Juvenile Form' der progressiven Muskelatrophie Ihre Beziehungen Zür Sogennanten Pseudohypertrophie Der Muskeln. Deutsches Archiv für Klinische Medizin, 34 467 - 519

Erdmann, E. and Hasse, W. (1975). Quantitative Aspects of Ouabain Binding to Human Erythrocyte and Cardiac Membranes. Journal of Physiology, 251, 671 - 682

Falk, R.S., Campion, D., Guthrie, D., Sparkes, R.S. and Fox, C.F. (1979). Phosphorylation of Red-Cell Membrane Proteins in Duchenne Muscular Dystrophy. New England Journal of Medicine, 300, 258 - 259

Farley, R.A. (1979). Ouabain-Dependent Conformation Changes of Mammalian (Na-K) ATPase. Biophysical Journal, 25, 93a

Fischer, S., Tortolero, M., Piau, J.P., Delaunay, J. and Schapira, G. (1978). Protein Kinase and Adenylate Cyclase of Erythrocyte Membrane from Patients with Duchenne Muscular Dystrophy. Clinica Chimica Acta, 88, 437 - 440

Fisher, E.R., Silvester, E., Vester, J.W., Nolan, S., Ahmad, V. and Danowski, T.S. (1976). Increased Erythrocytic Osmotic Fragility in Pseudohypertrophic Muscular Dystrophy. Journal of the American Medical Association, 236, 955

Fiske, C. and SubbaRow, Y. (1925). The Colorimetric Determination of Phosphorus. Journal of Biological Chemistry, 66, 375 - 400

Gale, A.N. and Murphy, E.A. (1978). The Use of Serum Creatine Phosphokinase in Genetic Counselling for Duchenne Muscular Dystrophy. 1 Analysis of Results from 29 Studies. Journal of Chronic Diseases, 31, 101 - 109

Gallup, B. and Dubowitz, V. (1973). Failure of "Dystrophic" Neurones to Support Functional Regeneration of Normal or Dystrophic Muscle in Culture. Nature, 243, 287 - 289

Gardner, J.D. and Conlon, T.P. (1972). The Effects of Sodium and Potassium on Ouabain Binding by Human Erythrocytes. Journal of General Physiology, 60, 609 - 629

Gardner-Medwin, D., Pennington, R.J. and Walton, J.N. (1971). The Detection of Carriers of X-linked Muscular Dystrophy Genes. Journal of the Neurological Sciences, 13, 459 - 474

Garst, J.B., Vignos, P.J., Hadaday, M. and Matthews, D.N. (1977). Urinary Sodium, Potassium and Aldosterone in Duchenne Muscular Dystrophy. Journal of Clinical Endocrinology and Metabolism, 44, 185 - 188

Golbus, M.S., Stephens, J.D., Mahoney, M.J., Hobbins, J.C., Haseltine, F.P., Caskey, C.T. and Barker, B.Q. (1979). Failure of Fetal Creatine Phosphokinase as a Diagnostic Indicator of Duchenne Muscular Dystrophy. New England Journal of Medicine, 300, 860 - 861

Gopinath, R.M. and Vicenzi, F.F. (1977). Phosphodiesterase Protein Activator Mimics Red Blood Cell Cytoplasmic Activator of $Ca^{2+}Mg^{2+}$ ATPase. Biochemical and Biophysical Research Communications, 77, 1203 - 1209

Goto, I. (1974). Creatine Phosphokinase Isozymes in Neuromuscular Disorders. Archives of Neurology, 31, 116 - 119

Goto, I. and Katsuki, S. (1970). Creatine Phosphokinase Isozymes in Pathological Human Serum. Clinica Chimica Acta, 30, 795 - 799

Gottlieb, M.H. (1976). The Limited Depletion of Cholesterol from Erythrocyte Membranes on Treatment with Incubated Plasma. Biochimica Biophysica Acta, 433, 333 - 343

Grassi, E., Lucci, B., Marchini, C., Ottonello, S., Parma, M., Reggiano, R., Rossi, G.L. and Tagliavini, J. (1978). Deformed Erythrocytes in Muscular Dystrophies. Neurology, 28, 842 - 844

Hanahan, D.J., Ekholm, J. and Hildenbrandt, G. (1973). Biochemical Variability of Human Erythrocyte Membrane Preparations as Demonstrated by $Na^{+}K^{+}Mg^{2+}$ and Ca^{2+} ATPase Activities. Biochemistry, 12, 1374 - 1387

Hanahan, D.J. and Ekholm, J.E. (1978). The Expression of Optimum ATPase Activities in Human Erythrocytes . Archives of Biochemistry and Biophysics, 187, 170 - 179

Harand, Y., Adair, R., Vignos, P.J. Jnr, Miller, M. and Kowall, J. (1973). Pyruvate Kinase Isoenzymes in Progressive Muscular Dystrophy and Acute Myocardial Infarction. Metabolism, 22, 493 - 501

Hardy, M.F., Gardner-Medwin, D. and Pennington, R.J.T. (1977). Serum Pyruvate Kinase in Carriers of Duchenne Muscular Dystrophy. Journal of the Neurological Sciences, 32, 137 - 139

Hathaway, P.W., Engel, K., Zellweger, H. (1970). Experimental Myopathy after Microarterial Embolization. Comparison with Childhood X-linked Pseudohypertrophic Muscular Dystrophy. Archives of Neurology, 22, 365 - 378

Hauser, S., Weiner, H., Ault, K., and Unanue, E. (1979). Lymphocyte Capping in Duchenne Muscular Dystrophy. New England Journal of Medicine, 300, 861

Haymond, M.W., Strobel, K.E. and Devivo, D.C. (1978). Muscle Wasting and Carbohydrate Homeostasis in Duchenne Muscular Dystrophy. Neurology, 28, 1224 - 1231

Heller, M. and Beck, S. (1978). Interactions of Cardiac Glycosides with Cells and Membranes. Properties and Structural Aspects of Two Receptor Sites for Ouabain in Erythrocytes. Biochimica Biophysica Acta, 514, 332 - 347

Hidalgo, C., Thomas, D.D. and Ikermoto, N. (1978). Effect of the Lipid Environment on Protein Motion and Enzymatic Activity of the Sarcoplasmic Reticulum Calcium ATPase. Journal of Biological Chemistry, 253, 6879 - 6887

Hobbs, A.S. and Dunham, P.B. (1978). Interaction of External Alkali Metal Ions with the Na-K Pump of Human Erythrocytes. Journal of General Physiology, 72, 381 - 402

Hodson, A. and Pleasure, D. (1977). Erythrocyte Cation-Activated Adenosine Triphosphatases in Duchenne Muscular Dystrophy. Journal of the Neurological Sciences, 32, 361 - 369

Hooshmand, H. (1975). Serum Lactate Dehydrogenase Isoenzymes in Neuromuscular Diseases. Diseases of the Nervous System, 36, 607 - 611

Horenstein, A.L. and Emery, A.E.H. In Press. Human Lymphocyte Capping in Duchenne Muscular Dystrophy.

Horvath, B. and Proctor, J.B. (1958). Muscular Dystrophy: Quantitative Studies on the Composition of Dystrophic Muscle. Research Publications. Association for Research in Nervous and Mental Disease, 38, 740 - 770

Howells, K.F. (1976). Structural Changes of Erythrocyte Membranes in Muscular Dystrophy. Research in Experimental Medicine, 168, 213 - 217

Howland, J.L. (1974). Abnormal Potassium Conductance Associated with Genetic Muscular Dystrophy. Nature, 251, 724 - 725

Howland, J.L. and Iyer, S.L. (1977). Erythrocyte Lipids in Heterozygous Carriers of Duchenne Muscular Dystrophy. Science, 198, 309 - 310

Hutchinson, J. (1879). On Ophthalmoplegia Externa or Symmetrical Immobility (partial) of the Eyes, with Ptosis. Transactions of the Medico-Chirurgical Society of Edinburgh, 62, 307 - 329

Ionasescu, V. (1975). Distinction Between Duchenne and Other Muscular Dystrophies by Ribosomal Protein Synthesis. Journal of Medical Genetics, 12, 49 - 54

Ionasescu, V., Zellweger, H. and Conway, T.W. (1971). Ribosomal Protein Synthesis in Duchenne Muscular Dystrophy. Archives of Biochemistry and Biophysics, 144, 51 - 58

Ionasescu, V., Zellweger, H. and Conway, T.W. (1971a). A New Approach for Carrier Detection in Duchenne Muscular Dystrophy. Neurology, 21, 703 - 709

Ionasescu, V., Zellweger, H., McCormick, W.F. and Conway, T.W. (1973). Comparison of Ribosomal Protein Synthesis in Becker and Duchenne Muscular Dystrophies. Neurology, 23, 245 - 253

Ionasescu, V., Zellweger, H. Shink, P. and Conway, T.W. (1973a). Identification of Carriers of Duchenne Muscular Dystrophy by Muscle Protein Synthesis. Neurology, 23, 497 - 502

Ionasescu, V., Radu, H. and Nicolescu, P. (1975). Identification of Duchenne Muscular Dystrophy Carriers. Archives of Pathology, 99, 436 - 441

Ionasescu, V., Zellweger, H. and Cancilla, P. (1978). Fetal Serum-Creatine-Phosphokinase not a Valid Predictor of Duchenne Muscular Dystrophy. Lancet, ii, 1251

Ionasescu, V., Stern, L.Z., Ionasescu, R. and Ruberstein, P. (1979). Stimulatory Effects of Drugs for Protein Synthesis on Muscle Cell Cultures in Duchenne Dystrophy. Annals of Neurology, 5, 107 - 110

Iyer, S.L., Katyare, S.S. and Howland, J.L. (1976). Elevated Erythrocyte Phospholipase A Associated with Duchenne and Myotonic Muscular Dustrophy. Neuroscience Letters, 2, 103 - 106

Iyer, S.L., Hoenig, P.A., Sherblom, A.P., and Howland, J.L. (1977). Membrane Function Affected by Genetic Muscular Dystrophy. 1. Erythrocyte Ghost Protein Kinase. Biochemical Medicine, 18, 384 - 391

Jarrett, H.W. and Penniston, J.T. (1976). Purification of the Ca^{2+} Stimulated ATPase Activator from Human Erythrocytes. Journal of Biological Chemistry, 253, 4676 - 4682

Jerusalem, F., Engel, A.G. and Gomez, M.R. (1974). Duchenne Dystrophy. 1. Morphometric Study of the Muscle Microvasculature. Brain, 97, 115 - 122

Jerusalem, F., Engel, A.G. and Gomez, M.R. (1974a). Duchenne Dystrophy ii Morphometric Study of Motor and Plate Fire Structure. Brain, 97, 123 - 130

Joiner, C.H. and Lauf, P.K. (1978). The Correlation Between Ouabain Binding and K Pump Inhibition in Human and Sheep Erythrocytes. Journal of Physiology, 283, 155 - 175

Jørgersen, P.L. (1974). Purification of (Na^+, K^+) ATPase: Active Site Determinations and Criteria of Purity. New York Academy of Sciences, 242, 36 - 52

Jørgerson, P.L., Hansen, O., Glynn, I.M. and Cavieres, J.D. (1973). Antibodies to Pig Kidney ($Na^+ + K^+$) ATPase Inhibit the Na^+ Pump in Human Red Cells Provided they have Access to the Inner Surface of the Cell Membrane. Biochimica, Biophysica Acta, 291, 795 - 800

Juliano, R.L. (1973). The Proteins of the Erythrocyte Membrane. Biochimica Biophysica Acta, 300, 341 - 378

Kalofoutis, A., Jullien, G. and Spanos, V. (1977). Erythrocyte Phospholipids in Duchenne Muscular Dystrophy. Clinica Chimica Acta, 74, 85 - 87

Kar, N.C. and Pearson, C.M. (1975). Glyoxalase Enzyme System in Human Muscular Dystrophy. Clinica Chimica Acta, 65, 153 - 156

Kar, N.C. and Pearson, C.M. (1975a). Lipase Activity in Normal and Dystrophic Human Muscle. Biochemical Medicine, 14, 135 - 137

Kar, N.C. and Pearson, C.M. (1976). A Calcium Activated Neutral Protease in Normal and Dystrophic Human Muscle. Clinica Chimica Acta, 73, 293 - 297

Kar, N.C. and Pearson, C.M. (1978). Muscular Dystrophy and Activation of Proteinases. Muscle and Nerve, 1, 308 - 313

Karagan, N.J. and Zellweger, H.V. (1978). Early Verbal Disability in Children with Duchenne Muscular Dystrophy. Developmental Medicine and Child Neurology, 20, 435 - 441

Kibrick, A.C., Hashiro, C., Walters, M. and Milhorat, A.T. (1964). Hydroxyproline Excretion in Urine of Patients with Muscular Dystrophy and Other Muscle Diseases. Proceedings of the Society for Experimental Biology and Medicine, 115, 662 - 665

Kirkpatrick, F.H., Woods, G.M., La Celle, P.L. and Weed, R.I. (1975). Calcium and Magnesium ATPases of the Spectrin Fraction of Human Erythrocytes. Journal of Supramolecular Structure, 3, 415 - 425

Klassen, G.A. and Blostein, R. (1969). Adenosine Triphosphatase and Myopathy. Science, 163, 492 - 493

Kleine, T.O. (1970). Evidence for the Release of Enzymes from Different Organs in Duchenne Muscular Dystrophy. Clinica Chimica Acta, 29, 227 - 231

Kleine, T.O. and Chlond, M., Von H (1967). Enzyme Distribution Patterns of Healthy Human Skeletal, Heart and Smooth Muscle and their Pathological Changes in Progressive Muscular Dystrophy and Other Conditions. Clinica Chimica Acta, 15, 19 - 33

Kobayashi, T., Mawatari, S. and Kuroiwa, Y. (1978). Lipids and Proteins of Erythrocyte Membrane in Duchenne Muscular Dystrophy. Clinica Chimica Acta, 85, 259 - 266

Kobayashi, T., Shinnoh, N. and Mawatari, S. (1979). Carnitine in Duchenne Muscular Dystrophy. Clinica Chimica Acta, 93, 157 - 159

Kohn, I., Missirlis, Y., McComas, A.J. and Brain, M.C. (1977). Increased Resistance to Deformation of Erythrocytes in Muscular Dystrophy. Clinical Research, 25, 708A

Koski, C.L., Jungalwala, F.B. and Kolodny, E.H. (1978). Normality of Erythrocyte Phospholipids in Duchenne Muscular Dystrophy. Clinica Chimica Acta, 85, 295 - 298

Kunze, D., Reichmann, G., Egger, E., Leuschner, G. and Eckhardt, H. (1973). Red Blood Cell Lipids in Progressive Muscular Dystrophy. Clinica Chimica Acta, 43, 333

Kunze, D., Reichmann, G. Egger, E., Olthoff, D. and Dohlerk (1975). Fatty Acid Pattern of Lipids in Normal and Dystrophic Human Muscle. European Journal of Clinical Investigation, 5, 471 - 475

Kyte, J. (1972). Properties of the Two Polypeptides of Sodium - and Potassium - Dependent Adenosine Triphosphatase. Journal of Biological Chemistry, 247, 7642 - 7649

Landouzy, L. and Dejerine, J. (1884). De ian Myopathie Atrophique Progressive (Myopathie Hereditaire débutante, dans l'enfance, par le Face, sans Altération du Système Nerveux). CR Academie Science (Paris), 98, 53 - 55

Lange, Y. and D'Alessandro, J.S. (1977). Characterisation of Mechanisms for Transfer of Cholesterol between Human Erythrocytes and Plasma. Biochemistry, 16, 4339 - 4343

Leyden, E. (1876). Klinik der Ruckenmarks - Krankheiten. Vol 2, 531 Hirschwald (Berlin)

Lin, G.S.B. and Macey, R.I. (1978). Shape and Stability Changes in Human Erythrocyte Membranes Induced by Metal Cations. Biochimica Biophysica Acta, 212, 270 - 283

Lindemayer, G.E. and Schwartz, A. (1973). Nature of the Transport Adenosine Triphosphatase Digitalis Complex. Journal of Biological Chemistry, 248, 1291 - 1300

Lishko, V.K., Malysheva, M.K. and Grevizirskaya, T.I. (1972). The Interaction of the (Na^+, K^+) ATPase of Erythrocyte Ghosts with Ouabain. Biochimica Biophysica Acta, 288, 103 - 106

Lloyd, S.J. and Nunn, M.G. (1978). Osmotic Fragility of Erythrocytes in Duchenne Muscular Dystrophy. British Medical Journal, 2, 252

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., (1951). Protein Measurement with the Folin Phenol Reagent. Journal of Biological Chemistry, 193, 265 - 275

Lumb, E.M. and Emery, A.E.H. (1975). Erythrocyte Deformation in Duchenne Muscular Dystrophy. British Medical Journal, 3, 467 - 468

Lundblad, A., Svensson, S., Yamashino, I. and Ohta, M. (1979). Increased Urinary Excretion of a Glucose-containing Tetrasaccharide in Patients with Duchenne Muscular Dystrophy. FEBS letters, 97, 249 - 252

Lux, S.E. (1979). Dissecting the Red Cell Membrane Skeleton. Nature, 281, 426 - 429

Lux, S.E., John, K.M. and Karnovsky, M.J. (1976). Irreversible Deformation of the Spectrin-Actin Lattice in Irreversibly Sickled Cells. Journal of Clinical Investigation, 58, 955 - 963

Lux, S.E., John, K.M. and Ukena, T.E. (1978). Diminished Spectrin Extraction from ATP Depleted Human Erythrocytes. Journal of Clinical Investigation, 61, 815 - 827

McComas, A.J., Sica, R.E.P. and Currie, S. (1971). An Electrophysiological Study of Duchenne Dystrophy. Journal of Neurology, Neurosurgery and Psychiatry, 34, 461 - 468

McComas, A.J., Sica, R.E.P. and Campbell, M.J. (1971). "Sick" Motoneurones A Unifying Concept of Muscle Disease. Lancet, i, 321 - 325

McComas, A.J., Sica, R.E.P. and Upton, A.R.M. (1974). Multiple Muscle Analysis of Motor Units in Muscular Dystrophy. Archives of Neurology, 30, 249 - 251

McGowan, E.B., Shafiq, S.A. and Stracher, A. (1976). Delayed Degeneration of Dystrophic and Normal Muscle Cell Cultures Treated with Pepstatin, Leupeptin and Antipain. Experimental Neurology, 50, 649 - 657

Mahoney, M.J., Haseltine, F.P., Hobbins, J.C., Banker, B.Q., Caskey, C.T. and Golbus, M.S. (1977). Prenatal Diagnosis of Duchenne Muscular Dystrophy. New England Journal of Medicine, 297, 968 - 973

Marchesi, V.T. and Steers, E. (1968). Selective Solubilisation of a Protein Component of the Red Cell Membrane. Science, 159, 203 - 204

Marchesi, V.T., Tillack, T.W., Jackson, R.L., Segrest, J.P. and Scott, R.E. (1972). Chemical Characterisation and Surface Orientation of the Major Glycoprotein of the Human Erythrocyte Membrane. Proceedings of the National Academy of Sciences, 69, 1445 - 1449

Masiak, S.J. and D'Angelo, G. (1975). Effects of N-Acetylimidazole on Human Erythrocyte ATPase Activity. Biochimica Biophysica Acta, 382, 83 - 91

Maskaleris, M.L., Gross, S. and Milhorat, A.T. (1969). Urinary Amino Acid and Peptide Excretion Patterns in Patients with Muscular Dystrophy (Duchenne). Clinical Chemistry, 15, 545 - 554

Matheson, D.W. and Howland, J.L. (1974). Erythrocyte Deformation in Human Muscular Dystrophy. Science, 184, 165 - 166

Matheson, D.W., Engel, W.K. and Derrer, E.C. (1976). Erythrocyte Shape in Duchenne Muscular Dystrophy. Neurology, 26, 1182 - 1183

Matsumoto, N., Yawata, Y. and Jacob, H.S. (1977). Association of Decreased Membrane Protein Phosphorylation with Red Blood Cell Spherocytosis. Blood, 49, 233 - 239

Mawatari, S., Takagi, A. and Rowland, L. (1974). Adenyl Cyclase in Normal and Pathological Human Muscle. Archives of Neurology, 30, 96 - 102

Mawatari, S., Schonberg, M. and Olarte, M. (1976). Biochemical Abnormalities of Erythrocyte Membranes in Duchenne Dystrophy. Archives of Neurology, 33, 489 - 493

Mendell, J.R., Engel, W.K. and Derrer, E.C. (1971). Duchenne Muscular Dystrophy: Functional Ischaemia Reproduces its Characteristic Lesions. Science, 172, 1143 - 1145

Mendell, J.R., Engel, W.K. and Derrer E.C. (1972). Increased Plasma Enzyme Concentrations in Rats with Functional Ischaemia of Muscle Provides a Possible Model of Duchenne Muscular Dystrophy. Nature, 239, 522 - 524

Mendell, J.R. and Wiechers, D.O. (1979). Lack of Benefit of Allopurinol in Duchenne Dystrophy. Muscle and Nerve, 2, 53 - 66

Menon, I.S., Gardner-Medwin, D., Weightman, D. and Dewar, H.A. (1971). Fibrinolytic Activity in Venous Blood of Patients with Muscular Dystrophy. Neurology, 21, 1019 - 1022

Miale, T.D., Frias, J.L. and Lawson, D.L. (1975). Erythrocytes in Human Muscular Dystrophy. Science, 187, 453

Milhorat, A.T. (1953). Creatine and Creatine Metabolism and Diseases of the Neuromuscular System. Proceedings of the Association of Research in Nervous and Mental Disease, 32, 400 - 421

Milhorat, A.T., Shafiq, S.A. and Goldstone, L. (1966). Changes in Muscle Structure in Dystrophic Patients, Carriers and Normal Siblings seen by Electron Microscopy; Correlations with Levels of Serum Creatine Phosphokinase. Annals of New York Academy of Sciences, 138, 246 - 291

Miller, S.E., Roses, A.D. and Appel, S.H. (1976). Scanning Electron Microscopy Studies in Muscular Dystrophy. Archives of Neurology, 33, 172 - 174

Mokri, B. and Engel, A.G. (1975). Duchenne Dystrophy; Electron Microscopic Findings Pointing to a Basic or Early Abnormality in the Plasma Membrane of the Muscle Fibre. Neurology, 25, 1111 - 1120

Monckton, G. and Nihei, T. (1969). A Correlation of Histology and Amino Acid Incorporation Studies in Duchenne Muscular Dystrophy. Neurology, 19, 415 - 418

Morse, P.F. and Howland, J.L. (1973). Erythrocytes from Animals with Genetic Muscular Dystrophy. Nature, 245, 156 - 157

Moser, H. and Vogt, J. (1974). Follow-Up Study of Serum Creatine Kinase in Carriers of Duchenne Muscular Dystrophy. Lancet, ii, 661 - 662

Nakao, M., Nakao, T. and Yamozoe, S. (1960). Adenosine Triphosphate and Maintenance of Shape in the Human Red Cells. Nature, 187, 945 - 946

Neerunjun, J.S., Allsop, J. and Dubowitz, V. (1979). Hypoxanthine-Guanine Phosphoribosyltransferase Activity of Blood and Muscle in Duchenne Dystrophy. Muscle and Nerve, 2, 19 - 23

Nicholson, G.A., Gardner-Medwin, D., Pennington, R.J.T. and Walton, J. (1979). Carrier Detection in Duchenne Muscular Dystrophy: Assessment of the Effect of Age on Detection Rate with Serum Creatine Kinase Activity. Lancet, i, 692 - 694

Niebroj-Dobosz, I. (1976). Erythrocyte Ghosts (Na^+, K^+) ATPase Activity in Duchenne's Dystrophy and Myotonic. Journal of Neurology, 214, 61 - 69

Norregaard Hansen, K., Lademann, A. and Norgaard-Pedersen, B. (1978). Potential of Radioimmunoassay of Myoglobin in Amniotic Fluid and Serum in Prenatal Diagnosis of Duchenne Muscular Dystrophy. Lancet, ii, 1250 - 1251

Oberc, M.A. and Engel, W.K. (1977). Ultrastructural Localisation of Calcium in Normal and Abnormal Skeletal Muscle. Laboratory Investigation, 36, 566 - 577

Oppenheimer, H. and Milhorat, A.T. (1961). Serum Proteins, Lipoproteins and Glycoproteins in Muscular Dystrophy and Related Diseases. Annals of the New York Academy of Sciences, 94, 308 - 319

Palek, J., Liu, S.C. and Snyder, L.M. (1978). Metabolic Dependence of Protein Arrangement in Human Erythrocyte Membranes. I. Analysis of Spectrin-Rich Complexes in ATP Depleted Red Cells. Blood, 51, 385 - 395

Panayiotopoulous, C.P., Scarpalezos, S., Papapetropoulos, T. (1974). Electrophysiological Estimation of Motor Units in Duchenne Muscular Dystrophy. Journal of the Neurological Sciences, 23, 89 - 98

Pearce, J.M.S., Pennington, R.J. and Walton, J.N. (1964). Serum Enzyme Studies in Muscle Disease. II. Serum Creatine Kinase Activity in Muscular Dystrophy and Other Myopathic and Neuropathic Disorders. Journal of Neurology, Neurosurgery and Psychiatry, 27, 96 - 99

Pearce, J.M.S., Pennington, R.J. and Walton, J.N. (1964a). Serum Enzyme Studies in Muscle Disease. III. Serum Creatine Kinase Activity in Relatives of Patients with the Duchenne Type of Muscular Dystrophy. Journal of Neurology, Neurosurgery and Psychiatry, 27, 181 - 185

Pearson, C.M., Kar, N.C., Peter, J.B. and Munsat, T.L. (1965). Muscle Lactate Dehydrogenase Patterns in Two Types of X-linked Muscular Dystrophy. American Journal of Medicine, 39, 91 - 97

Pearson, T.W. (1978). (Na^+, K^+) -ATPase of Duchenne Muscular Dystrophy Erythrocyte Ghosts. Life Sciences, 22, 127 - 132

Pennington, R.J. (1962). Some Enzyme Studies in Muscular Dystrophy. Proceedings of the Association of Clinical Biochemists, 2, 17 - 18

Pennington, R.J.T. (1974). Biochemical Aspects of Muscle Disease. p488 - 516. Disorders of Voluntary Muscle. Ed. J.N. Walton, Pub. Churchill Livingston

Pennington, R.J., Park, D.C. and Freeman, C.P. (1966). The Fatty Acid Composition of Infiltrating Fat in Muscle from a Case of Muscular Dystrophy. Clinica Chimica Acta, 13, 399 - 400

Pennington, R.J. and Robinson, J.E. (1968). Cathepsin Activity in Normal and Dystrophic Human Muscle. Enzymologia Biologica et Clinica, 9, 175 - 182

Percy, A.K. and Miller, M.E. (1975). Reduced Deformability of Erythrocyte Membranes from Patients with Duchenne Muscular Dystrophy. Nature, 258, 147 - 148

Perloff, J.K., De Leon, A.C. and O'Doherty, D. (1967). The Cardiomyopathy of Progressive Muscular Dystrophy. Circulation, 33, 625 - 648

Peter, J.B. and Worsfold, M. (1969). Muscular Dystrophy and other Myopathies: Sarcotubular Vesicles in Early Disease. Biochemical Medicine, 2, 364 - 371

Peter, J.B., Worsfold, M. and Pearson, C.M. (1969). Erythrocyte Ghost Adenosine Triphosphatase in Duchenne Dystrophy. Journal of Laboratory and Clinical Medicine, 74, 103 - 108

Pickard, N.A., Greumer, H.D., Verrill, H.L., Isaacs, E.R., Robinow, M., Nance, W.E., Myers, E.C. and Goldsmith, B. (1978). Systemic Membrane Defect in the Proximal Muscular Dystrophies. New England Journal of Medicine, 299, 841 - 846

Pilsum, Van, J.F. and Wolin, E.A. (1958). Guanidinium Compounds in Blood and Urine of Patients Suffering from Muscle Disorders. Journal of Laboratory and Clinical Medicine, 51, 219 - 223

Roelofsen, B. and Schatzmann, H.J. (1977). The Lipid Requirement of the (Ca, Mg) ATPase in the Human Erythrocyte Membrane as Studied by Various Highly Purified Phospholipases. Biochimica Biophysica Acta, 464, 17 - 36

Ronzoni, E., Berg, L. and Landau, W. (1958). Enzyme Studies in Progressive Muscular Dystrophy. Research Publications. Associations for Research in Nervous and Mental Disease, 38, 721 - 729

Rosalki, S.B. (1967). An Improved Procedure for Serum Creatine Phosphokinase Determination. Journal of Laboratory and Clinical Medicine, 69, 696 - 705

Rosalki, S.B. and Thomson, W.H.S. (1971). Serum Gamma-Glutamyl Transpeptidase in Muscle Disease. Clinica Chimica Acta, 33, 264

Roses, A.D. (1979). More on Phosphorylation of Red Cell Membranes in Muscular Dystrophy. New England Journal of Medicine, 300, 1114

Roses, A.D., Herbstreith, M.H. and Appel, S.H. (1975). Membrane Protein Kinase Alteration in Duchenne Muscular Dystrophy. Nature, 254, 350 - 351

Roses, A.D. and Appel, S.H. (1976). Erythrocyte Spectrin Peak II Phosphorylation in Duchenne Muscular Dystrophy. Journal of the Neurological Sciences, 29, 185 - 193

Roses, A.D., Roses, M.J., Miller, S.E., Hull, K.L. and Appel, S.H. (1976). Carrier Detection in Duchenne Muscular Dystrophy. New England Journal of Medicine, 294, 193 - 198

Roses, A.D., Roses, M.J., Nicholson, G.A. and Roe, C.R. (1977). Lactate Dehydrogenase 5 in Detecting Carriers of Duchenne Muscular Dystrophy. Neurology, 27, 414 - 421

Rowland, L.P. (1976). Pathogenesis of Muscular Dystrophies. Archives of Neurology, 33, 315 - 321

Rubin, C.S. and Rosen, O.M. (1975). Protein Phosphorylation. Annual Review of Biochemistry, 44, 831 - 887

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. Clinica Chimica Acta, 89, 99 - 110

Ruitenbeek, W. (1979). Membrane-Bound Enzymes of Erythrocytes in Human Muscular Dystrophy. Journal of the Neurological Sciences, 41, 71 - 80

Ruitenbeek, W., Edixhoven, M.J. and Scholte, H.R. (1979). Osmotic Stability of Erythrocytes in Human Muscular Dystrophy Before and After Phospholipase Treatment. Clinica Chimica Acta, 94, 259 - 266

Samaha, F.J. and Congedo, C.Z. (1977). Two Biochemical Types of Duchenne Dystrophy: Sarcoplasmic Reticulum Membrane Proteins. Annals of Neurology, 1, 125 - 130

Samaha, F.J. and Gergely, J. (1969). Biochemical Abnormalities of the Sarcoplasmic Reticulum in Muscular Dystrophy. New England Journal of Medicine, 280, 184 - 188

Sanyal, S.K., Johnsone, W.W., Thapar, M.K. and Pitner, S.E. (1978). An Ultrastructural Basis for Electrocardiographic Alterations Associated with Duchenne's Progressive Muscular Dystrophy. Circulation, 57, 1122 - 1129

Schapira, F., Dreyfus, J.C., Schapira, G. and Démos, J. (1960) Étude de la Creatine-Kinase Serique ches les Myopathes et leurs Familles. Revue Francaises d'Études Cliniques et Biologiques, 5, 384 - 386

Schotland, D.L., Bonilla, E. and Van Meter, M. (1977). Duchenne Dystrophy: Alteration in Muscle Plasma Membrane Structure. Science, 196, 1005 - 1007

Schrier, S.L. (1977). Human Erythrocyte Membrane Enzymes: Current Status and Clinical Correlates. Blood, 50, 227 - 237

Seay, A.R., Ziter, F.A., Wu, L.H. and Wu, J.T. (1978). Serum Creatine Phosphokinase and Pyruvate Kinase in Neuromuscular Disorders and Duchenne Dystrophy Carriers. Neurology, 28, 1047 - 1050

Sha'afi, R.I., Rodan, S.B., Hintz, R.L., Fernandez, S.M. and Rodan, G.A. (1975). Abnormalities in Membrane Microviscosity and Ion Transport in Genetic Muscular Dystrophy. Nature, 254, 525 - 526

Sheetz, M.P. and Singer, S.J. (1977). On the Mechanism of ATP Induced Shape Changes in Human Erythrocyte Membranes. I. The Role of the Spectrin Complex. Journal of Cell Biology, 73, 638 - 646

Sica, R.E.P. and McComas, A.J. (1978). The Neural Hypothesis of Muscular Dystrophy. Canadian Journal of Neurological Sciences, 5, 189 - 197

Siddiqui, P.Q.R. and Pennington, R.J.T. (1977). Effect of Ouabain Upon Erythrocyte Membrane Adenosine Triphosphatase in Duchenne Muscular Dystrophy. Journal of the Neurological Sciences, 34, 365 - 372

Siegel, G.J., Koval, G.J. and Albers, R.W. (1969). Sodium-Potassium Activated Adenosine Triphosphatase. VI. Characterisation of the Phosphoprotein formed from Orthophosphate in the presence of Ouabain. Journal of Biological Chemistry, 244, 3264 - 3269

Singer, S.J. (1974). The Molecular Organisation of Membranes. Annual Review of Biochemistry, 43, 805 - 833

Singer, S.J. and Nicholson, G.L. (1972). The Fluid Mosaic Model of the Structure of Cell Membranes. Science, 175, 720 - 731

Smith, I. and Thomson, W.H.S. (1977). Carrier Detection in X-linked Recessive (Duchenne) Muscular Dystrophy: Pyruvate Kinase Isoenzymes and Creatine Phosphokinase in Serum and Blood Cells. Clinica Chimica Acta, 78, 439 - 451

Solomons, C.C., Ringel, S.P., Nwuke, E.I. and Suga, H. (1977). Abnormal Adenine Metabolism of Erythrocytes in Duchenne and Myotonic Muscular Dystrophy. Nature, 268, 55 - 56

Solton, H.C. (1977). Echinocytes in Families with Duchenne Muscular Dystrophy. Journal of Medical Genetics, 14, 276 - 278

Somer, H., Donner, M., Murros, J. and Konttinen, A. (1973). A Serum Isozyme Study in Muscular Dystrophy. Archives of Neurology, 29, 343 - 345

Somer, H., Chien, S., Sung, L.A. and Thurn, A. (1979). Erythrocytes in Duchenne Dystrophy: Osmotic Fragility and Membrane Deformability. Neurology, 29, 519 - 522

Souweine, G., Bernard, J.C., Lasne, Y. and Lachanat, J. (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. Journal of Neurology, 217, 287 - 294

Steck, T.L. (1974). The Organisation of Proteins in the Human Red Blood Cell Membrane. Journal of Cell Biology, 62, 1 - 19

Steinberg, M.H., Eaton, J.W., Berger, E., Coleman, M.B. and Oelshlegel F.J. (1978). Erythrocyte Calcium Abnormalities and the Clinical Severity of Sickling Disorders. British Journal of Haematology, 40, 533 - 539

Stracher, A., McGowan, E.B. and Shafiq, S.A. (1978). Muscular Dystrophy: Inhibition of Degeneration in vivo with Protease Inhibitors. Science, 200, 50 - 51

Susheela, A.K. (1968). Free Fatty Acid Concentrations in Normal and Diseased Human Muscle and in Blood Sera from Patients with Neuromuscular Disease. Clinica Chimica Acta, 22, 219 - 222

Susheela, A.K., Kaul, R.D., Sachdeva, K. and Singh, N. (1975). Adenyl Cyclase Activity in Duchenne Dystrophic Muscle. Journal of the Neurological Sciences, 24, 361 - 363

Szibor, R., Redmann, K., Deike, H. and Müller, K. (1976). Der Einfluss Zon Ouabain auf die Electrophoretische Beweglich keit der Erythrocyte bei 7 patienten mit progressiver Muskeldystrophie Typ Duchenne. Helvetica Paediatrica Acta, 31, 249 - 256

Szibor, R., Steinbicker, V., Redmann, K. and Heuse, E. (1979). Lyon Phenomenom in Ouabain-treated Erythrocytes of Duchenne Muscular Dystrophy Carriers as Revealed by Cell Electrophoresis. Clinical Genetics, 15, 475 - 479

Takagi, A., Muto, Y., Takahashi, Y. and Nakao, K. (1968). Fatty Acid Composition of Lecithin from Muscles in Human Progressive Muscular Dystrophy. Clinica Chimica Acta, 20, 41 - 42

Takagi, A., Schotland, D.L. and Rowland, L.P. (1973). Sarcoplasmic Reticulum in Duchenne Muscular Dystrophy. Archives of Neurology, 28, 380 - 384

Takahashi, K., Shutta, K., Matsuo, B., Takai, T., Takao, H. and Imura, H. (1977). Serum Creatine Kinase Isoenzymes in Duchenne Muscular Dystrophy. Clinica Chimica Acta, 75, 435 - 442

Taniguchi, K. and Iida, S. (1972). Two Apparently Different Ouabain Binding Sites of (Na^+, K^+) ATPase. Biochimica Biophysica Acta, 288, 98 - 102

Thomson, W.H.S. and Smith, I. (1978). X-Linked Recessive (Duchenne) Muscular Dystrophy and Purine Metabolism: Effects of Oral Allopurinol and Adenylate. Metabolism, 27, 151 - 163

Thomson, W.H.S., Sweetin, J.C. and Elton, R.A. (1974). The Neurogenic and Myogenic Hypotheses in Human (Duchenne) Muscular Dystrophy. Nature, 249, 151 - 152

Thomson, W.H.S., Smith, I. and Elton, R.A. (1979). Carrier Detection Rate and Age in Duchenne Muscular Dystrophy. Lancet, i, 829

Tillman, W., Lenard, H.G., Wagner, D., Dönges, H. and Schröter, W. (1979). Rheologic Properties of Erythrocytes in Duchenne Muscular Dystrophy. Paediatric Research, 13, 152 - 155

Toop, J. and Emery, A.E.H. (1974). Muscle Histology of Foetuses at Risk for Duchenne Muscular Dystrophy. Clinical Genetics, 5, 230 - 233

Tzvetanova, E. (1978). Serum Creatine Kinase Isoenzymes in Progressive Muscular Dystrophy. Enzyme, 23, 238 - 245

Veen Van Der, K.J. and Willebrands, A.F. (1966). Isoenzymes of Creatine Phosphokinase in Tissue Extracts and in Normal and Pathological Sera. Clinica Chimica Acta, 13, 312 - 316

Verrill, H.L., Pickard, N.A. and Greumer, H-D (1977). Diminished Cap Formation in Lymphocytes from Patients and Carriers of Duchenne Muscular Dystrophy. Clinical Chemistry, 23, 2341 - 2343

Vickers, J.D., McComas, A.J. and Rathbone, M.P. (1978). Alterations of Membrane Phosphorylation in Erythrocyte Membranes from Patients with Duchenne Muscular Dystrophy. Canadian Journal of Neurological Sciences, 5, 437 - 442

Vickers, J.D., Rathbone, M.P. and Roses, A.D. (1978). Alterations of Erythrocyte Ghost Protein Phosphorylation in the Duchenne and Myotonic Muscular Dystrophies. Biochemical Medicine, 20, 434 - 439

Wacholtz, M.C., Raible, D.G., Jackowski, S., Rodan, S.B., Rodan, G.A. and Sha'afi, R.I. (1979). Adenylate Cyclase and ATPase. Activities in Red Cell Membranes of Patients and Genetic Carriers of Duchenne Muscular Dystrophy. Clinica Chimica Acta, 96, 255 - 259

Wakayama, Y., Hodson, A., Pleasure, D., Bonilla, E. and Schotland, D.L. (1978). Alteration in Erythrocyte Membrane Structure in Duchenne Muscular Dystrophy. Annals of Neurology, 4, 253 - 256

Wakayama, Y., Hodson, A., Pleasure, D., Bonilla, E. and Schotland, D.L. (1979). Freeze Fracture Studies of Erythrocyte Plasma Membrane in Human Neuromuscular Diseases. Neurology, 29, 670 - 675

Walton, J.N. (1973). Progressive Muscular Dystrophy: Structural Alterations in Various Stages and in Carriers of Duchenne Dystrophy. The Striated Muscle. Ed. Pearson C.M., Mostofi, F.K. Pub. Williams and Wilkins

Walton, J.N. and Nattrass, F.J. (1954). On the Classification, Natural History and Treatment of the Myopathies. Brain, 77, 169 - 231

Walton, J.N. and Pennington, R.J.T. (1966). Studies on Human Muscular Dystrophy with Particular Reference to Methods of Carrier Detection. Annals of the New York Academy of Sciences, 138, 315 - 328

Ways, P. and Hanahan, D.J. (1964). Characterisation and Quantification of Red Cell Lipids in Normal Man. Journal of Lipid Research, 5, 318 - 328

Weed, R.I., LaCelle, P.L. and Merrill, E.W. (1969). Metabolic Dependence of Red Cell Deformability. Journal of Clinical Investigation, 48, 795 - 809

Weinstock, I.M., Behrendt, J., Wiltshire Jnr, H.E., Keleman, J. and Louis, S. (1977). Pyruvate Kinase: Diagnostic Value in Neuromuscular Disease. Clinica Chimica Acta, 80, 415 - 422

Wieme, R.J. and Lauryssens, M.J. (1962). Lactate Dehydrogenase Multiplicity in Normal and Diseased Human Muscle. Lancet i, 433 - 434

Wilkerson, L.S., Perkins, R.C., Roelofs, R., Swift, L., Dalton, L.R. and Park, J.H. (1979). Erythrocyte Membrane Abnormalities in Duchenne Muscular Dystrophy Monitored by Saturation Transfer Electron Paramagnetic Resonance Spectroscopy. Proceedings of the National Academy of Sciences, 75, 838 - 841

Wilkinson, J.H. and Robinson, J.M. (1974). Effect of ATP on Release of Intracellular Enzymes from Damaged Cells. Nature, 249, 662 - 663

Williamson, J.R., Shanahan, M.O. and Hochmuth, R.M. (1975). The Influence of Temperature on Red Cell Deformability. Blood, 46, 611 - 624

Winkle Van, W.B., Allen, J.C. and Schwartz, A. (1972). The Nature of the Transport ATPase - Digitalis Complex. Archives of Biochemistry and Biophysics, 151, 85 - 92

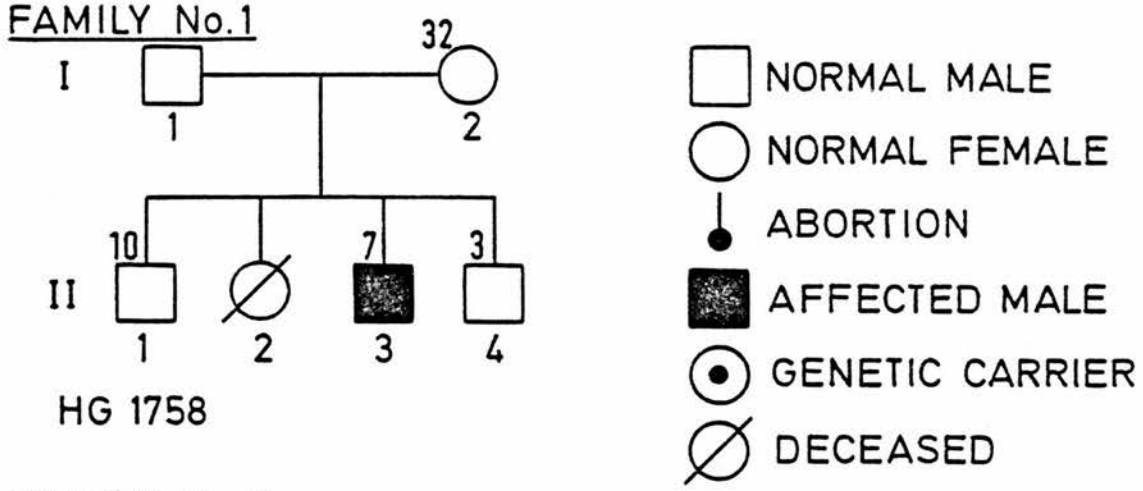
Yasmineh, W.G., Ibrahim, G.A., Abbasnezhad, M. and Awad, E.A. (1978). Isoenzyme Distribution of Creatine Kinase and Lactate Dehydrogenase in Serum and Skeletal Muscle in Duchenne Muscular Dystrophy, Collagen Diseases and Other Muscular Disorders. Clinical Chemistry, 24, 1985 - 1989

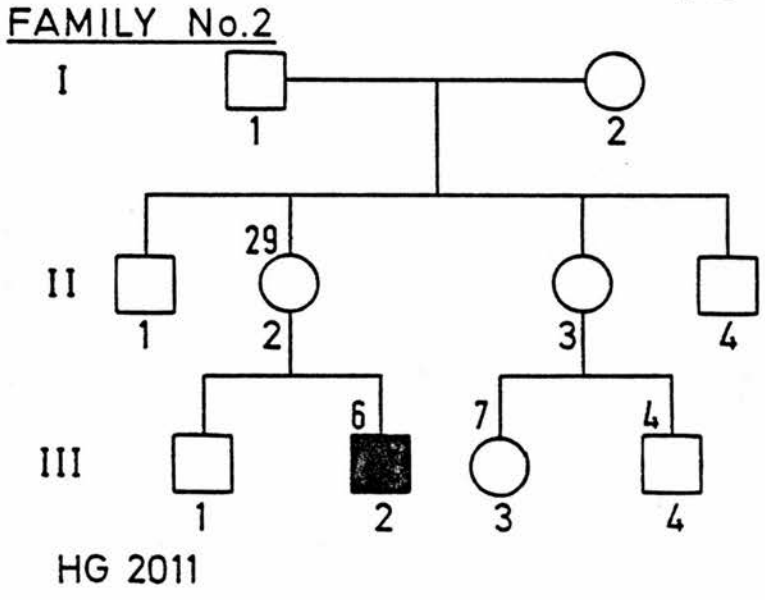
Zachowski, A., Lelieure, L., Aubry, J., Charlemagne, D. and Paraf, A. (1977). Roles of Proteins from the Inner Face of Plasma Membranes in Susceptibility of (Na^+, K^+) Stimulated, Mg^{2+}-Adenosine Triphosphatase to Ouabain. Proceedings of the National Academy of Sciences, 74, 633 - 637

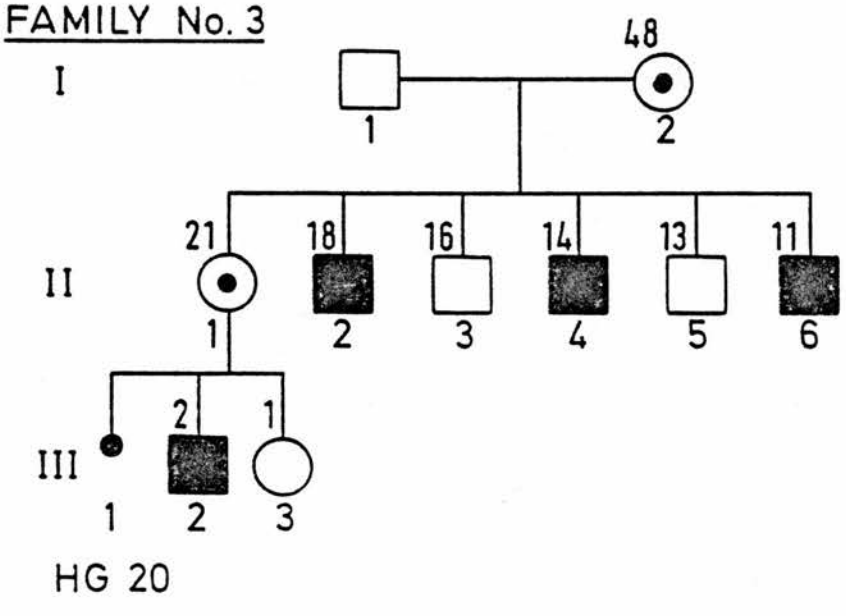
Zatz, M., Shapiro, L.J., Campion, D.S., Oda, E. and Kaback, M.M. (1978). Serum Pyruvate Kinase and Creatine Phosphokinase in Progressive Muscular Dystrophies. Journal of the Neurological Sciences, 36, 349 - 362

Ziparo, E., Lemay, A. and Marchesi, V.T. (1978). The Distribution of Spectrin Along the Membranes of Normal and Echinocytic Human Erythrocytes. Journal of Cell Science, 34, 91 - 101

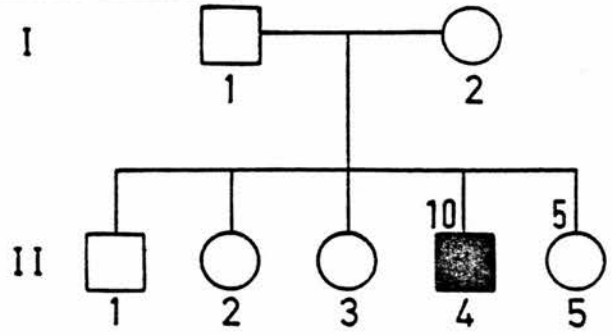
APPENDIX I


FAMILY No.1
I
32
1
2
II
10
7
3
1
2
3
4
HG 1758
NORMAL MALE
NORMAL FEMALE
ABORTION
AFFECTED MALE
GENETIC CARRIER
DECEASED


FAMILY No.2
I
1
2
II
29
1
2
3
4
III
6
7
4
1
2
3
4
HG 2011

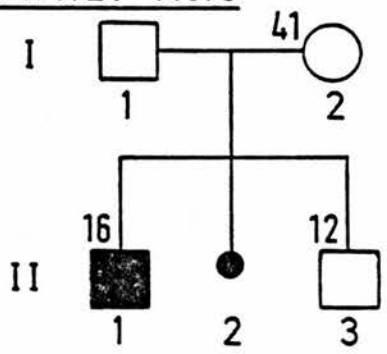

FAMILY No. 3
I
48
1
2
II
21
18
16
14
13
11
1
2
3
4
5
6
III
2
1
1
2
3
HG 20

FAMILY No. 4

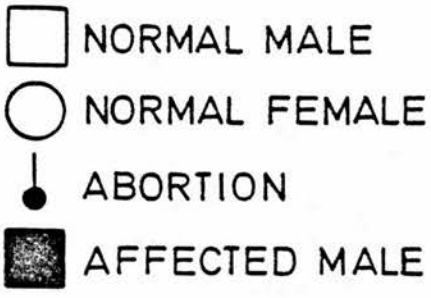


HG 787

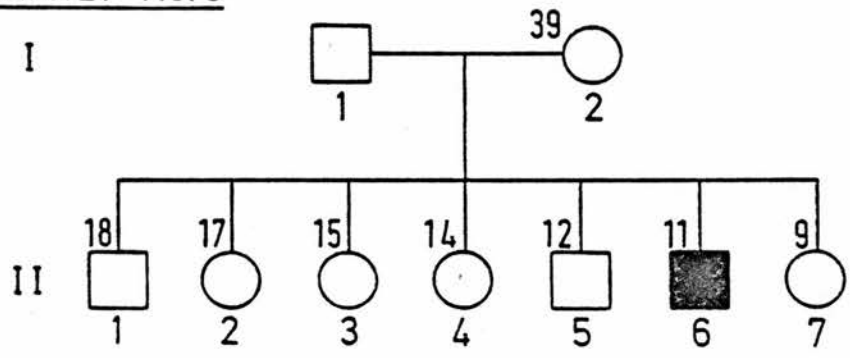
FAMILY No. 5



HG 68

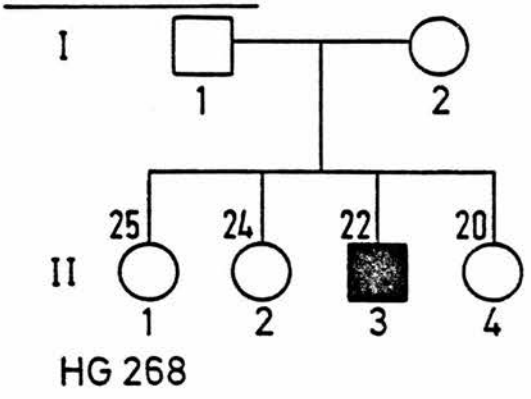


FAMILY No. 6

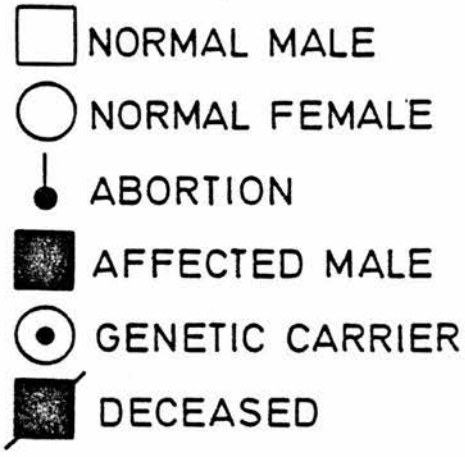


HG 612

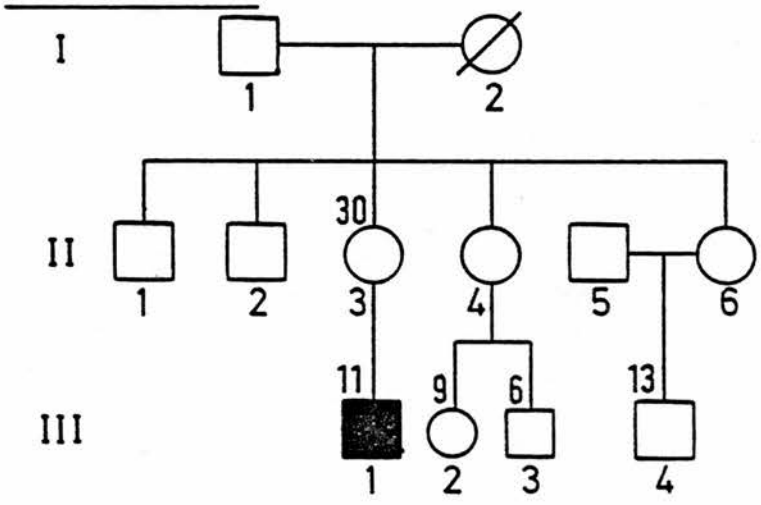
FAMILY No. 7



HG 268

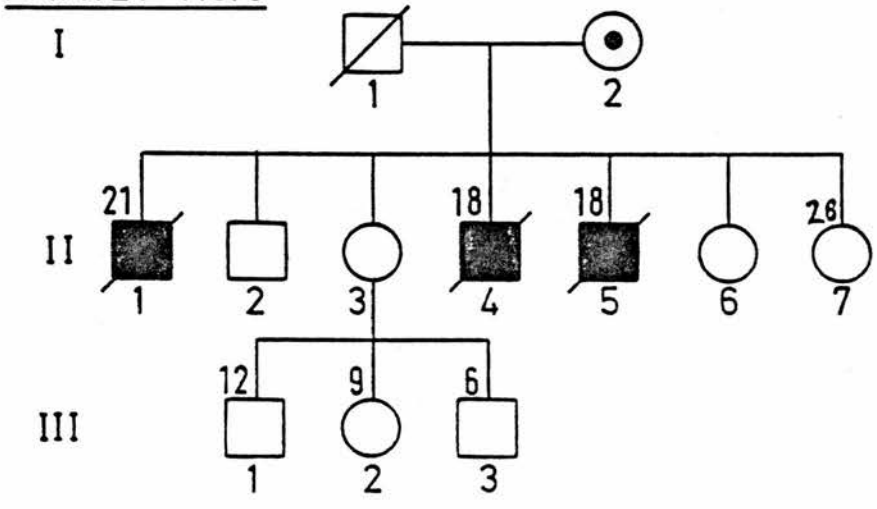


FAMILY No. 8

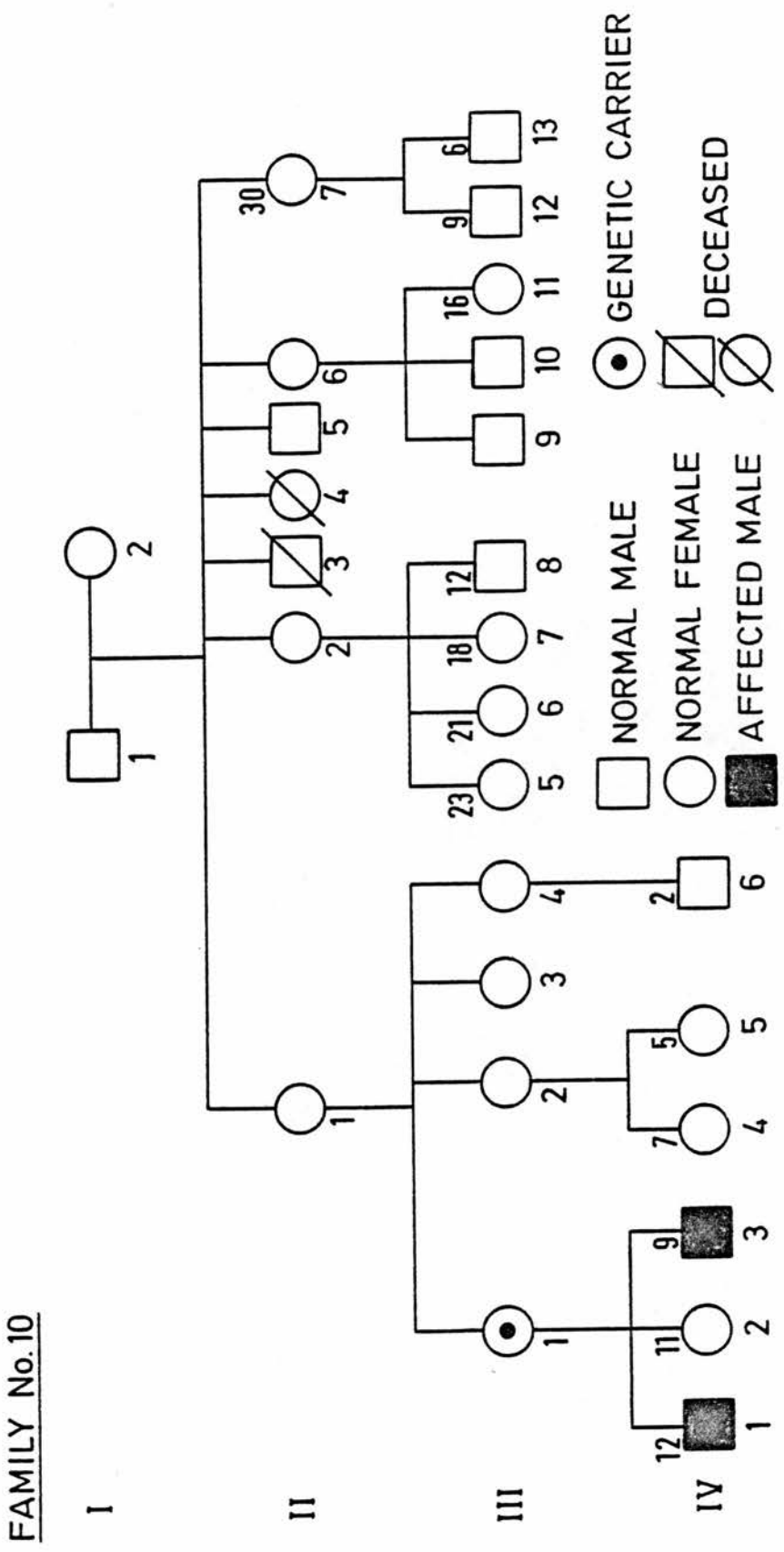


HG 1741

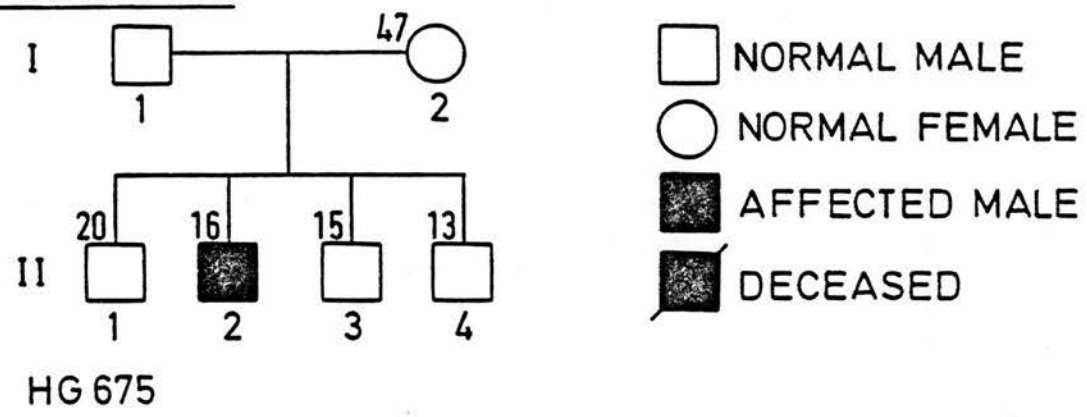
FAMILY No. 9



HG 1761

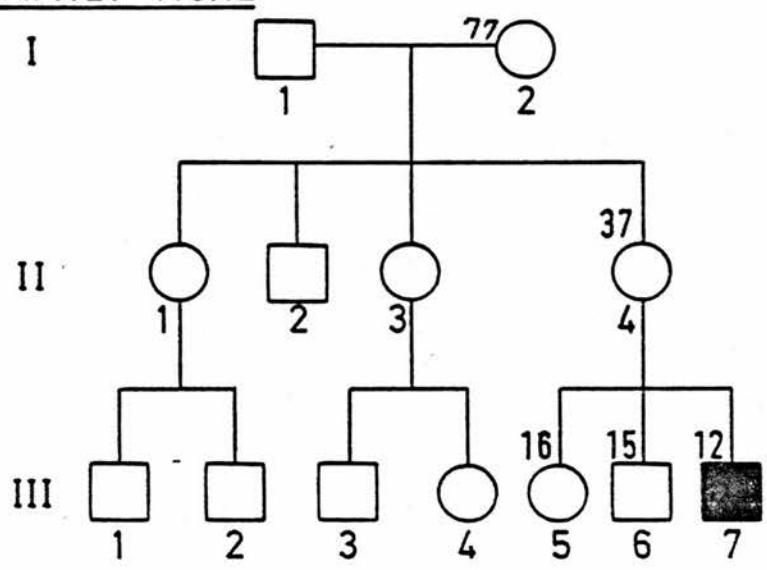

FAMILY No.10
I
II
III
IV
HG 1759
NORMAL MALE
NORMAL FEMALE
AFFECTED MALE
GENETIC CARRIER
DECEASED

FAMILY No.11



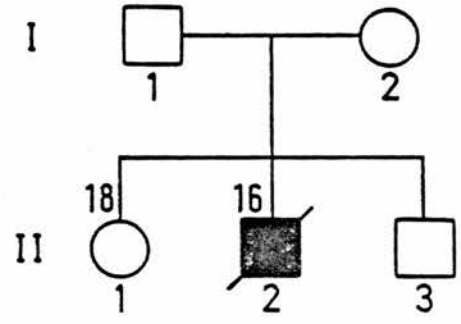
HG 675

FAMILY No.12



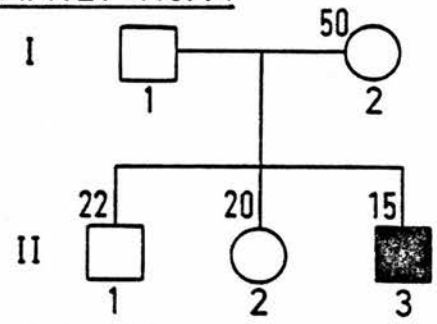
HG 2140

FAMILY No.13

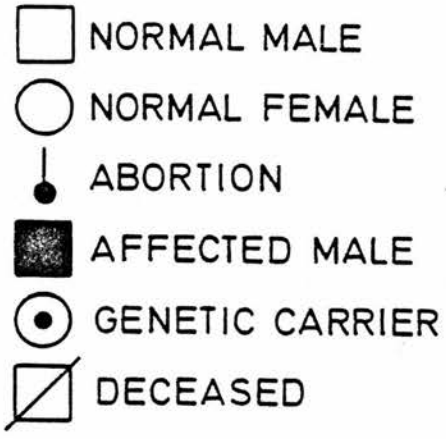


HG 16

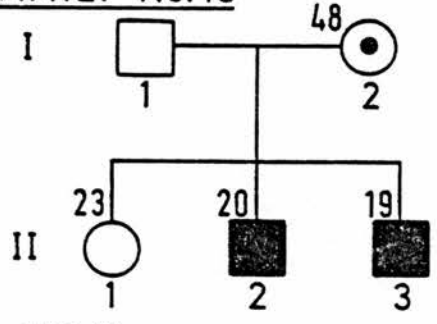
FAMILY No. 14



HG 1803

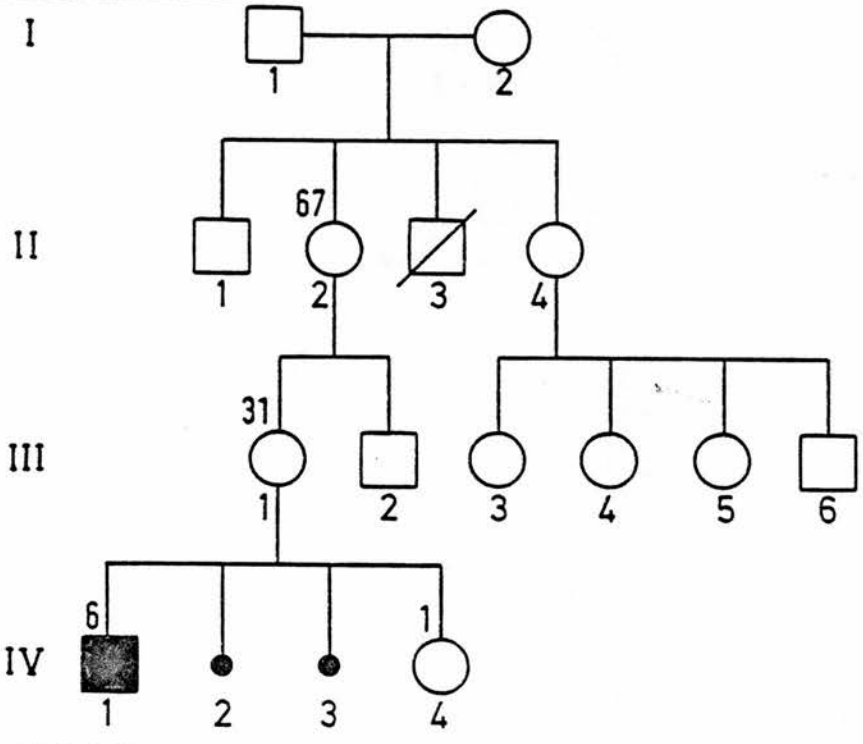


FAMILY No. 15



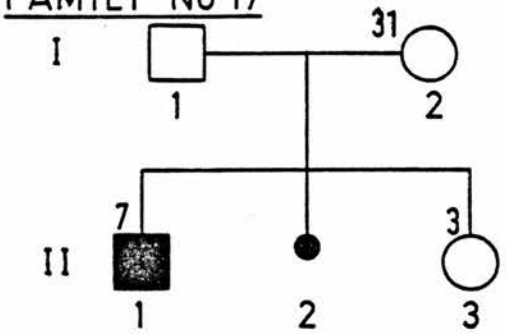
HG 11

FAMILY No. 16

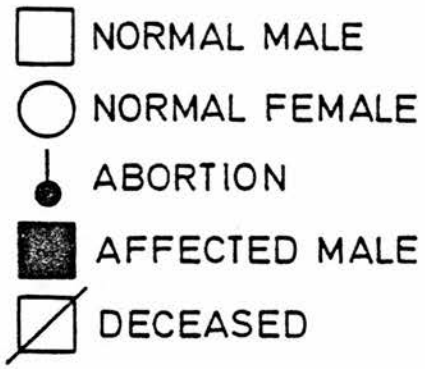


HG 2014

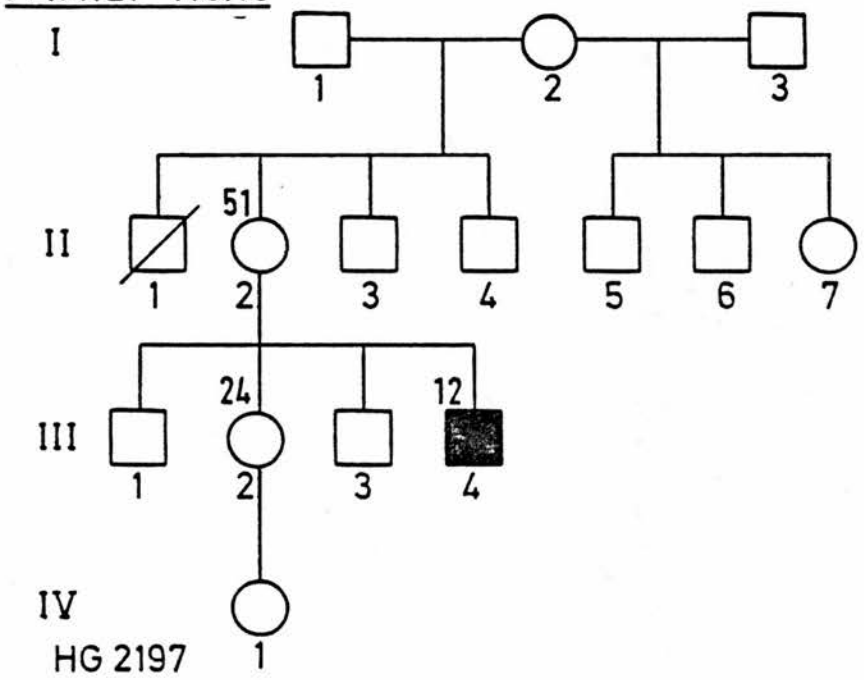
FAMILY No 17



HG 1870

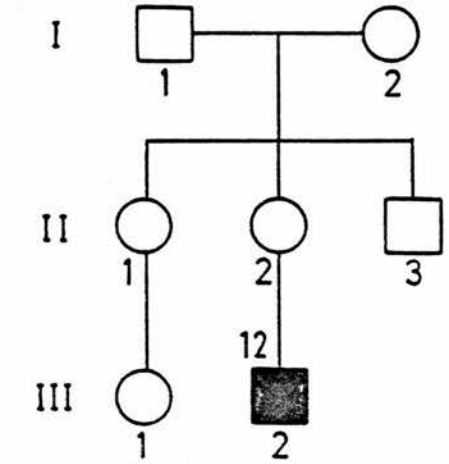


FAMILY No.18



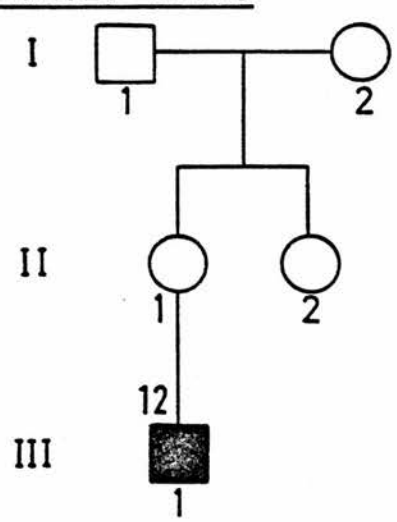
HG 2197

FAMILY No. 19

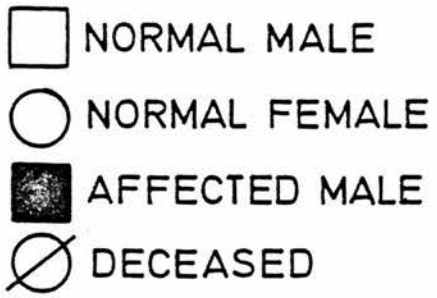


HG 1230

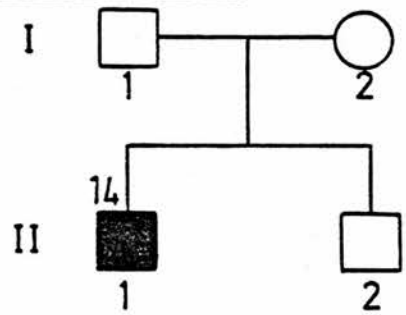
FAMILY No. 20



HG 2005

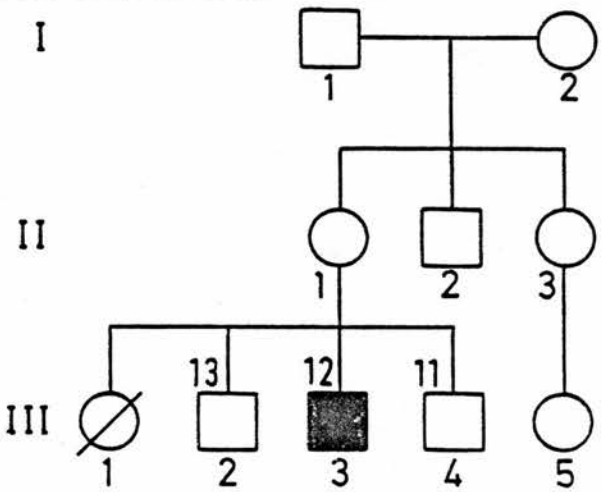


FAMILY No. 21



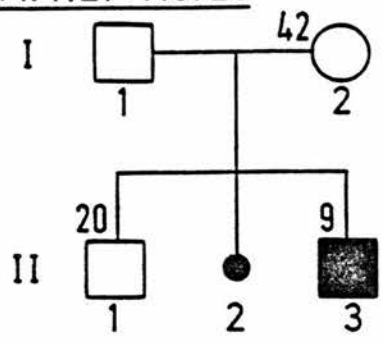
HG 774

FAMILY No. 22

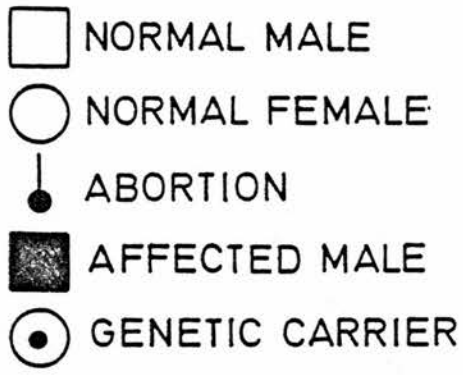


HG 626

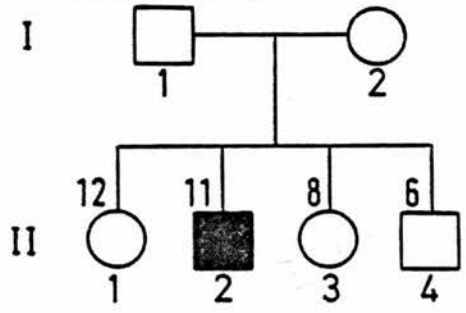
FAMILY No. 23



HG 1377

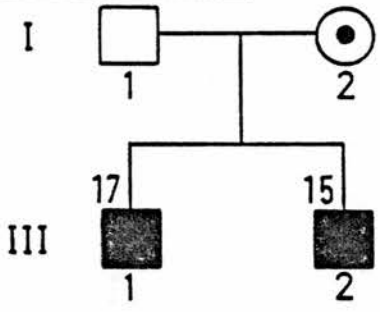


FAMILY No. 24



HG 1061

FAMILY No. 25



HG 2009

APPENDIX II

Echinocyte and stomatocyte investigations

Sodium chloride (0.9%) - dissolve 9.0 g NaCl in 1 litre H_2O

10 mM sodium cacodylate buffer, pH 7.4 - dissolve 2.1402 g sodium cacodylate trihydrate in H_2O, make up to 950 ml and adjust pH to 7.4 with HCl. Make up to 1 litre.

3% gluteraldehyde fixative - dilute 1.2 ml gluteraldehyde (special electron microscope grade - 25%) to 10 ml with 10 mM cacodylate buffer.

Buffered sucrose - dissolve 10 g sucrose in cacodylate buffer, make up to 100 ml with buffer.

Osmotic fragility investigations

Buffered saline - dissolve 13.65 g Na_2HPO_4 and 2.43 g $NaH_2PO_4 . 2H_2O$ in 1 litre of H_2O. Bring to pH 7.4. Add 90 g NaCl. Solution osmotically equivalent to 100 g/l NaCl. Dilute with H_2O for specific concentrations. Store at 4°C for one month.

Erythrocyte ghost membrane preparation

Hypotonic haemolysis buffer - dissolve 1.211 g Tris in 1 litre H_2O. Bring to pH 7.4 with HCl. Add 0.0584 g NaCl, 0.2033 g $MgCl_2$, 0.0746 g KCl and 0.1543 g dithiothreitol. Check pH. Store at 4°C for one month.

Protein solubilising fluid (PSF)

PSF - dissolve 0.9 g NaCl and 0.5 g NaOH in 100 ml H_2O. Store at 4°C for one month.

Protein estimation

Reagent I - dissolve 20 g Na_2CO_3 in 1 litre 0.1 M NaOH. Store at 4°C for two weeks.

Reagent II - dissolve 2 g KNa tartrate and 1 g $CuSO_4 . 5H_2O$ in 100 ml H_2O. store at 4°C for two weeks.

Reagent A - 1 ml of reagent II with 50 ml of reagent I. Prepare fresh.

Reagent B - Folin - Ciocalteu reagent diluted 1:1.1 with H_2O. Prepare fresh.

Bovine serum albumin stock - dissolve 10 mg of bovine serum albumin in 10 ml PSF. Concentration 1 mg/ml. Dilute with PSF for protein calibration curve. Store at 4°C for two weeks.

Inorganic phosphate estimation

Trichloroacetic acid - dissolve 5 g in 10 ml H_2O

Molybdate I - dissolve 25 g ammonium molybdate in 200 ml H_2O. Add 500 ml 5 M H_2SO_4 and make up to 1 litre with H_2O.

Molybdate II - dissolve 25 g ammonium molybdate in 200 ml H_2O. Add 300 ml 5 M H_2SO_4 and make up to 1 litre with H_2O.

Sodium bisulphite - dissolve 3.0 g $NaHSO_3$ in 200 ml H_2O. Prepare and stand for two days before use.

Sodium sulphite - dissolve 200 g $Na_2SO_3 . 7H_2O$ in 380 ml H_2O. Filter and keep well stoppered.

Aminonaphtholsulphonic acid - dissolve 0.5 g of the dry powder in 195 ml sodium bisulphite solution, add 5 ml sodium sulphite solution. Store in dark bottle for up to one month at 4°C.

Standard phosphate solution - dissolve 0.351 g KH_2PO_4 in 100 ml H_2O, add 10 ml 5 M H_2SO_4 and make up to 1 litre with H_2O. Solution keeps indefinitely. Dilute standard for use for phosphate calibration.

(Na^+, K^+) ATPase assay

Assay medium	- dissolve 3.633 g Tris in 300 ml H_2O. Bring to pH 7.4 with HCl. Add 0.0175 g NaCl, 0.0447 g KCl, and 0.0610 g $MgCl_2$. Check pH. Store at 4°C for one month. 4 mM Na_2ATP added to assay medium immediately prior to incubation.
Ouabain solution	- dissolve 0.007 g in 10 ml H_2O. Prepare fresh.

SDS - PAGE

Concentrated acrylamide and bis-acrylamide	- dissolve 10 g acrylamide and 0.375 g bis-acrylamide in H_2O and make up to 25 ml.
10 X buffer, pH 7.4 (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA)	- dissolve 24.2280 g Tris, 13.6080 g sodium acetate, and 3.7224 g EDTA in 200 ml H_2O. Adjust to pH 7.4 with glacial acetic acid. Make up to 500 ml with H_2O.
0.05% bromophenol blue	- dissolve 0.0252 g bromophenol blue in H_2O and make up to 50 ml.
0.7% TEMED	- dilute 70 µl TEMED to 10 ml with H_2O
1.5% ammonium persulphate	- dissolve 0.1500 g ammonium persulphate in 10 ml H_2O
Overlay solution for SDS - PAGE	- 10% SDS (according to concentration used in gel; 0.2 ml for 0.2% run), with 1 ml ammonium persulphate (1.5%), 0.5 ml TEMED (0.7%) and 8.3 ml H_2O.
Electrophoresis buffer	- to 90 ml 10 X buffer add 18 ml SDS (10%) for 0.2% run and make up to 900 ml with H_2O.
0.05% coomassie blue	- dissolve 0.0500 g coomassie blue in 25 ml propanol. Add 65 ml H_2O; and 10 ml acetic acid before use
Polyacrylamide gels	- mix 2.50 ml concentrated acrylamide bis-acrylamide solution, 2.00 ml 10 X buffer and 12.10 ml H_2O. De-airate. Add 0.40 ml SDS (10%) for 0.2% run, 2.00 ml ammonium persulphate (1.5%) and 1.00 ml TEMED (0.7%).

SDS - PAGE protein solubilising fluid (10 mM Tris, 20% sucrose, 1 mM EDTA and 80 mM dithiothreitol)	-	dissolve 0.0484 g Tris, 4.000 g sucrose, 0.0148 g EDTA and 0.2468 g dithiothreitol in 17.18 ml H_2O, adjust to pH 8.0 and make up to 20 ml with H_2O.

METHOD

Polyacrylamide gel solution was poured into 6.5 cm gel tubes. The gels were overlayed with PAGE solution for 40 minutes whilst polymerisation occurred. The overlay solution was then removed and the top of the gels washed three times in 10 X buffer. The SDS - PAGE PSF containing ghost membrane protein was incubated in a boiling water bath for three minutes, then cooled in ice. 50 µl (containing 3 µl 0.05% bromophenol blue and ghost membrane protein to a final concentration of 1 mg/ml) was layered onto each gel, and covered with electrophoresis buffer. A current of 2 mA/tube was applied until the samples ran into the gel, when the current was increased to 6 mA/tube. The duration of the run was approximately three hours. The gels were removed from the glass tubes, and stained in coomassie blue overnight. The gels were destained in 10% acetic acid for 48 hours.

PUBLICATIONS

PUBLICATIONS

1. Lloyd, S.J. and Nunn, M.G. (1978). Osmotic Fragility of Erythrocytes in Duchenne Muscular Dystrophy. British Medical Journal, 2, 252

Reprinted from the BRITISH MEDICAL JOURNAL,
22 *July* 1978, **2**, 252

Osmotic fragility of erythrocytes in Duchenne muscular dystrophy

Duchenne muscular dystrophy is an X-linked recessive disorder affecting young boys. It is one of the more severe forms of muscular dystrophy and is transmitted by healthy female carriers. Onset is usually at 3-5 years and affected individuals become chairbound by the end of the first decade. Death occurs in the late teens or early twenties from cardiac failure or pneumonia. The basic defect in this disorder is as yet unknown but several recent studies[1–4] have suggested that there may be a generalised membrane abnormality affecting erythrocytes as well as muscle. Fisher *et al*[4] have reported an increase in the osmotic fragility in cases of what they refer to as "pseudo-hypertrophic muscular dystrophy." We report here our findings in a series of 10 patients with confirmed Duchenne muscular dystrophy and four definite carriers. (A definite carrier is defined as the mother of an affected boy with another affected male relative. The four carriers studied here had serum creatinine kinase levels of 83, 132, 213, and 344 IU/l (normal upper limit 85 IU/l).)

Patients, methods, and results

Heparinised samples of venous blood were obtained from patients (aged 1-18), their unaffected brothers (aged 1-16), and female carriers (aged 23-56) when the families attended the muscular dystrophy clinic in the department. Control samples were obtained from young boys (aged 4-15) before ortho-

Per cent lysis (mean ± SD) in male controls, boys with Duchenne muscular dystrophy (DMD), their unaffected brothers, female controls, and definite carriers

Sodium chloride concentration (g/l)	Male controls (n = 10)	DMD patients (n = 10)	Brothers (n = 5)	Female controls (n = 36)	Definite carriers (n = 4)
1·0	100	100	100	100	100
3·6	95·1 ± 2·9	94·4 ± 5·9	93·4 ± 6·0	95·5 ± 3·6	91·6 ± 2·8
3·8	90·7 ± 3·0	91·2 ± 7·2	83·4 ± 13·2	89·9 ± 8·5	86·5 ± 3·5
4·0	79·4 ± 12·5	83·4 ± 13·6	63·6 ± 18·3	78·8 ± 14·4	75·7 ± 11·7
4·2	50·7 ± 17·6	66·7 ± 18·3	38·5 ± 11·9	64·4 ± 18·1	49·5 ± 20·1
4·4	13·9 ± 9·9	34·0 ± 19·6	9·5 ± 2·1	40·4 ± 17·7	18·1 ± 14·9
4·6	4·5 ± 4·2	16·3 ± 13·0	4·6 ± 2·1	18·6 ± 11·3	5·8 ± 7·1
4·8	1·7 ± 1·8	6·9 ± 7·0	1·3 ± 0·7	6·8 ± 6·7	2·1 ± 3·3
5·0	0·0	1·3 ± 1·5	0·0	1·2 ± 1·9	0·0

University Department of Human Genetics, Western General Hospital, Edinburgh EH4 2HU

SUSAN J LLOYD, BSC, research student
MURIEL G NUNN, medical student

paedic operations for disorders unrelated to neuromuscular abnormalities and healthy women volunteers (aged 17-67). The amount of lysis at various concentrations of sodium chloride was determined by the method of Dacie and Lewis.[5] The concentrations of sodium chloride resulting in 50% lysis of each sample were derived graphically.

Signs of lysis first became apparent in affected boys at a concentration of 5·0 g/l but in the controls and normal brothers at a concentration of 4·8 g/l. At all concentrations of sodium chloride from 4·4 to 5·0 g/l (see table) there was significantly greater lysis in affected boys than in either their unaffected brothers or controls ($P<0.05$). The concentration (mean $\pm$SD) of sodium chloride which resulted in 50% lysis was $4{\cdot}18\pm0{\cdot}10$ g/l for male controls, $4{\cdot}30\pm0{\cdot}13$ g/l for affected boys, and $4{\cdot}08\pm0{\cdot}12$ g/l for their unaffected brothers, the difference between affected boys and either their unaffected brothers or controls being statistically significant ($P<0.05$). There was no difference in erythrocyte osmotic fragility between definite carriers and the control series of normal women.

Comment

These results provide additional evidence that there is a probable defect in the erythrocyte membrane in patients with Duchenne muscular dystrophy.

We thank Professor A E H Emery for his encouragement and help with this study, which was supported by the Muscular Dystrophy Group of Great Britain. Miss S Lloyd holds a Fullerton postgraduate research fellowship at the University of Edinburgh. Miss M Nunn holds a Gunning Vacation Bursary.

[1] Kunze, D, *et al*, *Clinica Chimica Acta*, 1973, **43,** 333.
[2] Matheson, D W, and Howland, J L, *Science*, 1974, **184,** 165.
[3] Mokri, B, and Engel, A G, *Neurology*, 1975, **25,** 1111.
[4] Fisher, E R, *et al*, *Journal of the American Medical Association*, 1976, **236,** 955.
[5] Dacie, J V, and Lewis, S M, *Practical Haematology*, 5th edn. London, Churchill Livingstone, 1975.

(*Accepted 30 March 1978*)

Printed in Great Britain by Bourne Offset (Iver) Ltd., Iver, Bucks.